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

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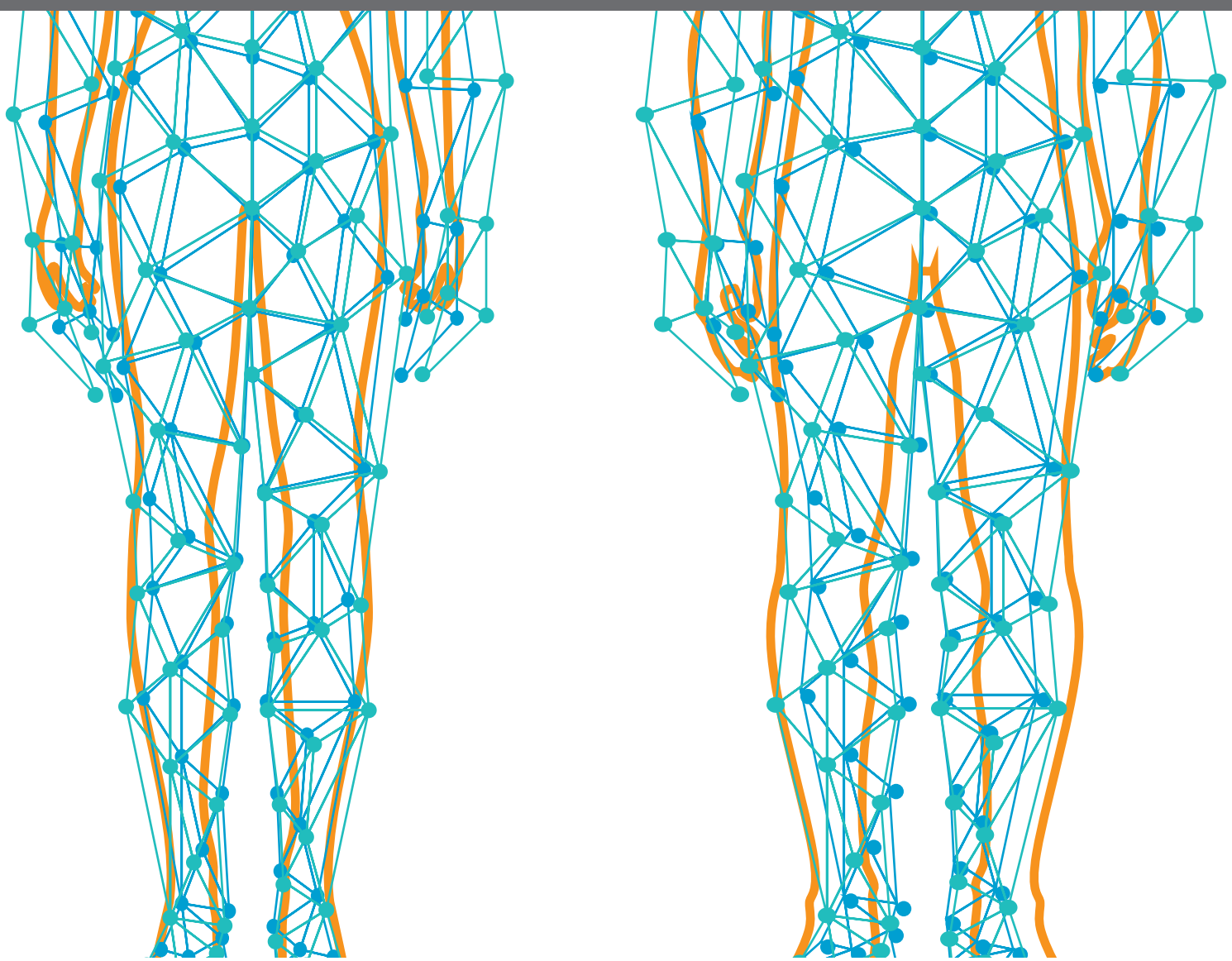

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EDITORIAL
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Editorial: Coronavirus Disease (COVID-19): Pathophysiology, Epidemiology, Clinical Management and Public Health Response, Volume II

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Editorial on the Research Topic

Coronavirus Disease (COVID-19): Pathophysiology, Epidemiology, Clinical Management and Public Health Response, Volume II

INTRODUCTION

Since the declaration of the SARS-CoV-2 outbreak a Public Health Emergency of International Concern by the World Health Organization (WHO), 6.2 million associated deaths have been reported and the multi-disciplinary work of researchers worldwide has provided a far deeper understanding of COVID-19 pathogenesis, clinical treatment and outcomes, mortality, dynamics governing disease spread, period of infectivity, and containment interventions. The required rapid processing and spread of accumulated scientific information would not have been possible if not for the special focus and attention given by scientific journals such as Frontiers in Public Health.

Following on from the success of the first Frontiers COVID-19 Research Topic, featuring 400 original research articles (Doolan et al.), Volume II of a dedicated COVID-19 Research Topic was opened for submissions. Relative to Volume I of the Research Topic, which was broad in scope, the follow-up volume, starting in August 2020, focused primarily on areas of public health and medicine, addressing the requirements of the pandemic at the time. Submissions were

solicited for the article types of Original Research, Review, Mini-Reviews, Systematic Reviews, Research Protocol, Opinion and Hypothesis, and special emphasis/invitation was promoted to the following topics: (1) Detection, investigation, surveillance, management and control of coronavirus outbreaks; (2) Determination of risk factors and prognostic markers; (3) Molecular and genomic epidemiology investigations of sources and modes of transmission; (4) Clinical trials of anti-infective therapeutics, companion diagnostics or patient care pathways; (5) Public health interventions for prevention, vaccine efficacy and immunization program effectiveness; (6) Natural history of COVID-19 clinical disease spectrum in different populations; (7) Systematic reviews and meta-analysis of COVID-19 epidemiological studies and surveillance data; (8) Pre-clinical development and clinical trials of therapeutic agents for COVID-19; (9) Pre-clinical development and clinical trials of COVID-19 candidate vaccines; (10) Clinical immunology of COVID-19; (11) Long-term sequelae from COVID-19 infections; (12) COVID-19 in pregnancy and potential long term impact on maternal and infant health; (13) Management of post-COVID-19 recovery and rehabilitation; (14) Community, culture, and technology-based interventions; (15) Analysis of social and behavior assumptions underpinning epidemiological models of viral transmission; (16) Understanding of social, economic and political costs of public health interventions such as lockdown, self-isolation and social distancing; (17) Implications of the current pandemic for governance and the social justice agenda worldwide; (18) Analyzing current social change to forecast post-pandemic futures; (19) Innovative delivering of teaching and training in medicine; (20) Post-COVID syndrome.

To provide a backdrop, at the time of the launch of Volume II of the Research Topic, 27 vaccines were undergoing human trials, but no vaccine had yet received authorization at that time, as countries worldwide were continuing to battle case numbers and prepare for resurgences. As of the closing of the Research Topic, nine vaccines had obtained Emergency Use Listing by the WHO, and an even higher number were being administered globally. In total, 385 manuscripts were submitted, 162 (42%) of which were accepted. As of May 2022, the Research Topic achieved ∼1,206,000 article views and 142,000 article downloads, with readership distributed across the globe. Frontiers, as the publisher of this Research Topic, made a significant contribution to the timely generation and distribution of peer-reviewed contemporary COVID-19 publications, and combined with Volume I, has produced 562 articles with more than 10,000,000 article views thus far.

Among the four areas covered by this Research Topic, primary focus of the accepted manuscripts was Epidemiology (62), followed by Clinical Management (38), Public Health Response (34), and Pathophysiology (25). The accepted submissions comprised of Original Research (83), Brief Research Report (17), Review (15), Systemic Review (11), Mini Review (3), Perspective (10), Opinion (5), General Commentary (1), Hypothesis and Theory (5), Case Report (4), Community Case Study (1), Study Protocol (3), and Methods (1).

EPIDEMIOLOGY

Of the articles primarily focused on Epidemiology, a clear area of interest was determination of risk factors and prognostic markers for mortality and/or morbidity. This should not come as a surprise given the extent of pressure observed in healthcare systems globally during the initial waves of the pandemic and the urgent need to identify populations at risk and intervention areas of priority. The goal of many investigations was achieving a better understanding of the individuals with increased risk, and prioritizing their needs during patient triage, ensuring timely medical interventions are implemented, and on a higher level, devise and implement public health interventions to decrease the transmission risk to susceptible populations. While some studies focused on general mortality/morbidity in specific geographical regions (Márquez-González et al.; Martins-Filho et al.), others focused on specific disease groups and more vulnerable patient populations, such as those with diabetes (Xiao Y-F. et al.), asthma and COPD (Pardhan, Wood et al.). Some researchers explored the impact of specific factors of interest such as Vitamin D deficiency (Pardhan, Smith et al.) and smoking (Miyara et al.); others explored the impact(s) of concurrent conditions such as acute kidney injury (Gutiérrez-Abejón et al.) and tuberculosis (Song et al.) on disease outcomes. Huang Y. et al., Bai et al., and Liu Z. et al. attempted to identify biological markers predicting mortality/morbidity.

The second most evaluated epidemiological Research Topic was disease surveillance in order to elucidate the geographical, demographic, health-characteristic, and behavioral distribution of confirmed cases at specific time points (or over time). Accurate disease surveillance is fundamental for governments and health care systems for timely implementation of tightening/relaxing measures as warranted, and for resource and treatment planning relative to available capacity. Healthcare workers are a crucial component of the mentioned resources—studies by Choudhry et al. and Feng et al. focused on disease surveillance among healthcare workers. Predicting the size and duration of future outbreaks and new waves was the ultimate goal; hence, many investigators (Yousefinaghani et al.; Shaharudin et al.; Català et al.; Pérez-Reche et al.; Kuhbandner and Homburg) studied prediction of epidemic curves to help guide implementation of timely public health measures. To complement the picture on symptomatic case identification, Li C. et al., Ambrosis et al., and Hashim et al. focused on investigating asymptomatic rates of infection/disease *via* routine testing and/or modeling. Asymptomatic and pre-symptomatic transmission has introduced a greater degree of challenge and uncertainty for monitoring the spread of infection into new clusters.

One of the challenges in controlling and projecting the course of the pandemic has been re-infection of individuals who have recovered from the disease. In order to obtain a better understanding of the factors leading to re-infection with SARS-CoV-2, Shastri et al., Zhu et al., and Xu et al. studied potential risk factors associated with re-infection, including exposure to a different variant of the virus and potential transmission mechanisms.

For measuring the prevalence of asymptomatic infection and vulnerability to re-infection, understanding the magnitude, variability, breadth, and duration of various types of immune responses is important. Many studies focused on evaluation of more easily (and cost-effectively) measurable humoral immune responses, *via* rapid home tests and/or laboratory tests [Ladage et al. (longitudinal); O'Kelly et al. (healthcare setting); Fujita et al. (healthcare workers); Cerino et al.] while Cremoni et al. analyzed humoral and cellular immune responses concurrently.

During the COVID-19 pandemic, frequent informal comparisons were made between SARS-CoV-2 and other SARS virus and influenza infections as well as between the current and previous pandemics in terms of symptoms experienced, severity of outcome, and general impact on public health. Hence, it was important to conduct formal analyses and reviews to better understand the similarities and differences as implemented by Mann et al., Liu L. et al., Nersesjan et al., and Ledberg.

CLINICAL MANAGEMENT

The topic of COVID-19 progression and the associated factors was approached from a clinical management perspective as well. Several studies on progression were in the context of an existing comorbidity or factor, such as hypertension (Mubarik et al.), cancer (Guo et al.; Lin et al.; Barranco et al.), smoking (Xie et al.), amongst others. Three studies generated nomograms to help predict patients' expected disease severity and progression based on patient characteristics, incoming medical status at hospital admission and biochemical/other test results (Tu et al.; Chen et al.; Yu et al.), and there were three other studies evaluating the role(s) of specific biomarkers in disease progression (Billoir et al.; Li L. et al.; Hu et al.) to enable use in clinical management if found relevant. There was also a study evaluating any progression of tinnitus in the context of COVID-19 (Beukes et al.).

Among the articles with a primary focus of clinical management, another main area of interest was the evaluation of various new or concomitant therapies with respect to benefits for COVID-19 progression and management. Treatments evaluated included non-invasive vagus-nerve stimulation (Azabou et al.), sodium copper chlorophyllin (Clark and Taylor-Robinson), corticosteroid use in critically ill patients (Li Y. et al.), spironolactone in patients with liver cirrhosis (Jeon et al.), humified warmed carbon dioxide (El-Betany et al.), cyclosporin A (Devaux, Melenotte et al.), lopinavir/ritonavir and darunavir/cobicistat in hospitalized patients (Castelnuovo et al.), integrated traditional Chinese and Western medicine therapy (Yin et al.) and statins (Fan et al.). The breadth of topics covered demonstrate the multitude of approaches considered, as well as indicating the many clinical specialties involved in those investigations.

Accurate, timely COVID-19 diagnosis and tools used for diagnosis was another research area of interest in clinical management. More than an acceptable threshold of false-positives or false-negatives can cause considerable damage both at individual and public health levels. Xiao A. et al. proposed a triage model for differential diagnosis between COVID-19 and Human Influenza A pneumonia *via* classification and regression tree analysis. Comins-Boo et al. discussed validation of a quick flow cytometry-based assay for acute infection based on CD64 and CD169 expression, which was proposed as a new tool for early diagnosis during the pandemic. Pană et al. studied the validity of measuring body temperature *via* non-contact infrared temperature monitors for triaging of possible COVID-19 among oncological and transplant patients. Yin et al. evaluated performance of four antigen rapid tests, one automated antigen dosing, and one molecular point-of-care test vs. gold-standard RT-PCR, while Caixeta et al. conducted a review summarizing the advantages and limitations of salivary tests for diagnosis of COVID-19.

Pregnant women do not seem to be at higher risk of SARS-CoV-2 infection; however, studies have shown an increased risk of developing severe COVID-19 if they are infected, compared with non-pregnant women of a similar age. Furthermore, COVID-19 during pregnancy has also been associated with an increased likelihood of preterm birth. Hence, pregnant women are considered part of the vulnerable patient population that requires detailed studying. There was one study evaluating impact of cesarean section or vaginal delivery on prevention of possible vertical transmission from a pregnant mother confirmed with COVID-19 to a neonate (Cai et al.). Hashim et al., meanwhile, conducted a systematic review to assess the justification for universal screening of pregnant women for COVID-19 prior to admission to labor.

During the pandemic, it was important to protect healthcare workers, another vulnerable population. Jiang et al. argued that automatic positioning technology applied to relocatable CT can minimize the close contact between technologists and patients and effectively improve the protection of medical staff without sacrificing image quality. Kurotschka et al. explored Italian general practitioners' care experiences and practices during the first wave of the pandemic and whether they were part of an organized emergency response. Vlacha et al. and Schöppenthau et al. explored the rate of infection in healthcare workers and associated factors in prevention of infection.

PUBLIC HEALTH RESPONSE

Comparison of public health responses has been vital in the quick adaptation of effective mitigation strategies. Sharma et al. and Basnet et al. shared details of the public health response in Nepal, Dorrucci et al. in Italy, Wang Z. et al. in China, Nam et al. in Vietnam, and Boccia in Europe/UK (during the holidays). Public education and awareness about virus transmission and protective practices were examined

by Hossain et al. in construction workers, Qarawi et al. in healthcare workers, Islam et al. for proper PPE-related waste disposal, Sewpaul et al. for complying with social distancing regulations and Iboi et al. in preventing outbreaks. Various studies covered strategic and logistic considerations for COVID-19 vaccines, including vaccine prioritization strategies (Zhang Y. et al.), acceptability (Ali et al.; Gerretsen et al.; Qattan et al.), administration logistics (Litaker et al.), and available emerging vaccines and their comparisons (Blumental and Debré).

Shortly after the beginning of the pandemic, it became clear that some of the individuals recovering from the acute phase of the disease have persisting, relapsing or even new onset symptoms over time. This general condition has been referred to in different contexts by multiple names, including "post COVID-19 condition," "chronic COVID-19 syndrome," or "long COVID," amongst others. The WHO suggested a global clinical case definition *via* Delphi consensus method, and included previously published/available case definitions in its publication as well. It was important to define and track this new condition and several studies focused on characterizing this new condition to understand the public health impact. Although individuals having a more severe version of the disease were more frequently impacted by post COVID-19 syndrome, this condition was observed among the mild cases as well. Chowdhury et al. characterized the symptoms experienced and the changes in the biochemical laboratory test values, recommending that both be examined as part of the routine clinical assessment post-disease. General symptom type, frequency and duration of symptoms were evaluated by Salamanna, Veronesi et al., while more specific symptoms and conditions of interest such as neuropsychiatric symptoms (Alper) and new-onset atherosclerosis (Liu and Zhang) were also explored by others. Mei et al. discussed the general impact of long COVID and its impact on healthcare systems; Kelly et al. generated a study protocol for a scalable rehabilitation pathway addressing the immediate requirements for those recovering from COVID-19 in the community.

It is well-known and demonstrated that the impact of the COVID-19 pandemic has not been limited to physical health. High mortality/morbidity rates observed along with the extended duration of the pandemic with new variants over time have had impact on emotional/mental health as well. Han et al. analyzed gender differences in the severity and psychological impact of COVID-19, while Alper presented a case study of an 18 year-old man with a mildly symptomatic illness that has subsequenlty developed depression and anxiety, disruptive interpersonal conflicts, and impairments in attention and motivation. A separate Frontiers Research Topic was specially devoted to "*Psychological, Behavioral, Interpersonal Effects, and Clinical Implications for Health Systems*". Overall, emotional/mental health and post COVID syndrome assessments should be part of the routine public health assessments and responses during the pandemic.

PATHOPHYSIOLOGY

Understanding the pathophysiology of COVID-19 is crucial in dealing with the severe forms of the disease, identifying individuals with increased risk, and taking timely action toward development and/or implementation of appropriate treatments. Many studies evaluated the genetic polymorphism and expression playing a role in pathophysiology, with primary focus on ACE2 (Devaux, Pinault et al.; Salamanna, Maglio et al.; Barash et al.; Zhang J. et al.). Hussman, Ruetsch et al., Koblischke et al., Qi et al., and Yang L. et al. explored inflammatory factors, mechanisms and pathways, with association to disease severity. In this research area, there were also studies focusing on disease manifestation in different body systems and organs—pulmonary (Busnelli et al.; Yang K. et al.; Qanadli et al.; Grippo et al.), gut and lung microbiome (Burchill et al.), liver (Wang X. et al.; Lou et al.), central nervous system (Xiang et al.), male reproductive system (He et al.), and skin (Jamshidi et al.). ElAbd et al., Kasozi et al., and Huang C. et al. discussed potentially effective treatments for disease outcomes along with anticipated or observed mechanisms for impact.

CONCLUSION

As the COVID-19 pandemic continues, with multiple smaller waves anticipated before gradually becoming endemic, many of the evaluated areas under this Research Topic remain relevant. In particular, epidemiological studies continue to feature strongly in the published scientific literature as understanding of the changing dynamics of COVID-19 remains a public health priority. Despite the development of efficacious vaccines and treatments that made a clear difference in addressing severe forms of the disease, more contagious and/or virulent forms of the virus that are able to evade the immune system persist, and are likely to be of concern in the near future. Therefore, concerted efforts on ongoing and new topics remain a crucial part of the continued fight against COVID-19, with accrued information helping prevent similar pandemics in the future as well.

AUTHOR CONTRIBUTIONS

BB compiled the individual article summaries and wrote the first draft. BB, ZK, MI, and TR edited for final revisions. All authors reviewed the draft, generated high-level summaries of the accepted Research Topic articles per their Editorial assignments, and approved the final version for publication.

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BRIEF RESEARCH REPORT
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The Pursuit of COVID-19 Biomarkers: Putting the Spotlight on *ACE2* and *TMPRSS2* Regulatory Sequences

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Diverse populations worldwide are differentially affected by coronavirus disease 2019 (COVID-19). While socioeconomic background has been studied extensively, little is known about the genetic variation underlying this phenomenon. This study is aimed at examining the genetic basis behind the great discrepancies among diverse ethnic groups in terms of COVID-19 susceptibility for viral infection, disease prognosis, and mortality. To this end, *in silico* analysis of single-nucleotide polymorphisms (SNPs) within regulatory sequences of the human angiotensin-converting enzyme 2 (*ACE2*) and transmembrane protease serine 2 (*TMPRSS2*)—the virus's gateway to host cells—and their plausible implications on expression levels was conducted. We provide indication that the variation in the human *ACE2* and *TMPRSS2* regulatory sequences is likely to be involved in and contribute to this phenomenon. SNPs that are abundant in the more susceptible populations introduce binding sites (BSs) for transcription factors or they may invalidate BSs for transcription repressor—both may enhance target gene (*ACE2* or *TMPRSS2*) expression in the relevant target tissues. SNPs that are abundant in the more resistant populations may invalidate BSs for a transcriptional repressor or they may introduce BSs for a transcriptional repressor or initiator of mRNA degradation, which may reduce target gene expression levels. This aspect, when added to the socioeconomic factors, can be a cause for the divergent prevalence of the disease and the different mortality rates within diverse populations. This demonstration may call for a shift in the paradigm of searching for COVID-19 biomarkers, such that SNPs within regulatory sequences should be of high importance.

Keywords: SNP, COVID-19, biomarkers, regulatory sequence elements, ACE2, TMPRSS2

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic, which began in late 2019 in Wuhan, Hubei province, China (1, 2), has spread throughout the world and affected every aspect of human life. The most common clinical signs and symptoms of the disease are fever, fatigue, dry cough, and breathlessness, while expectoration, headache, myalgia, diarrhea, nausea, vomiting, loss of taste or smell, cutaneous eruptions, and renal failure have also been reported (3). Countries throughout the world, and even subpopulations within countries, present great variation in death rate as well as in case fatality ratios (4). The important role of demography, particularly age structure of a

population, was demonstrated and may help explain differences in fatality rates across countries (5). These differences can also be caused by variations between countries in the number of people tested, characteristics of the local healthcare system, the tactics and actions taken to fight against COVID-19, the presence of possible subtypes of the virus, as well as inequalities in socioeconomic, ethnic, geographical, and social determinants of health (6, 7). The following risk factors have been associated with COVID-19: advanced age, obesity, male gender, heart diseases (8), diabetes and immunodeficiency, ethnicity/race (9, 10), and minorities. For example, in the USA (11) and the UK, COVID-19 death rates among African descent populations were higher than among Asian descent or white populations. Noteworthy, ethnicity is a complex entity composed of genetic makeup, social and economic constructs, cultural identity, lifestyle habits, and behavioral patterns (12). Thus far, disparities in COVID-19 disease burden and outcomes among racial and ethnic minorities were mostly associated with socioeconomic conditions, baseline health states, as well as social and health behaviors/behavioral risk factors (13–16), and a call for proper representation and race reporting in clinical trials has emerged (16, 17). Yet, data on COVID-19 by ethnicity/race are scant, and the genetic component has been largely overlooked in most studies.

COVID-19 infection depends on a specific interaction between host angiotensin-converting enzyme 2 (ACE2) as the entry receptor and the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus receptor binding domain of the surface spike glycoprotein (18–20). The cellular serine protease transmembrane protease serine 2 (TMPRSS2) is employed for the Spike protein priming, a cleavage that allows the fusion of viral and cellular membranes (21) and viral spread in the infected host (22). This process potentially involves other proteins, such as the human exopeptidase CD26 (23), also known as DPP4—a key immunoregulatory factor for hijacking and virulence, which are out of the scope of this paper.

ACE2 expression is highly abundant in the lungs and the epithelial cells of the gastrointestinal tract (GIT) and to a lesser extent in the kidney, liver, and male reproductive tissues (24, 25). Expression of TMPRSS2 is high in the GIT and proximal digestive tract and moderate in adult lungs—mainly in bronchial epithelial cells—and also abundant in the prostate gland, kidney, and urinary bladder (26). Both *TMPRSS2* and *ACE2* are expressed in human corneal epithelium, suggesting that ocular surface cells could serve as a potential entry point and as a reservoir for person-to-person transmission of this virus (27). Recently, the expression and function of coding regions and other variants in *ACE2* and *TMPRSS2* among different populations were systematically analyzed, implying different susceptibilities or responses to COVID-19 in different populations (28–34). In addition, variants located at regulatory regions of *TMPRSS2* were found to influence its expression (35). For example, delC allele (rs35074065, located in the shared 3′ regulatory region of TMPRSS2) leads to overexpression of TMPRSS2 [probably by disrupting a binding site (BS) for the repressor IRF2], thus facilitating entry of the D614G COV-19 subtype into host cells and accelerating its spread in Europe and North America where the allele is common (36). Moreover, a single-nucleotide polymorphism (SNP) within the androgen response element in an enhancer located 13 kb upstream of *TMPRSS2* transcription start site reduces binding and transactivation by the androgen receptor (37)—a signaling pathway that also modulates both TMPRSS2 and ACE2 expression and is associated with severe COVID-19 symptoms in men (38, 39).

Little attention has hitherto been given to polymorphism in the *ACE2* and *TMPRSS2* promoters and the possible association with COVID-19 infection, prognosis, and mortality in different ethnicities. Of note, while no association was observed between genetic variants located in or near *ACE2* and *TMPRSS2* genes and human quantitative phenotypes (40), some polymorphisms with relatively high frequencies in different human populations have possible functional effects of COVID-19 infection as they generate BSs for transcription factors (TFs) (41).

This study aims to propose possible variants in the regulatory regions of *ACE2* and *TMPRSS2* that may underlie the marked geographic and race variations in COVID-19 prevalence and mortality. These may further serve in genetic association studies in patients with SARS-CoV-2 infection.

METHODS

In order to gain insights on SNPs that might be relevant to the marked COVID-19 geographic and race variations, the following inclusion criteria were applied: (1) SNPs with a relatively high allelic frequency in specific populations; (2) SNPs for which there is a marked difference in their frequencies among Asian and Africans descents. The frequencies of each allele among diverse ethnic groups were obtained from the following studies: 1000 Genomes, gnomAD–Genomes, ExAc, and TopMed, and when the sample size was big enough, other studies of more specific populations were utilized. These SNPs and the relevant findings are described in detail in **Table 1**.

To examine the potential impact of the more abundant SNPs in *ACE2* and *TMPRSS2* regulatory sequences on their transcriptional regulation, expression, and mRNA stability, PROMO (42) was used to predict transcription factor binding sites (TFBSs) and their modifications in the presence of a given SNP. The diverse possible mechanisms through which these SNPs modulate ACE2 and TMPRSS2 levels are schematically described in **Figures 1A,B**, respectively. A summary of the expression pattern of ACE2, TMPRSS2, and the related key TFs, based on the Human Protein Atlas (43), is provided in **Table 2**.

Expression of the human *ACE2* gene is derived by alternative promoters; the former generates an alternative 5′-untranslated

Abbreviations: ACE2, angiotensin-converting enzyme 2; AP-2α, activating enhancer binding protein 2 alpha; C/EBPβ, CCAAT/enhancer binding protein beta; COVID-19, coronavirus disease 2019; GCF, GC-Rich Sequence DNA-Binding Factor; GIT, gastrointestinal tract; GR-α, glucocorticoid receptor alpha; NF-AT1, nuclear factor of activated T cells; PAX5, paired box 5; RXR-α, retinoid X receptor alpha; SNP, single-nucleotide polymorphism; TFBS, transcription factor binding site; TMPRSS2, transmembrane protease, serine 2; VDR, vitamin D receptor; XBP-1, X-box binding protein 1.

TABLE 1 | REFSEQ ID (rs), chromosomal location, occurrence, ethnicity, added/subtracted transcription factor (TF) prediction, and estimated expression outcome in the human angiotensin-converting enzyme 2 (*ACE2*) and transmembrane protease, serine 2 (*TMPRSS2*) proximal, and core promoter sequence.

Gene	SNP, rs	Location (GRCh38)	Frequency in ethnic populations			Modification in TFBS Added↑ Subtracted↓	Possible impact
			Global	Africans	South Asia	East Asia/Korea		
Human *ACE2*	rs4646114; C>T	chrX:15601259	0.2–2.2%	5.0–7.2%	0%	0%	NF-AT1, YY1**↑ c-Ets1**↓	Elevated transcription
	rs536092258, C>A	chrX:15601214	0.4%	0%	2.1%	0%	PR-B**, PR-A**↑, GR-α*↑	mRNA degradation
	rs4646115, T>C	chrX:15601146	0.1–0.6%	1.4–1.8%	0%	0%	C/EBPβ↑ GR-β**↓	Elevated transcription
	rs370596467, T>C	chrX:15600945	0.02–0.05%	0%	0.1–0.2%	0.1–0.4%	RXR-α, VDR↑ XBP1 ↓	Transcription suppression
Human *TMPRSS2*	rs61299115, delGGCGCAGCGC CGCGGCGCAGCGC>CGC	chr21:41508410-19	24.6–36.3%	29.7–32.5%	22.4%	0.9–1.8%	2X GCF ↓	Elevated transcription
	rs11088551, A>G	chr21:41508389	24.6–36.4%	29.7–32.6%	22.4%	1.0%-1.8%	AP2α ↑	Elevated transcription
	rs4303794, A>C	chr21: 41508379	24.6–36.4%	29.7–32.6%	22.4%	1.0–1.9%	PAX5 ↑	Elevated transcription

*Only single-nucleotide polymorphisms (SNPs) above 1/1,000 occurrences are included. *pre-mature mRNA. **A TF that is less relevant due to location/functional constraints.*

exon (44) and both encoding a similar 805 amino acid precursor: the predominant transcript ACE2-201 (*ENST00000252519.8)* and ACE2-202 (*ENST00000427411.1*). Additionally, alternative splicing may generate a shorter isoform due to termination after coding exon 12. The expression of the *TMPRSS2* gene is derived from a single promoter, yet two main transcripts are generated (45), of which the major one is TMPRSS2-201 (ENST00000332149.10).

RESULTS AND DISCUSSION

Single-Nucleotide Polymorphisms in Human *ACE2* Promoter

rs4646114 is the most abundant SNP (5–7.2%), mainly among African descent populations. It forms an additional TFBS to nuclear factor of activated T cells (NF-AT1). Viral infection activates T cells that induce NF-AT1 dephosphorylation, nuclear translocation, and transcriptional activation of target genes primarily involved in cell–cell interactions (46). NF-AT1 is expressed throughout the body, but especially in the lymphoid tissues, muscles, urinary bladder, kidneys, and lungs, all reported to be infected in many cases of COVID-19. Thus, following initial infection in certain cells expressing a high level of ACE2, NF-AT1 is proposed to further induce *ACE2* transcription during the immune response, which in turn enables substantial penetration and spread of COVID-19 to the other host cells during infection. This forms a positive feedback loop that accelerates penetration and spread of the virus in host cells.

rs536092258 is highly abundant in Asian populations (>2%). It forms a TFBS to the steroid nuclear receptor GR-α, which functions as an expression regulator of glucocorticoid-responsive genes. GR-α has a posttranscriptional role, acting as an RNA-binding protein and initiating mRNA degradation (47) and thus reducing protein levels. This potential effect is limited to the ACE2-202 variant, but not to the ACE2-201 variant, as only the former harbors this variation in the primary transcript.

rs4646115 is prevalent in African descent populations (1.4–1.8%). The SNP multiplies the TFBS of CCAAT/enhancer binding protein beta (C/EBPβ)—a leucine zipper-type TF that is involved in inflammation and acute-phase response and it is highly expressed in the lungs and liver. The multiplication of TFBSs has been shown to increase the expression of a given gene, and thus rs4646115 is likely to enhance ACE2 expression in the lungs and liver and facilitate COVID-19 infection that spreads through the lungs. Interestingly, C/EBPβ is also highly abundant in the adipose tissue, and a high-fat diet or saturated fatty acid exposure has been shown to directly activate C/EBPβ protein expression in the liver, adipocytes, and macrophages. It also influences the development of abdominal obesity and phenotypes related to the development of type 2 diabetes mellitus and cardiovascular disease, all reported as COVID-19 risk factors (8).

rs370596467 is quite rare though an interesting SNP. It is frequent in South and East Asian populations (0.1–0.4%). TFBSs

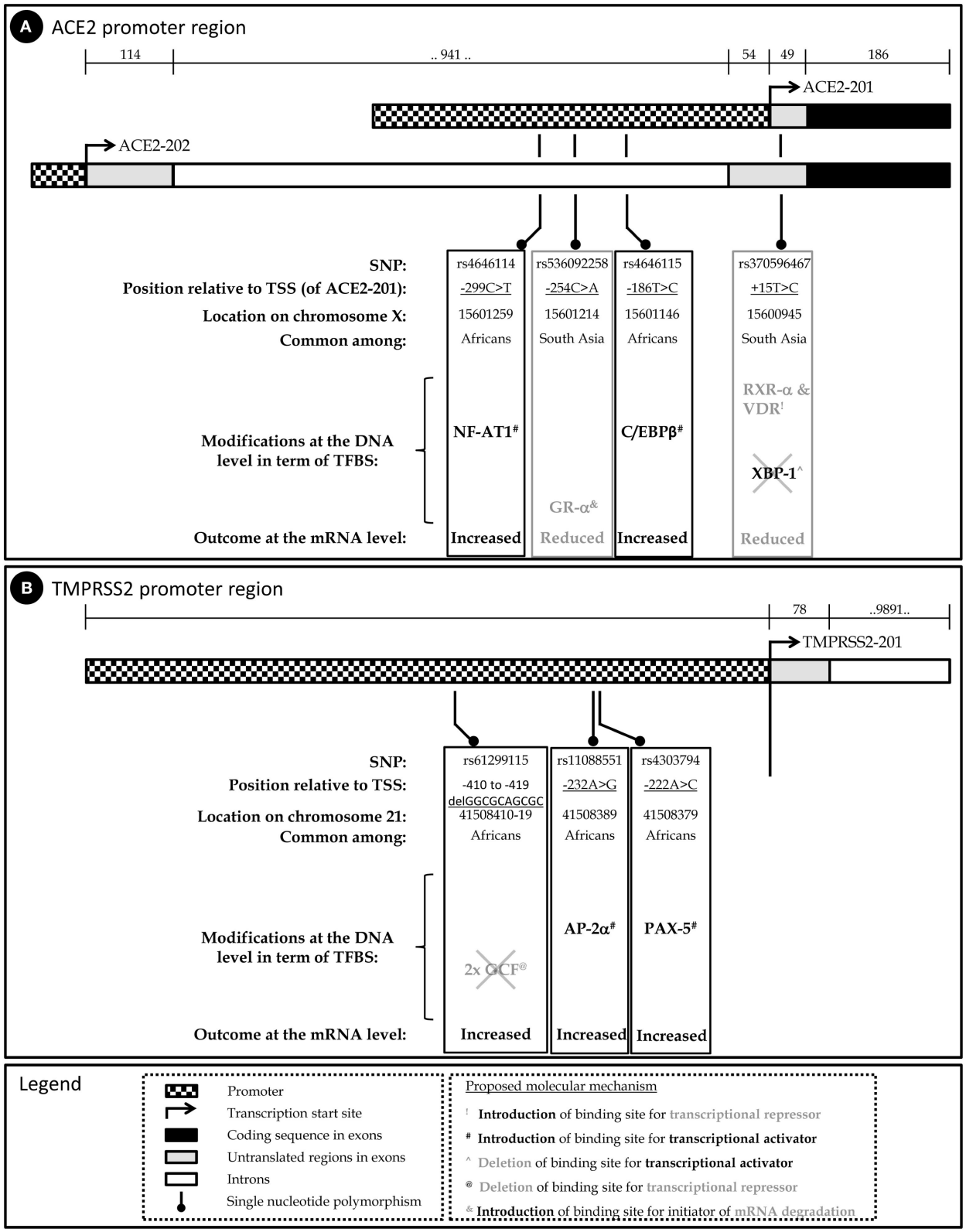

FIGURE 1 | Schematic representation of the human *ACE2* **(A)** and *TMPRSS2* **(B)** genes, the relevant SNPs and their predicted implication on mRNA level.

TABLE 2 | Expression levels of angiotensin-converting enzyme 2 (ACE2), transmembrane protease, serine 2 (TMPRSS2), and related transcription factors in different tissues.

	ACE2 and related transcription factors						*TMPRSS2* and related transcription factors			
Tissue	**ACE2**	**C/EBPβ**	**NF-AT1**	**GRα**	**VDR**	**RXRα**	**XBP1**	***TMPRSS2***	**GCF**	**AP-2α**	**PAX5**
Gastrointestinal and Proximal Digestive Tract	**High**	Moderate	Moderate	**High**	**High**	**High**	Moderate	**High**	Moderate	**High**	Moderate
Lung	Low	Moderate	Moderate	**High**	Low	**High**	Moderate	Moderate	Moderate	N/A	Low
Bone marrow, Lymphoid tissues, Thyroid gland	Low	**High**	**High**	**High**	Low	**High**	Moderate	Moderate	**High**	Moderate	**High**
Kidney and urinary bladder	Moderate	Moderate	Moderate	**High**	Low	**High**	Low	**High**	Moderate	N/A	Low
Liver and gall bladder	Moderate	Low	N/A	**High**	N/A	N/A	Moderate	Moderate	**High**	N/A	Low
Skin	Low	**High**	N/A	**High**	Low	**High**	Low	Low	**High**	**High**	Low
Male tissues	Moderate	N/A	Low	**High**	Low	**High**	Moderate	**High**	Moderate	Moderate	Low
Muscle tissue	N/A	Moderate	Moderate	**High**	Low	**High**	Low	N/A	**High**	N/A	Low

The level of expression was determined by normalized expression levels from three transcriptome datasets (HPA, GTEx, and FANTOM5). Tissues that are not relevant to COVID-19 are not noted.

to both retinoid X receptor alpha (RXR-α) (that is expressed in the lungs, skin, and GIT) and vitamin D receptor (VDR) (which is most abundant in the GIT) are introduced by this variation. VDR is a zinc finger protein containing a DNA-binding domain and two protein interaction surfaces. One of those surfaces is a site for the formation of a heterodimer with the partner protein, RXR-α. Together, this heterodimer suppresses gene activity, although the exact mechanism is currently unclear (48). The SNP also subtracts TFBSs for X-box binding protein 1 (XBP-1), a transcription activator that can increase *ACE2* activation. Together, the subtraction of activator (XBP-1) TFBS and the introduction of BSs to repressors can lead to *ACE2* gene repression and, consequently, lower *ACE2* expression.

Altogether, this analysis implies that carriers of SNPs rs4646114 and rs4646115, which are relatively more abundant among Africans, may present higher susceptibility to COVID-19. On the other hand, SNPs rs536092258 and rs370596467, which are relatively more abundant among individuals of South and East Asian origin, may provide tolerance, at least to some extent, against COVID-19 (**Figure 1A**).

Single-Nucleotide Polymorphisms in Human *TMPRSS2* Promoter

rs61299115, rs11088551, and rs4303794 are all highly frequent in the global population (25–36%); however, they appear in East Asian and Korean populations at a much lower extent (<2%).

rs61299115 introduces a deletion of 10 bp. Due to this deletion, an overlapping double BS for the transcriptional repressor GC factor [GC-Rich Sequence DNA-Binding Factor (GCF)] (49) is deleted, potentially enhancing *TMPRSS2* transcription. Therefore, among East Asian populations, where the minor allele is much less frequent compared to the rest of the world population, TMPRSS2 expression is expected to be relatively lower among the higher share of the population, conferring lower COVID-19 infection.

rs11088551 introduces a BS for activating enhancer binding protein 2 alpha (AP-2α), which belongs to a family of transcriptional regulators and involved in diverse developmental processes, apoptosis, and cell cycle (50, 51). AP-2α also interacts with inducible viral and cellular enhancer elements to regulate the transcription of selected genes. This suggests—similarly to rs61299115—that among East Asian populations, where the minor allele is much less frequent compared to the rest of the world population, TMPRSS2 expression is expected to be relatively lower among a higher share of the population, conferring lower COVID-19 infection.

rs4303794 introduces a BS for paired box 5 (PAX5), a pluripotent transcriptional activator of B-cell development and cancerous processes (52). This suggests that lack of rs4303794 is consistent with lower expression levels of TMPRSS2, and this scenario is prevalent among the East Asian populations.

Together, the three SNPs that are highly prevalent in the general population (25–36%) and are quite rare in the East Asian and Korean populations, hint toward a lower expression of TMPRSS2. This, in addition to the variations found in the

promoter of *ACE2*, can suggest a different COVID-19 etiology and prognosis in different populations (**Figure 1B**).

CONCLUSIONS AND STUDY LIMITATIONS

This study presents a novel approach and intriguing initial findings possibly underlying the relationship between genetic variations and ethnic susceptibility to COVID-19, which are of high and immediate interest, particularly to the biomedical community and more generally to civil societies worldwide. It brings to light five possible mechanisms by which the modification of TFBS (either production or subtraction) might impact mRNA levels of genes related to COVID-19 entry into host cells. Yet, the potential effects of the SNPs on ACE2 and TMPRSS2 expression levels should be further validated first by expression studies in diverse ethnic populations as well as in healthy and infected individuals, and also by mechanistic studies, to infer differential SNP-derived TF binding and activity in target host cells of the virus.

Noteworthy, as *ACE2* is located on chromosome X, allele distribution and impact are expected to be different among males and females. For instance, all males carrying a given SNP are considered hemizygous and would be affected, whereas only homozygote females carrying this SNP, but not heterozygote ones, would be affected. This should be further evaluated epidemiologically, while taking into account variations in the coding region of *ACE2*.

This study represents a proof of concept for a possible relationship of genetic variations within the *ACE2* and *TMPRSS2* regulatory sequences and COVID-19 etiologies, which, in addition to socioeconomic gaps, may explain discrepancies among diverse ethnic groups. It broadens the biological outlook on the COVID-19 pandemic to gene regulatory regions, rather than the more obvious and frequently investigated coding sequences. The variation presented in the human *ACE2* and *TMPRSS2* regulatory sequences is assumed, at least partially, to contribute to the different disease etiologies—including susceptibility to viral infection, disease prognosis, severity, and mortality—among, for example, African/African descent and Asian populations. Genetic evidence from human samples of infected and healthy individuals of diverse ethnicities around the world could further confirm and validate the proposed relationship. This approach should also be applied to other COVID-19-related human genes in the pursuit of COVID-19 biomarkers. Such information on variations in regulatory and coding sequences may pave the way for designing a diagnostic tool and perhaps also for formulating future population-sensitive government policies, i.e., setting priorities for preventive programs, quarantine, and (in the future) vaccination.

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

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.582793/full#supplementary-material

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Changes in Tinnitus Experiences During the COVID-19 Pandemic

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Introduction: The COVID-19 pandemic has disrupted delivery of healthcare, economic activity, and affected social interactions. Identifying and supporting those most affected by the pandemic is required. The purpose of this study was to determine the impact of the pandemic on individuals with tinnitus and to identify mediating factors.

Methods: This is a mixed-methods exploratory cross-sectional study, using data collected via an online survey from 3,103 individuals with tinnitus from 48 countries. The greatest representation was from North America (49%) and Europe (47%) and other countries were only marginally represented.

Results: Although the study was aimed at those with pre-existing tinnitus, 7 individuals reported having COVID-19 initiated tinnitus. Having COVID-19 symptoms exacerbated tinnitus in 40% of respondents, made no change in 54%, and improved tinnitus in 6%. Other mediating factors such as the social and emotional consequences of the pandemic made pre-existing tinnitus more bothersome for 32% of the respondents, particularly for females and younger adults, better for 1%, and caused no change to tinnitus for 67%. Pre-existing tinnitus was significantly exacerbated for those self-isolating, experiencing loneliness, sleeping poorly, and with reduced levels of exercise. Increased depression, anxiety, irritability, and financial worries further significantly contributed to tinnitus being more bothersome during the pandemic period.

Conclusions: These findings have implications for tinnitus management, because they highlight the diverse response both internal and external factors have on tinnitus levels. Clinical services should be mindful that tinnitus may be caused by contracting COVID-19 and pre-existing tinnitus may be exacerbated, although in the majority of respondents

there was no change. Additional support should be offered where tinnitus severity has increased due to the health, social, and/or emotional effects of the COVID-19 pandemic. Tinnitus may be more bothersome for those experiencing loneliness, having fewer social interactions, and who are more anxious or worried.

Keywords: COVID-19, public health, tinnitus, coronavirus, understanding, mental health–state of emotional and social well-being, loneliness, social isolation

INTRODUCTION

In March 2020, the World Health Organization (WHO) declared the COVID-19 outbreak a global pandemic (1). This pandemic has impacted the lives of millions of people around the globe, causing extraordinary disruption to the delivery of healthcare, economic activity, and social interactions (2). Due to the person-to-person transmission of COVID-19 (3), most countries introduced social distancing restrictions and advised people to stay at home where possible (4).

Although such measures reduced the spread of the virus, they can increase levels of depression and reduce well-being in the general population, as indicated by a systematic review, collating the current evidence (5). This review found lower psychological well-being and higher anxiety and depression compared to before COVID-19. Numerous factors including low-self rated health, poor sleep quality, higher perceived stress load, less family support, and unsteady family income, were associated with this increased risk of depression and anxiety.

Support should be directed toward those at higher risk of reduced well-being during the pandemic, such as those with existing mental health conditions (5). One such at-risk group are those with chronic tinnitus, due to already having an increased risk of reduced emotional well-being, depression, and anxiety (6, 7). Those experiencing tinnitus hear sounds in their head and/or ears in the absence of an external sound (8). It is one of the most frequently occurring chronic conditions, affecting 12–30% of the adult population (9). Although tinnitus occurs in all age groups, older adults have a higher incidence of tinnitus (10). This is also the age group most at risk of severe illness from COVID-19 (11). A complex bidirectional interaction exists between tinnitus and emotional distress, as they can trigger or exacerbate each other (12). Tinnitus frequently spikes or is even initiated during stressful periods (13). Also, due to the pandemic, it is more difficult to receive healthcare for conditions that are not seen as life-threatening, such as tinnitus. The pandemic has been shown to increase fear and worry in the general population (14) and may potentially worsen levels of tinnitus due to the clear relationship between emotional distress and severe tinnitus (15). This may, in turn, increase the societal cost of tinnitus, estimated to be £2.7 billion per annum in the United Kingdom (16). Further research into the impact of COVID-19 on tinnitus is required. Such studies are emerging, for example 122 tinnitus patients from a clinic in Germany indicated that although COVID-19 resulted in increased levels of stress only a small increase in tinnitus distress was found (17). Due to the highly heterogeneous nature of tinnitus (18), it is not known if these experiences would be similar in a non-clinical population. It is also not known if these experiences are unique to those living in Germany. A study targeting the general population experiencing tinnitus from more countries is desirable. The aim of this study was to investigate the impact of COVID-19 and factors that contribute to this impact. The hypothesis for this study is that tinnitus experiences will worsen during the pandemic.

METHODS

Study Design

A mixed-methods exploratory cross-sectional survey study design was used to explore the impact of the COVID-19 pandemic on experiences of tinnitus. Ethical approval was granted by the Faculty of Science and Engineering Research Ethics Panel at Anglia Ruskin University (Cambridge, UK, reference number FSE/FREP/19/927) for international data collection.

The Equator network Checklist for Reporting Results of Internet e-Surveys was used to report the methods and results of the survey (see **Supplementary Material**).

Survey Development

Items for the survey were identified through an iterative process by focusing on the current research identifying factors that could contribute to experiences during the pandemic. A list of possible theme questions was generated by the first author and members of the research team contributed to further themes (VM, DB, DS, JO). The first author drafted the survey consisting of 60 proposed questions. The number of questions were reduced by the research team by considering the appropriateness of each question for a tinnitus population. The final survey comprised of a maximum of 47 closed-ended questions and 3 open-ended questions and took approximately 10–15 min to complete. All questions except the open-ended questions were mandatory, although some of the questions were follow-up questions and only presented if responding "yes" to preceding questions by using skip logic. An example was if answering yes/no to having had COVID-19 symptoms.

The survey captured the following categories:

i) Demographic information such as ethnicity, tinnitus duration, and living situation (16 questions).
ii) Tinnitus severity during the pandemic was measured using the Tinnitus Handicap Inventory-screening version [THI-S; (19)] consisting of 10 questions and based on the full version consisting of 25 questions (20).

iii) COVID-19-related questions regarding following social isolation/distancing guidelines, experiencing COVID-19 symptoms and taking medication (11 questions).
iv) The effects of the restrictions imposed by the COVID-19 situation emotionally and financially (12 questions).
v) Strategies to cope with the current situation such as using social and professional support (10 questions).

The survey went through three stages of review before commencing data collection. Initially, two tinnitus associations (The American and British Tinnitus Associations) and their support groups consisting of individuals with tinnitus, reviewed the questionnaire. This was followed by three independent clinical audiologists reviewing the updated questionnaire. This process attempted to ensure (i) all functionality aspects of the online questionnaire were appropriate, such as progressing to subsequent questions and being able to select multiple responses where appropriate; (ii) the face validity of the questions, to assess whether they clearly capture the aspects they aimed to evaluate; and (iii) the interpretability regarding the wording of the questions (21). The suggestions made also improved the survey flow. In a third stage, the survey was then sent to three individuals experiencing tinnitus, to determine whether it was clear and easy to complete. Subsequently, errors identified were corrected and the comprehensibility of the questions was improved. This process indicated good face-validity of the survey. Although a fully psychometrically validated survey would be preferable, the time sensitive nature of this study did not allow for this and it was not the goal of the study to evaluate the study factor structure or internal consistency thereof.

The final survey items were inputted into Qualtrics (Qualtrics, Provo, UT) and were reviewed by team members to ensure functionality. No randomization of the items was used and respondents were unable to change their responses once submitted. No identifiable data were collected. The questionnaire focused on two main themes: tinnitus experiences, and support required for those with tinnitus during the pandemic. This paper focuses on tinnitus experiences during the pandemic. Results regarding the support required during COVID 19 will be reported separately.

Survey Translations

To improve accessibility the final English survey was translated into Dutch, Brazilian Portuguese, Portuguese, German, and Swedish. Translation guidelines (22) were followed where possible, but due to the timescale, both forward and backward translation was not possible. The translated versions were cross-checked and corrected by at least two native speakers of each language. Where possible healthcare professionals who had an understanding of hearing-related difficulties, were involved. Linguistic and cultural adjustments to the wording were made to suit each language.

Survey Distribution

Eligibility criteria included adults aged 18 years or older who provided informed consent. The survey was open to anyone meeting the inclusion criteria. Recruitment was mostly via patient organizations' social media outlets (Twitter, LinkedIn, and Facebook). The American Tinnitus Association (ATA) distributed the survey in the US, the British Tinnitus Association (BTA) in the UK, The Hörselskadades Riksförbund in Sweden, Tuut van Tegenwoordig in Belgium, and Hoorzaken in The Netherlands. The survey was launched on the 29th of April in the UK, the 7th of May in the USA, and the 12th of May 2020 in Belgium, and the Netherlands and later staggered across other European countries and was open for 6 weeks in each location. Online informed consent was required before undertaking the survey and only one submission from each IP address was permitted by the survey software.

Data Analysis

Data cleaning was initially undertaken to remove cases that did not meet study eligibility due to not having tinnitus, or not completing at least the questions relating to tinnitus on the questionnaire. Data analysis incorporated a mixed approach, including both quantitative and qualitative analysis. The Statistical Package for Social Sciences (SPSS) version 26.0 (IBM Corp, 2019) was used for descriptive statistics, including frequencies, means, and standard deviations. The Chi-Square test was used to test the relationships between categorical variables. Where significant, adjusted residuals were used for *post-hoc* analysis to identify which relationships were significant. Due to multiple testing, the p-value was adjusted (Bonferroni) to be significant for $p = 0.001$. Qualitative data from the open questions were analyzed separately using inductive thematic analysis and the themes identified were used to support quantitative analysis.

RESULTS

Representation of Individuals With Tinnitus

There were 3,400 respondents. Of these 38 did not provide consent and 259 responses were largely incomplete. The remaining 3,103 respondents represented 48 countries, although some countries were only marginally represented. The highest number (49%) were from North America (USA, Canada), followed by 47% from Europe (European Union and the United Kingdom). For comparative purposes, the whole sample was divided into four groups, a cohort from North America (49%), one from the United Kingdom and Ireland (UK = 24%), a combination of other European countries excluding the UK (e.g., Belgium, The Netherlands, Sweden, Norway, Portugal, France = 23%) and a group representing a combination of other countries located in South America, Oceania, Asia, and Africa (4%) as shown in **Figure 1**. The age range was 18–100 years with a mean of 58 years (SD:14.0; SE 0.3) with an even gender divide and ethnic distribution where the majority were white (92%) with other ethnic groups (Asian, Black, Hispanic, Indian, or mixed-ethnicity) each representing under 2% of the respondents, respectively.

Impact of the Pandemic on Tinnitus

The average tinnitus duration was 13.6 (SD:14.0; SE 0.3) years with a range of 0.3–80 years, indicating that most respondents

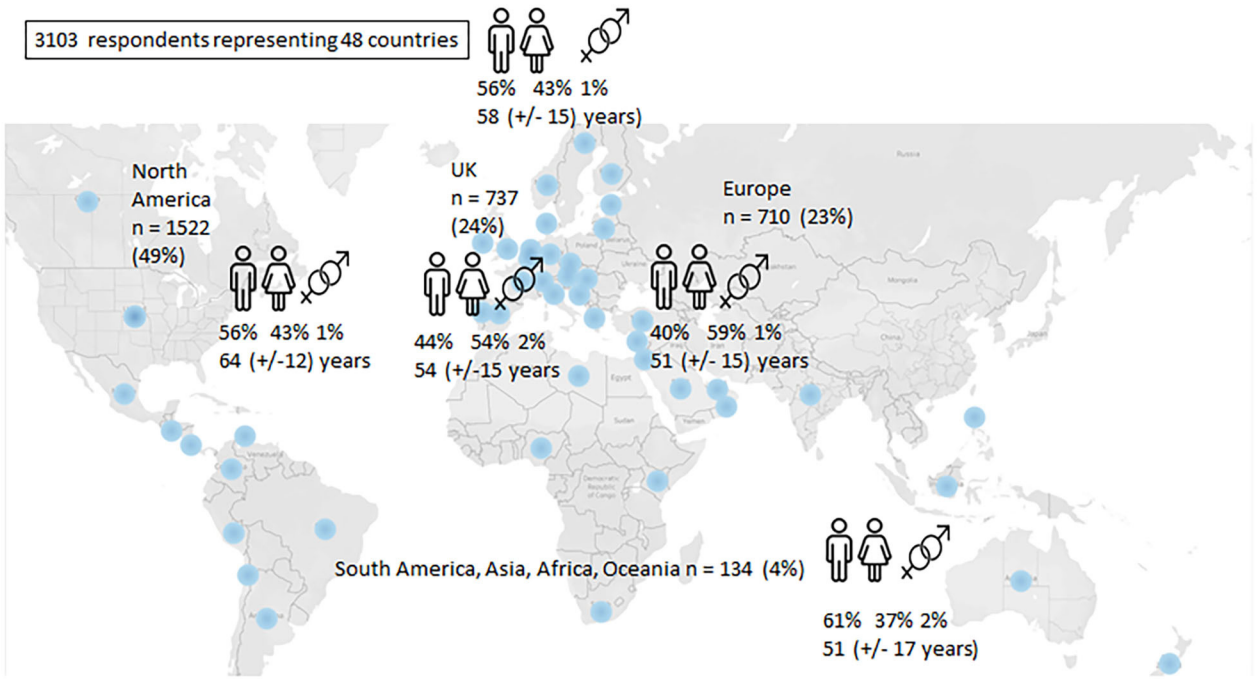

FIGURE 1 | Distribution of respondents.

had longstanding chronic tinnitus. When looking at tinnitus severity scores, the mean was 17 (SD:10; SE 0.2, range 0–40) out of 40 (with higher scores indicating more severity) on the Tinnitus Handicap Inventory Screening Version. Tinnitus was rated on average to be more bothersome during the pandemic. It was more frequently rated as "very" (24%) and "extremely bothersome" (13%) compared with before the pandemic (17 and 7%, respectively) as shown in **Figure 2**. For the majority, these changes were minor in either direction (e.g., from slightly to moderately bothersome). Tinnitus was rated to be stable during the pandemic for 67%, improved for 1%, whereas 32% rated tinnitus as more bothersome. Females (X^2 (15) = 57; $p = 0.001$) and those in age categories below 50 years of age, found tinnitus significantly more bothersome during the pandemic (X^2(15) = 91; $p = 0.001$). Mediating factors related to these reports were explored.

The Impact of the Health Concerns Stemming From COVID-19 on Tinnitus

When asked if health concerns stemming from COVID-19 (e.g., worried about getting ill) affected their tinnitus, 0.5% reported their tinnitus to be improved and 31.5% reported it had worsened (**Figure 3**).

COVID-19 symptoms were experienced by 8% (n = 237/2,952) and 8% (n = 249/2,952) were unsure if they had symptoms. Of those experiencing COVID-19 symptoms, tinnitus remained stable for 54% (n = 128/237), improved for 6% (14/237) and significantly (X^2 (15) = 345; $p = 0.001$) exacerbated tinnitus for 40% (n = 95/237). This was supported by statements such as: "I was too ill to notice at the time, but the tinnitus is definitely much worse now that I'm better enough to notice." (Female, 55 years, UK). Improvements in tinnitus following having COVID-19 symptoms were explained by "Being focused on getting better pushed the tinnitus issue into the background" (Male, 48 years, USA) and "I noticed the tinnitus less because the virus has shown me there are bigger problems than my tinnitus" (Male, 55 years, Belgium). Only 143/2,952 were tested for COVID-19 and of these 26 (18% of those tested) tested positive and of these, 58% (15/26) reported that their tinnitus was exacerbated by the virus. Being anxious about contracting the virus, also exacerbated tinnitus, as explained: "I'm constantly worrying if I've been exposed. This increased stress makes my tinnitus very loud" (Female, 31 years, UK).

It was not asked specifically whether tinnitus was initiated by having COVID-19, but in free-text, there were mentions of both tinnitus (n = 7) and hearing loss (n = 4) starting after contracting COVID-19. A respondent explained, "I did not have tinnitus before the virus. It came on when I was ill and is the only thing which has continued afterward" (Female, 52 years, UK) or "having the virus started my tinnitus" (Male, 36 years, The Netherlands).

Only 4 of the 28 (14%) diagnosed with COVID-19 were medicated in hospital, while most took medication at home, such as Ibuprofen, Paracetamol, Lorazepam, Methylprednisolone, Mortin, Tamiflu pills, Tylenol, Robitussin, Azelastine, Salbutamol, or Azythromycine. There were no reports of taking medication such as chloroquine or hydroxychloroquine, which can be ototoxic. Others described taking natural remedies

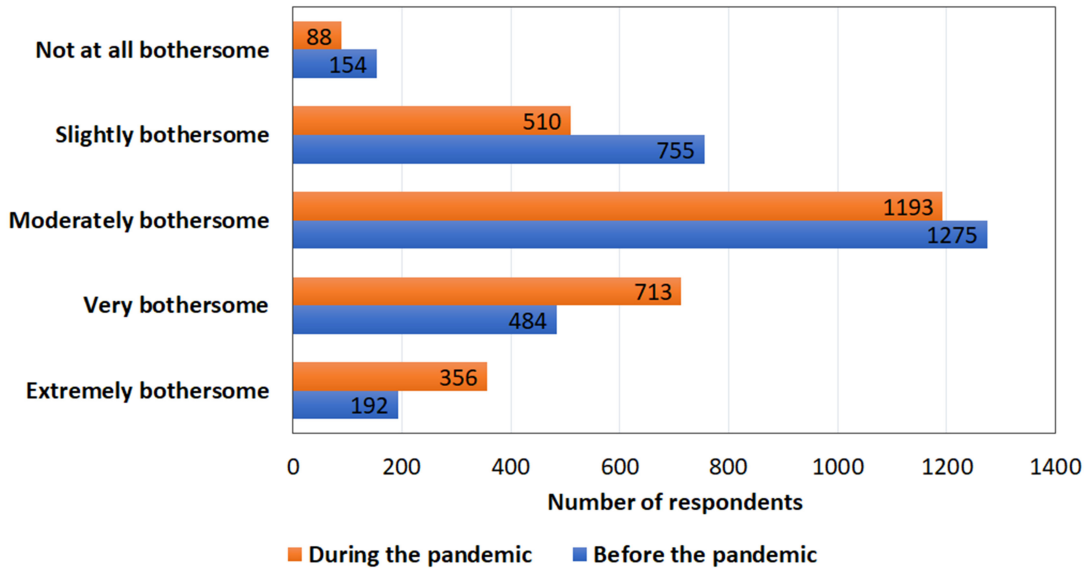

FIGURE 2 | Comparison of how bothersome tinnitus was before and after the COVID-19 pandemic.

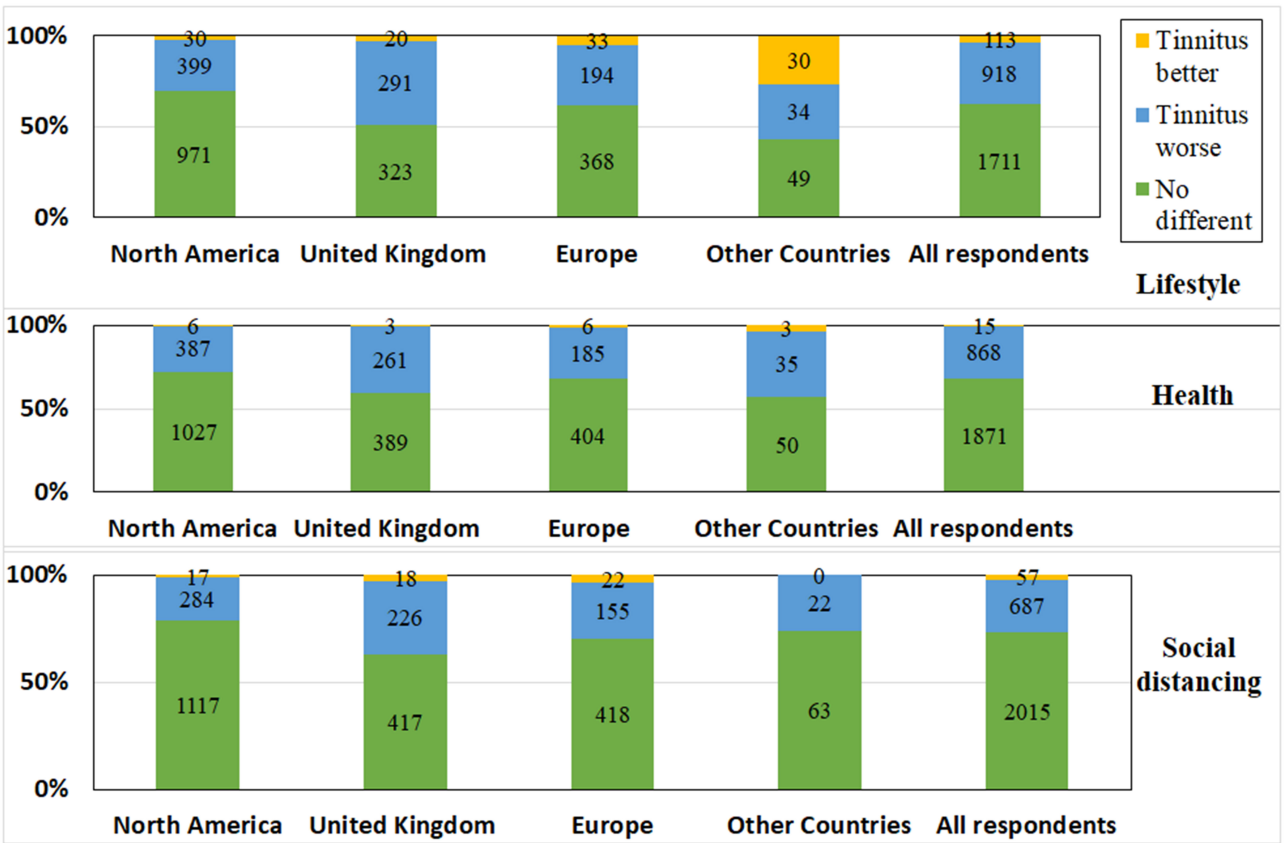

FIGURE 3 | The impact of lifestyle changes, social distancing restrictions, and health concerns during the pandemic on tinnitus experiences.

such as Chinese herbs, ginger, garlic, turmeric, honey, lemon, and zinc. Taking medication significantly increased the presence of tinnitus ($X^2(8) = 598$; $p = 0.001^*$). When asked which medications affected tinnitus, both prescribed medications e.g., "Steroid medication made my tinnitus worse" (Male, 66 years, USA) and using vitamins to try to boost the immune

response against the virus was reported to make tinnitus worse e.g., "I have experienced spikes in my tinnitus when taking vitamins/supplements such as Vitamin D to increase my immune system in response to COVID" (Male, 64 years, USA).

Respondents also had family members who had tested positive for COVID-19 (15%; $n = 37/243$), adding further concerns, expressed by comments such as "I think my current tinnitus spike is due to anxiety for myself and my family, since several of us are in the higher risk group" (Male, Norway, 36 years).

Respondents were asked if they had additional health concerns that may put them at risk for developing COVID. Significantly more respondents from North America reported additional health concerns (70%) compared with under 50% from other locations ($X^2(6) = 205$; $p = 0.001^*$). The presence of additional health concerns was not related to more bothersome tinnitus ($X^2(10) = 222$; $p = 0.02$).

The Impact of Social Distancing Restrictions During the Pandemic on Tinnitus

Social distancing restrictions stemming from COVID-19 did not affect tinnitus for 73% of respondents, improved it for 2%, and exacerbated tinnitus for 25% (**Figure 3**). Those reporting a positive impact, explained the reasons as follows: "I'm less frustrated not being in large crowds" (Female, 38 years, UK). In addition to the tinnitus being exacerbated, social distancing also made listening hard. This was explained by statements such as: "Having to understand 6 feet away and through a mask is so much harder and raises my irritation levels" (Male, 52 years, USA).

The impact of social distancing restrictions varied significantly between countries ($X^2(12) = 214$; $p = 0.001^*$) and had a significantly greater impact ($p = 0.001^*$) in the UK (34%) compared with North America (20%). Social distancing advice was followed by 44% of respondents, particularly from the UK (50%), as shown in **Figure 4**. Tinnitus was significantly more bothersome for those who were self-isolating ($X^2(35) = 550$; $p = 0.001$).

The Impact of Social Interactions

Figure 5 indicates that 86% of respondents reported fewer social interactions, 12% had a similar amount and 2% had more social interactions. More social interactions were desired by 84% of respondents, particularly in the UK as explained: "I notice my tinnitus more because I am stuck in my house all alone with nobody to speak to" (Female, 45 years, UK).

The Impact of Loneliness

When asked if respondents feel lonely because of pandemic related lockdown, most respondents (58%) reported being lonely. Tinnitus was significantly more bothersome for those reporting loneliness ($X^2(15) = 1{,}213$; $p = 0.001$). Experiences of loneliness may have been amplified during the pandemic due to the lockdown measures in place, as explained by the statement "So much time alone has just made me more aware of the tinnitus" (Female, 71 years, USA). When comparing locations, significant differences were found ($X^2(9 = 35$; $p = 0.003^*$) as those in North America reported significantly ($p = 0.001^*$) less loneliness (54%) than those in Europe (62%), the UK (60%), and combined other countries (58%).

The Impact of Lifestyle Changes Due to the Pandemic on Tinnitus

Lifestyle changes stemming from COVID-19 did not affect tinnitus for 62% of respondents, exacerbated tinnitus for 34%, and improved it for 4%. There were significant differences between locations ($X^2(12) = 232$; $p = 0.001^*$), as lifestyle changes impacted those in the UK (46%) significantly more ($p = 0.001^*$) than those in North America (29%), as seen in **Figure 3**. From the free text responses, some respondents reported tinnitus starting during the pandemic and assumed this was related to lifestyle changes saying "No one knows why my tinnitus started, it may be from staying at home, being out of routine or the stress" (Female, 44 years, UK).

The Impact of Living Demographics

To identify how lifestyle changes may have been affected, respondents were asked about their living demographics. The majority of respondents live in a city (48%, $n = 1{,}483$), a town (29%, $n = 896$) or small town (12%, $n = 370$) whereas 11% ($n = 354$) live rurally or in the countryside. Towns and cities were quieter than they would have been used to, which may have altered tinnitus experiences. Tinnitus being more noticeable due to life being quieter was often mentioned e.g., "I am now more aware of the tinnitus as my household is very quiet" (Female, 59 years, UK). For some being at home resulted in exposure to more noise, making tinnitus worse, such as "Increased noise from power tools/lawn equipment and kids playing on motorized toys have made my tinnitus worse" (Female, 43 years, USA).

The majority of respondents had access to a garden or park during the pandemic (89%, $n = 2{,}762$). The cohort from South America, Asia, Africa, and Oceania reported significantly less ($p = 0.001^*$) outdoor spaces (65%) in comparison to 88% from North America, 90% from Europe, and 94% from the UK. Having access to nature was reported to have a positive impact on tinnitus, explained by "I've been furloughed, so I am enjoying relaxing and being in nature more" (Male, 69 years, UK), although this association was not significant.

The Impact of the Pandemic on Sleep

Sleep problems such as waking up earlier or having less restful sleep were reported by 67% respondents, with 46% ($n = 1{,}819$) describing lower sleep quality, explained as "I've not been able to sleep because of a change in routine and worrying, which makes my tinnitus louder" (Male, 56 years, UK). More troubled sleep was related to tinnitus being significantly more bothersome ($X^2(5) = 113$; $p = 0.001$). Better sleep was reported by 6% ($n = 221$), for example, "Being at home means I have more time to sleep, meditate, do yoga, and eat healthy meals which all help me" (Female, 42 years, Canada).

The Impact of Exercise and Diet

Compared with before the pandemic, 38% of respondents reported doing more exercise and 46% reported doing less exercise, which contributed to tinnitus being significantly more bothersome ($X^2(15) = 323$; $p = 0.001$). When comparing current

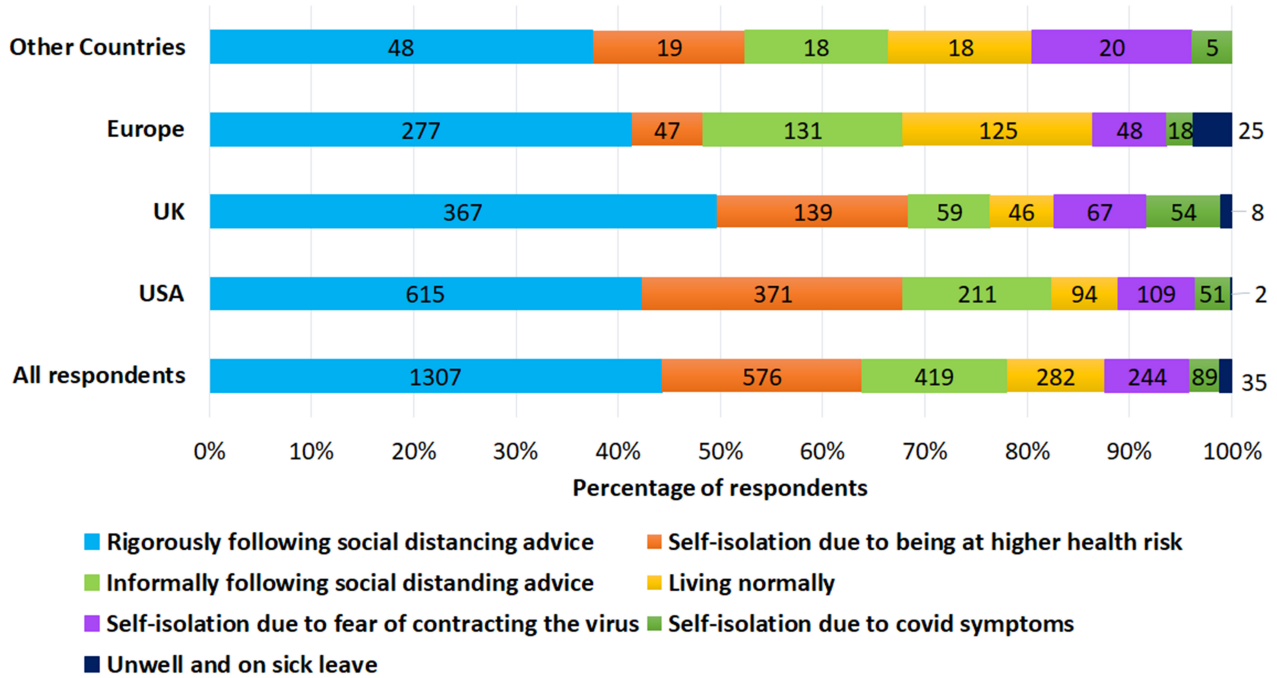

FIGURE 4 | The following of social distancing advice across different locations.

diet with that before the pandemic, it was similar for 59%, healthier for 17%, and less healthy for 24%, which also added to tinnitus being significantly more bothersome ($X^2(15) = 326$; $p = 0.001$). An increased intake of caffeine and alcohol was reported to alter tinnitus as follows, "I find myself drinking more coffee which makes the ringing stronger" (Female, 74 years, USA) or "I'm drinking more alcohol and this makes my night-time tinnitus worse" (Female, 41 years, UK).

The Impact of the Pandemic on Emotional State

Of the respondents, 34% reported being more anxious, 20% more depressed, 15% more irritable whereas 31% reported no change in their emotional state (**Figure 6**). Tinnitus was significantly more bothersome for those feeling more sad or depressed ($X^2(5) = 58$; $p = 0.001$); more anxious ($X^2(5) = 107$; $p = 0.001$); and more irritable ($X^2(5) = 48$; $p = 0.001$). Increased anxiety negatively impacted tinnitus as explained: "There is a lot of added stress and anxiety that make me less able to tolerate the tinnitus" (Male, 69 years, USA) or "So many more anxieties with household appliances breaking that can't be fixed, worrying about food supply, worrying about the virus. Any kind of worries has a negative effect on tinnitus" (Male, 73, UK). Frustration was often mentioned as impacting negatively on tinnitus such as "I'm frustrated with the confinement which I think makes my tinnitus seem extra loud" (Female, 72 years, USA), as well as relationship worries, "My tinnitus is really bad now. Maybe from relationship worries caused by my husband working from home. My cortisol level is on permanent red alert, it feels like in a war zone" (Female, 52 years, UK).

Emotional well-being experiences varied across locations as seen in **Figure 6** with those from the UK reporting more anxiety/depression and irritability during the pandemic (77%) and Europeans the least (67%).

The Impact of Financial Worries

The majority of respondents (51%) reported no financial worries, 41% were somewhat worried, and 8% were very worried about the impact of COVID-19 on finances. Tinnitus was significantly more bothersome ($X^2(15) = 345$, $p = 0.001$) where financial worries were reported. This was supported by statements such as "As a self-employed wedding photographer, all work has been canceled for the foreseeable future, and I have a massive loss in income which I think is contributing to my tinnitus being worse" (Female, 58 years, USA). Loss in investment income was a further factor causing anxiety "I'm retired and my investment income has dropped dramatically, causing stress" (Female, 63, USA).

Looking into reasons for financial worries, respondents were asked about changes in their employment situation as seen in **Figure 7**. Changes in employment patterns altered sound exposure which had a positive effect on tinnitus for some, supported by statements such as "working at home in quiet, instead of a crowded environment gives me less tinnitus" (Male, 53 years, France) and a negative effect for others, as explained "I think the increase in tinnitus is due to less noise from traffic and a busy office" (Female, 54 years, UK).

Although some reported benefits to working from home, there were numerous comments regarding how this change exacerbated tinnitus, such as "I now have a much higher workload, I'm more tired, this increases my tinnitus" (Female, 65

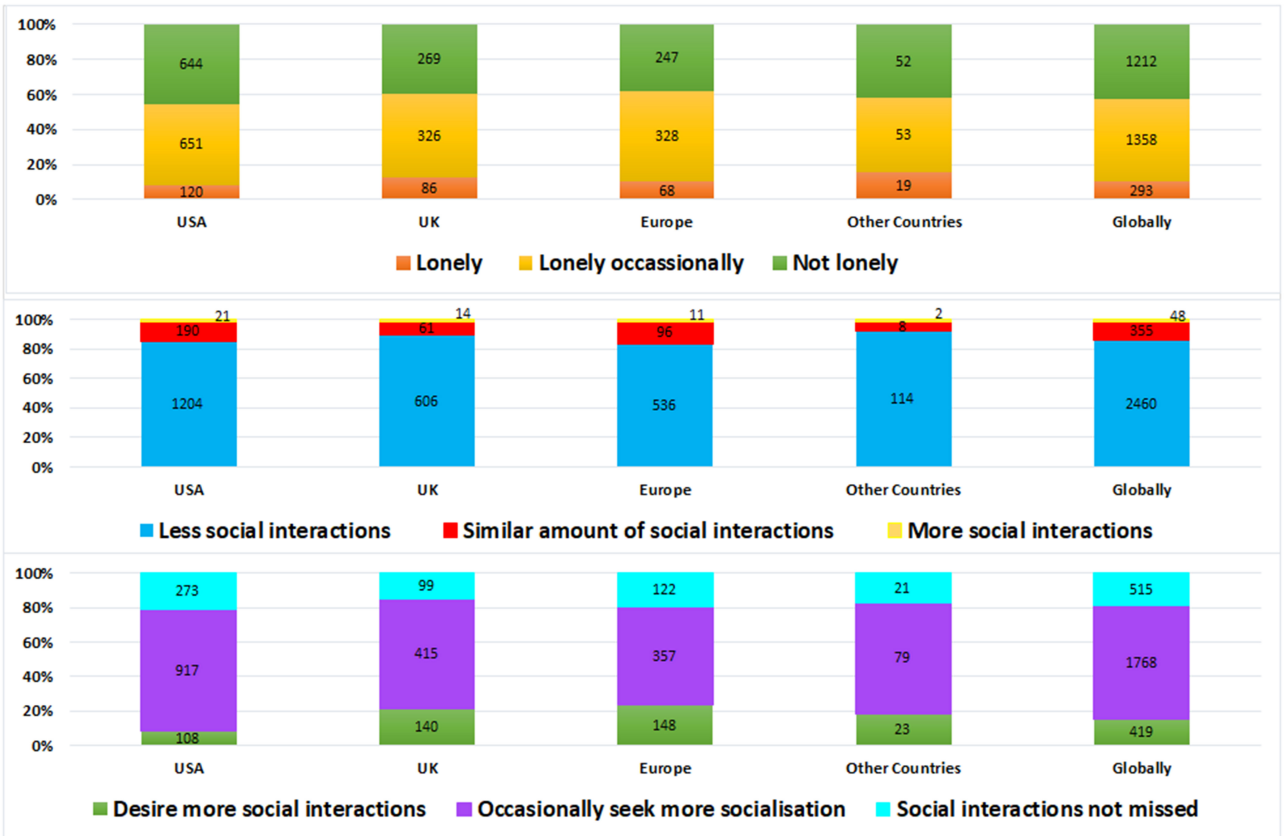

FIGURE 5 | The frequency of social interactions, desire for more interactions and loneliness experienced during the pandemic across different locations.

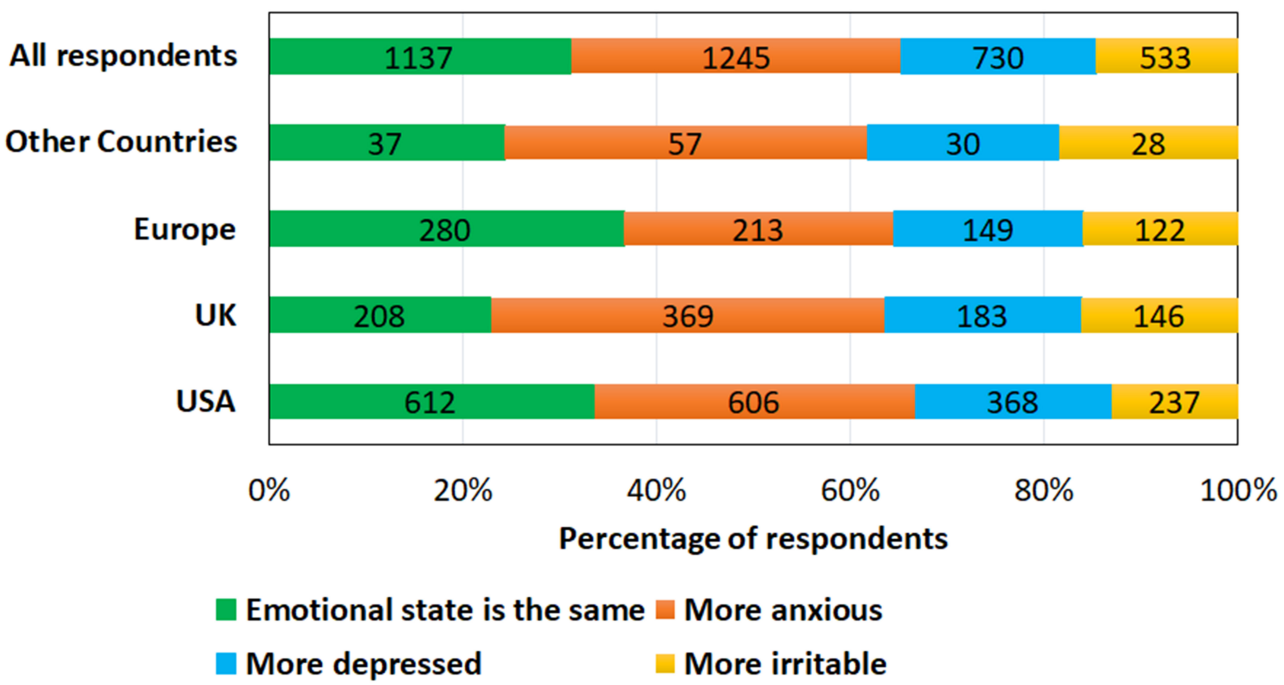

FIGURE 6 | Comparison of emotional state during the COVID-19 pandemic across different locations.

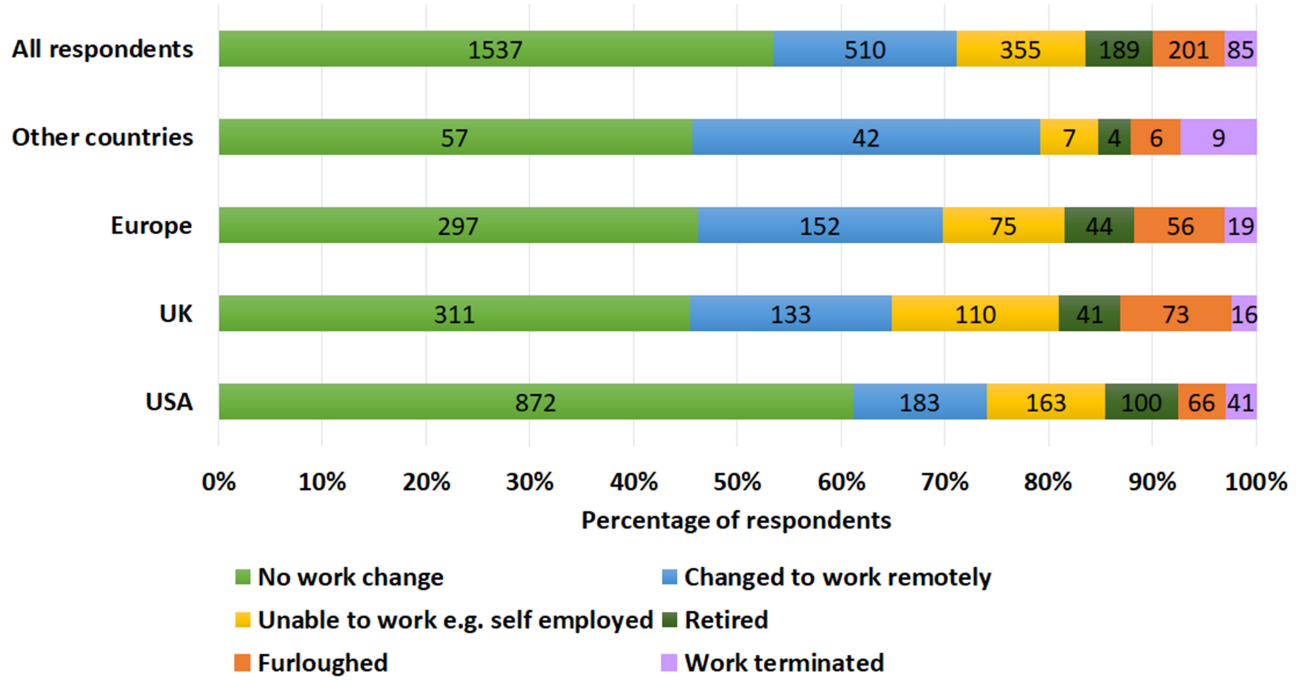

FIGURE 7 | Comparison of changes to work patterns during the COVID-19 pandemic across different locations.

years, USA). Working from home was associated with increased stress which aggravated tinnitus, for example, "Struggling to work at home with nobody to ask when I'm stuck makes me panic and then the tinnitus a lot worse" (Female, 46 years, UK). Working from home together with home-schooling and an increase in household chores, were further factors affecting tinnitus, explained by "It is very busy because my child is at home, and in addition to work, housekeeping, teacher and entertainment. There is little time to relax" (Female, 36 years, The Netherlands).

Participants were asked to explain their responses regarding their current tinnitus experiences in free text. Thematic analysis for these responses identified diverse and overlapping factors that contributed to these experiences as are summarized in **Table 1**. Overall tinnitus was more bothersome during the pandemic than before the pandemic.

DISCUSSION

This survey, completed by 3,103 individuals from 48 countries, provides insights regarding tinnitus experiences during the COVID-19 pandemic. Experiencing COVID-19 symptoms did not impact upon tinnitus for 54%, improved tinnitus experiences for 6% and exacerbated it for 40% of the respondents. Of the 26 that were tested positive for COVID-19, 58% reported that their tinnitus was exacerbated by the virus. For some, focusing on surviving the virus helped reframe problems and push tinnitus into the background of their thoughts. Having COVID-19 was antidotally reported to initiate tinnitus ($n = 7$) and hearing loss ($n = 4$). Tracking and managing hearing-related changes due to having the COVID-19 will be important as clinical services resume (23). A rapid systematic review indicated only a few reports of audio-vestibular symptoms in confirmed COVID-19 cases but emphasized the need for understanding the longer-term risks (24). A change in hearing status has been reported by 1 in 10 COVID-19 adults after discharge from one hospital in Manchester in the UK (25) highlighting the need for further monitoring. This is particularly important for those who may have been treated with ototoxic medications (26).

Overall tinnitus was rated as more bothersome during the pandemic than before. An increase in the number of individuals complaining of heightened tinnitus severity since access to their clinics was reinstated post lockdown has also been reported in Italy (27). Those who were female and younger were more likely to find tinnitus more bothersome. From the explanations provided, it appears this may be partly attributed to greater lifestyle changes in these groups during the pandemic, such as changes in employment, increased childcare, and household responsibilities. A study involving tinnitus patients at a clinic in Germany found that COVID-19 brought increased grief, frustration, stress, and nervousness, although there was only a small increase in tinnitus distress compared with 2 years prior to lockdown. Tinnitus distress was worst for those with high neuroticism, indicating that both external and internal factors contribute to tinnitus distress (17).

Lifestyle changes imposed by the pandemic appeared to be one of the factors making tinnitus worse, as reported by a third of the respondents and conversely the greatest factor in improving tinnitus for a small minority. The same experience such as working from home for some worsened tinnitus, and

TABLE 1 | Factors related to tinnitus being stable, better, or exacerbated during the pandemic that were identified through thematic qualitative analysis of free text responses.

	Health-related factors	Social distancing restrictions	Lifestyle changes	Emotional state
Tinnitus exacerbated (31%)	• Health concerns • Family health concerns • Concerned about contracting the virus • Effects of having the virus • Future healthcare • Difficulty accessing healthcare • Reduced ability to access hearing healthcare • Taking medication/ vitamins • Fluctuations in the tinnitus sounds heard	• Rigorously following social distancing advice • Fewer engagements • Fewer social interactions • Housebound • Loneliness • Listening difficulties	• Less exercise • Noisier at home • Too quiet • Increased alcohol intake • Increased caffeine intake • Diet less healthy than prior to the pandemic • Higher workload • Busier • Decreased activity levels • Less exercise compared with before the pandemic • Poor sleep	• Frustrations • Relationship problems • Stress, worrying and anxiety • More depressed • More irritable • Financial worries • More jobs (work, schooling, household) • Lack of relaxation time • Work terminated or furloughed
Tinnitus better (2%)	• Reframing problems • Fighting the virus	• Reduced listening frustration	• Healthier than prior to the pandemic • Increased relaxation • Sleeping better • More peaceful lifestyle • Quieter • More time in nature • More exercise • Better diet	• Working from home
Tinnitus stable (67%)	• No additional health concerns • Tinnitus not severe • Not had virus • Family healthy	• Acceptance of new routine • Not self-isolating • Continuing social interactions	• Access to outdoor spaces • Diet unchanged	• No additional mental health concerns • No financial changes • Similar work patterns

for others, improved tinnitus, highlighting the heterogeneous nature of tinnitus experiences. Being away from all the noise associated with crowded places lowered tinnitus levels for some respondents, whereas due to it being quieter working from home, many found their tinnitus was more bothersome. Tinnitus is often reported to be more noticeable in quiet and has led to tinnitus treatments often using some form of background noise to help prevent the starkness of the tinnitus percept (28). Others reported that working from home exposed them to more noise than usual from neighbors, electrical tools, and children, which aggravated tinnitus. Those with tinnitus often report trying to avoid noisy situations (29).

Diverting attention by focusing on other activities and being physically active is a common strategy used to cope with tinnitus (29). Thus, not being distracted by these activities due to the lockdown restrictions was found to exacerbate tinnitus. Being too busy, however, resulted in more stress and less sleep, which appeared to aggravate tinnitus. The majority of respondents reported lower sleep quality, more trouble sleeping, waking up more during the night, and being less rested, which was significantly associated with tinnitus being worse, whereas 6% reported better sleep due to having more time to relax. Tinnitus related distress has also previously been related to insomnia (30). Some respondents reported having a more relaxed and peaceful lifestyle that made their tinnitus less noticeable. This highlights the importance of relaxation techniques and mindfulness training during the provision of tinnitus therapies (31). Similar to the present survey, it has previously been reported that tinnitus is worse in both quiet and noisy situations when stressed, and due to lack of sleep (32). Tinnitus was significantly worse for those with less healthy diets. Drinking more coffee, alcohol, and taking more supplements to build immunity were reported to negatively impact tinnitus for some respondents. Although no clear relationship exists, previous studies have also noted that caffeine may exacerbate tinnitus (32). Diversity in the factors found to improve or worsen tinnitus across respondents, amplify the difficulty in attempting to subtype tinnitus (33).

Increased levels of anxiety, depression, and irritability were reported. These emotional factors are associated with exacerbated tinnitus annoyance (15). Different worries and frustrations exacerbated these negative feelings including more relationship problems due to being confined, worrying about food supply, and concerns regarding contracting the virus. Financial worries also made tinnitus significantly worse, due to being made redundant, being furloughed, and reduced value of investments. These findings mirror a recent systematic review regarding the impact of COVD-19 on mental health that indicated lower psychological well-being and higher levels of anxiety and depression in the general public compared to before COVID-19 (5). This emphasizes the support needed for both those experiencing tinnitus and the general population to reduce anxiety and depression and improve well-being during the pandemic. This support is likely to be needed after the pandemic as well, due to the likely continued financial difficulties and impact on many aspects of society. A common theme was that concerns regarding contracting the virus made tinnitus worse. These

concerns could trigger additional fear-avoidance behaviors (34), where respondents rigorously followed social distancing advice or self-isolated, to avoid risks of contracting COVID-19, despite a desire for more social interactions. This may have, however, contributed to fewer social interactions and feeling lonelier, which was associated with tinnitus being significantly worse. This aligns with previous literature indicating that social distancing (35) and self-isolation results in negative psychological effects (36) and poorer mental health outcomes (37). When social interactions were possible, having to stand further away and follow the conversation while people were wearing a mask was reported to make conversing more difficult.

Around half of the respondents reported occupational changes due to the pandemic. Having to wear headphones and participate in video conferencing calls was often mentioned as negatively impacting tinnitus. Neck strain from working on computers all day had an additional negative impact. For others, juggling homeschooling, work, and more household tasks resulted in more stress and little relaxation time. For some, working remotely meant not commuting and having more time to relax, which positively impacted tinnitus.

This survey has highlighted how emotional state, health concerns, social contact, and lifestyle contribute to tinnitus distress. The relationship between tinnitus and these internal and external factors is complex. Interestingly, respondents living in the UK were most likely to report how the pandemic negatively affected their tinnitus, which may be linked to the rapid spread of the virus and the high death rate in the UK during the time of the survey. The knowledge of these factors is of value during the clinical management of those with tinnitus and special attention should be placed to fully explore health, social, occupational, and emotional factors that may contribute to tinnitus severity.

Limitations and Future Studies

Although this study attempted to capture a range of views regarding the impact of the COVID-19 pandemic on tinnitus experiences world wide, it is not fully representative. Most responses were from the USA and Europe. Furthermore, not all ethnic minority groups are represented as white ethnic representation dominate this survey, although similar ethnic dominance has previously been found from other surveys [e.g., (38)]. A further drawback is that it was not possible to use a psychometrically validated questionnaire for this survey due to the time sensitive nature of the study. The timescale did also not allow scope to perform both forward and backward translation and fully pilot the translated questionnaires. The study design did not allow for longitudinal comparison as there were no comparative scores (e.g., of tinnitus severity) before the pandemic. A further limitation of this study is that there was no clinical evaluation of the respondents. Findings are based only on self-reported survey data without any clinical data. Additionally, standardized self-reported questionnaires were not included to measure levels of anxiety, depression, hearing loss, and hyperacusis. Moreover, limited data were collected regarding dimensions of the tinnitus, such as its location, type, and number of sounds heard. The cross-sectional design limits causal relationships, although this was countered by the use of direct quotes to formulated probable paths for causal relationships. This study also did not capture the population who may have developed tinnitus due to COVID-19 or during the pandemic, as those with chronic tinnitus were targeted. Furthermore, it is not presently known if the tinnitus would become chronic if it developed after having COVID-19, and therefore should be monitored. However, as there were a few accounts of tinnitus and hearing loss initiation after contracting COVID-19, exploring how frequently this happened in those with COVID-19 symptoms should be investigated. Identifying how people with tinnitus are coping during the COVID-19 pandemic and what support is required is important and is currently being investigated. Future studies should focus on whether these effects change over time as the true impact of COVID-19 experienced or as lockdown restrictions are lifted.

Clinical Implications

As the COVID-19 pandemic may remain for the foreseeable future, the health, social, and emotional implications are likely to continue for some time. Ways of supporting those experiencing the most profound effects, such as individuals who are socially isolated, should be prioritized by patient organizations and support services. Although in-person support may be restricted for the foreseeable future for non-essential healthcare concerns, online interventions such as Internet-based cognitive behavioral therapy (39) or other remote tinnitus interventions could be valuable for those in need. Investment should be increased for services that provide tinnitus support. A careful case history is required as survivors of COVID-19 treated with certain ototoxic medications, such as chloroquine or hydroxychloroquine, may be at an increased risk for developing hearing loss, tinnitus, or balance problems (40). Those who have had COVID-19 should be monitored for changes in hearing-related problems, such as initiation or worsening of tinnitus. There is most likely also a cohort of patients who experienced an onset of tinnitus during this period and who will need access to clinical care for their tinnitus. Those who are most socially isolated, lonely, as well as those with poor sleep, are at most risk for tinnitus severity increasing. It highlights the heterogeneity of tinnitus with many people responding well, and others struggling during the pandemic and in need of additional support.

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 Anglia Ruskin University, Cambridge, UK, reference number FSE/FREP/19/927. The patients/participants provided online informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

This study was conceptualized by EB and designed by EB, VM, DB, PA, DS, and JO. Data collection was by EB, VM, GA, VK, LJ, ML, JO, and DS. Data analysis and interpretation and drafting

the article was done by EB. All authors critically revised the article and approved the version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.592878/full#supplementary-material

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Bilirubin Levels as Potential Indicators of Disease Severity in Coronavirus Disease Patients: A Retrospective Cohort Study

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Objectives: The coronavirus disease (COVID-19) pandemic has caused a large number of deaths. Some patients with severe or critical COVID-19 have been observed to have elevated bilirubin levels. Studies on the association of bilirubin level and mortality in patients with COVID-19 are limited. This study aimed to examine the role of bilirubin levels in COVID-19 severity and mortality.

Methods: A retrospective cohort study was conducted in patients hospitalized with COVID-19 in Leishenshan Hospital in Wuhan, China. Cox regression analyses and logistic regression analyses were conducted to investigate the risks for mortality and disease severity, respectively. Kaplan–Meier analyses with log-rank tests were performed to assess the association between bilirubin level and survival.

Results: In total, 1,788 patients with COVID-19 were included in the analysis. 5.8% (4/69) of patients in the elevated serum total bilirubin (STB) group died, compared to 0.6% (11/1,719) of patients in the non-elevated STB group. The median alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the elevated STB group were 29 U/L [interquartile range (IQR): 16–45 U/L] and 22 U/L (IQR: 13–37 U/L), respectively, which were significantly higher than the median ALT (median: 23, IQR: 15–37) and AST (median: 20, IQR: 16–26) activities in the non-elevated STB group (both $p < 0.05$). Patients with an elevated STB level showed increased mortality [hazard ratio (HR): 9.45, $P = 0.002$], elevated conjugated bilirubin (CB) levels (HR: 4.38, $P = 0.03$), and an elevated ratio of CB to unconjugated bilirubin (UCB, CB/UCB) (HR: 2.49, $P = 0.01$). CB/UCB was positively correlated with disease severity (odds ratio: 2.21, $P = 0.01$).

Conclusions: COVID-19 patients with elevated STB and CB levels had a higher mortality, and CB/UCB was predictive of disease severity and mortality. Thus, it is necessary to pay special attention to COVID-19 patients with elevated bilirubin levels in clinical management.

Keywords: COVID-19, bilirubin, length of hospitalization, mortality, disease severity

INTRODUCTION

In December 2019, coronavirus disease (COVID-19), caused by severe acute respiratory syndrome (SARS) coronavirus 2 (SARS-CoV-2), emerged in Wuhan, China (1, 2). Soon thereafter, it became a pandemic. More than 200 countries and territories have reported confirmed cases. However, there are currently no specific drugs for treatment. As of October 10, 2020, over 36 million people had been infected with SARS-CoV-2 and over one million people had died of COVID-19 worldwide (3).

Lungs are recognized as the primary target organs for COVID-19 (4). However, COVID- 19 patients frequently show evidence of damage to other organs. Furthermore, those with pre-existing liver disease or newly occurred evidence of liver injury have an increased likelihood of a poor prognosis (5, 6). A number of studies have suggested that liver disease is one of the most common comorbidities of COVID-19 patients and a number of patients infected with SARS-CoV-2 can develop different degrees of liver injury (7, 8). To monitor liver damage, bilirubin levels are a universally accepted marker.

Most studies on COVID-19 are descriptive and focus on its epidemiological and clinical characteristics (9–11). Studies on the risk factors associated with mortality in patients with COVID-19 are controversial. Elevation of bilirubin levels has been observed in some COVID-19 patients with severe or critical disease. Previous studies have proposed a link between bilirubin levels and disease severity, but they have had relatively small sample sizes and have not explored the relationship between bilirubin levels and the survival of patients with COVID-19 (8, 12, 13). Therefore, we aimed to determine the association between bilirubin levels and disease severity and mortality in patients with COVID-19 based on a large sample.

PATIENTS AND METHODS

Setting

The Leishenshan Hospital, Wuhan, China, was rapidly built as a designated hospital for patients with COVID-19. The hospital contains 1600 beds. On February 8, 2020, the Leishenshan Hospital treated its first patients. By April 15, 2020, it had served its purpose and was officially closed. During the time that it was in operation, a total of 1,880 patients with confirmed COVID-19 were admitted.

Study Design and Participants

This retrospective study was approved by the Research Ethics Commission of Zhongnan Hospital of Wuhan University (No. 2020074), and considering the rapid spread of COVID-19, the requirement for patient consent was waived by the ethics commission. Patients without data on serum total bilirubin (STB) levels were excluded. We included 1,788 patients: 1,785 patients admitted from February 8 to March 18 and 3 patients with a missing admission date. The follow-up period lasted until April 14, 2020.

Data Collection

We obtained patient information including that of demographic and clinical characteristics, laboratory findings, computed tomography (CT) images, clinical management, and outcomes from the electronic medical record system. All information was typed in a pre-designed data collection form. All data were independently examined by three investigators to ensure data accuracy.

Definitions

Patients enrolled were divided into elevated STB group and non-elevated STB group according to their STB levels on admission. The reference range for the physiological STB concentration was 5–21 μmol/L. The cut-point was the upper limit of reference range. We regarded the survival (alive or dead) and disease severity of patients with COVID-19 as the primary outcome variables. Disease severity was classified as Mild, Common, Severe, or Critical based on the seventh version of the guidelines for the diagnosis and treatment of COVID-19 published by the National Health Commission of China (14). Mild disease was characterized by mild clinical symptoms with no findings of pneumonia on imaging. Common disease was characterized by fever, respiratory symptoms, and other symptoms, with signs of pneumonia revealed on imaging. Severe disease was characterized by the presence of any one of the following factors: (1) shortness of breath or a respiratory rate of ≥30 beat per minute (BPM); (2) oxygen saturation of ≤93% at rest; (3) arterial partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2) ≤300 mmHg; and (4) a lesion that has progressed by more than 50% within 24–48 h as seen on pulmonary imaging. Critical disease was characterized by the presence of any one of the following factors: (1) respiratory failure requiring mechanical ventilation; (2) shock state; and (3) organ failure requiring intensive care. In one patient, the highest level of severity at hospitalization was mild disease; thus, we classified the patient as having both mild and common disease. CT findings were also a vital outcome in our study. Two experienced radiologists assessed all chest CT images and reached a consistent rating after discussion. In the early stage, chest CT images mainly showed ground-glass opacities (GGO) and reticulation or cord changes. Consolidation was a manifestation observed in the progression stage, and pleural effusion was rarely observed (15). Score 1 was calculated according to whether the CT images showed GGO, reticulation or cord changes, consolidation, and pleural effusion. One point was assigned for each presentation, and the sum of these points was considered Score 1. The area of lung lobe involvement was reflected by Score 2: no involvement, 0; <25%, 1; 26–50%, 2; 51–75%, 3; and 76–100%, 4. The total score was the sum of scores 1 and 2. The days of CT scores was calculated as the duration from onset to CT scans.

Abbreviations: COVID-19, The coronavirus disease; ROC, Receiver operating characteristic; STB, Serum total bilirubin; HR, Hazard ratio; CB, Conjugated bilirubin; UCB, Unconjugated bilirubin; CB/UCB, Conjugated bilirubin to unconjugated bilirubin; SARS, Severe acute respiratory syndrome; SARS–CoV−2, Severe acute respiratory syndrome coronavirus 2; CT, Computed tomography; GGO, Ground–glass opacities; IQR, Interquartile range; ECMO, Extracorporeal membrane oxygenation; INR, International normalized ratio; CI, Confidence interval; OR, Odds ratio; ACE2, Angiotensin converting enzyme 2; MERS, Middle East respiratory syndrome; ICU, Intensive care unit.

TABLE 1 | Demographic and clinical features of 1,788 patients with COVID-19.

Covariate		Total (n = 1,788)	Non-elevated STB group(n = 1,719)	Elevated STB group (n = 69)	P-value
Age, year		59 (49–68)	59 (49–68)	56 (46–67)	0.38
Sex					0.001
Female	936 (52.3)	914 (52.3)	22 (31.9)		
Male	852 (47.7)	805 (47.7)	47 (68.1)		
Disease severity on admission					0.07
Mild	670 (37.5)	653 (38)	17 (24.6)		
Common	808 (45.2)	768 (44.7)	40 (58)		
Sever	285 (15.9)	275 (16)	10 (14.5)		
Critical	25 (14)	23 (1.3)	2 (2.9)		
The highest level of severity at hospitalization					0.04
Mild and Common	931 (52.2)	900 (52.5)	31 (45.6)		
Sever	804 (45.1)	772 (45)	32 (47.1)		
Critical	48 (2.7)	43 (2.5)	5 (7.4)		
The highest level of oxygen support					<0.001
Low flow oxygen therapy	257 (82.9)	245 (83.3)	12 (75)		
High flow oxygen therapy	47 (15.2)	45 (15.3)	2 (12.5)		
Tracheal intubation	5 (1.6)	4 (1.4)	1 (6.3)		
ECMO	1 (0.3)	0	1 (6.3)		
Symptoms when admitted to the hospital					
Fever or Myalgia	621 (79)	597 (79.7)	24 (64.9)	0.03	
Respiratory system symptoms	635 (80.8)	605 (80.8)	30 (81.1)	0.96	
Digestive system symptoms	82 (10.4)	79 (10.5)	3 (8.1)	0.64	
Nervous system symptoms	27 (3.4)	26 (3.5)	1 (2.7)	0.80	
Other system symptoms	26 (3.3)	24 (3.2)	2 (5.4)	0.46	
Antiviral therapy		869 (54.2)	840 (48.9)	29 (42.0)	0.16
Antibiotic therapy		521 (29.1)	500 (29.1)	21 (30.4)	0.12
The appliance of vitamin C		248 (13.9)	236 (13.7)	12 (17.4)	
Traditional Chinese medicine therapy		1,533 (85.7)	1,478 (82.5)	55 (79.7)	
Anticoagulation treatment		131 (7.3)	120 (7.0)	11 (15.9)	0.01
Use of corticosteroid		106 (5.9)	101 (5.9)	5 (7.2)	0.60
Use of antimalarial		139 (7.8)	135 (7.9)	4 (5.8)	0.05
Length of hospitalization, day		18 (13–24)	18 (13–24)	19 (14–24)	0.78
CT scores					1.00
0–4	78 (39.8)	75 (40.1)	3 (33.3)		
5–7	118 (60.2)	112 (59.9)	6 (66.7)		

Statistical Analysis

We used median (interquartile range) and frequency (percentage) to present continuous and categorical variables, respectively. To discern the differences in baseline characteristics between the groups with non-elevated STB levels and elevated STB levels, continues variables were analyzed by the Mann-Whitney test and Chi-square test, and Fisher's exact test was adopted for categorical variables accordingly. Univariate and multivariate Cox regression analyses were conducted to investigate the risk of survival and disease severity in patients with COVID-19 according to STB levels. Univariate and multivariate logistic regression analyses were performed to identify the relationship between disease severity and the ratio of conjugated bilirubin to unconjugated bilirubin (CB/UCB). The survival of patients with COVID-19 according to STB status was assessed using Kaplan–Meier analyses and log-rank tests. A receiver operating characteristic (ROC) curve was used to determine the relationship between CB/UCB and disease severity or length of hospitalization. Curve fitting analyses were performed to detect the relationship between days from onset and CT scores. All statistical analyses were performed using SPSS, version 22.0 (IBM Corp., Armonk, NY, United States). Statistical significance was defined using a two-sided $P < 0.05$.

RESULTS

Participant Baseline Characteristics

This study cohort included 1,788 patients with COVID-19 who were admitted to the Leishenshan Hospital. Among the 1,788

TABLE 2 | Laboratory results of 1,788 patients with COVID-19.

Covariate	Total (n = 1,788)	Non-elevated STB group (n = 1,719)	Elevated STB group (n = 69)	*P*-value	Reference range
Interleukin-6, pg/mL	1.5 (1.5–4.0)	1.5 (1.5–3.75)	5.7 (1.5–20.9)	<0.001	0–7.0
Procalcitonin, ng/mL	0.04 (0.03–0.05)	0.04 (0.03–0.05)	0.05 (0.03–0.10)	<0.001	<0.05
Alanine aminotransferase, U/L	23 (15–37)	23 (15–37)	29 (16–45)	0.04	9–50
Aspartate aminotransferase, U/L	20 (16–27)	20 (16–26)	22 (17–37)	0.02	15–40
Albumin, g/L	37.7 (35.0–40.0)	37.7 (35.0–39.9)	38.5 (35.1–40.6)	0.46	40.0–55.0
Creatinine, μmol/L	64.3 (54.5–74.2)	64.1 (54.3–76.1)	68.2 (60.9–79.4)	0.01	64.0–104.0
Ureanitrogen, mmol/L	4.8 (3.9–5.8)	4.8 (3.9–5.8)	5.1 (4.1–6.4)	0.11	2.8–7.6
INR	1.0 (0.9–1.0)	1.0 (0.9–1.0)	1.0 (1.0–1.1)	<0.001	0.8–1.3
Prothrombin time, s	11.3 (10.9–11.8)	11.3 (10.9–11.7)	11.7 (11.1–12.4)	<0.001	9.4–12.5
Thrombin time, s	17.6 (17.0–18.4)	17.7 (17.0–18.4)	17.4 (16.9–18.6)	0.44	10.3–16.6
Activated partial thromboplastin time, s	27.2 (24.6–30.4)	27.2 (24.6–30.4)	27.3 (24.1–30.1)	0.82	25.1–36.5
Fibrinogen, g/L	3.0 (2.5–3.7)	3.0 (2.5–3.7)	2.7 (2.3–3.8)	0.07	2.38–4.98
D-dimer, ng/mL	0.38 (0.21–0.90)	0.38 (0.21–0.89)	0.65 (0.20–1.47)	0.05	0–0.50
Leucocyte count, $\times 10^9$/L	5.7 (4.7–6.9)	5.7 (4.7–6.9)	5.7 (4.9–7.6)	0.35	3.5–9.5
Neutrophil count, $\times 10^9$/L	3.3 (2.5–4.3)	3.3 (2.5–4.2)	3.4 (2.6–5.2)	0.24	1.8–6.3
Lymphocyte count, $\times 10^9$/L	1.6 (1.2–2.0)	1.6 (1.3–2.0)	1.6 (1.1–2.0)	0.51	1.1–3.2
Erythrocyte count, $\times 10^9$/L	4.1 (3.8–4.5)	4.1 (3.8–4.5)	4.2 (3.6–5.0)	0.20	4.3–5.8
Hemoglobin, g/L	126.0 (115.0–137.0)	126.0 (115.0–137.0)	128.0 (111.0–144.0)	0.59	130.0–175.0
Platelet count, $\times 10^9$/L	229.0 (187.0–277.2)	230.0 (189.0–278.0)	195.0 (148.5–265.5)	0.001	125.0–350.0
IgM of SARS-CoV-2	219 (35.7)	209 (35.6)	10 (37.0)	0.88	
IgG of SARS-CoV-2	531 (91.6)	508 (91.5)	23 (92.0)	1.00	

TABLE 3 | Comorbidities in 1,788 patients with COVID-19.

Comorbidity	Total (n = 1,788)	Non-elevated STB group (n = 1,719)	Elevated STB group (n = 69)	*P*-value	Non-elevated CB group (n = 1,686)	Elevated CB group (n = 102)	*P*-value
Cardiovascular disease	356 (19.9)	340 (19.8)	16 (23.2)	0.87	326 (19.3)	30 (29.4)	0.01
Pulmonary disease	89 (5.0)	86 (5.0)	3 (4.3)	0.74	82 (4.9)	7 (6.9)	0.37
Nervous system disease	56 (3.1)	54 (3.1)	2 (2.9)	0.76	52 (3.1)	4 (3.9)	0.64
Endocrine disease	137 (7.7)	132 (7.7)	5 (7.2)	0.66	125 (7.4)	12 (11.8)	0.11
Malignancy	64 (3.6)	61 (3.5)	3 (4.3)	0.89	59 (3.5)	5 (4.9)	0.46
Digestive system disease	45 (2.5)	40 (2.3)	5 (7.2)	0.37	39 (2.3)	6 (5.9)	0.03

patients, 69 were assigned to the elevated STB group [median age: 56 years, interquartile range (IQR): 46–67 years; 68.1% male] and the remaining 1,719 were assigned to the non-elevated STB group [median age: 59 (IQR: 49–68) years; 47.7% male]. The demographic and clinical features are shown in **Table 1**. The elevated STB group included a higher proportion of patients who required tracheal intubation and extracorporeal membrane oxygenation (ECMO) than the STB normal or decreased group ($P < 0.001$). Moreover, the elevated STB group also had a higher rate of critical disease ($P = 0.04$). Laboratory testing results are shown in **Table 2**. Levels of interleukin-6, procalcitonin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, and D-dimer and international normalized ratio (INR), prothrombin time, and platelet count all differed significantly according to STB level. The median ALT and AST activities in the elevated STB group were 29 U/L (IQR: 16–45) and 22 (IQR: 13–37), respectively, which were significantly higher than the median activities of ALT (median: 23, IQR: 15–37) and AST (median: 20, IQR: 16–26) in the non-elevated STB group (both $p < 0.05$). The underlying comorbidities are presented in **Table 3**. The number of patients with cardiovascular diseases, malignancies, and digestive system diseases was higher in the elevated STB group than in the non-elevated STB group. However, these differences were not statistically significant. Patients with elevated CB levels had significantly high rates of having concurrent cardiovascular diseases ($p = 0.01$) and digestive system diseases ($p = 0.03$).

Bilirubin Levels and Mortality

The association between bilirubin level and mortality is shown in **Table 4**. The elevated STB group had higher CB and UCB levels than the non-elevated STB group (both $P < 0.001$). Mortality was considerably higher in the elevated STB group (5.8%) than in the non-elevated STB group (0.6%, $P < 0.001$). Patients with elevated

CB levels exhibited a similar result, while no deaths occurred in the elevated UCB group. The dynamic changes after the onset of increased bilirubin levels have been depicted using curve fitting analysis in **Supplementary Figure 1**.

Survival Analyses

The Kaplan–Meier analyses revealed that the elevated STB group had significantly higher mortality than the non-elevated STB group ($P < 0.001$, **Figure 1**). In the univariate Cox regression analyses, STB levels, CB levels, and CB/UCB were found to be related with COVID-19 survival. After adjusting for age, history of cardiovascular disease, leucocyte count, platelet count, lymphocyte count, and creatinine levels, multivariate Cox regression analysis revealed that patients with elevated STB levels had a significantly higher risk for COVID-19-related mortality [hazard ratio (HR): 9.45, 95% confidence interval (CI): 2.21–40.47, $P = 0.002$]. Elevated CB (HR: 4.38, 95% CI: 1.78–16.29, $P = 0.03$) and CB/UCB (HR: 2.49, 95% CI: 1.32–4.71, $P = 0.01$) were also regarded as potential factors influencing survival. Univariate and multivariate analyses showed that survival did not differ between patients with elevated and non-elevated UCB levels (both $P > 0.05$). The results are shown in **Table 5**.

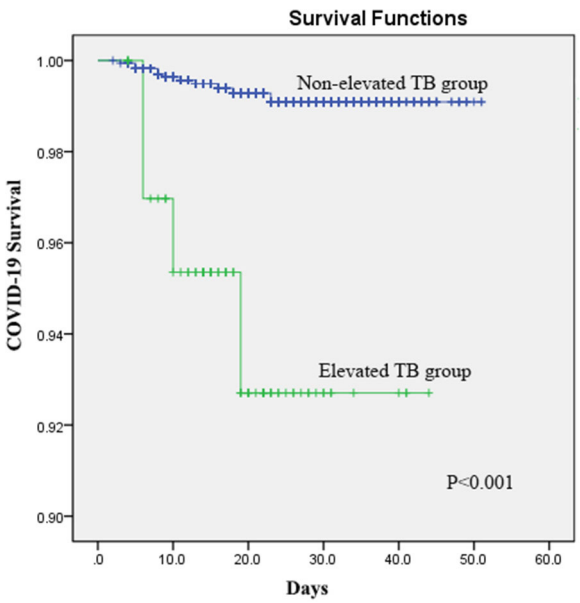

FIGURE 1 | Kaplan–Meier curves for survival in patients with COVID-19 according to elevated serum total bilirubin (STB) levels.

Association Between CB/UCB and Disease Severity

In the univariate logistic regression analysis, the risk for critical disease increased with CB/UCB (**Table 6**). We found a similar trend in multivariate analysis [odds ratio (OR): 2.21, 95% CI: 1.20–4.07, $P = 0.01$, **Table 6**). The area under the ROC curve (AUC) of CB/UCB for critical disease was 0.71 ($P < 0.001$, **Figure 2A**). Therefore, there was a relation between CB/UCB and critical disease. According to ROC curve analysis, CB/UCB showed a correlation with hospitalization length >30 days (AUC: 0.60, $P < 0.001$, **Figure 2B**).

Evaluation of CT Images

The results of curve fitting analyses are given in **Figure 3**. The peak of Score 1 was 2.5 on Day 20 for all patients, 2.5 on Day 19 for patients with non-elevated STB levels, and 2.6 on Day 23 for patients with elevated STB levels. Score 2 of all patients reached the peak on Day 14 (Score = 2.4), and the peak for patients with non-elevated STB levels was 2.4 on Day 14. However, Score 2 in patients with elevated STB levels showed a significant delay with regard to the inflection point (2.5 on Day 27). For all patients (4.9) and patients with non-elevated STB levels (4.9), the total score reached the peak on Day 18. Patients with elevated STB levels reached a delayed inflection point (4.9) on Day 27.

TABLE 4 | Bilirubin levels and mortality in 1,788 patients with COVID-19.

Covariate	Total (n = 1,788)	Non-elevated STB group (n = 1,719)	Elevated STB group (n = 69)	P-value	Mortality	Reference range
DB/IB	0.53 (0.44–0.71)	0.53 (0.44–0.70)	0.55 (0.43–0.96)	0.29		
Serum total bilirubin, μmol/L	9.1 (7.0–12.0)	9.0 (6.9–11.6)	26.0 (22.8–31.1)	<0.001		5.0–21.0
5.0–21.0	1,595 (89.2)	1,595 (92.8)	0	<0.001	10/1,585	
<5.0	124 (6.9)	124 (7.2)	0		1/124	
>21.0	69 (3.9)	0	69 (100.0)		4/69	
Conjugated bilirubin, μmol/L	3.1 (2.4–4.3)	3.1 (2.4–4.1)	9.2 (7.5–14.0)	<0.001		0–7.0
0–7.0	1,686 (94.3)	1,675 (97.4)	11 (15.9)	<0.001	10/1,686	
>7.0	102 (5.7)	44 (2.6)	58 (84.1)		5/102	
Unconjugated bilirubin, μmol/L	5.7 (4.3–7.8)	5.6 (4.2–7.5)	16.1 (13.6–20.3)	<0.001		1.5–18.0
1.5–18.0	1,590 (98.2)	1,547 (99.4)	43 (68.3)	<0.001	13/1,590	
<1.5	10 (0.6)	9 (0.6)	1 (1.6)		1/10	
>18.0	19 (1.2)	0	19 (30.2)		0	
Mortality	15 (0.8)	11 (0.6)	4 (5.8)	<0.001		

TABLE 5 | Univariate and multivariate Cox regression analyses for mortality in patients with COVID-19.

	Group	HR	95% CI		*P*-value
Cox regression analysis					
Univariate analysis	Non-elevated STB	ref			
	Elevated STB	9.02	2.87	28.32	<0.001^
Multivariate analysis*	Non-elevated STB	ref			
	Elevated STB	9.45	2.21	40.47	0.002^
Univariate analysis	Normal CB	ref			
	Elevated CB	8.37	2.86	24.5	<0.001^
Multivariate analysis*	Normal CB	ref			
	Elevated CB	4.38	1.78	16.29	0.03^
Univariate analysis	Non-elevated UCB	ref			
	Elevated UCB	0.05	<0.001	1.80E+08	0.79
Multivariate analysis*	Non-Elevated UCB	ref			
	Elevated UCB	<0.001	<0.001	/	0.99
Univariate analysis	CB/UCB	4.42	2.64	7.42	<0.001^
Multivariate analysis*	CB/UCB	2.49	1.32	4.71	0.01^

**Adjusted for age, history of cardiovascular disease, leucocyte count, platelet count, lymphocyte count, and creatinine level.*
/Limit value.
^P < 0.05.

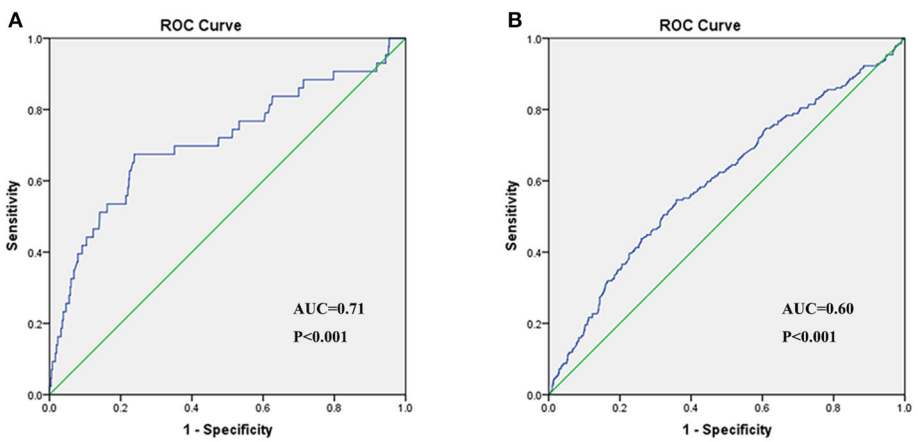

FIGURE 2 | Receiver operating characteristic (ROC) curve of CB/UCB for disease severity **(A)** and hospitalization length **(B)** in patients with COVID-19.

DISCUSSION

Based on our clinical experience during the outbreak of COVID-19, a small group of patients experienced increased bilirubin levels, regardless of the presence of pre-existing liver diseases. Patients with elevated bilirubin tended to have worse prognoses and more severe disease. Therefore, we conducted this study to determine whether bilirubin levels in COVID-19 patients were related to disease progression and prognosis. We found that 5.8% of patients in the elevated STB group died, compared to 0.6% of patients in the normal STB group. In the Cox regression analyses, those with elevated STB levels had a higher risk of mortality (unadjusted and adjusted). Results of the analyses of CB and CB/UCB showed a similar trend. However, UCB levels were not significantly associated with the survival of COVID-19 patients in either the univariate or multivariate Cox regression analyses. Univariate and multivariate logistic regression analyses demonstrated that the risk of critical infection increased with the elevation of CB/UCB. We used ROC curves to confirm the existence of a relationship between CB/UCB and disease severity. The ROC curves also showed a relation between CB/UCB and hospitalization length. Curve fitting analyses revealed that lung involvement progressed initially in all patients and then improved. However, patients with elevated STB levels showed a delay in reaching the turnaround point.

Angiotensin converting enzyme 2 (ACE2) receptor has been discovered to be a necessary entry receptor of SARS-CoV-2 in previous studies, which presents a wide distribution (16, 17). COVID-19 may affect many organs including the liver. SARS-CoV-2, the virus that causes COVID-19, is reported to share

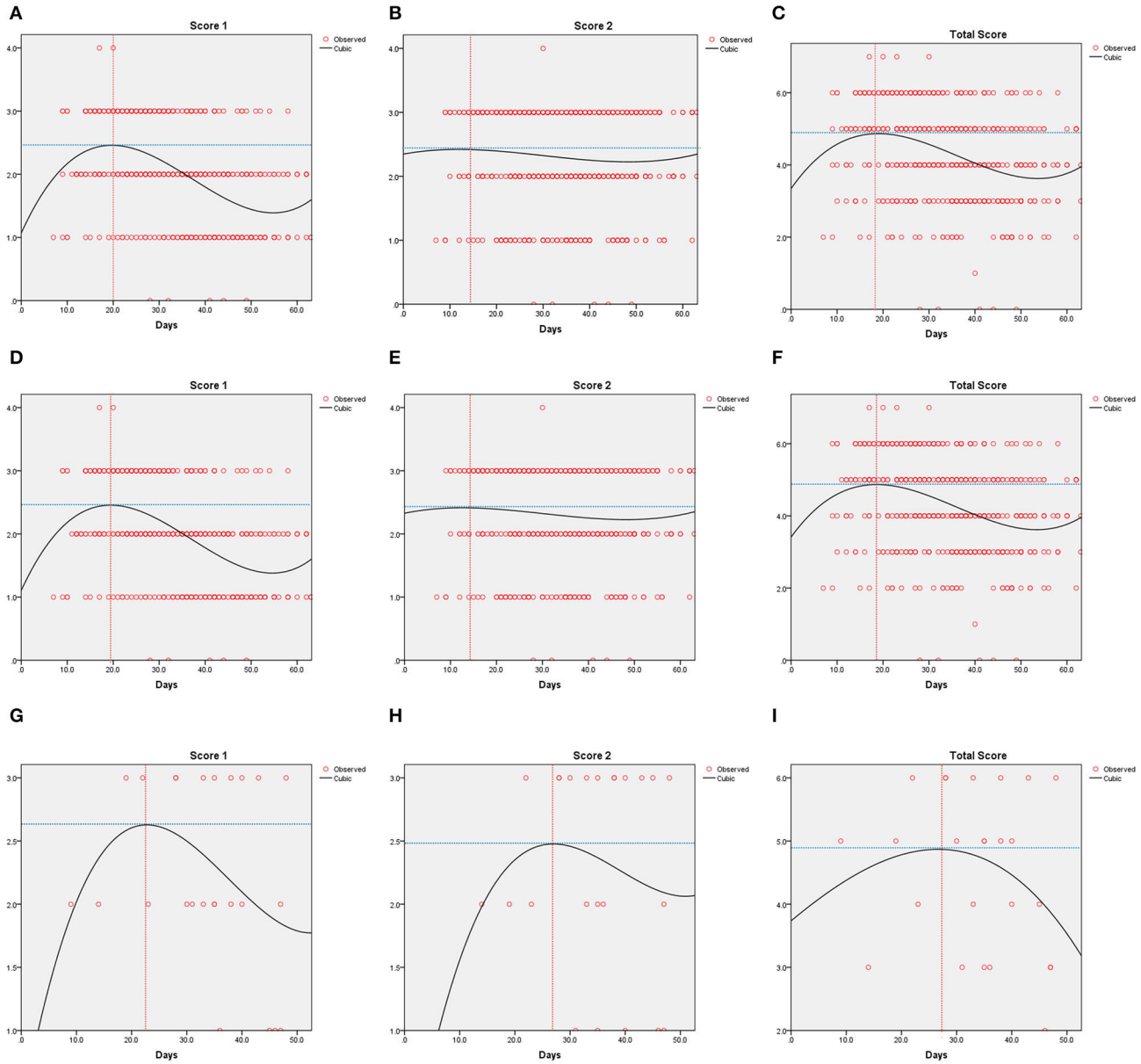

FIGURE 3 | Curve fitting analysis for all patients **(A–C),** the normal or decreased serum total bilirubin (STB) group **(D–F)** and the elevated STB group **(G–I)** in patients with COVID-19.

82 and 50% of its genome with SARS-CoV and Middle East respiratory syndrome (MERS)-CoV, respectively. SARS-CoV and MERS-CoV both cause severe respiratory symptoms (18–20). Coincidentally, liver impairment has been reported in up to 60% of patients with SARS and also in patients with MERS (21, 22). Free bilirubin is derived from catabolism of heme, predominantly hemoglobin heme (23). Once produced, free bilirubin quickly binds to albumin, and this complex of free bilirubin bound to albumin in blood is called UCB. Therefore, free bilirubin only represents the fraction of bilirubin that remains unbound to any solubilizing compound in the vascular bed. Then, UCB enters the liver along with the blood stream and is rapidly absorbed by hepatocytes after isolated from albumin. After that, UCB turns into CB in hepatocytes through a sequence of biochemical reactions and CB is excreted into the bile ducts. So, an elevated CB level can be an important manifestation of liver injury.

Gong et al. demonstrated that patients with severe COVID-19 tended to have higher bilirubin levels. In another study, Wang et al. found a significantly higher level of bilirubin in intensive care unit (ICU) patients with COVID-19 than in non-ICU patients. Their results were consistent with our findings. An elevated bilirubin level is regarded as a vital marker of

TABLE 6 | Univariate and multivariate logistic regression analysis for critical disease in patients with COVID-19.

	Group	OR	95% CI		P value
Logistic regression analysis					
Univariate analysis	CB/UCB	4.42	2.63	7.42	<0.001^
Multivariate analysis*	CB/UCB	2.21	1.2	4.07	0.01^

**Adjusted for age, history of cardiovascular disease, leucocyte count, platelet count, lymphocyte count, and creatinine levels.*
^P < 0.05.

altered liver function, indicating liver damage. Chai et al. (24) demonstrated that the expression of ACE2 receptors in bile duct epithelial cells was relatively high and even equivalent to that in alveolar type II cells. However, hepatocytes may express ACE2 receptors at only one-twentieth the concentration found in bile duct epithelial cells. These findings suggest that epithelial cell damage in the bile duct may represent another mechanism of liver tissue injury, besides the direct infection of hepatocytes by SARS-CoV-2.

Due to the metabolic pathway of bilirubin, elevated UCB is common in hemolytic disease while elevated CB is usually related to damage to hepatocytes. Elevation of CB/UCB may indicate acute hepatitis. Thus, it is understandable that elevated STB levels, CB levels, and CB/UCB are associated with disease progression and prognosis. However, an increased UCB concentration is not only a marker of hemolysis but may also indicate a disorder of the bilirubin metabolism within the liver tissue. This parameter is probably associated with disease progression, but we did not obtain statistically significant results for the association between UCB concentration and COVID-19 survival. Further studies may be needed in this regard.

The study has several limitations. First, although 1,788 patients were enrolled in the study, there were only 69 patients in the elevated STB group. Second, some medical data which available in some patients and missing in other patients may exist some contrary effect on our primary outcomes. Third, owing to the retrospective nature of the study, we could not avoid sample heterogeneity and some parameters associated with disease prognosis were not recorded in the medical record system. Fourth, absence of data on cholestatic enzymes and data on pre-hospitalization bilirubin was a great pity. Fifth, only 4 patients died in the elevated STB group, thus the number is low and further larger epidemiological studies are required. Finally, Gilbert syndrome, known as a common genetic unconjugated bilirubinemia, may confound the possible association of bilirubin and severity of COVID-19 disease. Therefore, more attention should be focused on this issue in the future, and multicenter studies with a bigger sample is needed. Moreover, continuously monitoring to the changes of bilirubin levels may be of great value.

In conclusion, elevated STB levels, CB levels, and CB/UCB were associated with a higher risk of COVID-19 mortality. Moreover, CB/UCB was associated with disease severity and length of hospitalization; thus, it may be useful as a prognostic indicator. Patients with elevated STB levels tended to have more severe pneumonia and took longer to recover than patients with normal STB; thus, it is necessary to pay special attention to COVID-19 patients with elevated bilirubin levels in clinical management.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

ZLiu, JL and LG contributed conception and design of the study. WL performed the statistical analysis and wrote the first draft of the manuscript. WZ, RG, ZLi and XW contributed to data acquisition. GZ, DC, SW, QL and DH contributed to manuscript revision. All authors contributed to data interpretation and approved the final version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.598870/full#supplementary-material

Supplementary Figure 1 | Curve fitting analysis for the dynamic changes of bilirubin in patients with COVID-19.

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Dynamics of CD4 T Cell and Antibody Responses in COVID-19 Patients With Different Disease Severity

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Disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) ranges from mild illness to severe respiratory disease and death. In this study, we determined the kinetics of viral loads, antibody responses (IgM, IgG, neutralization) and SARS-CoV-2-specific CD4 T cells by quantifying these parameters in 435 serial respiratory and blood samples collected from a cohort of 29 COVID-19 patients with either moderate or severe disease during the whole period of hospitalization or until death. Remarkably, there was no significant difference in the kinetics and plateau levels of neutralizing antibodies among the groups with different disease severity. In contrast, the dynamics of specific CD4 T cell responses differed considerably, but all patients with moderate or severe disease developed robust SARS-CoV-2-specific responses. Of note, none of the patients had detectable cross-reactive CD4 T cells in the first week after symptom onset, which have been described in 20–50% of unexposed individuals. Our data thus provide novel insights into the kinetics of antibody and CD4 T cell responses as well as viral loads that are key to understanding the role of adaptive immunity in combating the virus during acute infection and provide leads for the timing of immune therapies for COVID-19.

Keywords: SARS-CoV-2, COVID-19 patients, adaptive immunity, SARS-CoV-2-specific antibodies, SARS-CoV-2-specific T cells

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INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has recently emerged as a new human-to-human transmissible pathogen, causing a pandemic with serious global health consequences. Most infected patients present with mild-to-moderate symptoms and approximately 20% develop severe disease (1). Older people as well as persons with underlying chronic diseases appear to be predisposed to a poor clinical outcome, and male patients have a greater risk of death (2–4). As of June 30, 2020, the World Health Organization (WHO) reported 10.2 million confirmed cases of coronavirus disease (COVID-19), including 503.862 deaths.

SARS-CoV-2 is a lipid-enveloped virus with a positive-stranded RNA genome and four structural proteins (spike glycoprotein, S; envelope protein, E; membrane protein, M; nucleocapsid protein, N). The target of neutralizing antibodies (nAbs) is the S protein, forming prominent projections at the virus surface and mediating viral entry functions. S-specific antibodies directed to both sub-units of S (S1, S2) that prevent these functions can therefore inhibit entry of coronaviruses into cells and potentially protect from disease (5, 6). Passive immunization with convalescent plasma containing such antibodies or strongly neutralizing monoclonal antibodies (mabs) are pursued as a therapeutic option for severe cases [reviewed in (7)]. In addition, most of the current efforts of developing vaccines rely on the use of the S protein as an immunogen.

Infection with SARS-CoV-2 activates innate and adaptive immune responses, including the induction of virus-specific T and B cells, but dysfunctional immune responses, such as inflammatory cytokine storms, are probably associated with the severity of COVID-19 [reviewed in (8)]. CD4 T cells play essential roles in coordinating immune responses via the help to B cells for nAb production. They also promote effector activity of CD8 T cells and the establishment of B and T cell memory (9). SARS-CoV-2-specific CD4 T cells produce IL-2 and IFN-γ, suggesting that COVID-19-recovered individuals exhibit a TH1 cell response (10–12).

Experimental data obtained in non-human primate models indicate that pre-existing virus-specific nAbs and T cells can mediate protection against virus challenge (13, 14). It is unknown, however, how the time course of nAbs as well as T cells correlate with virus clearance and to which extent adaptive immune responses contribute to resolution of disease in the course of infection. We addressed these questions in a comprehensive study of three well-characterized groups of COVID-19 patients with different disease outcomes (moderate, severe, deceased) by quantifying virus loads, Ab responses as well as CD4 T cell responses over the entire time of hospitalization. The goal of this study was to analyze the kinetics of viral load and SARS-CoV-2-specific immune responses. We found that viral loads declined significantly faster in patients with less severe disease, but all patients developed comparable levels of neutralizing antibodies with similar kinetics. In contrast to the antibody response, the dynamics of specific CD4 T cell responses differed considerably, but all patients with moderate or severe disease developed robust antiviral responses.

MATERIALS AND METHODS

Study Cohort

Human blood samples from all patients have been collected under the approval of the Ethics committee of the Medical University of Vienna, Austria (EK 2283/2019). All patients provided written informed consent. The use of anonymized healthy control samples for the validation of serological assays has been approved by the Ethics committee of the Medical University of Vienna, Austria (EK 2156/2019). Between March, 11, 2020 and April, 14, 2020, 29 patients with blood samples available for 14 consecutive days or longer after symptom onset were included (**Table 1**). The median interval between symptom onset and collection of first blood sample collection was 7 days (IQR 4–11). Of the 29 patients, 13 had moderate disease, requiring low-flow oxygen and were admitted to the normal ward (NW; group 1), nine were severe cases, of whom all required supplemental oxygen (high-flow nasal cannula, non-invasive ventilation, or invasive ventilation), were admitted to the intensive care unit (ICU) and survived (group 2), and seven patients (4 ICU, 3 NW) deceased (group 3). Antiviral treatment included remdesivir, lopinavir/ritonavir, hydroxychloroquine, or human recombinant soluble angiotensin converting enzyme-2 (**Supplementary Table 1**).

Detection of SARS-CoV-2 RNA

Viral RNA load was determined in endotracheal aspirates (if available in ICU patients) and nasopharyngeal swabs. Briefly, SARS-CoV-2 RNA was extracted from respiratory specimens using NucliSENS easyMAG extractor (BioMérieux, Marcy l'Etoile, France). SARS-CoV-2 real-time TaqMan PCR was performed with WHO recommended primers and probe located in the E-gene, as described previously (15).

SARS-CoV-2 Virus Isolation

The SARS-CoV-2 strain was isolated from a nasopharyngeal swab from a COVID-19 patient. Vero E6 cells (ATCC® CRL-1586) were infected with the specimen and incubated at 37°C until a cytopathic effect (CPE) occurred. Cell culture supernatant (SN) was harvested and the presence of SARS-CoV-2 was confirmed by PCR. The SN was negative for other human coronaviruses, rhinovirus, metapneumovirus, parainfluenzavirus, influenza A/B viruses, respiratory syncytial virus as well as enteroviruses. The virus isolate was then passaged two more times in Vero E6 cells. The sequence was determined by next generation sequencing and uploaded to the GISAID database (EPI_ISL_438123/hCoV-19/Austria/CeMM0360/2020).

SARS-CoV-2 Neutralization Test (NT)

Two-fold serial dilutions of heat-inactivated serum or plasma samples were incubated with 50–100 $TCID_{50}$ SARS-CoV-2 for 1 h at 37°C before the mixture was added to Vero E6 cell monolayers (starting dilution of samples 1:10). Incubation was continued for 2–3 days. NT titers were expressed as the reciprocal of the serum dilution required for 100% protection against virus-induced cytopathic effects. NT titers $\geq$10 were considered positive. For two initially seropositive cases (nAb titers $\geq$240) with unknown disease onset, the earliest time point of symptom onset was set, assuming that the time to seroconversion was 10 days. Two negative (historical) and three positive (PCR-confirmed patients, 10–14 days after symptom onset) serum samples were included in each assay as controls. The NT was validated with 45 serum samples from healthy controls, including five samples with a prior PCR-confirmed infection with other human coronaviruses (HCoV-OC43 or HCoV-229E, 154–441 days after disease), which all yielded a negative result (NT titer <10).

TABLE 1 | Demographic data and comorbidities among groups of patients with COVID-19.

	Group 1 moderate disease ($n = 13$)	Group 2 severe disease ($n = 9$)	Group 3 deceased ($n = 7$)	*P*-value
Age, years	71.9 (29-98)	56.6 (12-77)	77.5 (63–84)	0.025
SEX				
Female	9 (69%)	4 (44%)	2 (22%)	0.19
Male	4 (31%)	5 (56%)	7 (78%)	0.19
CHRONIC COMORBIDITIES				
Hypertension	4 (31%)	3 (33%)	5 (56%)	0.18
Chronic lung disease	0 (0%)	1 (11%)	1 (11%)	0.41
Diabetes	2 (15%)	2 (22%)	2 (22%)	0.78

Data are median (range) or numbers (%). P-values derived from Mann Whitney U-test for continuous variables and Fishers exact test for categorical variables.

Generation of the Recombinant SARS-CoV-2 Spike Protein

The coronavirus spike ectodomain of SARS-CoV-2 (strain Wuhan-Hu-1; residues 1–1213; GenBank: QHD43416.1) was expressed transiently in COS-1 cells (ATCC® CRL-1650) with a C-terminal trimerization motif and a strep-tag using the pCAGGS expression plasmid, kindly provided by Berend Jan Bosch (16, 17). COS-1 cells were electroporated with 5 μg DNA using a Bio-Rad GenePulser apparatus (settings: 1.5 kV, 25 μF, infinity) and were grown for 20–22 h in Dulbecco's modified eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS) and 1% Penicillin-Streptomycin-Glutamine (both from Gibco). The medium was then replaced with DMEM containing 2% FCS and 25 mM HEPES (Gibco). Incubation was continued for another 72 h. Ninety-six hours after electroporation the supernatant (SN) was harvested and cleared by centrifugation (10,000 rpm; 30 min; 4°C: Beckmann JA 14). To confirm the presence of the strep-tagged ectodomain of the spike, serial dilutions of the SN were added to Strep-Tactin coated microplates (IBA GmbH, Göttingen, Germany) and were incubated for 1 h at 37°C in phosphate-buffered saline (PBS) pH 7.4, 2% sheep serum, 2% Tween 20. A rabbit mab recognizing the S1 subunit of SARS-CoV-2 (Sino Biologicals, Spike S1 Antibody, Rabbit mab, # number 40150-R007) was then added and incubated for 45 min at 37°C. Bound mab was detected with DAR-HRP (Anti-rabbit IgG, horseradish peroxidase (HRP)-linked species-specific whole antibody from donkey, GE Healthcare, # NA 934).

SARS-CoV-2 IgM and IgG ELISA

COS-1 SN containing the strep-tagged spike protein was diluted 1:3 in PBS pH 7.4, 2% sheep serum, 2% Tween 20 and was added to Strep-Tactin coated microplates (IBA GmbH, Göttingen, Germany) that were blocked for 30 min with 1% bovine serum albumin (BSA) in PBS pH 7.4. Antigen incubation was carried out for 60 min at 37°C. Serial dilutions of human serum or plasma samples (starting dilution 1:100) were added and incubated for 45 min at 37°C. In the case of the IgM ELISA, samples were pre-incubated with rheumatoid-factor-IgG-absorbent (RF absorbent, Siemens Healthcare Diagnostics GmbH, # OUCG15/10446434). Bound human antibodies were detected either with goat anti-human IgM or IgG labeled with HRP (Thermo Fisher Scientific: Goat anti-Human IgM Secondary Antibody, HRP, # 31415. Goat Anti-Human IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP, # 31412). Absorbance was measured at 450 nm. Titers were determined by curve fitting with a four-parameter logistic regression using GraphPad Prism 8 (GraphPad Software Inc.). A positive control serum was included in each test. This control serum was obtained from a COVID-19 patient (16 days after disease onset) with an NT titer of 960. For cut-off determination, we used 30 plasma samples from healthy blood donors. The cut-off for titer determinations was set as the mean absorbance value from these negative controls at a 1:100 dilution plus three standard deviations.

Preparation of Blood Samples

Peripheral blood mononuclear cells (PBMCs) were separated from whole-blood samples using Ficoll-Paque Plus™ (GE Healthcare) and were cryopreserved in liquid nitrogen, as previously described (18). PBMCs were thawed and depleted of CD8-positive cells using magnetic beads coupled with anti-CD8 antibody and LD columns (Miltenyi Biotec GmbH, Germany), as previously described (19). The depleted PBMCs were incubated overnight in serum-free medium (AIM-V; Gibco) at 37°C in 5% CO_2. For use in ELISpot assays, cells were resuspended at a final concentration of 2×10^6 cells/ ml in AIM-V. The purity and viability of CD8-depleted PBMCs in each sample was assessed using anti-CD8-APC, anti-CD3-PE, anti-CD4-PacificBlue™, and 7-aminoactinomycin D (BD Bioscience) and flow cytometry (18). Purity of CD8-depleted PBMCs was usually >99%. Plasma and serum samples were stored at −20°C.

Peptides

For T cell stimulation, four PepMix™ SARS-CoV-2 peptide pools (product codes: PM-WCPV-VEMP, PM-WCPV-VME, PM-WCPV-S, and PM-WCPV-NCAP) were purchased from JPT (Berlin, Germany). The pools comprise 15 mer peptides overlapping by 11 amino acids and cover the entire sequences of the SARS-CoV-2 structural proteins: envelope (E), membrane

(M), spike (S), and nucleoprotein (N). The S pool is composed of two sub-pools S1 (aa 1-643) and S2 (aa 633-1273). Peptides were dissolved in dimethyl sulfoxide and diluted in AIM-V medium for use in ELISpot assays.

IFN-γ ELISpot Assay

IFN-γ ELISpot assays were performed as previously described (12, 13). Briefly, plates (MSIPS4W10, Merck-Millipore) were coated with 1.5 μg anti-IFN-γ antibody (3420-3-1000, Mabtech) per well. For blocking, PBS/5% BSA (11930, Serva) was used. The CD8-depleted PBMCs of COVID-19 patients (1×10^5 cells/well) were incubated at 37°C and 5% CO_2 for about 45 h with SARS-CoV-2 peptides (2 μg/ml; duplicates), AIM-V medium (negative control; 2–4 wells), or leucoagglutinin (PHA-L; L4144, Sigma; 0.5 μg/ml; positive control). After washing, spots were developed with 0.1 μg biotin-conjugated anti-IFN-γ antibody (3420-6-250, Mabtech), streptavidin-coupled alkaline phosphatase (ALP; 3310-10, Mabtech; 1:1,000) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT; B5655, Sigma). The number of spots was evaluated using a Bio-Sys Bioreader 5000 Pro-S/BR177. Spots were counted using automatically calculated spot-size thresholds (upper and lower gates) to distinguish spots produced by antigen-specific T cells from cell clusters and from non-specific background spots with Bioreader v 10 software. Responses to SARS-CoV-2 peptide pools were defined positive if at least two-fold above the mean +3 SD of spots from 5 healthy controls who tested negative for coronavirus S-specific IgG (≥50 spots). The ELISpot assay was validated by comparing IFN-γ responses between undepleted PBMC controls, CD4-depleted, and CD8-depleted PBMCs, as described previously (20). FACS analysis revealed that cell depletion by magnetic bead separation was complete (**Supplementary Figure 1**). The sums of responses from CD4- and CD8-depleted fractions were comparable to PBMC controls.

Statistics

Statistical analysis was performed using SPSS version 26.0 and Prism version 5.0. Combined correlation coefficient was calculated by using Fisher's z′ transformation and averaging over patients to assess the relation between virus loads from nasopharyngeal swabs and endotracheal aspirates. Decline of virus loads in nasopharyngeal swabs and endotracheal aspirates was assessed by Generalized Estimation Equations (GEE) model applying an unstructured correlation matrix. This analysis was restricted to the first 30 days after disease onset and only the first negative test result was included. First, a model with homogeneous slope was fit (**Figure 1A**), however, a model with heterogeneous slope fit the data better according to Akaike's criterion and was applied to compare groups with respect to decline behavior. The GEE model Walsh chi^2 test was conducted to analyse variables (age, sex or comorbidities) potentially associated with differences in vRNA decline, IgG, IgM, NT titer, and CD4 T cell response. Pearson's correlation analyses was performed to assess the relationship between nAb titers or CD4 T cell levels and anti-S IgM, IgG, and between viral RNA and nAb titers or CD4 T cell levels. Dunn's multiple comparisons following a Kruskal Wallis test were performed for analysis of IFN-γ ELISpot assays. Statistical significance was determined as $P < 0.05$ ($^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

RESULTS

Patients and Clinical Outcome of Disease

We analyzed viral loads, virus-specific antibody, and CD4 T cell responses in 29 COVID-19 patients over the entire period of their hospitalization. The basic characteristics of these patients are displayed in **Table 1** and more specific information (including therapies) are shown in **Supplementary Table 1**. The patients

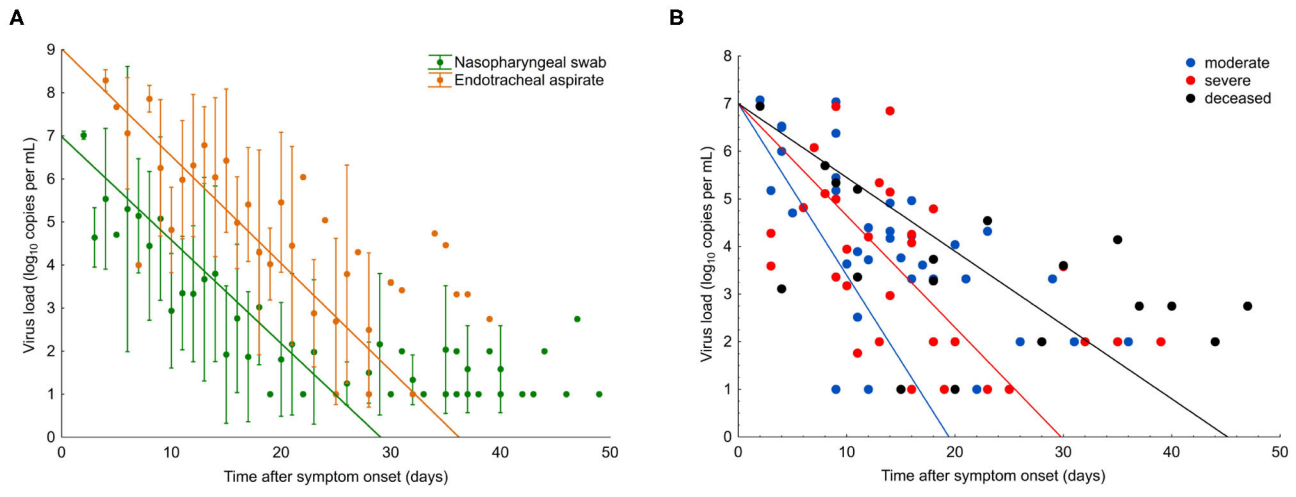

FIGURE 1 | SARS-CoV-2 viral load in COVID-19 patients with different disease severity. **(A)** Viral load from nasopharyngeal swabs (green) and endotracheal aspirates (orange). Data points are mean; error bars indicate SD; slopes represent best fit. **(B)** Viral load from nasopharyngeal swabs from all patients ($n = 29$). Data points indicate viral load in individual samples; slopes represent viral RNA decline in patient groups, as assessed by Generalized Estimation Equations (GEE) applying an unstructured correlation matrix. Group one, moderate (blue); group two, severe (red), and group three, deceased (black).

were divided into three groups, according to disease outcome, classified as "moderate disease," "severe disease," and "deceased."

Thirteen cases had moderate disease, but still required hospitalization and were admitted to the normal ward (NW; group 1), nine were severe, of whom all were admitted to intensive care unit (ICU) and survived (group 2), and seven patients (4 ICU, 3 NW) deceased (group 3). The median age of all patients was 71.9 years (range 29–98).

Viral RNA Load

For the comparison of viral RNA (vRNA) loads over time in the three different patient groups, we analyzed 271 respiratory specimens, including 203 nasopharyngeal swabs and 68 endotracheal aspirates collected between 2 and 49 days after symptom onset. In nasopharyngeal swabs, the overall median viral load at the time point of presentation was 5.1 $\log_{10}$ copies/ml (interquartile range, IQR 4.0–6.5) and continuously declined over the course of disease (**Figure 1A**). Endotracheal aspirates (collected from 10 patients, six from group 2, and four from group 3) had, on average, 100 times higher copy numbers/ml than nasopharyngeal swabs (**Figure 1A**). A significant correlation ($r = 0.71, p < 0.01$) was found between the vRNA copy numbers in the two materials during the time course of disease (**Figure 1A**).

In the first samples, collected within a median of 8 days after symptom onset (IQR 4–10), viral loads were not significantly different between the three patient groups ($p = 0.15$). The decline of vRNA, however, was significantly slower in groups 2 and 3 than in group 1 ($p < 0.01$), as determined by a generalized estimating equation model (**Figure 1B**). Significantly more patients in groups 2 (7/9) and 3 (4/7) received antiviral treatment than group 1 (2/13) ($p = 0.0115$). There was no significant difference in vRNA decline among the patients who received different antiviral therapies, including remdesivir (vRNA halflife, 4.0; IQR 2.6–8.4), lopinavir/ritonavir (vRNA halflife, 3.3; IQR 2.5–4.9), and hydroxychloroquine (vRNA halflife, 4.0; IQR 2.6–8.4). Analysis of vRNA loads by age, sex or chronic comorbidities in generalized estimating equation model, Walsh chi^2 tests revealed that vRNA decline was significantly slower in patients older than 65 years ($p = 0.024$) and in patients with chronic lung disease ($p = 0.03$), whereas no effect was seen with hypertension ($p = 0.228$) or diabetes ($p = 0.900$).

Neutralizing Antibody Titers and Correlation With Anti-S IgM and IgG Titers

To assess whether there was a correlation between the extent of viral loads as well as disease outcomes and a specific humoral immune response, we first quantified neutralizing antibodies in 161 sequential serum/plasma samples from the three patient groups (median 5 serum specimens per patient). The results are shown in **Figures 2A–C**. In the first samples (median day 7 after onset of symptoms, IQR, 4–11), 16 patients already had detectable nAbs, and 13 patients were seronegative. No association was seen between seropositivity at presentation and severity of illness (Chi square = 2.1; $p = 0.4$). In the course of disease, all patients developed nAbs, which were negatively correlated with vRNA loads (Pearson r –0.446; $p < 0.0001$; **Figure 2D**). The titers showed a steep rise between days 6–11 and reached a plateau between days 15 and 22 after symptom onset. The plateau titers were quite high (median, 640; IQR 440–720), and there was no significant difference of these titers among the three patient groups ($p = 0.32$, Kruskal-Wallis test).

To determine the correlation between neutralizing antibody titers and IgM and IgG responses to the spike (S) protein, we performed corresponding ELISAs (using the whole ectodomain of S as an antigen) with sequential samples ($n = 158$) of the 29 patients (**Figures 2E,F**). Fourteen patients were already either IgM, IgG or IgM, and IgG positive in the first samples obtained between 2 and 14 days after symptom onset, and 11 of these samples were also NT positive. Seroconversion for anti-S IgM or IgG was observed on days 11 (IQR 9–14) and 12 (IQR 10–18) after symptom onset, respectively. In the assays used, plateau titers of IgM (log 3.1, IQR 2.8–3.4) were similar to NT titers (log 2.8, IQR 2.6–2.9), and IgG were about ten-fold higher than IgM titers (log 4.0, IQR 3.7–4.2). There was a positive correlation between total anti-S Ab (IgM and IgG) titers and nAb titers (Pearson r 0.792, 95% CI 0.73–0.84; R^2 0.627; $p < 0.0001$; **Figure 2G**). The correlation between anti-S IgG titers and nAb titers (Pearson r 0.817, 95% CI 0.76–0.86; R^2 0.667; $p < 0.0001$) was stronger than between anti-S IgM titers and nAb titers (Pearson r 0.516, 95% CI 0.39–0.62; R^2 0.266; $p < 0.0001$; **Figures 2H,I**).

SARS-CoV-2 Specific CD4 T Cell Responses

To investigate whether the extent and time course of specific CD4 T cell responses correlated with disease outcome, we analyzed peripheral blood mononuclear cells (PBMCs) from 21 patients (eight from group 1; eight from group 2; five from group 3). For this purpose, CD8-positive cells were depleted from PBMCs, and CD4 T cell responses were quantified by IFN-γ enzyme-linked immunosorbent spot (ELISpot) assays. IFN-γ ELISpot assays were performed using pools of peptides covering the entire sequences of all four viral structural proteins S, M, N, and E. Sequential samples were available from 17 of the 21 patients.

As shown in **Figure 3A**, no specific CD4 T cell reactivity was detectable in the first week after symptom onset. After this initial delay, all, except two deceased patients developed detectable antiviral CD4 T cell responses. Overall, the magnitude of CD4 T cell responses increased until week 3 after symptom onset (**Figure 3A**). The contribution of viral proteins to overall CD4 T cell responses is displayed in **Figure 3B** and shows that S (including S1 and S2) and M dominated the response, contributing 45% and 33% to measured reactivities, respectively. The contribution by N was somewhat lower (21%) and that of E was only marginal (1%), corresponding to the amounts of the proteins in the virus particle (21).

Since corticosteroid therapy can have a profound T cell suppressive effect (22), the kinetics of CD4 T cell responses from patients with or without corticosteroid therapy were studied separately. The analysis of response kinetics from patients not receiving corticosteroid therapy revealed considerable individual variation, as displayed in **Figure 3C**. All patients from groups 1

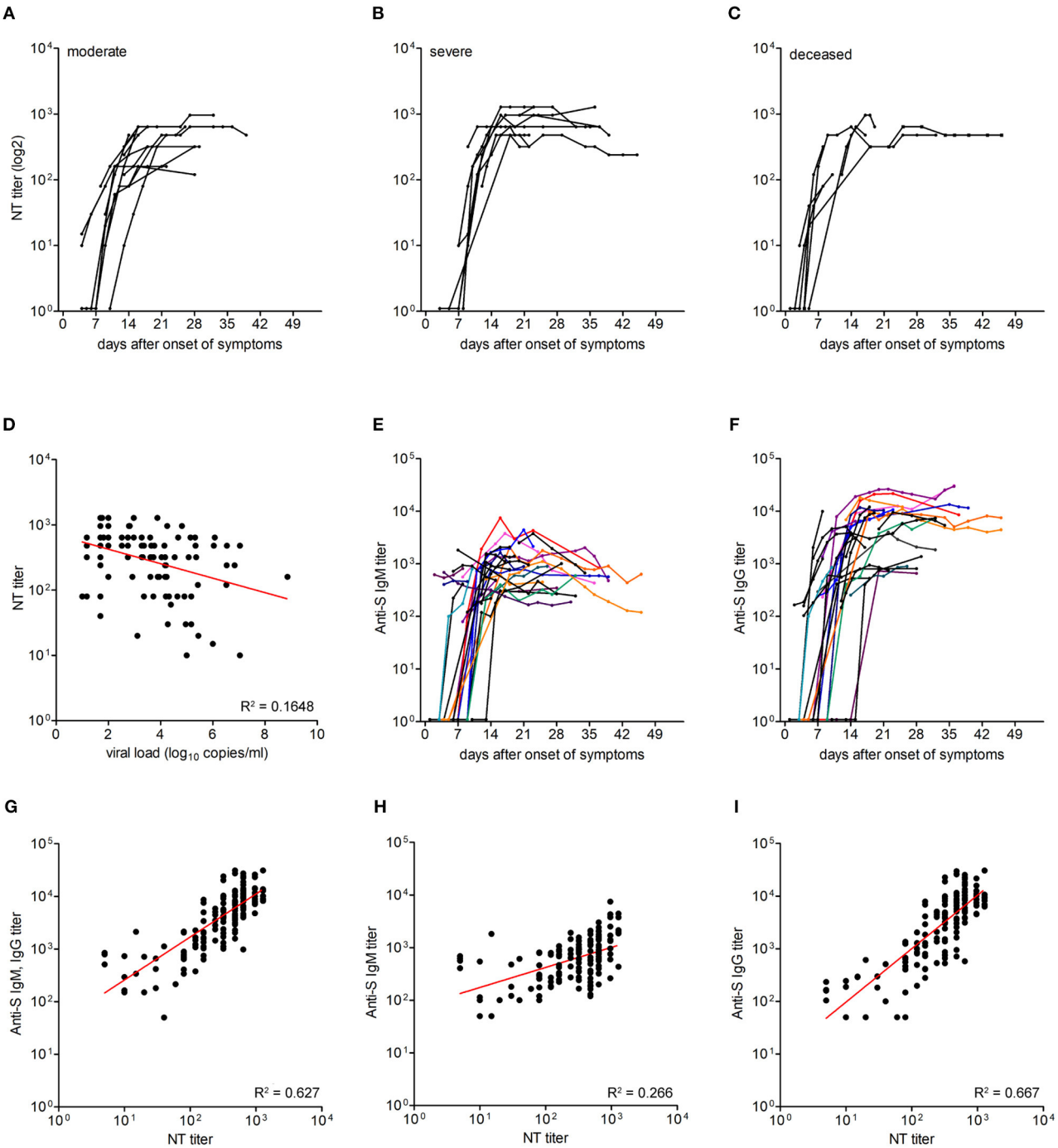

FIGURE 2 | Titers of neutralizing antibodies and correlation with viral load and IgM and IgG titers against the spike protein. **(A–C)** Neutralizing antibody titers in moderate ($n = 13$), severe ($n = 9$), and deceased ($n = 7$) patients. **(D)** Correlation between neutralizing antibody titers and virus RNA loads was assessed using Pearson correlation. **(E,F)** IgM and IgG ELISA titers against the spike protein in 29 patients. Each line represents an individual patient. **(G–I)** Correlation between neutralizing antibody titers and anti-S IgM and IgG ELISA titers, anti-S IgM ELISA titers and anti-S IgG ELISA titers, as assessed by Pearson correlation.

and 2 mounted a robust CD4 T cell response, reaching at least 10 times the cut-off of the ELISpot assay. However, differences were observed with respect to the time point when strong responses became detectable, ranging from 14 to 24 days after symptom onset (**Figure 3C**). Of the deceased patients, one had no response at days 4 and 21 after symptom onset, and the second patient mounted a low response at day 19, which dropped toward the cut-off at day 32 after symptom onset (**Figure 3C**). For 4 of the 5

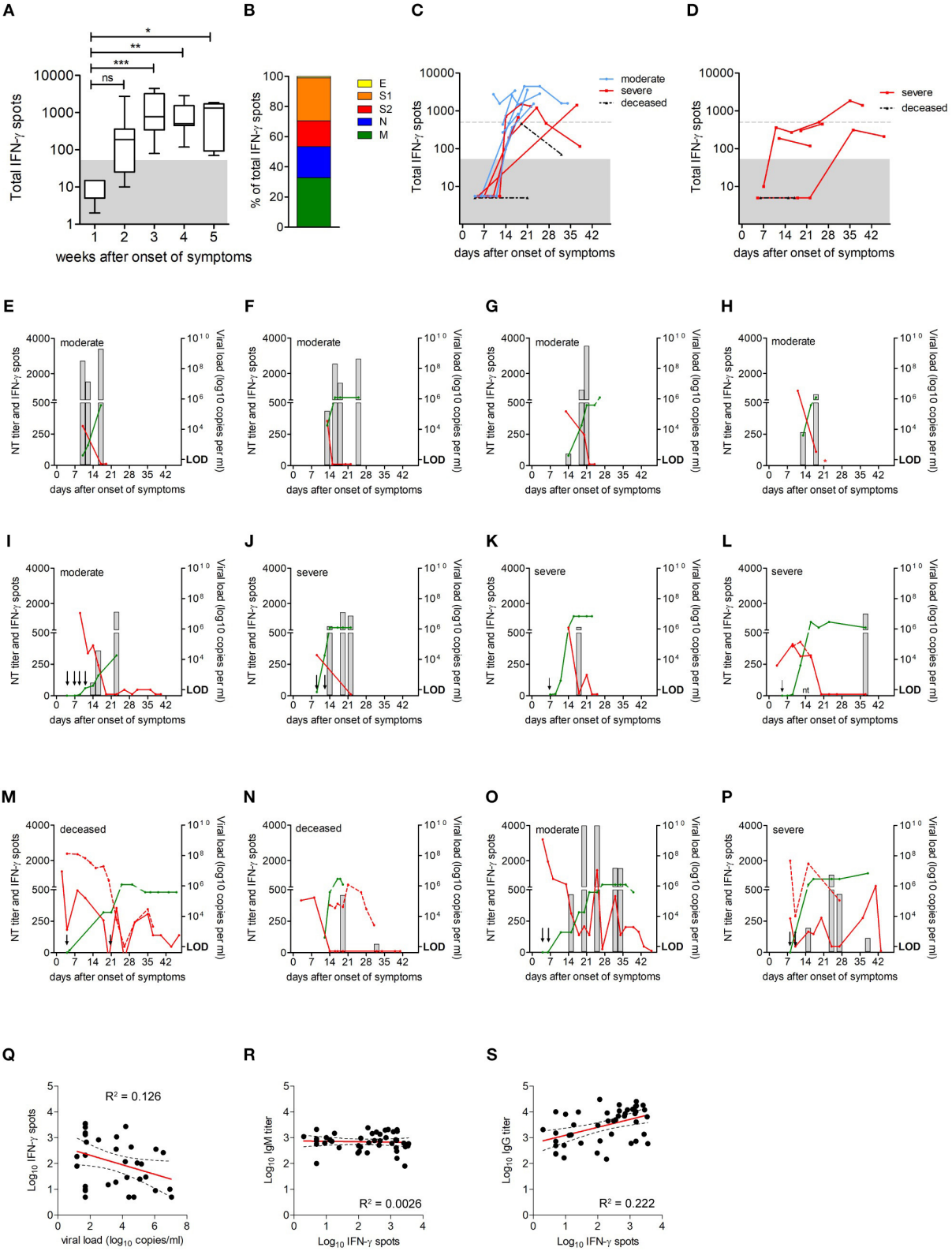

FIGURE 3 | Extent of SARS-CoV-2-specific CD4 T cell responses over time. **(A)** Extent of CD4 T cell responses to the four SARS-CoV-2 structural proteins, as determined by IFN-γ ELISpot assays ($n = 21$); data are presented as box and whiskers plots, with bounds from 25th to 75th percentile,plots, with bounds from 25th

(Continued)

FIGURE 3 | to 75th percentile, median line, and whiskers ranging from minimum to maximum of total IFN-γ spots. Significance was determined by Kruskal Wallis test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Area below cut off in IFN-γ ELISpot assay (<50 spots per 106 PBMCs) is shaded gray. **(B)** Percentage of spots contributed by S1, S2, M, N, and E. **(C)** Kinetics of CD4 T cell responses in patients with moderate or severe disease and in deceased patients; group one, moderate (blue circles); group two, severe (red squares) and group three, deceased (black triangles). **(D)** Kinetics of CD4 T cell responses in patients with corticosteroid therapy ($n = 5$); group two, severe (red squares) and group three, deceased (black triangles). Dotted gray lines indicate 500 spots (i.e., 10 times the cut-off of the ELISpot assay). Area below cut off in IFN-γ ELISpot assay (<50 spots per 10^6 PBMCs) is shaded gray. **(E–P)** CD4 T cell responses (gray columns), neutralizing antibody titers (green lines), and virus loads in nasopharyngeal swabs (red lines) or endotracheal aspirates (dotted red line) in individual patients. Arrows indicate time points of ELISpot assays with no detectable CD4 T cell reactivity; red star indicates discharge, negative PCR result was not obtained; nt, not tested; LOD, limit of detection. **(Q–S)** Correlations between virus-specific CD4 T cell levels and vRNA loads, anti-S IgG, or IgM ELISA titers were assessed using Pearson correlation.

patients who received corticosteroids, no or low antiviral CD4 T cell responses were detected, and one patient mounted a robust response >500 spots, but only 5 weeks (days 35 and 39) after symptom onset (**Figure 3D**).

We next analyzed the time course of CD4 T cell responses in relation to viral clearance for all patients shown in **Figure 3C**. The most common pattern in patients from group 1 and group 2 was characterized by a robust CD4 T cell response followed by viral clearance (**Figures 3E–L**). Of the two deceased patients, one had detectable CD4 T cells after vRNA clearance, and one did not mount a detectable response until week 3 after symptom onset and had several virus rebounds until finally deceased (**Figures 3M,N**). In accordance with recent studies (12), CD4 T cell levels were negatively correlated with vRNA loads (Pearson r −0.3555; $p = 0.0390$; **Figure 3Q**). In addition, strikingly different patterns were observed in two cases. Specifically, one patient from group 1 and one from group 2 developed strong CD4 T cell responses, but nevertheless had an early virus rebound and prolonged infection (**Figures 3O,P**). There were no significant differences in CD4 T cell response kinetics in relation to patients sex or age (sex, $p = 0.469$; age >65 years, $p = 0.943$; generalized estimating equation model, Wald chi^2 test).

Because CD4 T cells play an important role in promoting efficient antibody production through support of antibody class switch and the development of high-affinity antibody-secreting B cells, we correlated CD4 T cell levels with antibody titers. As shown in **Figures 3R,S**, there was a positive correlation between CD4 T cell levels and anti-S IgG titers (Pearson r 0.4714; $p = 0.0011$), whereas no correlation was observed between CD4 T cell responses and anti-S IgM titers (Pearson r −0.0511; $p = 0.7388$), indicating that the development of IgG is correlated with the activation of virus-specific CD4 T cells.

DISCUSSION

In this study, we provide a comprehensive quantitative analysis of the time course of viral loads, neutralizing antibody and CD4 T cell responses in 29 COVID-19 patients with different disease outcomes over the whole period of hospitalization or until death. In line with previous reports [reviewed in (23)], all patients developed high levels of SARS-CoV-2 S-specific antibodies. Remarkably, there was neither a significant difference in the kinetics nor in the plateau levels of nAb responses among the patients with different outcomes, even in those succumbing to the disease, indicating that antibody levels are not predictive for the outcome of the disease. People with an asymptomatic SARS-CoV-2 infection were reported to have lower titers of virus-specific antibodies or were even seronegative compared to patients with severe disease (24, 25), indicating that other arms of the immune system control infection in these people. Challenge studies with non-human primates have demonstrated a protective role of nAbs when present before SARS-CoV-2 infection (13, 14, 26, 27). In acute infection, however, the production of nAbs thus appears to be too late for contributing to virus clearance and/or resolving disease.

The observed kinetics of virus and antibody titers have implications for therapies based on antibodies, administered as either convalescent plasma or mabs [reviewed in (7, 28)]. As already deduced from preliminary trials (28), the success of passive antibody therapy requires a good timing of administration. Our data based on a tight sampling schedule during hospitalization indicate that the therapeutic window is at (or very early after) symptom onset, when virus titers are still high, but Abs are not yet detectable. A further important parameter for convalescent plasma therapy is the use of preparations with confirmed high titers of nAbs, thus probably limiting the donors to people recovered from symptomatic disease. In this respect, it is good news that nAb responses showed an excellent correlation with those obtained in an ELISA using the trimeric ectodomain of S (**Figure 2F**), in agreement with other studies in which either the whole spike and/or its receptor-binding domain were used (11, 17, 29–32). In some early phase samples, we observed neutralization when IgG were not yet detectable but IgM were already present, indicating that IgM Abs alone can neutralize the virus. Assays detecting S-specific IgG as well as IgM antibodies might thus be valuable surrogate tools for predicting nAb levels of patients in early convalescence.

The picture of antiviral CD4 T cells is more heterogeneous as compared to antibodies, but important features can be discerned. Specific CD4 T cells were not detected in the first week after onset of symptoms, but then increased over time. All patients with moderate and severe disease developed robust antiviral CD4 T cell responses, which were negatively correlated with vRNA loads, consistent with a recent report (12). The data based on multiple sequential samples indicate that the kinetics of the response was highly variable. The measured CD4 T cell activity can therefore be strongly influenced by the timing of sample collection, which points to possible pitfalls that could arise from data collected at single time points only. In one deceased patient, we even did not detect any specific CD4 T cells. The difference in responses might be due to an inflammation-triggered sequestration of antigen-specific cells to the infected tissue, which may eventually

reduce detectability in the peripheral blood (33, 34). In our study, we did not observe significant differences in immune response kinetics in relation to age, sex or co-morbidities. However, it is important to note that the sample size in the present study was small, and the combined effects of these factors on immune response kinetics will have to be clarified in larger cohort studies.

Recent data indicate the presence of SARS-CoV-2-reactive T cells in healthy controls, not previously exposed to SARS-CoV-2. These cells could be cross-reactive and the result of previous infection with other human coronaviruses (10, 12, 35, 36). None of the patients in our groups of moderate or severe disease had detectable antiviral CD4 T cells in the samples obtained in the first week, indicating that there was no pre-existing immunity in these cases. Whether the presence of pre-existing cross-reactive CD4 T cells may affect disease outcome and prognosis needs to be addressed in future prospective studies.

In conclusion, our data elucidate the dynamics of adaptive immune responses during the course of hospitalization with moderate or severe COVID-19. Since prolonged virus shedding and virus rebound was observed in patients with moderate and severe disease despite the presence of high titers of neutralizing antibodies and robust CD4 T cell responses, these arms of the immune response do not appear to be able to prevent progression to severe disease. Due to ethical reasons, the blood volume that could be collected for multiple sequential samples at different time points was limited. Thus, we were not able to analyze the kinetics of CD8 T cells or other cytokine-producing CD4 T cell subsets in parallel to Abs, and the possible beneficial or detrimental role of these cells in viral clearance or the pathogenesis of COVID-19 will have to be resolved in future studies. It is likely that the efficient interplay between helper CD4 T cells and B cells to promote the production of high-affinity and potently neutralizing antibodies is essential for inducing post-infection immunity. How long such immunity is maintained and whether sufficiently durable immunity can be induced by active immunization are key questions in the search for an effective vaccine and for understanding the epidemiology of COVID-19 in the future.

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 the Medical University of Vienna, Austria. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JA, KS, and FH: conceptualization and writing. MKo, IM, KS, SA, FS, CS, LW, AB, and MKu: methodology. MKo, JA, KS, MT, IM, and AB: investigation. EP-S, MT, AZ, TS, WH, and MF: resources. JA: funding acquisition. KS and JA: supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.592629/full#supplementary-material

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HYPOTHESIS AND THEORY
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The Need for Ocular Protection for Health Care Workers During SARS-CoV-2 Outbreak and a Hypothesis for a Potential Personal Protective Equipment

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SARS-CoV-2 is a coronavirus with high infectivity and has caused dramatic pressure on health systems all over the world. Appropriate personal protection for medical staffs is critical. For ocular protection, there is ongoing hot debate and concern for potential ocular transmission of SARS-CoV-2. Ocular manifestations and positive detection of viral RNA in ocular samples were only reported in very small number of patients infected with SARS-CoV-2. However, health care workers need to face patients more closely and have higher risk of aerosol contamination. Thus, appropriate ocular protection for medical workers is still recommended by organizations such as WHO and American Academy of Ophthalmology. Although eye goggles provide excellent protection and are mandatory for medical practitioners with high risk of exposure, they are not ideal for common clinical practice, because they can disturb vision due to extensive formation of water droplets and frequently cause moderate to severe discomfort after longtime wearing, which have been reported to interfere with working status. For the majority of medical workers who don't deal with high risk patients, they are not advised to wear goggles in daily practice. However, they also face the risk of infection due to the presence of asymptomatic carriers. Especially in situations with high risk of ocular exposure, such as close physical examination, eye surgery, dental clinics and surgery, ocular protection may be needed. Griffithsin has been shown to directly bind to spike proteins and has anti-viral activity against a broad spectrum of viruses, including coronavirus. Griffithsin is found to inhibit the entry of SARS-CoV at relatively low concentration and is stable and non-toxic. SARS-CoV-2 and SARS-CoV share the same entry receptors and their spike proteins are similar in conformation. We hypothesize that contact lenses containing nanoparticles loaded with griffithsin may provide sufficient ocular protection for medical staffs without high risk of exposure during the outbreak period of SARS-CoV-2. If proven effective, griffithsin-loaded contact lens can be considered as a supplementary ocular protective equipment for medical workers who can tolerate well. The daily disposable contact lens should be applied as needed and refrain from extended wearing in order to reduce potential side effects.

Keywords: SARS-CoV-2, ocular protection, sustained releasing contact lens, personal protective equipment, griffithsin

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INTRODUCTION

The Risk of Ocular Transmission of SARS-CoV-2 in Health Care Workers

The novel coronavirus "SARS-CoV-2" is now causing global pandemic and has claimed more than 800,000 lives until July, 2020. Although SARS-CoV-2 is principally a respiratory virus, there is concern that the ocular surface may serve as potential route of SARS-CoV-2 transmission.

The entry of SARS-CoV-2 into host cells relies on protein-protein interaction of its spike protein (S protein) with host surface receptors (ACE2 or CD147) (1, 2). The critical motif for receptor recognition and binding is found in receptor binding domain (RBD) of S protein. After binding, proteolytic cleavage by membrane protease TMPRSS2 is needed to allow for fusion of virus and cell membrane and subsequent entry, a process called "protein priming"(3, 4). Therefore, ACE2/CD147 and TMPRSS2 are all essential for virus entry and transmission. Previous studies have illuminated that ACE2, TMPRSS2, and CD147 were all expressed on ocular surface, including cornea and conjunctiva (5–7). Thus, theoretically the eye can serve as the entry route for SARS-CoV-2, as this area is likely to be contaminated by aerosol, droplets or direct touching (8). In addition, the ocular surface is anatomically connected with the respiratory tract *via* the nasolacrimal duct. The nasolacrimal duct drains tear in the conjunctival sac continuously into the inferior nasal meatus and is thought to play important roles in the spreading of ocular virus into the respiratory system. Thus, the eye theoretically possesses dual routes for virus spread: lacrimal drainage-based spread and direct infection *via* ocular cell receptors (9). Although currently no evidence of intraocular infection of SARS-CoV-2 is available, some common beta coronaviruses can penetrate inside and lead to retinitis and uveitis (10). Besides, the special region of limbus also provide potential routes for the spread of virus *via* blood circulation or trigeminal nerve branches (11). In one animal study of SARS-CoV-2 on rhesus monkeys, virus inoculated on conjunctival surfaces caused characteristic interstitial pneumonia and was detected in a variety of organs by autopsy (12). Thus, these evidence indicates ocular surface has the structural and physiological foundation for SARS-CoV-2 infection.

However, according to clinical data and RT-PCR tests of ocular samples, ocular involvement and positive isolation of viral RNA were only reported in a very small number of patients infected with SARS-CoV-2 (**Tables 1**, **2**). The main ocular manifestations were symptoms related to conjunctivitis. The reported rates of patients with ocular symptoms were 1.5%-31.6% in different studies (13–18). Particularly to be noted were some medical staffs who were not protected with goggles when they were exposed to SARS-CoV-2 and became infected (13, 17). Zhang et al. reported an emergency nurse infected with SARS-CoV-2 and tested positive in conjunctival swabs. The patient was protected with N95 mask during the whole practice time but found her goggle dislocated. Conjunctivitis was the initial symptom, and the conjunctival sample was tested positive on the second day and turned negative at day 9 (17). Xia et al. also reported a patient presented with conjunctivitis and watery secretions as initial symptoms and virus was detected at the early phase of infection (16). A large cross sectional study of 535 patients showed that ocular symptoms were present in 5.05% of patients, and the average duration of conjunctivitis was 5.9 days (14). According to a recent systematic review which included 11 studies on the topic of ocular involvement in SARS-CoV-2, 3 patients with conjunctivitis had positive PCR test, 8 patients had positive tear-PCR in the absence of conjunctivitis, and 14 patients with conjunctivitis but were tested negative by RT-PCR (21). These clinical results indicated that for the generally population, the link between ocular involvement and SARS-CoV-2 infection is still controversial. At least the ocular surface is not a major route for SARS-CoV-2 transmission.

It has been well-recognized that the ocular surface possesses a variety of mechanisms to protect from viral infection, which may explain the low rate of ocular involvement and RNA detection. Many mechanical activities, like tearing, blinking and barrier function of eyelid and lashes may all prevent landing of virus-containing droplets on ocular surface (22). In one experiment on model man, particles of 0.6–5.0 μm were emitted from a jet set 20 cm from the nose. The amount of particles landing on ocular surface was only 1/8 to 1/4 of those on lips, which indicated ocular surface is an uncommon landing area for droplets (8). In addition, the ocular surface possesses multiple innate and acquired immune compounds and actions to defend against viral infection, including lactoferrin, β-lysin, secretory IgA, complement, interferons, *etc.*(23).

TABLE 1 | Clinical reports of ocular involvement of SARS-CoV-2.

References	Total number of patients	Number of patients with ocular symptoms	Ocular symptoms as the initial presentation	Types of ocular manifestations
Zhou et al. (13)	67	1	1	Conjunctival injection, watery secretion
Chen et al. (14)	535	27	4	Conjunctival injection
Wu et al. (15)	38	12	1	Conjunctival injection, chemosis, epiphora, increased secretion
Xia et al. (16)	30	1	1	Conjunctival injection
Zhang et al. (17)	72	2	1	Conjunctival injection, epiphora
Xu et al. (18)	30	1	1	Eye itching

TABLE 2 | Summary of reviewed articles for the detection of SARS-CoV-2 in tears or conjunctival secretions.

References	Participants	Demographic characteristics	Positive Detection rate	Ocular manifestations	Detection phase	Duration of positive detection
Fang et al. (19)	32	F:M=1:1 Mean age=41 (34–54)	15.6%	None	During admission time	N.A.
Zhou et al. (13)	67	F:M=1.68 Mean age=36 (22–78)	1.5% positive 3.0% suspected	1.5%	During admission time	N.A.
Zhang et al. (17)	72	F:M=1.00 Mean age=59	1.4%	2.8%	Before and during admission time	Conjunctival swab turned positive 1 day after conjunctivitis, and became negative at day 9
Xu et al. (18)	30	F:M=1.14 Mean age=48	0%	3.3%	During admission time	N.A.
Deng et al. (20)	114	F:M=0.84 Mean age=61 (10–88)	0%	0%	During admission time	N.A.
Xia et al. (16)	30	F:M=0.43 Mean age=55 (13–83)	3.3%	3.3%	During admission time	Detected at day 2 and day 4 after the onset of symptoms

Although ocular involvement is infrequent in patients infected with SARS-CoV-2, there is evidence for higher risk of ocular transmission for first-line medical workers and the need for ocular protection during high risk procedures. Many procedures such as tracheal intubation, dental surgery and electrocautery generate high concentration of aerosols which may contain the virus and increase the possibility of ocular landing and transmission (24). For ophthalmological surgeons at high risk of ocular transmission, lack of appropriate personal protection results in reduced amount of surgical interventions and potential delay of necessary operations during SARS-CoV-2 outbreak (25). In one previously published study during the outbreak of SARS-CoV, nurses caring for intubated patients who didn't use eye protection had 8 times higher infection rate than those wearing goggles (8 vs. 1%) (26). Thus, we think although ocular involvement is not common in patients infected with SARS-CoV-2, but still can serve as potential transmission route especially for medical workers. The American Academy of Ophthalmology has recognized the risk of ocular transmission in the beginning and called for appropriate eye protection for ophthalmology workers (27).

Griffithsin Can Block the Entry of Coronavirus and Other Enveloped Viruses

Griffithsin is a small lectin consisting of 121 amino acids and is derived from *Griffithsia* spp. (28). Grifithsin has been found to be able to block the entry of a variety of enveloped viruses, including HIV, MERS-CoV, SARS-CoV and HCV and efficiently inhibit viral entry, because it has high affinity to bind to multiple sites of glycoproteins on the virus envelope (29–32). In the previous efficacy studies, griffithsin has been tested either as prophylactic agents or therapeutic drugs against viral infection and showed high potency (33). In an *in vitro* study, griffithsin was found to prevent cell fusion and cell-to-cell transmission of HIV at a concentration of <1 nM by binding to its envelop protein gp120 (34). In mice models, intra-vaginal application of gel containing 0.1% griffithsin prevented spread of HSV-2 and significantly reduced disease scores (35). Griffithsin is found to specifically bind to monosaccharides (mannose, glucose, and N-acetylglucosamine) and oligosaccharide moieties of glycoproteins of virus, thus can theoretically work on any virus whose surface proteins are glycosylated, such as S protein of coronavirus (32). In addition, one molecule of griffithsin possesses three identical carbohydrate-binding domains (36). On crystal structures, the three binding sites are located in an equilateral triangle, and each possesses an aspartic acid residue which makes extensive contact with saccharides (36). Thus griffithsin is multivalent and can work at low concentration, and the estimated EC_{50} value to block the activity of SARS-CoV is 0.28–0.96 μM (36). On mice inoculated with lethal doses of SARS-CoV, concomitant administration of 5 mg/kg intranasal griffithsin improved survival rate to 100% and dramatically reduced lung injury (32). Based on the the action of griffithsin and previous studies, we can infer that this small peptide can also block the entry of SARS-CoV-2, because the S protein of SARS-CoV-2 and SARS-CoV are similar in conformation and both glycosylated with high-mannose glycan (37–39). Moreover, griffithsin is very stable and resistant to the degradation of protease and detergent (40). *In vitro* and *in vivo* toxicology studies demonstrate that griffithsin has no cytotoxicity (41). In summary, griffithsin is a safe anti-viral agent and has been shown to block the entry of a wide variety of coronavirus. It is reasonable to hypothesize that griffithsin is a good candidate for SARS-CoV-2 prevention, which has been suggested by several researchers (42, 43).

Sustained-Releasing Therapeutic Contact Lenses

As the ocular surface is continuously exposed to the environment, a prolonged eye protection is needed. Traditional eye drops may not provide sufficient protection due to blinking and drainage by nasolacrimal duct. It is estimated that drugs administrated via eye drops only reside in tears for 1–3 min and have very low bioavailability (44). Thus, sustained-releasing therapeutic contact lenses containing griffithsin may be the optimal option for the protection of ocular surfaces against SARS-CoV-2. As griffithsin is a small protein, it can be entrapped in nanoparticles which can enable sustained delivery. The technique was first describe by Gulsen et al. who dispersed drug-laden nanoparticles in hydroxyethyl methylacrylate (HEMA) monomers before polymerization to make therapeutic contact lenses (45). The contact lenses containing drug-laden nanoparticles are able to release drugs for an extended period of time, and show reasonably good tolerability, transparency and permeability (46).

THE HYPOTHESIS

The ocular surface is a possible transmission route of SARS-CoV-2, especially for medical staffs who work in close contact with infected patients. Theoretically, griffithsin can bind to S protein on virus envelop and inhibit the entry of SARS-CoV-2. Contact lenses with nanoparticles releasing griffithsin may be a way to protect the ocular surface from SARS-CoV-2 infection and provide a supplementary protection method for health care workers in daily practice.

DISCUSSION

The global pandemic of SARS-CoV-2 in 2020 has caused tremendous pressure on the health systems of almost every country in the world. Due to inappropriate protection and shortage of medical supplies, many medical staffs got infected (47). SARS-CoV-2 has relatively high infectivity and mainly spreads *via* close contact and droplets. There is ongoing hot debate on the potential role of ocular surface in the transmission of SARS-CoV-2, and some clinical and laboratory findings support that ocular involvement was observed in a minority of patients. For medical workers with high risk of aerosol exposure and close contact with patients, ocular surface may be a potential and overlooked site of contamination. WHO has alarmed medical stuffs to wear protective goggles during the whole contact period with patients who were suspected or confirmed to be infected (48).

However, for daily medical practice in ordinary clinics, wearing eye goggles is not mandatory or always practical. Although eye goggles seem to provide the best protection and not harmful to ocular surface, they have several disadvantages. First of all, goggles are generally uncomfortable to use, and very likely to disturb vision due to extensive formation of water droplets. Thus protective goggles are very inconvenient for doctors who require precise vision, including ophthalmologists, dentists, surgeons and so on. Besides, long-term use of eye goggles is reported to disturb working status and may lead to increased medical errors. In a recent survey conducted during SARS-CoV-2 outbreak on 231 nurses in China, use of eye goggles caused headache, skin pressure injury and dizziness in 79%, 66%, and 49% of nurses, respectively. 82.7% of nurses subjectively reported that use of eye goggles negatively impacted their working status, and events of medical errors were reported in 19.5% of nurses wearing goggles (49). Third, foggy goggles may interfere with vision and need frequent adjustment during use, which was reported in 59.7% of nurses in China (49). The adjustment may lead to increased risk of being infected. In addition, SARS-CoV-2 infection due to dislocation of eye goggles has also been reported in an emergency nurse (17). Due to long incubation period and relatively high proportion of asymptomatic infection of SARS-CoV-2, it is difficult to identify infected patients in the beginning (50). So during the outbreak period, any medical workers are at risk of being infected, because they may be likely to contact closely with an asymptomatic patient in the outpatient clinics or during physical examinations. For example, during the slit lamp or direct fundoscopy examination, an ophthalmology doctor need to directly face the patient at a distance of 3–10 cm. There is also huge risk of aerosol exposure during processes such as dental repair, open surgery, tracheal intubation and so on (51, 52). A recent survey conducted in British ophthalmology practitioners showed that they were very unconfident about no ocular protection in the daily work and called for more eye protection (53). Thus, it is necessary to provide adequate eye protection for medical workers during the outbreak period, as medical workers are at higher risk of aerosol exposure which can potentially result in risk of ocular contamination.

Based on the broad spectrum antiviral activity of griffithsin, we proposed a theoretical device of contact lenses with griffithsin nanoparticles as a potential alternative personal protective equipment against SARS-CoV-2. Although no previous data of the antiviral efficacy of griffithsin on SARS-CoV-2 is available, we made the hypothesis based on the efficacy study of griffithsin on other common viruses, including MERS-CoV, SARS-CoV, HIV, HCV, and so on (29–32). Griffithsin is continuously released onto the ocular surface and can bind directly to the S protein of coronavirus to block the entry of virus. The sustained releasing system enables prolonged protection time. Besides, contact lens doesn't disturb vision and is relatively well-tolerated by regular users. It can be served as voluntary choice for those who tolerate well and need precise vision during clinical practice. Based on current available results, ocular involvement is found in a small number of patients confirmed to be infected by SARS-CoV-2. We consider the ocular surface is likely to be a minor transmission route, so contact lenses containing griffithsin may provide sufficient protection for medical workers not directly facing high risk patients. Besides, as griffithsin has anti-viral activity against a broad spectrum of enveloped virus, this therapeutic contact lenses can be further applied in a variety of situations which require eye protection for medical practitioners. In addition, Decker et al. proposed a low cost lab-scale production method of griffithsin with engineered *E. coli*, which could generate more than 20 tons of griffithsin per year at the cost of below 3,500$ (42).

This would make the griffithsin-loaded contact lens affordable to the medical systems.

Despite the potential benefit for griffithsin-loaded contact lens to act against ocular transmission of SARS-CoV-2, special attention should be paid to the safety concerns associated with contact lens wear. Incidence of infectious keratitis, Acanthamoeba and fungal infections related to contact lens use is on the rise in recent years (54). According to a survey of contact lens users in USA, nearly a third of them reported previous contact lens-related red or painful eye requiring a doctor's visit (55). Thus, infection risk is a potential limitation for our proposed protection method. However, several ways can be taken to control the risk of bacterial keratitis. First of all, griffithsin-loaded contact lens is basically designed for health care workers, who generally have higher awareness of the importance of hand hygiene before applying (56). Second, the contact lens should be designed as daily disposable use to reduce infection associated with overnight wear, long-term use and case pollution (57, 58). As reported in a study in Australia, the rate of microbial keratitis associated with daily disposable contact lens wear is relatively low (1–2 per 10,000 wears per year) (59).

As therapeutic contact lens can only cover the corneal portion of the eye, there is potential risk of uncovered part to be infected. However, griffithsin can dissolve into tear film and spread over the ocular surface. This will expand its protection area beyond the covered part. Besides, as shown in previous studies, griffithsin is a highly potent antiviral agent and is effective at very low concentration, which indicates that griffithsin dissolved in tear film may also have antiviral activity (34, 36). As for SARS-CoV-2, no data of inhibition efficacy is currently available. Thus, pharmacokinetic studies of tear concentration after application of the therapeutic contact lens need to be compared with the antiviral concentration of SARS-CoV-2 in order to decide the longest protection time.

Another potential limitation of griffithsin-loaded contact lens is its potential ocular toxicity associated with the medication. Although lectin is commonly used in ocular formulation to improve drug retention time, currently no ocular formulation and safety profile of griffithsin on ocular tissues is available. As indicated in the inhibition study of SARS-CoV, griffithsin is multivalent and can effectively inhibit the virus at low concentration of 0.28–0.96 μM. A previous safety study showed that mucosal or systemic administration of 2 mg/kg griffithsin on mice should no systemic toxicity *in vivo* (60). An *in vitro* study showed that compared with other anti-viral lectins, application of griffithsin showed minimal effects of toxicity, T cell activation and alteration of gene expressions, which indicated excellent safety profile (41). To date, the safety of griffithsin has been tested in two phase 1 clinical trials on human (NCT04032717 and NCT02875119), but the results have not been published. In the two clinical trials, griffithsin was applied as either vaginal gel (at variable doses) or rectal enema (4.2 ml in volume containing 9.6 mg/ml of griffithsin) to prevent HIV-1 infection. As ocular surface is a special area and more sensitive to drug irritation, more *in vitro* and *in vivo* preclinical studies on the ocular safety of different doses of griffithsin are preliminarily required. The safety issues regarding long-term ocular application of griffithsin *via* contact lens need to be verified and the concentration of griffithsin need to be set at minimal inhibition concentration in order to avoid supratherapeutic toxicity. The protection benefits and potential adverse effects of griffithsin-loaded contact lens should be balanced and considered before applying for use in clinics.

Overall, griffithsin-loaded contact lens can be considered as a supplementary choice for ocular protection besides eye goggles for health care workers during SARS-CoV-2 outbreak.

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

LW conducted literature search. YD provided guidance and approved the final manuscript. Both authors proposed and discussed about the idea of the hypothesis and contributed to manuscript writing and editing.

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REVIEW
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Clinical Characteristics, Diagnosis, and Treatment of Major Coronavirus Outbreaks

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Human coronavirus infections have been known to cause mild respiratory illness. It changed in the last two decades as three global outbreaks by coronaviruses led to significant mortality and morbidity. SARS CoV-1 led to the first epidemic of the twenty first century due to coronavirus. SARS COV-1 infection had a broad array of symptoms with respiratory and gastrointestinal as most frequent. The last known case was reported in 2004. Middle East respiratory syndrome coronavirus (MERS-CoV) led to the second outbreak in 2012, and case fatality was much higher than SARS. MERS-CoV has a wide array of clinical presentations from mild, moderate to severe, and some patients end up with acute respiratory distress syndrome (ARDS). The third and recent outbreak by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) started in December 2019, which lead to a global pandemic. Patients with SARS-CoV2 infection can be asymptomatic or have a range of symptoms with fever, cough, and shortness of breath being most common. Reverse transcriptase-Polymerase chain reaction (RT-PCR) is a diagnostic test of choice for SARS CoV-1, MERS-CoV, and SARS CoV-2 infections. This review aims to discuss epidemiological, clinical features, diagnosis, and management of human coronaviruses with a focus on SARS CoV-1, MERS-CoV, and SARS CoV-2.

Keywords: COVID-19, MERS, SARS, SARS-CoV-2, clinical

INTRODUCTION

Coronaviruses (CoV) are the largest group of viruses in Nidovirales order with spike-like projections, which led to the name "Coronavirus." The CoVs have caused three global outbreaks in the last 20 years, with coronavirus disease-2019 (COVID-19) being the latest. The first epidemic of the twenty first century was Severe Acute Respiratory Syndrome (SARS) caused by SARS-CoV (SARS-CoV-1), which was first reported in November 2002 in Guangdong China, leading to 8,098 laboratory-confirmed cases with a case fatality rate of 9.6% globally (1, 2). The Middle East Respiratory Syndrome (MERS) caused by MERS-CoV was the second outbreak, first reported in Saudi Arabia in 2012 with 2,521 laboratory-confirmed cases with a case fatality rate of 36% (3). SARS-CoV-2 causes the third and most recent CoV outbreak (COVID-19). It first originated

in Wuhan, China, after a cluster of patients presented with atypical pneumonia-like respiratory symptoms with a shared history of visits to a local Wuhan seafood market. Initially, the virus was thought to be a novel CoV and was labeled as 2019-novel CoV (2019 nCoV) (1, 4, 5). The outbreak was declared as a public health emergency by the World Health Organization (WHO) on Jan 30th, 2020 (6). It continued to spread globally and was declared a pandemic on March 11th, 2020, by WHO. The 2019-nCoV was later identified and renamed as SARS-CoV-2. SARS-CoV-2 is a zoonotic disease that most likely originated in bats. It primarily causes respiratory illness, very similar to SARS-CoV and MERS-CoV, with a much higher rate of transmission (7). The number of cases of COVID-19 continues to increase around the world, with more than 34.5 million cases and >1 million deaths worldwide as of October 2, 2020.

These outbreaks of SARS, MERS, and COVID-19 share many similarities, including the clinical presentation, transmission, and management. Although acute respiratory tract infections are the most common clinical manifestations, extrapulmonary symptoms are increasingly recognized (8–10). In a retrospective analysis of 138 SARS patients in Hong Kong, 28% of patients had watery diarrhea as their presenting complaint (11). In a meta-analysis based on COVID-19 patients, the pooled prevalence of gastrointestinal (GI) symptoms was found to be 17.6% (95% confidence interval [CI], 12.3–24.5%), and the RNA virus was detected in stool samples in about 48.1% (95% CI, 38.3–57.9%) of the patients (8). The case fatality of MERS (36%) is much higher than SARS (9.5%) and COVID-19 (2.3%) (3, 12).

SARS, MERS, and COVID-19 all have a zoonotic origin. Respiratory droplets also spread SARS infection. SARS was contained by public health measures like isolation of patients, tracing and strict quarantine of contacts, community quarantine, surveillance, and social distancing. The primary reservoir for MERS-CoV in dromedary camels. Although it is human to human transmission, most have the primary case started by acquiring infection from the camel. Most human to human transmission cases of MERS occurs while in close contact with infected persons like healthcare settings, households, and workplaces. Systematic and strict infection control measures in these situations have helped to limit the spread. Compared to SARS and MERS, COVID-19 is more transmissible but lower mortality, which led to wide transmission. Most cases are asymptotic to mild symptoms, and this, along with increased globalization since MERS and SARS infection, led to the spread of COVID-19 more rapidly. Based on lessons learned from SARS and MERS outbreaks, there is an increased international collaboration between various governments and organizations, which led to the rapid development of diagnostic tests after the Chinese Ministry of Health shared the genetic sequence SARS-CoV-2 virus.

This review aims to discuss the epidemiology, classification of CoV, clinical features, diagnosis, and management along with vaccine options for SARS, MERS, and COVID-19.

CORONAVIRUSES

The CoVs are RNA viruses of the Coronavirinae subfamily, Coronaviridae family, and Nidovirales order (International Committee of Taxonomy of Viruses) (**Figure 1**). Coronavirus is a group of large, single positive-sense, enveloped, highly diverse RNA viruses. The RNA genome is 27–32 kb in size, largest among RNA viruses, capped, and polyadenylated in nature (14–16). Under cryo-electron tomography and cryo-electron microscopy, CoV virions have a spherical shape around 125 nm in diameter, club-shaped spike projections arising from the virion's surface. These crown-like spikes give the appearance of a solar corona, thus naming them as coronavirus. The nucleocapsid is in the virion's envelope, and these nucleocapsids are helically symmetrical, which is not a common finding in positive-sense viruses (17).

The CoV genome has 6 to 10 open reading frames (ORFs). Spike (S) protein (trimeric), membrane (M) protein, envelope (E) protein and nucleocapsid (N) protein are structural proteins of CoV. Beta-CoVs also have hemagglutinin esterase (HE) glycoprotein. RNA has a cap structure at the 5' end and polyadenyl sequences at the 3' end. The 5' end codes for polymerase, followed by genes for envelope proteins and the nucleocapsid protein. The CoV genetic material is very susceptible to frequent mutations, leading to new strains of the virus with differing virulence (14, 18). Virions of CoV attach to the host cell surface receptors via its protein spikes and through the viral envelope's infusion with the plasma membrane of an endocytic vesicle releasing its genome into the host cell. The entire replication cycle occurs in the cytoplasm, involving the production of subgenome-sized (sg) minus-strand and full-length RNA intermediates. The viral genome serves as mRNA for the replicase polyproteins and a template for minus-strand synthesis (19).

Coronavirus Classification

Coronavirinae is subdivided into four genera based on protein sequences, genomic structures, and phylogenetic relationships. Four genera are Alphacoronavirus (Alpha-CoV), Betacoronavirus (Beta-CoV), Gammacoronavirus (Gamma-CoV), and Deltacoronavirus (Delta-CoV) (15, 20). While Alpha-CoV and Beta-CoV are known to infect mammals, Gamma-CoV and Delta-CoV infect both birds and mammals. The primary host for Alpha-CoV and Beta-CoV are bats and rodents, while birds are the primary host for Gamma-CoV and Delta-CoV. Coronaviruses cause infections in avian and mammalian species manifesting in the form of respiratory illness (pneumonia, acute respiratory distress syndrome), GI symptoms (diarrhea, nausea, vomiting), hepatitis, encephalomyelitis, vasculitis, and coagulopathy. These viruses account for almost 30% of the common cold cases in human beings, mainly due to HCoVs (HCoV-OC43, HCoV-HKU1, HCoV-229E, and HCoV-NL63). The SARS, MERS, and COVID-19 can present with both respiratory and gastrointestinal symptoms (14, 18).

Human Coronavirus (HCoV)

There are seven known HCoVs. All of these HCoVs have an animal origin and are found primarily in rodents or bats based on the current sequence databases (21). Out of seven, HCoV-229E and HCoV-NL63 are alpha-CoVs. HCoV-OC43, HCoV-HKU1, SARS-CoV-1, MERS-CoV, and SARS-CoV-2 are

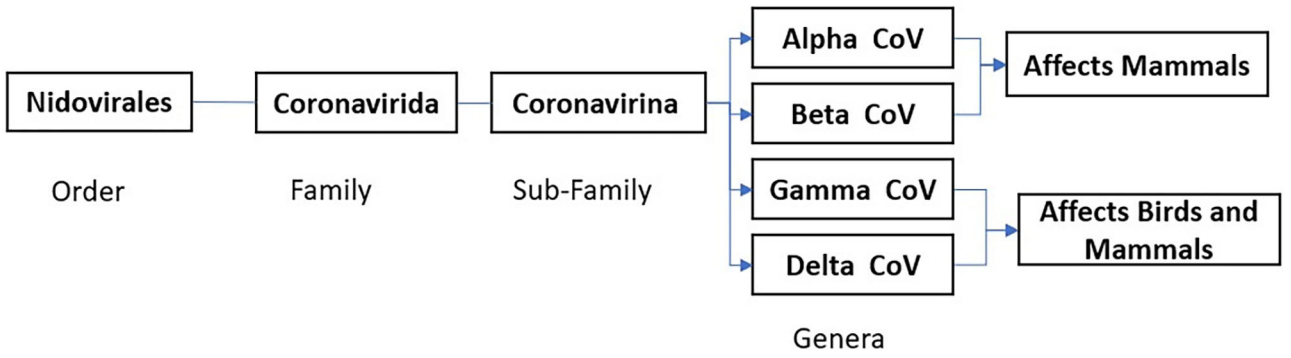

FIGURE 1 | Corona Virus Classification based on International Committee Taxonomy of Virus (ICTV) ninth report 2011 (13).

beta-CoVs (**Figure 2**) (7). SARS-CoV-1, SARS-CoV-2 MERS-CoV, HCoV-NL63, and HCoV-229E originated in bats, whereas HCoV-OC43 and HKU1 likely originated in rodents (20). The last three CoVs (SARS-CoV-1, MERS-CoV, and SARS-COV-2) have led to major outbreaks causing significant mortality and morbidity.

HCoV-229E

In 1966, HCoV-229E strain B814 was the first-ever isolated HCoV identified from the nasal passage of a patient who presented with the common cold. The patients infected with HCOV-229E present with symptoms of the common cold (sneezing, sore throat, headache, malaise, and 20–30% patients also have fever and cough). The incubation period is 2–5 days. HCoV-229E peaks during the winter season in tepid climates (7).

HCoV-OC43

HCoV-OC43 was first reported in 1967. While it has a similar clinical presentation, time of incubation, and epidemiology with HCoV-2294, but it has no serological cross-reactivity with HCoV-229E. The symptomatology due to these two viruses mimics those of influenza and rhinovirus. HCoV-OC43 has been shown to have infected neurons in *in-vivo* studies in mice and also neuroinvasive features clinically. It also peaks during the winter season in tepid climates (7, 22).

HCoV-NL63

The first case of HCoV-NL63 was reported from a 7 months-old girl in the Netherlands in 2004. Children under the age of 5 years are most commonly infected, but it can infect all age groups. The patient infected with HCoV-NL63 typically presents with coryza, fever, bronchiolitis, fever, and may even present with croup in some rare cases. The incubation period is typically 2–4 days. Patients with HCoV-NL63 have co-infection with other respiratory viruses in about 71% cases. It is globally widespread and peaks during early summer, spring, and winter seasons (7, 22).

HCoV-HKU1

HCoV-HKU1 was first discovered in 2004. HCoV-HKU1 presents as mild respiratory symptoms. It also peaks in the winter season, and the incubation period is 2–3 days (7). HCoV-229E, HCoV-OC43, HCoV-HKU1, and HCoV-NL63 are all transmitted by respiratory droplets and fomites. It accounts for up to 15–30% of respiratory infections in a year and causes more severe disease in the elderly, immunocompromised individuals (such as those with underlying co-morbidities and neonates) (17).

SARS-CoV

SARS-CoV or SARS-CoV-1 is the first coronavirus known to cause severe acute respiratory distress syndrome (ARDS). After the discovery of the SARS-CoV-2 virus in 2019, SARS-CoV is also referred to as SARS-CoV-1. SARS was first reported in 2002 and then spread globally with the last reported case in 2004. Infected patients presented with myalgias, malaise, fever, chills, cough, dyspnea, and respiratory distress as a late symptom. In severe cases, multi-organ involvement was reported (GI, liver, and kidney) (7). Diarrhea was reported in 40 to 70% of SARS-CoV-1 cases (9, 11, 23). Abnormal liver chemistries, elevated creatinine kinase, and lymphopenia were common laboratory findings. The route of transmission included respiratory droplets, fomites, and fecal-oral routes. The Chinese horseshoe bat was found to be a natural host of SARS-CoV-1 with the civet as an intermediate host. SARS-CoV-1 utilizes angiotensin-converting enzyme 2 (ACE2) receptors, which are almost omnipresent in the body (7, 17, 24).

MERS-CoV

MERS-CoV was first reported from Saudi Arabia in 2012. Patients present with fever, cough, chills, sore throat, myalgias, arthralgias, dyspnea, pneumonia, and acute renal failure. In up to 30% of patients, gastrointestinal symptoms like vomiting and diarrhea can be seen. The route of transmission is by respiratory droplets and fomites. Bats are likely the animal reservoir host, and dromedary camels are likely the intermediate host for human transmission. MERS-CoV utilizes Dipeptidyl peptidase 4 (DPP4) as its receptor (7, 17, 24).

SARS-CoV-2

Patients primarily present with fever, cough, and dyspnea. A systematic review and pooled analysis of 45 studies showed that fever (81.2%), cough (62.9%), loss of appetite (33.7%),

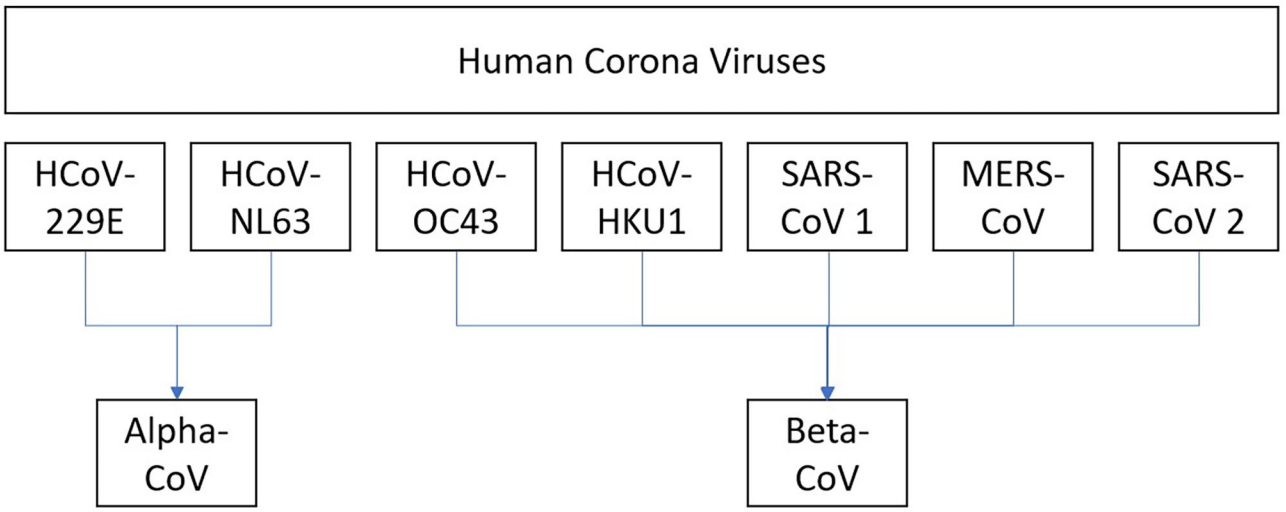

FIGURE 2 | Human coronaviruses.

shortness of breath (26.9%), loss of taste (25.4%), and sputum production (24.2%) were common symptoms reported by patients (25). Another systematic review and meta-analysis showed that fever (76.70%), cough (67.76%), olfactory (44.40%), gustatory (38.16%), dyspnea (37.49%), fatigue (29.93%), sputum production (17.85%), sore throat (16.7%), and headache (15.49%) were common symptoms observed in COVID-19 patients (26). The prevalence of gastrointestinal symptoms like diarrhea (9.1%), nausea/vomiting (5.2%), and abdominal pain (3.5%) were reported in COVID-19 positive patients (27). ARDS, acute respiratory failure, arrhythmias, septic shock, acute cardiac injury, cardiomyopathy, acute renal failure are common complications observed in these patients (25, 26). The primary transmission route is respiratory droplets, but there are reports of transmission via fomites or fecal-oral route have been seen (7, 21). SARS-CoV2 uses human ACE2 receptors, which is utilized by SARS-CoV-1, but it was found to have a higher affinity for these receptors than SARS-CoV-1, which in turn can partly explain why SARS-CoV-2 is more infectious than SARS-CoV-1 (28, 29).

SEVERE ACUTE RESPIRATORY SYNDROME (SARS)

The first case of the severe acute respiratory syndrome (SARS) was found in Foshan city of Guangdong province in China on November 16th, 2002, and it spread to more than 30 countries across five continents. There has been a total of 8,098 cases and 774 deaths caused by SARS-CoV-1 (30, 31). WHO declared the end of the SARS epidemic in July 2003. Four more SARS-related incidents occurred from July 2003 to January 2004. Three of those incidents were due to laboratory biosafety breaches in Singapore, Taipei, and Beijing leading to the occurrence of seven cases. There were four sporadic community-acquired cases reported in China. No new cases of SARS have been reported since January 2004 (32). SARS-CoV-1 had a mortality rate of 9%, and mortality reached up to 50% in patients who were older than 60 years (33).

Multiple studies were performed to investigate the role of primary animal hosts and intermediary hosts as the outbreaks typically started in live animal markets in China. In a seroprevalence study conducted in Guangdong, China, 9.1% were tested positive for the SARS-CoV-1 IgG antibody. These positive IgG antibodies were higher in the animal trader group (13%) when compared to 1–3% of persons in control groups. Further investigation showed that these animal trader groups predominately traded "masked palm civets" among other animals (34). Another study showed that SARS-CoV-1 was isolated from other animals such as raccoon dogs and in humans working in the same market. All the animal isolates retained a 39-nucleotide sequence (35). Despite these findings, widespread SARS-CoV-1 infection was not noted in the civet cats suggesting that it was most likely an intermediate host (36). In 2005, one of the horseshoe bats species was found to have an 88–92% nucleotide sequence with SARS-CoV-1. This indicated that bats were more likely the natural host for this virus (37).

Incubation Period

The estimated mean incubation period for SARS-CoV-1 infection was 4.6 days (95% Cl, 3.8–5.8 days), with 95% of cases having disease onset within 10 days, which could extend as long as 16 days (32, 36, 38). A study from Hong Kong on 1,755 patients showed that the average time from symptom onset to need for invasive mechanical ventilation and death was 11 and 23.7 days, respectively (38). The diagnosis is made by contact history, laboratory tests along with clinical manifestations (39). The WHO proposed five criteria to assist in the diagnosis, as depicted in **Figure 3**. Patients have suspected SARS if they meet criteria 1 to 4 (or) 2 to 5 unless they have an alternative diagnosis to explain their illness (36).

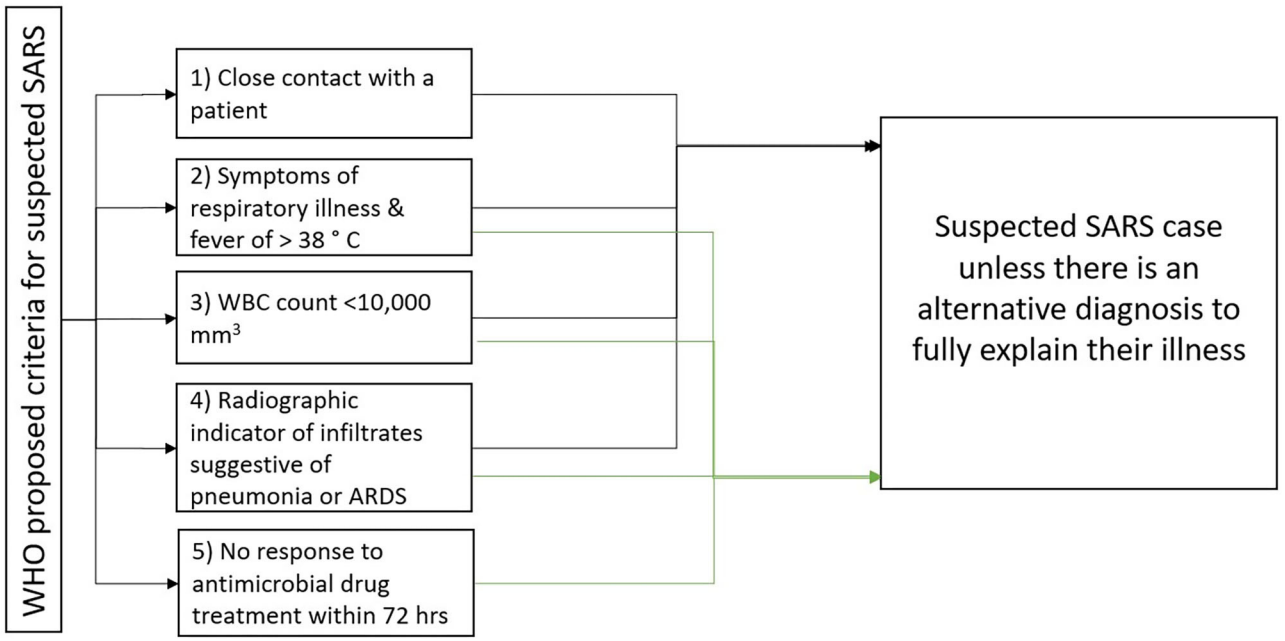

FIGURE 3 | WHO criteria for suspected SARS case (36).

Clinical Manifestations

Clinical symptoms of SARS include fever, chills, myalgia, malaise, dry cough, shortness of breath, and headache. Nausea, vomiting, dizziness, and upper respiratory symptoms like sore throat, rhinorrhea were less frequent (**Table 1**) (40, 41). In more than 60% of cases, radiographic changes were observed to be present on initial presentation, and in 41% of cases, the radiographic changes occurred before lower respiratory tract symptoms (39). Patients manifest symptoms in different stages. Fever, dry cough, myalgia, and malaise were presenting symptoms in the first week, which were shown to improve most patients. Returning of fever, along with worsening lung consolidation and respiratory failure, were observed during the second week in about 20% of the patients, which could potentially result in acute respiratory distress syndrome (ARDS) (32).

Diarrhea was one of the common symptoms observed in patients with SARS (32, 39). In a retrospective study with confirmed SARS cases in Hong Kong, 28% of patients had watery diarrhea as presenting symptoms. Furthermore, 38.4% of patients developed diarrhea during illness. Diarrhea lasted for a mean duration of 3.7 days and resolved spontaneously in most cases. Moreover, SARS-CoV-1 RNA was detected in the stool for up to 10 weeks after the onset of symptoms (11). In children under the age of 12 years, symptoms were much milder than adults, but the teenage individuals had similar presentations as adults. Fortunately, there was no known mortality in young children and teenagers. The mortality rate increased with age, especially those with multiple comorbidities (32, 39). Elderly patients sometimes presented with atypical symptoms such as decreased well-being, confusion, and falls (32). Epidemiologic showed that asymptomatic infections were common in SARS. A meta-analysis showed that the overall seroprevalence among humans (except animal handlers) was 0.10% (95% Cl, 0.02–0.18). Healthy blood donors and individuals recruited from the health-care setting showed a seroprevalence of 0.16% (95% CI, 0–0.37) compared to overall prevalence (42). Furthermore, healthcare workers and individuals who had close contact with SARS patients had a higher seroprevalence of 0.23% (95% Cl, 0.02–0.45). Transmission of the virus occurred predominately after the fifth day of illness, probably due to low viral load in the upper respiratory tract (especially during the early phase of the illness). Unlike COVID-19, the lack of a large number of asymptomatic carriers and paucity of transmission in the early phase of illness (first 5 days) assisted in aggressive case detection, contact isolation, and control of this global outbreak (39) (**Table 1**).

Diagnosis

Laboratory Diagnosis

Reverse transcriptase PCR (RT-PCR) assay for the detection of viral RNA is the test of choice for SARS diagnosis (**Table 1**). Viral RNA has been found in both upper and lower respiratory tract secretions, serum, stools, and urine specimens, enabling RT-PCR to be performed on all these samples (32, 39). As viral load is low during the first 5 days of illness, a negative specimen during this time does not exclude the diagnosis. Furthermore, the lower respiratory tract (sputum, tracheal aspirate, and bronchoalveolar lavage) samples have a higher viral load than those of the upper respiratory tract (nasal, pharyngeal, and nasopharyngeal). Therefore, a single specimen from the upper respiratory tract also does not rule out the diagnosis. Testing multiple specimens improves the rate of detection (39). Viral cultures could be used

TABLE 1 | Epidemiological and clinical features of SARS, MERS, and COVID-19.

Disease	SARS	MERS	COVID-19
First reported case [Year]	2002	2012	2019
Country of diagnosis	China	Saudi Arabia	China
Human Corona Virus [HCoV]	SARS-CoV-1	MERS-CoV	SARS-CoV2
Genera	Beta-CoV	Beta-CoV	Beta-CoV
Mode of transmission	Human to Human	Human to Human and Contact with infected camel	Human to Human
Natural reservoir	Bats	Bats	Bats
Intermediate host	Civet	Dromedary camels	Pangolins
Common clinical features	Fever, chills, malaise, dry cough, shortness of breath, headache, nausea, vomiting, diarrhea	Fever, chills, headache, runny nose, dry cough, sore throat, abdominal pain, nausea, vomiting, diarrhea	Cough, fever, shortness of breath, abdominal pain, diarrhea, vomiting
Laboratory findings	Marked lymphopenia, elevated ALT, elevated lactate dehydrogenase (LDH), pro-inflammatory cytokines	Leukopenia or lymphocytosis with lymphopenia, elevated transaminases, elevated LDH, elevated creatinine	Lymphopenia, elevated CRP, elevated AST, elevated procalcitonin level, elevated PT, aPTT, D-dimer, and ESR
Radiographic findings	Normal appearance, interstitial thickening, focal to multilobular airspace opacity with airspace opacities most common	Focal to multilobar airspace disease, ground-glass opacities, and occasional pleural effusions with ground-glass opacities being most common	Ground glass opacities (GGO), consolidation, paving stone sign, pleural thickening, vascular thickening, and fibrinous lesions common findings
Case fatality (%)	9.5	36	2.3
Number of cases and deaths	8,098 cases, 774 deaths	2,521 cases, 919 deaths (by Jan 16th 2020)	More than 8 million cases, 438,000 deaths (by June 16th, 2020)

for diagnosis but takes a long time and require processing in biosafety level 3 facilities. Hence, they are restricted to special cases or for research purposes only (32, 39).

Marked lymphopenia involving both B and T lymphocytes (CD4 and CD8 subsets), and natural killer (NK) cells are observed in SARS patients (39). Low levels of CD4 and CD8 on presentation are associated with worse clinical outcomes (43). Pro-inflammatory cytokines and chemokines like interleukin 1 (IL-1), IL-6, IL-8, IL-12, C-C motif chemokine ligand 3 (CCL3), and CCCL10 levels also elevated (39). High Lactate dehydrogenase (LDH) level on admission is associated with higher mortality (38). Reactive hepatitis has been reported as a common complication in SARS patients. In a study of 294 SARS patients, 24% (70/294) had elevated alanine transaminase (ALT) on admission, and 69% (209/294) developed ALT elevation during the course of hospitalization (44). Liver function with elevated ALT increased further in patients who received systemic corticosteroid and ribavirin for treatment (32). Spontaneous recovery in the elevation of ALT was noticed in most patients with improvement in the disease. Though precise etiology for this abnormal ALT is unclear, cytokine release from inflammatory cells is the probable culprit (44). Other common laboratory abnormalities included acute kidney injury, elevated creatine kinase, and thrombocytopenia (45).

Radiographic Diagnosis

The common Chest X-ray findings are unilateral, or bilateral peribronchial thickening or airspace infiltrates (32, 46). High-resolution computer tomography (HRCT) can detect early lung parenchymal changes. Some of these include interlobular septal and intralobular interstitial thickening, consolidation, and ground-glass opacification, predominantly involving peripheral lung fields and lower lobes (32). While these findings are not pathognomonic, they are supplementary to the diagnosis of SARS patients.

Treatment

Antiviral Therapy

Ribavirin

Ribavirin is a synthetic nucleoside analog that was used empirically for the treatment of the SARS patients during the outbreak in 2003. Clinical studies, including a retrospective case series, and one randomized clinical trial with multiple clinical arms, were performed to determine the effectiveness of ribavirin in SARS patients. However, no conclusive determination could be made (32, 47). In a study conducted in the Greater Toronto area with 144 patients, 126 patients were treated with a higher dose of ribavirin, about half the patients developed drop of hemoglobin (>2 g/dl), and 40% of patients had 1.5-fold increase transaminases (32, 46). Although the exact cause of the drop in hemoglobin is uncertain, the hemolysis was proposed to be the likely cause. Other adverse effects noticed with ribavirin included bradycardia and teratogenicity (48). There is no conclusive data that ribavirin was effective in SARS, and significant side effects were seen.

SARS-CoV protease inhibitors

Protease inhibitors block virus entry and/or inhibit protease (cathepsin L) lysis (49). A combination of Lopinavir and ribavirin showed clinically significant synergistic *in-vitro* activity against SARS-CoV-1 prototype HKU39849. It was used clinically in addition to a standard treatment protocol (50, 51). When compared with the standard treatment regimen (ribavirin and

steroids) treatment, combination therapy with lopinavir and ribavirin showed a decrease in the overall mortality rate (15.6% vs. 2.3%, $P < 0.05$) and intubation rate (11% vs. 0%, $P < 0.05$) (51).

Other protease inhibitors like Nelfinavir, Calpain inhibitor VI (Val-Leu-CHO), and calpain inhibitor III (Z-Val-Phe-Ala-CHO) were studied *in-vitro* for potential effects in SARS (47). Nelfinavir is an HIV-1 protease inhibitor with a safety profile already established in humans, and it showed to inhibit the replication of SARS-CoV-1 in Vero E6 cells (52). Calpain inhibitor VI (Val-Leu-CHO) and calpain inhibitor III (Z-Val-Phe-Ala-CHO), which are cellular cysteine proteases, were found to be potent inhibitors for SARS-CoV in Vero Cell (53).

Viral binding inhibitors

The angiotensin-converting enzyme 2 (ACE2) is a cellular receptor that interacts with the S1 domain of the spike protein. Compounds and peptides that bind to ACE2 can be theoretically used as an agent for the treatment and prevention of SARS (47). Sui et al. showed that recombinant single-chain variable region fragments (scFvs) against the S1 domain of SARS spike protein could be used as a target to inhibit the virus. One such human monoclonal antibody includes 80 R, which can inhibit syncytia formation between ACE2 and spike protein. This agent has been studied *in-vivo* in animal studies to determine its clinical use for emergency prophylaxis and treatment of SARS (54).

Fusion inhibitors

In-vitro evidence shows that fusion inhibitors could be potentially used against SARS-CoV-1 as it prevents the attachment (fusion) of the viral envelope to the host cell membrane. Bosch et al. tested peptides derived from the membrane-proximal (HR2) and membrane-distal (HR1) (heptad repeat region) of the spike protein as inhibitors of SARS-CoV-1. HR2 but not HR1 peptides were found to be inhibitory against SARS-CoV-1 (55). Similarly, another *in-vitro* study showed that one peptide, CP-1 derived from the HR 2 region, inhibited SARS-CoV-1 infection at the molecular level (56). This inhibitory potency of the HR2 peptides against SARS-CoV-1 was initially promising, but none of them made it to the clinical trials.

RNA Interface

RNA interference treatment (RNAi) technology has been used to target human immunodeficiency virus (HIV), Hepatitis B, and Hepatitis C viral infections. It is a process by which small interfering RNA (siRNA) is administered, leading to mRNA degradation (47). In an *in vivo* study conducted by Zhang and colleagues, specific siRNAs targeting the S gene in SARS-CoV-1 were constructed, and it showed that siRNA could effectively and specifically inhibit gene expression of Spike protein in SARS-CoV-1 infected cells (57). SiRNA inhibitors were studied in 21 rhesus macaques, 20 of them in 5 groups ($n = 4$) infected with SARS-CoV-1 strain PUMCO1, and one individual was for observation (without infection). Five groups included two control groups (infection control, non-specific SiRNA control) and three treatment groups (prophylactic treatment, co-delivery, and post-exposure treatment). Over the next 20 days, they were observed for SARS-like symptoms, SARS-CoV-1 RNA presence, lung histopathology, and immunochemistry changes. Macaques in the treatment group had less severe SARS-like symptoms with the relief of fever, decreased viral levels, and lower acute diffuse alveolar damage. This study suggested that siRNA may be used to reduce the severity of disease and decrease viral load (58). Other compounds like glycyrrhizin, a component of liquorice root, nitric oxide, niclosamide (antihelmintic drug) have shown *in-vitro* activity against SARS-CoV-1 by inhibiting replication of the virus, and no clinical studies have been performed using these agents (47).

Steroids

Systemic steroids were administered as one of the mainstay therapy during the SARS outbreak. Although multiple reasons exist for their use, the primary mechanism appears to be the anti-inflammatory role of steroids. First, multiple patients affected with SARS show clinical features consistent with cryptogenic organizing pneumonia (COP), which respond to steroids and are likely caused by immune hyperactivity and cytokine dysregulation. Second, in patients with severe SARS, there was evidence of hemophagocytosis in the lung, attributed to cytokine dysregulation. Additionally, steroids might play a role in mitigating the clinical progression of pneumonia and respiratory failure association with a peak level of SARS-CoV-1 viral load mediated by the host inflammatory response (32, 47).

Steroids are used as adjunctive therapy to ribavirin treatment in most cases. If the patient's respiratory status deteriorated, pulse dose steroids were added in studies reporting improved clinical outcomes (47). Overall, data on the use of steroids is controversial and adverse events were noted. A retrospective cohort analysis showed that the use of pulse methylprednisolone was associated with an increased risk of 30 day mortality (adjusted odds ratio [aOR] 26.0; 95% CI, 4.4–154.8) (59). Furthermore, a systemic review concluded that systemic steroids were not associated with any definite benefits but had potentially adverse effects like infectious complications, avascular necrosis, and steroid-induced psychosis (60). Prolonged use of steroids can also increase the risk of nosocomial infections, such as disseminated fungal disease, metabolic derangements, psychosis, and osteonecrosis (32).

Interferon

Interferon-alfa (IFN-α) has been used in the treatment of Hepatitis B and C. A similar approach was tried in *in-vitro* studies against SARS-CoV-1 replication (47). Pegylated (PEG) IFN-α is shown to significantly reduce viral replication, excretion, and expression by type-1 pneumocytes when given prophylactically to macaques before experimental infection with SARS-CoV-1. Postexposure treatment with PEG IFN-α showed intermediate results only (61). In a study of 22 patients with SARS infection, patients who received IFN-alfacon-1 along with corticosteroid (combined approach) showed rapid resolution of radiographic lung abnormalities, lower levels of creatine kinase, rapid normalization of lactate dehydrogenase level, improved oxygen saturation ($p = 0.02$), and lower rates of tracheal intubation (11.1% vs. 23.1%) and death (0.0% vs. 7.7%) compared with the corticosteroid monotherapy group. When combination

therapy was given during the late-stage to six critically ill patients, four died despite therapy. This suggests that treatment during the early stages of the disease is essential (62).

Convalescent Plasma

During the outbreak, one of the initially proposed hypotheses was to use convalescent plasma from a patient fully recovered from SARS to treat patients having active SARS infection (32, 47). A retrospective study comparing convalescent plasma and pulsed steroids showed that patients in the plasma group had a higher discharge rate (77% vs. 23%, $p = 0.004$) and lower mortality (0% vs. 23.8% $p = 0.049$) when compared to the steroid group (63). In another study, patients who received convalescent plasma before day 14 had a higher day 22 discharge rate than those who received after day 14 (58.3% vs. 15.6%; $P < 0.001$). Similarly, a higher discharge rate was observed in patients with PCR positive and seronegative for CoV at the time of plasma infusion compared to seropositive patients (66.7% vs. 20%; $P = 0.001$) (64). Monoclonal antibodies obtained from immortalized B-lymphocytes isolated from patients with SARS during the convalescence period were shown to neutralize virus infection *in-vitro* and prevent replication *in vivo* in the mouse model of SARS-CoV-1 infection (65). These studies implicated that convalescent plasma is more effective if given early during disease. It can be given during the early phase of SARS if there is another outbreak (51, 52).

Prevention

Vaccines

Severe morbidity and mortality associated with SARS make it crucial to develop a safe and successful vaccine to prevent re-emergence and spread of disease (36). It is vital to develop protective immune responses, including neutralization antibody and cytotoxic T lymphocytes generation (66).

Inactivated vaccine

Inactivated vaccines consist of whole or a specific component derived from pathogen by killing or inactivating through various chemicals (formalin, β-propiolactone, and diethylpyrocarbonate) or radiation, which make the viral genome non-infectious while maintaining the structure of the virus and thus preserving antigenicity. Compared to a live vaccine, the inactivated vaccines are easy to prepare and cannot propagate disease in immunocompromised patients (67). Various studies on SARS-CoV-1 research showed that inactivated vaccines induce the production of neutralizing antibodies (68–70). The inactivated vaccine was administered to humans and was well-tolerated and elicited SARS-CoV-1 specific neutralizing antibodies (71). However, no data on vaccine efficacy is available due to a lack of a natural challenge (72).

Viral vector vaccines

In viral vector vaccines, vaccine antigen is produced *in situ* upon infections of cells. Vector virus can be either an attenuated virus or genetically alerted virus which cannot replicate (73). These vaccines have several features that make them induce efficiently both innate and B cell- and T-cell-mediated immune responses, including their ability to persist in the host as genetic material, ability to infect directly antigen-presenting cells. Adenovirus vectors have both spike and nucleocapsid proteins. Adenovirus vectors show variable results depending on the preparation, route of administration, and animal model used, but the challenge experiment has not been performed yet (67, 72).

Subunit vaccines

Subunit vaccines are comprised of purified antigen and only utilize antigenic components from the virus of interest. In the subunit vaccine, antigenic components are grown *in-vitro* and then harvested for vaccine use. This vaccine either contains a spike protein component or nucleocapsid protein. It induces a high level of B-cell and T-cell-mediated immune response and generates high titers of antibodies. However, there is no *in-vivo* experiment performed yet (67, 72).

DNA vaccines

DNA vaccines consist of plasmid DNA that code for viral antigen components, which are directly injected or otherwise inoculated in the vaccine. DNA vaccine induces both humoral and cellular immune responses. It also uses spike peptides to induce high titers of neutralizing antibodies. Although DNA vaccines have shown promise in preclinical models, their success in the clinical studies has been unsatisfactory (67, 72).

Live attenuated vaccines

These vaccines are made by decreasing or removing the virulence of live virus by using chemical or site-directed mutagenesis. This process makes the virus an attenuated pathogen capable of producing a subclinical infection. The live vaccine will result in an innate and adaptive immune response, which can last life-long. The efficacy and immunogenicity of a live attenuated vaccine consisting of a recombinant SARS-CoV-1 lacking E gene were studied (67, 72). In a study, Hamsters immunized with recombinant SARS-CoV-1 without E gene developed a high level of serum-neutralizing antibody titers, and they were protected from replication of homologous (SARS-CoV Urbani) and heterologous (GD03) SARS-CoV-1 in both upper and lower respiratory tract (74). Thus, the deletion of a gene may be the first step toward developing a live attenuated SARS-CoV-1 vaccine (72).

MIDDLE EASTERN RESPIRATORY SYNDROME

Epidemiology

MERS-CoV was first isolated from the sputum of a 60 year male from the city of Jeddah in Saudi Arabia on September 20th, 2012. A pancoronavirus RTPCR assay was used to isolate this virus (75). This patient died due to renal failure and severe respiratory disease due to MERS-CoV (76). MERS became an epidemic with 2521 laboratory-confirmed cases and 919 deaths (case fatality rate 36%) (3). MERS-CoV cases are predominately reported from the Arabian Peninsula, with around 84% from Saudi Arabia (3). Twenty-seven countries have reported cases of MERS. All cases

outside the Arabian Peninsula had either history of travel to the region or contact with someone who traveled to the region (3, 77).

The primary host of MERS-CoV remains unknown, and there is no definitive epidemiologic evidence linking MERS-CoV infection and bats. When more than 1,000 samples from *Taphozous perforates* bats (also called Egyptian tomb bat, species of Emballonuridae family) were analyzed, only a small amount of MERS-CoV closely matching to a human MERS-CoV was found (77). Dromedary camels (*Camelus dromedarius)* are major reservoir/intermediate hosts for MERS-CoV. Although there are cases of human-to-human transition, especially in health care settings due to close contact, while delivering unprotected care to a patient, the virus does not pass easily from the human-to-human (78). The WHO data shows that men are being affected more compared to women. The 50–59 years and 30–39 years age groups are at the highest risk of acquiring infection of primary and secondary cases, respectively (79).

Incubation Period

The median incubation period is estimated to be around 5.2 days, ranging from 1.9 to 14.7 days. The time interval between symptom onset in a patient and symptoms in contact was about 7.6 days (95% CI, 2.5 to 23.1) (80). Approximately 4 days is the median time from illness onset to hospitalization with a median length of stay of 41 days (76). The incubation period was also found to be correlated with the severity of the disease. The mean incubation period was shorter for patients who died compared to those who survived (81).

Clinical Manifestations

Pulmonary Symptoms

MERS has no specific signs and symptoms but mainly presents with respiratory manifestations. Clinical presentation ranges from asymptomatic cases to mild, moderate, severe disease with ARDS, multi-organ failure, and death (76, 77). These patients initially present with mild symptoms of low-grade fever, chills, headache, runny nose, dry cough, sore throat, dyspnea, and myalgia (**Table 1**) (76, 77). Patients can also have other respiratory tract symptoms like sputum production, wheezing, chest pain, headache, and malaise (80). Patients can deteriorate rapidly with progression to ARDS within a few days (80, 82, 83). Severe cases can present with pneumonia, ARDS, encephalitis, myocarditis, acute renal failure, secondary bacterial infection, or other life-threatening complications (83, 84).

Extrapulmonary Symptoms

Various extrapulmonary manifestations have been reported in patients with MERS, including acute renal impairment, which was present in up to half of patients. About 1/3rd of severely ill patients have GI symptoms. Anorexia, abdominal pain, nausea, vomiting, and diarrhea are common GI symptoms seen in patients with MERS (76, 77, 82). Other extrapulmonary manifestations include neurological, cardiac manifestations, hepatic and hematological complications. Cardiac complication includes pericarditis, arrhythmias, and hypotension. Neurological complications like ataxia, confusion, coma, and focal neurological symptoms were seen in a retrospective study of three patients in ICU from Saudi Arabia (85). In a single-center retrospective study of 70 patients, the majority of patients were old with a median age of 62 years, and 95.7% of patients with confirmed MERS-CoV infections were symptomatic. Studies also found arrhythmias in 15.7%, disseminated intravascular coagulation (DIC) in 14.7%, liver dysfunction in 31.4%, and acute kidney injury in 42.9% of the patients (86).

TABLE 2 | WHO released the last update for case definition (confirmed and probable case) for classification and reporting purposes on July 26th, 2017 (88).

Updated case definition by WHO July 26th, 2017	
Confirmed case	1. Patient with laboratory-confirmed MERS, regardless of clinical signs and symptoms
Probable cases	1. Patient with febrile acute respiratory illness with clinical, radiological, or histopathological evidence of pulmonary parenchymal disease, and a direct epidemiologic link with case of laboratory-confirmed MERS case; and laboratory testing for MERS-CoV is unavailable, negative on a single inadequate specimen or inconclusive
	2. Patient with febrile acute respiratory illness with clinical, radiological, or histopathological evidence of pulmonary parenchymal disease that cannot be explained entirely by any other etiology; and patient resides or traveled to the Middle East or another country where MERS-CoV is known to be circulating in dromedary camels or where human infections have recently occurred; and laboratory testing for MERS-CoV is inconclusive
	3. Patient with an acute febrile respiratory illness of any severity; and has a direct epidemiologic link with a confirmed MERS-CoV case, and laboratory testing for MERS-CoV is inconclusive

Risk Factors

Risk factors associated with severe MERS include old age, male gender, existing co-morbid conditions, low serum albumin, superimposed bacterial infections, and weaker immune system. About 76% of patients with MERS reported having at least one underlying co-morbid condition. The most common co-morbid conditions seen in hospitalized MERS patients were obesity, diabetes, hypertension, cardiovascular diseases, or end-stage renal disease, and these chronic diseases are thought to attenuate innate immunity response by down-regulating production of pro-inflammatory cytokines such as interferon-gamma (IFN-g) and interleukins (ILs) (76, 77, 84). The patients who died had increased frequency of comorbid conditions when compared with recovered or asymptomatic cases (86.8% vs. 42.4%, $p < 0.001$). The most commonly reported co-morbid condition included chronic renal failure (13.3%), diabetes (10.0%), and heart disease (7.5%) (87). Lungs of smoker patients have shown upregulation of DPP4 receptors, making them more prone to have severe disease than a non-smoker (77).

Diagnosis

No specific clinical features or radiographic features differentiate MERS from other respiratory viral infections, and diagnosis relies on laboratory findings (**Table 2**).

TABLE 3 | WHO interim guidance, Jan 2018: MERS-CoV Detection by NAAT/PCR (89).

MERS diagnosis based on nucleic acid amplification test (NAAT) testing	
Laboratory confirmed case	Two positive NAAT assays with different targets/sequencing on the MERS-CoV genome or One positive NAAT result for a specific target on the MERS-CoV genome and MERS-CoV sequence confirmation from a separate viral genomic target
Probable	Patients with a positive NAAT result for a single specific target without further testing but with a history of potential exposure and consistent clinical signs with MERS

Laboratory Diagnosis

Real-time reverse-transcription polymerase chain reaction (rRT-PCR) is a diagnostic test that is widely used for MERS infection as it is highly sensitive with a short turnaround time (**Table 3**) (77, 80). Three rRT-PCR assays are developed and routinely used for the detection of MERS-CoV. Assays target upstream of the E protein gene (UpE), the open reading frame 1b (ORF 1b), and 1a (ORF 1a). The assays for the UpE and ORF-1a targets have 100% sensitivity (95% CI, 91.1–100%) in detecting the infection (90). UpE assay is recommended for screening and ORF-1a or ORF-1b assay for confirmation (89).

Sample can be collected from upper respiratory tract specimens (nasopharyngeal and oropharyngeal) and lower respiratory tract specimens (sputum, tracheal aspirate, or lavage). Lower respiratory tract specimens have higher viral load than upper respiratory tract specimens as Dipeptidyl peptidase 4 (DPP4) receptors are expressed on non-ciliated bronchial epithelial cells and alveolar epithelial cells but not in upper respiratory tract epithelium. DPP4 are cellular receptors for MERS-CoV. Swabs from nasopharyngeal and oropharyngeal specimens should be collected on kits, which contain viral transport medium and both swabs from nasopharyngeal and oropharyngeal specimen should be placed in the same tube to increase the viral load (89).

If the first test, particularly upper respiratory tract specimen, comes negative in a patient with suspected MERS, a repeat test should be done, especially from lower respiratory tract specimens. In order to confirm the clearance of the virus, respiratory samples should be tested until there are two consecutive negative samples, and samples should be taken at least 2–4 days apart (89) (**Table 3**).

The infectious MERS-CoV virus can also be isolated from blood, urine, and fecal sample by culture but takes longer than RT-PCR (76, 80). MERS-CoV has also been isolated from environmental objects such as bedsheets, bedrails, intravenous fluid hangers, and X-ray devices in healthcare settings (76, 80). For antibody detection, paired serum samples are needed for the confirmation of infection. A single sample can provide information regarding prior infections or identifying probable cases, provided that the sample was taken at least 21 days after onset of illness. For paired samples, the first sample should be collected during the first week of illness, and two samples should be collected 3–4 weeks apart. Viral cultures are not recommended as a routine diagnostic test (89). Furthermore, viral culture and antibody detection assay using the whole virus should be done in specific laboratories that are biosafety level 3 (BSL-3) laboratories in the WHO Laboratory Biosafety Manual (80, 89).

Similar to SARS, laboratory abnormalities in MERS include leukopenia, thrombocytopenia, and elevated transaminases, lactate dehydrogenase, and creatinine levels. These are non-specific and can be found in other coronaviruses. Occasionally anemia, creatine kinase, C-reactive protein, and procalcitonin elevation, and hyponatremia are noted (76, 77, 80).

Radiologic Diagnosis

Abnormal chest radiograph findings are found to be more common in patients with MERS (90–100%) than with SARS (60–100%) (91). Airspace opacity was the most common abnormality in SARS patients, whereas ground-glass opacities were found more commonly in MERS patients (45). Chest X-ray findings are non-specific and similar to various viral pneumonia associated with ARDS. In severely ill MERS patients, chest radiograph and computed tomographic (CT) scan showed abnormalities in almost all patients, and it ranges from a mild unilateral focal lesion, bilateral multilobar airspace disease, ground-glass opacities, and occasional pleural effusions (76, 80). Thoracic imaging is usually normal in mild cases. The most common features seen on thoracic CT scans are bilateral, predominantly basilar, and subpleural air space involvement, with extensive ground-glass opacities and pleural effusions. Thoracic CT imaging done 3 weeks after onset of symptoms could reveal fibrotic changes, traction bronchiectasis, and architectural distortion (80, 82).

Treatment

The treatment is mostly supportive with the goal of reducing the risk of complications like a secondary bacterial or viral infection, respiratory failure, and multiorgan failures in MERS. Supportive care includes rest, intravenous fluids, analgesics, and also broad-spectrum antimicrobial, antivirals, and antifungals to minimize the risk of co-infection with opportunistic pathogens if needed. Other supportive care is based on organ dysfunction and management of complications like using a ventilator for patients with respiratory failure (76, 77).

Although there are some treatments available, they are not specific to treat MERS-CoV (77).

Antibiotics

Broad-spectrum antibiotics are commonly given empirically during the management of MERS to treat bacterial pneumonia. A retrospective study of 93 patients reports 23.6% bacterial infection in patients with MERS, Legionella *pneumophila*, and Streptococcus *pneumoniae* are the most common agents, and so broad-spectrum antimicrobial should be considered for MERS patients (92). In critically ill patients, macrolide therapy was not associated with a difference in clearance of MERS-CoV RNA and improvement in 90 day mortality (93). Teicoplanin is a glycopeptide antibiotic isolated from Actinoplanes teichomyceticus and known to be active against

gram-positive bacterial infections. *In-vitro*, it has been shown to inhibit the entry of MERS-CoV pseudotyped viruses into host cellular cytoplasm. There are no pharmacodynamic studies of this antibiotic specific to MERS-CoV, which are required to understand its antiviral efficacy (94, 95).

Antivirals

Ribavirin

Ribavirin is a nucleoside analog activated to a nucleotide by host kinases. Ribavirin was shown to inhibit MERS-CoV replications *in-vitro* (vero cells), but the dose is too high to be achieved *in vivo*. The 50% inhibitory concentration (IC50) of ribavirin was 41.45 microgram/ml, whereas a 1,000 mg intravenous dose of ribavirin can only achieve a level of up to 24 microgram/ml in human beings (95, 96). Ribavirin and interferon combinations inhibit MERS-CoV replication *in-vitro*. When used in combination, the required dose for IFN-α2b and ribavirin decreased by 8- and 16-folds, respectively. The combination also was shown to improve clinical outcomes in non-human primates (rhesus macaques and common marmoset) infected with MERS-CoV within 8 h of virus inoculation (76, 95). When this combination was tested in a severely ill patient, it showed improvement in survival at 14 days but not at 28 days, which was most likely due to administration in the advanced stages of the disease (97). A retrospective cohort study looked at a combination of ribavirin with IFN-α2a or IFN-β1a to treat MERS-CoV infection. Mortality rate was 85% vs. 64% ($p = 0.24$) in IFN-α2a and IFN-β1a, respectively (98). Although most of the data is available from small studies, a combination of ribavirin and interferon may be considered in MERS patients, especially in the early stages of the disease.

Protease inhibitors

Protease inhibitors are a well-known anti-retroviral agent, being used in the treatment of HIV. Lopinavir and Nelfinavir were shown to inhibit MERS-CoV *in-vitro*. Mean 50% effective concentration (EC50) of lopinavir using Vero E6 and Huh7 cells was 8.0 μM (96). An ongoing randomized controlled trial comparing the efficacy of treatment with a combination of lopinavir/ritonavir and recombinant IFN-β1b provided with standard supportive care with placebo and standard supportive care treatment in patients with laboratory-confirmed MERS requiring hospitalization (99).

Mycophenolic Acid

Mycophenolic acid (MPA) is an inhibitor of cellular inosine monophosphate dehydrogenase and inhibits purine synthesis in lymphocytes. In an *in-vitro* study, MPA showed strong inhibition of MERS-CoV with an IC50 of 2.87 μM. Similarly, IFN-β showed the most robust inhibition of MERS-CoV *in vitro*, with an IC50 of 1.37 U ml-1 compared to other interferon products (IFN-a2b, IFN-c, IFN-universal, IFN-a2a, and IFN-b). IFNβ, MPA alone, or in combination may be a useful post-exposure intervention in high-risk patients with known exposures to MERS-CoV or treatment of MERS-CoV (100). In a retrospective chart review study involving 51 patients, patients with MERS-CoV infection received different treatments, including broad-spectrum antibiotics, steroids, various antivirals, and mycophenolate mofetil. Eight patients who received mycophenolate mofetil and IFN-β survived, but this group of patients had low lower Acute Physiology and Chronic Health Evaluation II (APACHE-II) scores compared to other groups (101).

Resveratrol

Resveratrol has shown antiviral properties against many human viruses like the influenza virus, Epstein–Barr virus, herpes simplex virus, respiratory syncytial virus. Antiviral effects of resveratrol against MERS-CoV observed *in-vitro* due to observed inhibition of MERS-CoV nucleocapsid (N) protein expression. It can also prolong cellular survival due to the downregulation of apoptosis induced by MERS-CoV. However, there are adverse effects also reported with resveratrol like increasing viral RNA replication during Hep-C virus infection *in-vitro* (OR6 cells), and potent cytotoxicity in cultured cells. This drug needs to be studied further for its antiviral properties, with careful consideration to be given for potential adverse events (76).

Fusion Inhibitors

Fusion inhibitors are antiviral peptides, which prevents MERS-CoV entry into host cells by targeting various S protein areas. Camostat, a serine protease inhibitor and the heptad repeat 2 peptide (HR2P), a synthesized peptide are two MERS-CoV fusion inhibitors that were tested *in vitro*. Camostat suppressed MER-CoV viral entry into human bronchial submucosal gland-derived Calu-3 cells by 10-fold but was not efficacious against the immature lung tissue. HR2 blocks MERS-CoV replication and the spike protein-mediated cell-cell fusion (95, 96). Although fusion inhibitors have shown effects *in vitro*, and no *in vivo* clinical data available.

Interferon

In vitro, IFN-β has higher antiviral activity on MERS-CoV when compared to SARS-CoV (102). ORF4a inhibits IFN-β production through inhibitions of interferon regulatory transcription (IRF-3) factors and nuclear factor (NF)-κB actions (103). Among *in-vitro* studies, IFN-β is more potent that IFN-α2b, IFN-α2a, IFN-γ, IFN-universal type 1 with IC50 of 1.37 U/ml (96). Animal and *in-vitro* studies showed that IFNs have synergistic effects when used in combination with ribavirin, mycophenolate, which is discussed above in the mycophenolate and ribavirin sections.

Corticosteroids

High-dose systemic corticosteroids were given to treat many patients with severe MERS-CoV disease with the intention to reverse the progression of respiratory distress and to prevent lung fibrosis but turned out to be futile (87). A multicenter retrospective study of 309 critically ill ICU patients with MERS-CoV infection showed that patients who got corticosteroids were more likely to be on a ventilator (93.4% vs. 76.6%, $P < 0.05$) compared to patients who did not receive steroids. After adjusting for time-varying confounders, corticosteroid therapy was not significantly associated with 90 day mortality (aOR 0.75; 95% CI, 0.52–1.07) but was associated with delayed MERS-CoV

RNA clearance (adjusted hazard ratio [HR], 0.35; 95% CI, 0.17–0.72; $P = 0.005$) (104). Steroids should be avoided in patients with MERS unless they are indicated for other clinical conditions as their safety is not clear in patients with MERS-CoV (82).

Convalescent Plasma

Convalescent plasma therapy involves the use of plasma or whole blood from patients with MERS-CoV infection who recovered fully from the disease. During the MERS outbreak in Korea in 2015, 3 of 13 patients with MERS infection with respiratory failure received four convalescent plasma infusion from recovered MERS patients. However, only two of four donor plasma showed neutralizing activity; therefore, the donor plasma should be tested for neutralizing activity. Only the donor plasma with a plaque reduction neutralization test (PRNT) titer 1:80 showed meaningful serologic effects after convalescent plasma infusion. ELISA IgG can be used as a substitute for neutralization tests in limited resource situations as it can predict PRNT titer $\geq$1:80 with >95% sensitivity and 100 % specificity with OR of 1.6 and 1.9, respectively (105).

Monoclonal Antibodies (mAbs)

Monoclonal antibodies are commonly used in various diseases, including infectious diseases. Mersmab1, first developed by Du et al., binds to the MERS-CoV spike protein receptor-binding domain (RBD) and thus competitively blocks the binding of the RBD to its cellular receptor, DPP4 (106). Three human monoclonal antibodies m336, m337, and m338 were identified from a large naïve-antibody library, and these antibodies target the receptor (CD26/DPP4) binding domain (RBD) of the MERS-CoV spike glycoprotein. All three human monoclonal antibodies have neutralizing activity and highest with m336 (107). Given the above results, mAbs can be developed as one of the treatment options against MERS-CoV in humans. A phase 1 randomized, double-blinded, placebo-controlled, first-in-human trial has been performed to study the safety, tolerability, pharmacokinetics, and immunogenicity of single ascending doses of a co-administered REGN3048 and REGN3051 monoclonal antibody but results have not been published yet (108).

Multiple other drugs like chloroquine, chlorpromazine, loperamide, Nitazoxanide, and cyclosporin, have also shown activity against MERS-CoV *in-vitro* but no *in-vivo* studies are available (95, 96).

Prevention

Vaccines

For the development of vaccines against MERS-CoV, viral enveloped protruding spike (S) glycoprotein and its RBD and/or the nucleocapsid (N) protein are primary targets (109, 110). Various vaccines are under development, and it includes subunit, DNA, recombinant vector, and live attenuated vaccines.

Subunit vaccines

Protein subunit vaccine has defined one or more immunogenic components, and subunit antigen induces antibody responses with primarily CD4 T-cell responses. These vaccines have low risk *in vivo* compared to other vaccine types and are generally well-tolerated (110, 111). A recombinant protein containing residues 377–588 in the truncated receptor-binding domain of MERS-CoV spike (S) protein was fused with human IgG Fc fragment (S377-588-Fc) in an *in-vitro* culture of transfected 293T cells. In vaccinated mice, recombinant S377-588-Fc induced strong MERS-CoV S-specific antibodies, which blocks binding of RBS to DPP4 receptors and thus inhibits MERS-CoV infection. It shows that truncated RBD can be a potential candidate for a future safe vaccine against MERS-CoV (112).

DNA vaccines

DNA vaccines are safe, yield stable antigen expression, and cause only low-grade adverse effects like local pain at the injection site, and malaise or fever (110, 111). Although DNA vaccines induce lower immune response compared to other vaccines type, it induced both humoral and cellular immune response at low cost than others (111). Phase 1 open-label clinical study of GLS-5300 MERS-CoV DNA vaccine was conducted, and 75 healthy adults aged 18–50 years were enrolled in this study. These individuals were divided into three groups of 25, and each group received different doses (0.67, 2, or 6 mg) of the vaccine. The most common adverse effect in all groups was the injection site reaction (93%). As measured by S1-ELISA, seroconversion occurred in 66, 86, and 94% participants after first, two, and three vaccination, respectively. Neutralizing antibodies against MERS-CoV EMC-2012 infection of Vero cells were seen in 43, 39, and 3% at week 14, week 24, and at the end of the study, respectively. The B-cell and T-cell responses were 77 and 64%, respectively, at week 60. This vaccine should be tested further in MERS endemic area for efficacy (113).

Vector vaccine

Vector vaccines ChAdOx1 MERS, replication-deficient simian adenovirus vector (ChAdOx1), and modified vaccinia virus Ankara (MVA) based vaccine is known as MVA-MERS-S and already went through phase 1 clinical trial. Phase 1 open-labeled, non-randomized, uncontrolled trial for ChAdOx1 MERS was conducted between March 14 and August 2018 at Oxford, UK. Twenty-four healthy people aged 18–50 years with negative pre-vaccination tests for HIV antibodies, hepatitis B surface antigen, and hepatitis C antibodies received a single intramuscular injection of ChAdOx1 MERS at three different doses (5×10^9 viral particles, 2.5×10^{10} viral particles, and 5×10^{10} viral particles for low, intermediate and high dose group, respectively). No serious adverse effects were reported in all three groups with different doses during 12 months follow-up. Seroconversion was 75, 92, and 68%, respectively in all groups at 14, 56 days, and 1 year after vaccination. From baseline, a significant increase in both T-cell ($p < 0.003$) and IgG ($p < 0.0001$) to the MERS-CoV spike antigen was seen at all doses. These results support the clinical development progression of phase 1b and 2 trials, especially in the endemic area (114).

In Germany, an open-label phase 1 clinical trial was done for the MVA-MERS-S vaccine, and this trial included healthy aged 18–55 years individuals with no clinically significant health problems with key exclusion criteria of previous MVA

vaccination. Individuals were allocated to two different doses groups as one being the low-dose group (1 × 107 plaque-forming unit p) and the other being the high-dose group (1 × 108 PFU). These individuals received two doses of vaccine 28 days apart via the intramuscular route. No severe or serious adverse effects were noted. After the second dose of vaccine, seroconversion using a MERS-CoV S1 ELISA at any timepoint during the study was found to be 75% in the low dose group and 100 in the high-dose group. MERS-CoV spike-specific T-cell responses were detected in 83 and 91% of participants in the low-dose and high-dose group, respectively (115).

Live attenuated vaccine

Live attenuated vaccines can induce a potent immune response as they present antigens to the host immune system similar to natural infection. In animal models, a live attenuated vaccine for MERS-CoV has shown efficacy (110, 111). An engineered mutant virus lacking structural E protein, rMERS-CoV-ΔE genome replicated after cDNA clone was transfected into cells and was only efficiently disseminated in cells expressing the E protein in trans. The rMERS-CoV-ΔE mutant virus can be a potential vaccine candidate for MERS-CoV (116). Live attenuated vaccine CoV accessory proteins, and nsp16-deficient MERS-CoV vaccine have also been considered (110).

CORONAVIRUS DISEASE 2019 (COVID-19)

The first cases of COVID-19 were reported from Wuhan, China. In December 2019, cases of pneumonia of unknown cause occurred in Wuhan, Hubei Province of China, who had exposure to animals sold in the local Hunan seafood market (117–119). On January 7th, 2020, a new CoV type was isolated from these patients with pneumonia. Within a few days, the genetic sequence of this novel CoV (SARS-CoV-2) was identified (120). On January 30th, 2020, WHO declared the SARS-COV-2 outbreak as a Public Health Emergency of International Concern (PHEIC), and on March 11th, 2020, COVID-19 was declared as a global pandemic (121).

Human-to-human transmission due to close contact further caused the spread of the virus to other provinces during the Spring festival season in China. Within a span of a few weeks, It spread globally to multiple nations throughout the World (122). The first case outside China was reported on Jan 13th, 2020, in Thailand. As of July 4th, 2020, there are more than 11 million cases and 530,000 deaths worldwide. As of today, the United States (US) has the maximum number of cases followed by Europe (123). Human-to-Human transmission occurs due to direct contact or through respiratory droplets spread by coughing/sneezing or directly through fomites (124). SARS-CoV-2 can be detected in respiratory secretion up to 2 weeks after disease symptoms resolve. A study of 73 patients from china showed that 54.3% of patients were found to have positive SARS-CoV-2 RNA in the stool samples (125).

More than 75% of CoV infections have animals as a source of infection, and bats are considered as a reservoir for all human coronaviruses. There is still uncertainty about the intermediate host that led to human transmission (122). Pangolins are considered as a probable intermediate host for SARS-CoV-2 as Pangolin-CoV is 91 and 90.55% identical to SARS-CoV-2 and BatCoV RaTG13, respectively. BatCoV RaTG13 from *rhinolophus affinis* shares a 96% whole-genome identity with SARS-CoV-2 (126).

Incubation Period

In a study of initial cases from Wuhan, China, the median age of these patients was 59 years, ranging from 15 to 89 years. The mean incubation period was estimated to be 5.2 days (95% CI, 4.1–7.0) (127). A study of publicly reported cases outside Hubei province found a median incubation period of 5.1 days (95% Cl, 4.5–5.8), and symptom onset was within 11.5 days (95% Cl, 8.2–15.6 days) in 97.5% of infected patients (128). Given the above information, 14 days quarantine or medical observation will identify an active case in more than 97% of exposed patients. These studies have limitations; they included mostly hospitalized patients who can confound results, as the incubation period may differ in mild cases.

Clinical Features

Patients with SARS-CoV-2 infection can be asymptomatic or have a wide range of symptoms (**Table 1**). Mild cases are reported to recover within 1 week, and severe cases developed progressive respiratory failure leading to death (118). In a prospective study of 16,749 patients with COVID-19, cough (70%), fever (69%), and shortness of breath (65%) were the most common symptoms. Almost 29% of patients presented with enteric symptoms along with respiratory symptoms, and only 4% have just enteric symptoms alone (129).

A meta-analysis of 47 studies showed pooled prevalence of diarrhea, nausea/vomiting and abdomen as 7.7%(95% Cl = 7.2–8.2%), 7.8 %(95% Cl = 7.1–8.5%) and 3.6%(95% Cl = 3.0–4.3%), respectively (130). In a retrospective study of COVID-19 patients, when comparing digestive-only, respiratory-only, and digestive and respiratory groups, stool RNA was positive in 60, 14.3, and 80% patients, respectively. It took a long time to clear the virus in a patient with positive viral RNA in stool compared to those with a negative test (44.2 vs. 33.7 days, $P = 0.003$). The diarrhea duration in COVID-19 can last up to 14 days, with an average duration of 5.4 ± 3.1 days (131).

Recently more symptoms are being reported like loss of smell and taste sensation (132). A meta-analysis of 27 studies showed a pooled prevalence of loss of smell and taste in these patients to be 41.47% (95% Cl 3.13–31.03%) and 35.04% (95% Cl 22.03–49.26%), respectively (133). Both of these symptoms presented in patients on average on the fourth day after initial symptoms of the disease, but 13–15.5% of patients had a loss of smell and taste sensation as the first symptom (134, 135).

COVID-19 is a prothrombotic state leading to both microvascular and macrovascular thromboembolic events in pulmonary and extrapulmonary organs (136). Venous thromboembolism, particularly pulmonary embolism, is the most common coagulopathic manifestation in COVID patients (137). Several proposed mechanisms for thrombosis in COVID-19 patients include angiotensin-converting enzyme-2 receptor-mediated endothelial damage leading to cytokine

storm, intussusceptive angiogenesis, and macrophage activation syndrome leading to activation of the coagulation cascade (136–139). The incidence of thrombotic events in COVID patients is 7.7–49% in various retrospective and prospective studies (140–144).

About 47% of patients with COVID-19 were without any comorbidities (129). A systematic review of thirty-one articles with comorbidity-specific data showed that diabetes mellitus (8.55%), cardiovascular/cerebrovascular disease (8.03%), respiratory disease (6.19%), and hypertension as most prevalent comorbidities in COVID-19 positive patients (145). Another systematic review of ten studies found 33.9% of the overall prevalence of obesity in hospitalized patients with COVID-19. Patients with obesity (defined by BMP> = 25) had higher odds of poor outcomes compared to a better outcome with a pooled odds ratio of 1.88 (95% CL:1.25–2.80, $p = 0.002$) (146). A meta-analysis of 212 studies showed that patients with severe disease were much older than (60.4 years, 95% Cl = 57.8–63.1) than patients with non-severe disease (44.6 years, 95% Cl = 42.8–46.3), $p < 0.0001$. It also showed that more men were in severe group (60.8%, 95% Cl = 57.2–64.2) compared to the non-severe group (47.6, 95% Cl = 44.9–50.4%), $p < 0.0001$ (147).

Diagnosis

Laboratory Diagnosis

Table 4 outlines the case definitions used by WHO for surveillance. It is crucial to make a rapid and accurate diagnosis, especially in the current pandemic situation. The RT-PCR, real-time RT-PCR (rRT-PCR), and reverse transcription loop-mediated isothermal amplification (RT-LAMP) are currently available diagnostic tests, which detects unique sequences of virus RNA by nucleic acid amplification test (NAAT) to make the diagnosis (**Table 1**). RT-PCR assays target the RNA-dependent RNA polymerase (RdRp)/helicase (Hel), spike (S), and nucleocapsid (N) genes of SARS-CoV-2 (149, 150). NAAT test can be done on upper respiratory specimens (nasopharyngeal and oropharyngeal swab or wash), lower respiratory specimens [sputum (if produced) and/or endotracheal aspirate or bronchoalveolar lavage], blood and stool samples (149). Although upper and lower respiratory specimens are most commonly used for the test, a study of 73 hospitalized patients with COVID-19, stool SARS-COV-2 RNA test was positive for 53.4% patients, and in 23% cases stool test remained positive even after a negative respiratory test (125).

Case Definition

Defining the COVID-19 case is essential not only at the individual level but also from the public health perspective. WHO gave guidelines for defining a case as a laboratory-confirmed case of COVID-19 in the area with no known COVID-19 virus circulation and also in the area with established virus circulation.

a. *In an area with no known COVID-19 virus exposure*

- A case considered as laboratory-confirmed by NAAT: If a patient has positive NAAT result for at least two different targets on the COVID-19 virus genome, of which at least one target is preferably specific for COVID-19 virus using a validated assay; (OR)
- One positive NAAT result for the presence of beta coronavirus and COVID-19 virus further identified by sequencing the partial or whole genome of the virus as long as the sequence target is larger or different from the amplicon probed in the NAAT assay used (149).

b. *In an area with established COVID-19 virus exposure*

A screening by rRT-PCR using a single discriminatory target can be sufficient to consider a case laboratory-confirmed by NAAT.

One or more negative tests do not rule out the possibility in a patient with a high suspicion of COVID-19. Some of the factors which could explain at least in part for negative results include poor quality of the specimen, specimen not handled appropriately, collected very early or late in infection, use of only upper respiratory tract sample. In these cases, a sample should be collected and tested again, including a lower respiratory tract sample, if possible (149). Serological tests can be used to identify asymptomatic cases, diagnosis, and study the extent of outbreak retrospectively. In a patient with a negative NAAT and high suspicion for COVID-19, paired serum samples (in the acute and convalescent-phase) can be used to make the diagnosis (149). In a study of 285 patients with COVID-19, Immunoglobulin-G (IgG) and IgM levels were checked for patients. Hundred percentage of patients had positive virus-specific IgG within 17–19 days after symptom onset, and 94.1% of patients had IgM positive within 20–22 days after symptom onset. IgM and IgG levels plateaued within 6 days after seroconversion (151). Viral cultures are not recommended as a routine diagnostic test (149).

There are non-specific laboratory abnormalities observed in patients with COVID-19 infection. The most common laboratory findings include lymphopenia, elevated C-reactive Protein (CRP), elevated aspartate aminotransferase,

TABLE 4 | WHO case definitions for surveillance and last updated on March 20th, 2020.

WHO Case Definitions for Surveillance March 20, 2020	
Suspected case	• A patient with acute respiratory illness, AND a history of travel to or residence in a location reporting community transmission of COVID-19 during the 14 days prior to symptom onset OR • A patient with any acute respiratory illness AND having been in contact with a confirmed or probable COVID-19 case (see definition of contact) in the last 14 days prior to symptom onset OR • A patient with severe acute respiratory illness (fever and at least one sign/symptom of respiratory disease, e.g., cough, shortness of breath; AND requiring hospitalization) AND in the absence of an alternative diagnosis that fully explains the clinical presentation
Probable case	• A suspect case for whom testing for the SARS-CoV-2 is inconclusive OR • A suspect case for whom testing could not be performed for any reason
Confirmed case	A person with laboratory confirmation of COVID-19, irrespective of clinical signs and symptoms

It includes the definition for the suspected, probable, and confirmed case, and these definitions may need to adapt further based on epidemiological situations (148).

hypoalbuminemia, elevated procalcitonin level, elevated D-dimer and erythrocyte sedimentation rate (ESR) (152–154). Serum levels of pro-inflammatory cytokines (interleukins, MCP1, MIP1A, MIP1BTNFα, IFNγ, IP10, and MCP1) were found to be elevated in patients with COVID-19. Furthermore, a higher concentration of GCSF, IP10, MCP1, MIP1A, and TNFα were noted in critically ill individuals requiring treatment in the intensive care unit (155). Along with the clinical presentation of COVID-19, elevated serum CRP may be used as a marker for the presence and severity of the disease (152).

Radiographic Diagnosis

Chest CT scan is the primary screening imaging modality for COVID-19. Ground glass opacities (GGO), consolidation, paving stone sign (finding ground-glass opacities with lobular interval thickening and interlobular interval lines), pleural thickening, and vascular thickening, and fibrinous lesions are common CT chest findings seen in a patient with COVID-19 (156, 157). Pleural effusion, pericardial effusion, and lymphadenopathy are rarely observed on CT scans in these patients (157). In a study comparing CT scan findings of COVID-19 and non-COVID pneumonia were GGOs (100% vs. 90.0%), mixed GGO (63.6% vs. 72.7%) and consolidation (54.5% vs. 77.3%), respectively. Pulmonary opacifications were more common in the peripheral area in COVID-19 than non-COVID-19 groups (100% vs. 31.8%, $p = 0.05$) (158).

Although NAAT is a gold-standard test for COVID-19 diagnosis due to high specificity, its sensitivity is 30–50%. Expectedly, diagnosis can be falsely missed if NAAT is the only test used for diagnosis. Patients with epidemiological features and positive CT scan findings should be isolated, and the NAAT test to be repeated (153, 156). COVID-19 group had ground-glass opacity (GGO) or GGO with consolidation more frequently, whereas the non-COVID-19 pneumonia group has consolidation as a common finding on CT scan ($P < 0.05$) (153). Therefore, patients should be isolated and rRT-PCR to be repeated in case there is a high suspicion of COVID-19 on CT imaging but a negative initial rRT-PCR test.

Treatment

The mainstay treatment for COVID-19 is supportive management, with oxygen and mechanical ventilation, if needed (159). Empiric antibiotics have been used to prevent superimposed infections (160). FDA gave emergency use authorization for Remdesivir on May 1st, 2020, and there are no other FDA-approved medications available for COVID-19 (159–162). WHO announced the launch of an international clinical trial called SOLIDARITY trial on Match 18th, 2020, to help find an effective treatment of COVID-19. This trial will compare various options against the standard of care to assess the efficacy of these treatments. It will also add other drugs based on emerging evidence. This trial started to compare four treatment options (Remdesivir; Lopinavir/Ritonavir; Lopinavir/Ritonavir with Interferon beta-1a; and Chloroquine or Hydroxychloroquine) to the standard of care and study efficacy of these treatments. Hydroxychloroquine vs. standard of care and lopinavir/ritonavir vs. standard of care trials were discontinued on July 4th, 2020 by WHO based on the evidence presented at WHO Summit on COVID-19 research and innovation on July 1st and 2nd 2020. Overall, over 100 countries are participating in this trial (163).

The following treatments are currently being used for COVID-19 due to the effects seen *in vitro*.

Protease Inhibitors

Lopinavir-Ritonavir

For the treatment of COVID-19, the NIH panel recommends against the use of lopinavir/ritonavir and other HIV protease inhibitors unless it is for a clinical trial (159). Lopinavir is a highly potent inhibitor of the HIV protease essential for intracellular HIV assembly, and its half-life increases when combined with ritonavir via cytochrome P450 inhibition (161, 164). Lopinavir/ritonavir inhibits SARS-CoV-2 3CLpro *in-vitro* and thus suppress the cleavage of polyproteins into multiple functional proteins like RNA polymerase and a helicase (159, 160). In a randomized, controlled, open-label trial of 199 hospitalized patients with SARS-CoV-2 infection, patients were randomized in a 1:1 ratio to either lopinavir-ritonavir (400 and 100 mg, respectively) twice a day for 14 days along with standard care, or standard care alone. There was no difference in time for clinical improvement, mortality at 28 days, and detectable viral load was seen in the lopinavir-ritonavir group compared to standard treatment. Severe adverse events were seen more commonly in the standard treatment group, but the lopinavir-ritonavir group showed more gastrointestinal (nausea, vomiting, and diarrhea) adverse effects (165).

Darunavir/Cobicistat

Darunavir/Cobicistat is another protease inhibitor used in HIV patients. No clinical trials have been conducted yet in the US. A single unpublished trial from China showed that it was not effective in COVID-19 treatment as darunavir has low affinity for coronavirus protease (159).

Remdesivir

It is an analog of adenosine, nucleotide prodrug, which inhibits viral RNA replication by interfering with the activity of viral RNA-dependent RNA polymerase (RdRp) (150, 161). It has shown activity against Ebola in rhesus monkeys, and other RNA viruses, including arenaviruses and coronaviruses (161, 164). Remdesivir has inhibitory activity against SARS-CoV-2 infection at EC90 of 1.76 μM, in *in-vivo* non-human primate models (164). It also has inhibitory effects against SARS-CoV-2 infection of Human Liver cells, Huh-7 cells (160, 164). In a study of 53 patients who received at least one dose of remdesivir on a compassionate-use basis, clinical improvement was noticed in 68% (36/53) patients. 57% (17/30) patients were extubated who were receiving mechanical ventilation. The overall mortality rate was 13%, but it was higher (18%) in patients receiving mechanical ventilation (166).

A preliminary update from a randomized controlled trial involving 1,063 patients called Adaptive COVID-19 Treatment Trial (ACTT) sponsored by the National Institute of Allergy and Infectious Diseases (NIAID) indicates that patient who received

remdesivir showed a 31% faster time to recovery than the placebo group ($p < 0.001$). It also suggested a lower mortality rate of 8% in the remdesivir group compared to 11.6% in the placebo group but did not reach statistical significance ($p = 0.059$) (167, 168). FDA gave emergency use authorization for Remdesivir use on May 1st, 2020, after preliminary results from the ACTT trial. Multiple clinical trials are in development to study remdesivir use in COVID-19 patients (169).

Chloroquine and Hydroxychloroquine

NIH panel recommends against the use of chloroquine or hydroxychloroquine for the treatment of COVID-19 in hospitalized patients. NIH panel also recommends against chloroquine or hydroxychloroquine for COVID-19 treatment in non-hospitalized patients, except in the context of a clinical trial. NIH panel also recommends against the use of hydroxychloroquine with azithromycin for COVID-19 treatment, except in the context of a clinical trial (170). Chloroquine and Hydroxychloroquine are immunomodulatory drugs that inhibit terminal phosphorylation of ACE2 and elevate pH in endosomes involved in virus cell entry. Hydroxychloroquine metabolizes into chloroquine *in-vivo* and may have lower adverse effects than chloroquine (159, 164).

Hydroxychloroquine was more potent than chloroquine *in-vitro* in SARS-CoV-2 infected Vero cells using physiologically-based pharmacokinetic (PBPK) models. This model also recommended an oral loading dose of 400 mg twice daily on day 1, followed by an oral maintenance dose of 200 mg twice daily for 4 days of hydroxychloroquine for patients with SARS-CoV-2 (171). For chloroquine, a dose of 500 mg is needed to achieve an EC90 value of 6.90 μM in Vero E6 cells (172). In a study conducted in China, 22 patients were randomized into two groups with one treated with chloroquine 500 mg orally twice daily for 10 days, and others treated with Lopinavir/Ritonavir 400/100 mg orally twice daily for 10 days. On day 10, 90% of patients in the Chloroquine group were SARS-CoV-2 RT-PCR negative compared to 75% in Lopinavir/Ritonavir group. CT scan improvement was 100% in the Chloroquine group and 75% in Lopinavir/Ritonavir group (173). In a randomized controlled study of 62 patients with two parallel groups with one assigned to receive 5 days of Hydroxychloroquine (400 mg/day) along with standard treatment and other assigned to control group receiving standard treatment, 80.6% of patients in the Hydroxychloroquine (HCQ) group compared to 54.8% in the control group showed improvement in pneumonia on CT imaging. HCQ group had 2.2 days vs. 3.2 days of mean duration fever and 2.0 days vs. 3.1 days of cough compared to the control group (174).

An observational study from France of 80 confirmed COVID-19 patients who received a combination of HCQ and azithromycin for at least 3 days and then followed for at least 6 days showed that the majority (81.3%) of patients were discharged from the unit as they had a favorable outcome. Rapid fall in nasopharyngeal viral load was noticed with 83% negative on Day 7 and 100% negative on Day 12 (175). Eighty-four COVID-19 positive patients were given a combination of HCQ and azithromycin as treatment. Eighteen percentage of these patients had an increase in QTc interval by 40 to 60 ms, and another 12% had an increase in Qtc by >60 ms. Acute renal failure (OR 19.45, 95% CI, 2.06–183.88, $P = 0.01$) was a strong predictor of extreme QTc prolongation instead of baseline QTc level (176).

Convalescent Plasma

NIH panel states that there is insufficient data to recommend either for or against the use of convalescent plasma or hyperimmune immunoglobulin for the treatment of COVID-19. Convalescent plasma has been used in the past for the treatment of various diseases, including SARS. In the United States, FDA had issued guidance for the use of convalescent plasma collected from individuals who have recovered from COVID-19 (COVID-19 convalescent plasma) for administration to a patient with COVID-19 and investigational studies during the public health emergency (177). A case series of 5 patients with laboratory-confirmed COVID-19 and ARDS received convalescent plasma infusion. In these patients, SOFA score decreased, PaO_2/FiO_2 increased, and viral load deceased and became negative within 12 days after transfusion. The ARDS resolved in 4 patients at 12 days after transfusion (178). Clinical trials are in development regarding the evaluation of the use of both convalescent plasma and SARS-CoV-2 IVIG to treat COVID-19 (179).

Antibodies

The Spike protein of CoV is a primary inducer of neutralizing antibodies. Cross-reactivity of the anti-SARS-CoV-1 antibody was checked with SARS-CoV-2 spike protein due to the similarity between the receptor-binding domain (RBD) in SARS-CoV-1 and SARS-COV-2. SARS-CoV-1 specific human monoclonal antibody CR3022 binds to SARS-CoV-2 RBD very strongly. A similar affinity was not seen with other SARS-CoV-1 RBD-directed antibodies 230, m396, and 80R. Given the above information, CR3022 can be a potential candidate for the treatment of COVID-19 infection (180).

Interleukins Inhibitors and JAK-Inhibitors

NIH Panel recommends against the use of Janus kinase (JAK) inhibitors (e.g., baricitinib) to treat COVID-19 unless it is for a clinical trial. There is insufficient data in favor of or against the use of Interleukin-1 inhibitors (e.g., anakinra) and IL-6 inhibitors (e.g., sarilumab, siltuximab, or tocilizumab)in the treatment of COVID-19. Interleukin inhibitors are therapies directed against the inflammatory cytokines or other parts of the innate immune response. It is proposed that significant tissue damage, including in lungs and other organs, is caused by exacerbated immune response and cytokine release (181). Interleukin-1 is a pro-inflammatory cytokine that induced IL-6 in macrophages and monocytes. It is elevated in patients with COVID-19, and other conditions, such as macrophage activation syndrome (MAS), severe chimeric antigen receptor T-cell (CAR-T) mediated cytokine release syndrome (CRS). Janus kinase (JAK) enzymes regulate signal transduction in immune cells (159). Interleukin inhibitors are thought to act by suppressing cytokine processes, which causes tissue damage (159, 181). Similarly, the JAK inhibitor can block the cytokine release. Thus,

IL-1 and IL-6 blockades and JAK inhibition proposed a potential treatment option for patients with COVID-19 infection (159).

A phase 2/3 open-label, randomized parallel-group, three arms, multicenter study is underway in Italy to assess the efficacy and safety of intravenous Administrations of Emapalumab, an Anti-interferon Gamma (Anti-IFNγ) Monoclonal Antibody, and Anakinra, and Interleukin-1(IL-1) Receptor Antagonist, vs. Standard of Care, in Reducing Hyper-inflammation and Respiratory Distress in Patients With SARS-CoV-2 Infection. It was started in April with an estimated date of completion in Sept 2020 [141]. In a retrospective study conducted in China with 15 patients, Tocilizumab (TCZ), a monoclonal antibody against IL-6 was given to all patients. Eight patients received methylprednisolone along with TCZ. C-reactive protein (CRP) and IL-6 levels were checked before and after TCZ therapy. CRP level decreased significantly after TCZ therapy, dropped from 126.9 (10.7–257.9) to 11.2 (0.02–113.7) mg/L ($P < 0.01$). However, in four critically patients who received only one dose of TCZ, three of them died, and CRP did not return to normal within a week. IL-6 level spiked first before decreasing after receiving TCZ. Again, all four critically patients had a persistent increase in IL-6 even after getting TCZ. Given the above results, repeated doses might improve the condition in critically ill patients. IL-6 can be used to know the severity and prognosis of the disease. Since it was a small study, the results should be interpreted with caution (182).

Interferons

NIH panel recommends against the use of interferons for the treatment of COVID-19, except in the context of a clinical trial as there are no clinical trials and no proven benefits of interferons in other coronavirus infection and potential adverse effects outweigh benefits (159).

Corticosteroids

Both WHO and NIH panels recommend using systemic corticosteroids for patients with critical (mechanically ventilated patient) and severe (requiring supplemental oxygen) COVID-19 disease. Whereas, WHO and NIH panel recommends against corticosteroids in patients with non-severe (not requiring supplemental oxygen) COVID-19 disease (170, 183). These recommendations are based on a preliminary report from the Randomized Evaluation of COVID-19 Therapy (RECOVERY) trial. In this trial, 2104 patients were assigned to receive dexamethasone (6 mg once daily) oral or intravenous for up to 10 days and 4,321 to receive usual care alone. Dexamethasone group found to have lower mortality at 28 days after randomization than the usual care group with reported deaths 482/2,104 patients (22.9%) and 1,110/4,321 patients (25.7%), respectively (age-adjusted rate ratio, 0.83; 95% CI, 0.75–0.93; $P < 0.001$). Furthermore, the incidence of death was lower in the dexamethasone group compared to usual care group in patients on mechanical ventilation (29.3% vs. 41.4%; rate ratio, 0.64; 95% CI, 0.51–0.81) and one receiving supplemental oxygenation (23.3% vs. 26.2%; rate ratio, 0.82; 95% CI, 0.72–0.94) but no clear effects were seen in patients without any supplemental oxygen (17.8% vs. 14.0%; rate ratio, 1.19; 95% CI, 0.91–1.55) (184). In a systemic review and meta-analysis, 23 randomized trials reported mortality and showed lower mortality in the group randomized to glucocorticoids (odds ratio 0.87, 95% credible interval 0.77 to 0.98; risk difference 31 fewer per 1,000, 95% credible interval 55 fewer to 5 fewer; moderate certainty) than standard care (185).

Anticoagulation

Given the risk of thrombotic events in patients with COVID-19, the American Society of Hematology and the International Society on Thrombosis and Hemostasis recommends thromboprophylaxis with antithrombotic agents in all hospitalized COVID-19 patients unless there are contradictions (186, 187). Various societies like the American College of Chest Physician, American College of Cardiology, Anticoagulation Forum, American Society of Hematology, and CDC recommends against using therapeutic anticoagulation unless there is a confirmed or high suspicion of thrombotic events and other indications of anticoagulation like atrial fibrillation, mechanical cardiac valves and secondary venous thromboprophylaxis (170, 187–190). A single-center, open-labeled randomized controlled study of 20 COVID-19 positive patients requiring mechanical ventilation were randomized to either therapeutic or prophylactic dose of enoxaparin. Patients in the therapeutic enoxaparin group showed a significant increase of PaO_2/FiO_2 ratio of 163, 209, and 261 at baseline, after seven days and 14 days, respectively ($p = 0.0004$). Whereas, in the prophylactic enoxaparin group, no statistically significant difference in PaO_2/FiO_2 was noticed over time. Similarly, the therapeutic enoxaparin group (15 days [interquartile range, IQR 6–16)] had higher ventilator-free days compared to the prophylactic enoxaparin group (0 days [IQR 0–11)], $p = 0.028$. No difference was found in all-cause mortality and in-hospital mortality between the two groups. Although this study shows that therapeutic enoxaparin improves gas exchange and ventilator-free day in severe COVID-19 patients, further large randomized clinical trials are needed as it was a single-center study with a small sample (191).

Prevention

Vaccines

The genetic sequence of SARS-CoV-2 was revealed on 11 January 2020. It provides the basis of further studies to develop treatment and vaccines against SARS-CoV-2. Based on vaccine development pathways for other coronaviruses like MERS and SARS, pathways like nucleic acid, subunit vaccines, inactivated or live attenuated vaccines, and virus vector-based, are being investigated., The majority of vaccines in development are targeting S protein (150, 192). WHO is coordinating and directing global efforts to develop and evaluate vaccine candidates through global collaboration, development of robust methods, accelerating progress and avoiding duplication of research efforts, and coordinating efforts to rapidly and simultaneously assessing many vaccines (193) (**Table 5**). As of July 7th, 2020, there are 21 vaccine candidates in clinical evaluation and 139 candidates in the preclinical evaluation as per WHO (193).

TABLE 5 | Eight candidate vaccines in clinical evaluation- obtained from WHO DRAFT landscape of COVID-19 candidate vaccines−11 May 2020 (193).

Platform	Type of candidate vaccine	Developer	Current stage
Non-replicating viral vector	Adenovirus Type 5 vector	CanSino Biological Inc./Beijing Institute of Biotechnology	Phase 2 ChiCTR2000031781 Phase 1 ChiCTR2000030906
Inactivated	Inactivated	Wuhan Institute of Biological Products/Sinopharm	Phase1/2 ChiCTR2000031809
Inactivated	Inactivated	Beijing Institute of Biological Products/Sinopharm	Phase 1/2 ChiCTR2000032459
Inactivated	Inactivated + alum	Sinovac	Phase 3 NCT04456595 Phase 1/2 NCT04352608 NCT04383574
DNA	DNA plasmid vaccine	Candila Healthcare Limited	Phase 1/2 CTR1/2020/07/026352 (not yet recruiting)
Non-replicating viral Vector	ChAdOx1-S	University of Oxford/AstraZeneca	Phase 3 ISRCTN89951424 Phase 2b/3 2020-001228-32 Phase 1/2 PACTR202006922165132 2020-001072-15
RNA	3 LNP-mRNAs	BioNTech/Fosun Pharma/Pfizer	Phase 1/2 2020-001038-36 NCT04368728
DNA	DNA plasmid vaccine with electroporation	Inovio Pharmaceuticals	Phase 1/2 NCT04447781 NCT04336410
Protein subunit	Full length recombinant SARS CoV-2 glycoprotein nanoparticle vaccine adjuvanted with Matrix M	Novavax	Phase 1/2 NCT04368988
DNA	DNA Vaccine (GX-19)	Genexine Consortium	Phase 1 NCT04445389
DNA	DNA plasmid vaccine +Adjuvant	Osaka University/AnGes/Takara Bio	Phase 1 JapicCTI-205328
Inactivated	Inactivated	Institute of Medical Biology, Chinese Academy of Medical Sciences	Phase 1 NCT04412538
Non-replicating viral vector	Adeno-based	Gamaleya Research Institute	Phase 1 NCT04436471 NCT04437875
Protein subunit	Native like trimeric subunit Spike Protein vaccine	Clover Biopharmaceuticals Inc./GSK/Dynavax	Phase 1 NCT04405908
Protein subunit	Adjuvanted recombinant protein (RBD-Dimer)	Anhui Zhifei Longcom Biopharmaceutical/Institute of Microbiology, Chinese Academy of Sciences	Phase 1 NCT04445194
Protein subunit	Recombinant spike protein with Advax™ adjuvant	Vaxine Pty Ltd/Medytox	Phase 1 NCT04453852
RNA	LNP-nCOVsaRNA	Imperial College London	Phase 1 ISRCTN17072692
RNA	mRNA	Curevac	Phase 1 NCT04449276
RNA	mRNA	People's Liberation Army (PLA) Academy of Military Sciences/Walvax Biotech	Phase 1 ChiCTR2000034112
VLP	Plant-derived VLP	Medicago Inc./Universite Laval	Phase 1 NCT04450004 (not yet recruiting)
RNA	LNP-encapsulated mRNA	Moderna/NIAID	Phase 2 NCT04405076

CONCLUSION

Over the last 20 years, three coronaviruses have been transmitted from animals to humans who have resulted in epidemic or pandemic. SARS-CoV-1 led to the first epidemic of the twenty first century crippling the healthcare system of the affected countries. A WHO-led global response to this disease through a virtual network of laboratories and health systems worldwide helped limit its spread. There have been no new cases since 2004, but it remains a potential threat in the future. MERS emerged in 2012 and still exists in dromedary camels, and it has the potential to infect people who have close contact with them. The majority of human cases of MERS occurred due to human-to-human transmission in the healthcare setting. Hence, early recognition of a case and implementation of internationally recommended infection control measures are needed to prevent healthcare facility associated outbreaks. COVID-19 is the latest deadly respiratory illness that is believed to have originated in a live animal market in China. Its rapid spread has become a pandemic and continues to threaten the healthcare system and the world's economy. Stringent public health measures such as social distancing, contact tracing, testing, quarantines, and travel restrictions are of paramount importance to control the spread. Scientists are working to find medications to treat the disease

and to develop a vaccine. Multiple vaccines are currently in various trials. As now, there is no specific treatment or vaccine for COVID-19; therefore, prevention measures are critical. These zoonotic infections are the consequences of urbanization, agricultural work, and other human activities. There are currently no specific antiviral medications for SARS, MERS, or COVID-19. There are still knowledge gaps in understanding the pathophysiology, viral kinetics, and duration of viral shedding of COVID-19, which is a significant limitation in developing effective treatment and vaccines. Moreover, there is a significant lack of knowledge about natural history and clinical courses in special populations like pregnant patients and children. Therefore, well-coordinated international collaborative research needs to be done on the pathogenesis of human coronaviruses, which is needed to develop treatment and preventative measures against coronaviruses.

AUTHOR CONTRIBUTIONS

HG: conception and design. MG: formal analysis. AP, MG, and HG: literature search. RM: first draft. All authors critical revision and editing and final approval.

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Perception and Attitudes Toward PPE-Related Waste Disposal Amid COVID-19 in Bangladesh: An Exploratory Study

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Personal protective equipment (PPE) is an essential item to protect from exposure to infectious pathogens or contaminants, which is frequently used at health care settings and public spheres since the coronavirus disease 2019 (COVID-19) outbreak. There is no prior study investigating public perception and attitudes toward PPE-related waste disposal in Bangladesh. Hence, an online survey was carried out among 1,303 Bangladeshi adult residents to explore the issue. Results stated that face mask and hand gloves were the widely used PPE, where around 45.50% mask and 31.60% hand gloves were disposable. Approximately 94.50% of the participants percepted to use at least one type of PPE while going outside. Only 18.65% of the respondents percepted to burn the PPE-related waste, while most of them reported other less protective disposal measures. Females, urban residents, and participants with higher education were found to have better perception and attitudes toward PPE-related waste disposal. To the best of the authors' knowledge, being the first exploratory study in the country, the present findings are anticipated to be helpful at policy levels with respect to arranging awareness programs.

Keywords: COVID-19 pandemic, PPE waste disposal, environmental pollution, environmental health risk, public attitudes and practices, medical waste in Bangladesh

INTRODUCTION

Since the coronavirus disease 2019 (COVID-19) outbreak, personal protective equipment (PPE) (e.g., face masks, gloves, goggles, gowns, etc.) is being widely used in health care settings and public spheres, which rapidly accumulates potential infectious waste in the solid waste streams throughout the world (1). Proper disposal of these wastes is essential for the control of the reemergence of viral infection, and environmental protection (2), as well as to meet the Sustainable Development Goals, especially SDG3, SDG6, SDG8, SDG12, and SDG13 (3). Bangladesh reported the first COVID-19 case on March 8, 2020, and as of September 7, 2020, a total of 327,359 cases are reported (4). To control the COVID-19 transmission, the government encouraged people to use PPE through public awareness programs, and a rule concerning mandatory mask use was enforced on July 21, 2020 (5). The country is already alleged to have the worst waste management system, and the sudden rise of

COVID-19-related waste load and its improper disposal increased the risk of re-transmission and had consequences on the environment as well (1, 6, 7).

For having no recognized treatment available for COVID-19 patients, along with other public health measures, PPE is being recommended for use to escape from the potential virus infection (1, 8). Consistent with personal safety measures, Bangladeshi people have not fled out of the scenario. But, proper disposal of PPE-related waste is indispensable to reduce disease transmission (1, 6, 9). The haphazard disposal of these wastes may create clogging in waterways (e.g., municipal drain, canal, etc.) and enhance environmental pollution load, especially in poor urban areas (10). Plastic-based face masks and other PPE are known as a potential source of micro-plastic fibers in the environment (11). It is suggested that proper disposal and segregation of household waste with plastic-based healthcare waste and mix-up of these wastes increase the risk of disease transmission to waste workers (2). **Figure 1** illustrates the probable environmental and human health risk of PPE-related waste. Therefore, it is urgent to properly dispose of used PPE to lessen unwanted infectious sources (2, 6). Therefore, the present study, for the first time in Bangladesh, investigated the perception and attitudes toward PPE-related waste disposal, which may help government authorities to rethink policy levels.

METHODS

An online survey was conducted from May 20 to June 19, 2020, among a total of 1,303 Bangladeshi adult residents. Consistent with the study aims, a self-administered questionnaire was applied based on the national and international guidelines and literature regarding PPE waste disposal. The questionnaire included sociodemographics and safety equipment's use and disposal perception- and attitude-related questions. PPE waste disposal perception and attitudes were assessed with a total of five items based on a 5-point Likert scale (1 = strongly disagree to 5 = strongly agree), whereas overall score was based on summing all items (what the extract items were asked is presented in the **Table 1** footnote). The data were analyzed through Microsoft Excel (2010) and Statistical Package for the Social Sciences (version 25.0). Descriptive statistics such as frequency and percentage were used along with the ANOVA tests to test for PPE use perception and attitude mean differences with the variables. The level of statistical significance was $p < 0.05$ for all tests. Frequency and descriptive statistics were used to determine mean scores and standard deviations of the study variables.

RESULTS

A total of 1,303 responses were recorded in this study (mean age = 27.16 ± 7.78 years); 57.20% were male ($n = 745$), and 72% were from urban or semi-urban areas ($n = 937$). Most of the respondents were educated, and education levels were graduate level or above (70.0%), and 24.40% had higher secondary education level. Nearly 64% of them were students, along with other professions including teacher (3.20%), service holder (16.80%), businessman (2.30%), housewife (3.70%), unemployed (5.60%), and others (4.50%) (**Table 1**).

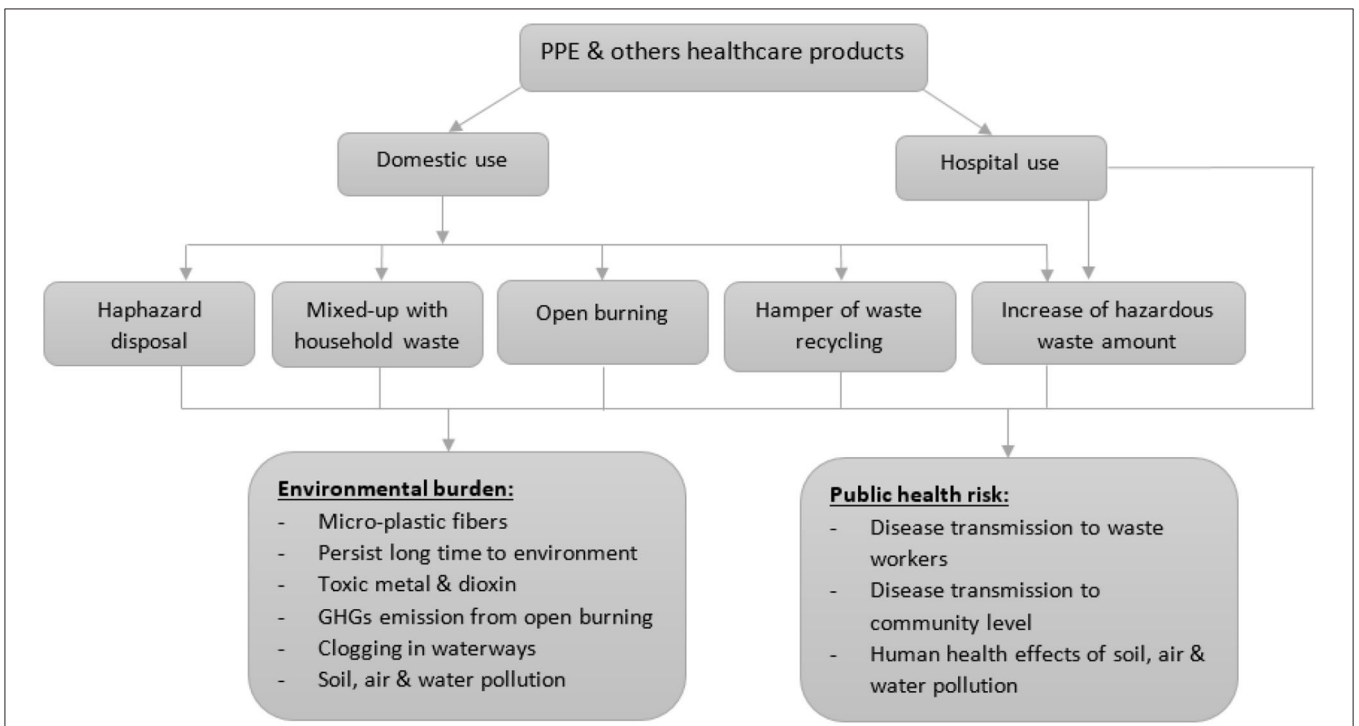

FIGURE 1 | Figure showing the environmental and public health risk of improper disposal of personal protective equipment (PPE) and others healthcare wastes (2, 11).

TABLE 1 | Mean difference of personal protective equipment (PPE)-related waste disposal perception attitudes.

Variables (*n*; %)	Overall perception and attitudes	P&A-1	P&A-2	P&A-3	P&A-4	P&A-5
Total mean ± SD	17.754 ± 3.342	4.673 ± 0.749	3.399 ± 1.357	3.291 ± 1.459	2.360 ± 1.275	4.031 ± 1.161
Gender						
Male (745; 57.20%)	17.431 ± 3.475***	4.663 ± 0.766	3.258 ± 1.351***	3.268 ± 1.456	2.323 ± 1.296	3.918 ± 1.240***
Female (558; 42.80%)	18.186 ± 3.106	4.686 ± 0.727	3.589 ± 1.342	3.321 ± 1.463	2.408 ± 1.247	4.181 ± 1.029
Age group						
18–30 years (1,138; 87.3%)	17.728 ± 3.374	4.676 ± 0.751**	3.395 ± 1.363	3.241 ± 1.465***	2.401 ± 1.286*	4.016 ± 1.159
31–40 years (99; 7.6%)	18.37 ± 3.151	4.778 ± 0.581	3.586 ± 1.317	3.747 ± 1.296	2.111 ± 1.186	4.151 ± 1.248
41–50 years (31; 2.4%)	17.645 ± 3.136	4.677 ± 0.599	3.419 ± 1.385	3.581 ± 1.500	2.000 ± 1.211	3.967 ± 1.277
More than 51 years (35; 2.7%)	16.943 ± 2.786	4.286 ± 1.073	3.029 ± 1.175	3.371 ± 1.457	2.057 ± 1.109	4.200 ± 0.867
Residence						
Rural (365; 28.0%)	16.644 ± 3.573***	4.430 ± 0.957***	3.096 ± 1.300***	3.008 ± 1.409***	2.564 ± 1.204***	3.545 ± 1.256***
Urban (938; 72.0%)	18.187 ± 3.145	4.768 ± 0.626	3.518 ± 1.360	3.401 ± 1.464	2.280 ± 1.294	4.219 ± 1.065
Education						
Primary (23; 1.8%)	15.913 ± 2.678*	4.174 ± 1.072*	2.869 ± 1.140**	2.826 ± 1.614	2.174 ± 1.193	3.869 ± 0.967
Secondary (50; 3.8%)	17.420 ± 3.643	4.640 ± 0.875	3.240 ± 1.302	3.120 ± 1.466	2.500 ± 1.344	3.920 ± 1.047
Higher secondary (318; 24.4%)	17.616 ± 3.194	4.679 ± 0.722	3.286 ± 1.354	3.211 ± 1.442	2.384 ± 1.292	4.056 ± 1.116
Graduate (650; 49.9%)	17.917 ± 3.225	4.692 ± 0.720	3.521 ± 1.343	3.295 ± 1.461	2.377 ± 1.265	4.031 ± 1.156
Postgraduate (262; 20.1%)	17.744 ± 3.735	4.668 ± 0.783	3.313 ± 1.398	3.450 ± 1.450	2.279 ± 1.278	4.034 ± 1.266
Occupation						
Business (30; 2.3%)	17.700 ± 4.036	4.700 ± 0.794***	3.200 ± 1.186	3.533 ± 1.408	2.400 ± 1.003	3.867 ± 1.224
Service (219; 16.8%)	17.612 ± 3.428	4.667 ± 0.780	3.365 ± 1.389	3.425 ± 1.458	2.210 ± 1.246	3.945 ± 1.312
Student (832; 63.9%)	17.829 ± 3.301	4.686 ± 0.733	3.454 ± 1.354	3.215 ± 1.479	2.433 ± 1.304	4.041 ± 1.127
Teacher (42; 3.2%)	18.381 ± 3.882	4.881 ± 0.328	3.452 ± 1.451	3.643 ± 1.574	2.238 ± 1.411	4.167 ± 1.286
Housewife (48; 3.7%)	17.312 ± 3.149	4.187 ± 1.065	3.083 ± 1.235	3.500 ± 1.288	2.375 ± 1.248	4.167 ± 0.975
Unemployed (73; 5.6%)	17.479 ± 3.420	4.671 ± 0.765	3.219 ± 1.315	3.411 ± 1.245	2.274 ± 1.133	3.904 ± 1.227
Others (59; 4.5%)	17.508 ± 2.873	4.746 ± 0.575	3.305 ± 1.417	3.169 ± 1.440	2.051 ± 1.121	3.237 ± 0.953

$^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$.

P&A-1, wearing PPE (e.g., mask, hand gloves, etc.) while going outside of home; P&A-2, disposing PPE and other healthcare waste in separate covered bins or bags; P&A-3, disposing PPE and other healthcare waste in household waste bins; P&A-4, burning PPE and other healthcare waste individually; P&A-5, disposing PPE and other healthcare waste in community containers or disposal areas.

Among the available PPE, face mask and hand gloves were highly used. Of these, nearly 45.50 and 31.60% of people used disposable face masks and hand gloves, respectively. Approximately 94.50% of the participants percepted to use at least any type of protective equipment as a preventive measure of COVID-19 while going outside of home for working, shopping, or any other purpose. Only half of the respondents (49.35%) perceived to disposed of the used mask, hand gloves, and others healthcare waste in separate covered bins or bags, whereas 54.56 and 75.60% reported to have the attitudes of disposing PPE in household bins and in the community container or disposal area, respectively. Only 18.65% of participants percepted to burn their used mask, hand gloves, tissues, and other bio-waste to reduce disease transmission (**Figure 2**).

In **Table 1**, the relationship between PPE-related waste disposal perception and attitudes and sociodemographics are presented. Within the total sample, the PPE-related waste disposal perception and attitude mean score was 17.754 (± 3.342). However, PPE waste disposal perception and attitude mean scores were higher in female gender (18.186 ± 3.106 vs. 17.431 ± 3.475; $f = 16.502$, $p < 0.001$), urban residence (18.187 ± 3.145 vs. 16.644 ± 3.573; $f = 58.473$, $p < 0.001$), and higher education level ($f = 2.402$, $p = 0.048$). Besides, significant mean differences of perception and attitude components, i.e., disposing in separate covered bins or bags ($f = 19.360$, $p < 0.001$) and disposing in community containers or disposal areas ($f = 16.535$, $p < 0.001$) were also found to be higher in females. Whereas, participants from urban areas highly percepted across all of the components. Lastly, education level was significantly associated with wearing PPE while going outside ($f = 2.707$, $p = 0.029$) and disposing PPE-related waste in covered bins ($f = 3.207$, $p = 0.012$), whereas it was only wearing PPE while going outside that associated with occupation status ($f = 4.103$, $p < 0.001$; **Table 1**).

DISCUSSION

To the best of the authors' knowledge, the present study for the first time provides an initial observation on PPE-related waste disposal perception and attitudes amid the COVID-19 outbreak among the Bangladeshi sample. Based on the findings, a higher portion of the participants reported to have the perception

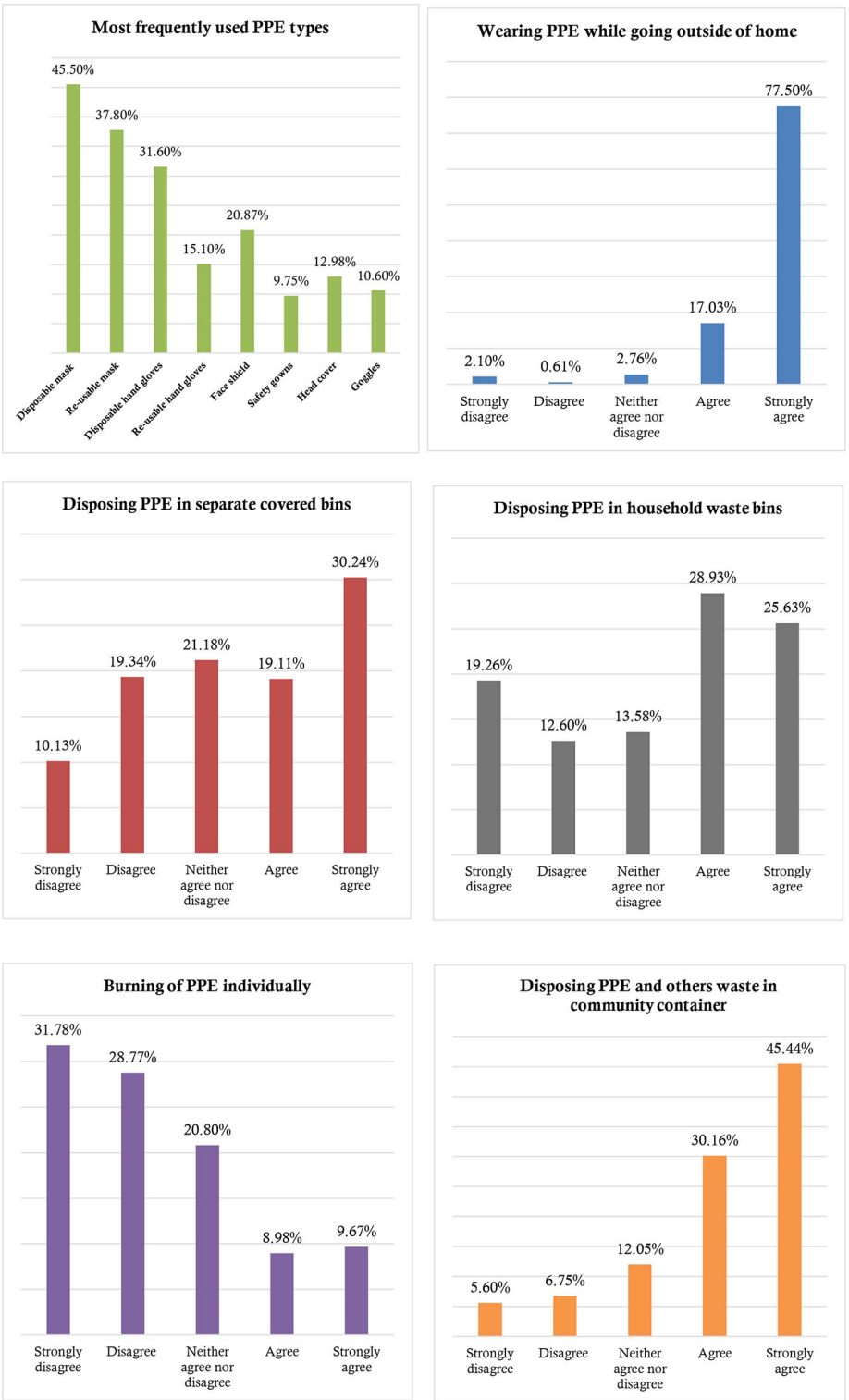

FIGURE 2 | Distribution of personal protective equipment (PPE) use and PPE-related waste disposal perception and attitudes.

and attitudes of disposing PPE-related waste within household waste and in community containers or disposal areas, which may be negligibly effective against virus reinfection for the country. Bangladesh has been reported to mismanage handling healthcare waste in either household or community areas despite proper rules and regulations (6). As a result, healthcare waste is

mostly disposed of in unauthorized places without any separation or proper treatment by untrained, unprotected, and unaware cleaners (6).

Higher literacy is commonly regarded as the protective factor against occurring negative effects; similar assertions can be made for COVID-19-related issues. For instance, the study reported that higher education, more specifically, literacy related to COVID-19, increases the positive attitudes and practices toward the COVID-19 issue that are reported in other countries like the present finding (12, 13). Besides, the urban residents are reported to have more positive PPE-related waste disposal perception and attitudes, which can be explained by the sociodemographic condition of the country. That is, in Bangladesh, the urban people's literacy rate is far higher than the rural ones as reported by UNESCO (14). An Egyptian study observed the positive effect of safety and waste management literacy on the laboratory technician's knowledge, attitudes, and practices after implementing an intervention program (15), which reflects the urgent need of literacy awareness programs in Bangladeshi people. Moreover, it is found that females are more concerned regarding disposing of PPE-related waste compared to males. This finding may be because of their responsibilities of taking care of the family members' PPE-related waste disposal. Besides, females are usually considered as more cautious than males in terms of infectious disease prevention practices, e.g., hand hygiene, PPE use, etc., that is reported in other countries for their higher positive attitudes toward COVID-19 issues (12, 16).

Since the COVID-19 outbreak, biomedical waste generation rate has increased globally, including Bangladesh, which creates extra public health burden and becomes a challenge to waste management authorities (17). It is reported that, on average, 1.63–1.99 kg/bed/day medical waste is generated in Dhaka City, the capital of Bangladesh, whereas there are nearly 141,903 hospital beds in the country (6). Approximately, 40,000 informal waste collectors work across the country; they are at high risk of COVID-19 infection due to lack of adequate protection (6, 7, 18). Poor management of COVID-19 wastes in Bangladesh increases the risk of infection and environmental hazards. Polypropylene is the common material of protective equipment like N-95 masks, and Tyvek is used for protective suits, hand gloves, and medical face shields, which can persist for a long time and pollute the environment (1). Due to the disruption of routine municipal waste management and plastic waste recovery and recycling activities for the pandemic, it increases the landfilling and environmental pollutants like dioxins and toxic metals (1, 11).

As aforementioned, improper PPE-related waste disposal and management can be the source of reemergence of the virus infection. Therefore, ensuring public better attitudes and practices toward PPE-related waste disposal along with the time-oriented policy should be implemented. The present findings, being an initial observation, may help policy makers in facilitating public awareness programs.

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 Institute of Allergy and Clinical Immunology of Bangladesh, Dhaka, Bangladesh. The patients/participants provided their online informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SI, MB-D, and MM conceptualized the study, implemented the project, collected data, and wrote the first draft. MS and MM reanalyzed the data, interpreted the data, rewrote the draft, and addressed the reviewer comments. All authors approved the final version.

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Association of Statin Use With the In-Hospital Outcomes of 2019-Coronavirus Disease Patients: A Retrospective Study

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Background: Statins have multiple protective effects on inflammation, immunity and coagulation, and may help alleviate pneumonia. However, there was no report focusing on the association of statin use with in-hospital outcomes of patients with coronavirus disease 2019 (COVID-19). We investigated the association between the use of statins and in-hospital outcomes of patients with COVID-19.

Methods: In this retrospective case series, consecutive COVID-19 patients admitted at 2 hospitals in Wuhan, China, from March 12, 2020 to April 14, 2020 were analyzed. A 1:1 matched cohort was created by propensity score-matched analysis. Demographic data, laboratory findings, comorbidities, treatments and in-hospital outcomes were collected and compared between COVID-19 patients taking and not taking statins.

Result: A total of 2,147 patients with COVID-19 were enrolled in this study. Of which, 250 patients were on statin therapy. The mortality was 2.4% (6/250) for patients taking statins while 3.7% (70/1,897) for those not taking statins. In the multivariate Cox model, after adjusting for age, gender, admitted hospital, comorbidities, in-hospital medications and blood lipids, the risk was lower for mortality (adjusted HR, 0.428; 95% CI, 0.169–0.907; $P = 0.029$), acute respiratory distress syndrome (ARDS) (adjusted HR, 0.371; 95% CI, 0.180–0.772; $P = 0.008$) or intensive care unit (ICU) care (adjusted HR, 0.319; 95% CI, 0.270–0.945; $P = 0.032$) in the statin group vs. the non-statin group. After propensity score-matched analysis based on 18 potential confounders, a 1:1 matched cohort (206:206) was created. In the matched cohort, the Kaplan-Meier survival curves showed that the use of statins was associated with better survival ($P = 0.025$). In a Cox regression model, the use of statins was associated with lower risk of mortality (unadjusted HR, 0.254; 95% CI, 0.070–0.926; $P = 0.038$), development of ARDS (unadjusted HR, 0.240; 95% CI, 0.087–0.657; $P = 0.006$), and admission of ICU (unadjusted HR, 0.349; 95% CI, 0.150–0.813; $P = 0.015$). The results remained consistent when being adjusted for age, gender, total cholesterol, triglyceride, low density lipoprotein cholesterol, procalcitonin, and brain natriuretic peptide. The favorable outcomes in statin users remained statistically

significant in the first sensitivity analysis with comorbid diabetes being excluded in matching and in the second sensitivity analysis with chronic obstructive pulmonary disease being added in matching.

Conclusion: In this retrospective analysis, the use of statins in COVID-19 patients was associated with better clinical outcomes and is recommended to be continued in patients with COVID-19.

Keywords: COVID-19, statin, outcome, mortality, ARDS

INTRODUCTION

Coronavirus disease 2019 (COVID-19) has already been a global pandemic since early December 2019. Since then, infection with COVID-19 has rapidly spread throughout the world, even causing widespread social and economic disruption (1, 2). At the time of submission, the total number of infected patients has risen to 43, 251, 698 around the world, with 1,154,214 associated deaths. However, thus far, there are no specific therapies or vaccines available for COVID-19. Most of the currently used clinical interventions are symptomatic supportive therapies, which have exhibited limited therapeutic effects for COVID-19.

It is worth noticing that patients with common comorbidities, including hypertension and cardiovascular diseases are at greater risk for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and its related acute respiratory distress syndrome (ARDS) and mortality (3). Most of these patients are taking statins routinely based on cardiovascular guidelines. Statins, as one of the inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA), are a class of lipid-lowering medications and are frequently used in patients with cardiovascular diseases or to prevent cardiovascular events (4–6). Statins are also well-known for their potential immunomodulatory and anti-inflammatory effects in pneumonia (7, 8). An earlier retrospective cohort study showed that, in bacterial pneumonia patients, in-hospital mortality was significantly reduced after using statins (9). Many researchers have focused on statins in the treatment of infections (10–12). In 2014, some researchers suggested that statins might be used for treatment of patients with Ebola virus disease (13). Although most of these studies argue that statins are advantageous to outcomes and prognosis of patients with pneumonia, Fernandez et al. demonstrated that hospital mortality was significantly higher after statin therapy (14). Therefore, whether statin use was associated with reduced mortality for patients with pneumonia is still in debate. To the best of our knowledge, there is no clinical or experimental data focusing on the effects of statin use on the in-hospital outcomes of COVID-19 patients. The main purpose of the present study was to investigate the association of the statin use with the in-hospital outcomes of COVID-19 patients.

METHODS

Study Design and Participants

This retrospective study was performed at Zhongnan Hospital of Wuhan University and Leishenshan Hospital in Wuhan, China, which were designated hospitals to treat patients with COVID-19. Leishenshan Hospital was taken over by Zhongnan Hospital of Wuhan University during the epidemic period. The inclusion criteria included patients with COVID-19 who were admitted to the 2 hospitals from March 12, 2020 to April 14, 2020 and who were either discharged with following recovery or died during hospitalization. The exclusion criteria included incomplete medical records, loss of follow-up due to being transferred to other hospitals and discontinuation of use of statins. COVID-19 was diagnosed according to the interim guidance of the World Health Organization (15). The treatment strategies for COVID-19 in the 2 hospitals were based on Chinese Guideline of Clinical Management for COVID-19 (1st−7th version). Patients with critical illness (severe respiratory failure, shock, and multiple organ dysfunction) were transferred to the intensive care unit (ICU). This study complied with the edicts of the 1975 Declaration of Helsinki (16) and was approved by the institutional ethics board of Zhongnan Hospital of Wuhan University (No. 2020026). The patients' consents were obtained from individual participant or their relatives.

Data Collection

The electronic medical records of the patients with confirmed SARS-CoV-2 infection by real-time reverse-transcription PCR (RT-PCR) were extracted and reviewed by a trained team of physicians from the 2 hospitals during the epidemic period. The data including demographics, medical history, laboratory examinations, comorbidities, complications, treatments, and outcomes were collected and analyzed. The researchers were responsible to contact the patients or their families in case of uncertainties about the data to ensure to maximum the accuracy of our data.

In-hospital Outcomes

The main in-hospital outcomes included COVID-19 related death or discharge. Successful treatment toward hospital cure for the patients with COVID-19 comprised all of the following criteria: relieved clinical symptoms, normal body temperature, significant resolution of inflammation as shown by chest radiography and at least 2 consecutive negative results assessed by RT-PCR assay for SARS-Cov-2 (17). The secondary outcomes included development of ARDS and requirement of ICU care. The definition of ARDS required bilateral infiltrates on chest radiograph consistent with pulmonary edema and partial pressure of arterial oxygen/fraction of inspired oxygen $<$300 mmHg.

Propensity Score-Matched (PSM) Analysis and Sensitivity Analysis

To validate the findings, propensity score-matched (PSM) cohort was created based on 18 baseline variables which were expected to be potential confounders. Statin and non-statin users were paired according to the propensity scores using nearest matching with a caliper size of 0.05. The balance of covariates was evaluated by estimating standardized differences (SD) and *p*-values before and after matching, and SD absolute value <0.1 was considered perfect balancing between the two groups. The imbalanced variables with SD $\geq$ 0.1 in PSM analysis were adjusted in the following multivariate Cox model.

We performed two sensitivity analyses to evaluate the robustness of propensity score-matched cohort analyses. In the first sensitivity analysis, comorbid diabetes was not excluded in matching. In the second sensitivity analysis, chronic obstructive pulmonary disease (COPD) was added in matching.

Statistical Analysis

Categorical variables are shown as frequencies and percentages, and continuous variables as mean ± standard deviation, or median (interquartile range). The means for continuous variables were compared using independent group *t*-tests when the data were normally distributed, otherwise, the Mann–Whitney *U*-test was used. Proportions for categorical variables were compared using the X^2-test, although Fisher's exact test was used when data were limited. A Kaplan–Meier plot was used for survival data. We compared the in-hospital outcomes of patients who did and did not use statins by using Cox proportional hazards models to calculate hazard ratios and 95% confidence intervals. In the unmatched cohort, the variables which were considered to confound the association of statin use with the clinical outcome were adjusted for in the Cox regression model. Additionally, we adjusted for imbalanced variables with SD $\geq$ 0.1 in PSM analysis in following multivariate Cox model. All statistical analyses were performed with SPSS19.0 for Windows. A two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

Demographics Data

A total of 2,351 consecutive patients hospitalized with COVID-19 who were successfully treated and discharged or died at Zhongnan Hospital of Wuhan University or Leishenshan Hospital from March 12 to April 14, 2020 were analyzed (**Figure 1**). By reviewing all electronic medical records, 128 patients with incomplete data, 55 patients who were on statins prior to admission but interrupted after admission and 21 patients who had been transferred to other hospitals and lost follow-up were excluded. Finally, 2,147 patients with COVID-19 were enrolled in this study. Of which, 250 patients were on statins prior to admission and continued their use during hospitalization including 162 (64.8%) atorvastatin (20 mg every day) and 75 (30.0%) rosuvastatin (10 mg every day), and the remaining 13 (5.2%) patients used other statins such as pravastatin. The remaining 1,897 patients never used statins.

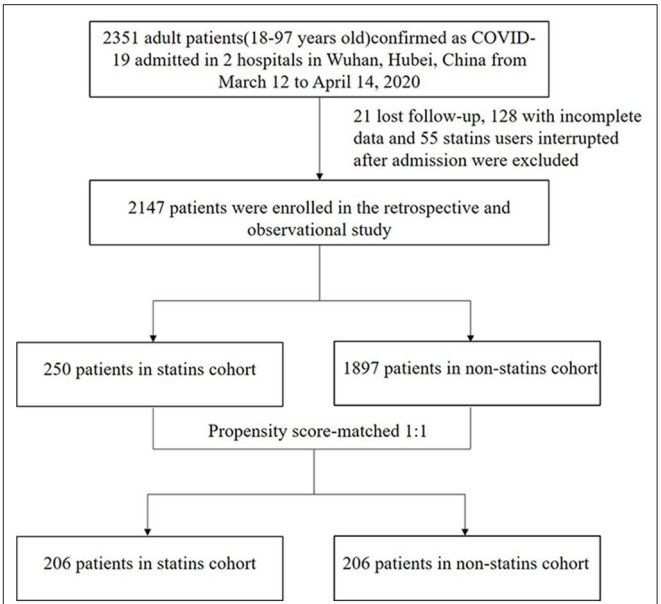

FIGURE 1 | The flowchart showing the strategy of participant enrollment COVID-19, coronavirus disease 2019.

Clinical Characteristics on Admission Before and After PSM Analysis

The comorbid coronary heart disease, hypertension, diabetes, cerebrovascular diseases were more frequent in the statin users than the non-statin users (**Table 1**). Many laboratory results including neutrophil count, prothrombin time, activated partial thromboplastin time, D-dimer, total cholesterol (TC), triglyceride, low density lipoprotein cholesterol (LDL-C), procalcitonin, creatine kinase-MB, high-sensitivity troponin I (hs-TnI), and brain natriuretic peptide (BNP) showed significant ($p < 0.05$) or marginal significant ($p < 0.10$) differences between the two groups on admission (**Table 2**). Since many significantly imbalanced variables existed in the baseline state on admission, the outcomes could not be directly compared between the two groups. Then the PSM analysis was conducted to account for these 18 potential confounding factors (**Supplementary Table 1**). A new cohort of patients were matched in the statin group *vs.* non-statin group at a ratio of 1:1 with 206 patients in each group. In the matched cohort, all the variables in **Table 1** did not show significant differences (all $p > 0.05$), however, the variables with SD > 0.1 including TC, triglyceride, LDL-C, procalcitonin, and BNP did not achieve a perfect matching, therefore, along with age and sex, these variables entered in the multivariate COX model for adjustment (**Table 3**). One hundred and forty-four (69.9%) patients were on atorvastatin (20 mg every day), 61 (29.6%) were on rosuvastatin (10 mg every day) and the other one patient was on pravastatin. The treatments were comparable in the unmatched and matched cohorts (**Table 1**).

In-hospital Outcomes

In the unmatched cohort, the mortality is 2.4% (6/250) for patients taking statins while 3.7% (70/1,897) for those not taking

TABLE 1 | Demographics, clinical characteristics, and treatments of COVID-19 patients.

	Unmatched			Matched		
	Non-statin	**Statin**	***P*-value**	**Non-Statin**	**Statin**	***P*-value**
Number of patients	1,897	250		206	206	
Male-counts (%)	926 (48.8)	115 (46.0)	0.403	80 (38.8)	90 (43.7)	0.317
Age-years	58 (48, 68)	66 (57, 72)	<0.001	66 (57, 73)	64 (57, 72)	0.556
Smoker-counts (%)	133 (7.0)	21 (8.4)	0.423	18 (8.7)	19 (9.2)	0.863
Heart Rate, bpm	83 (75, 92)	85 (77, 99)	0.661	84 (76, 94)	85 (75, 98)	0.780
SBP, mmHg	130 (124, 140)	133 (122, 145)	0.311	132 (124, 141)	133 (123, 145)	0.564
DBP, mmHg	81 (73, 95)	83 (73, 98)	0.552	82 (72, 97)	83 (73, 97)	0.785
Fever-counts (%)	1,332 (70.2)	167 (66.8)	0.269	144 (69.9)	139 (67.5)	0.595
Hospitalization-days[a]	17 (12, 24)	16 (12, 21)	0.589	16 (12, 23)	16 (16, 20)	0.799
Comorbidities-count (%)						
Hypertension	575 (30.3)	130 (52.0)	<0.001	106 (51.5)	102 (49.5)	0.693
Coronary heart disease	97 (5.1)	68 (27.2)	<0.001	44 (21.4)	41 (19.9)	0.715
Diabetes	239 (12.6)	54 (21.6)	<0.001	41 (19.9)	41 (19.9)	>0.999
Cerebrovascular diseases	43 (2.3)	27 (10.8)	<0.001	13 (6.3)	16 (7.8)	0.563
COPD	35 (1.8)	4 (1.6)	>0.999	10 (4.9)	3 (1.5)	0.087
Chronic hepatic dysfunction	55 (2.9)	9 (3.6)	0.540	10 (4.9)	9 (4.4)	0.814
Chronic renal dysfunction	63 (3.3)	10 (4.0)	0.578	10 (4.9)	7 (3.4)	0.457
Malignancy	45 (2.4)	4 (1.6)	0.651	11 (5.3)	4 (1.9)	0.112
Treatments-count (%)						
Antiviral therapy	1,726 (90.9)	230 (92.0)	0.596	181 (87.9)	187 (90.7)	0.543
Antibiotic therapy	813 (42.9)	110 (44.0)	0.732	91 (44.2)	91 (44.2)	>0.999
Glucocorticoid therapy	173 (9.1)	24 (9.6)	0.805	21 (10.2)	16 (7.8)	0.389
Immunoglobulin therapy	61 (3.2)	7 (2.8)	0.724	4 (1.9)	5 (2.4)	0.751
ACEI/ARB	106 (5.6)	38 (15.2)	<0.001	22 (10.7)	33 (16.0)	0.111
Mechanical ventilation	180 (9.4)	26 (10.4)	0.646	22 (10.6)	17 (8.2)	0.400
CRRT	47 (2.5)	3 (1.2)	0.267	6 (2.9)	2 (1.0)	0.284
ECMO	9 (0.5)	0 (0)	0.610	4 (1.9)	0 (0)	0.123

Values are median (interquartile range), mean ± standard deviation or n (%). [a]Hospitalization indicates days from admission to death/discharge. COVID-19, coronavirus disease 2019; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; SBP, systolic blood pressure; DBP, diastolic blood pressure; COPD, chronic obstructive pulmonary disease; CRRT, continuous renal replacement therapy; ECOM, extracorporeal membrane oxygenation.

statins. In the multivariate Cox model, after adjusting for age, gender, admitted hospital, comorbidities (hypertension, coronary heart disease, diabetes, cerebrovascular diseases), in-hospital medications (ACEI/ARB, glucocorticoid) and blood lipids (TC, LDL-C), statin use was associated with lower mortality (adjusted HR, 0.428; 95% CI, 0.169–0.907; $P = 0.029$), ARDS (adjusted HR, 0.371; 95% CI, 0.180–0.772; $P = 0.008$) or ICU care (adjusted HR, 0.319; 95% CI, 0.270–0.945; $P = 0.032$) vs. non-statin use.

In the matched cohort, as shown in the Kaplan-Meier survival curves, the use of statins was associated with better survival ($P = 0.025$; **Figure 2**). In a Cox regression model, the use of statins was associated with lower risk of in-hospital mortality (unadjusted HR, 0.254; 95% CI, 0.070–0.926; $P = 0.038$), development of ARDS (unadjusted HR, 0.240; 95% CI, 0.087–0.657; $P = 0.006$), and admission of ICU (unadjusted HR, 0.349; 95% CI, 0.150–0.813; $P = 0.015$). The results remained consistent when being adjusted for age, gender, TC, triglyceride, LDL-C, procalcitonin, and BNP (**Table 3**).

Sensitivity Analyses

Additional sensitivity analyses aiming to further assess the robustness of the association between statin use and outcomes were performed, the results remained consistent and statistically significant in the first sensitivity analysis with comorbid diabetes being excluded in matching and in the second sensitivity analysis with COPD being added in matching (**Supplementary Table 2**).

DISCUSSION

Main Findings

The present study demonstrated for the first time that, the continuous use of statins was associated with lower mortality, less development of ARDS and less requirement of ICU care. Even after matched or adjusted for the blood lipids, the results remained consistently significant, indicating that this association was independent of its lipid-lowering effect.

Association of the Statin Use With the In-hospital Outcomes for Patients With COVID-19

The observations regarding the association of statin use with the outcomes of types of pneumonia had been reported previously (18, 19). Overall, significant decrease in mortality

TABLE 2 | Laboratory results among different groups on admission.

	Unmatched			Matched		
	Non-statin	**Statin**	***P*-value**	**Non-statin**	**Statin**	***P*-value**
Number of patients, *n*	1,897	250		206	206	
White blood cell count, $\times 10^9$/L	5.21 (3.58, 6.64)	5.41 (3.94, 6.93)	0.203	5.21 (3.40, 6.65)	5.27 (3.89, 6.82)	0.530
Neutrophil count, $\times 10^9$/L	3.28 (2.53, 4.39)	3.49 (2.60, 4.72)	0.049	3.29 (2.48, 4.21)	3.43 (2.59, 4.65)	0.218
Lymphocyte count, $\times 10^9$/L	1.54 (1.16, 1.93)	1.59 (1.10, 1.96)	0.610	1.46 ± 0.61	1.60 ± 0.66	0.028
Hemoglobin, g/L	126 (114, 136)	123 (114, 135)	0.214	122 (110, 130)	124 (115, 135)	0.016
Platelet, $\times 10^9$/L	225 (182, 274)	224 (182, 290)	0.608	216 ± 74	239 ± 78	0.002
D-dimer, μg/mL	0.45 (0.23, 1.26)	0.62 (0.30, 1.58)	0.001	0.47 (0.31, 1.28)	0.47 (0.32, 1.10)	0.767
Total cholesterol, mmol/L	4.23 (3.62, 4.81)	4.16 (3.45, 5.36)	0.455	4.15 (3.48, 4.98)	4.22 (3.50, 5.15)	0.386
Triglyceride, mmol/L	1.17 (0.79, 1.70)	1.39 (0.87, 2.10)	<0.001	0.68 (0.45, 1.00)	1.37 (0.89, 1.91)	<0.001
HDL-C, mmol/L	1.13 (0.94, 1.36)	1.13 (0.94, 1.32)	0.910	1.30 ± 0.40	1.17 ± 0.29	<0.001
LDL-C mmol/L	2.54 ± 0.70	2.61 ± 0.95	0.284	2.41 (1.87, 2.92)	2.50 (1.98, 3.11)	0.131
Serum potassium, mmol/L	4.33 (4.03, 4.62)	4.34 (4.00, 4.67)	0.510	4.34 ± 0.50	4.35 ± 0.54	0.888
Serum calcium, mmol/L	2.18 (2.10, 2.24)	2.18 (2.09, 2.25)	0.637	2.18 (2.10, 2.25)	2.15 (2.07, 2.21)	0.001
CRP, mg/L	1.62 (0.53, 6.11)	1.50 (0.53, 7.20)	0.834	2.28 (0.65, 13.7)	1.50 (0.54, 6.14)	0.042
Procalcitonin, ng/mL	0.04 (0.03, 0.06)	0.04 (0.03, 0.07)	0.042	0.04 (0.03, 0.06)	0.04 (0.03, 0.06)	0.425
Creatine kinase, U/L	53.0 (36.8, 79.0)	55.0 (35.0, 77.8)	0.185	54.5 (39.0, 83.1)	57.4 (35.0, 87.8)	0.091
Creatine kinase–MB, ng/mL	1.16 (0.80, 1.94)	1.36 (0.98, 2.04)	0.012	1.19 (1.19,1.21)	1.19 (1.19,1.49)	0.256
hs-TnI, ng/mL	0.01 (0.010, 0.011)	0.01 (0.010, 0.013)	0.084	0.01 (0.010, 0.013)	0.01 (0.010, 0.013)	0.261
BNP, pg/mL	0.01 (0.01, 44.6)	14.4 (0.010, 83.2)	0.025	10 (10.0, 21.9)	10 (10.0, 22.2)	0.792
Alanine aminotransferase,U/L	23.0 (15.0, 38.0)	22.1 (15.0, 38.0)	0.781	19.0 (12.3, 34.9)	21.9 (15.0, 36.0)	0.026
Aspartate aminotransferase,U/L	20.0 (16.0, 28.0)	20.0 (16.0, 28.0)	0.766	20.0 (16.0, 28.8)	20.0 (16.0, 26.7)	0.376
Creatinine, μmol/L	64.6 (54.2, 77.2)	64.9 (55.5, 78.3)	0.496	63.9 (56.4, 77.7)	63.9 (55.6, 77.9)	0.864
PaO_2/FiO_2, mmHg	351 (312, 387)	335 (303, 372)	0.113	366 (318, 389)	335 (309, 370)	0.075

Values are median (interquartile range), mean ± standard deviation. HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; CRP, C-reactive protein; hs-TnI, high-sensitivity troponin I; BNP, brain natriuretic peptide; PaO2/FiO2, partial pressure of arterial oxygen to the fraction of inspired oxygen.

TABLE 3 | Hazard ratio for In-hospital outcomes of COVID-19 patients in matched cohort.

	Total	**Non-statin**	**Statin**	***P*-value**	**HR**	**95% CI**
Unadjusted outcomes-count (%)						
Death-count	13 (3.2)	10 (4.9)	3 (1.5)	0.038	0.254	0.070–0.926
ARDS	22 (5.3)	16 (7.8)	6 (2.9)	0.006	0.240	0.087–0.657
ICU admission	26 (6.3)	17 (8.3)	9 (4.4)	0.015	0.349	0.150–0.813
Adjusted outcomes-count (%)						
Death-count	13 (3.2)	10 (4.9)	3 (1.5)	0.037	0.251	0.068–0.923
ARDS	22 (5.3)	16 (7.8)	6 (2.9)	0.034	0.232	0.060–0.894
ICU admission	26 (6.3)	17 (8.3)	9 (4.4)	0.031	0.381	0.158–0.915

Values are n (%). P-value was acquired by using the COX model. COVID-19, coronavirus disease 2019; ARDS, acute respiratory distress syndrome; ICU, intensive care unit; HR, hazard ratio; CI, confidence interval.
Adjusted variables included age, gender, TC, triglyceride, LDL-C, procalcitonin, and BNP.

was demonstrated in the hospitalized pneumonia patients taking statins. A previous retrospective cohort study showed that statin use was associated with a decreased risk of mortality in patients hospitalized with community-acquired pneumonia (20). In another study, continuous use of statins was also correlated with a decreased risk of mortality or intubation in patients with COPD (21) Consistent with above observations, we found a decrease in mortality, less development of ARDS, and less requirement of ICU care in patients with continuous use of statins during their hospitalization for COVID-19.

The current study further showed some laboratory indicators reflecting inflammation (C-reactive protein, procalcitonin) and myocardial injury (hs-TNI, BNP) on admission could not be perfectly matched by PSM analysis. They might be still resistant confounders which had been adjusted in the following multivariate Cox model. However, there is another possibility

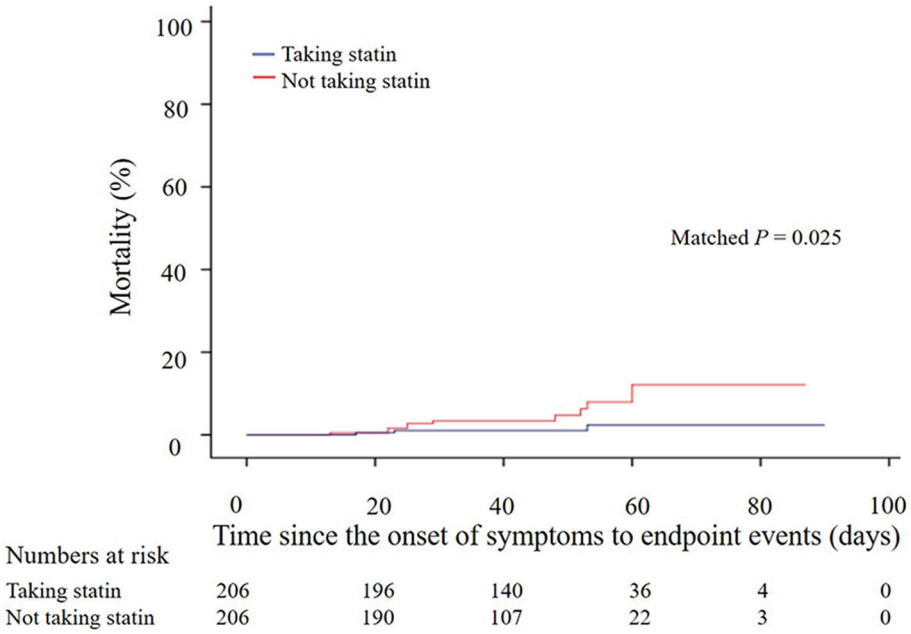

FIGURE 2 | Kaplan–Meier survival curves of 412 COVID-19 patients with and without taking statins (206:206) after propensity score-matched analysis. COVID-19, coronavirus disease 2019.

that, they might be mediators. Due to routine use of statins before admission, the pharmacological actions of statins on COVID-19 patients may be already significant since the patients were initially infected with COVID-19. This postulation might be supported by the findings of elevated alanine transaminase and creatine kinase and decreased calcium concentration on admission, which were frequently-observed side effects in long-term use of statins, especially in those with complications of liver injury and rhabdomyolysis.

Possible Mechanisms for the Association Between Statin Use and Favorable Outcomes of COVID-19

The potential benefits of statins for patients with COVID-19 are presumably related to their multiple effects. In addition to their benefits in patients with cardiovascular or cerebrovascular diseases, the pleiotropic effects including anti-inflammatory, anti-thrombotic, immunomodulatory, and reducing reactive oxygen species have also been reported (22). The evidence for the anti-inflammatory properties of statins in the lung and their potential role as novel treatments for respiratory diseases have been noticed (23). Our findings were consistent with these reports and demonstrated that the use of statins was associated with less development of ARDS. This association was beyond its lipid-lowering effect.

Increased level of angiotensin II was observed in COVID-19-affected individuals, which was demonstrated to be correlated with viral load and severity of illness of COVID-19 (24). As the isoenzyme of angiotensin converting enzyme (ACE), ACE2 plays a protective role in generating angiotensin-(1-7) (Ang 1-7) from angiotensin II in renin-angiotensin-aldosterone system (RAAS). When patients are infected with SARS-CoV-2, their ACE2 is further down-regulated by binding it with SARS-CoV-2 resulting in a deterioration of the imbalance of ACE2/ACE, and subsequently induces a sharp release of AngII by over-activation of RAAS. The upregulation of ACE2 by the use of statins was reported previously (25, 26), and may be another important mechanism of statin benefits for COVID-19. However, the exact mechanisms underlying the association between statin use and in-hospital outcomes need to be validated by further experimental studies and clinical observations.

Comparisons to Other Studies

Statins are well-known for their anti-inflammatory effects, and some hospitals included them in the COVID-19 treatment protocol (27). However, whether statin use was associated with reduced mortality for patients with COVID-19 is still in debate. Especially, some scholars have an opinion that statins should be used with caution in COVID-19 patients because it could cause myalgia, myopathies, or rhabdomyolysis, thereby exacerbating the disease (28), yet it should be noted that both of statin induced myopathies and the deterioration of SARSCoV-2 infection are characterized as elder, and liver and kidney dysfunction. Thus, it is hard to differentiate the appearance of myalgia, rhabdomyolysis, increased creatine phosphokinase, and acute kidney injury in COVID-19 from statin therapy (29). Furthermore, there were other studies showed that COVID-19 patients could not benefit from the administration of statin (30–32). This discrepancy may result from the limited

sample size and the heterogeneity of study population. Such as in the study from Dreher et al. only 50 patients with COVID-19 were included (31), and in the case series of 1,000 COVID-19 patients, they included all mild-to-critical patients with tested positive for COVID-19 (32). Nevertheless, a retrospective analysis of 154 COVID-19 patients reported that the use of statin could significantly reduce the severity of COVID-19 among nursing home residents (33), which is consistent with our findings. Overall, based on our results, continuation of statin therapy among COVID-19 patients with a history of atherosclerotic cardiovascular disease or diabetes was recommended.

Study Limitations

Our study has several limitations. First, with all the limitations of a retrospective study and relatively small sample size, further randomized prospective studies with more patients are required to verify the findings in our study. However, the propensity score-matched analysis, sensitivity analyses, and multivariate Cox model have been further conducted to limit potential bias or exclude possible confounders. The results from these analyses showed consistent findings and strengthened our conclusion. Even so, a cause-and-effect relationship between statins and survival cannot be inferred and only an association between statins and favorable outcomes was reported in the present study. Second, some specific information regarding cardiovascular complications and inflammation such as echocardiography and interleukin-6 were not included in the study due to the limited conditions in the isolation ward and the urgency of constraining the COVID-19 epidemic.

Conclusion

The continuous use of statins was associated with favorable outcomes in patients with COVID-19. The statin use is recommended to be continued in patients with COVID-19. However, given that this study was a retrospective analysis, further prospective studies and randomized clinical trials are warranted to verify the beneficial effect of statin in COVID-19 patients and in its different subgroups.

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

This study was approved by the Institutional Ethics Board of Zhongnan Hospital of Wuhan University (No. 2020026). The patients' consents were obtained from individual participant or their relatives.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.584870/full#supplementary-material

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REVIEW
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Impact of SARS-CoV-2 on Male Reproductive Health: A Review of the Literature on Male Reproductive Involvement in COVID-19

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Coronavirus Disease 2019 (COVID-19) has created a global pandemic. Global epidemiological results show that elderly men are susceptible to infection of COVID-19. The difference in the number of cases reported by gender increases progressively in favor of male subjects up to the age group ≥60–69 (66.6%) and ≥70–79 (66.1%). Through literature search and analysis, we also found that men are more susceptible to SARS-CoV-2 infection than women. In addition, men with COVID-19 have a higher mortality rate than women. Male represents 73% of deaths in China, 59% in South Korea, and 61.8% in the United States. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is the pathogen of COVID-19, which is transmitted through respiratory droplets, direct and indirect contact. Genomic analysis has shown that SARS-CoV-2 is 79% identical to SARS-CoV, and both use angiotensin-converting enzyme 2 (ACE2) as the receptor for invading cells. In addition, Transmembrane serine protease 2 (TMPRSS2) can enhance ACE2-mediated virus entry. However, SARS-CoV-2 has a high affinity with human ACE2, and its consequences are more serious than other coronaviruses. ACE2 acts as a "gate" for viruses to invade cells and is closely related to the clinical manifestations of COVID-19. Studies have found that ACE2 and TMPRSS2 are expressed in the testis and male reproductive tract and are regulated by testosterone. Mature spermatozoon even has all the machinery required to bind SARS-CoV-2, and these considerations raise the possibility that spermatozoa could act as potential vectors of this highly infectious disease. This review summarizes the gender differences in the pathogenesis and clinical manifestations of COVID-19 and proposes the possible mechanism of orchitis caused by SARS-CoV-2 and the potential transmission route of the virus. In the context of the pandemic, these data will improve the understanding of the poor clinical outcomes in male patients with COVID-19 and the design of new strategies to prevent and treat SARS-CoV-2 infection.

Keywords: SARS-CoV-2, COVID-19, ACE2, tmprss2, gender differences, male fertility

INTRODUCTION

In early December 2019, several cases of pneumonia of unknown etiology were reported in China (Wuhan City of Hubei Province). The pathogen was confirmed as a novel coronavirus (2019-nCoV) by the Chinese authorities on January 7, 2020 (1). At present, the International Research Committee on Taxonomy of Pathogens and Viruses officially named the pathogen as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). SARS-CoV2 has emerged as a novel β-coronavirus and is the causative agent of Coronavirus Disease 2019 (COVID-19) (2). Since the outbreak of COVID-19 in December 2019, the number of infected cases has increased exponentially, and it was declared a pandemic by the World Health Organization (WHO) on March 11, 2020. By August 1, 2020, SARS-CoV-2 has infected 17,786,110 people and caused 683,491 deaths (https://www.worldometers.info/coronavirus/). In the past two decades, coronaviruses have caused two serious pandemics, including SARS in 2002 and Middle East Respiratory Syndrome (MERS) in 2012 (3). Although they all belong to the β-coronavirus cluster, SARS-CoV-2 has caused more infections, deaths and economic disruptions. According to recent reports, COVID-19 is primarily transmitted through respiratory droplets and contact, and its main symptoms and signs include fever, dry cough, nasal congestion, fatigue, ageusia, lymphopenia, and dyspnea (1). The disease spectrum of COVID-19 ranges from mild and self-limiting respiratory tract illness to severe progressive pneumonia, multi-organ failure, and death (4). Notably, male individuals seem to be susceptible to SARS-CoV-2 infection, and their mortality rate is also high (5, 6). Coronavirus infection is known to cause orchitis in cats, and based on reports, orchitis is a complication of SARS (7, 8). Ebola virus and Zika virus can cause sexual transmission of the virus by contaminating semen (9, 10). However, similar findings have not been reported in SARS-CoV-2.

In this review, we comprehensively review COVID-19 with regard to its pathogenic mechanism, clinical manifestations, and gender differences from related literature. In addition, we explain the relationship between COVID-19 and the previous two coronavirus pandemics. The possible mechanism of orchitis caused by SARS-CoV-2 and the potential transmission route of the virus are explored, emphasizing the challenges faced by male reproductive health in this pandemic.

PATHOGENIC MECHANISM OF SARS-CoV-2

Virion Structure

Coronavirus (CoV) is an enveloped positive-sense RNA virus with special glycoprotein spikes around the viral envelope, showing a crown-like appearance under an electron microscope (2). With regard to genes, CoV is categorized into four important genera (*Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*), which are the largest groups of viruses that cause respiratory and gastrointestinal infections. The α-CoV and β-CoV can infect mammals, whereas γ-CoV and δ-CoV predominantly infect birds (1). Structurally, coronavirus is composed of hemagglutinin esterase (only found in some β-CoVs), envelope, nucleocapsid, membrane, and spike (S) protein. S protein is an immense multipurpose viral transmembrane protein, and the entry of coronavirus into host cells is mediated by the interactions between S protein and its receptor (11). On mature viruses, the S protein exists as a trimer and contains two functional subunits, which mediate the binding to the host cell receptor (S1 subunit) and the fusion of the viral membrane and the cell membrane (S2 subunit) (12). Studies have shown that SARS-CoV has a receptor-binding domain (RBD) at the C-terminus of S1 (13). In addition, different coronaviruses use distinct domains within the S1 subunit to recognize various attachments to entry receptors (**Figure 1**) (12). A recent study has determined the crystal structure of SARS-CoV-2 RBD complexed with the receptor, revealing the subtle but important difference in receptor recognition between SARS-CoV-2 and SARS-CoV (14).

Cellular Receptor for Coronavirus

Based on previous reports, the S protein of SARS-CoV binds to angiotensin-converting enzyme 2 (ACE2) as a host cell receptor. Given the genome sequence similarity between SARS-CoV and SARS-CoV-2, other studies have validated that SARS-CoV-2 also uses ACE2 as its receptor (12, 15, 16). On the other hand, proteases recruited by the virus to facilitate membrane fusion, especially TMPRSS2 (a type II transmembrane serine protease), which can cleave ACE2 and S protein, eliminate the structural constraint of S1 on S2, and releasing the internal membrane fusion peptide, thereby enhancing viral entry (**Figure 1**) (17). The full-length ACE2 consists of an N-terminal peptidase domain (PD) and a C-terminal Collectrin-like domain (18). The structure of the claw-like ACE2-PD alone and that complexed with the RBD of the S protein of SARS-CoV reveal the molecular basis of the interaction between the RBD of S protein and PD of ACE2 (19, 20).

ACE2 is a membrane exopeptidase, which is expressed in multiple organ systems, including the type I and type II alveolar epithelial cells, enterocytes of the small intestine, heart, kidneys, and testes (21, 22). The sequence of ACE2 is 41.8% identical to the domain of ACE (21). In addition, ACE2 plays a crucial role in the renin-angiotensin-aldosterone system (RAAS) (23). After angiotensinogen is produced in the liver, it is cleaved by renin to angiotensinogen (Ang) I, which is then converted to Ang II by ACE. Ang II induces bronchial smooth muscle contraction, pulmonary fibroblast proliferation, alveolar epithelial cell apoptosis, and pulmonary vascular permeability (24). By contrast, ACE2 acts as an angiotensin II-degrading enzyme to generate angiotensin (1-7), which has vasodilation, antihypertensive, and diuretic effects (25). Moreover, ACE2 participates in the absorption of neutral amino acids in the intestine (24). ACE2 has protective effects in multiple pathophysiological processes. The lack of protection of ACE2 leads to dysfunctional RAS and causes acute lung pathologies. Researchers found that infection of avian influenza H5N1, H7N9, and SARS-CoV results in a remarkably reduced ACE2 expression and subsequently elevates Ang II, which is associated with disease progression, severity, and lethality (26, 27). Furthermore, ACE2 can protect the lungs from acute lung injury. This protection is

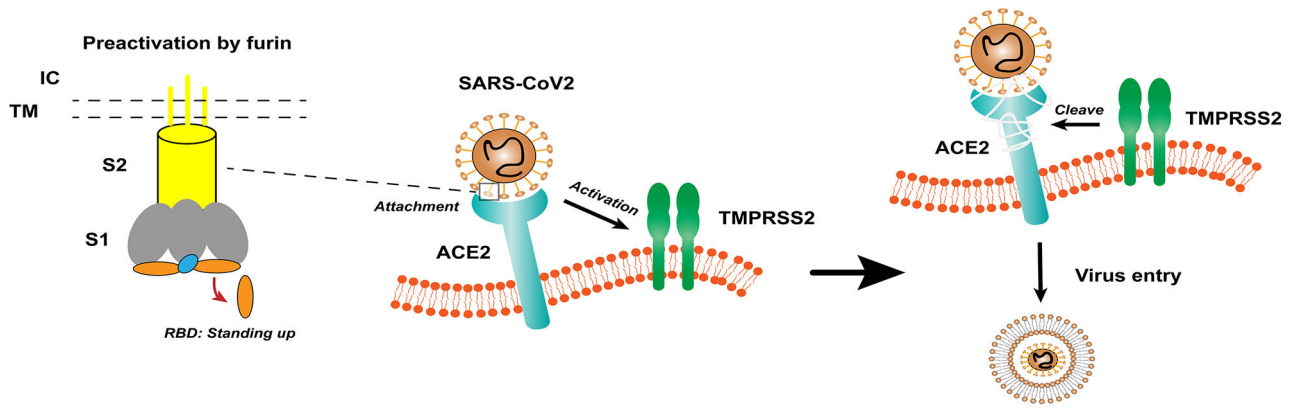

FIGURE 1 | The structure of coronavirus spikes includes: S1, S2, TM, IC, RBD. The RBD hidden in the spikes for immune evasion. When recognizing the ACE2 receptor, the RBD stands up to bind the receptor. The furin pre-activation of the spike can enhance the ability of the virus to enter certain cells. At the cell membrane, SARS-CoV-2 recognizes the ACE2 receptor and recruits TMPRSS2. TMPRSS2 facilitates viral entry and spreads into the host cell by cleaving ACE2 and S protein. S1, receptor-binding subunit 1; S2, membrane fusion subunit 2; TM, transmembrane anchor; IC, intracellular tail; RBD, receptor-binding domain; ACE2, angiotensin-converting enzyme 2; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; TMPRSS2, type II transmembrane serine protease.

achieved by inactivating Ang II to negatively regulate RAS. The key positive role of ACE2 is not only in the respiratory system, but also in the modulation of heart function, kidney protection, and absorption of tryptophan in the epithelium of the small intestine (21, 22, 24). However, ACE2 plays an indispensable role in facilitating the cellular entry of SARS-CoV-2 and SARS-CoV. The duality of ACE2 has become the focus of recent research.

Relationship With SARS-CoV and MERS Coronaviruses

In the past two decades, coronaviruses have caused two severe pandemics, including SARS in 2002 and MERS in 201 (3), both of which belong to the β-coronavirus cluster. The WHO has affirmed that SARS has caused 8,096 morbidities and 774 deaths in 2003, with a case fatality rate of 9.6%. By contrast, MERS has caused 2,494 cases and 858 deaths with a case fatality rate of 34.4% (1). SARS-CoV-2 is a novel β-coronavirus. Genomic analysis has shown that SARS-CoV-2 is ∼79% identical to SARS-CoV (16), and it is the third zoonotic coronavirus disease and the third major medical crisis.

Despite the high case fatality rate of SARS-CoV and MERS-CoV, SARS-CoV-2 has caused more infections, deaths, and economic disruptions. By August 1, 2020, a total of 17,786,110 COVID-19 cases and 683,491 deaths were reported (https://www.worldometers.info/coronavirus/). Surface plasmon resonance technology was used to quantify the interaction kinetics of SARS-CoV-2-ACE2. The results show that the ectodomain of SARS-CoV-2 S protein binds to the PD of ACE2 with approximately 15 nM affinity, which is about 10- to 20-fold higher than that of SARS-CoV and ACE2 (28). A study has elucidated the structural and biochemical mechanisms of SARS-CoV-2 receptor recognition. The researchers have found that compared with SARS-CoV, the four residues responsible for coronavirus receptor binding in SARS-CoV-2 RBD have structural changes (residues 482–485: Gly-Val-Glu-Gly). The 3D structure of such residues shows a more compact configuration and form better contact with the N-terminal helix of ACE2 (14). In the loop conformation of the ACE2-binding ridge, the flexible glycyl residues of SARS-CoV-2 replace the rigid prolyl residues in SARS-CoV. The phenylalanine Phe486 of SARS-CoV-2 RBD is inserted into the hydrophobic pocket to provide an additional binding force (29). In addition, previous studies have identified two virus-binding hotspots (hotspot Lys31 and hotspot Lys353) and compared with SARS-CoV, both virus-binding hotspots are stabilized at the SARS-CoV-2–ACE2 interface (14). Finally, SARS-CoV-2 also has a multi-base (FURIN) cleavage site that can increase the ability of the virus to internalize into cells, thereby reducing its dependence on target cell proteases for entry (15). Based on the abovementioned findings, SARS-CoV-2 exhibits a high affinity with human receptors and notable contagiousness, and its consequences are more serious than other coronaviruses. Intervention strategies based on the SARS-CoV-2 receptor recognition structure are currently studied.

CLINICAL MANIFESTATIONS OF COVID-19

Relationship of ACE2 Distribution and Clinical Manifestations

The clinical manifestations of COVID-19 have a strong correlation with the tissue distribution of ACE2, and its initial clinical manifestations are usually fever, dry cough, shortness of breath, and pneumonia (28). As the prominently targeted organ, ACE2 in normal lung tissue is expressed in type I and type II alveolar epithelial cells (22). The interaction between SARS-CoV2 and ACE2 may cause symptomatic infection. In the second or third week of a symptomatic infection, the infection can develop into a severe disease with dyspnoea and chest symptoms (30). Pathological changes show diffuse alveolar injury with cellular fibromyxoid exudates, pulmonary edema, and hyaline

membrane formation, leading to acute respiratory distress syndrome (ARDS) (31). Clinical data show decreased oxygen saturation, and the radiological characteristic is progressive pneumonia (6). Laboratory findings indicate that lymphopenia with or without leukocyte abnormalities is the major para-clinical criterion for patients with COVID-19 infection. SARS-CoV2 can indirectly infect and destroy immune cells (mostly T cells) and macrophages, causing a decrease in lymphocytes, particularly $CD8^+$ T cells, and neutrophils may increase, and blood C-reactive protein and erythrocyte sedimentation rate increase (32). Notably, COVID-19 patients have elevated D-dimers. Researches have reported that the prevalence of venous thromboembolism (VTE) in patients with COVID-19 is 25%, and VTE often leads to unfavorable prognosis (25). Severe COVID-19 patients also have elevated levels of pro- and inflammatory cytokines, and cytokine storm may be the primary phenomenon of virus pathogenesis, which can lead to inflammation, lung injury, ARDS, and other organ failures (33). Some patients show involvement of other organs, and some patients presented with cardiovascular system symptoms as their first complaints, such as palpitation and chest distress. In a clinical study involving 41 COVID-19 patients, 12% of patients have developed acute fulminant myocarditis (34). Whether the pathophysiological mechanism of myocardial injury is due to the direct attack on the heart after SARS-CoV-2 interacts with ACE2 still needs further research. The Human Protein Atlas database shows that ACE2 protein has a high expression level in the kidneys (35). A recent study showed that 23 of 85 COVID-19 patients have developed acute renal failure. The autopsy results show that six of the patients have severe acute tubular necrosis (28). Intracellular virus arrays are observed in proximal renal tubular epithelial cells by electron microscopy, indicating that SARS-CoV-2 directly infects human renal tubules and causes acute tubular damage (35). Consequently, deterioration of renal function will increase the burden on the heart and the risk of infection, affecting the poor prognosis. Researchers have explored the expression of ACE2 in the digestive system by scRNA-seq analysis, and the results show that ACE2 is expressed not only in cholangiocytes but also in absorptive enterocytes in the ileum and colon (36). Although gastrointestinal symptoms are not as common as respiratory symptoms, they can also manifest as initial symptoms.

Potential Transmission Mode of SARS-CoV-2

COVID-19 is primarily spread through respiratory droplets, direct and indirect contact, and has the characteristics of human-to-human transmission. Another mode of transmission is "hidden transmission," which is defined as asymptomatic virus carriers who become the source of infection and transmit SARS-CoV-2 to close contacts (1). SARS-CoV-2 RNA has also been detected in other biological samples, such as stool, urine, and blood. In particular, stool contains viral RNA in a high percentage of cases, and virus clearance in stool takes longer than pharyngeal swabs. Moreover, the proportion of patients with viral RNA detected in the urine and blood is fairly low (37, 38). Recent studies have reported that SARS-CoV-2 is present in saliva, and the viral load lasts for a long duration; the study has also speculated that the salivary glands may act as a reservoir for SARS-CoV-2 to increase the viral load in saliva (39). Lu et al. reported that SARS-CoV-2 can also be transmitted through the mucous membranes, including conjunctival secretions and tears (40). Therefore, in addition to the respiratory tract and lungs, SARS-CoV-2 transmission raises questions about viral shedding in other body fluids (including seminal fluid) and other modes of transmission. The expression of ACE2 and TMPRSS2 in the testis and male genital tract indicates that the testis is a high-risk organ susceptible to SARS-CoV-2 infection (41). Wang et al. reported that CD147 was another possible SARS-CoV-2 virus invasion pathway. Liu et al. analyzed the expression level of BSG (CD147) and found that BSG was expressed in all types of testis cells (42). The expression of genes involved in multiple pathways provided more possibilities for virus invasion. If the virus can infect human testes, it may involve multiple pathways and even lead to viral contamination of the seminal fluid (42, 43). In previous reports, researchers found that semen samples from survivors of Ebola virus disease remained positive for up to 272 days after the onset of symptoms (44). Some viruses may also be persistent, such as the Zika virus, which can be detected in the semen of a cured male patient for up to 1 year (41). The persistence of the virus indicates that semen can act as a virus reservoir for Ebola and Zika viruses and can be sexually transmitted (9, 10). Is this potential transmission route suitable for SARS-CoV-2? This issue has been under-investigated so far. Examination of existing proteomic databases and sperm surface surveys with monoclonal antibodies revealed that, literally, these cells hold all of the ACEs, including ACE2 (45–48). In addition to ACE2, the fusion between SARS-CoV-2 and human sperm also requires the presence of TMPRSS2. This protease is known to be present in prostasomes that are released into the seminal fluid from the prostate gland at ejaculation (49). These exosome-like structures seem to incorporate TMPRSS2 into sperm (50). A close examination of the human sperm proteomic database also reveals the presence of related proteases TMPRSS11B and TMPRSS12 as well as FURIN (45, 46), in these cells, all of which are thought to serve as activating proteases for viral infection including coronaviruses (51–53). The presence of these activating proteases and ACE2 in the sperm plasma membrane provides the possibility for the sexual transmission of the virus. However, it remains to be seen whether SARS-CoV-2 can replicate in large quantities after entering the cell, and then, release themselves out of host cells causing damage and further spread, just like it does in the lungs. In terms of clinical results, Paoli et al. reported the absence of viral RNA in the semen of a male who was cured by COVID-19 (54). Pan et al. investigated semen samples from 34 Chinese male patients and confirmed the absence of the virus in all samples. Pan et al. also pointed out that in the scRNA-seq dataset of human testicular cells, ACE2, and TMPRSS2 are sparsely expressed in human testes, and there is almost no overlapping gene expression. Therefore, ACE2-mediated viral entry of SARS-CoV-2 into target host cells is unlikely to occur within the human testicle (55). Unfortunately, men with COVID-19 in this study are more likely to have demonstrated milder symptoms. It is plausible that viremia or a

certain viral threshold is not achieved to cross the blood-testis barrier (56). Previous studies have shown that higher viral load is associated with more severe disease symptoms. Song et al. tested 12 Chinese patients with COVID-19 at the rehabilitation stage. None of these patients showed viral RNA in their semen samples. Notably, the authors tested the testis tissue of a patient who died of COVID-19 and did not detect viral RNA (57). Contrary to previous results, Li et al. reported the detection of six SARS-CoV-2-positive semen samples in semen collected from 38 severe and recovering Chinese COVID-19 patients (58). The authors cited 12 comatose or dying subjects. As we hypothesized, more severe diseases may correspond to higher blood viral load and a higher chance of crossing the blood-testis barrier. However, the methodological issues of the study have raised some concerns. According to the results of recent clinical studies, SARS-CoV-2 showed only a minor risk of virus shedding into the semen. Nevertheless, even a minor risk is unacceptable in the light of treating otherwise healthy couples for infertility reasons. Therefore, the American Society for Reproductive Medicine and the Society for Assisted Reproductive Technology has issued warning that prospective parents, ART patients, gamete donors, and gestational carriers who meet the SARS-CoV-2 diagnostic criteria must avoid pregnancy or participate in any fertility programs (59). Current studies are limited by the small sample size and short follow-up time. Therefore, detailed information about virus shedding and survival time requires further research. If it could be proved that SARS-CoV-2 can be transmitted sexually in the future studies, the sexual transmission might be a critical part of the prevention of transmission. Based on the abovementioned considerations, patients recovering from SARS-CoV-2 should monitor testicular function, including testosterone and sperm concentration. Unprotected sexual relations must be avoided to prevent from possible infection.

IMPACT OF GENDER ON COVID-19 OUTCOMES

For the first time in China, gender differences in COVID-19-detected cases and mortality has been reported (5, 6). Consistent with the global situation, the difference in the number of cases reported by gender increases progressively in favor of male subjects up to the age group ≥60–69 (66.6%) and ≥70–79 (66.1%). However, in the age group of 20–39, the detection rate of women is slightly high (60). In addition, male individuals are more susceptible to the infection and have the highest mortality rate of SARS-CoV-2 (5). Male represents 73% of deaths in China, 59% in South Korea (25), and 61.8% in the United States (https://www.worldometers.info/coronavirus/coronavirus-age-sexdemographics/). A recent study has collected epidemiological data available to 59,254 patients from 11 different countries, and the results also show an association between male and high mortality rate (61). Several studies have shown that a substantial percentage of COVID-19 occurs in patients with underlying comorbidities. In particular, in elderly male patients with comorbidities, the mortality rate of COVID-19 appears to be higher. Common comorbidities include cardiovascular disease, diabetes, chronic respiratory disease, hypertension, and cancer. Men with pre-existing cardiovascular conditions have the highest case fatality rate (6, 33, 34). Thus, some researchers have considered male sex as a poor prognostic factor (25). Although the research data of Wu et al. (62), Nogueira et al. (63), and Korea Centers for Disease Control and Prevention showed that the proportion of women infected with SARS-CoV-2 was higher, the majority of death cases were men (64). Through literature search and analysis, we found that men are more susceptible to SARS-CoV-2 infection than women (**Figure 2**) (62–94). Why are men more affected in this pandemic? In this context, analyzing the pathological mechanism of SARS-CoV-2 binding to various tissue cells under different hormone environments is important. Based on previous studies, SARS-Cov-2 interacts with the ACE2 receptor and TMPRSS2 to enter the cell (15). Physiologically, the expression of ACE2 is negatively correlated with age, and men have higher expression than women of comparable age (34). Some reports indicate that healthy and diabetic men and men with renal disease have higher levels of ACE2 circulation than women (95). Sex hormones affect many components of tissue-based RAAS, including ACE2 (96). Although the genes coding for ACE2 are located on the X chromosome, many reports of preclinical studies agree that the expression of ACE2 in males under pathological conditions is frequently higher than that in females (23, 96). Data from experimental animal models has shown that sex hormones can affect the expression and activity of ACE2 in the mouse adipose tissue, kidneys, and myocardium. In normal mice, the activity of ACE2 in the kidneys of male mice is higher than that of female mice; spontaneously hypertensive male mice also show higher ACE2 expression than female mice, and the difference may be due to the secretion of female estradiol (E2) (97, 98). Some studies also show that, after ovariectomy, the ACE2 expression in the kidneys and adipose tissue of women increases, whereas estradiol replacement decreases the expression of ACE2. Male orchiectomy can reduce ACE2 activity. Thus, testosterone maintains high levels of ACE2 expression in the heart and kidneys, whereas estrogen reduces ACE2 expression in these organs (23). Recently, it has been confirmed that the expression level of ACE2 in male lungs is higher than that in females (31). TMPRSS2 belongs to the type II transmembrane serine protease family, which is considered as a critical host cell factor for the spread of a variety of clinically relevant viruses, including influenza A virus, SARS-CoV, and MERS-CoV coronaviruses (3, 99). TMPRSS2 is highly expressed in the prostate epithelium, localized, and metastatic prostate cancer (100). TMPRSS2 expression has also been detected in airway epithelial cells (31). Androgen receptor (AR) activity is considered as a requirement for TMPRSS2 gene transcription (30). AR has been shown to modulate TMPRSS2 expression in non-prostate tissues (including the lungs). *In vitro* and *in vivo* studies have shown that androgen administration increases TMPRSS2 expression in human lung epithelial cells, and androgen deprivation reduces the transcription of TMPRSS2 in the murine lung (101). Moreover, inhibiting TMPRSS2 may prevent SARS-CoV-2 infection (102). Data from a study in the Veneto region of Italy show that among patients with SARS-CoV-2 infection in 68 hospitals, the risk of infection

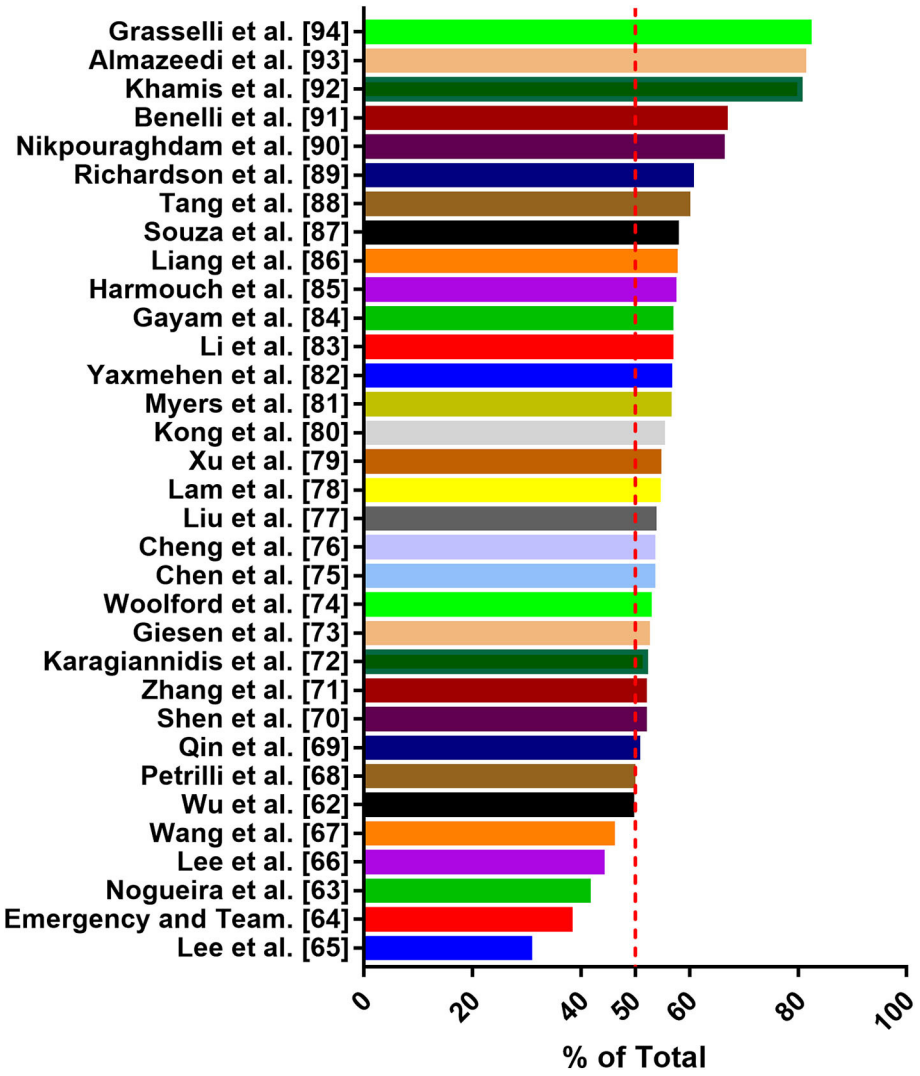

FIGURE 2 | Data on the proportion of males infected with SARS-CoV-2 from 33 articles.

for SARS-CoV-2 in-patients with prostate cancer who received androgen-deprivation therapy (ADT) is significantly reduced (OR 4.05; 95% CI 1.55-10.59) compared with patients who did not receive ADT (100). Collectively, the modulation of testosterone on the expression of ACE2 and TMPRSS2 is considered as a contributor to the dominant male COVID-19 infection (30). However, no thorough analysis of the specific mechanism of this difference has been conducted. In addition to the aforementioned factors, body immune response, viral load, lifestyle differences, and other potentially unknown mechanisms may jointly affect the progress and prognosis of COVID-19. Men and women are known to have differences with regard to the risk and severity of diseases involving the immune system. Women are susceptible to autoimmune diseases, whereas men are disproportionately affected by infectious diseases. In particular, age may lead to gender differentiation in the outcome of COVID-19 cases. Compared with women, men's age-related decline in T cell function accelerates (103). Globally, smoking and drinking rates are higher among men than women, and smoking is associated with increased activity of ACE2. Gender differences in behavior such as smoking and drinking may cause men to have an increased risk of comorbidities, such as chronic lung disease, hypertension, and cardiovascular disease, which may provide a possible explanation for the higher mortality in men (23).

SARS-CoV-2 AND MALE FERTILITY

Similarly, scRNA-seq analysis documents that ACE2 is highly expressed in seminiferous tubule cells, spermatogonia, adult Leydig, and Sertoli cells of the human testis, and Leydig cells may be involved in the regulation of steroidogenesis (104). TMPRSS2 is highly expressed in the prostate epithelial cells and the apical plasma membrane of prostate luminal cells (15).

These findings imply a potential risk associated with SARS-CoV-2 infection in the male reproductive system. Based on previous studies, viruses such as HIV, HBV, mumps, human herpes, Ebola, and Zika can invade the human testes and cause viral orchitis, and in some cases, lead to male infertility and testicular tumor (105). Xu et al. described the pathological changes of the testis in the autopsy reports of six men who died of SARS-CoV complications. The testes of the deceased showed extensive germ cell destruction, with few or no sperm in the seminiferous tubules. The basement membrane of the testis was thickened, and peritubular fibrosis was observed. Leukocyte infiltration and vascular congestions were present in the interstitial tissue (8). At present, such occurrence has not yet been described for SARS-CoV-2. A recent study has reported the characteristics of the 34 Chinese men recovering from COVID-19 and found that six patients (19%) have scrotal discomfort around the time of COVID-19 confirmation (55). However, no testicular investigation was conducted in these patients to rule out this aspect and the possibility of viral orchitis remains unclear. We propose hypotheses based on previous studies. Testicular injury in COVID-19 patients may involve multiple possible mechanisms: (1) Fever raises the temperature of the testis, leading to apoptosis of meiotic germ cells (106). Fever also plays an important role in mumps orchitis. (2) Similar to other viral orchitides, SARS-CoV-2 may cross the blood-testis barrier and trigger an immune response in the testis or a secondary autoimmune response, leading to autoimmune orchitis (8, 55). (3) The combination of SARS-CoV-2 and ACE2 may directly impair testicular function and cause epididymal orchitis. (4) COVID-19 has been associated with abnormalities in coagulation, and the segmental vascularization in the testis can account for an orchitis-like syndrome (43). Recently, the world's first case of priapism in a COVID-19 patient was reported, and the presence of dark blood clots at cavernosal blood aspiration supports ischemia-related priapism (107). Previous studies have found that orchitis is a complication of SARS (8). Due to the strict relation between the two viruses, it may be generalized to SARS-CoV-2, but it should be emphasized that the current evidence is limited and contradictory. As mentioned earlier, sperm cells hold all of the ACEs, including ACE2, which converts angiotensin II to angiotensin (1-7). Recent publications indicate that human sperm also express angiotensin II type 1 receptor (AT1R), angiotensin II type 2 receptor (AT2R), and the angiotensin (1-7) MAS receptor (48, 108). These cells, therefore, possess the complete repertoire of ligand-processing enzymes and receptors needed to support RAAS. By analogy with somatic cells, a SARS-CoV-2 attack on human spermatozoa would be expected to impact ACE2 activity leading to an increase in the availability of angiotensin II relative to angiotensin (1-7). Since angiotensin II stimulates the acrosome reaction in sperm cells, it is possible that prolonged exposure to elevated levels of angiotensin II might lead to premature acrosomal exocytosis and sperm senescence (109). Angiotensin II also further affects sperm fertilization and motility by stimulating AT1R and AT2R (108). The recent discovery of MAS receptors in the principal piece of the sperm tail and the acrosomal domain of the sperm head further emphasizes the importance of ACE2. Angiotensin (1-7) activates MAS receptors to maintain sperm in a viable motile state. However, SARS-CoV-2 attack may affect the generation of angiotensin (1-7), thereby initiating a truncated apoptotic cascade characterized by rapid motility loss (110). A cohort study showed that although SARS-CoV-2 RNA was not detected in the semen samples of recovered or acutely infected patients, patients with a moderate infection have statistically significant impairment of sperm quality (sperm concentration, total number of sperm per ejaculate, total number of progressive motility, total number of complete motility) compared with men recovered from a mild infection and the control group (111). The impact of SARS-CoV-2 on male reproductive function is still unclear, and the abovementioned possible pathogenesis needs further research.

Recently, how the COVID-19 pandemic will affect fertility has received widespread attention. Considering the previous pandemic experience and the scale of the COVID-19 pandemic, fertility decline seems to be possible, particularly in high-income countries and in the short term (112). Given the possible impact of SARS-CoV-2 on male fertility, COVID-19 may directly or indirectly affect the world's demographics in the future.

CONCLUSION

COVID-19 is a zoonotic coronavirus disease that has constituted a pandemic, endangering human lives, and the global economy (32). Compared with women, men are more susceptible to infections in this outbreak, and their mortality of COVID-19 is also higher (5). SARS-CoV-2 is the etiological agent of COVID-19, which is primarily spread through respiratory droplets, direct and indirect contact (1). Genomic analysis shows that SARS-CoV-2 is 79% identical to the SARS-CoV, and both use ACE2 as their receptor. In addition, TMPRSS2 can enhance ACE2-mediated viral entry (15, 16). The structural basis of SARS-CoV-2 receptor recognition indicates that it has a higher affinity with human ACE2, and the consequences are more serious than other coronaviruses (14). Although ACE2 acts as a "gate" for viruses to invade cells, it also has protective effects on multiple pathophysiological processes (28). The clinical manifestations of COVID-19 have a strong correlation with tissue distribution of ACE2. Apart from the lung tissue, ACE2 is also expressed in the heart, kidney, intestine, and testis and causes corresponding clinical symptoms (21, 22). Testosterone can increase the expression of ACE2 and TMPRSS2, and apart from human immune response, lifestyle differences and other factors affect the progress and prognosis of COVID-19, providing a possible explanation for the male-dominated infection and higher mortality (23, 30, 103). Studies have found that the expression of ACE2 and TMPRSS2 in the testes and male genital tract indicate that the testis is also an organ susceptible to SARS-CoV-2 infection (41). A close examination of the human sperm proteomic database reveals that these cells not only hold all of the ACEs (including ACE2), but also have related proteases TMPRSS11B, TMPRSS12, and FURIN (45–48). The presence of these activating proteases and ACE2 in the sperm plasma membrane provides the possibility for the sexual transmission of the virus. Based on previous studies,

many viruses can invade the human testes, cause viral orchitis (8, 105), and even lead to viral contamination of seminal fluid (43). For example, seminal fluid can serve as a virus reservoir for Ebola and Zika viruses, and they can be sexually transmitted (9, 10). But it is still quite unclear for SARS-CoV-2. A study has reported that six Chinese male patients (19%) recovering from COVID-19 have scrotal discomfort around the time of COVID-19 confirmation (55). Li et al. reported the detection of six SARS-CoV-2-positive semen samples in semen collected from 38 severe and recovering Chinese COVID-19 patients (58). However, more studies have reported the opposite result (41, 57). The risk of testicular damage and sexual transmission caused by SARS-CoV-2 infection requires further in-depth studies. How the COVID-19 pandemic will affect fertility has received widespread attention. Recent publications indicate that human sperm also express angiotensin II type 1 receptor (AT1R), angiotensin II type 2 receptor (AT2R), and the angiotensin (1-7) MAS receptor (48, 108). SARS-CoV-2 attacking human sperm may interact with these receptors, affecting sperm fertilization and motility in many ways, leading to male infertility.

This article describes the pathogenic mechanism and clinical manifestations of SARS-CoV-2 infection according to published literature. Based on the epidemiological results, the susceptibility of men to SARS-CoV-2 needs further exploration. The possible mechanism of orchitis caused by SARS-CoV-2 and the potential transmission route of the virus are proposed, raising concerns about male reproductive health in the context of COVID-19.

AUTHOR CONTRIBUTIONS

WH and XL searched the literature and conceived and wrote the review. LF, SX, YulL, LC, YuL, GW, DL, and BF critically appraised the literature and made a intellectual contribution to the work. All authors approved the final version of the manuscript for publication.

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Assessing the Severity of Illness in Patients With Coronavirus Disease in Saudi Arabia: A Retrospective Descriptive Cross-Sectional Study

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Objectives: We aimed to describe the epidemiological and clinical characteristics of patients with COVID-19 in Saudi Arabia in various severity groups.

Methods: Data for 485 patients were extracted from the medical records from the infectious disease center of Prince Mohammed bin Abdul Aziz Hospital in Riyadh. Patients' basic information, laboratory test results, signs and symptoms, medication prescribed, other comorbidities, and outcome data were collected and analyzed. Descriptive data were reported to examine the distribution of study variables between the severe and not severe groups.

Results: Of 458 included patients, 411 (89.7%) were classified as not severe, 47 (10.3%) as severe. Most (59.1%) patients were aged between 20 and 39 years. Patients with severe conditions were non-Saudi, with a chronic condition history, and tended to have more chronic conditions compared with those without severe disease. Diabetes, hypertension, and thyroid disease were significantly higher in patients with severe disease. Death was reported in only 4.26% of severe patients. Only 16 (34.04%) patients remained in the hospital in the severe group.

Conclusions: Severe cases were more likely to have more comorbidities, diabetes, hypertension, and thyroid disorders were most common compared with non-severe cases.

Keywords: COVID-19, characteristics, severe, non-severe, Saudi Arabia

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a new human disease. The rapid spread of this epidemic has led to increased morbidity, mortality, and economic loss worldwide (1, 2). In the last month of 2019, many cases of acute respiratory illness had been reported in Wuhan, Hubei Province, China, now known as novel coronavirus–infected pneumonia (NCIP) (3, 4). As of January 31, 2020, some 9,692 NCIP cases in China had been confirmed. Globally, cases have been reported in the most of countries (5).

COVID-19 was recognized in the tests of bronchoalveolar lavage liquid from a patient in Wuhan and was affirmed as the etiology of the NCIP. Full-genome sequencing and phylogenic investigation indicated that COVID-19 was a clade distinct from the beta coronaviruses related to human severe acute respiratory syndrome and Middle East respiratory syndrome (6).

COVID-19 has features common to the coronavirus family and was classified as having beta coronavirus 2b ancestry. Given it closely resembles bat coronaviruses, it has been hypothesized that bats were the essential source of the virus. Although the starting point of COVID-19 is as yet being researched, growing evidence suggests spread to humans occurred by means of transmission from wild animals illegally sold in the Huanan Seafood Wholesale Market (6). Huang et al. (7) had reported the first 41 cases of NCIP, in which most patients had visited this market. Patients' clinical signs included fever, non-productive cough, dyspnea, myalgia, fatigue, normal or decreased leukocyte counts, and radiographic evidence of pneumonia. Organ dysfunction [e.g., shock, acute respiratory distress syndrome (ARDS), acute cardiac injury, acute kidney injury] and death can occur in severe cases (7).

Coronavirus diseases can range from not severe to very severe and even fatal respiratory infections. A retrospective study of 1,099 patients with COVID-19 from 552 hospitals and 30 provinces in China had found that 87.9% of patients had a fever and 67.7% had a cough, whereas diarrhea (3.7%) and vomiting (5.0%) were rare. Also, significantly more patients with severe disease received mechanical ventilation (non-invasive: 32.37 vs. 0%, $P < 0.001$; invasive: 13.87 vs. 0%, $P < 0.001$) compared with non-severe cases. The study showed that the most common complications of patients admitted to hospital were pneumonia (79.1%), followed by ARDS (3.37%), and shock (1.00%) (8).

The COVID-19 spread by human-to-human transmission with the same disease severity (including oxygen saturation, respiratory rate, blood leukocyte/lymphocyte count, and chest X-ray/computed tomography manifestations) (9). Subsequently, Guan et al. (8) had reported 99 instances of NCIP from the same hospital and suggested that COVID-19 infection clustered within gatherings of people in close contact, appeared to affect older men with comorbidities, and could result in ARDS.

On March 9, 2020, the Saudi Ministry of Health (MOH) had declared 4 new patients infected with COVID-19 (10). On June 10, 2020, according to the Saudi MOH, there were 3,717 confirmed cases of COVID-19, with the total number of the active cases standing at 33,515. There were 1,693 critical cases among the total active cases (11). Be that as it may, the distinction in clinical attributes between severe and non-severe cases was not reported in Saudi Arabia. This study aims to describe the epidemiological and clinical characteristics of patients with COVID-19 in Saudi Arabia in various severity groups. Strengthening the evidence base with regard to the severity of the illness could help healthcare providers better address this vulnerable population.

MATERIALS AND METHODS

Study Design

We conducted a descriptive, cross-sectional study of all confirmed cases of patients with COVID-19 between March 1, 2020 and May 20, 2020, adhering to STROBE (strengthening the reporting of observational studies in epidemiology) guidelines for cross-sectional studies (10).

Study Setting

We conducted a study of all patients with COVID-19 admitted to the infectious disease center of Prince Mohammed bin Abdul Aziz hospital in Riyadh, which is a MOH hospital and is one of the major referral hospitals in Riyadh, Saudi Arabia. The hospital is located east of Riyadh, on a 10,000 m^2 plot, in a built-up area of 105,000 m^2, comprising 5 floors with a 500-bed capacity. The hospital is equipped with 120 beds for intensive care, 63 rooms for emergencies, 15 rooms for surgery, a pavilion for radiology, some of the top laboratories in the world with American Association for Laboratory Accreditation, and outpatient clinics (12). In preparation for the pandemic, this hospital was one of the leading hospitals designated as a COVID-19 center, and as such, patients with COVID-19 were transferred to this hospital.

Study Population

The population included males and females of all ages with confirmed COVID-19 infection in the laboratory using real-time reverse transcriptase-polymerase chain reaction (RT-PCR) who were admitted to the hospital during the study period. Admission to the ICU was for patients with confirmed COVID-19 infection who required rapidly increasing oxygen supplementation, oxygen via high-flow nasal cannula, non-invasive positive pressure ventilation, mechanical ventilation, or vasopressors (13). Therefore, as in previous studies (8, 14), cases were classified as severe or not severe. For the present paper, no exclusion criteria were applied.

Variable Definitions

The variable of obesity was determined according to body mass index (BMI). BMI is computed as weight (kg)/height (m^2), and obesity is classified as BMI $\geq$30, as per the World Health Organization (WHO) weight classification (15). Other chronic conditions were identified according to whether the patients had had any of the following diagnosed conditions: diabetes mellitus (defined as current use of diabetic-lowering medication associated with HbA1c levels $\geq$7% in accordance with the recommendations from the American Diabetes Association) (16), hypertension (being previously diagnosed as having hypertension by any medical professional and taking antihypertensive medication), asthma, pneumonia, kidney disease (i.e., urinary albumin creatinine ratio $\geq$30 mg/g and/or estimated glomerular filtration rate $<$60 mL/min/1.73 m^2), cardiovascular disease (e.g., angina, myocardial infarction, stroke, or heart failure), cancer of any type, any psychiatric disease, dyslipidemia (i.e., total cholesterol $\geq$200 mg/dl, triglycerides $\geq$150 mg/dl, low-density lipoprotein cholesterol $\geq$100 mg/dL, or high-density lipoprotein cholesterol $\leq$40 mg/dL

in males and ≤50 mg/dL in females), and thyroid disease. The number of comorbidities was categorized into *none*, *1*, and, ≥*2* comorbidities. The presence of any symptoms was classified as yes or no. The result of chest X-rays were classified as normal or abnormal. Required mechanical ventilation was classified as yes or no, and patient outcomes were classified into died, home isolation, recovered, discharged, stable, still positive, and still in the hospital.

The available data from the patient laboratory test results were also analyzed. These included body temperature, heart rate, respiratory rate, white blood cell count, platelet count, hemoglobin, international normalized ratio, creatinine, sodium, and chloride. Systolic and diastolic blood pressure measured on admission were collected and categorized as following: systolic BP <100, 100–119, 120–140, and >140 mmHg subgroups and diastolic BP <80, 80–89, 90–100, and >100 subgroups (17).

Data Source

Data were collected from patients' medical records by trained medical personnel. A patient's medical record consists of data on patients' basic information (age, sex, smoking status, nationality, history of any chronic conditions), laboratory test results, signs and symptoms, medications prescribed, other comorbidities, and outcomes. A well-designed and organized checklist was used to obtain and extract information from patients' medical records.

Statistical Analysis

The data obtained were entered and analyzed using SAS version 9.4. Descriptive data were reported as dichotomous, polychotomous, and as frequencies and percentages to examine the distribution of study variables among the severe group (transferred to ICU) and the not severe group (not transferred to ICU); the chi-squared or Fisher's exact test, as appropriate, was used to compare categorical variables between groups. Continuous variables are presented as median with interquartile range (IQR) and compared, if normally distributed, using Student's *t*-test; otherwise, the Mann–Whitney *U*-test (Wilcoxon rank-sum test) was used. No imputation was performed for all tests, and a *P*-value of <0.05 was considered to be statistically significant.

Ethical Considerations

This research was reviewed and approved by the Institutional Review Board at King Fahad Medical City, Riyadh, Saudi Arabia, under the IRB log number 20-156. Permissions from the MOH and hospital management were obtained to conduct this study.

RESULTS

From March 1, 2020 to May 20, 2020, 458 patients infected with COVID-19 were reported and included in the analysis. Characteristics and comorbid underlying conditions of the included patients are presented by severity in **Table 1**. Of these 458 patients, 411 (89.7%) were classified as not severe, 47 (10.2%) as severe. Patients aged between 20 and 39 years made up the majority of the study population at 59.4%, followed by those aged between 40 and 59 years at 30.6%, older than 60 at 7.4%, and younger than 20 at 2.6%; a male and non-Saudi with the predominance of 86.9 and 80.4% of the study population, respectively. Some 21.8% had a history of chronic conditions; 324 (70.7%) patients had one chronic condition and 96 (20.9%) had 2

TABLE 1 | Study population baseline characteristics for patients with COVID-19 by severity of illness.

Characteristics	Total patients (*N* = 458)	Non-severe (*N* = 411)	Severe (*N* = 47)	*P*-value
Age, years				0.381
<20	12 (2.62)	6 (1.46)	6 (12.8)	
20–39	272 (59.4)	231 (56.2)	41 (87.23)	
40–59	140 (30.57)	140 (34.06)	0 (00.00)	
>60	34 (7.42)	34 (8.27)	0 (00.00)	
Gender, *n* (%)				0.079
Female	60 (13.1)	50 (12.2)	10 (21.3)	
Male	398 (86.9)	361 (87.8)	37 (78.7)	
Nationality, *n* (%)				0.025
Non-Saudi	368 (80.35)	336 (81.8)	32 (68.1)	
Saudi	90 (19.65)	75 (18.3)	15 (31.9)	
Smoker	14	14.29	10	12.2
History of chronic conditions	98	21.83	56	13.86
Number of chronic conditions				<0.001
None	38 (8.3)	38 (9.3)	0 (00.00)	0.487
1	324 (70.7)	323 (78.6)	1 (2.13)	
≥2	96 (20.9)	50 (12.2)	46 (97.9)	
Comorbidity				
Diabetes mellitus	62 (13.6)	33 (8.1)	29 (61.7)	<0.001
Hypertension	50 (10.94)	14 (3.41)	36 (76.6)	<0.001
Asthma	16 (3.51)	12 (2.93)	4 (8.51)	0.071
Pneumonia	7 (1.53)	5 (1.22)	2 (4.26)	0.108
Cardiovascular disease	9 (1.97)	6 (1.46)	3 (6.4)	0.055
Cancer	2 (0.44)	1 (0.24)	1 (2.13)	0.195
Psychiatric disease	4 (0.88)	3 (0.73)	1 (2.13)	0.353
Obesity (BMI ≥25/≥30 kg/m^2)	236 (57.1)	235 (63.51)	1 (2.23)	<0.001
Dyslipidemia	4 (0.88)	2 (0.49)	2 (4.26)	0.054
Thyroid disease	11 (2.41)	6 (1.47)	5 (10.64)	0.002
Kidney disease	6 (1.32)	5 (1.23)	1 (2.13)	0.482
Any symptoms				0.685
No	50 (11.01)	44 (10.81)	6 (12.8)	
Yes	404 (88.9)	363 (89.2)	41 (87.23)	
The result of chest X-Ray				0.008
Normal	398 (88.05)	363 (98.41)	35 (76.09)	
Abnormal	54 (11.9)	43 (10.6)	11 (23.91)	
Required mechanical ventilation				<0.001
No	413 (90.6)	404 (98.8)	9 (19.2)	
Yes	43 (9.43)	5 (1.22)	11 (80.6)	
Patient status				<0.001
Died	2 (0.44)	0 (00.00)	2 (4.26)	
Home isolation	201 (43.9)	183 (44.53)	18 (38.3)	
Recovered	7 (1.53)	6 (1.46)	1 (2.13)	
Discharged	11 (2.4)	7 (1.7)	4 (8.51)	
Stable, still positive	207 (45.2)	201 (48.9)	6 (12.8)	
Still in hospital	30 (6.55)	14 (3.41)	16 (34.04)	

TABLE 2 | Reported signs and symptoms in patients with COVID-19 by age group.

Signs and symptoms	Total patients	Age, years				*P*-value
		<20 (%)	20–39 (%)	40–59 (%)	>60 (%)	
Fever	324 (70.74)	9 (75)	196 (72.1)	101 (72.14)	18 (52.94)	0.128
Dry cough	277 (60.5)	8 (66.7)	168 (61.8)	86 (61.43)	15 (44.12)	0.236
Dyspnea	128 (27.9)	2 (16.7)	69 (25.4)	48 (34.3)	9 (26. 5)	0.213
Sore throat	62 (13.54)	1 (8.33)	39 (14.34)	15 (10.7)	7 (20.6)	0.419
Diarrhea	44 (9.61)	1 (8.33)	26 (9.56)	14 (10)	3 (8.82)	0.995
Fatigue	29 (6.33)	1 (8.33)	20 (7.4)	6 (4.3)	2 (5.9)	0.668
Vomiting	23 (5.02)	0 (00.00)	17 (6.3)	4 (2.9)	2 (5.9)	0.403
Runny nose	17 (3.71)	0 (00.00)	8 (2.9)	7 (5)	2 (5.9)	0.569
Chest pain	14 (3.06)	1 (8.33)	6 (2.21)	3 (2.14)	4 (11.8)	0.012
Abdominal pain	11 (2.4)	1 (8.33)	7 (2.6)	2 (1.43)	1 (2.94)	0.485
Myalgia	7 (1.53)	0 (00.00)	5 (1.8)	2 (1.43)	0 (00.00)	0.826
Nausea	7 (1.53)	0 (00.00)	6 (2.2)	1 (0.71)	0 (00.00)	0.539
Systolic blood pressure, mm Hg						<0.001
<100	15 (3.6)	0 (00.00)	0 (00.00)	13 (12.8)	2 (6.3)	
100–119	171 (41.01)	6 (54.6)	116 (42.7)	38 (37.3)	11 (34.4)	
120–140	180 (43.17)	3 (27.3)	135 (49.6)	25 (24.5)	17 (53.13)	
>140	51 (12.23)	2 (18.2)	21 (7.72)	26 (25.5)	2 (6.3)	
Systolic blood pressure, mm Hg	122.74–15.63	127.81–14.94	123.09–14.89	121.45–15.66	123.67–21.43	0.0521
Diastolic blood pressure, mm Hg						0.046
<80	86 (20.6)	3 (27.3)	58 (21.32)	19 (18.63)	6 (18.8)	
80–89	34 (8.2)	1 (9.1)	25 (9.19)	8 (7.84)	0 (00.00)	
90–100	291 (69.8)	7 (63.64)	189 (69.5)	70 (68.63)	25 (78.13)	
>100	6 (1.44)	0 (00.00)	0 (00.00)	5 (4.9)	1 (3.13)	
Diastolic blood pressure, mm Hg	75.87–10.44	79.27–8.85	75.72–10.35	76.69–10.47	71.92–11.04	0.1105
Body temperature, °C, Median (IQR)	37 (36.7–37.7)	36.8 (36.6–37.2)	36.9 (36.7–37.7)	37.2 (36.8–37.8)	36.9 (36.7–37.3)	0.1444
Heart rate, beats per minute, Median (IQR)	86 (78–99)	92 (84–101)	84 (77–99)	90 (82–99)	82 (64–96)	0.1534
Respiratory rate, breaths per minute, Median (IQR)	20 (18–20)	19 (19–20)	20 (19–20)	19 (18–20)	20 (19–20)	0.0054

or more. The most common types of comorbidities were obesity (BMI $\geq$30 Kg/m^2) according to the international WHO criteria for BMI, accounting for 97.8% of the study population, followed by diabetes in 13.5% of the cases and hypertension contributing to 10.9% of cases.

The chi-squared test and fisher's exact test in **Table 1** showed that patients with severe conditions were remarkably non-Saudi ($P = 0.025$), with a chronic condition history ($P < 0.001$), and tended to have a higher number of chronic conditions ($P < 0.001$) compared with those without severe disease The percentage of patients with diabetes, hypertension, or thyroid disease were significantly higher ($P < 0.001$, $P < 0.001$, $P = 0.002$, respectively) in the severe condition group. Significantly more patients with severe disease received mechanical ventilation (80.85 vs. 1.22%). In terms of patient outcomes, death was reported in only 4.26% of severe patients. Only 16 (34.04%) patients remained in the hospital in the severe group.

Reported signs and symptoms for the study population were compared according to age group using the chi-squared test and fisher's exact test (**Table 2**). For the entire study population, fever, dry cough, and dyspnea were the most common symptoms upon admission. Chest pain was found to be significantly higher among patients in the age group 60 years and older. More than 43% of patients had a systolic blood pressure of 120–140 mm Hg, and 69.8% had a diastolic blood pressure of 90–100 mm Hg. Of patients aged 60 years and older, 53.1% had systolic blood pressure of 120–140 mm Hg and 78.1 had diastolic blood pressure of 90–100 mm Hg. A higher respiratory rate was observed among patients between 20–39 and older than 60 years old (median respiratory rate, 20 [IQR 19–20] for both age groups; $P = 0.0054$).

According to Student's *t*-test and the Mann–Whitney *U*-test, there were numerous significant differences in laboratory findings between patients in the severe and non-severe groups (**Table 3**). Patients with severe disease showed increased white blood cells (median 8.4 [IQR 6–12.3] $\times$ 10^9/L vs. 6 [IQR 4.8–7.7] $\times$ 10^9/L; $P < 0.001$), and platelet counts (median 244 [IQR 198–342] $\times$ 10^9/L vs. 226 [IQR 180–277] $\times$ 10^9/L; $P = 0.046$). Most patients in the severe group had reduced hemoglobin levels (median concentration 136 g/L [IQR 126–147] vs. 150 g/L [IQR 140–161]; $P < 0.001$) and higher international normalized ratio (median 1.03 [IQR 0.99–1.14] vs. 1 [IQR 0.97–1.05]; $P = 0.004$).

Antibacterial drugs, antipyretic drugs, antimalaria drugs, antithrombotic drugs, and antiviral treatment drugs were the main treatments prescribed to patients with COVID-19.

TABLE 3 | Laboratory findings of patients with COVID-19 by the severity of illness.

Laboratory findings	Total			Non-severe			Severe			*P*-value
	N	Median	IQR	*N*	Median	IQR	*N*	Median	IQR	
White blood cell count (WBC), $\times 10^9$/L	335	6.2	4.9–8	288	6	4.8–7.7	47	8.4	6–12.3	<0.0001
Platelet count (PLT), $\times 10^9$/L	332	229.5	182.5–284.5	285	226	180–277	47	244	198–342	0.0466
Hemoglobin (Hgb), g/dl	334	148.5	136–159	287	150	140–161	47	136	126–147	<0.0001
Hematocrit (Hct), L/L	333	0.454	0.421–0.484	286	0.459	0.428–0.487	47	0.428	0.394–0.453	<0.0001
Prothrombin time (PTT), S	235	35.1	32.1–38.1	188	34.9	31.9–37.75	47	37.2	34–43.3	0.0123
International Normalized Ratio (INR)	257	1.01	0.97–1.07	210	1	0.97–1.05	47	1.03	0.99–1.14	0.0044
Creatinine, μmol/L	79	80.8	64.9–89.6	70	81.3	66.8–89.6	9	63.4	62.3–70	0.1306
Sodium (Na), mmol/L	306	138	136–139	263	138	136–139	43	137	134–139	0.0505
Chloride (Cl), mmol/L	319	104	101–106	272	104	102–106	47	103	100–106	0.0525

TABLE 4 | Treatments of patients with COVID-19.

Laboratory findings	Total patients using a treatment	Non-severe (*N* = 329)	Severe (*N* = 122)
Antimalarial drugs	92 (20.3)	54 (16.4)	38 (31.15)
Antiviral drugs	10 (2.1)	10 (3.04)	0 (00.00)
Antibacterial drugs	153 (33.9)	108 (32.83)	45 (36.9)
Antithrombotic drugs	91 (20.2)	70 (21.3)	21 (17.2)
Antipyretic drugs	105 (23.3)	87 (26.4)	18 (14.8)

Treatment for non-severe and severe cases is shown in **Table 4**. Antibacterial drugs were the most commonly used class overall. A higher percentage of patients with severe conditions were treated with antibacterial drugs compared with those without (95.7 vs. 54.3%). Antimalarial drugs were the second most common type of antibiotic administered for severe cases.

DISCUSSION

COVID-19 is currently a major infectious disease, causing substantial morbidity and mortality worldwide. A better understanding of patient characteristics in terms of illness severity could lead to increased adoption of appropriate care in this group, consequently reducing the burden of infection. In this retrospective study, a total of 458 patients with COVID-19 were included, of whom 47 were recognized as having severe disease. The overall mortality rate was 0.44% by May 20, 2020, all in the severe group. Most of the patients were in stable condition or were discharged from the hospital, suggesting that the COVID-19 had been controlled and treated effectively.

Few systematic reviews and meta-analyses have been conducted to describe the clinical characteristics of COVID-19 patients in China (18–22). Among those reviews, Cao et al. (21) and Hu et al. (22) were the most inclusive, with a total of 46,959 and 47,344 patients, respectively. These reviews included all published retrospective cohort studies and case series until March 10, 2020. The mean age in the Cao et al. review was 46 years, whereas Hu et al. had reported an average age older than 40 years. In both reviews, there was an equal distribution of both sexes. The most frequently reported clinical manifestations in both reviews were fever (85%) and cough (65%), followed by dyspnea (38%) (21) and fatigue (42%) (22). Cao et al. had found that hypertension and diabetes were the most commonly reported comorbidities, accounting for 18 and 10%, respectively (21); similar figures had been reported by Hu et al. (22).

Lechien et al. had described the clinical characteristics of patients with non-severe COVID-19 from multiple European countries (23). This retrospective study included 1,420 patients (mean age 39, 67% female). The most reported symptoms were headache and loss of smell, accounting for 70% of all cases; only 45% of patients reported fever. As for comorbidities, 9% of the patients had hypertension. This study reported differences in clinical presentation between males and females and among different age groups. For example, female patients tended to have headaches and loss of smell, whereas male patients tended to have fever and cough. As for age, younger patients had ear, nose, and throat complaints, whereas older patients reported having fever and fatigue.

We found few similarities between meta-analysis findings (21, 22) and our findings in terms of mean age, and the most commonly reported clinical manifestation. In our study, however, the male to female ratio was higher, and fever and cough were also commonly reported, which is in line with the findings of Lechien et al. (23). The proportion of patients with diabetes among the severe cases was significantly higher than among the non-severe. Likewise, the proportion of patients with hypertension tended to be higher among the severe cases than in the severe group. Evidence from multiple meta-analyses suggests that hypertension and diabetes have been strongly associated with disease severity and poor prognosis (19, 24–26). In Saudi Arabia, 28% of the population has been considered obese (27). Hypertension and diabetes have also been found to affect 15 and 24% of the Saudi population, respectively (28–30). The prevalence of such conditions is expected to be even higher due to obesity and a sedentary lifestyle (31–33). These characteristics can lead to longer hospitalization and ICU admission, which increases the burden on the healthcare system.

One of the worth noting findings in this study, which was not previously been reported elsewhere, is the number of expatriates,

which represents 80% of the samples. A possible reason for this high percentage is low economic status. In a study conducted by Al Khamis et al. (34) two-thirds of expatriates have low incomes (less than SR2000 per month). Al Khamis et al. have also reported that 65% of expatriates are married but without their families (34). Despite the majority of non-Saudis not having severe disease, the majority live in shared accommodations and worker housing units, where infection spread is expected to be higher. In addition, the MOH has started an "active screening" program, also called "active surveillance," in overpopulated areas, which are typically inhabited by expatriates, for early detection of COVID-19 cases; this program could explain the higher prevalence among expatriates (35).

To our knowledge, this study is one of few studies that describes the characteristics of patients with COVID-19 in Saudi Arabia. In our study, we collected all available variables for patients to enable a clear picture of the characteristics of patients with COVID-19 in terms of severity of illness in the Riyadh city population. In terms of symptoms, in our sample, roughly 11% of patients were asymptomatic. Estimating the asymptomatic percentage of COVID-19 patients is an essential measurement for decision makers. Despite the strengths of our study, it also has limitations. First, this study was performed in one hospital in Riyadh; thus, we cannot generalize these results to the entire COVID-19 population in Riyadh. Moreover, the sample size was 458 patients, which could have led to some non-significant differences in the subgroup analysis because the power of the study was limited by sample size. Finally, because the laboratory tests are only performed upon request by the physician according to the patient's condition, we have observed that some test results have a very high percentage of missing values.

In this study, People with Diabetes, hypertension, and thyroid disease were at higher risk of severe disease requiring hospitalization and subsequently ICU admission. Therefore, our study results could help both healthcare providers and decision makers during this pandemic. For healthcare providers, these results can help them identify those high-risk patients and provide them with early intervention to prevent complications. Additionally, patients at high risk should be targeted for screening in order to diagnose them as soon as possible to start providing care. Not only healthcare providers but also decision makers might be able to use the results of this study to apply procedures to reduce the outbreak of this virus and other infections. This can be achieved by enforcing workplace policies which prohibit the physical attendance for people at higher risk of infection and arrange for distant working schedule where appropriate. Additionally, hospital outpatient follow-up visits for people with higher risk should be performed using Teleclinics and or phone calls where appropriate. Medication refills and collection also need be arranged with a courier. Applying these measures could potentially reduce the spread of infection to people at high risk and subsequently reduce admission and future complication.

According to our study, ∼80% of patients with COVID-19 patients were non-Saudi, which might be associated with living in overpopulated areas. According to these study results, the government should adopt new regulations to reduce overpopulated residences. By adopting these regulations, reducing the spread of infection in these places not only helps reduce the burden during the COVID-19 pandemic but also during all possible future infectious diseases.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors after obtaining approval from the local ethical committee, without undue reservations.

AUTHOR CONTRIBUTIONS

AMA: study design, data collection, and writing. ZA: study design, data analysis, and writing. RA: data collection and writing. SHA and ASA: manuscript drafting. MA and AAA: data collection. SA: writing and critical revision of the manuscript. All authors approval of the final version.

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OPINION
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Neuro-Oncology During the COVID-19 Outbreak: A Hopeful Perspective at the End of the Italian Crisis

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Since December 2019, when severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) had been identified for the first time in Wuhan, Hubei, China, the outbreak has quickly become a worldwide pandemic with disruptive health, social, and economic impact. As of August 2020, more than 21,600,000 cases and 775,000 confirmed deaths have been reported by the WHO across all continents, with exponential spread initially in Europe, and currently in the East, United States, and Latin America. Italy has been hurt dramatically, being the first European country involved, and the epicenter of the pandemic for a few months (1). The regions of northern Italy (Lombardy, Veneto, Emilia-Romagna, and Piedmont) were particularly affected, requiring immediate and tight emergency measures to contain the infection followed by a national lockdown set since the beginning of March (2). In this complex scenario, the Italian cancer community have faced many arising tough challenges (3). Cancer patients are more susceptible to infections because of their immunosuppressive status and, at least in theory, at major risk of developing severe complications from the coronavirus disease (COVID-19), including adult respiratory distress syndrome (ARDS), intensive care unit admission, and even death. The first reports from China seem to confirm this hypothesis suggesting that patients with cancer are more likely to contract the virus and to develop COVID-19-related complications (4). More recent works indicated with strong evidence that cancer patients are at an increased risk of mortality and severe illness due to SARS-CoV-2 infection, regardless of whether they have active cancer, are on anticancer treatment, or both (5, 6). A recent multicenter European study involving 890 cases with confirmed COVID-19 demonstrated a worsening gradient of mortality from breast cancer to hematological malignancies and identified male gender, older age, and number of comorbidities as negative prognostic variables (7). Patients diagnosed with primary brain tumors (PBTs) are considered one of the most fragile and vulnerable category due to several factors: the older age along with the multiple age-related frailties and comorbidities, the frequent presence of neurological deficits causing loss of autonomy in the activities of daily living and increased risk of thromboembolic events, the often severe lymphopenia both disease and treatment-related (e.g., alkylating agents such as temozolomide and nitrosourea), and finally the chronic use of steroids to control brain edema leading to further immunosuppression and to an increased susceptibility to infection (8). Moreover, preliminary clinical data suggest that the SARS-CoV-2 infection can commonly involve the central nervous system (CNS) especially in those patients with lower lymphocyte counts, causing neurological manifestations both central such as dizziness, headache, acute cerebrovascular disease, depressed level of consciousness, ataxia, and seizures, or peripheral such as hypogeusia, and hyposmia (9). The first reports from autopsies of patients with COVID-19 revealed that the brain tissue was often hyperemic and edematous, with some

areas of neuronal degeneration (9). As for other coronaviruses, including SARS-CoV and MERS-CoV, the main pathogenic mechanism may be the direct CNS invasion of SARS-CoV-2. The olfactory nerve has been recently described as the potential neural pathway used by the virus to gain entry into the CNS (10, 11). In addition, the SARS-CoV-2 virus seems to exploit the angiotensin-converting enzyme 2 receptor to entry inside the cells, and these receptors have been detected into the brain over glial cells and neurons (12). Another pathogenic mechanism under current investigation potentially leading to neurological damages in patients with COVID-19 is represented by endothelial ruptures in cerebral capillaries followed by bleeding within the brain parenchyma (12). For all these reasons, it is almost conceivable that PBTs patients, with an already injured brain, could be more exposed in terms of frequency and seriousness to these symptoms. In the context of the COVID-19 pandemic, the need for close and continuous assistance by caregivers, usually family members, could increase exponentially the risk of interfamily infection. Moreover, travel restrictions imposed to mitigate the SARS-CoV-2 spread limit the possibility of patients to move around the country, the attendance for repeat appointments, and continuity in care in the referral centers where they are usually managed and treated. Given the paucity of effective treatments available, the difficult access to clinical trials represents another relevant issue. The opening of new sponsored, multicenter, early-phase (Phases I–II) clinical studies previously scheduled at our institutions has been postponed, as well as the recruitment into the ongoing ones has been placed on hold, denying our patients a potentially effective therapeutic option. Finally, the drug-to-drug interactions between antiepileptic drugs and antiviral agents or chloroquine derivates, often used as empirical therapy for COVID-19 infection, potentially leading to toxic effects, represent a new concern that we faced. At the time of writing, in northern Italy, pressure on hospitals and intensive care units has sharply declined, while the contagion curve has settled on a plateau with a low number of newly infected cases. As neuro-oncologists working in three Institutions known as national referring hubs for the management of PBTs (Humanitas Cancer Center, Milan; Department of Medical Oncology, Bellaria Hospital, Bologna; and Veneto Institute of Oncology, Padua) and located frontline in the most affected endemic regions of Italy, we have directly experienced the dramatic impact of this pandemic. Moreover, two of the three centers being part of academic, general hospitals with an emergency department admitting SARS-CoV-2 positive subjects on a daily basis were forced to completely revolutionize their organization in just a few days, redefining spaces and reallocating medical resources (13). Several COVID-19 isolated wards have been created, and new intensive care beds have been built up wherever possible. Clinical activities not strictly necessary have been temporarily suspended and physicians working in these sectors redistributed. In our three institutions, oncologists continued to do their job full time giving care to their patients with the best intensity, limiting unnecessary hospital access and implementing COVID-free paths and all the safety procedures required to protect patients and medical staff. Now, thanks to the strict lockdown measures the biggest storm is moving away behind us and it is the right time to take a first balance, reflecting on the choices made and planning the future, with the concrete possibility of a second wave of infections in the next autumn. In the present paper, we would like to discuss some critical aspects involving management of PBT patients during the pandemic, sharing our personal experience on how we have modified our neuro-oncological daily practice to ensure either the safety and the continuum-of-care of our patients. We also present some preliminary data about the prevalence of the infection among the patient population of our three referral Neuro-Oncology Centers, showing the features and the clinical course of PBTs patients who got infected by SARS-CoV-2.

First of all, we have educated all our patients and their caregivers on the importance to strictly respect the correct behavior rules, as maintaining social distance, limit all unnecessary interactions, wearing mask, and frequent handwashing. Most of these recommendations have been given by phone/video contact or emails, and then reinforced directly during the onsite visits for those patients for whom it was necessary to come to the centers. We deferred all tumor assessment and clinical evaluations of asymptomatic or clinically stable, low-risk, PBT patients such as meningiomas and IDH-mutant low-grade glioma (WHO grade II). Taking decisions whether or not to start oncologic treatments must be carefully evaluated case-by-case, weighing potential risks of delaying and benefit gained for every proposed therapeutic intervention. For example, we considered reasonable and safe the postponing of adjuvant treatments (radiotherapy and chemotherapy) in the case of slow growth IDH-mutant low-grade gliomas, planning a new brain imaging in a 4- to 6-month period after surgery. We spared chemotherapy in likely non-responder patient population such as elderly or frail glioblastoma (GBM) patients with unmethylated MGMT promoter, as well as taking into account an hypofractionated radiotherapy approach to reduce the number of patients' access to the Hospital (14, 15). We avoided second or third line potentially immunosuppressive treatments in patients with poor performance status. Surgical indications were discussed in remote multidisciplinary tumor boards and avoided when the survival benefit expected is likely to be marginal. Due to the detrimental effects of inhibiting antiviral immunity and general immunosuppression, we carefully weighed the use of steroids, administering the lowest possible dose (ideally 10 mg of prednisone or equivalent that is an anti-inflammatory dose without immunosuppressive effects) for preventing or control brain edema and neurological symptoms (16). We limited the length of corticosteroid treatment to the shortest period of time, planning a fast tapering after clinical improvement. In patients taking high-dose steroids, we implemented prophylaxis with trimethoprim/sulfamethoxazole to reduce the risk of development of other interstitial pneumonias, and the concomitant use of low-dose diuretics with a steroid-sparing effect, such as furosemide. For those patients, in the case of onset of respiratory symptoms, we recommended performing a chest CT scan, even in the absence of fever. Given the aforementioned difficulties to travel and reach referral centers, we empowered cooperation with local institutions often lacking a particular expertise in these rare

TABLE 1 | Brain tumor patients infected by SARS-CoV-2.

Case	Age* (years)	Sex	Histology and molecular profile	Last treatment (A/C)	Lymphocyte count* ($\times 10^3/mm^3$)	Steroid Therapy* (DEX mg dose)	SARS-CoV-2 diagnosis (NFS/BAL)	COVID-19 Clinical manifestations
1	44	male	IDH wild-type GBM	Second surgery (C)	2.3	YES (2 mg)	NFS	NO asymptomatic
2	54	male	IDH wild-type GBM	metronomic Temozolomide (A)	0.4	YES (8 mg)	BAL	YES interstitial pneumonia
3	43	male	IDH mutant astrocytoma	metronomic Temozolomide (A)	0.1	YES (16 mg)	NFS	YES interstitial pneumonia
4	56	male	IDH wild-type GBM	Regorafenib (C)	0.5	YES (4 mg)	NFS	YES interstitial pneumonia
5	64	male	IDH wild-type GBM	Regorafenib (C)	1.43	YES (2 mg)	NFS	YES interstitial pneumonia

A, active; BAL, bronchoalveolar lavage; C, concluded; DEX, dexamethasone; GBM, glioblastoma; IDH, isocitrate dehydrogenase; NFS, nasopharyngeal swab.
**At the time of SARS-CoV-2 infection.*

tumors and territorial primary care to ensure continuum-of-care in patients receiving active treatments. We ensured case-by-case direct communication with local physicians giving them all the necessary support to manage both cancer treatments and supportive care, including antiepileptic drugs. We implemented the use of direct phone calls, email contacts, or telemedicine with patients to check results of blood tests or give guidance on adverse events or disease-related symptom management. All our three centers followed the same SARS-CoV-2 testing policy. We tested all patients before hospitalization, outpatients with fever or respiratory symptoms before or during any active anticancer treatment, or patients with family members or caregivers with a known positivity for SARS-CoV-2. If a patient without symptoms or with mild symptoms tests positive, we can evaluate starting or continuing an oncologic treatment only after at least 1 month and the negativity of two nasopharyngeal swab for polymerase chain reaction (PCR) analysis of SARS-COV-2 performed 24 h apart. Neuro-oncologists must play a crucial role even in the multidisciplinary management of COVID-19-positive PBT patients during an eventual hospitalization, supporting pulmonologists, intensivists, and other specialists in clinical decision making. Indeed, we should help our colleagues in defining prognosis of our patients and accordingly the indication to invasive respiratory support and resuscitation. Finally, given the inability to access the COVID-19 wards, we took charge of communication with close relatives, keeping them constantly informed about patients' clinical conditions and the course of hospitalization. Among approximately 800 patients referring to our three centers between the end of February and the end of May, we reported a total of only five cases of SARS-CoV-2 infection assessed through the positivity of nasopharyngeal swab or bronchoalveolar lavage: four patients have a diagnosis of IDH wild-type GBM, one of IDH-mutant anaplastic astrocytoma. All patients infected were male, with a median age of 54 (range: 42–64), and were treated in Lombardy and Veneto. At the time of positivity to the test, the median dose of steroids assumed was 4 mg of dexamethasone (range: 2–16), and in three cases, the total lymphocyte count was $\leq$500/μL. Two patients were on active third-line treatment both with a metronomic temozolomide schedule (100 mg/m^2, 3 weeks on and 1 week off), two were diagnosed with COVID-19 after an interruption of their second-line treatment with regorafenib, and one just came out of a second-surgery, and a re-radiation had been scheduled. In four out of five cases, presentation symptoms were fever, with respiratory insufficiency and radiological evidence of interstitial pneumonia, whereas only one patient was asymptomatic at the time of diagnosis. Demographic and clinical features of our patients infected by SARS-CoV-2 are summarized in **Table 1**.

Three patients died due to COVID-19-related pneumonia; one patient recovered from the infection but was unable to continue oncological treatments due to the severe worsening of his clinical conditions, while the last one, after 1 month in home isolation and two consecutive negative swabs received a second radiation therapy (25 Gy in five fractions). Interestingly, four out of five cases experienced a significant worsening of their neurological status concurrently with the development of typical COVID-19 symptoms, despite the evidence of stability by brain MRI. This observation may reinforce the hypothesis of a potential direct CNS involvement by SARS-COV-2 causing a neurological deterioration not dependent on brain tumor progression.

Other international cooperative groups of experts proposed their insights and recommendations on neuro-oncological patients' management amid the COVID-19 outbreak (17, 18). This sort of guidelines reflect most of the actions that we effectively took during the peak of infections, being in some parts even more severe and stringent. Now that we are in a post-emergency phase, with the Italian lockdown recently ended, we can look back at our experience trying to assess the real impact of COVID-19 outbreak on our neuro-oncological community. Given the likely presence of asymptomatic cases or patients already in supportive care who died in long-term care facilities who were never tested, it will be really difficult to define the exact prevalence of the infection among our patients. However, the small number of cases who had severe or fatal complications from the SARS-COV-2 infection in our heavily hit regions somehow

reassures us and shows the sustainability of carrying out a standard neuro-oncology practice from now on.

Since we currently do not know when this pandemic will end, or if the pathogen spread could even be controlled through a vaccination strategy or effective antiviral drugs, it is imperative to focus all our efforts on implementing safe COVID-free pathways into our centers that can guarantee the full safety of patients and staff. In this complex and dynamic scenario, where at least initially the coexistence with the SARS-CoV-2 will be obliged, we strive to not abandon the intensity of care for our PBT patients.

AUTHOR CONTRIBUTIONS

All authors equally contributed to the conception and writing of the present manuscript.

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No Evidence of Re-infection or Person-to-Person Transmission in Cured COVID-19 Patients in Guangzhou, a Retrospective Observational Study

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Objectives: To clarify the clinical characteristics of cured patients with coronavirus disease (COVID-19), and to clarify the re-infection and person-to-person transmission in the cured.

Methods: A total of 187 cured COVID-19 patients with antibody test were followed up every 2 weeks in this retrospective observational study. Assessment for general condition, symptoms, epidemiological contact history, polymerase chain reaction (PCR) assay, and antibody tests were performed and recorded. Information from Guangzhou CDC was also screened.

Results: There were 33 (17.6%) patients with negative results for IgG and 35 (18.7%) patients with positive results for IgM. The average days of antibody detection from disease onset were 53.0. PCR assay was positive in 10 (5.3%) patients during the follow-up. Neither IgG nor IgM results showed a relationship with PCR test results (all $P > 0.05$). Neither re-infection nor person-to-person transmission was found in the cured patients. Factors associated with appearance of antibody comprised hospitalization days (OR: 1.06, 95%CI: 1.02–1.11, $P = 0.006$) and antibiotics treatment (OR: 3.50, 95%CI: 1.40–8.77, $P = 0.007$).

Conclusions: In our study, no evidence of person-to-person transmission was found in cured COVID-19 patients. There seemed to be no re-infection in the cured COVID-19 patients in Guangzhou. These finding suggest that the cured do not cause the spread of disease. Additionally, neither IgG nor IgM can be used to replace the PCR test in cured patients.

Keywords: COVID-19, cured patients, re-infection, person-to-person transmission, antibody

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INTRODUCTION

Coronavirus disease (COVID-19) is an acute infectious disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and is characterized by high morbidity and mortality (1, 2). COVID-19 outbreak began in China in December 2019 and spread rapidly worldwide, with the World Health Organization declaring it a pandemic on March 11, 2020. At present, 4,000,000 confirmed cases of COVID-19 have been detected in more than 200 countries, resulting in more than 280,000 deaths (3), and additional patients with COVID-19 are expected to be cured and discharged over time. Prevention remains the focus for control of COVID-19 (4), but the cured or recovered patients should not be ignored. Currently, little is known about cured COVID-19 patients, and there are no studies to clarify the infectious of the cured or guidelines regarding the management of these patients. However, it is very important to understand the clinical characteristics of cured patients, especially with respect to re-infection and person-to-person transmission.

During the immune response activated by the infection, IgM levels are usually elevated earlier, indicating recent infection and infectivity, while elevated IgG levels indicate adaptive immunity (5). However, in patients with COVID-19, the relevance of IgM and IgG antibodies has not been clarified. Researches demonstrated that IgM and IgG could be identified during the middle and later stage of COVID-19, and thus could have a high diagnostic value in patients with acute infection (6–8). Compared with real-time reverse transcriptase polymerase chain reaction (RT-PCR), the detection of antibodies by ELISA is faster, less expensive, and easier to perform. Therefore, antibody detection might be widely used to assist in the diagnosis of SARS-CoV-2 infection. Till date, no study has evaluated the clinical significance of IgM and IgG detection in terms of re-infection and person-to-person transmission, especially in COVID-19 patients who were cured and discharged home.

In this retrospective observational study, we investigated the clinical significance of IgM and IgG in cured patients after SARS-CoV-2 infection. Furthermore, we clarified the re-infection risk and reported person-to-person transmission of the cured patients. We expect that a deeper understanding the characteristics of cured patients with COVID-19 would be of great value in preventing the spread of the disease.

METHODS

This retrospective observational study was conducted from January 20 to March 10, with follow up till April 10, 2020. All cured adult patients with COVID-19 who performed antibody test were enrolled in our study. Patients were followed up in Guangzhou Eighth People's hospital, a government-designated hospital which admitted nearly 80% of the COVID-19 cases in Guangzhou, the capital city of Guangdong Province in southern China. This study was approved by the ethics committee of the Guangzhou Eighth People's Hospital. Because of the retrospective nature of the study design and the grim scenario of COVID-19 pandemic, the Ethics Committee assented to exempt of all informed consents.

Definition

COVID-19 was diagnosed as per the World Health Organization's interim guidelines (9). High throughput sequencing or RT-PCR were only performed in subjects with the following features: 1. with a confirmed or suspected contact history of COVID-19; 2. presented with symptoms; 3. with abnormal chest computed tomography (CT) imaging related to COVID-19. A positive result on high throughput sequencing or RT-PCR assay together with at least two of the above three clinical features, confirmed the diagnosis of COVID-19. **Criteria for cured** and discharged to home were as follows: vital signs were stable for more than 3 days; the PCR test was negative two times consecutively 24 h apart; and the acute exudative lung lesions were absorbed or cured on chest CT. **Re-infection** criteria were as follows: typical clinical symptoms; chest CT indicative of new infiltration; and two positive repeat PCR tests performed consecutively at an interval of more than 24 h. All confirmed re-infection cases were reviewed by two senior COVID-19 experts. **Person-to-person transmission** criteria were as follows: The cured were supposed to be the carriers. New confirmed COVID-19 cases occurred after one with unprotected exposure to the cured within 2 weeks. Since all new confirmed COVID-19 cases in Guangzhou were reported to Guangzhou CDC, and Guangzhou CDC released the cases including the exposure to source of transmission daily, the person-to-person transmission was assessed from the reports of CDC.

Follow Up

All recovered or cured patients with COVID-19 were quarantined at home for 2 weeks after being discharged. They were free to go anywhere in Guangzhou after 2 weeks. The cured patients were followed up every 2 weeks. Follow-up consisted of assessing the general condition, symptoms, living area, PCR assay, and antibody test. Additionally, these recovered patients were required to report if people close to them had been diagnosed with COVID-19. For patients with a positive PCR test, a chest CT was performed immediately, and PCR test was re-performed consecutively at an interval of more than 24 h. The PCR assay and antibody test were performed on the same day. If positive, IgM antibody test would be repeated within 2 weeks. During the study, the researchers screened the report from CDC in Guangzhou every day to determine whether there were any new confirmed COVID-19 cases linked to transmission by the cured patients.

IgM and IgG Testing

The serum SARS-CoV-2 antibodies (IgM and IgG) were detected using colloidal Gold-based Immunoassays (Colloidal gold kits, Livzon Inc, Zhuhai, China). First, the kit was removed and kept for 30 min at room temperature. Subsequently, 10 μl of plasma sample and 20 μl of whole blood sample were added into the reaction pore until the liquid was fully absorbed. Lastly, two drops of sample diluents were added into the reaction

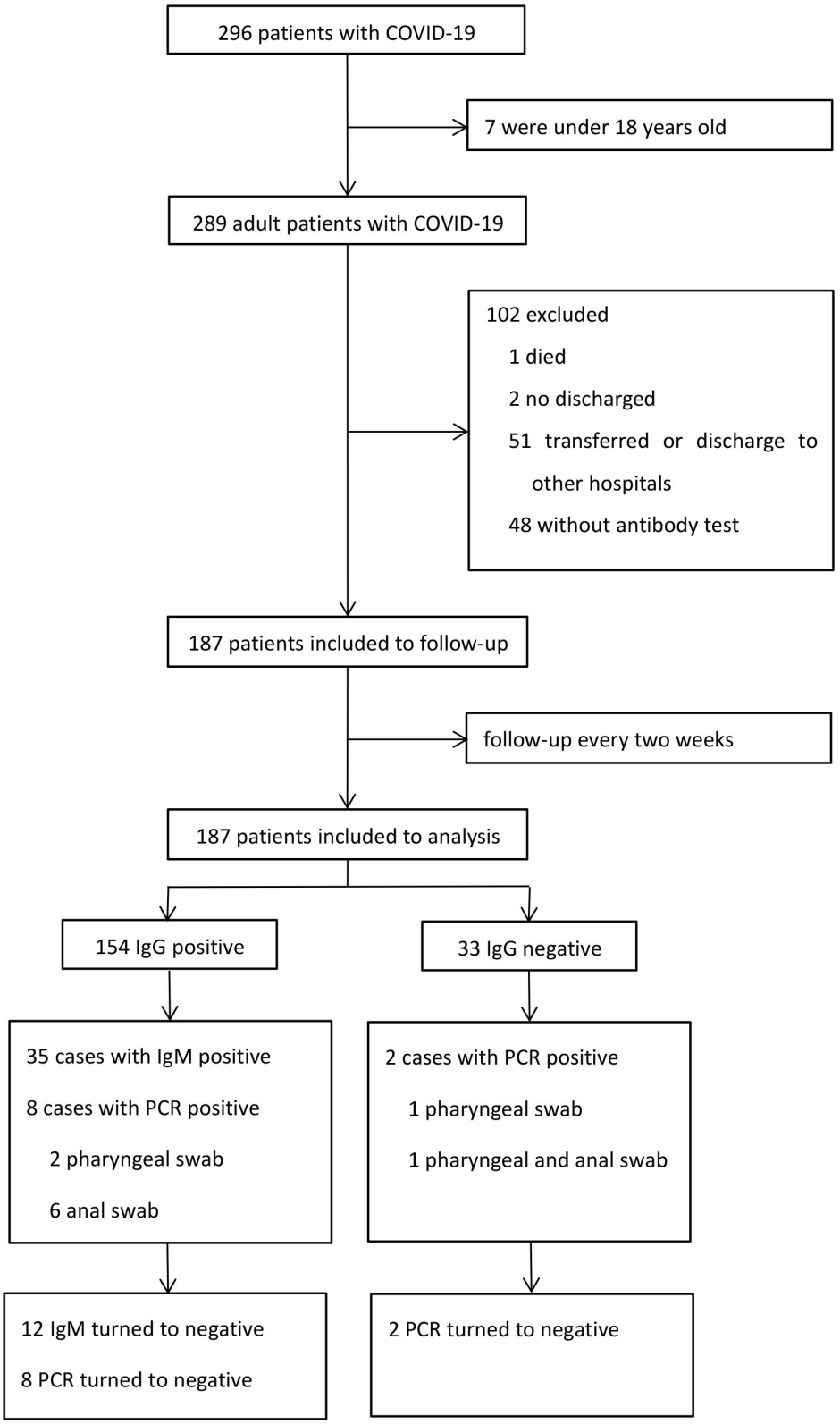

FIGURE 1 | Flow of patients through the study.

hole until the liquid was fully absorbed. The result could be read in 15 min.

Statistical Analyses

Shapiro-Wilk normality test was used to assess for normal distribution of data. Continuous variables with normal distribution were expressed as mean ± standard deviations (SD), while those with non-normal distribution were expresses as median and inter quartile range (IQR). Categorical variables were summarized as counts and percentages. For continuous variables, Independent *t*-test or Wilcoxon rank sum test were used. For comparison of categorical variables, Chi-square test and Fisher's exact test were used. Logistic regression analyses were performed to examine the relationship between independent variables and presence of IgG. Determinants with a *P*-value of 0.10 or less in univariate models were initially included in the multivariate model and were then discarded using backward selection. A *P*-values < 0.05 means statistically significant. All data were processed with SPSS version 22.0 for Windows (SPSS, Chicago, IL, USA).

RESULTS

A total of 296 patients were diagnosed with COVID-19 from January 20, 2020 to March 10, 2020. Among these patients, one died, two were still hospitalized, seven were under 18 years old, 48 refused to perform antibody test, and 51 were transferred or discharged to other hospitals for treatment (**Figure 1**). Altogether, 187 patients were screened and followed up at least once in our hospital and subsequently followed up till April 10, and they were included in the final analysis. The mean follow-up time was 45.7 days. No re-infection occurred in any patient after discharge and no medical staffs were infected during the treatment.

Altogether, 128 of newly diagnosed COVID-19 cases in Guangzhou were reported by CDC from March 11, 2020, to April 10, 2020 (**Figure 2**). Among these patients, 115 were imported from outside China, 13 were with a contact history with imported COVID-19 patients from outside China, and no one were in contact with the cured patients.

We found that the patients in the IgG positive group were older (49.1 vs. 43.2, $P = 0.031$), hospitalized longer (21.0 vs. 14.0, $P < 0.001$), had more severe disease (18.2 vs. 3.0, $P = 0.049$), and with higher proportion of antibiotics treatment (88.3 vs. 63.6, $P = 0.001$) than in the negative group (**Table 1**). There was no difference between the two groups in terms of transmission source, incubation period, and comorbidities (all $P > 0.05$). The complications of COVID-19 included acute respiratory distress syndrome (ARDS), septic shock, acute liver failure, acute renal failure, and acute heart injury. There was no difference between the IgG positive group and negative group with regard to complications (all $P > 0.05$). No differences were found in

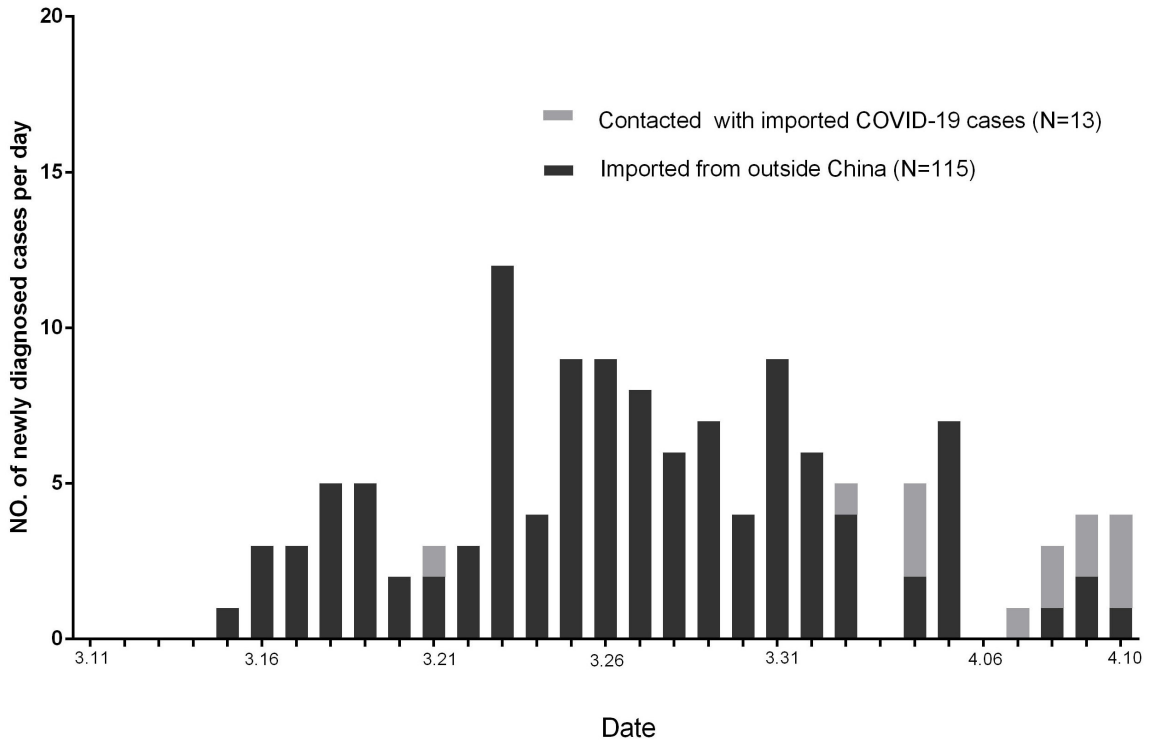

FIGURE 2 | All of newly diagnosed COVID-19 cases in Guangzhou from March 11 to April 10.

TABLE 1 | Baseline characteristics of patients with COVID-19.

Baseline characteristics	IgG positive (*N* = 154)	IgG negative (*N* = 33)	P
Age, year	49.1 ± 14.4	43.2 ± 12.8	0.031*
Female Sex, *N* (%)	86 (55.8)	19 (57.6)	0.856
Incubation period, day	4.0 (8.0)	4.0 (7.0)	0.501
Interval from diagnosis to hospitalization, day	1.0 (2.3)	2.0 (3.8)	0.046*
Hospitalization days, day	21.0 (19.0)	14.0 (8.5)	< 0.001*
Exposure to source of transmission			0.289
Contact with Hubei residents, *N* (%)	94 (61.0)	16 (48.5)	
Contact with COVID-19 patients, *N* (%)	38 (24.7)	9 (27.3)	
Others, *N* (%)	22 (14.3)	8 (24.2)	
Severe disease, *N* (%)	28 (18.2)	1 (3.0)	0.049*
Comorbidities			
Any, *N* (%)	67 (43.5)	14 (42.4)	0.999
Cardiovascular disease, *N* (%)	31 (20.1)	6 (18.2)	0.799
Diabetes, *N* (%)	7 (4.5)	4 (12.1)	0.204
Malignancy, *N* (%)	3 (1.9)	0 (0)	0.999
Chronic respiratory disease, *N* (%)	3 (1.9)	2 (6.0)	0.463
Chronic kidney disease, *N* (%)	2 (1.3)	0 (0)	0.999
Chronic liver disease, *N* (%)	7 (4.5)	3 (0.9)	0.385
Cerebrovascular disease, *N* (%)	4 (2.6)	0 (0)	0.999
White blood cell counts, 109/L	5.1 (2.3)	5.3 (3.4)	0.225
Ureanitrogen, mmol/L	3.7 (1.4)	3.6 (1.4)	0.234
Creatinine, μmol/L	60.7 (29.6)	60.0 (22.6)	0.565
Procalcitonin > 0.25 μg/L, *N* (total N)	62 (100)	7 (16)	0.167
Albumin, g/L	39.7 ± 5.7	40.6 ± 3.7	0.404
CRP > 10ng/L, *N* (total N)	59 (134)	5 (18)	0.190
ALT, U/L	25.0 (23.0)	18.9 (6.5)	0.011*
AST, U/L	19.3 (12.7)	16.6 (7.0)	0.008*
Abnormal chest CT, *N* (%)	151 (98.1)	29 (87.9)	0.183
Complications			
Any, *N* (%)	34 (22.1)	4 (12.1)	0.197
ARDS, *N* (%)	22 (14.3)	1 (3.0)	0.135
Acute cardiac injury, *N* (%)	5 (3.2)	1 (3.0)	0.999
Septic shock, *N* (%)	3 (1.9)	0 (0)	0.999
Acute kidney injury, *N* (%)	1 (0.6)	0 (0)	0.999
Acute liver injury, *N* (%)	17 (11.0)	3 (9.1)	0.777
Treatments			
Antibiotics, *N* (%)	136 (88.3)	21 (63.6)	0.001*
Mechanical ventilation, *N* (%)	13 (8.4)	0 (0)	0.129
Systemic glucocorticoids, *N* (%)	6 (3.2)	0 (0)	0.375
ICU Admission, *N* (%)	6 (3.9)	1 (3.0)	0.999
IgM positive, *N* (%)	35 (22.7)	0 (0)	0.001*

*CRP, C-reactive protein; ALT, alanine transaminase; AST, aspartate transaminase; CT, computed tomography; ARDS, acute respiratory distress syndrome; ICU, intensive care unit. *P-values < 0.05. Reference range of Procalcitonin, < 0.05 μg/L. Reference range of CRP, 0–10 ng/L.*

the treatment comprised mechanical ventilation, glucocorticoids, intensive care between the two groups (all $P > 0.05$).

Potential variables, including age (OR, 1.03; 95% CI, 1.00–1.06; $P = 0.033$), hospitalization days (OR, 1.08; 95% CI, 1.03–1.13; $P = 0.003$), severe disease (OR, 7.11; 95% CI, 0.93–54.26; $P = 0.058$), abnormal chest CT (OR, 6.94; 95% CI, 1.48–32.67; $P = 0.014$), and antibiotics treatment (OR, 4.32; 95% CI, 1.82–10.23; $P = 0.001$), that might be associated with antibody production were screened by using univariate logistic regression analyses (**Table 2**). In the multivariate logistic regression model, determinants associated with antibody production comprised hospitalization days (OR: 1.06, 95%CI: 1.02–1.11, $P = 0.006$) and antibiotics treatment (OR: 3.50, 95%CI: 1.40–8.77, $P = 0.007$).

Out of these 187 patients, 35 (18.7%) patients showed positive results and 152 (81.3%) showed negative results for IgM (**Table 3**). There were 154 (82.4%) patients with positive results and 33 (17.6%) patients with negative results for IgG. The antibody tests were performed after 53 days on an average from the date of disease onset. Of the 35 IgM positive cases, 12 cases turned negative during the follow up. PCR assays were undertaken in all patients using both pharyngeal and anal swabs. They yielded two positive pharyngeal swabs, seven positive anal swabs, and one positive result for both pharyngeal and anal swabs. On further retesting, all the positive results of PCR assays were found to be negative.

In the IgG positive group, eight patients demonstrated positive results on PCR from two pharyngeal swabs and six anal swabs. In the IgG negative group, one patient had positive pharyngeal swabs and one both pharyngeal and anal swabs. We found no relationship between IgG test and PCR assay. Of the 35 IgM positive patients, two had positive anal swabs and no pharyngeal swabs. There was no relationship between IgM test and PCR assay.

TABLE 2 | Determinants associated with appearance of antibody in cured COVID-19 patients.

	Antibody positive		
Determinants	**OR**	**95% CI**	***P***
Univariate modle			
Age	1.03	1.00–1.06	0.033*
Hospitalization days	1.08	1.03–1.13	0.003*
Severe cases	7.11	0.93–54.26	0.058
Abnormal chest CT	6.94	1.48–32.67	0.014*
Antibiotics Treatment	4.32	1.82–10.23	0.001*
Multivariate modle			
Hospitalization days	1.06	1.02–1.11	0.006*
Antibiotics Treatment	3.50	1.40–8.77	0.007*

*CT, computed tomography. *P-values < 0.05.*

DISCUSSION

In this retrospective observational study, we investigated the clinical features of the cured or recovered COVID-19 patients for the first time. Although they were PCR or IgM positive, these patients displayed no clinical manifestations of infection, and no signs of new acute infection were found on chest CT, indicating that these patients did not meet the re-infection criteria. Based on these findings, a positive result on PCR or IgM assay should not be considered indicative of COVID-19 re-infection. There might be several reasons for absence of re-infections. Firstly, the patients with COVID-19 were discharged from hospitals after following strict criteria, and the duration of hospital stay was more than 14 days, far exceeding that in community acquired pneumonia (10), which means that the SARS-CoV-2 was more likely to be have been eradicated. Secondly, 17.6% of the patients were negative for antibody, which might prevent a repeat infection by the virus. Thirdly, an effective prevention and control strategy ensured that the cured patients were kept away from other confirmed COVID-19 patients. Finally, the medical staffs working in the front line have not been infected till date, which effectively prevented secondary infections and spread of the disease in the hospital (11). Re-infection cases were reported in Hong Kong and the United States (12, 13). Based on the known literatures and our research, we believe that patient immunity is helpful to avoid infection, but not all patients can produce immunity after infection. Therefore, the prevention and control strategy is still the key point (14).

The antibodies can be observed in the middle and later stage of COVID-19, and performed well in the diagnosis of COVID-19 (7, 8, 15). For those who have recovered, the clinical significance of the PCR and antibody tests has not been clarified. Our study found that was resurgence of positive results of PCR or IgM tests in some patients after being discharged home. Among people who were in contact with the cured patients, no one was diagnosed with COVID-19 as reported by the Guangzhou CDC. The incubation period of COVID-19 is 3–14 days, and our follow-up period for cured patients was more than 14 days. This might have helped in excluding the cases in the incubation period

TABLE 3 | Outcomes of cured patients with COVID-19.

Outcomes	**Total (*N* = 187)**	**PCR positive (*N* = 10)**	**PCR negative (*N* = 177)**	***P***
IgG positive, *N* (%)	154	8 (80.0)	146 (82.4)	0.999
IgM positive, *N* (%)	35	2 (20.0)	33 (18.6)	0.999
First antibody tests from onset, day	53.0 ± 9.9	50.3 ± 16.5	53.2 ± 9.4	0.369
Follow-up time, day	45.7 ± 11.2	48.7 ± 11.7	45.5 ± 11.1	0.380
Re-infected, *N*	0	0	0	N/A
Fever during follow-up, *N*	0	0	0	N/A
Transmission after discharge, *N*	0	0	0	N/A
Reported by the cured, *N*	0	0	0	N/A
Reported by CDC, *N*	0	0	0	N/A
Contact with newly diagnosed patients, *N*	0	0	0	N/A

PCR, polymerase chain reaction; CDC, Centers for Disease Control and Prevention.

of the infection. Based on these findings, we believe that the cured patients cannot cause person-to-person transmission. They also indicate that a positive result of the PCR or IgM assay does not mean that the cured patient is infectious.

IgG antibodies usually appears 3–40 days after the onset of symptoms (8). In our study, 82.4% patients produced IgG antibodies. However, IgG antibodies were not detected in 17.6% patients when tested after 53 days on an average from the onset of the disease, which means that these patients might not produce IgG antibodies. IgM antibodies appeared in 35 patients when tested after 53 days on an average following the onset of symptoms, and disappeared in 12 patients during the follow up period. Therefore, IgM antibodies might be present in some COVID-19 patients for a long time.

All COVID-19 patients were discharged home after they had negative PCR test results on two consecutive occasions, 24 h apart. However, positive results of PCR or IgM were again observed in some patients during the follow up period. The positive PCR turned to negative in the subsequent retest. Current research has not been able to explain the cause of the positive PCR retests, or confirm whether it is caused by a virus residue. Interestingly, the percentage of positive anal swabs in the cured patients was much higher than the positive pharyngeal swabs. PCR positivity of anal swabs was reported in several studies, which has led to a discussion on the possibility of fecal-oral transmission (16, 17). The reason for PCR positive anal swabs may be that the virus enters the digestive tract from the patient's mouth. However, whether the virus remains active is unknown. During the follow-up, we did not find any new confirmed COVID-19 cases that came into contact with the cured patients who demonstrated positive PCR test results from anal swabs. Although PCR positive, fecal-oral transmission could not be confirmed in our study, and further research is needed.

Compared with the IgG negative group, the IgG positive group patients were older, with longer hospital stay, higher proportion of antibiotic use, higher proportion of severe cases, and higher proportion of CT abnormalities. Further logistic regression analysis showed that the treatment of antibiotic and length of stay were risk factors for antibody production. The mechanism of antibody production associated with antibiotic treatment and long-term hospitalization is not clear. Although diabetes, cancer, and other diseases may cause a decline in immunity, they do not affect the production of antibodies. Similarly, although the use of glucocorticoids may inhibit the immune system, it also has no effect on the production of antibodies.

Studies found that IgG and IgM have a good diagnostic value in the middle and later stage of the disease (6–8, 15). However, the value of IgG and IgM in the diagnosis of cured COVID-19 patients is not clear. In our study, we found that both IgM and IgG have no relationship with PCR. Therefore, for the cured patients, IgG and IgM neither have a diagnostic value, nor can they be used to replace the PCR test. Since neither re-infection nor person-to-person transmission was found in the cured patients, IgG and IgM cannot be used to guide the prevention and control of COVID-19.

This study has the following limitations. Firstly, since this was an observational study, no interventions such as re-exposure of the cured patients to SARS-CoV-2 were performed. Therefore, it is hard to judge whether the cured patients were immune to the virus. Secondly, at the beginning of COVID-19 outbreak, there is no effective antibodies test, and the testing of antibodies were not performed at that time. So we could not compare the levels of antibody between hospitalization and follow-up. Thirdly, this was a single center study carried out in Guangzhou, a mild epidemic area. Accordingly, the conclusions of this study might not be suitable for extrapolation to other areas. Fourthly, our conclusions were based on a small sample size, which need to be further verified in a study with a large sample size. Nevertheless, our study results clarified some clinical features of the cured patients and maybe be of considerable importance for the prevention and control of COVID-19.

CONCLUSIONS

In our study, no evidence of person-to-person transmission was found in cured COVID-19 patients. There seemed to be no re-infection in the cured COVID-19 patients in Guangzhou. These finding suggest that the cured do not cause the spread of disease. Additionally, neither IgG nor IgM can be used to replace the PCR test in cured patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the Corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of the Guangzhou Eighth People's Hospital (No. 202001134). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

GX, FL, MY, JZ, QL, YT, and WM: study concept and design. YL, HS, and FZ: acquisition of data and patient recruitment. CF and YH: analysis and interpretation of data. GX, FL, MY, JZ, and QL: drafting of the manuscript. YT and WM: revising the manuscript. All authors contributed to the article and approved the submitted version.

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Thirty-Day Mortality and Morbidity in COVID-19 Positive vs. COVID-19 Negative Individuals and vs. Individuals Tested for Influenza A/B: A Population-Based Study

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Background: As of October 2020, COVID-19 has caused 1,000,000 deaths worldwide. However, large-scale studies of COVID-19 mortality and new-onset comorbidity compared to individuals tested negative for COVID-19 and individuals tested for influenza A/B are lacking. We investigated COVID-19 30-day mortality and new-onset comorbidity compared to individuals with negative COVID-19 test results and individuals tested for influenza A/B.

Methods and findings: This population-based cohort study utilized electronic health records covering roughly half (n = 2,647,229) of Denmark's population, with nationwide linkage of microbiology test results and death records. All individuals ≥18 years tested for COVID-19 and individuals tested for influenza A/B were followed from 11/2017 to 06/2020. Main outcome was 30-day mortality after a test for either COVID-19 or influenza. Secondary outcomes were major comorbidity diagnoses 30-days after the test for either COVID-19 or influenza A/B. In total, 224,639 individuals were tested for COVID-19. To enhance comparability, we stratified the population for in- and outpatient status at the time of testing. Among inpatients positive for COVID-19, 356 of 1,657 (21%) died within 30 days, which was a 3.0 to 3.1-fold increased 30-day mortality rate, when compared to influenza and COVID-19-negative inpatients (all $p < 0.001$). For outpatients, 128 of 6,263 (2%) COVID-19-positive patients died within 30 days, which was a 5.5 to 6.9-fold increased mortality rate compared to individuals tested negative for COVID-19 or individuals tested positive or negative for influenza, respectively (all $p < 0.001$). Compared to hospitalized patients with influenza A/B, new-onset ischemic stroke, diabetes and nephropathy occurred more frequently in inpatients with COVID-19 (all $p < 0.05$).

Conclusions: In this population-based study comparing COVID-19 positive with COVID-19 negative individuals and individuals tested for influenza, COVID-19 was

associated with increased rates of major systemic and vascular comorbidity and substantially higher mortality. Results should be interpreted with caution because of differences in test strategies for COVID-19 and influenza, use of aggregated data, the limited 30-day follow-up and the possibility for changing mortality rates as the pandemic unfolds. However, the true COVID-19 mortality may even be higher than the stated 3.0 to 5.5-fold increase, owing to more extensive testing for COVID-19.

Keywords: COVID-19, SARS-CoV-2, coronavirus, ischemic heart disease, morbidity, mortality, neurology, psychiatry

INTRODUCTION

COVID-19 has led to a worldwide healthcare crisis with >30,000,000 confirmed infected people, resulting in 1,000,000 deaths as of October 2020 (1, 2). Governmental initiatives including lockdowns and social distancing are aiming to restrict the spread of the virus. Yet, critical voices (3) have argued the socioeconomic consequences may be unjustified given that little is known about how the pandemic compares with annual influenza epidemics in terms of mortality and morbidity. According to the WHO seasonal influenza A/B may result in 290,000–650,000 deaths worldwide annually (4, 5). Substantially higher mortality rates for COVID-19 will result in even more adverse impact on global health without strict preventive measures. However, large-scale studies including follow-up of individuals tested for COVID-19 and influenza A/B from the same cohort are lacking.

Of further concern, COVID-19 might not only be a respiratory disease but a multi-organ disorder because of the wide expression of the angiotensin-converting enzyme-2 receptor to which SARS-Cov-2 binds (6), leading among others to thromboembolic complications (7), severe inflammatory responses (8), and possibly diabetes (9). Neurological and psychiatric complications will likely constitute a major health burden as well (10, 11). But how COVID-19 morbidity compares to similarly severe influenza morbidity is equally poorly understood.

Here, for the first time, we utilized population-based electronic health records (EHR) from Denmark linked with nationwide databases on test results for infections and death records, to investigate mortality in people with COVID-19 compared to people with influenza and to people tested negative for COVID-19. For secondary outcomes we estimated COVID-19-associated new-onset comorbidity, including cardiovascular, neurological and psychiatric events, compared to influenza and individuals tested COVID-19-negative. Analyses were stratified according to age, sex and in- and outpatient status. We hypothesized that COVID-19 would be associated with higher mortality and increased rates of novel comorbidities compared to influenza A/B.

MATERIALS AND METHODS

This retrospective Danish study was based on EHR covering two well-defined administrative regions: Capital Region (i.e., Greater Copenhagen and Bornholm) and Region Zealand, comprising roughly 50% of the Danish population. Denmark has an almost exclusively public health care sector based on catchment areas.

Registers and Study Population

The EHR system of the Capital and Zealand Regions, which is called EPIC (version 2019, Verona, Wisconsin, USA), consists of data from all hospital contacts in these regions. From implementation in 2016 to June 30, 2020, 2,647,229 individuals were registered. Diagnoses are defined according to ICD-10 (12). Registration of death in the EHR is synchronized with the Danish national population registry, updated every 24 h. Accuracy of test results for influenza and SARS-CoV-2 virus is ensured by synchronization of EPIC with the nationwide Danish Microbiology Database (13). All individuals ≥18 years tested for COVID-19 between March 1-June 1, 2020, and all individuals tested for influenza A/B between November 1, 2017-June 1, 2020, were followed for mortality and new-onset comorbidities 30-days after the test until June 30, 2020. Included individuals in this study were hospitalized patients who were tested for COVID-19 or influenza during admission (from now on referred to as *inpatients*); and non-hospitalized patients screened during ambulatory visit, as well as healthy individuals screened in hospital-based testing facilities created for the purpose of screening the general population (from now on referred to as *outpatients*).

Assessment of COVID-19 and Influenza Test Results

COVID-19

All individuals tested for COVID-19 during March 1-June 1, 2020 with laboratory tests CORONAVIRUS 2019-NCOV and/or CORONAVIRUS SARS-COV-2 RNA via nasal, pharyngeal and/or tracheal samples with reverse-transcriptase-polymerase-chain reaction (RT-PCR) assays were included. These specific tests cover all performed COVID-19 tests in the catchment areas and are available from the Danish Microbiology Database (13).

Influenza A/B

We included all individuals tested for influenza A/B during November 1, 2017 to March 1, 2020, using 9 different RT-PCR laboratory tests (**Supplementary Table 1**), covering all available influenza tests based on nasal, pharyngeal and/or tracheal samples.

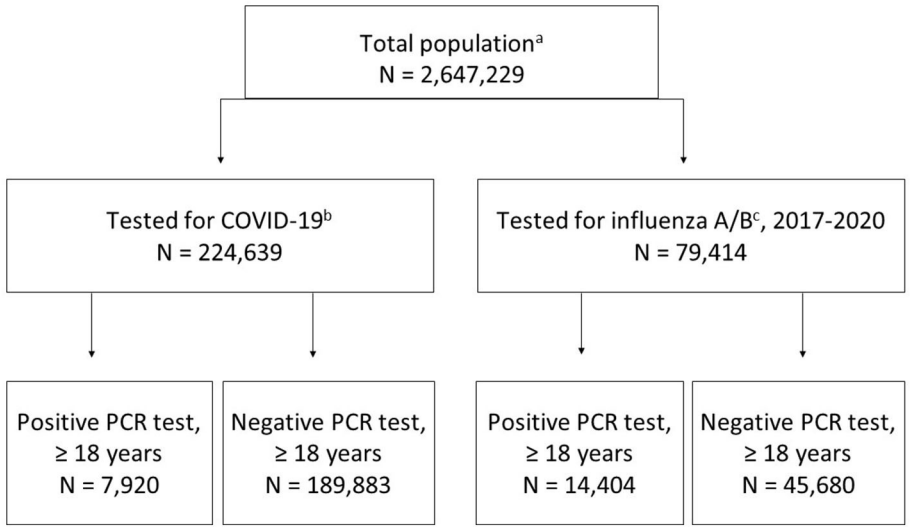

FIGURE 1 | Flow chart of study populations. (a) total population in EHR registered per June 30, 2020; (b) tested between March 1-June 1, 2020; (c) tested between November 1, 2017-March 1, 2020.

Outcome Measures

Primary Outcome Measures

Thirty-day mortality among the group of inidividuals tested positive for COVID-19, compared to 30-day mortality of the group of individuals with COVID-19-negative tests. Additional comparisons were made to the group of individuals tested influenza-positive or influenza-negative.

Secondary Outcome Measures

*N*ew-onset (i.e., 30 days after COVID-19 or influenza test) comorbidity diagnoses, including neurological, psychiatric and cardiovascular disease, pulmonary embolism, venous thrombosis, renal failure, diabetes and rheumatoid arthritis, in all populations. ICD-10 codes are listed in **Supplementary Table 1**.

Data Collection, Statistical Analysis, and Ethics

Anonymized retrospective aggregate-level data on sex, age, prior comorbidities and population mortality 30 days after test results were extracted for individuals ≥18 years for each groups, using the EPIC Slicer-Dicer function. For search strategies see **Supplementary Table 1**. As individuals could be tested multiple times, individuals were only included in the COVID-19-negative, respectively, influenza-negative populations, when all their tests had been negative. Individuals tested for influenza during March 1-June 1, 2020 (i.e., FLU-19) were included for sensitivity analysis (see below). To avoid overlap, we removed COVID-19-positive individuals from the FLU-19 group.

Main analysis was the relative risk (RR) of mortality rates 30 days after a test, in the overall populations and stratified according to in- and outpatient status, sex, and age. Secondary analysis was RR of cumulative 30 days post-test incidence of new-onset comorbidities, after exclusion of individuals who already had the investigated comorbidity before the test. We compared COVID-19 positive with COVID-19-negative and influenza-positive individuals. To validate mortality data, absolute mortality rates extracted from electronic health records (EPIC) were compared with official Danish statistics numbers (**Supplementary Table 2**). Sensitivity analysis was conducted by comparing individuals ≥18 years with a positive or negative influenza test from the same time period as the COVID-19 population, i.e., March 1-June 1, 2020 (FLU-19), in order to investigate the possible influence of the COVID-19 pandemic, including lockdown and social distancing measures, on mortality rates in individuals tested for influenza. Chi-squared statistics were used to calculate odds ratio (OR), RR and 95% confidence intervals (CI) using SPSS (version 25; IBM, Armonk, NY, USA). Two-sided $p \leq 0.05$ was considered significant.

The Ethics Committee of the Capital Region of Denmark waives approval for register-based studies on aggregated anonymized data (Section 14.2 of the Committee Act. 2; http://www.nvk.dk/english). Use of anonymized aggregate-level data was approved by the Danish Data Protection Agency. Results from ≤5 patients were displayed as "≤5" to ensure data privacy.

RESULTS

A total of 224,639 individuals of any age were tested for SARS-CoV-2 between March 1-June 1, 2020; positive results were found in 7,920 individuals ≥18 years (i.e., our case population). A negative COVID-19 test occurred in 189,883 individuals ≥18 years. Between November 1, 2017-March 1, 2020, we identified 79,414 individuals, who were tested for influenza A/B. Positive results were found in 14,404 individuals aged ≥18 years. Negative influenza A/B tests were identified in 45,680 individuals ≥18 years (**Figure 1**). Demographics are displayed in **Table 1** and **Supplementary Tables 3–5**. The proportion of inpatients at the time of COVID-19 or influenza tests was lower

TABLE 1 | Demographics and prior comorbidities among individuals tested for COVID-19 or influenza as in- or outpatient.

	Inpatients			Outpatients		
	COVID-19 positive (*N* = 1,657)	**COVID-negative (*N* = 31,483)**	**Influenza-positive (*N* = 7,200)**	**COVID-19-positive (*N* = 6,263)**	**COVID-negative (*N* = 158,400)**	**Influenza-positive (*N* = 7,204)**
Age – years, no. (%)						
Mean, years	65	60	66	47	48	49
18–39	182 (11.0)	7,456 (23.7)‡	931 (12.9)	2,310 (36.9)	54,659 (34.5)‡	2,390 (33.2)‡
40–59	427 (25.8)	6,790 (21.6)‡	1,413 (19.6)‡	2,509 (40.1)	62,701 (39.6)	2,637 (36.6)‡
60–80	636 (38.4)	11,261 (35.8)‡	2,876 (39.9)	1,043 (16.7)	33,166 (20.9)‡	1,762 24.4)‡
> 80	412 (24.9)	5,976 (19.0)‡	1,980 (27.5)	401 (6.4)	7,874 (5.0)‡	415 (5.8)
Sex (%)						
Women	737 (44.5)	17,099 (54.3)‡	3,844 (53.4)‡	3,931 (62.8)	99,364 (62.7)	4,272 (59.3)‡
Prior medical diagnoses - no. (%)#						
Neurological, any	275 (16.6)	4,998 (15.9)	1,025 (14.2)	505 (8.1)	14,074 (8.9)‡	474 (6.6)‡
Cerebrovascular, any	141 (8.5)	2,819 (9.0)	485 (6.7)	145 (2.3)	3,698 (2.3)	125 (1.7)‡
Ischemic stroke incl. TIA	78 (4.7)	1,739 (5.5)	165 (2.3)‡	85 (1.4)	2,302 (1.5)	130 (1.8)‡
Psychiatric, any	202 (12.2)	6,581 (20.9)‡	821 (11.4)	383 (6.1)	12,264 (7.7)‡	305 (4.2)‡
Ischemic heart disease	147 (8.9)	2,631 (8.4)	547 (7.6)	90 (1.4)	3,719 (2.3)‡	155 (2.2)‡
Heart failure	100 (6.0)	1,861 (5.9)	422 (5.9)	45 (0.7)	1,581 (1.0)‡	68 (0.9)
Diabetes	199 (12.0)	3,291 (10.5)‡	827 (11.5)	209 (3.3)	4,903 (3.1)	255 (3.5)
Chronic lower respiratory disease	182 (11.9)	3,943 (12.5)	1,362 (18.9)‡	223 (3.6)	7,934 (5.0)‡	366 (5.1)‡
Obesity	46 (2.8)	1,296 (4.1)‡	167 (2.3)	132 (2.1)	3,929 (2.5)	141 (2.1)

COVID-19, positive COVID-19 PCR test between March 1-June 1, 2020; influenza, positive influenza A/B PCR test between November 1, 2017 to March 1, 2020; COVID-neg, negative COVID-19 PCR test between March 1-June 1, 2020. Each patient was followed for a total of 30 days from positive test until end of follow-up or death. ‡Indicates statistically significant difference ($p < 0.05$) compared to COVID-19 populations. #Established medical diagnoses, registered in the medical files, prior to testing for COVID-19 or influenza. TIA, transitory ischemic attack.

in the COVID-19-positive (20.9%) and the COVID-19-negative (16.6%) populations compared to influenza-positive (50%) and influenza-negative (57.7%) populations. We therefore analyzed mortality and comorbidities both in the overall populations and stratified according to in- and outpatient status, sex, and age.

Primary Outcome: Mortality

Overall Mortality Rates

Overall 30-day mortality in COVID-19-positive individuals was 484 of 7,920 (6.1%), whereas 30-day mortality for COVID-19-negative individuals was 2,654 of 189,883 (1.4%), corresponding to an increased mortality by RR 4.37 (95% CI = 3.98–4.80).

Mortality Rates of Inpatients Tested for COVID-19 and/or Influenza

Thirty-day mortality for hospitalized COVID-19 patients ≥18 years was 356 of 1,657 (21.5%), which was higher than in COVID-19-negative individuals (30-day mortality 2,185/31,483; 6.9%; $p < 0.001$) (**Figure 2**, **Table 2**, and **Supplementary Tables 6–9**). The corresponding numbers for individuals tested positive for influenza were 516/7,200 (7.2%) and for influenza-negative individuals 2,873/26,366 (11%). Mortality for COVID-19-positive inpatients was increased by RR 3.10 (95% CI = 2.80–3.42) compared to COVID-19-negative patients, and by RR 3.00 (95% CI = 2.65–3.39) and RR 1.97 (95% CI = 1.79–2.18) compared to influenza-positive, respectively, influenza-negative inpatients (all $p < 0.001$).

When mortality rates were stratified according to age, 30-day mortality rates for hospitalized COVID-19 patients were 16/427 (3.7%, age 40–59 years), 150/636 (23.6%, 60–80 years) and 190/412 (46%, >80 years). The corresponding numbers for COVID-19-negative individuals were 158/6,790 (2.3%), 1,004/11,261 (8.9%), and 1,008/5,976 (16.9%) and for influenza-positive individuals 26/1,413 (1.8%), 214/2,876 (7.4%), and 271/1,980 (13.7%), respectively. Mortality for COVID-19-positive inpatients was significantly increased with age 60–80 years (RR = 2.65; 95% CI = 2.27–3.08) and >80 years (RR = 3.17; 95% CI = 2.62–3.83), when compared to COVID-19-negative individuals (RR 2.73; 95% CI = 2.42–3.08) and influenza-positive individuals (RR 3.37 (95% CI = 2.89–3.92). When mortality rates were stratified according to sex, 30-day mortality rates for hospitalized COVID-19 patients were 143/738 (19.4%, female) and 213/919 (23.2%, male). The corresponding numbers for COVID-19-negative individuals were 1,027/17,134 (6.0%, female) and 1,158/14,349 (8.1%, male) and for influenza-positive individuals 240/3,851 (6.2%, female) and 276/3,349 (8.2%, male). Mortality for COVID-19-positive inpatients was significantly increased in females and males compared to COVID-19-negative females (RR 3.23; 95% CI = 2.76-3.79) and males (RR 2.87; 95% CI = 2.52–3.27) and influenza-positive females (RR 3.11; 95% CI = 2.57–3.77) and males (RR 2.81; 95% CI = 2.39–3.31). See **Table 2** and **Supplementary Table 6** for a full outline of inpatient mortality rates stratified according to sex and age groups.

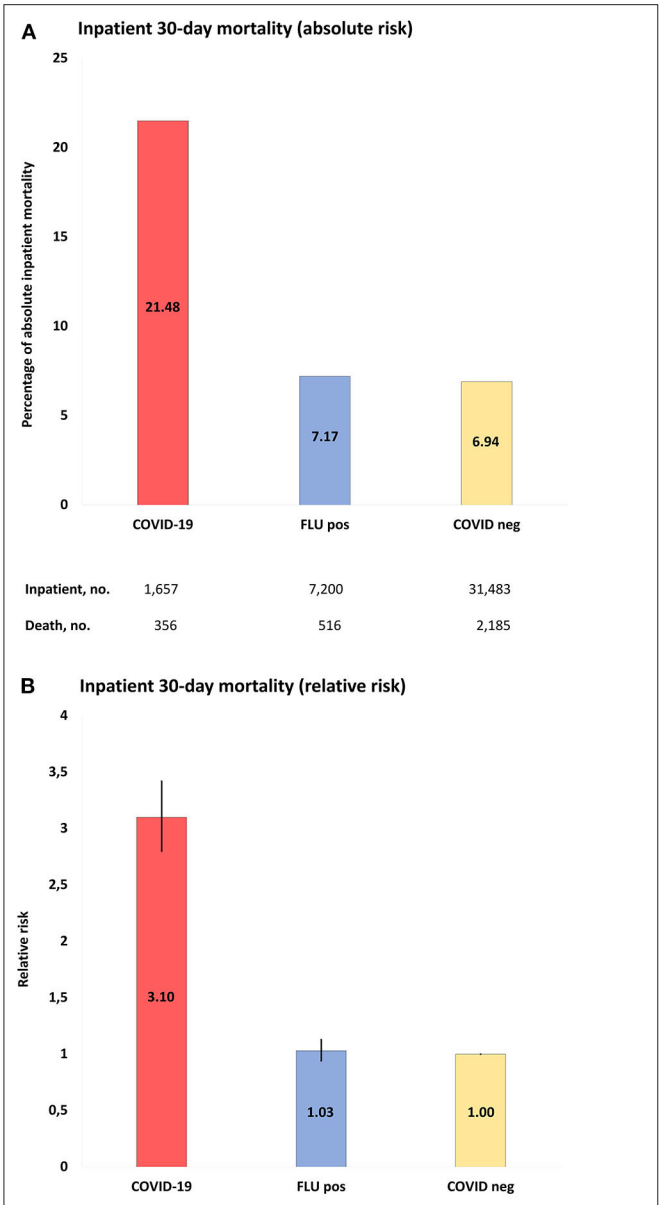

FIGURE 2 | (A), Absolute risk with 95% CI of COVID-19 inpatient 30-day mortality, when compared to populations of the study: COVID-19, positive COVID-19 test between March 1-June 1, 2020; FLU pos, positive influenza A/B test between November 1, 2017-March 1, 2020; COVID-neg, negative COVID-19 test between March 1-June 1, 2020. **(B)**, RR with 95% CI of inpatient mortality of study populations compared to COVID-19 negative population as reference.

Mortality Rates of Outpatients Tested for COVID-19 and/or Influenza

Regarding outpatients, positive COVID-19 tests were associated with 128 deaths in 6,263 people (2% 30-day mortality) and negative COVID-19 tests with 469 deaths in 158,400 people (0.3%), whereas the corresponding numbers for influenza-tested people were 27/7,204 (0.4%; positive test) and 129/19,314 (0.7%; negative test). Mortality rates for COVID-19-positive outpatients were increased by RR 6.90 (95% CI = 5.69–8.38) compared to COVID-19-negative outpatients, by RR 5.45 (95% CI = 3.61–8.25) compared to influenza-positive outpatients, and by RR 3.06 (95% CI = 2.40–3.90) compared to influenza-negative outpatients. **Figure 3** and **Supplementary Tables 6–9** show details.

The 30-day mortality rates for outpatients with COVID-19 were 20/62,701 (0.03%), 33/1,043 (3.2%), and 92/401 (22.9%) for age groups 40–59, 60–80, and >80 years, respectively. The corresponding numbers for COVID-19 negative individuals were 161/33,166 (0.5%) and 288/7,874 (3.7%) in the age groups 60–80 and >80 years, and for influenza-positive individuals ≤5/1,761 and 20/415 (4.8%), respectively. The case numbers were too low in the remaining age groups for statistics. The 30-day mortality rates for outpatients with COVID-19 were 75/3,937 (1.9%) and 53/2,326 (2.3%) in females and males, respectively. The corresponding numbers for COVID-19 negative individuals were 261/99,512 (0.3%) and 208/58,888 (0.4%) for females and males, respectively, and for influenza-positive individuals 17/4,284 (0.4%) and 10/2,920 (0.3%), respectively. Outpatient 30-day mortality was significantly increased in COVID-19 males and females compared to COVID-19 negative and Influenza-positive and negative individuals. See **Table 2** and **Supplementary Table 6** for full details of outpatient mortality rates stratified according to age and sex.

Secondary Outcomes: New-Onset Comorbidities

Figure 4 and **Supplementary Tables 10**, **11** display data regarding novel diagnoses after COVID-19 and influenza tests.

New-Onset Comorbidities Among COVID-19-Positive and COVID-19-Negative Individuals

Pulmonary embolism 30 days after testing was more frequent in COVID-19-positive compared to COVID-19-negative individuals [RR 2.47 (95% CI = 1.60–3.78)], **Supplementary Table 10**. Diabetes and renal failure were also more frequent in COVID-19-positive compared to negative individuals (0.6 vs. 0.2% and 0.6 vs. 0.1%, respectively; both $p < 0.001$). Neurological disorders (excluding vascular disorders) and ischemic heart disease were less frequent in COVID-19-positive than in COVID-19-negative people (0.2 vs. 0.5% and 0.1 vs. 0.3%, respectively; both $p < 0.05$). Rates of new-onset cerebrovascular disorders, venous thrombosis and psychiatric disorders were not significantly different between the two populations.

New-Onset Comorbidities in Inpatients Tested Positive for COVID-19 vs. Influenza-Positive Individuals

Incident ischemic stroke 30 days after a test was more frequent in COVID-19-positive inpatients compared to those with influenza, RR 3.10 (95% CI = 1.56–6.08), **Supplementary Table 11**. New-onset diabetes and nephropathy were more frequent in COVID-19 positive compared to influenza-positive inpatients (1.9 vs. 1.2% and 1.8 vs. 0.9%, respectively; both $p < 0.05$). Rates

TABLE 2 | Relative risk of 30-day mortality after a COVID-19 or influenza test among in- or outpatients.

	Inpatients			Outpatients		
	Total (N)	**Death (N)**	***RR (95% CI)***	**Total (N)**	**Death (N)**	***RR (95% CI)***
COVID-19-positive vs. COVID-negative						
Overall	1,657 vs. 31,483	356 vs. 2,185	**3.10** (2.80–3.42)†	6,263 vs. 158,400	128 vs. 469	**6.90** (5.69–8.38)†
Female	738 vs. 17,134	143 vs. 1,027	**3.23** (2.76–3.79)†	3,937 vs. 99,512	75 vs. 261	**7.26** (5.63–9.37)†
Male	919 vs. 14,349	213 vs. 1,158	**2.87** (2.52–3.27)†	2,326 vs. 58,888	53 vs. 208	**6.45** (4.79–8.70)†
18–39 years	182 vs. 7,456	≤5 vs. 15	N/A	2,310 vs. 54,659	≤5 vs. ≤5	N/A
40–59 years	427 vs. 6,790	16 vs. 158	1.61 (0.97-2.67)	2,509 vs. 62,701	≤5 vs. 20	N/A
60–80 years	636 vs. 11,261	150 vs. 1,004	**2.65** (2.27–3.08)†	1,043 vs. 33,166	33 vs. 161	**6.52** (4.50–9.43)†
> 80 years	412 vs. 5,976	190 vs. 1,008	**2.73** (2.42–3.08)†	401 vs. 7,874	92 vs. 288	**6.27** (5.07–7.76)†
COVID-19 positive vs. FLU-positive						
Overall	1,657 vs. 7,200	356 vs. 516	**3.00** (2.65–3.39)†	6,263 vs. 7,204	128 vs. 27	**5.45** (3.61–8.25)†
Female	738 vs. 3,851	143 vs. 240	**3.11** (2.57–3.77)†	3,937 vs. 4,284	75 vs. 17	**4.80** (2.84–8.11)†
Male	919 vs. 3,349	213 vs. 276	**2.81** (2.39–3.31)†	2,326 vs. 2,920	53 vs. 10	**6.65** (3.39–13.05)†
18–39 years	182 vs. 931	≤5 vs. ≤5	N/A	2,310 vs. 2,390	≤5 vs. ≤5	N/A
40–59 years	427 vs. 1,413	16 vs. 26	**2.04** (1.10–3.76)‡	2,509 vs. 2,638	≤5 vs. ≤5	N/A
60–80 years	636 vs. 2,876	150 vs. 214	**3.17** (2.62–3.83)†	1,043 vs. 1,761	33 vs. ≤5	N/A
> 80 years	412 vs. 1,980	190 vs. 271	**3.37** (2.89–3.92)†	401 vs. 415	92 vs. 20	**4.76** (2.30–7.57)†
COVID-19 positive vs. FLU-negative						
Overall	1,657 vs. 26,366	356 vs. 2,873	**1.97** (1.79–2.18)†	6,263 vs. 19,314	128 vs. 129	**3.06** (2.40–3.90)†
Female	738 vs. 13,456	143 vs. 1,275	**2.05** (1.75–2.39)†	3,937 vs. 11,773	75 vs. 60	**3.74** (2.67–5.24)†
Male	919 vs. 12,910	213 vs. 1,598	**1.87** (1.65–2.12)†	2,326 vs. 7,541	53 vs. 69	**2.49** (1.75–3.55)†
18–39 years	182 vs. 3,867	≤5 vs. 40	N/A	2,310 vs. 6,664	≤5 vs. ≤5	N/A
40–59 years	427 vs. 4,757	16 vs. 224	0.79 (0.48–1.31)	2,509 vs. 6,454	≤5 vs. 6	N/A
60–80 years	636 vs. 10,939	150 vs. 1,307	**1.97** (1.70–2.30)†	1,043 vs. 4,963	33 vs. 51	**3.08** (2.00–4.75)†
> 80 years	412 vs. 6,803	190 vs. 1,302	**2.41** (2.15–2.70)†	401 vs. 1,233	92 vs. 71	**3.98** (2.98–5.32)†

COVID-19, positive COVID-19 test between March 1-June 1, 2020; COVID-neg, negative COVID-19 test between March 1-June 1, 2020; FLU positive, positive influenza A/B test between November 1, 2017-March 1, 2020; FLU negative, negative influenza A/B test between November 1, 2017-March 1, 2020. Each included patient was followed for a total of 30 days from positive test until end of follow-up or death. N/A, not applicable due to low number of patients (N≤5). †$p < 0.001$; ‡$p < 0.05$. The bold values indicate statistically significant values.

of new-onset pulmonary embolism, neurological disorders and psychiatric disorders were not statistically different.

New-Onset Comorbidities in Outpatients Tested Positive for COVID-19 vs. Influenza-Positive Individuals

Incidence diagnoses 30 days after positive tests in outpatients yielded either too low numbers for meaningful statistics or were not statistically different (**Supplementary Table 11**).

Sensitivity Analysis

The COVID-19-positive population was compared to a population of influenza-tested individuals from the same time period, March 1-June 1, 2020, i.e., outside the influenza peak season (FLU-19). In total, 12,502 people were tested for influenza A/B (56% inpatients; 566 positive and 8,318 negative). Inpatient mortality in FLU-19-positive and -negative populations was 26/317 (8.2%), respectively, 578/5,058 (11.4%). Inpatient mortality was significantly increased in COVID-19 compared to FLU-19-positive and -negative individuals (RR 2.62 (95% CI = 1.79–3.83), respectively, RR 1.88 (95% CI = 1.67–2.12); both $p < 0.001$).

DISCUSSION

To our knowledge, this is the first population-based study comparing mortality rates and new-onset comorbidities of COVID-19 patients with those of COVID-19-negative controls and individuals tested for influenza A/B. 30-day mortality was 3.0 to 6.9-fold higher in the group of individuals tested positive for COVID-19 compared to individuals tested COVID-19 negative and when compared with individuals tested for influenza. The largest difference in mortality between COVID-19 and influenza was observed in outpatients. Equally important, new-onset ischemic stroke, renal failure and diabetes occurred at increased rates in COVID-19-positive inpatients compared to influenza patients.

Previous studies have reported widely varying overall COVID-19 mortality rates, e.g., 1.4% among 1,099 cases in Wuhan, China (14), and 7.2% among 22,512 in Italy (15). In our study, the overall 30-day COVID-19 mortality was 6.1% and males over 60 years of age were overrepresented, which is well in line with previous data from COVID-19 patients from Denmark (16). Importantly, mortality rates are very different among in-

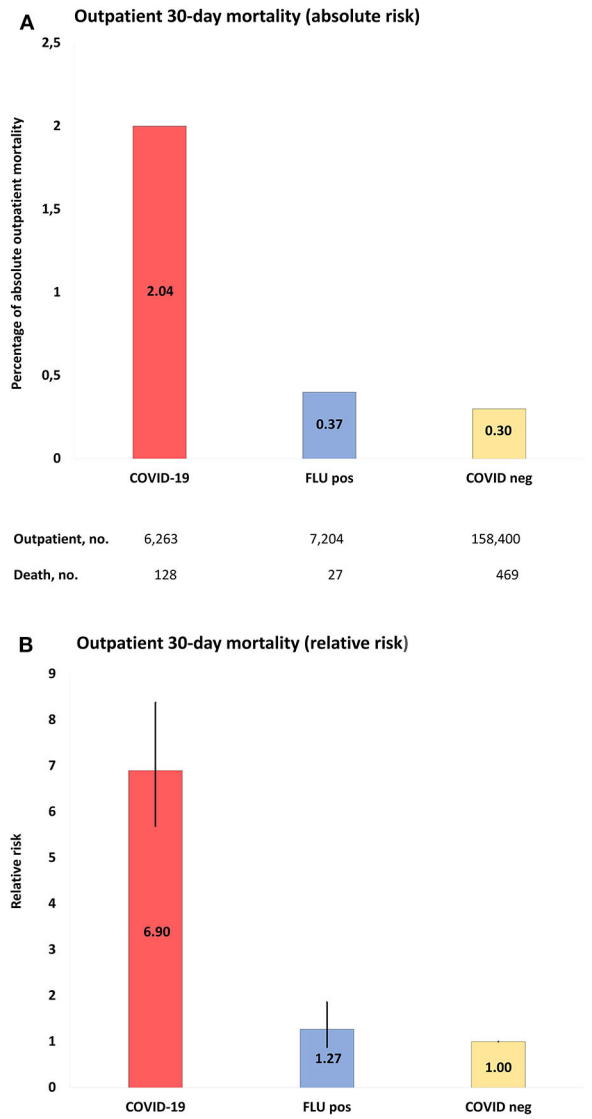

FIGURE 3 | (A), Absolute risk with 95% CI of COVID-19 outpatient 30-day mortality, when compared to populations of the study: COVID-19, positive COVID-19 test between March 1-June 1, 2020; FLU pos, positive influenza A/B test between November 1, 2017-March 1, 2020; COVID-neg, negative COVID-19 test between March 1-June 1, 2020. **(B)**, RR with 95% CI of outpatient mortality of study populations compared to COVID-19 negative population as reference.

and outpatients. In the COVID-19-positive inpatient population 30-day mortality was 21%, corresponding well-again with a mortality of 28% in 191 inpatients reported by Zhou et al. (17), respectively, a median 14-day mortality of 26% in 140 inpatients from Xie et al. (18). These numbers are much higher than the 2% mortality in outpatients tested COVID-19 positive (i.e., individuals from the general public not requiring hospitalization) in the present study, indicating that, not surprisingly, inpatients with COVID-19 are doing worse than outpatients.

Compared to individuals tested positive for influenza and individuals tested COVID-19-negative, COVID-19 30-day mortality was increased 3.0 to 3.10-fold for inpatients and 5.5 to 6.9-fold for outpatients. This is somewhat in contrast with an estimated 20-fold mean increase of COVID-19 mortality compared to influenza, based on indirect estimated numbers from the general public in the US (19). This discrepancy could be explained by the higher proportion of sick individuals in our influenza tested populations, as the influenza testing in Denmark is primarily done on individuals at risk due to chronic conditions. If testing for influenza A/B in Denmark had been equally widespread as for COVID-19, the excess COVID-19 mortality gap would likely have been even larger.

Thromboembolic complications in COVID-19 are assumed to be frequent (20). New-onset ischemic stroke was indeed more frequent in COVID-19 than in influenza inpatients. Increased rates of ischemic stroke in COVID-19 compared to influenza were also found in another study based on retrospective medical charts review from 2 academic centers in New York (21). Given that signs and symptoms of stroke – especially minor stroke – may be obscured by systemic illness as well as sedation and ventilation, the true risk may even be higher than the 3- to 7-fold increase reported here and in the cited work (21). We also found that the risk of new-onset diabetes was 3-fold elevated in COVID-19-positive individuals compared to negative controls and 2-fold elevated compared to influenza-positive patients. These results substantiate concerns of diabetogenic effects of COVID-19 (22), including the possibility of ketoacidosis (23). Similarly, nephropathy was frequent in our COVID-19 population, and renal failure may lead to more complications and higher in-hospital mortality (24). Ischemic heart disease appeared equally prevalent in inpatients with COVID-19 and those with influenza. Finally, pulmonary embolism occurred more often in our COVID-19 positive population compared to negative controls (albeit not compared to influenza populations).

All these comorbidities, alone or in combination, may put patients with COVID-19 at risk for multiorgan failure. This, together with hypoxemia owing to pulmonary changes, including diffuse alveolar damage with fibrin membranes, thickened alveolar walls, lymphocytic infiltration (25), and pulmonary thrombosis (25), complicated by cardiac arrhythmias, hypotensive shock (26), and possibly brainstem dysfunction (27), is being proposed as the final pathway to death in COVID-19 (28), Many of these mechanisms are unlikely to be specific enough to be reliably captured by diagnostic coding in EHR-based studies such as ours. Large prospective multicenter registries and autopsy studies comparing COVID-19 patients with COVID-19-negative controls and influenza victims are required to dissect the exact contribution of each of these factors.

Concerns for neurological and psychiatric complications in COVID-19 are increasingly being raised (11). Yet, most [albeit not all (10)] reports have revealed a predominance of relatively unspecific symptoms such as altered mental state in highly selected groups without control groups (11, 29, 30), while we report on EHR-registered diagnoses. Our results show decreased or similar frequencies of new-onset neurological and psychiatric diagnoses in COVID-19 individuals within 30 days

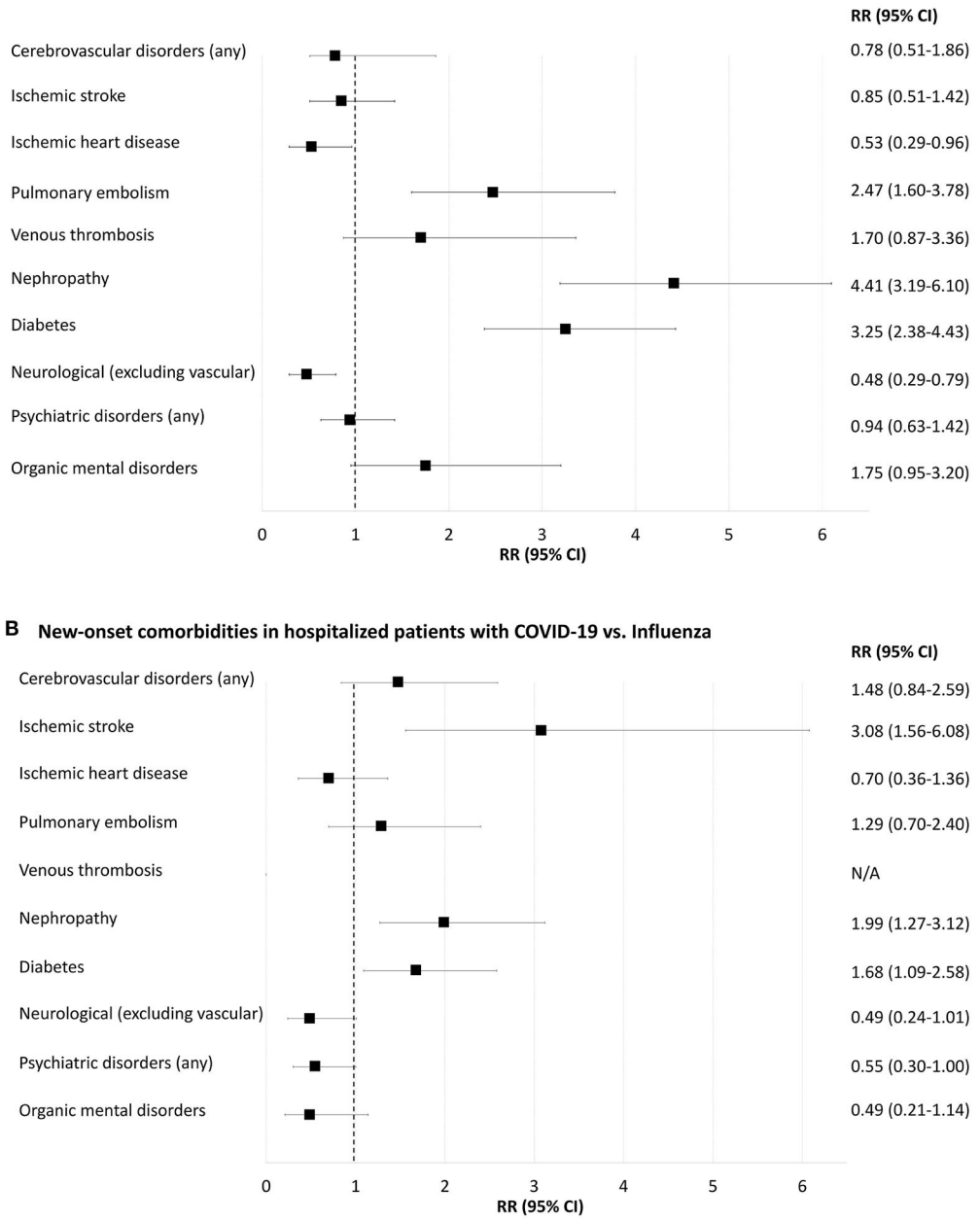

FIGURE 4 | Forest-plot showing relative risk of new-onset comorbidities 30 days after positive or negative COVID-19 or influenza A/B tests (i.e., prior comorbidities excluded). **(A)**, COVID-19 positive compared to COVID-19 negative individuals. **(B)**, Inpatients with positive COVID-19 compared to inpatients with positive influenza. N/A, not applicable because of too few cases for meaningful statistics (≤ 5 individuals). New-onset delirium, neurotic and anxiety disorders, affective disorders, schizophrenia and delusional disorders and rheumatoid arthritis yielded too few cases for meaningful statistics (data shown in **Supplementary Tables 10**, **11**).

of testing compared to influenza, which suggests either that these complications in COVID-19 are no more frequent than for severe influenza or that the nationwide lockdown in Denmark resulted in fewer contacts to the health care system by people with COVID-19 but relatively mild comorbid symptoms, including neurological and psychiatric ones. Indeed, observations from California, Italy and Denmark (31–33) indicate a lower incidence of hospitalization of patients with e.g., cardiac disease during

the COVID-19 lockdown. Further, mild cognitive and emotional symptoms are not likely to be reported within 30 days, and thus the potential long-term consequences of COVID-19 could not be investigated in this study, where we investigated the acute short-term comorbidities.

Strengths and Limitations

Strengths of our study are related, among others, to the large population numbers and the catchment area-based approach. The extracted general mortality data during years 2018–2020 corresponded well to Danish statistics mortality data (**Supplementary Table 2**). Numbers of COVID-19 and influenza tests, test results, admissions, and mortality rates in this study were equally consistent with the official Danish numbers (34) Further, test results of SARS-CoV-2 and influenza swabs are synchronized with the Danish national microbiology database (13), which is again linked with nationwide mortality data. Therefore, the mortality outcome can be considered virtually complete. We validated our data extraction strategy by ensuring that two individual searches supervised by two independent Epic Slicer-Dicer experts yielded identical results.

As to limitations, we were unable to adjust for confounding factors such as socioeconomics, lifestyle, ethnicity and comorbidities, owing to the use of aggregated EHR data. Instead, we performed stratified analyses according to age- and sex-groups. Of note, people who died of COVID-19 without being tested (i.e., without being recognized as COVID-19 victims) were for obvious reasons not included in our results, which might have led to an underestimation of COVID-19 mortality. Further, we could not adjust for influenza immune prophylaxis given to 10–15% of the Danish population annually, primarily patients in at risk-groups due to chronic conditions (35). Vaccination reduces influenza rates and increases the chance of a milder course of influenza, leading to a lower probability of new-onset comorbidities and decreased mortality. These effects depend on the effectiveness of the vaccine which varies each year and was particularly low during the 2017/2018 season owing to a mismatch between strains used in the production of the vaccine and those causing the seasonal epidemic (36). In the 2017/2018 peak influenza season, influenza B was identified in 68% patients tested positive for influenza, while only 17% were tested positive for influenza A (H3N2) and 14% for influenza A (H1N1). Vaccine effectiveness was 30–33%, 0–13%, and 45–50% for influenza B, A (H1N1) and A (H3N2), respectively (36). When comparing COVID-19 with influenza A/B, it must thus be kept in mind that influenza vaccination likely has had a decreasing effect on overall morbidity and mortality in the influenza population. Selection bias might also be considerable because individuals were tested in hospital settings (even as outpatients), and the testing strategy of COVID-19 in Denmark has been much more comprehensive compared to influenza. Furthermore, we only investigated the individuals tested for COVID-19 or influenza, whereas if instead comparing with the entire background population in the capture area, the mortality and morbidity ratios would likely be more increased as the tested population likely have more symptoms and comorbidities than the population not tested.

CONCLUSIONS

In this first population-based study comparing individuals with COVID-19 positive test results with individuals tested negative for COVID-19 and individuals with influenza, COVID-19 was associated with substantially higher mortality. Due to use of aggregated data with limited ability to adjust for confounders, results must be interpreted with caution, but this mortality is likely even higher than the stated 3.0 to 5.5-fold increase owing to more extensive testing for COVID-19. In addition, we observed higher rates of new-onset ischemic stroke, diabetes and renal failure. Next, middle- and long-term follow-up data are required to investigate mortality trajectories in COVID-19 vs. influenza populations, and molecular and genetic studies will have to elucidate the specific biological mechanisms behind COVID-19's higher mortality and morbidity compared to influenza.

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

DK and MB contributed to the conception, design of the study, and contributed equally as senior authors. VN and MA contributed equally as first authors. All authors contributed to the intellectual conception, revision of important intellectual content, and approval of the final version of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.598272/full#supplementary-material

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REVIEW
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Body Localization of ACE-2: On the Trail of the Keyhole of SARS-CoV-2

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The explosion of the new coronavirus (SARS-CoV-2) pandemic has brought the role of the angiotensin converting enzyme 2 (ACE2) back into the scientific limelight. Since SARS-CoV-2 must bind the ACE2 for entering the host cells in humans, its expression and body localization are critical to track the potential target organ of this infection and to outline disease progression and clinical outcomes. Here, we mapped the physiological body distribution, expression, and activities of ACE2 and discussed its potential correlations and mutal interactions with the disparate symptoms present in SARS-CoV-2 patients at the level of different organs. We highlighted that despite during SARS-CoV-2 infection ACE2-expressing organs may become direct targets, leading to severe pathological manifestations, and subsequent multiple organ failures, the exact mechanism and the potential interactions through which ACE2 acts in these organs is still heavily debated. Further scientific efforts, also considering a personalized approach aimed to consider specific patient differences in the mutual interactions ACE2-SARS-CoV-2 and the long-term health effects associated with COVID-19 are currently mandatory.

Keywords: SARS-CoV-2, COVID-19, ACE2, ACE2 receptor, body localization

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INTRODUCTION

SARS-CoV-2 Clinical Characteristics

Since its discovery in December 2019 the coronavirus disease (COVID-19), caused by the transmission of a novel coronavirus known as SARS-CoV-2 induced pneumonia, infected more than 37,800,000 people worldwide and caused more than 1,080,000 deaths until October, 2020. COVID-19 patients mainly displayed pneumonia-associated symptoms, such as fever, shortness of breath, cough, sputum production, and myalgia or fatigue (1, 2). However, despite SARS-CoV-2 infection is manifested as a respiratory tract infection, it may causes symptoms associated to multiple organs, including intestine and stomach (diarrhea, anorexia, nausea, vomiting, and abdominal pain), liver (abnormal enzymes levels), pancreas (pancreatitis), kidney (protein and blood in their urine, abnormal creatinine level), brain (strokes, seizures, confusion, and brain inflammation), heart and blood vessels (elevations of cardiac injury biomarkers, palmus, chest distress, cardiac inflammation and injury, arrhythmias, and blood clots), eyes (conjunctivitis, inflammation of the membrane that lines the front of the eye, and inner eyelid), nose (anosmia), ect (3–11). This multiple organ involvement can lead to a poorer outcome to the viral infection and often result in hospitalization and intensive care unit (ICU) admittance (12–14). Despite the mechanisms for high morbidity and mortality induced by SARS-CoV-2 are currently unknown, based on available literature data in public databases, it is known that the risk of infection and mortality increases with advancing age and also seems to show a sexual dimorphism, male elderly

subjects are at higher risk of infection, as well as death (1, 2). In addition, despite COVID-19 is a non-discriminatory disease, involving both healthy individuals and those with comorbidity conditions, it is well-documented that mortality further increases in presence of pre-existent pathologies, such as cardiovascular disease, hypertension, diabetes, obesity, chronic pulmonary disease, and cancer (12–14). Despite, the biological mechanisms behind these observations are still unclear, virus/host cell interaction, immunological differences, and sex-based hormonal differences are likely to be involved.

Interaction Between SARS-CoV-2 and ACE2

Mechanisms implicated in SARS-CoV-2/host cell interaction are of key importance for cell infection and replication that in turn lead to disease and related damage. In this context, the angiotensin converting enzyme 2 (ACE2), an enzyme important in the renin-angiotensin-aldosterone system (RAAS), scarcely present in the circulation, but widely expressed in organs and able to regulate blood pressure and fluid balance, has been seen to play a key role (15, 16). ACE2 operates as an ACE counterpart: it acts as a carboxypeptidase, removing single amino acids, converting Ang II to its metabolite angiotensin-(1–7) (Ang1–7), balancing the effects of Ang II. ACE2 is found to the apical surface of epithelial cells, differently from ACE, which is located between the apical and basolateral membranes in polarized cells. ACE2 plays its pivotal role in regulating blood pressure and consequently hypertension. This activity is mediated by the ACE2/Ang-(1–7)/Mas receptor axis, through which the regulation of angiotensin and Ang-(1–7) and nitric oxide (NO) availability control blood pressure alterations, which cause damages to vascular tissue as atherosclerosis, hypertrophy and more in general, endothelial alterations (17). Operatively, there are two forms of ACE2: (1) the full-length ACE2, that presents a structural transmembrane domain able to anchor its extracellular domain to the plasma membrane, and (2) the soluble form of ACE2, that lacks the membrane anchor and circulates in small amounts in the blood (15, 16). SARS-CoV-2 enters cell by the binding of spike (S) viral protein, an amino acid long protein that belongs to the viral envelope and leans outwards with a "corona" like form, to the ACE2 receptor (16, 18). The initial step of viral entry is represented by the binding of the N-terminal domain of the viral protein unit S1 to a pocket of the ACE2 receptor. After this, the receptor transmembrane protease serine 2 (TMPRSS2), a member of the Hepsin/TMPRSS subfamily that is stechiometrically contiguous to ACE2 receptor, induces the cleavage of the protein between the S1 and S2 units, with the help of Furin which facilitates the entry of the virus into the cell after binding (19, 20). Furin [also termed paired basic amino acid cleaving enzyme (PACE)], a member of the subtilisin-like proprotein convertase family that processes protein of the secretory pathway, is expressed in multiple organs, such as in lungs, liver, and small intestines. Following the binding of the S glycoprotein to ACE2, furin-mediated proteolytic cut of the S protein is necessary for viral entry into the cell (18, 21). Thus, both TMPRSS2 and Furin are crucial for S activation. The key role of these two proteases was also demonstrated by a recent study that showed that multicycle replication of SARS-CoV-2 in Calu-3 human airway cells was strongly suppressed by inhibiting TMPRSS2 and Furin activity (22). However, virtually, other human proteases, e.g., cathepsin L and B, elastase, trypsin and factor X, may be involved in the entry of SARS-CoV-2 into the human cell and in the shedding of ACE2. A critical cell membrane protease involved in the endogenous shedding of ACE2 from membranes is the disintegrin metalloproteinase 17 (ADAM17), also known as tumor necrosis factor-α converting enzyme (TACE) (23). While TMPRSS2 cleaves both ACE2 and the S protein of SARS-CoV-2, leading to membrane fusion and cellular uptake of the virus, ADAM17 acts directly and solely on ACE2 and leads to ACE2 shedding into the extracellular cellular space. Thus, ADAM17 and TMPRSS2 may have opposite effects on ACE2 shedding. Evidences have shown that the expression of TMPRSS2 inhibits ADAM17-shedding of ACE2 (24). However, it is unclear how TMPRSS2 transcends ADAM17 to cleave ACE2 during SARS-CoV-2 infection.

Despite numerous information has been obtained up to now, the exact role of ACE2 in SARS-CoV-2 cellular infection and of proteases that process SARS-CoV-2 S protein is not yet defined. Certainly, genetics and demographic characteristics, lifestyle, comorbidities, and medication usage may have an impact on ACE2 expression and activity in SARS-CoV-2 cellular infection.

Risk Factors for COVID-19 Severity and ACE2 Expression

ACE2 is regulated by a gene which maps on the X chromosome (Xp22.2), thus suggesting that some differences may exist in the expression of ACE2 between men and women (25). In women to prevent the redundant expression of the products of the genes present in double copy on the X chromosomes, a physiological random inactivation occurs in one of the two chromosomes (25). The remained chromosomal portions that escape to the inactivation and the genes present in these areas (∼15%) can be over-expressed in women (25). ACE2 is encoded precisely in these regions of the X chromosome which escape the inactivation of one of the two X chromosomes, supporting the hypothesis of a greater ACE2 expression in women (25). There is evidence that ACE2 tissue levels are also regulated by estrogens that can increase the presence of ACE2 receptor (26). Thus, if, as reported by several commentary in literature, the presence of ACE2 throughout the body could make tissues more vulnerable to SARS-Cov-2 infection women should be more predisposed to the virus than men (26). On the contrary, epidemiological data of the World Health Organization (WHO) highlighted gender-based clinical differences in SARS-CoV-2, with a higher mortality rates in male patients, in particular elderly patients (27). Even this latest information appears to be in contrast with the hypothesis that ACE2 throughout the body could make tissues more vulnerable to SARS-Cov-2 infection. In fact, it was demonstrated that ACE2 level decrease with age and seem to be higher in young people that commonly develop a less severe COVID-19 form (26). It is important to underline that also the opposed hypothesis, that a mild/moderate

ACE2 deficiency may protect from SARS-CoV-2 invasion, seems improbable considering the high affinity of the virus for ACE2 receptor. In addition, this latter hypothesis is also unlikely because different degree of ACE2 deficiency are related with specific diseases, i.e., diabetes, obesity and cardiovascular disease, that characterize individuals more prone to be infected and to have severe complications related to SARS-CoV-2. These inconsistencies highlight that other factors, such as for example organ-specific ACE2 distribution and expression levels and potential co-expression and interaction with specific proteases, may contribute to the severity of SARS-CoV-2.

Although it is demonstrated that lungs inflammation is one of the main symptom during SARS-CoV-2 infection, the lungs, among all organs, present a moderate expression of ACE2 and, as reported above, SARS-CoV-2 may affect other organs, organs that have a high to moderate expression of ACE2. In this context a detailed map of the physiological organ-specific distribution, expression, and activities of ACE2, also considering organ-specific gender biases and organs often poorly considered (specific brain regions, oral cavity, thyroid, pancreas, duodenum, colon, rectum, gallbladder, male -testis and seminal vesicle- and female tissues -ovary, oocyte, uterus, vagina-, skin, and others), and a complete overview on the potential link between these organs and SARS-CoV-2 may contribute to understand the potential infection routes as well as the clinical symptoms and mechanisms of the virus susceptibility.

ACE2 IN HUMAN PHYSIOLOGY: BODY LOCALIZATION, EXPRESSION, FUNCTION AND ACTIVITIES

About 20 years ago, the first paper reported the mapping of ACE2 in 72 tissues (28). Over the years, it has become more and more clear that ACE2 localization can be quite tricky (28). Starting from the localization in the renal and cardiovascular tissues, over time it has become evident that ACE2 is also present in tissues and organs where initially no trace of it was detected (**Figure 1**), as in the gastrointestinal tract, up to recent studies that report slight positivity even in locations so far considered ACE2 free, such as in circulating leukocytes (29–31).

There is no question that the ACE2 receptor is also expressed at the level of epithelia of the respiratory system (tracheal and bronchial epithelial cells, alveolar epithelial cells, type 2 pneumocytes), cardiovascular system (endothelium of coronary arteries, myocites, epicardial adipocites, vascular endothelial, and smooth cells), gastrointestinal tract (esophagus keratinocytes, gastrointestinal epithelial cells, intestinal epithelial cells, duodenum, small intestine, rectum), urogenital system (kidney proximal tubules, bladder urothelial cells, luminal surface of tubular epithelial cells, testis, seminal vesicle), as well as in the liver and gallbladder and in the nervous system. (25, 28, 32) However, it is important to underline that, while mRNA seems to be expressed homogeneously in all tissues, the same is not always certainly for protein expression (**Figure 1**) (28).

Many studies over the years have focused on the role of ACE2 in the cardiovascular system, both for the functions of the renin–angiotensin system (RAS) system and for the study of new therapeutic targets in cardiac pathologies (15, 16). ACE2 is recognized as a protector of vascular tissues, balancing angiotensin II effects, protecting endothelia, and promoting mechanisms of regeneration (15). Intuitively, ACE2 impairments leads to severe cardiac dysfunction, with increased atherosclerosis, and endothelial damage. ACE2 is studied also in hypertension models, as genetic variation affects systolic function in men and ventricular mass in women (33). Not by chance, increased levels of ACE2, both at the gene level and protein expression, but also in circulating soluble forms, are detected after myocardial injury, suggesting a potential role as cardiac biomarker (15). Cardiac alterations result to be usually correlated with thyroid dysfunction, particularly to hyperthyroidism (34). Thyroid hormones also seem to act on ACE2 expression both influencing the receptor gene expression and conditioning the release of ACE from lung endothelium (35). ACE2 has been investigated also as cancer marker, as it has been observed an increase of ACE2 expression in thyroid cancer with an increase of ACE2/ACE *ratio* proportional to the differentiation grade of the cancer (36). The activity of ACE2 in cardiovascular system is strictly related to those in brain, as ACE2 is expressed in the neuronal area deputy to cardiovascular control, so that is result to be less expressed in case of cardiac injury, while an over-expression in brain leads to a protective action, *via* reduction of pro-inflammatory cytokines and augmentation of NO activity (37). Many animal models have been used for the study of ACE2 role in the brain. Data highlighted the antihypertensive and sympatholytic action of ACE2 in the hypothalamus *via* reduction in Ang II and increase in Ang-(1–7) levels, and a positive effect of ACE2 in the neuronal recovery from stroke (38). ACE2 is also involved in mechanism of memory, *via* regulation of brain-derived neurotrophic factor expression, and the production of reactive oxygen species, in stress response, regulating corticotropin releasing hormone at hypothalamus level, and in neurogenesis related to serotonin level, secondary to the availability of its precursor tryptophan (39, 40). The fil rouge between tryptophan synthesis and ACE2 crosses the activity in many systems and binds their functionality. In fact, ACE2, involved in the RAS mediated homeostasis, plays at intestinal level regulating the microbiome, acting on amino acid uptakes, and expression of antimicrobial peptides (41). ACE2 acts as amino acid transporter, binding B0AT1, a neutral amino acid transporter, in the small intestine, and in animal model of ACE2 deficiency a reduction of tryptophan levels in the blood has been demonstrated (42). This reduction is reflected in the intestine with greater inflammation at the level of the colon, endothelium alteration and reduced ability to damage response, involving also mammalian target of rapamycin (mTOR) pathway, a member of the phosphatidylinositol 3-kinase-related kinase family of protein kinases (43). During the years attention was addressed also to the intestine, since it is known to express the highest level of ACE2 (28). In addition to ACE2 localization in the intestine, ACE2 was found in smooth muscle cells and endothelium of vessels from the stomach, and colon, smooth muscle cells of the muscularis mucosae, and the muscularis propria (28). ACE2 was also copiously present in the enterocytes of all parts of the small

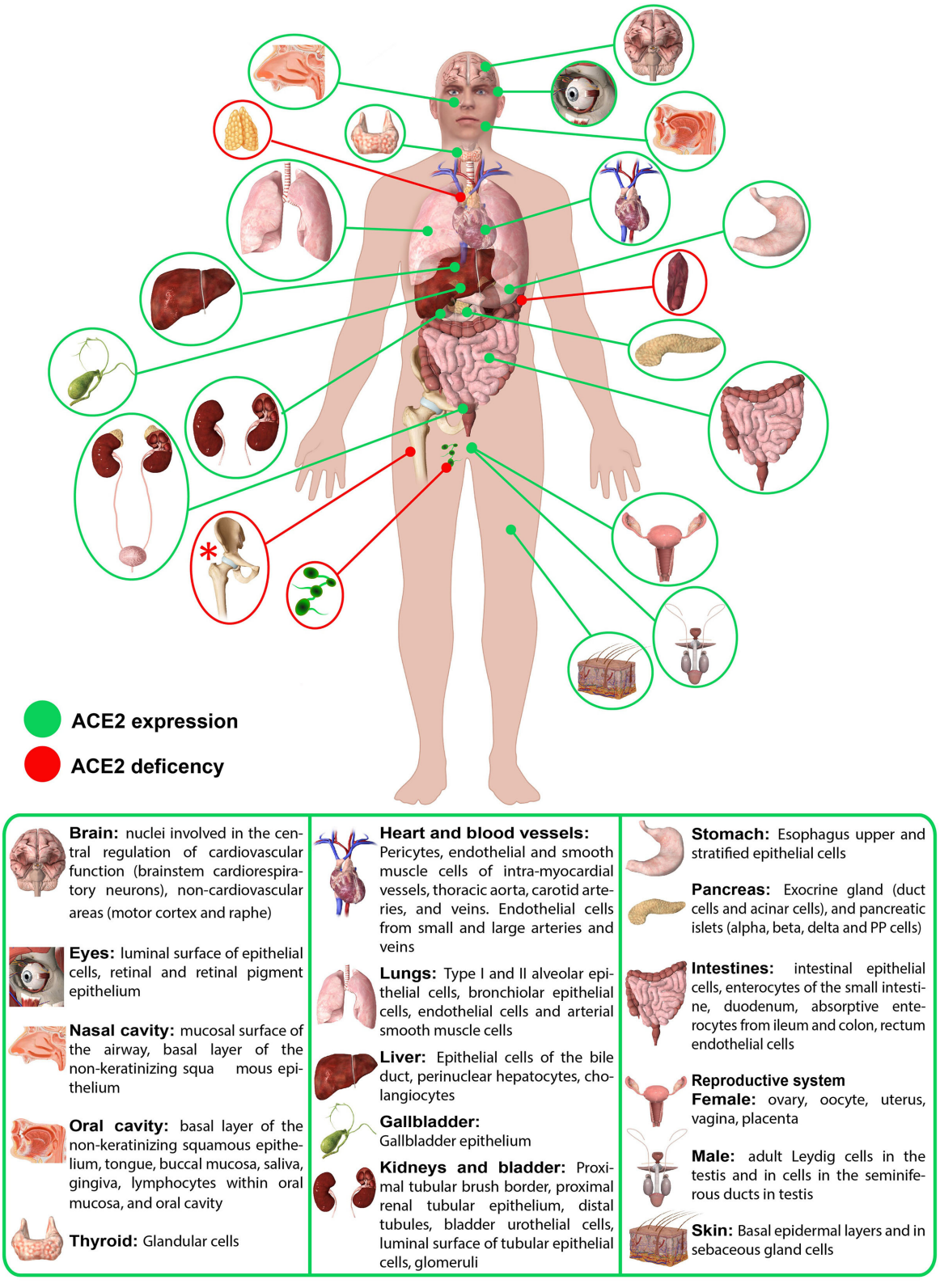

FIGURE 1 | Schematic representation of ACE2 expression in human organs. ACE2 mRNA is present in all organs (28). ACE2 protein expression is present in heart, kidney, testis, lung (type I and type II alveolar epithelial cells), nasal, and oral mucosa and nasopharynx (basal layer of the non-keratinizing squamous epithelium),

(Continued)

FIGURE 1 | smooth muscle cells and endothelium of vessels from stomach, small intestine and colon, in smooth muscle cells of the muscularis mucosae and the muscularis propria, in enterocytes of all parts of the small intestine including the duodenum, jejunum, and ileum (but colon), skin (basal cell layer of the epidermis extending to the basal cell layer of hair follicles smooth muscle cells surrounding the sebaceous glands, cells of the eccrine glands), endothelial, and smooth muscle cell of the brain (28). Red asterisk (*): ACE2 deficiency only hypothesized.

intestine including the duodenum, jejunum, and ileum, but not in enterocytes of the colon (28). In addition to the gastrointestinal tract, ACE2 has been found in kidney and in pancreas, with dislocation similar to those of ACE2 that is in kidney apical surface area of the proximal tubules and pancreatic acini and islets (28). As for pancreas, the presence of ACE2 influences islets status *via* regulation of blood pressure and NO release, as well as acting on tissue fibrosis (44). The role of ACE2 has been widely investigated also for the onset of diabetes, as ACE2 deficiency has been associated with impairment of first-phase insulin secretion and of glucose tolerance (45–47). The alteration of RAS system and specifically of ACE2 activity induces an alteration of pancreatic islets, due to unbalance NO production, which in turn influences blood flow, also secondary to glucose availability (45–47). The wide expression of ACE2 in kidney is not so surprisingly, considering the pivotal role of RAS system in this organ, in which it regulates the electrolytic equilibrium *via* reabsorption of sodium and water into the blood, while causing excretion of potassium (48). ACE2 acts balancing the RAS activity, regulating renal homeostasis and it is postulated that its activity is more related to a local control than a systemic regulation of blood pressure (48). Effects of reduced ACE2 are described as promoting proteinuria, in particular albuminuria, glomerular disease and are related to diabetic nephropathy, with lower ACE2 expression at tubular level (48). Despite the role of ACE2 in hepatic glucose metabolism is not completely investigated, the alteration of the ACE2 pathway is, also in this localization, related to the development of impairment of metabolic activity, and in particular of insulin resistance (48). ACE2 in liver has been found expressed in endothelial cells, bile duct cells, and perinuclear hepatocytes and it was mostly elevated in hepatic fibrogenic resistance (28). Notably, insulin resistance correlates with endothelium-dependent and insulin-mediated vasodilatation (46, 49). In addition, a recent RNA-seq data in the human protein atlas database have shown the highest expression of ACE2 in liver cholangiocytes, followed by hepatocytes (50)

ACE2 expression seems to be correlated to the sensory organs. However, the real expression of ACE2 at ocular level, instead, seems to be still object of debate. Although it is the least widely expressed among the RAS system components, ACE2 is detectable in the aqueous humor (51, 52). Some papers declare a not significant mRNA presence and immunoreactivity of ACE2 in human conjunctiva (53), while according to others, ACE2 gene expression is detectable both in human conjunctiva and primary pterygium tissues, even if in a reduced cohort of patient (54). ACE2 is expressed at the oral level in particular at the oral tongue than at the buccal and gingival and could be found in epithelial cells, T cells (<0.5%), B cells (<0.5%), and fibroblast (<0.5%) (31). In addition, in the oral and nasal mucosa and in the nasopharynx, ACE2 expression was found in the basal layer of the non-keratinizing squamous epithelium (55). Human ACE2 was detected in ciliated airway epithelial cells of human airway tissues derived from nasal regions (55). Concerning ACE2 presence at the ear level no data were present on human. However, a recent online study found high expression of ACE2 in the cat ear tip (56). Another sensory organ where ACE2 was also found is at the skin level (57). The activity of RAS system in controlling cell proliferation and differentiation, also in case of tissues injury in the mechanism of self-renewed of damaged cells and neo-angiogenesis, is reflected also in the skin, where the epidermal stem cells express the different players of this system, including ACE2 (57). Immunohistochemical evaluation of ACE2 presence in healthy and oncologic patients showed ACE2 in basal cell layer of normal epidermis and sebaceous glands and a reduction of ACE2 reactivity in patients affected by pre-malignant lesions (actinic keratosis) and non- melanoma malignant skin cancers (basal cell carcinoma and squamous cell carcinoma), suggesting an involvement in the pathogenesis of the disease (58).

Considering the role of Angiotensin II in the menstrual cycle, the presence of ACE2 in the female reproductive systems appear quite intuitive. In fact, AngII acts on follicular, ovulatory and luteinic phases, influencing follicle development, oocytes maturation, and corpus luteum progression, balancing the levels of steroid hormones (59). In addition, it promotes spiral artery vasoconstriction and endometrium regeneration at the uterus level. Angiotensin II has been identified also as a player in endometrium fibrosis and endometrial metastases (59). Not surprisingly, during pregnancy, ACE2/AngII/Ang 1–7 axis is involved in maintenance of blood pressure and alterations of this pathway correlate with disorders like pre-eclampsia and eclampsia, while reduction of ACE2 expression negatively influences gestation and fetus birth (60). In parallel, ACE2 expression has been detected also in testis, particularly in spermatogonium and Leydig and Sertoli cells, with possible correlation with spermatogenesis and maintenance of functional and structural integrity of the apparatus (61).

Finally, despite the presence of ACE2 in numerous organs, tissues and cells have not been completely clarified and in many of them not yet investigated, ACE2 seems to be absent in the spleen, thymus, lymph nodes, bone marrow, and in several cells of the immune system (15, 62). However, it is important to underlined that numerous studies on ACE2 expression in bone marrow are currently in progress since all the players of RAS system are present in the bone marrow, acting on cell lineages proliferation and also in hematopoietic restoration after myelosuppression, and ACE2 seems to have in particular a role in CD34+ proliferation (63).

In this moment, with the ongoing COVID-19 pandemic, this rapid overview related to the distribution, expression and

activities of the ACE2 in human body could help and improve our understanding on potential infection routes of SARS-CoV-2 through the body. Thus, in the next section we discuss how the presence, distribution and abundance of ACE2 in specific target organs may be related to the COVID-19 clinical symptoms and manifestations.

SARS-COV-2 CLINICAL IMPLICATION AND POTENTIAL MUTUAL INTERACTIONS WITH ACE2

Nasal Cavity

On October 5, 2020 searching on PubMed "COVID-19 OR COVID-2019 OR severe acute respiratory syndrome coronavirus 2 OR severe acute respiratory syndrome coronavirus 2 OR 2019-nCoV OR SARS-CoV-2 OR 2019nCoV OR (Wuhan AND coronavirus) AND (Nose OR Nasal Cavity)" we found 388 papers. Most of the studies were guidelines on how to perform nasal and oropharyngeal swab procedure for the screening of COVID-19 infection. The other studies detected, analyzed and discussed the different nasal manifestations in COVID-19 patients (64). Orhinolaryngological symptoms resulted common manifestations of COVID-19, particularly in mild or moderate form of the disease (65, 66). The nasal cavity and turbinates have important physiological functions in filtering, warming, and humidifying inhaled air and these functions are critical during SARS-CoV-2 infection since the nasal cavity is the principal gateway for virus entrance. In fact, epithelial cells in this region are considered suitable clinical sample for early virus detection. Increasing number of reports on SARS-CoV-2 positive patients described olfactory dysfunction, such as loss of smell, cacosmia, phantosmia nasal obstruction or rhinorrhea, and nasal congestion (1, 67–73)[1]. Some data also reported SARS-CoV-2 positive patients with isolated anosmia, without any other symptoms, suggesting these patients as a potential source of rapid virus spread (68, 69, 74). Anosmia in SARS-CoV-2 positive patients can be present as primary symptom or as an early symptom, with different percentage among the examined studies, percentages that can range from 6 to ∼80% (1, 70–73, 75). Lechien et al. demonstrates that about 87% patients with an anosmia duration ≤12 days were also PCR SARS-CoV-2 positive (73). Additionally, Kaye et al. analyzing a court of 237 COVID-19 patients showed that 73% of patients reported anosmia and 26.6% reported loss of sense of smell as initial symptom (70). Patients below 40 years, particularly female, seem to be the more prone to develop SARS-CoV-2 form with only hyposmia/anosmia manifestations (1, 69–72). However, an Asian study reported a lower percentage of patients with olfactory dysfunctions in comparison to European patients (73). This aspect may be probably due to the diverse ACE2 polymorphisms and expression level between Asian and European individuals (74). The loss of smell in SARS-CoV-2 patients may be caused by different factors, such as localized olfactory cleft oedema, architectural deformity of the olfactory neuroepithelium, or direct neuro-invasion of the olfactory nerve pathways. As above described, it is important to underline that gene expression databases highlighted a moderately/high expression of ACE2 in human olfactory mucosa (76). However, to date, whether ACE2 expression in the olfactory epithelium is neuronal or non-neuronal or if it occurs in both cell types it is not completely clear (77, 78). SARS-CoV-2 brain infection could be facilitated by the neuronal expression of the host receptors through absorption in ciliated dendrites/soma and consequent axonal anterograde transport along the olfactory nerve (79, 80). Concerning the non-neuronal expression of ACE2, it could be due to the nasal cavity olfactory epithelium that would work as virus reservoir (79, 80). Several RNaseq transcriptome reports conducted in human and murine olfactory epithelium suggested a non-neuronal expression of ACE2 as well as of TMPRSS2 (79–82), but further studies are mandatory to confirm these finding. It was also shown that nasal brushing epithelial cells, nasal turbinate epithelial cells, and nasal airway epithelial cells contained ACE2-expressed and TMPRSS2-expressed cell clusters (82).

Oral Cavity

On October 5, 2020 searching on PubMed "COVID-19 OR COVID-2019 OR severe acute respiratory syndrome coronavirus 2 OR severe acute respiratory syndrome coronavirus 2 OR 2019-nCoV OR SARS-CoV-2 OR 2019nCoV OR (Wuhan AND coronavirus) AND oral cavity" we found 218 papers. Several studies evaluated the presence of SARS-CoV-2 in saliva through entry into the oral cavity with several potential pathways, *via* a direct infection of oral mucosa lining cells, *via* droplets from the respiratory tract, from the blood circulation by gingival crevicular fluid, or *via* extracellular vesicles released from infected cells and tissues (83). To et al. confirmed that SARS-CoV-2 can be detected by PCR in about 92% of saliva samples, indicating the saliva as a potential source of SARS-CoV-2 spreading (84). The presence of SARS-CoV-2 in patients' saliva also suggested the likelihood of salivary gland infection. Chen et al. collecting saliva directly from salivary gland, found SARS-CoV-2 nucleic acid, hypothesizing that salivary glands were SARS-CoV-2 infected (85). This hypothesis is further reinforced by the fact that the ACE2 epithelial cells of the salivary glands have been shown to be an early target for the SARS-CoV-2 (86). In addition, high levels of mRNA and protein levels of the cellular protease Furin as well as of TMPRSS2 have also been found in the salivary glands (86). Thus, the possible role and function of salivary gland cells in the initial SARS-CoV-2 entry and progress must be further considered and validated as well as their potential function as virus reservoir, able to establish a persistent infection which could last also for months (87). Furthermore, it should be underlined that the saliva samples not only contain saliva secreted from the salivary glands but also the secretions from the nasopharynx and from the lung *via* the action of cilia lining the airway. Thus, more studies are needed to delineate the real sources and functions of SARS-CoV-2 in saliva.

Another point related to the oral cavity is represented by the fact that numerous studies reported an acute loss of taste (hypogeusia/ageusia) as a frequent symptom of SARS-CoV-2 infection, particularly common among females and younger individuals (∼20–39 years) (1, 84, 85). A recent case series presented several cases of SARS-Cov-2 infection where the

[1]https://news.joins.com/article/23738003?cloc=joongang-mhomegroup6 (accessed July 15, 2020)

loss of taste was also associated with oral lesions (88). These lesions presented two distinct patterns, one resembling aphthous-like ulcers in young patients with mild cases of COVID-19 and another with more widespread patterns resembling Herpes Simplex Virus 1 necrotic ulcers in the more severe and immunosuppressed older individuals (89). Whether these lesions were due directly by SARS-CoV-2 or were an associated manifestation resulting from the severe compromised state of the patient remains to be determined. However, what is known is that taste disorders are linked to an extensive variety of viral infections (90). Upper respiratory tract infection can lead to acute onset ageusia because of viral damage to the olfactory epithelium (90). Furthermore, as previously reported for the nasal cavity, viruses can also use the olfactory nerve as a route into the central nervous system (CNS). Thus, ageusia may be a secondary result of olfactory dysfunction. However, it is important to underline that ACE2 is not only extensively express in the salivary glands and in oral tissues and its expression was higher in tongue than buccal or gingival tissues (https://gtexportal.org). Furthermore, ACE2 positive cells were enriched in epithelial cells, thus damage of mucosal epithelial cells of the oral cavity may explain ageusia, oral mucosal ulcerations, and necrosis detected in SARS-CoV-2 patients (31, 91). In addition, it was reported that the ACE2 within oral mucosa is also expressed in lymphocytes, and comparable results were also reported for other organs of the digestive system (31). However, since the ACE2-positive lymphocytes is quite few whether this aspect could indicate that SARS-CoV-2 attacks the lymphocytes and leads to the severe disease in patients' needs further studies (31). More in generally SARS-CoV-2-mediated gustatory disturbances has not yet been definitively identified.

Eyes

On October 5, 2020 searching on PubMed "COVID-19 OR COVID-2019 OR severe acute respiratory syndrome coronavirus 2 OR severe acute respiratory syndrome coronavirus 2 OR 2019-nCoV OR SARS-CoV-2 OR 2019nCoV OR (Wuhan AND coronavirus) AND (eyes OR ocular manifestations)" we found 820 papers. Most of the studies were official recommendations of ophthalmological societies for precaution and prevention of SARS-CoV-2 infection or studies on the impact of COVID-19 outbreak on eye care. Currently, the presence and prevalence of ocular manifestations in SARS-CoV-2 infection, consistent with conjunctivitis and including conjunctival hyperemia, chemosis, epiphora, or increased secretions, are still controversial (92–95). Despite it was reported that only a small percentage (from about 1 to 6%) of SARS-CoV-2 positive patients developed signs of conjunctivitis, other studies showed that up to 31% of SARS-CoV-2 hospitalized patients presented conjunctivitis (96–99). Wu et al. showed that about 31.6% of COVID-19 patients had ocular abnormalities, and ocular symptoms were more frequent in severe cases of COVID-19 patients (97). In fact, about 50% of COVID-19 patients with ocular abnormalities were classified as critical, 16.7% were classified as severe, and 33.3% were classified as moderate severity (97). In addition to conjunctivitis other ocular abnormalities directly correlated with the COVID-19 severity seem to be alterations in retina and in its vasculature (100). A recent study evaluating the retina of patients with COVID-19, within 30 days from the onset of systemic symptoms, found an enlargement of retinal arteries and veins in more severe cases and showed an inverse correlation with time to symptoms onset (100). In this context, Casagrande et al. demonstrate the existence of SARS-CoV-2 nucleic acid in the human retina COVID-19 patients (101). Additional studies also highlighted the presence of SARS-CoV-2 RNA in tear film and/or conjunctival swabs of COVID-19 patients with conjunctivitis but not in patients without ocular symptoms (98, 102–105). Differently, Xie et al. demonstrated that the SARS-CoV-2 RNA was present also in the normal ocular surface of COVID-19 patients without conjunctivitis (106). Despite this point is still debated, it is critical to underline that ocular surfaces have a great tropism for respiratory viruses and also for coronavirus (107, 108). Whether specifically SARS-CoV-2 may infect retina and conjunctival cells in human remains unclear. Based on the current literature, several reports hypothesized that the exposure of the ocular surface to SARS-CoV-2 could lead to infection probably due to the drainage of virus particles *via* the nasolacrimal duct, specifically through the lacrimal canaliculi that drain tears from the eye surface into the nasal cavity, into the respiratory tract (109, 110). In this context it is important to emphasized that others reports also considered the presence of ACE2 and TMPRSS2 on the cornea and conjunctiva as a possible virus route (56, 111, 112). The presence of the ACE2 and TMPRSS2 on the corneal cells may allow the virus to cross the ocular surface, and then spread from the eye to other parts of the body through the blood stream and/or through the nervous system (ophthalmic branch of trigeminal nerve) (112, 113). However, to date, there are no clear evidences that SARS-CoV-2 virus, in humans, can enter inside the eye or spread to the brain through corneal nerves (114).

Lungs

On October 5, 2020 searching on PubMed "COVID-19 OR COVID-2019 OR severe acute respiratory syndrome coronavirus 2 OR severe acute respiratory syndrome coronavirus 2 OR 2019-nCoV OR SARS-CoV-2 OR 2019nCoV OR (Wuhan AND coronavirus) AND lungs" we found 4,138 papers. While SARS-CoV-2 was detected in many organ systems, the lungs seems to be the main organs affected by the virus (96, 115). In fact, it is known that the upper respiratory tract and lungs serve as predominant site of virus entry and replication and that SARS-CoV-2 patients showed the symptoms of pneumonia and alveolar damage (116). The most common and severe complication in patients with SARS-CoV-2 patients is acute hypoxemic respiratory failure or acute respiratory distress syndrome that lead to oxygen and ventilation therapies (1, 117–135). Some of these critically ill patients also required intubation and invasive ventilation. Lungs radiological images and computed tomography (CT) scans of SARS-CoV-2 positive patients provided numerous information about the severity of the infection and showed abnormal results in about 86% of patients (1, 117–135). The most common patterns of radiological images and CT scans were ground-glass opacities, consolidation, centrilobular nodules, architectural distortion, bronchial wall thickening, vascular enlargement,

traction bronchiectasis, reticulation, crazy paving pattern, intrathoracic lymph node enlargement, and subpleural bands, that cause pulmonary discomfort and require rapid diagnosis and treatment (1, 117–135). In addition, autoptic results revealed that in about 48% of cases the predominant histopathological finding were capillary congestion, microthrombi as well as moderate intra-alveolar fibrin exudation resultant in exudative disseminate alveolar damage and superimposed bronchopneumonia (135). A more widespread histological pattern of alveolar damage with greater fibrotic evolution in the lungs was observed in patients who died after a long period of mechanical ventilation (136). In few cases, an intra-alveolar deposition of neutrophilic granulocytes, probably due to superimposed bacterial infection, was also detected (137). Since the distribution of ACE2 in different organs seems to be notably linked to the clinical symptoms of SARS-CoV-2 infection and since the acute respiratory distress syndrome is a potential deadly complication of SARS-CoV-2, research studying lung complications of ACE2 down-regulation are of key significance in this context. Several studies on lung injury highlighted that ACE2 receptors down-regulation lead to critical inflammatory lesions in the respiratory tract (alveolar wall thickening, edema, infiltrates of inflammatory cells, bleeding) which seem to be carried out by angiotensin II (135, 138–142). A key point to remark is that the wide surface of alveolar epithelial cells might explain the vulnerability of lungs to the virus invasion. As previously explained ACE2 are principally expressed in type II pneumocytes, little cylindrical cells that correspond to the 5% of all pneumocytes (1). These type of pneumocytes exert immunoregulatory functions and are of key importance for alveolar surfactant production and they also function as stem cells, progenitors of type I pneumocytes, that represent the 95% of all pneumocytes and that are responsible of gas exchanges (142). Thus, the damage of type II pneumocytes owing to the binding of SARS-CoV-2 to ACE2 receptors is critical for several factors, i.e., for the local unopposed ACE→ Angiotensin II→ AT1 receptor axis over-activity, for the reduced production of alveolar surfactant by injured type II pneumocytes that lead to reduced lung elasticity and, finally, for the reduced repair of type I pneumocytes that bring to impaired gas exchanges and fibrosis (143). While ACE2 is expressed in the bronchial epithelium and in type 2 pneumocytes, TMPRSS2 results strongly expressed in the cytoplasm of bronchioles and alveolar epithelial cells (144). Since ACE2 was found to exist on alveolar epithelial cells at approximately similar level as in the whole lung, Sato et al. found that the expression level of TMPRSS2 was considerably different between the peripheral and central parts of the lung (145). Thus, since that peripheral parts of the lung strongly express TMPRSS2, along with ACE2, the SARS-CoV-2 may be considered to damage the peripheral area at the beginning of infection. These data explain why chest CT revealed consolidation and ground glass opacities in the bilateral peripheral lobes in COVID-19 cases (146). However, these factors would not even prevent the simultaneous role of other mechanisms including an altered immune response to initial viral invasion, or a genetic susceptibility to hyper-inflammation and thrombosis (8, 147). In SARS-CoV-2 pneumonia, thrombosis may play a direct, and critical role in gas exchange abnormalities and in multisystem organ dysfunction. Unfortunately, to date, as for all the other organs affected by SARS-CoV-2, the lungs impairment during this new infection remain to be further clarified.

Heart and Blood Vessels

On October 5, 2020 searching on PubMed "COVID-19 OR COVID-2019 OR severe acute respiratory syndrome coronavirus 2 OR severe acute respiratory syndrome coronavirus 2 OR 2019-nCoV OR SARS-CoV-2 OR 2019nCoV OR (Wuhan AND coronavirus) AND (cardiovascular system OR heart OR blood vessels)" we found 3,170 papers. In most of these reports cardiovascular complications emerged among the most significant manifestations in SARS-CoV-2 infection (148–155). Different cardiovascular complications, such as myocarditis, acute coronary syndrome, decompensated heart failure, pulmonary embolism, cardiogenic shock, and infection of a heart transplant recipient, accompanied by altered levels of creatine kinase isoenzyme-MB, myohemoglobin, cardiac troponin I, and N-terminal pro-brain natriuretic peptide were currently reported (1, 149–155). In addition, a high prevalence of pre-existing cardiovascular morbidities, including hypertension, and coronary artery diseases, has been detected among patients with severe SARS-CoV-2 (1, 149, 156). In COVID-19 patients, the highest mortality rates were also observed in case of pre-existing cardiovascular disease and elevated cardiac troponin levels (137, 157). Furthermore, patients with higher troponin levels had also increased markers of inflammation, including C-reactive protein, interleukin (IL)-6, ferritin, lactate dehydrogenase (LDH), high neutrophil count, and high amino-terminal pro-B–type natriuretic peptide (158). Despite it was initially hypothesized that COVID-19 patients with pre-existing cardiovascular morbidities and treated with ACE inhibitors (ACEi) or angiotensin receptor blockers (ARBs) (155, 159, 160). could be at increased risk for severe SARS-CoV-2 infection, a recent retrospective study on COVID-19 patients with hypertension showed that ACEI/ARB therapy attenuated the inflammatory response (161). In addition, a study on SARS-CoV-2 patients with hypertension showed no difference in the percentage of patients treated with ACEi/ARBs between those with severe and non-severe infection and between survivors and non-survivors (162). However, understanding the positive or negative effect of ACEi/ARB in COVID-19 appears to be complex, and this could also be due to the clinical stage of the virus (viral contamination phase vs. tissue inflammation phase). Several clinical trials on this question are forthcoming (NCT04329195, NCT04331574, NCT04351581, NCT04353596). To date, the mechanisms by which SARS-CoV-2 leads to cardiac manifestations is currently unclear. These mechanisms would involve several factors such as a direct viral damage and an immune-mediated damage by inflammatory cytokines (i.e., a systemic cardiotoxic cytokine-storm), and cytotoxic immune cell response. As reported in the previous section, the cardiac tissue presents a high ACE2 expression level (163). Specifically, it was shown that cardiomyocytes from the heart contain about 6% ACE2-expressed cells and 0.8% TMPRSS2-expressed cells, and the cardiovascular progenitor cells contain 12.5%

ACE2-expressed cells and 0.4% TMPRSS2-expressed cells, thus SARS-CoV-2 could directly infect the myocardial tissue (82). In addition, Furin can also be considered a critical molecule that makes SARS-CoV-2 cause adverse cardiovascular events through the ACE2 receptor. This speculation is supported by the occurrence of high level of Furin in the peripheral blood of COVID-19 patients (164). Additionally, PCR analyses also identified SARS-CoV-2 in the cardiac tissue of ∼35% of infected patients, further supporting that a direct viral damage can occur (165). Kuba et al. by using a mouse model showed that SARS-CoV pulmonary infection leads to an ACE2-dependent myocardial infection (138). This infection can lead to a localized inflammatory response with resulting myocarditis that bring to acute cardiac injury and the prospective for arrythmias or heart failure (166). Autoptic data on SARS-CoV-2 positive patients showed the existence of mononuclear inflammatory myocardial infiltrate, thus supporting this hypothesis also for this new coronavirus (3). Numerous studies also reported immunological derangements in SARS-CoV-2 positive patients (167, 168). This altered immunologic status has been related with an increased risk of cardiovascular disease and could be also an indirect mechanism of immunological dysfunction that lead to cardiac sequelae (166–168). In addition, numerous SARS-CoV-2 positive patients showed respiratory distress that lead to hypoxemia that could cause cardiac injury secondary to an oxygen mismatch (166–168). Other systemic consequences of cardiac injury in SARS-CoV-2 patients could also be related to sepsis and disseminated intravascular coagulation (DIC) that vary from minimal change to interstitial inflammatory infiltration and myocyte necrosis vasculature microthrombosis and vascular inflammation (166–168). However, to date whether SARS-CoV-2 infection impair the heart remains to be further demonstrated.

Kidney and Bladder

On October 6, 2020 searching on PubMed "COVID-19 OR COVID-2019 OR severe acute respiratory syndrome coronavirus 2 OR severe acute respiratory syndrome coronavirus 2 OR 2019-nCoV OR SARS-CoV-2 OR 2019nCoV OR (Wuhan AND coronavirus) AND (kidney OR urinary system)" we found 1,031 papers. The kidney is one of the major organs which play a key role in the filters which excrete toxins, waste products, and extra water from our body. Despite most of the work were focused on kidney transplantation and on the management of dialysis patients during SARS-CoV-2 infection, several studies reported an increased incidence of acute renal injury during the infection (169, 170). The bladder may also be affected and may ultimately lead to multiple-organ failure and death (169, 170). Although initial reports suggested that the burden of acute kidney injury during SARS-CoV-2 infection was moderately low (about 0.5%), recent studies reported an incidence going up to 56.9% (115, 169, 171–174). In critically ill patients, this incidence was remarkably higher, ranging from 61 to 76% (175). A higher incidence of acute renal injury has been reported in USA and UK than in China (96, 150, 174, 176). Several studies also showed that patients with acute renal injury have a higher mortality rate compared to other patients and this is particularly true for those in the ICU (177–179). In a recent study, it was shown a high incidence of renal dysfunction (46%) and acute renal injury (29%) also in hospitalized children with COVID-19 (180). Patients with acute renal injury also showed elevated levels of serum creatinine and blood urea nitrogen associated to higher leukocyte count and lower lymphocyte and platelet counts (169). Prolonged activated partial thromboplastin time and higher D-dimer, both coagulation parameters, were also more common in these patients (169). In addition, a high percentage of SARS-CoV-2 patients with acute renal injury had proteinuria albuminuria and hematuria, along with isolation of viral RNA from urine, all factors that support the potential viral tropism for the kidney (181, 182). This tropism was also confirmed from an autopsy study by Su et al. that demonstrated by electron microscopy SARS-CoV-2 presence in the renal tubular epithelium of seven of 26 SARS-CoV-2 patients (176). This study also showed the presence of a diffuse proximal tubule injury with the loss of brush border, non-isometric vacuolar degeneration, necrosis, and occasionally hemosiderin granules and pigmented casts (176). In addition, a prominent erythrocyte aggregates obstructing the lumen of capillaries without platelet or fibrinoid material were also detected (176). Clusters of coronavirus-like particles with distinctive spikes in the tubular epithelium and podocytes were also detected. Post-mortem examination of the viral nucleocapsid protein *in situ* in the kidney also showed that SARS-CoV-2 antigens is accumulated in kidney tubules, suggesting that SARS-CoV-2 may infects kidney directly, leading to acute renal injury and potentially contributing to viral spread (183–185). This direct route of SARS-CoV-2 may be due to an ACE2-dependent pathway. It was found that both proximal tubular cells or tubular progenitor cells in the kidney co-expressed ACE2 and TMPRSS2 and their expression levels resulted high in nephron epithelial cells, epithelial cells, endothelial cells, and mesangial cells of the kidney (82, 186). Additionally, Pan et al. showed that the TMPRSS2 gene was co-expressed with ACE2 in kidney podocytes (170). These cells are particularly vulnerable to viral infection and their injury easily induces heavy proteinuria that was detected in about 43.9% of SARS-CoV-2-infected patients (181). The co-expression of ACE2 and TMPRSS2 in renal tubular cells could imply that SARS-CoV-2 may directly bind to ACE2-positive cells in the kidney and destroy the function of renal tubules. However, kidney disease involvement in SARS-CoV-2 patients is likely to be multifactorial and may be also due to cytokine damage (high levels of IL-6), organ crosstalk (Lung-kidney) and other systemic effects (187, 188).

Stomach and Intestines

On October 6, 2020 searching on PubMed "COVID-19 OR COVID-2019 OR severe acute respiratory syndrome coronavirus 2 OR severe acute respiratory syndrome coronavirus 2 OR 2019-nCoV OR SARS-CoV-2 OR 2019nCoV OR (Wuhan AND coronavirus) AND (stomach OR intestines OR gastrointestinal system OR digestive system)" we found 977 papers. A lot of studies showed that the gastrointestinal tract represents a common target organ of SARS-CoV-2 infection (1, 2, 29, 96, 150–152, 189–198). A recent study suggests that the gastrointestinal symptoms in COVID-19 patients can be present up to 50% (39.6–50%), with symptoms including nausea, diarrhea, anorexia,

abdominal pain, belching, and emesis (199, 200). Anorexia appears to be the most common gastrointestinal symptom (26.8%), but the mechanism of its onset in COVID-19 patients remains unclear (4). However, this symptom can be due to gustatory dysfunction, which was found in a high percentage of COVID-19 patients (201). Several data reported that these gastrointestinal manifestations during SARS-CoV-2 infection can be associated with a poor disease course; comparing patients with non-severe disease with those with severe infection it was shown a higher risk of developing gastrointestinal symptoms in patient with severe infection (29, 202). The occurrence of these gastrointestinal symptoms can not only coexist with other symptoms, but also precedes the typical phenotype of SARS-CoV-2 infection (203). It was shown that also pediatric patients and children with SARS-CoV-2 infection may present digestive symptoms, most commonly diarrhea, in the absence of respiratory symptomatology (203, 204). Although different clinical features, such as milder disease course symptoms are present in pediatric patients and children with SARS-CoV-2, the gastrointestinal symptoms appear to be similar to those found in adult individuals (204). Despite gastrointestinal symptoms were frequently observed in SARS-CoV-2 patients, to date, the exact significance of these manifestations are still unclear. An autopsy report, with details of gastrointestinal pathology in a SARS-CoV-2 patient, showed the presence of segmental dilatation and stenosis in the small intestine (205). To date, autopsy data and reports with a full description of the gastrointestinal appearance associated to SARS-CoV-2 infection are still few to allow a clear conclusion. In addition to the clinical symptoms induced by the gastrointestinal disorders during SARS-CoV-2 infection, these manifestations can highlight one more route of virus transmission, i.e., the fecal-oral transmission. An increasing number of data showed that stool samples contain high concentration of SARS-CoV-2 RNA during infection for a relatively long period of time (from 1 to 12 days) (193, 204, 206). These data were also confirmed in pediatric patients and in children where ∼80% of patients resulted positive on rectal swabs even after negative nasopharyngeal tests (204). This aspect suggests a potential replication of SARS-CoV-2 virus in the gastrointestinal tract. This hypothesis is partially confirmed by Lin et al. that analyzing by endoscopy severe and non-severe SARS-CoV-2 patients with gastrointestinal manifestations detected the presence of SARS-CoV-2 RNA in esophagus, stomach, duodenum, and rectum of severe patients while only in the duodenum on one of four non-severe patients (4). Although, there are numerous data on gastrointestinal symptoms during SARS-CoV-2, the exact mechanism by which the virus affects the gastrointestinal tract is still not so clear. The occurrence of several mechanisms has been hypothesized. One mechanism may involve the presence of ACE2 receptors in the gastrointestinal tract. Liang et al. found that ACE2 was highly expressed in the small intestine especially in proximal and distal enterocytes (207). In addition, Zhang et al. found that ACE2, TMPRSS2, and Furin, all critical for fusion of viral and the cellular membranes, were co-expressed in esophageal upper epithelial and gland cells and also in the enterocytes from ileum and colon, thus speculating exactly these organs as potential targets for SARS-CoV-2 (208). In addition, Guo et al. suggested that TMPRSS2 was highly expressed in almost all organs of the digestive tract including colon, stomach, small intestine, and esophagus (209). The co-expression of ACE2 and TMPRSS2 in the intestinal enterocytes may explain the disruption of intestinal absorption that leads to diarrhea. However, a second mechanism could involve a direct injury of the gastrointestinal system due to an inflammatory response (cytokine storm) (208). Absorptive enterocytes may be infected and destroyed by the virus, probably leading to malabsorption, disturbed intestinal secretion, and an activated enteric nervous system ensuing symptoms like diarrhea (210).

Liver

On October 6, 2020 searching on PubMed "COVID-19 OR COVID-2019 OR severe acute respiratory syndrome coronavirus 2 OR severe acute respiratory syndrome coronavirus 2 OR 2019-nCoV OR SARS-CoV-2 OR 2019nCoV OR (Wuhan AND coronavirus) AND liver" we found 1,319 papers. Several data reported that approximately half of SARS-CoV-2 patients show liver biochemistry abnormalities, with increased levels of aminotransferases, gamma-glutamyl transferase, bilirubin, and alkaline phosphatase (116, 211–218). Median aspartate aminotransferase-dominant aminotransferase increase seems to indicate the disease severity and seems to be an index of hepatic injury (211). Concerning hepatic injury, Bloom et al. reported that about 1 in 5 patients developed grade 3 or 4 hepatocellular injury during hospitalization (212). In addition, it was reported that liver abnormalities seem to be more common in patients with severe disease upon presentation (212). In fact, a recent meta-analysis including 20 retrospective studies with 3,428 COVID-19 patients revealed that higher levels of alanine aminotransferase, aspartate aminotransferase and bilirubin were associated with a significant increase in the severity of COVID-19 infection (219). A recent meta-analysis also linked elevated admission levels of these markers to patient mortality (220). Other common factors linked with liver injury were decreased lymphocyte count, increase neutrophil count, and male gender (213). However, to date, the exact changes that lead to the altered liver biochemistries in SARS-CoV-2 patients remains unclear. Post-mortem liver biopsy showed the presence of a moderate microvascular steatosis and a mild lobular and portal activity (116). Another study suggested collateral liver damage from viral-induced cytotoxic T-cells (221). Additionally, since also abnormal coagulation markers have been reported in SARS-CoV-2 patients it is possible that the presence of microthrombi lead to an altered hepatic perfusion and consequent hepatocyte injury and aspartate aminotransferase increase (214, 215, 222). Whether these changes can be due to direct viral cytopathic effect, to cytokine release linked with SARS-CoV-2, to ischemia, to a preexisting condition, or to other causes, such as drug-induced liver injury, are currently unknown, also because studies on mechanisms of SARS-CoV-2 related liver dysfunction are limited. What we know currently is that ACE2 receptor are highly expressed in cholangiocytes (59.7%) and low expressed in hepatocytes (2.6%), thus some studies hypothesized a cholangiocytes mediating viral-associated injury (216). However, Zhou et al. showed that TMPRSS2 is

highly expressed in hepatocytes (223). In fact, it was shown that alkaline phosphatase, an index of cholangiocytes injury, was the liver parameter less subject to significant alterations during SARS-CoV-2 infection while, aminotransferases and gamma-glutamyl transferase, indicators of hepatocyte injury, were the more common and almost always altered liver parameters in severe SARS-CoV-2 patients (116). In fact, autoptic analyses of liver tissue from SARS-CoV-2 patients do not demonstrate a cholangiocyte damage (116). As just described, liver injury in COVID-19 may be the direct insult to the liver or bile cells via receptors of ACE2 but it is further aided by hyper-inflammation, cytokine storm or bystander hepatitis and drug-induced damage. Another hypothesis is that since the SARS-CoV-2 RNA was also present in stool, it would be possible a transmission from the gut to liver by portal circulation (224). To date the exact mechanism of viral-associated liver injury needs further investigation.

Gallbladder

On October 7, 2020 searching on PubMed "COVID-19 OR COVID-2019 OR severe acute respiratory syndrome coronavirus 2 OR severe acute respiratory syndrome coronavirus 2 OR 2019-nCoV OR SARS-CoV-2 OR 2019nCoV OR (Wuhan AND coronavirus) AND gallbladder" we found 21 papers. Despite few articles were found on gallbladder during SARS-CoV-2 infection, several information on its alteration during the new viremia were found in manuscripts on liver injury (225, 226). Gallbladder is a storage pouch for bile that is continually produced by liver, thus their functions are strictly related. Specific right upper quadrant ultrasounds on gallbladder of SARS-CoV-2 patients detected gallbladder sludge and distention in about 54% of patients, suggesting the presence of cholestasis (226, 227). Cholestasis in these patients seem to be not associated with age, gender, ICU admission, or gastrointestinal symptoms at presentation (226). The fatality rate seems to be higher among patients with cholestasis than those without cholestasis (228). As for liver, the gallbladder was found susceptible to the infection probably due to the high ratio of gallbladder epithelium cells expressing ACE2 (28). Also in this case the mechanism of viral-associated gallbladder alterations is unclear, although it is obvious that its alterations during SARS-CoV-2 infection are associated with liver injury.

Pancreas

On October 7, 2020 searching on PubMed "COVID-19 OR COVID-2019 OR severe acute respiratory syndrome coronavirus 2 OR severe acute respiratory syndrome coronavirus 2 OR 2019-nCoV OR SARS-CoV-2 OR 2019nCoV OR (Wuhan AND coronavirus) AND pancreas" we found 77 papers. Currently, data on pancreas involvement in SARS-CoV-2 infection are scarce. However, several case reports showed pancreatic injury in COVID-19 patients and it was reported that about 1–2% of non-severe and 17% of severe patients with SARS-CoV-2 infection presented pancreatic injury (5, 229–231). Several of these patients also presented abnormal blood glucose, suggesting that the pancreatic injury might be due directly to cytopathic effect by local SARS-CoV-2 replication (5, 229–231). Additionally, pancreatic injury might be caused indirectly by systemic responses to respiratory failure or to the harmful immune response induced by SARS-CoV-2, which led also to the damage in multiple organs (5). Similar results were also found by Liu et al. that detected elevated levels of amylase and lipase associated to focal enlargement of the pancreas or dilatation of the pancreatic duct based on CT scans (232). Hadi et al. also described SARS-CoV-2 patients with severe acute pancreatitis, which itself may lead to multi-organ failure including adult respiratory distress and kidney failure as seen the patients examined in the study (231). Considering the proportion of SARS-CoV-2 patients with pancreatic injury and theexpression of ACE2 and TMPRSS2 in the pancreas (particularly in pancreatic islet cells), researcher and clinicians should pay attention to the possibility of damage caused by SARS-CoV-2.

Brain

On October 7, 2020 searching on PubMed "COVID-19 OR COVID-2019 OR severe acute respiratory syndrome coronavirus 2 OR severe acute respiratory syndrome coronavirus 2 OR 2019-nCoV OR SARS-CoV-2 OR 2019nCoV OR (Wuhan AND coronavirus) AND brain" we found 1,293 papers and most of them showed that SARS-CoV-2 invades the CNS, developing neurological impairments such as stroke, epilepsy, anosmia and hypogeusia, seizures, and encephalitis (1, 66, 233–236). Specifically, a retrospective analysis by Mao et al. (237) underlined that about 40% of SARS-CoV-2 patients developed headache, disturbed consciousness, and other brain dysfunction symptoms (1), and an autopsy study reported the presence of edema in brain tissue of SARS-CoV-2 patients (66). Several case-series and two retrospective studies also reported critical stroke conditions related to COVID-19 (238, 239). In this context, Beyrouti et al. examining a small cohort of COVID-19 patients, also underlined that ischemic stroke (confirmed by reverse-transcriptase PCR) linked to severe SARS-CoV-2 patients occurs in the context of a systemic highly prothrombotic state, as shown by large vessel occlusion and elevated D-dimer levels (240), conditions that can make patients more prone to acute cerebrovascular events. Moriguchi et al. reported the first case of meningitis related to COVID-19 underling that SARS-CoV-2 RNA was not present in nasopharyngeal swab, but it was detected in cerebrospinal fluid sample (241). Numerous cases of encephalitis/encephalopathy associated with SARS-CoV-2 infection were also described and were confirmed by post-mortem analyses, where acute disseminated encephalomyelitis and neocortical micro-infarcts were detected (242–244). Neurologic complications associated to COVID-19 patients are not limited to the CNS. In fact, several authors also reported a correlation between SARS-CoV-2 and Guillan-Barrè syndrome, an acute/sub-acute immune-mediated polyradiculoneuropathy with diverse degrees of limbs or cranial-nerves weakness, lack of deep tendon reflexes, sensory, and dysautonomic symptoms cause by peripheral nerves and roots demyelination and/or axonal injure (245–248). Other studies also described Miller Fisher syndrome as another neurologic complication of SARS-CoV-2 infection (249–251). These neurological manifestations in the brain of SARS-CoV-2 infected patients were confirmed and recognized by CT scan images and magnetic resonance

imaging (MRI) scan, where presence of necrotizing hemorrhagic encephalopathy, brain thrombosis and acute infarction, eptomeningeal enhancement, perfusion abnormalities, and cerebral ischemic stroke, demyelinating lesions, right temporal lobe edema, and brainstem inflammation, were recognized (252–255). In addition, the presence of SARS-CoV2 was identified in frontal lobe tissue by using transmission electron microscopy (237) and by genome sequencing in cerebrospinal fluid of SARS-CoV-2 patients, supporting that this new pneumonia virus can cause nervous system damage (241). In addition to the above described neurological manifestations, several SARS-CoV-2 infected patients showed delirium and/or mental status changes. These symptoms may be a manifestation of direct CNS invasion, induction of CNS inflammatory mediators but may be also a secondary effect of other organ system failure, an effect of sedative strategies, a prolonged mechanical ventilation time, or environmental factors, including social isolation (256). Despite these data clearly highlighted the involvement of the brain in SARS-CoV-2 infection, the exact mechanism for virus neurotoxicity is not yet straightforward, since this depend on the brain entry route of the virus, which, to date, has not been fully elucidated (257). The pathway of the virus into the brain could be primarily linked to the route of transmission and distribution of intracellular receptors of SARS-CoV-2. Mao et al. hypothesized that SARS-CoV-2 virus may interact with ACE2 in the capillary endothelium and caused blood–brain-barrier destruction, thus promoting the entry of the virus into CNS (237) and next causing neuroinfection. In fact, it was found that ACE2 and TMPRSS2 were expressed in the oligodendrocyte precursor cells and the astrocytes of the substantia *nigra* and cortex (82). COVID-19 can potentially damage the capillary endothelium within the brain and contribute to elevated blood pressure. The risk of SARS-CoV-2 cerebral hemorrhage through an ACE2 receptor can result in abnormally high blood pressure and increase cerebral hemorrhage. However, although ACE2 and TMPRSS2 are present in the nervous system, additional pathways were also hypothesized for the entry of SARS-CoV-2 into the nervous system, including the direct intranasal entry to the brain *via* olfactory nerves, the indirect entry to the brain go through the blood-brain barrier via hematogenous or lymphatic spread, the hypoxic injury, and finally the immune-related injury (7, 258). It is known, that coronaviruses can enter to the nervous system straight through the olfactory nerve, potentially causing loss of smell and taste, and enter the nervous system through blood circulation and neuronal pathways. In addition, coronaviruses, including SARS-CoV-2, trigger harmful effects in the lung tissue leading to several lung lesions and consequent hypoxia, that can be responsible of the brain disease progression. These data highlighted that awareness, management and timely analysis of infection-related neurological complications of SARS-CoV-2 patients are key to improve the prognosis of severe ill patients.

Skin

On October 7, 2020 searching on PubMed "COVID-19 OR COVID-2019 OR severe acute respiratory syndrome coronavirus 2 OR severe acute respiratory syndrome coronavirus 2 OR 2019-nCoV OR SARS-CoV-2 OR 2019nCoV OR (Wuhan AND coronavirus) AND (skin OR cutaneous manifestation) we found 771 reports. Skin manifestations due to SARS-CoV-2 infection are of different types and currently reported in numerous case reports, case series, and literature reviews (259–264). The first case study on skin manifestations was published by Recalcati et al. and included 88 patients that showed widespread urticaria, erythematous rush and chickenpox-like vesicles (265). Subsequently, other authors described urticarial rash petechial also in association with decrease platelet count and sometimes also with eosinophilia (265–270). Zhang et al. evaluating 140 patients with SARS-CoV-2 infection, stated that urticaria were self-reported by 1.4% of patients (268). Despite, the majority of studies reported that urticarial skin manifestations were not correlated with SARS-CoV-2 severity (265, 268), a prospective cohort study reported that the presence of urticaria and maculopapular skin lesions were associated with higher morbidity and higher mortality rate (2%) (271). In addition to urticarial skin manifestations, Manalo et al. also described a transient livedo reticularis as potential skin manifestation linked to SARS-CoV-2 (272). Other described skin manifestations are related to acral ischemia often related to an hypercoagulation status of SARS-CoV-2 patients, that have a negative prognostic implication in virus evolution (273–275). These manifestations could be caused by direct injury of vascular endothelium by SARS-CoV-2, which could lead to DIC, antiphospholipid syndrome, and vasculitis mimics. Case series showed purpuric skin involvement in severe SARS-CoV-2 patients, in detail retiform purpura on the buttocks, dusky purpuric patches on the palms and soles, and livedo reticularis on the chest and limbs were detected (261, 273, 276). Tissue biopsies from skin and lung detected thrombogenic vasculopathy and deposits of C5b-9 and C4d complement proteins (273). This was in line with widespread activation of both alternative and lectin pathways of complement, suggesting that severe SARS-CoV-2 patients can suffer thrombotic microvascular injuries that can involve not only the lungs but also the skin, and probably other organs (273). Skin manifestations were found also in pediatric patient where the skin lesions commonly happen in asymptomatic or mildly symptomatic children and adolescents (277–279). Skin biopsy of acral perniosis lesion in SARS-CoV-2 pediatric patients revealed a superficial and deep lymphocytic infiltrate, where vacuolar change and purpura were also present (280, 281). Hemorrhagic parakeratosis in the stratum corneum were also detected and as well as dermal infiltrate strongly perivascular and perieccrine and lymphocytic vasculitis in the thin muscular walls of small vessels (4, 205). Similar results were also found in skin biopsies from SARS-CoV-2 adult patients that showed a lymphocytic perivascular and perieccrine infiltrate (282, 283). To date, there are still not enough studies to define which are the skin manifestations of SARS-CoV-2 infection, and why they occur. As reported by Recalcati et al. these dermatological manifestations "are similar to cutaneous involvement occurring during common viral infections" (265). Several hypotheses could be formulated from integration of the clinical observations and data from literature, but to date whether these skin manifestations were neurogenic, microthrombotic, or immune complex mediated is unclear. However, examine the tissue samples to understand

if SARS-CoV-2 can be detected in the skin itself could be of key importance also considering that ACE2 is present in basal epidermal layers and in sebaceous gland cells of the skin (58). In addition, a recent study detected that ACE2 and TMPRSS2 were co-expressed at the epithelial sites of the skin, highlighting the potential roles of these molecules in SARS-CoV-2 (284). However, so far it is not known if skin manifestations (non-pruritic, erythematous rashes, urticaria, or varicella-like lesions) in COVID-19 patients are a place of viral replication or just a local reaction to systemic infection.

Male and Female Reproductive System and Pregnancy

On October 7, 2020 searching on PubMed "COVID-19 OR COVID-2019 OR severe acute respiratory syndrome coronavirus 2 OR severe acute respiratory syndrome coronavirus 2 OR 2019-nCoV OR SARS-CoV-2 OR 2019nCoV OR (Wuhan AND coronavirus) AND (reproductive system OR ovaries OR testis OR pregnancy)" we found 1,301 reports. Most of these reports described high levels of ACE2 expression in the testes, spermatids, ovaries, fallopian tubes, placenta, and uterus, thus highlighting a potential high risk of SARS-CoV-2 infection in the human reproductive system (61, 285–288). However, data on the presence of SARS-CoV-2 in male reproductive system are conflicting. A study carried out by Li et al. revealed that SARS-CoV-2 was found in the testes of infected cases (289). A post-mortem study on 91 COVID-19 victims also showed varying degrees of spermatogenic cell reduction and damage, and presence of SARS-COV-2 RNA and virus particles in the testes (290). Conversely, some clinical studies did not detect SARS-CoV-2 in semen or testicular biopsy of COVID-19 patients (291–293). Thus, it is possible to speculate that SARS-CoV-2 gains access to the male reproductive system in some but not in every COVID-19 patient. However, since SARS-CoV-2 can lead to systematic effect it can have also other consequences on the reproductive system. Several studies reported testicular discomfort and devastation to the testicular parenchyma in COVID-19 patients even when the testes were SARS-CoV-2 negative (291, 293).

As known, the reproductive health issues may not be restricted to men, but woman may also have consequences. What seems to be quite clear is the distribution and function of ACE2 in the female reproductive system. Jing et al. clearly reported the ACE2 expression in the ovary, uterus, vagina, and placenta (60). Moreover, since Ang II, ACE2, and Ang-(1–7) regulate follicle development and ovulation, modulate luteal angiogenesis, and degeneration, and influenced the regular changes in endometrial tissue and embryo development SARS-CoV-2 infection may disturb the female reproductive functions, resulting in infertility, menstrual disorder and fetal distress (60). Although these data suggested that there are potential routes for SARS-CoV-2 to compromise female fertility, currently no studies on damage to female COVID-19 patients' reproductive system were reported. For SARS-CoV-2 role in female reproductive system, the latest evidences were mainly focused on pregnant women. The simultaneous expression of ACE2 and TMPRSS2 seems to lack at the cellular level of maternal- fetal interface. Despite the clinical manifestation in COVID-19 pregnant women seems to remain the same as in non-pregnant patients, several studies suggest that pregnant women infected with COVID-19 may be at risk for preterm delivery (294–296). Recent papers also reported cases of pre-eclampsia and manifested gestational hypertension in COVID-19 pregnant women (297–299). An analysis of the WAPM study on COVID-19 reported that early gestational age at infection, maternal ventilatory supports and low birthweight are the main determinants of adverse perinatal outcomes in fetuses with maternal COVID-19 infection (300). However, significant neonatal respiratory diseases appear to be rare in presence of SARS-CoV-2 positivity (301). In this context, the key question is whether SARS-CoV-2 can be transmitted to fetuses from a woman infected with COVID-19. The evidence of infection in infants in the time immediately following birth (since few hours to a couple of days) suggests the possibility of maternal fetal transmission, via intrauterine vertical infection or mediated by breastfeeding. In this latter case, evidence of the presence of Sars- Cov2 in maternal milk is still controversial, and in the close contact between mother and child in these phases could lie the true way of transmission. Instead, despite primary reports from China suggested that vertical transmission was unlikely, several case series revealed the possibility of vertical transmission from positive SARS-CoV-2 woman (302, 303). Two conditions are mandatory for transplacental transmission to be possible: (1) SARS-CoV-2 must reach the placenta and (2) ACE2 must be present in the placenta. Regarding the first condition, several papers supported the presence of SARS-CoV-2 in placental tissue. In particular, histopathological signs of placenta alteration have been observed in pregnant women affected by Sars-CoV-2, with evidence of inflammatory state and alteration in vascular supply. (304–308). Regarding the second condition controversial results are still present (309, 310). However, a recent study indicated that trophoblastic cells, which are in direct contact with the maternal blood in the intervillous space, showed a strong expression of ACE2 throughout pregnancy, supporting that SARS-CoV2 is able to infect the placenta *via* a receptor-mediated mechanism (311). A further study investigated the potential transmission routes in the first trimester, and they found expression of ACE2 and co-expression of TMPRSS2 in the trophoblast, blastocyst, and hypoblast (312). However, other proteases such as Furin, trypsin and cathepsins B and L could be also implicated (16, 313, 314). Thus, despite the lack of clinical evidence, SARS-CoV-2 infection may carry a potential risk of reproductive system.

Thyroid

On October 7 2020 by searching on PubMed "COVID-19 OR COVID-2019 OR severe acute respiratory syndrome coronavirus 2 OR severe acute respiratory syndrome coronavirus 2 OR 2019-nCoV OR SARS-CoV-2 OR 2019nCoV OR (Wuhan AND coronavirus) AND thyroid" we found 112 papers. Data on direct thyroid involvement in SARS-CoV-2 infection arescarce and most of the reports are focused on identifying a possible association between hypothyroidism and outcomes related to COVID-19. A consensus statement regarding issues specific to thyroid dysfunction during SARS-CoV-2 pandemic was

issued by the British Thyroid Association and the Society for Endocrinology (315). The consensus suggested to patients with hypothyroidism or hyperthyroidism to continue their medications, however, it underlined that patients on anti-thyroid drugs are at a risk of agranulocytosis, symptoms that often overlap with those of SARS-CoV-2 (315). However, recently, van Gerwen et al. evaluated 3,703 COVID-19 patients of which 251 patients (6.8%) had pre-existing hypothyroidism and received thyroid hormone therapy (316). They found that hypothyroidism was not associated with increased risk of hospitalization, mechanical ventilation, and death (316). A direct thyroid involvement associated with COVID-19 was highlighted by Campos-Barrera et al. that identified a subacute thyroiditis associated with a very mild presentation of COVID-19 in a healthy 37-year-old female (317). Subacute thyroiditis was not the only thyroid condition associated with COVID-19. In fact, cases of thyroxine thyrotoxicosis have been also described (318). Several case reports and a case series were focused on the prevalence of subacute thyroiditis and thyroxine thyrotoxicosis in patients with severe presentation of COVID-19 from ICU (319–324). More recently in a retrospective study on 50 COVID-19 patients it was found a decrease in total T3 and TSH concentrations in 56% of patients (325–327). The decrease in T3 concentration resulted more pronounced in patients with the severe SARS-CoV-2 (325). Despite the few data related to the thyroid involvement during SARS-CoV-2 infection, it is important to emphasize that, as previously reported ACE2 expression levels were high in thyroid and its expression were positively and negatively associated with immune signatures in males and females (328). Additionally, TMPRSS2 was also expressed in thyroid (82). Therefore, surely further studies would be important to understand a potential involvement of the thyroid in SARS-CoV-2 infection.

DISCUSSION

Since it has been demonstrated that the novel SARS-CoV-2, which affected a very high number of people all over the world, entry into the cell exploiting ACE2, more and more research and studies are focusing their attention on ACE2 role, function, and distribution and on its interaction with specific proteases that assist SARS-CoV-2 infection. In fact, it is known that following the entry of the virus into the human cell through the binding with ACE2, the S protein is cleaved by TMPRSS2, with the help of Furin which facilitates the entry of the virus into the cell after binding. However, theoretically, also other human's proteases (cathepsin L and B, elastase, trypsin and factor X) could be involved in this complex process and numerous studies are currently ongoing.

Our overview highlighted that ACE2 receptors, being ubiquitous, and extensively expressed in numerous human tissues and organs, such as in the heart, vessels, gut, lung, kidney, testis, and brain and many other, may play a key role in the involvement and subsequent impairments of various organs during the SARS-CoV-2 infection. ACE2 is typically bound to cell membranes and poorly present in the soluble form in circulation. In addition to its negative role in SARS-CoV-2 infection, and in other virus, membrane-bound and soluble ACE2 also perform beneficial biological functions, the main represented by the degradation of angiotensin II to angiotensin 1–7. Thus, ACE2 receptors cut down some harmful effects consequential to the bind of angiotensin II to AT1 receptors, which comprise vasoconstriction, increase inflammation, and thrombosis (329). However, the entry of SARS-CoV-2 in the cells by membrane fusion down-regulates ACE2 receptors, thus SARS-CoV-2 seems to entry into the cell with the membrane receptor, which is functionally detached from the membrane external site. This phenomenon can cause the detrimental effects in SARS-CoV-2 infection. It is important to underline that several other factors, such as genetics, demographic, lifestyle, co-morbidities and drugs usage could have a potential impact on ACE2 expression and activity. In fact, it was extensively reported that SARS-CoV-2 patients present several features associated with infection and severity of the disease, such as older age, hypertension, diabetes and cardiovascular disease, that share a different degree of ACE2 deficiency and that can produce bias in the evaluation of the effective damages caused by the virus (1). However, despite during SARS-CoV-2 infection ACE2-expressing organs may become direct targets, leading to critical pathological manifestations and subsequent multiple organ failure or even death, the exact mechanism and effective action through which ACE2 act on these organs is still heavily debated. Another point at the center of the clinical and scientific debate is represented by the potentially beneficial effect (or not) of soluble form of ACE2, the form that lacks the membrane anchor and circulates in small amounts in the blood. A paper by Battle et al. hypothesizes that the soluble form of ACE2 might behave like a competitive interceptor of SARS-CoV avoiding the binding of the virus to the surface-bound, full-length ACE2, the form that contains a structural transmembrane domain, which anchors its extracellular domain to the plasma membrane (330). This evidence is in line also with in *vitro* studies (331, 332). A preclinical model of Vero-E6 cells, infected with SARS-CoV-2, isolated from a nasopharyngeal sample of COVID-19 patient, demonstrated the efficacy of human recombinant soluble ACE2 (hrsACE2) in inhibiting viral replication in a dose-dependent manner. Such activity was also confirmed in human capillary organoids cultures and in kidney organoids cultures generated from human embryonic stem cells (331). In addition, the soluble ACE2 form seems to be also involved in blocking SARS-CoV-2 replication and in immune response against the virus, in concert with Fc portion of immunoglobulin (331). The administration of rhACE2 also seems to induce a reduction of IL-6 levels in severe COVID-19 patients (333). The increased production of IL-6 and other inflammatory cytokines (IL-1β, IL-2, IL-7, IL-8, IL-10, granulocyte-colony stimulating factor, granulocyte macrophage-colony stimulating factor, interferon-inducible protein-10, monocyte chemotactic protein 1, macrophage inflammation protein-1α, IFN-γ, and TNF-α,2,3,12,15) together with the presence of lymphopenia, lymphocyte activation and dysfunction, abnormalities of granulocytes and monocytes, increased production of immunoglobulin G (IgG) and total antibodies in COVID-19 patients points out how

the SARS-CoV-2 is able to disrupt also the normal immune responses, leading to an impaired immune system (334–339). Lymphopenia is a key feature of patients with severe COVID-19 (334). A marked reduction in the number of CD4+ T, CD8+ T, NK, and B cell was detected in these patients (335). In addition, a high expression of CD69, CD38, and CD44 on CD4+ and CD8+ T cells was seen (336). Virus-specific T cells from severe COVID-19 patients also highlight a central memory phenotype with high levels of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and IL-2. Nevertheless, lymphocytes have an exhaustion phenotype with programmed cell death protein-1 (PD1), T cell immunoglobulin domain and mucin domain-3 (TIM3), and killer cell lectin-like receptor subfamily C member 1 (NKG2A) upregulation (337). Unlike eosinophils, basophils, and monocytes percentage that were reduced in severe COVID-19 patients the level of neutrophils resulted increased (338). Thus, the damage and inefficiency of the immune system caused by lymphopenia, T cell exhaustion and cytokine release syndrome, and organ specific ACE2 expressing cells (endothelial, alveolus in lungs, proximal tubule, and glomerulus in kidneys, pericytes in heart, ect) could potentially lead to complications like acute respiratory disease syndrome and multi-organ failure. These complications not only can lead to a poorer outcome to the SARS-CoV-2 infection but can also lead to permanent alterations that can persist long after viremia (*long-term COVID-19*), such as pulmonary fibrosis, neurodegenerative deseases, cardiovascular and kidney diseases (340, 341). Highlighting the pathological basis and mechanisms of COVID-19 and all the functions and activities of ACE2 during the virus would be essential for our understanding of the pathophysiology of the disease. A great help to our understanding could come from pathological studies of larger series of autopsy findings. Furthermore, the development of advanced and alternative preclinical models could help to discover more about the SARS-CoV-2 infection process itself, to analyze specific aspects of ACE2 in relation to SARS-CoV-2 pathophysiology and, most importantly, to learn the disease progression pattern observed in humans. In addition, more exhaustive and systematic studies on the physiological localization and activity of ACE2 might help in the comprehension of the mechanisms underlying the infection. In this regard, attention should be paid to the investigation of the cells of the immune system, in consideration of preliminary scientific evidence on the identification of ACE2 in immune cells residing in the tissues. This could open further scenarios on both the virus spreading mechanisms and tissues damage.

We believe that devote scientific efforts for the clinical management of SARS-CoV-2 patients, also considering a personalized strategy aimed to provide individually tailored treatment for each patient, are currently mandatory. As showed in this report this aspect should also considered specific patient differences in the mutual interactions ACE2-SARS-CoV-2 with their consequences for the disease pathophysiology. Another interesting aspect that could be explored in patients who have overcome the disease is the possible onset or persistence of the alterations above described in the organs and systems and the evaluation of whether they are transient or permanent (*long-term COVID-19*), to assess the extent of ACE2 activity impairment due to SARS-CoV-2 infection.

AUTHOR CONTRIBUTIONS

FS, MF, and ML designed the manuscript. FS and MM collected and analyzed literature, wrote the manuscript, edited, and prepared manuscript for submission. ML and MF revised the manuscript. All authors read and approved the final manuscript.

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Quantitative SARS-CoV-2 Antibody Screening of Healthcare Workers in the Southern Part of Kyoto City During the COVID-19 Pre-pandemic Period

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Fujita K, Kada S, Kanai O, Hata H, Odagaki T, Satoh-Asahara N, Tagami T and Yasoda A (2020) Quantitative SARS-CoV-2 Antibody Screening of Healthcare Workers in the Southern Part of Kyoto City During the COVID-19 Pre-pandemic Period. Front. Public Health 8:595348. doi: 10.3389/fpubh.2020.595348

Background: The coronavirus disease-2019 (COVID-19) pandemic is associated with a heavy burden on the mental and physical health of patients, regional healthcare resources, and global economic activity. While understanding of the incidence and case-fatality rates has increased, there are limited data concerning seroprevalence of antibodies against the severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) in healthcare workers during the pre-pandemic period. This study aimed to quantitatively evaluate seroprevalence of SARS-CoV-2 antibodies in healthcare workers in the southern part of Kyoto city, Japan.

Methods: We prospectively recruited healthcare workers from a single hospital between April 10 and April 20, 2020. We collected serum samples from these participants and quantitatively evaluated SARS-CoV-2 IgG antibody levels using enzyme-linked immunosorbent assays.

Results: Five (5.4%), 15 (16.3%), and 72 (78.3%) participants showed positive, borderline, and negative serum SARS-CoV-2 IgG antibody status, respectively. We found the mean titer associated with each antibody status (overall, positive, borderline, and negative) was clearly differentiated. Participants working at the otolaryngology department and/or with a history of seasonal common cold symptoms had a significantly higher SARS-CoV-2 IgG antibody titer ($p = 0.046$, $p = 0.046$, respectively).

Conclusions: Five (5.4%) and 15 (16.3%) participants tested positive and borderline, respectively, for SARS-CoV-2 IgG antibody during the COVID-19 pre-pandemic period. These rates were higher than expected, based on government situation reports. These findings suggest that COVID-19 had already spread within the southern part of Kyoto city at the early stage of the pandemic.

Keywords: COVID-19, seroprevalence, SARS-CoV-2, ELISA, antibody

INTRODUCTION

Coronavirus disease-2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). COVID-19 was first reported in Wuhan, China, in December 2019, and the outbreak was subsequently declared a pandemic by the World Health Organization (WHO) on March 11, 2020 (1). The disease course varies from mild and self-limiting upper respiratory infection symptoms to severe respiratory failure, which might require respiratory support (2, 3). By mid-March 2020, pandemic centers were located in China, the United States, and several European countries. In Japan, the government announced a state of emergency on April 4, 2020. At the end of July 2020, >750,000 people worldwide had died of COVID-19 (1, 4). COVID-19 is associated with a heavy burden on the mental and physical health of patients, regional healthcare resources, and global economic activity. Effective policies to deal with the pandemic are required and should be founded on reliable epidemiological data. The diagnosis of COVID-19 is based on viral nucleic acid detection using a reverse-transcription polymerase chain reaction (RT-PCR) assay for SARS-CoV-2. Whereas, an RT-PCR assay is accurate at detecting an active case of COVID-19, identifying individuals who have recovered from SARS-CoV-2 infection has been challenging. In contrast to tracking active cases, antibody detection can provide information on individual and herd-acquired immunity against SARS-CoV-2. Furthermore, an antibody assay can help to estimate the number of people within a community who remain potential cases, assisting governments in effective decision-making. To date, data concerning the seroprevalence of SARS-CoV-2 antibodies in healthcare workers worldwide are limited. During the pre-pandemic period, we quantitatively evaluated the seroprevalence of SARS-CoV-2 antibodies in healthcare workers in the southern part of Kyoto city, an area famous for its heritage status and a popular tourist destination.

PARTICIPANTS AND METHODS

Participants

This study was conducted at the National Hospital Organization Kyoto Medical Center (600 beds), located in southern Kyoto, Japan. In response to the pandemic, our hospital formed an infectious disease department dedicated to COVID-19, involving medical staff such as internal medicine physicians, chest physicians, general and thoracic surgeons, cardiologists, nephrologists, otolaryngologists, and emergency physicians. We prospectively recruited medical doctors, nurses, and ward clerks employed at our hospital between April 10 and April 20, 2020. All participants were asymptomatic and worked within any of the following departments: infectious disease, respiratory medicine, otolaryngology, or emergency medicine. Healthcare workers from these departments were selected as they were considered more likely to treat patients with suspected COVID-19, of which they might not have been aware. Additionally, we collected the following questionnaire-based data: a history of seasonal common cold from winter 2019 to early spring 2020 and a history of regular contact with children aged <12 years. The questionnaires were created based on previous studies involving behavior patterns during the H10N8 avian influenza outbreak (5).

ELISA Assay

We collected 6 ml of blood from each participant between April 10 and April 20, 2020. After extracting serum, we deep froze and stored the samples at −80°C. We used an enzyme-linked immunosorbent (ELISA) assay, using COVID-19 IgG ELISA kits (DRG international, Inc. Springfield, NJ, USA), to evaluate the presence of serum IgG antibody against SARS-CoV-2, in accordance with the manufacturer's instructions. Briefly, 1:100 diluted human serum samples were placed onto a 96-well microplate (coated with SARS-CoV-2 recombinant full-length nucleocapsid protein) and then incubated for 30 min at room temperature (20–25°C). After washing, 100 μl HRP-labeled anti-IgG tracer antibody was added into the wells and the samples were incubated for 30 min at room temperature (20–25°C). Following the second wash cycle, 100 μl substrate was added into the wells and the samples were incubated for 20 min at room temperature (20–25°C). Last, stop solution was added into the wells to terminate the reaction. The optical density of each well was determined using a microplate reader set to 450 nm within 10 min. For IgG detection, the cut-off value was modified through using an internal negative and positive control of Japanese samples, because of the differences in ethnicity between ELISA kits (Chinese controls) and our samples (Japanese). We interpreted the results as positive, borderline, and negative, according to the manufacturer's instructions.

Statistical Analysis

The data were analyzed using JMP version 14.0.0 (SAS institute Inc. Cary, NC). A Fisher's exact test was used to compare proportions among occupations, wards, questionnaires, and SARS-CoV-2 IgG antibody status. Wilcoxon rank sum or Kruskal-Wallis tests, as appropriate, were used to compare SARS-CoV-2 IgG antibody titers between groups, and p-values < 0.05 were considered statistically significant.

Ethical Approval

This study was approved by the relevant institutional review boards (approval number: 20-009) and written informed consent was obtained from all study participants.

RESULTS

In total, 92 healthcare workers were recruited for this study. Medical doctors, nurses, and medical clerks comprised 42 (45.7%), 48 (52.2%), and 2 (2.2%) participants, respectively. Of 92 participants, 59 (64.1%) were women, and most participants were aged between 20 and 39 years. Among the participants, the otolaryngology department was the most common place of work, followed by the respiratory and emergency medicine departments. Of 92 participants, 47 (51.1%) had a history of seasonal common cold symptoms from winter 2019 to early spring 2020, and 19 (20.7%) participants had a history of regular contact with children aged <12 years (**Table 1**).

TABLE 1 | Clinical and demographic characteristics of participating healthcare workers.

	n = 92
Sex (female)	59 (64.1)
Age group (years)	
20–29	30 (32.6)
30–39	29 (31.5)
40–49	21 (22.8)
≥50	12 (13.0)
Occupation	
Medical doctor	42 (45.7)
Nurses	48 (52.2)
Medical clerk	2 (2.2)
Department	
Department of Infectious Diseases	18 (19.6)
Respiratory Medicine Ward	22 (23.9)
Otolaryngology Ward	30 (32.6)
Emergency Medicine Ward	22 (23.9)
Questionnaire	
*History of seasonal common cold symptoms	47 (51.1)
*History of regular contact with children	19 (20.7)
**History of exposure to a viral infection	84 (91.3)
SARS-CoV-2 antibody status	
Positive	5 (5.4)
Borderline	15 (16.3)
Negative	72 (78.3)

Data are shown as counts (%). SARS-CoV-2, severe acute respiratory syndrome coronavirus-2.
**Covered period from winter 2019 to early spring 2020, including history of regular contact with children aged <12 years.*
***Participants considered exposed to viral infection were defined as those with their own history of seasonal common cold symptoms and/or examining outpatients with common cold symptoms.*

Seroprevalence of Antibodies Against SARS-CoV-2

In total, 92 serum samples collected between April 10 and April 20, 2020 were tested for antibodies against SARS-CoV-2. Of 92 participants, 5 (5.4%), 15 (16.3%), and 72 (78.3%) showed positive, borderline, and negative SARS-CoV-2 IgG antibody test results, respectively (**Table 1**). There were no significant differences in antibody status between the professional groups (**Table 2**). We identified 2 and 3 participants with a positive antibody status in the respiratory disease and otolaryngology departments, respectively. The highest proportion of participants with a positive and borderline SARS-CoV-2 IgG antibody status worked at the otolaryngology department, whereas the lowest proportion were working at the emergency medicine department (**Table 3**).

Participants with a history of seasonal common cold from winter 2019 to early spring 2020 showed a higher rate of positive SARS-CoV-2 IgG antibody test results than participants with no such history ($p = 0.046$). A history of regular contact with children or of exposure to a viral infection did not affect the seroprevalence of SARS-CoV-2 IgG antibody (**Table 4**).

TABLE 2 | SARS-CoV-2 IgG antibody seroprevalence among healthcare workers according to occupation.

		IgG against SARS-CoV-2						*p*-value
Occupation	***n***	**Positive**		**Borderline**		**Negative**		
Medical doctor	42	2	(4.7%)	6	(14.0%)	34	(76.2%)	0.9236
Nurse and Medical clerk	50	3	(6.0%)	9	(18.0%)	38	(76.0%)	

Data are shown as counts (%). The p-value was estimated using the Fisher's exact test, with $p < 0.05$ considered statistically significant.
SARS-CoV-2, severe acute respiratory syndrome coronavirus-2.

TABLE 3 | SARS-CoV-2 IgG antibody seroprevalence among healthcare workers according to department.

		IgG against SARS-CoV-2						*p*-value
	n	**Positive**		**Borderline**		**Negative**		
Department of Infectious Diseases	18	0	(0.0%)	3	(16.7%)	15	(83.3%)	0.2102
Respiratory Diseases Ward	22	2	(9.1%)	4	(18.2%)	16	(72.7%)	
Otolaryngology Ward	30	3	(10.0%)	7	(23.3%)	20	(66.7%)	
Emergency Medicine Ward	22	0	(0.0%)	1	(4.6%)	21	(95.5%)	

Data are shown as counts (%). The p-value was estimated using a Fisher's exact test, with $p < 0.05$ considered statistically significant.
SARS-CoV-2, severe acute respiratory syndrome coronavirus-2.

TABLE 4 | Seroprevalence of SARS-CoV-2 IgG antibody according to exposure status as determined using a questionnaire.

		IgG against SARS-CoV-2						*p*-value
	n	**Positive**		**Borderline**		**Negative**		
*History of seasonal common cold symptoms	47	5	(10.6%)	9	(19.2%)	33	(70.2%)	0.0458
*History of regular contact with children	19	1	(5.3%)	1	(5.3%)	17	(89.5%)	0.3294
**History of exposure to a viral infection	84	5	(6.0%)	14	(16.7%)	65	(77.4%)	>0.99

Data are shown as counts (%). The p-value was estimated using a Fisher's exact test, with $p < 0.05$ considered statistically significant.
SARS-CoV-2, severe acute respiratory syndrome coronavirus-2.
**Covered period from winter 2019 to early spring 2020, including history of regular contact with children aged <12 years.*
***Participants considered exposed to viral infection were defined as those with own history of seasonal common cold symptoms and/or examining outpatients with common cold symptoms.*

Serum SARS-CoV-2 IgG Antibody Titer

The mean antibody titer of all participants was 0.120 ± 0.0372 (**Figure 1A**). The mean titer of the antibody positive, borderline, and negative groups was 0.219 ± 0.051, 0.161 ± 0.0101, and 0.105 ± 0.018, respectively (**Figure 1B**). Mean antibody titers stratified according to occupation and department are shown in **Figures 2A,B**. There were no significant differences in mean antibody titers between doctors, nurses, and medical clerks (0.119 ± 0.0326 and 0.121 ± 0.0058, $p = 0.994$; **Figure 2A**). The mean

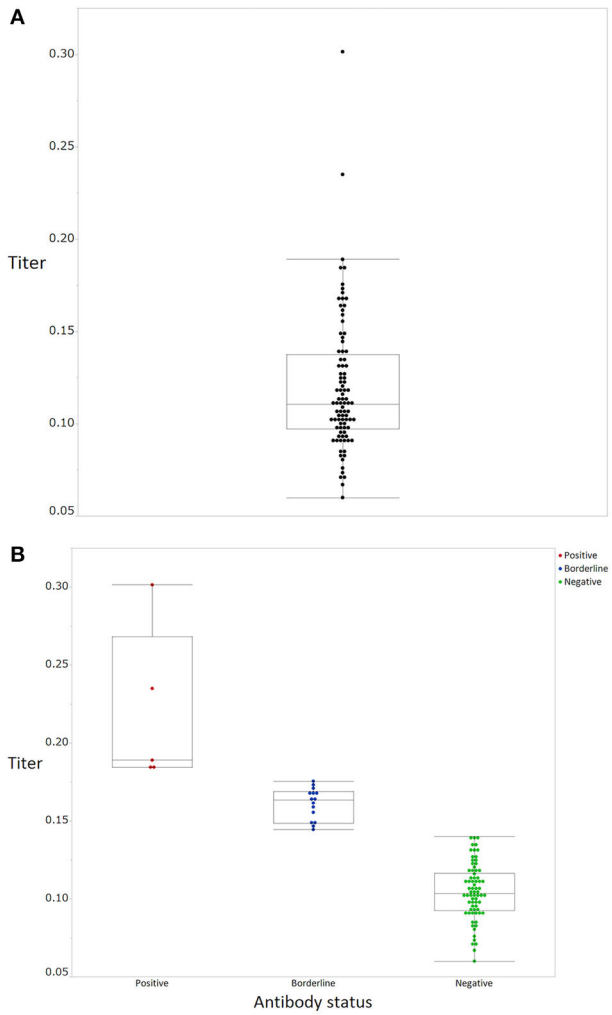

FIGURE 1 | SARS-CoV-2 IgG antibody titers of the participants. **(A)** All participants (black), **(B)** stratified according to positive (red), borderline (blue), and negative (green) status. Boxes correspond to the interquartile range of values for each group; error bars show the 90th percentile range.

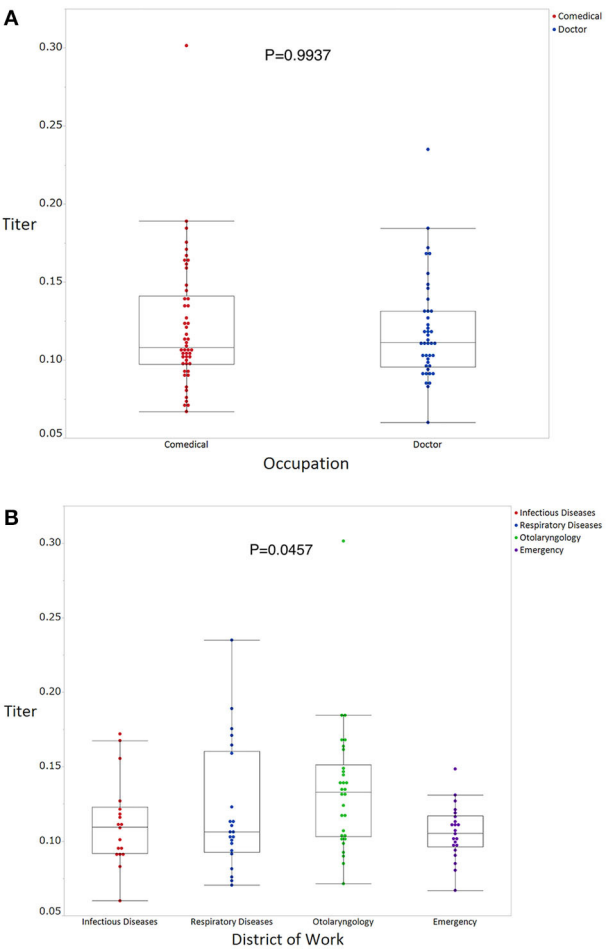

FIGURE 2 | SARS-CoV-2 IgG antibody titers stratified according to occupation **(A)** and department **(B)**. A Wilcoxon rank sum test **(A)** or a Kruskal-Wallis test **(B)** was used to compare the titer of SARS-CoV-2 IgG antibody levels between the groups, with p-values < 0.05 considered statistically significant.

antibody titer among workers at the otolaryngology department was significantly higher than that among workers in the other three departments (0.112 ± 0.029, 0.121 ± 0.043, 0.134 ± 0.043, and 0.11 ± 0.018, $p = 0.046$; **Figure 2B**). Participants with a history of seasonal common cold symptoms had a significantly higher titer of SARS-CoV-2 IgG antibody than those with no such history (0.13 ± 0.044 and 0.11 ± 0.026, $p = 0.046$; **Figure 3A**). There were no significant differences in the mean antibody titer between participants with and without a history of regular contact with children or with a history of exposure to a viral infection ($p = 0.304$, **Figure 3B**; $p = 0.418$, **Figure 3C**).

DISCUSSION

In this study, 5 (5.4%) and 15 (16.3%) healthcare workers were positive and borderline, respectively, for the presence of SARS-CoV-2 IgG antibodies. The mean antibody titer among the borderline group was clearly distinct from that of the negative group. Participants with borderline antibody results might have been latently sensitized by patients with COVID-19. As our hospital accepted patients with confirmed COVID-19 after April 15, 2020, the antibody status of our study participants might reflect community-acquired immunity, resulting from unwitting exposure in daily medical practice.

The mean antibody titer was significantly higher among workers at the otolaryngology department than among those working in other departments. This suggests that healthcare workers within the otolaryngology department were more likely to be exposed to SARS-CoV-2 than their counterparts working in other departments.

According to official statements from Kyoto city authorities, confirmed incidence and fatality associated with COVID-19 in Kyoto city at the end of April 2020 comprised 215 and 11 cases, respectively (6). However, antibody seroprevalence observed in this study was much higher than that expected, based on

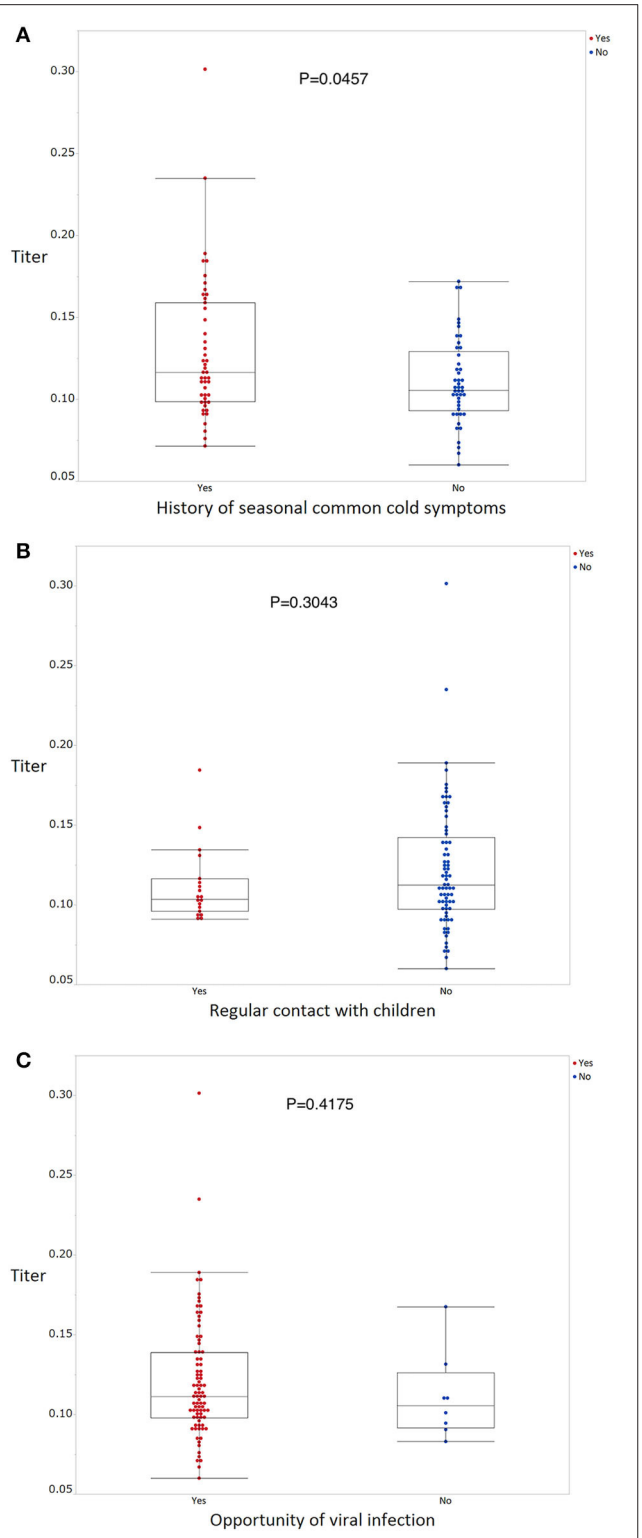

FIGURE 3 | SARS-CoV-2 IgG antibody titers stratified according to exposure status as determined using questionnaires concerning behavior patterns **(A–C)**. Wilcoxon rank sum tests were used to compare the titer of SARS-CoV-2 IgG antibody levels between the groups, with p-values < 0.05 considered statistically significant.

data from government reports. Furthermore, the number of participants with borderline antibody status in our study was nearly 3 times that of participants with a positive antibody status.

Recently, several studies have reported that the population-wide seroprevalence of SARS-CoV-2 antibodies was higher than expected, based on the number of confirmed cases (7, 8). For example, in Kobe city, Japan, 3.3% of outpatients tested SARS-CoV-2 IgG antibody positive (8). Using quantitative methods, our results showed there were >3 times the number of people sensitized with SARS-CoV-2 than those found to have positive SARS-CoV-2 IgG antibody. Given that the pathogenicity of SARS-CoV-2 appears to be similar to SARS-CoV, most patients infected with SARS-CoV-2 will express specific IgG antibodies within 1 week−3 months after infection (9). In light of this timeline of seroconversion, the study participants with a positive or borderline antibody status were likely to have been exposed to SARS-CoV-2 between December 2019 and March 2020. These findings suggest that COVID-19 was already present in Kyoto at the early stages of pandemic. The period between December and March is a time of heightened tourist activity in Kyoto, in particular, involving tourists from China and Taiwan who are celebrating the Chinese New Year spring festival. After the spring festival, on March 5, 2020, the Japanese government implemented a strict ban on travelers arriving from China.

According to epidemiological data provided by the WHO (1) and by Johns Hopkins University (4), incidence and case-fatality rates in major European countries (Germany, United Kingdom, France, Italy, and Spain) and the United States are much higher than those in major Asian countries (China, Japan, South Korea, and Taiwan). There are several possible explanations for this phenomenon. Differences in lifestyle and behavioral habits between Western and Asian populations might explain some of the variability in these rates. Some studies have shown a correlation between universal BCG vaccination policy, and morbidity and mortality associated with COVID-19 (10, 11). Although this hypothesis has resulted in clinical trials to evaluate the efficacy of BCG vaccination against COVID-19 (NCT04327206 and NCT04362124), restricted basic and clinical evidence makes this association difficult to evaluate. Meanwhile, other authors have suggested that differences in viral genotypes and virulence may affect morbidity and mortality associated with COVID-19; however, this explanation requires further elaboration. The National Institute of Infectious Diseases of Japan reported that the first wave of the COVID-19 pandemic emerged from Wuhan, China, and flattened toward the end of March; however, a second wave emerged from European countries, spreading across Japan after the end of March (12). Nevertheless, even during the second wave, Japan retained its much lower morbidity and mortality rates compared to those of Western countries, as reported at the end of April 2020 (13). Kamikubo and Takahashi hypothesized that the pre-pandemic spread of a low-virulence type of SARS-CoV-2 and subsequent exposure to a mild-virulence type of SARS-CoV-2 induced herd immunity, which reduced the severity of a high-virulence type of SARS-CoV-2 in Japan (14). The findings concerning borderline antibody titers in the present study offer some support

for this hypothesis. In this study, participants with a history of seasonal common cold from winter 2019 to early spring 2020 had a significantly higher SARS-CoV-2 antibody titer than those with no such history, which might have resulted from pre-pandemic exposure to low- and middle-virulence types of SARS-CoV-2. Importantly, a history of exposure to seasonal cold should not be interpreted as equivalent to a history of subclinical exposure to SARS-CoV-2 virus, since this virus was supposedly absent from the environment during the last season of the common cold. Further research is required to verify this hypothesis.

This study had several limitations. First, this was a single-center study; therefore, selection bias might have affected our findings. Second, the small sample size restricted the statistical power of our analyses. Third, as our participants were recruited from departments where exposure to COVID-19 was more likely, the reported seroprevalence might be an overestimate relative to that of workers in other departments or within the general population. Fourth, because the COVID-19 pandemic is ongoing, a significant proportion of available research results that we have referred to might be premature. Fifth, we did not evaluate the IgM antibody. A negative serum for IgG antibody might nevertheless contain a specific IgM antibody, especially as samples were obtained at the early stages of the pandemic. The interpretation of these results would likely be affected by the quantitation of IgM.

In conclusion, our study findings indicated a relatively high frequency of healthcare workers with a positive or borderline SARS-CoV-2 antibody status in the southern part of Kyoto city, an area frequented by tourists. Our results suggest that COVID-19 might already have been present in Kyoto at the early stage of the pandemic. Several previous studies have evaluated SARS-CoV-2 antibody profiles in patients with COVID-19 (15–17); however, our study is the first to quantitatively evaluate antibody levels in healthcare workers involved with patients during the COVID-19 pre-pandemic period. Serial evaluation of SARS-CoV-2 IgG antibody status is likely to reveal risk factors associated with COVID-19 susceptibility and mechanisms of disease spread. Finally, these results should be approached with caution, as there remains a lack of evidence regarding the role of antibodies present after recovery from COVID-19 in developing immunity against subsequent infections.

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 NHO Kyoto Medical Center IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KF and SK designed the study and collected blood samples. KF, SK, OK, HH, and TO recruited the participants and collected the data. TT, NS-A, and AY managed the ELISA tests and interpreted the results. KF, SK, and OK participated in the statistical analysis. KF and SK drafted and revised the manuscript. All authors contributed to the article and approved the submitted version.

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OPINION
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Preparing for Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Self-Testing Implementation: Lessons Learned From HIV Self-Testing

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INTRODUCTION

The number of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) cases and associated death continue to rise globally. Widespread testing for SARS-CoV-2 infection is crucial in order to identify individuals who need to need to be isolated, thereby reducing their chances to infect others and allowing them to seek treatment earlier which can prevent further negative health outcomes and mortality (1). Currently, the most common testing method for SARS-CoV-2 diagnosis is Real-Time Polymerase Chain Reaction (RT-PCR) from nasopharyngeal, throat or saliva specimens (2). However, SARS-CoV-2 testing has been hampered in many countries due to inadequate test kits, uncomfortable testing procedures, shortages of personal protective equipment (PPE) for health care workers, and low demand among people to seek testing for SARS-CoV-2 at health facilities (3–7).

In response, the United States Food and Drug Administration (FDA) provided Emergency Authorization Use for several SARS-CoV-2 self-sampling kits (SARS-CoV-2SS) that allow individuals to self-collect nasal swabs and saliva specimens and send to a lab for testing (8). Other efforts to increase testing include drive-through methods that include both self-sampling and health care- collected samples (9, 10). The National Institute of Health has also launched the Rapid Acceleration of Diagnostics (RADx) program to accelerate the development of, scale up, and deploy innovative point-of-care of technologies, support the scale-up of more advanced technologies, and nontraditional approaches for testing as well as establish community-engaged implementation projects to improve access to testing in underserved and vulnerable populations (11). Similar research and programmatic activities to increase testing capacity SARS-CoV-2 are also being implemented in other regions (12–16).

The efforts to increase testing capacity for SARS-CoV-2 diagnosis testing will be enhanced with the availability and widespread promotion of self-sampling and eventually SARS-CoV-2 self-testing (SARS-CoV-2ST) (17–19). The benefits of self-sampling and self-testing include their abilities to help decentralize care, promote social distancing, conserve PPEs, address transportation and privacy barriers for individuals who do not want to test at a clinic or a drive-through setting, and

reach more individuals who are not reached with current testing modalities (12, 17, 20). Unlike self-sampling, SARS-CoV-2ST will allow individuals to receive their results at home without the need to ship their specimens to a laboratory for testing (21, 22). To our knowledge, there is only one FDA-approved SARS-CoV-2ST diagnostic kit for individuals to use and receive their results at home as of November, 17, 2020 (23). However, a number of other different at-home SARS-CoV-2ST kits are being developed and evaluated to either detect antibodies or active viral infections (24). Antibody SARS-CoV-2ST kits reveal markers of immune response that show up in blood more than a week after a person has been infected whereas active infections will be detected with nucleic acid SARS-CoV-2ST kits through the virus' genetic materials (24, 25).

As researchers, federal health agencies, and public health practitioners prepare to implement SARS-CoV-2ST, lessons learned from the global implementation and scaling up efforts for HIV self-testing (HIVST) can prove useful. We describe research related to questions that emerged regarding HIVST and how they are similar or different to the questions that will need to be addressed for SARS-CoV-2ST before this crucial strategy can be implemented and scaled up successfully. We also discuss the findings of the first antibody SARS-CoV-2ST acceptability and usability study (22) and the public health implications of and recommendations people who obtain a positive SARS-CoV-2ST result for antibodies or active viral infections. Lastly, we identify key structural inequities in communities that are most affected by COVID-19 that need to be addressed during future SARS-CoV-2ST implementation efforts.

IN-HOME HIV SELF-TESTING HISTORY

In 2012, the US FDA approved the first over-the-counter rapid HIVST kit, the OraQuick In-Home HIV Test which allows users to test for antibodies using saliva sample, similar to the new saliva-based SARS-CoV-2ST kit (26), and receive a preliminary result at home in 20 min (27). The benefits of HIVST include privacy, an increase of access to HIV testing, earlier diagnosis of HIV, confidentiality of results, and reducing queues for facility-based HIV testing (28). HIVST can also help bypass social barriers such as stigma and discrimination that deter people from accessing facility-based HIV testing (28). Since the approval of HIVST, several questions emerged about its accuracy, acceptability, feasibility, the lack of pre-and-post-test counseling, whether users would seek a confirmatory test, and link to care (29). There is now overwhelming evidence that HIVST is accurate, acceptable, feasible, and effective with minimal social harms (29). As a result from these studies, the World Health Organization (WHO) now recommends HIVST as one of the testing strategies for HIV prevention efforts (30). These studies, including our own (31–39), have provided evidence on different distribution strategies from online platforms, peers to sexual partners, community health workers (40–42). Similarly, these studies have assessed different approaches to verify HIVST results either through direct supervision by health provider, requesting participants to return used HIVST kits, electronic transmission of photographs, or using Bluetooth sensors (43).

ANTIBODY SARS-CoV-2 SELF-TESTING ACCEPTABILITY AND USABILITY

In the first published SARS-CoV-2ST study, researchers in England examined the acceptability and feasibility of two types (i.e., Guangzhou Wondfo Biotech Co Ltd and Fortress Orient Gene Biotech Co Ltd) of SARS-CoV-2ST lateral flow immunoassays (LFIAs) or rapid point-of-care tests that use a blood sample from a finger-prick and produce a self-read result after 10 or 15 min for detection of SARS-CoV-2 antibodies (Immunoglobin M and Immunoglobin G) (22). Participants received LFIAs by mail and recorded their interpretation of their results in an online survey with the option to upload a photograph of the results (22). To assess participants' ability to correctly interpret the test results, a clinician reviewed all the samples of the uploaded photographs that were reported as positive and unable to read as well as a random sample of 200 participant-reported negative or invalid results. Acceptability in the national study was high with 99.3% (8,693/8,754) and 98.4% (2,911/2,957) of participants reporting that they attempted to use the two LFIA types (22). Feasibility was also high in the pilot and national studies with 86.5% (225/260) of pilot participants and 97.5 and 97.8% of participants in the national study reporting they completed all the steps for the tests successfully, respectively (22). The majority of participants 85.8% (7,272/8,475) and 84.8% (2,416/2,848) uploaded the photographs of their results with substantial agreement between participant and clinician interpreted results for both test types (22). However, there were differences between some of the self-reported results and those reported by the clinician and some participants reported some difficulties with using the lancet and pipette of the test kits (22).

DISCUSSION

The scientific and clinical fields involved in HIV prevention have provided extensive experience, amassed over decades, regarding the value of testing and the added benefits of in-home self-testing (30). This experience can be brought to bear for SARS-CoV-2ST, including strategies that can help avoid repeating the pitfalls encountered during the path toward implementing and scaling up HIVST. For example, limited evidence on the public health impact and cost-effectiveness of HIVST, uncertain levels of consumer demand and concerns about potential social harms amongst others delayed the roll out of HIVST (44). Global efforts and collaborations between WHO, researchers, local health agencies, donors, and policy makers have addressed some of these limitations. Initiatives such as but not limited to the Self-**T**esting **AfR**ica (STAR), the largest HIVST implementation science project to date (44), 4 Youth by Youth crowdsourced HIVST interventions (45, 46), and **S**elf-**T**esting **E**ducation and **P**romotion (STEP) project (28, 33), have created a market for HIVST in sub-Saharan Africa. These initiatives combined with other studies around the globe have accelerated access to

HIVST by gathering the necessary acceptability, feasibility, and fidelity data, creating an enabling environment with regards to HIVST policies, generating diverse demand through multiple distribution channels, and creating advocacy for additional financing, as well as accelerate market entry for suppliers at affordable and sustainable prices (44, 45, 47–49). Similar initiatives are needed swiftly to gather additional accuracy, acceptability, feasibility, and programmatic data to encourage policy makers, donors, and local health agencies to support for SARS-CoV-2ST implementation and scale up.

While the findings from the first antibody SARS-CoV-2ST acceptability and usability study in England were promising, some participants reported difficulties using the pipette and applying the blood drop to the cassette (22). Thus, more studies are needed to assess ease of use of SARS-CoV-2ST and how to provide the support that potential users may need. One potential strategy to support SARS-Co-V-2ST users is online real-time instructions, which has been evaluated with HIVST and found to be acceptable and successful in increasing HIV testing (50). A recent SARS-CoV-2SS study has shown that participants are willing to self-collect specimens [saliva, oropharyngeal swab (OPS), and dried blood spot (DBS) card] at home while being observed by a clinician through a telehealth session (51). A total of 159 participants were mailed kits and 153 scheduled a video appointment with the majority of the ($n = 143$) completing all three self-collected samples (52). A similar approach can be assessed for SARS-CoV-2ST to move beyond simply observing potential users to providing additional instructions and post-test supports in the self-testers receive a positive result.

The public health implications of potential positive antibody SARS-CoV-2ST results extend beyond treatment since individuals with antibodies for SARS-CoV-2 are considered to have recovered from COVID-19 and should less symptomatic. However, a positive antibody SARS-CoV-2ST result will allow individuals, including skeptics, to learn indeed whether they had a COVID-19 infection—increasing their perception of risk and potentially positively influencing future behaviors to prevent COVID-19 re-infection. Alternatively, a positive antibody SARS-CoV-2ST result has the potential to help individuals make informed decisions about their risk levels as they consider returning to work or interact with infected individuals (19). In addition, antibody SARS-CoV-2ST results can help identify qualified individuals who may be interested in donating blood for convalescent plasmaexternal icon as a treatment for COVID-19. The Centers for Disease Control and Prevention (CDC) describe general recommendations for positive antibody test results that people who receive a positive antibody SARS-CoV-2ST result can follow such as continuing with normal activities, washing hands often, avoiding close contact, wearing a mask when around others, and continued use of PPE if the person is a health care worker or first responder (53, 54). On the other hand, a nucleic acid SARS-Co-V2ST kit will provide individuals with active infections an instant preliminary positive result that can allow them to follow recommendations for people who are sick such as self-isolate in order to prevent potential transmission and seek confirmatory diagnostic testing and early treatment (19).

We must also be mindful that the SARS-CoV-2 transmission profile is not the same as HIV and it presents immense new challenges that will require us to envision and test new ways for its easy and reliable detection and its equitable access among marginalized racial groups, sexual minority ages, incomes and the multitude of intersections between them. As is the case with HIV, Black communities are disproportionately affected by COVID-19 (55). This population has experienced extensive barriers to facility-based HIV testing and HIVST (35) and the pattern seems to be repeating for COVID-19. We need novel ways to ensure the most vulnerable populations, who are also the most likely to be infected with and die from COVID-19, have access to affordable SARS-CoV-2ST. It is important for investigators who are validating SARS-CoV-2ST kits in community settings to design the studies in a way that ensures adequate representation from the populations most vulnerable to COVID-19 infection and mortality. To promote adequate representation of special populations (elderly aged 65 years and older, youth aged 17 years and younger, Black, Latinx, Tribal communities indigenous to North America, and Spanish-speaking and francophone populations) in SARS-CoV-2ST research and programs, there are several lessons learned from HIV research and HIVST that can be applied to COVID-19.

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DFC conceived the idea for the manuscript and drafted it before all authors reviewed and edited the manuscript into this final form. All authors contributed to the article and approved the submitted version.

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Effect of Face Masks on Interpersonal Communication During the COVID-19 Pandemic

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Interpersonal communication has been severely affected during the COVID-19 pandemic. Protective measures, such as social distancing and face masks, are essential to mitigate efforts against the virus, but pose challenges on daily face-to-face communication. Face masks, particularly, muffle sounds and cover facial expressions that ease comprehension during live communication. Here, we explore the role of facial expressions in communication and we highlight how the face mask can hinder interpersonal connection. In addition, we offer coping strategies and skills that can ease communication with face masks as we navigate the current and any future pandemic.

Keywords: SARS-CoV-2, coronavirus, communication, social distancing, pandemic (COVID-19), pandemic

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INTRODUCTION

The COVID-19 pandemic has severely affected the way people communicate with each other. Precautionary measures to limit the spread of the virus necessitated a shift in the communication paradigm when it comes to greetings and handshakes. The arising situation required people to adopt salutations that do not entail physical contact, such as the "peace sign," the "hand on chest," and the "namaste" (1). In addition, emphasis on personal spaces and social distancing markedly increased, with telecommunication witnessing a huge rise, as business meetings, conferences, and educational activities shifted to virtual communication via social applications, such as Zoom, Cisco Webex, Skype, and Microsoft Teams.

Face-to-face communication, specifically, was majorly affected by the pandemic. The need for face masks, as an important protective measure to decrease the spread of the virus, had a huge toll on interpersonal communication. Facial expressions and gestures play a major role in facilitating interpersonal communication, comprehension, and the delivery of intended messages. As such, wearing face masks hindered the ability of seeing and understanding people's expressions during conversations, and decreased the impact of communicated material.

In this piece, we explore the role of facial expressions in communication and we highlight how the face mask can affect it. In addition, we offer coping strategies to enhance the quality of interpersonal communication while wearing protective face masks.

ROLE OF FACIAL EXPRESSIONS IN COMMUNICATION

Facial expressions play a prominent role in communication and relay of emotion across individuals. People perceive facial expressions off one another, and this helps them forecast events and

situations, and develop responses to them (2). The face, as an anatomical figure, can be separated into upper, middle, and lower portions, with each playing an important role in expressing the feelings and moods of an individual (3). For example, actions like smiling and grimacing involve lower facial structures, like the mouth, the lips, and the cheeks, and these are often included in our daily conversations.

Facial expressions of different emotions involve action units, or elementary changes in facial appearance recognized by the Facial Action Coding System, which is a system that taxonomizes human facial movements by their appearance on the face. These facial expressions are produced by a set of facial muscles (4). The middle face involves the "nose wrinkle," an action unit that wrinkles and pulls the skin upward along the sides of the nose; this is used to convey disgust (4, 5). The lower face involves multiple action units, and these include the "chin raiser," the "lip stretcher," the "lip tightener," the "lips part," and the "jaw drop," and each is associated with a set of facial muscles that convey a specific emotion (4, 5). The "chin raiser" pushes the boss of the chin and the lower lip upward, while the "lip tightener" causes lips to appear narrower; both action units are used to convey anger (4, 5). The "lips stretcher" stretches lips horizontally, and the "lips part" separates them to a limited extent; both action units are used to convey fear (4, 5). In addition, the "jaw drop" parts lips so that the space between the teeth is visible and this is used to convey surprise (4, 5).

The middle and lower face are noted to be very influential with regards to emotional recognition. Kestenbaum explored the modes of processing of emotional expression in children and showed that the mouth can be used to recognize a neutral expression and is best for recognizing the emotion of happiness (6). Gagnon et al. investigated children's ability to recognize fear, surprise, disgust, and anger based on information from the upper, middle, or lower face, and found that children can recognize fear, surprise, and anger using expressions involving the lower face, and disgust using expressions involving the middle face (5). While the upper face is also pivotal for the development of emotional expressions, the roles of the middle and lower face cannot be understated.

MASKING FACIAL COMMUNICATION

The high infectivity of SARS-CoV-2 and the increasing rates of COVID-19 infection pushed physicians and health experts to recommend wearing facemasks during the pandemic. This measure combined with social distancing and handwashing helps in slowing the spread of the virus and decreasing its transmission, especially between people that are designated as asymptomatic carriers (7, 8). Previous studies comparing non-fit-tested P2 masks, surgical masks, and no masks in fighting influenza for households had shown that masks may reduce the transmission of viruses during pandemics (9).

Despite its crucial protective role, the face mask poses challenges on daily face-to-face communications. Interpersonal communication describes the interaction between two individuals or more through oral or physical (gestures) interactions. Proper application of the protective mask involves covering the mouth and the nose, which muffles sound and makes it challenging to understand speech and some higher-pitched voices. Furthermore, face masks eliminate the roles of the middle and lower face in emotional expression, rendering its action units invisible to the receiving individual (**Figure 1**). For example, in the physician-patient setting, positive facial expressions play an important role in decreasing the patient's anxiety (10). Therefore, the physician-patient relationship is affected by wearing face masks. Covering the face will reduce the ability of determining the patient's feelings and emotions and affect the physician's measured response to the situation (10). Likewise, the physician's expression of empathy can be missed by the patient. Furthermore, people with special needs and hearing disabilities rely on sign language to communicate. Covering the lower part of the face (nose, cheeks, mouth, tooth, nose, and chin) will adversely affect their understanding of communicated information and make them feel more disabled and ostracized. As a result, emotional perception decreases and the role of the upper face in emotional expression increases in significance.

Nonverbal communication, such as facial gestures and expressions, constitutes 55% of our overall communication (11). The eyes and the mouth are the two main organs that help in reading other's faces. By wearing face masks, people are inclined to focus more on the eyes to be able to understand the facial expressions intended. Eye contact can be used to show empathy and concern for others, to manage feelings, to express interest, or to help with communication. Nevertheless, prolonged eye contact can result in uncomfortable feelings sometimes (11), as it can magnify actual interest in communicated material or convey signs of aggression.

There are a number of populations globally that veil the face for religious or cultural reasons (12). In addition, surgical or cloth face masks have been worn in several East Asian countries since the early 20th century (13). During the 1918 flu pandemic, face masks were commonly worn around the globe (14). After Japan's Great Kanto Earthquake of 1923, firestorms and thick smoke and ash in the air also necessitated face masks. Singapore and Hong Kong suffered flu pandemics in the 1950s and 1960s, and the SARS outbreak of the early 2000s was particularly troublesome for China, Hong Kong, and Taiwan (15). Wearing a face mask became a cultural sign of respect and a social contract toward others. Nevertheless, in the West, the subtraction of nose, mouth, and cheeks during interpersonal communication will necessitate further adaptation.

ENHANCING COMMUNICATION WITH FACE MASKS

Given the importance of face masks in mitigating the spread of COVID-19, communication adjustments are needed to adapt to the new "normal." Here, we highlight coping measures that can enhance the quality of interpersonal communication while wearing a face mask:

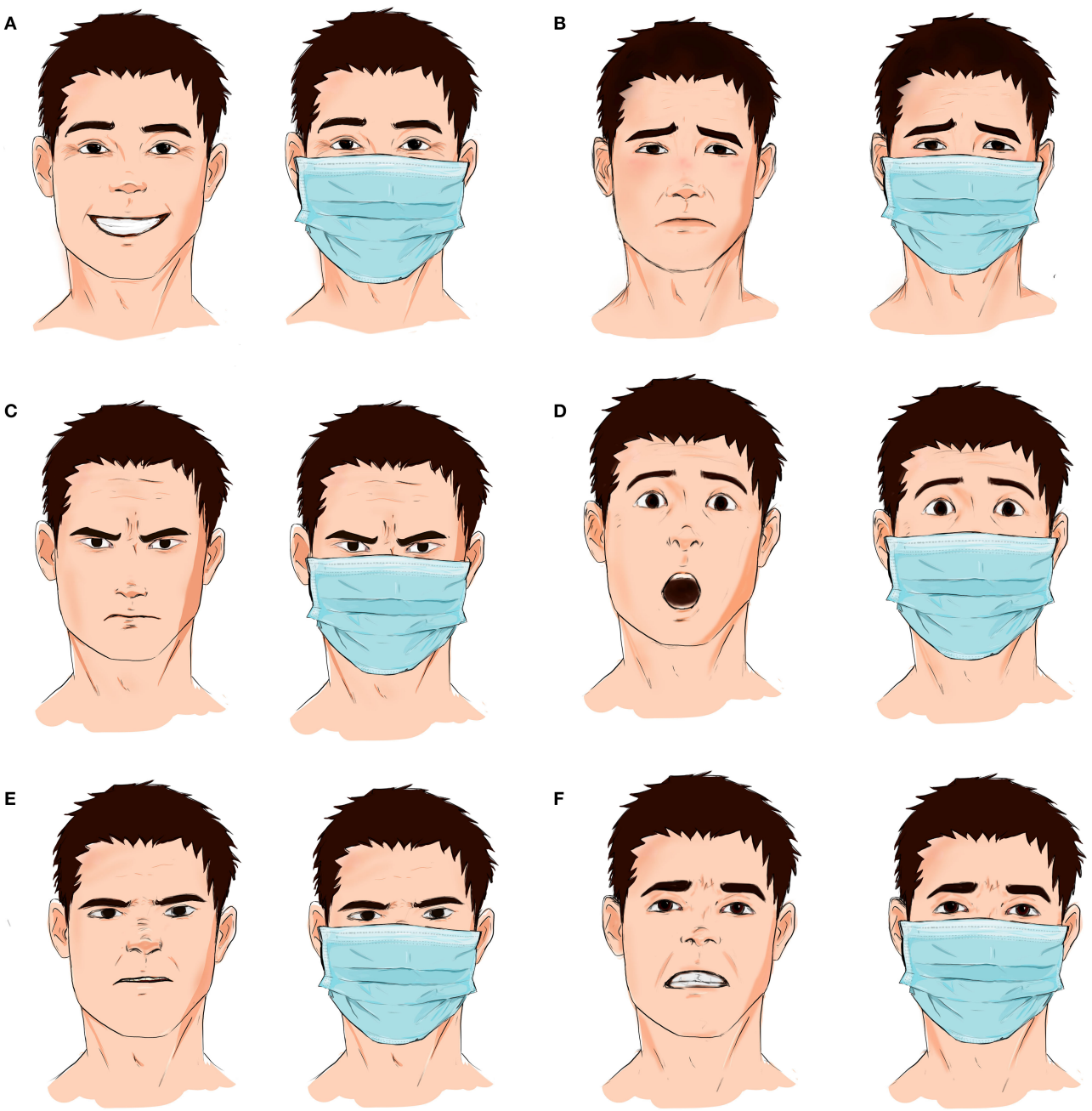

FIGURE 1 | Face masks cover the middle and the lower portions of the face. As such, facial expressions involving the mouth, lips, teeth, and nose are masked during interpersonal communication. **(A)** Happiness is usually perceived when the corners of the lips rise upward. With face masks, happiness can be caught on the face by focusing on the wrinkles at the edge of the eyes. **(B)** Sadness involves movement of the eyebrows, the nasolabial folds, and the corners of the lips; however, the last two are masked by face masks. **(C)** Facial expression of anger emphasizes the downward and central movement of eyebrows, the glaring eyes, and narrowing of the corners of the lips, with the latter getting covered by face masks. **(D)** Expressions of surprise and shock are usually formed of elevated eyebrows and a raised upper lip; only the latter is covered by protective masks. **(E)** Nose wrinkling and raising of the upper lip convey feelings of disgust; however, face masks cover both expressions. **(F)** Feelings of guilt are usually portrayed by slightly upping eyebrows together and stretching the mouth, with the latter getting covered with a face mask.

1. **Raising awareness on the use of face masks and acknowledging the communication challenges that arise as a result in an objective manner**.

It is important for experts to address the underlying problems and concerns regarding face masks while highlighting their importance as protective equipment

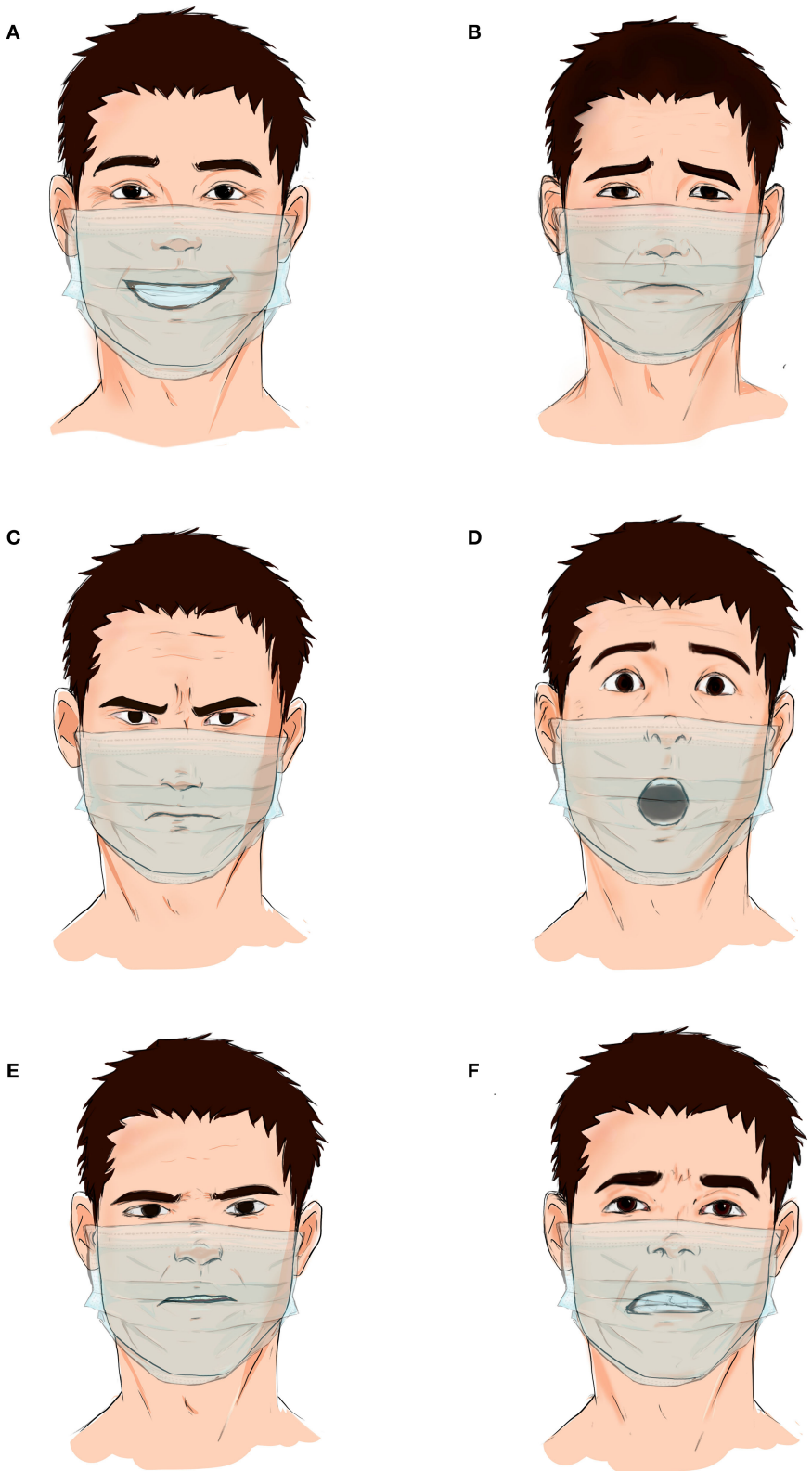

FIGURE 2 | Transparent protective face masks and face shield preserve the importance of facial expressions during interpersonal communication. Feelings of happiness **(A)**, sadness **(B)**, anger **(C)**, surprise **(D)**, disgust **(E)**, and fear **(F)** can easily be noted and picked up through the individual's facial reactions and expressions.

against infection (16). This will ease people's acceptance of and commitment to the face mask. Scientists and experts can prevent the spread of false assumptions and empower people by raising awareness on several health challenges and topics through social media, interviews, and podcasts (16).

2. **Utilizing and recognizing the upper face through the eyebrows, eyes, and upper cheeks during interpersonal communication**.

   For example, closing the eyes when agreeing and raising eyebrows when opposing can be adopted in interpersonal settings. The eyebrows, specifically, have received little attention in communication research. Past work has examined the role of eyebrows in emotional expression, nonverbal communication, facial aesthetics, and sexual dimorphism (17–19). For face recognition, the eyebrows may be at least as influential as the eyes. The absence of eyebrows in familiar faces leads to a significant disruption in recognition performance (20). In fact, a significantly greater decrement in face recognition is observed in the absence of eyebrows than in the absence of eyes (20).
3. **Emphasizing the importance of non-verbal communication, such as body language, during communication**.

   For example, people can express their ideas using hand gestures to facilitate the communication process. Non-verbal communications are essential in facilitating the communication process, have a vast influence on the social environment, and can come in different forms, such as facial expressions, body movements, and eye messages, which can support or substitute verbal communication (21).
4. **Paying more attention during interpersonal settings and facing the communication partner directly**.

   This ensures that the communicator has the receiver's attention while nothing is blocking the visual field between them. Synchronous communication is an intended and direct form of communication, which focuses on capturing attention and conveying the needed message. It has been reported that people who communicate through synchronous communication, such as phone or face-to-face communication, perceive the urgency of a situation quicker than those receiving official messages through asynchronous channels, such as text messages (22).
5. **Talking louder and slower in quieter settings**.

   Articulating speech and increasing its volume in a calm setting helps communicators overcome the sound muffling that can result from the face mask. The hierarchy hypothesis asserts that when an individual initially fails to reach social goals through communication, they will continue to try to attain them, but will alter their speech rate and vocal intensity (23).
6. **Relying more on telecommunication for interpersonal interactions**.

   Technological advancements can play a central role in facilitating live connections and interactions between individuals (24). Telecommunication via Skype, Zoom, Facetime, and Cisco Webex was key in keeping the educational, economic, and health sectors alive during the outbreak.
7. **Manufacturing transparent face masks or face shields**.

   People will be able to see each other's facial expressions and emotions without threatening their personal protection (**Figure 2**). This will also allow people with special needs to communicate easily and understand conversations. The elderly and individuals with hearing impairment rely heavily on facial expressions for communication. Cloth and surgical facemasks hinder their ability to understand and indulge in meaningful conversations (25). The use of transparent face masks will help those individuals read lips and have proper dialogues.
8. **Conducting cross-sectional surveys exploring the effect of face masks on communication**.

   This will help in measuring the impact of the pandemic and wearing face masks on interpersonal communication, quantitatively and qualitatively (26, 27). Research must take into account the cultural differences in communication and the impact of face masks on different societal groups.

CONCLUSION

For the time being, face masks are here to stay, as we continue to make efforts to stop the spread of SARS-CoV-2. Nevertheless, identifying the problems and challenges that affect healthy communication while wearing face masks is vital to adapt better to the ensued norm. In addition, developing coping strategies and skills that can ease our communication with face masks is crucial in our efforts to navigate the COVID-19 pandemic and any other pandemic that might erupt in the future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

NM and JF conceived the study. NM, MF, and JF wrote the first draft. All authors contributed to the article and approved the submitted version.

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REVIEW
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Bee Venom—A Potential Complementary Medicine Candidate for SARS-CoV-2 Infections

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is characterized by severe cytokine storm syndrome following inflammation. SARS-CoV-2 directly interacts with angiotensin-converting enzyme 2 (ACE-2) receptors in the human body. Complementary therapies that impact on expression of IgE and IgG antibodies, including administration of bee venom (BV), have efficacy in the management of arthritis, and Parkinson's disease. A recent epidemiological study in China showed that local beekeepers have a level of immunity against SARS-CoV-2 with and without previous exposure to virus. BV anti-inflammatory properties are associated with melittin and phospholipase A2 (PLA2), both of which show activity against enveloped and non-enveloped viruses, including H1N1 and HIV, with activity mediated through antagonist activity against interleukin-6 (IL-6), IL-8, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α). Melittin is associated with the underexpression of proinflammatory cytokines, including nuclear factor-kappa B (NF-κB), extracellular signal-regulated kinases (ERK1/2), and protein kinase Akt. BV therapy also involves group III secretory phospholipase A_2 in the management of respiratory and neurological diseases. BV activation of the cellular and humoral immune systems should be explored for the application of complementary medicine for the management of SARS-CoV-2 infections. BV "vaccination" is used to immunize against cytomegalovirus and can suppress metastases through the PLA2 and phosphatidylinositol-(3,4)-bisphosphate pathways. That BV shows efficacy for HIV and H1NI offers opportunity as a candidate for complementary therapy for protection against SARS-CoV-2.

Keywords: bee venom, complementary medicine and alternative medicine, SARS-CoV-2 (2019-nCoV), pharmokinetics of bee poison, COVID-19 and complementary medicine, bee venom in clinical trials

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causal agent of coronavirus disease 2019 (COVID-19), a respiratory infection that emerged in Wuhan province of China in late 2019 (1, 2), becoming a global pandemic in 2020. By April 1, 2020, global mortality rates were reaching 5% (3). Within weeks, global mortality rates increased to 6.7% (5% for the African region, 4.4% for the Americas, 5% in the Eastern Mediterranean region, 4.4% for Southeast Asia, 8.9% for the European region, 4.4% in the Western Pacific region) (4). The public health challenges imposed by COVID-19 are immense, including management of the high number of asymptomatic cases (5). The disease has exacerbated existing socioeconomic disparities, especially in vulnerable communities in developing countries, including Africa, that have disproportionately been affected by the consequences of extreme preventative measures (6).

Severe SARS-CoV-2 infections are characterized by cytokine storm syndrome, hyperinflammation, and multiorgan failure (2, 7). Host cells are infected through the angiotensin-converting enzyme 2 (ACE-2) receptor (8, 9), associated with both innate and acquired immunity (10). ACE2 is considered to enhance viral replication and potentiate host cell invasion (10) and is a major component of the renin-angiotensin-aldosterone system (RAAS), interacting with enzymes of the CVS to cascade cardiovascular disease (11, 12). ACE2 may be the reason SARS-CoV-2 patients require pharmacological thrombosis prophylaxis (13, 14); the pathogenesis of SARS-CoV-2 involves viral binding to epithelial cells and local propagation with minimal innate immune response (15). The second stage of infection exhibits increased viral propagation, an active immune response, viral spread to the lower respiratory system, and may include cardiovascular and digestive systems (16). The third stage involves hypoxia, infiltration of the entire respiratory system, and finally acute respiratory distress syndrome (ARDS), which is potentially fatal (15). SARS-CoV-2 is associated with coagulopathies, thrombotic events (17) and lymphocyte exhaustion (18).

At present, there is no globally accepted alternative medical treatment protocol against SARS-CoV-2, although administration of polyclonal antibodies shows some promise (19). The efficacy of chloroquine and its derivatives continue to be explored for prevention of COVID-19 (20, 21) as well as Famotidine, an antiulcer drug, administered at high dosage (10× normal) for 14 days for control of SARS-CoV-2 infection (7). Remdesivir, which has previously been used to manage the Middle East Respiratory Syndrome-Cronavirus (MERS-CoV) has been explored as a candidate drug against SARS-CoV-2 (22–24). Combinations of Lopinavir/ritonavir, commonly used to prevent HIV/AIDS are also under investigation for efficacy against SARS-CoV-2 (25, 26). Neutrophil extracellular traps (NETs), common in snakes, insects, arachnids and myriapods have also been considered for SARS-CoV-2 (27, 28). Bee venoms (BVs) can act as ACE2 inhibitors or angiotensin-receptor blockers (ARBs), although studies on BVs and SARS-CoV-2 are sparse. Snake venom is known to act through phospholipase A2 (PLA2) to produce arachidonic acid, which induces hypotension (29). In humans, hymenoptera venom lowered key parameters in the RAAS (30). A combination of BV and propolis has been associated with hypotension in laboratory animals through a reduction in serum angiotensin levels (31), demonstrating a close relationship between BV and the ACE2 pathway.

BEE VENOM THERAPY

Bee venom (BV) therapy dates back to the era of Hippocrates, where it was deployed to alleviate joint pain and arthritis (32). In contemporary medicine, BV is deployed for treatment of multiple sclerosis (33), arthritis and Parkinson's disease (34). Activity is based on anaphylactic reaction benefits on metabolism and on organelles, especially those of the respiratory system (35). Allergens may offer benefits against COVID-19 (36, 37); BV can induce elevation of specific IgE and IgG antibodies (38) and leads to production of IgE antibodies, which can respond to a variety of antigens (39) (**Table 1**). Although IgE are responsible for allergic outbursts, they also offer host protective roles over a wide range of allergens (39). BV can act as an adjuvant when combined with Toll-like receptor (TLR) ligands (40) and modulate the immune system, enhance the differentiation of foxP3-expressing cells and increase circulating regulatory T cells (41, 42). BV triggers an increase in CD25, CD4+ T cells and foxP3 mRNA, resulting in a shift in the BV-specific IgG4/IgE ratio (43). BV regulates the immune response and phsiopathological changes (44) and supports clinical observations in Apitherapy, where beekeepers were shown to mount immunity against COVID-19 in Wuhan province, PR China (45).

The *bv*PLA2 can trigger mast cell maturation (46), is important in cell signaling and for production of key lipids and may act as a receptor ligand (47). PLA2 can inhibit the flow of inflammatory cells to targets (48). BVs may lead to lasting induced tolerance to related allergens (49), as a function of reducing IgG4 and activating IL-10, modulating the immune system and inducing deviation from TH2 to TH1(50–52). Melittin (APi M 1) can be used to develop mimotopes (49). APi M 10 (icarapin), a BV component, activates effector cells of honey bee venom allergic patients (53). Since IgE possesses an epitope for APi M 10, this may offer opportunity for adjuvant development. BV antigens can be used as adjuvants in the treatment of pain (54) and the action of melittin on cell membrane pore formation (54, 55), leading to apoptosis serves to strengthen adjuvant properties. BV also has antiviral properties (56). BV can desensitize mast cells and basophils (57) and suppress innate lymphoid cells. BV materials can inhibit proteinsynthesis, induce angiogenesis (58) and activate caspase-3-8-9 (59) (**Table 1**).

CONDITIONS THAT ALLOW BEE VENOM USE DESPITE ITS TOXICITY

Bee venom is cytotoxic at high doses, however, non-cytotoxic concentrations of BV range from 1 to 3 μg/ml, show significant therapeutic potential (60). Low doses, controlled concentrations,

TABLE 1 | Bee venom enzymes and peptides and their functions in mammalian systems.

References	Component	Compound	Properties/mode of action	% BV	Properties / mode of action for mammalian analog
Dams and Briers (130) Wehbe et al. (103)	Enzyme	Hyaluronidases	Breakdown of hyaluronic acid to increase tissue permeability, accelerated distribution of toxins "spreading factor" Increases bioavailability of drugs, used in therapy of extravasations, management of complications associated with aesthetic injection of hyaluronic acid-based fillers	1–3	Ubiquitous in somatic tissues Six forms in humans (HYAL1-4, HYAL-P1, and PH-20) PH-20 has highest activity, highest in testicles and involved in fertilization process Breaks down tissue hyaluronic acid and chondroitin sulfate increasing tissue permeability e.g., of sperm during adhesion and penetration to cumulus oophorus
Boens et al. (131) Szulc and Bauer, (131, 132)	Enzyme	Acid phosphatases	Hydrolyzing monophosphate esters to release products associated with pain and inflammation, Potent releaser of histamine in human basophils, thus relevant in allergic process	1	In prostate, erythrocytes, macrophages, platelets, bones, spleen, lungs, testes Hydrolyzes phosphate Enzyme dysregulation is associated with pathophysiological conditions e.g., prostatic acid phosphatase (cancer of prostate); tartrate-resistant acid phosphatase (abnormal bone resorption in osteoporosis) Released by platelets during clotting. Binding to α2-macroglobulin leads to a reduction in its activity
Murakami et al. (133) Stahelin (134)	Enzyme	Phospholipase A2 (PLA_2)	Most lethal enzyme in BV Formation of melittin-PLA_2 complex referred to as the bee hemolytic factor that cleaves cellular membrane phospholipids and cellular lysis Potent allergen Trypanocidal, antibacterial, neuronal protection, anti-tumor properties. Hepato-protective in acetaminophen-induced liver damage	10–12	Ubiquitous in many cells and tissues (pancreas, spleen, liver, intestines, spleen, lung, heart, testis, brain, macrophages, inflamed tissues, and inflammatory cells). Involved in inflammation: generation of precursors of eicosanoids (prostaglandins, leukotrienes), platelet-activating, factor; cell activation via a specific receptor; digestion and metabolism of dietary phospholipids; host defense and signal transduction, exocytosis, antimicrobial activity, anticoagulation, ischemia, brain development Overproduction of lipid mediators associated with PLA_2 activity can cause inflammation and tissue disorders PLA_2 is expressed in alveolar macrophages during inflammation to clear lung exudates, and by cytokine induction and airway dysfunction
Connolly et al. (135) Lima and Brochetto-Braga (91)	Enzyme	Phosphomonesterase	Acid phosphatase with similar properties	1	Found in accessory reproductive organs (prostate and seminal vesicles) and in other parts of the genital tract (testis, vas deferens, epididymis) Hydrolyses choline-O-phosphate Involved in calcium metabolism during blood clotting Alkaline phosphomonesterases involved in wound healing Activity increased in kidney from dioxydin accumulation.
Brás et al. (136)	Enzyme	α-glucosidase	Associated with honey production by bees	0.6	Four human forms in digestive system [salivary and pancreatic α-amylases (endohydrolase); α-maltotriose (oligoglucans); α-maltase-glucoamylase and α-sucrose-isomaltase (exohydrolases)] Essential for digestion of starch to glucose Facilitates glucose absorption especially by enterocytes Involved in metabolic disorders such as type 2 diabetes and obesity due to hyperglycemia Application for anti-diabetic agents

(Continued)

TABLE 1 | Continued

References	Component	Compound	Properties/mode of action	% BV	Properties / mode of action for mammalian analog
Holtsberg et al. (137) Karamitros and Konrad (137, 138)	Enzyme	Lysophospholipase	Increases PLA2 activity. PLA2 degrades phospholipids into lisophospholipids that are cleaved by the lysophospholipases into glycerophosphocoline and anionic fatty acids	1	Found in eosinophils, pancreas, brain, liver, lactating mammary glands, and most (if not all) cells Breaks down phosphatidylcholine to glycerophosphate-choline to release choline. Hydrolyses lysophospholipids and attenuates lysophosphatidic acid-mediated signal transduction in nervous tissues Pancreatic form is involved in digestion Eosinophilic form is involved in immunologic function Those with an N-terminal L-Asparaginase domain have role in amino acid metabolism useful in medical and therapeutic applications e.g., antileukemic protein drug
Soliman et al. (57, 139) Pucca et al., (57, 139)	Peptide	Melittin	Most toxic component Attacks lipid membranes causing cell lysis, haemolysis, cytotoxicity, and biodegradation Forms melittin-PLA2 complex that causes cellular injury Induces mild allergic but severe pain reactions In cancer therapy due to its cytotoxic activity on cancer cells Control of excessive immune responses Anti-inflammatory, antimicrobial, antiviral, fungicidal, and anti-cancer properties	40–60	
Pucca et al. (57, 140) Issa et al., (57, 140)	Peptide	Apamin	Inhibits Ca^{2+}-dependent K^{+} channels (blocks potassium permeabilities) facilitating the crossing of the blood brain barrier Causes neurotoxic effects such as intense local pain, hyperactivity, seizures, tonic-clonic convulsions, jerks, spasms Potential treatment for neurological disabilities such as learning deficit disorder, Parkinson's disease by activating of inhibitory muscarinic receptors of motor nerve terminals	1–3	Tissues with acceptor receptors for apamin in lower mammals: rat brain, rat neuroblastoma, rat and guinea pig liver, guinea pig colon, synaptosomes, rat myotubes
Moreno and Giralt (85).	Peptide	Mast cell degranulating peptide	Inflammatory and anti-inflammatory properties: inflammation/allergy: at low concentration it induces massive release of histamine, serotonin and vasoactive amines from mast cells -anti-inflammatory/ anti-allergic: in high quantity it inhibits mast cell degranulation by inhibiting histamine Can cause hyperexcitability in mammalian neurons (convulsant) Potential to induce allergy and inflammation by inducing secretion of mast cells, basophils, and leukocytes is of value in designing therapeutic compounds	2–3	
Seo et al. (77) Cherniack and Govorus (76) Gu et al. (78)	Peptide	Adolapin	Inhibits cyclooxygenase activity and blocks prostaglandin synthetase system leading to antipyretic, anti-inflammatory and anti-nociceptive/analgesic cascades Inhibits lipoxygenase from human platelets Elevates the c-GMP level in rat spleen and brain and inhibits phospholipase A2, c-AMP in rats' spleen Utilized in bee venom acupuncture to successfully manage musculoskeletal diseases (lumbar disc disease, osteoarthritis of the knee, rheumatoid arthritis, adhesive capsulitis, and lateral epicondylitis)	1	

(Continued)

TABLE 1 | Continued

References	Component	Compound	Properties/mode of action	% BV	Properties / mode of action for mammalian analog
Elieh et al. (141) Rady et al. (79, 141)	Amines	Histamine	Mediators of allergic and inflammatory reactions. Can cause anaphylactic responses, sometimes leading to cerebral or myocardial ischemia Can cause pain by affecting neurons or through release of pain-inducing chemicals	1.5	Mediators of local allergic and inflammatory reactions Physiological modulators in tissues/organs of gut, nervous system, blood etc. Act as neurotransmitters Role in fight-or-flight response (adrenaline/noradrenaline)
		Dopamine		0.1–1	
		Noradrenaline		0.1–0.7	
		Serotonin		0.1–0.5	
		Adrenaline		0.1–1	
	Pheromones	Iso-pentyl acetate; n-butyl acetate; iso-pentanol; n-hexyl acetate; n-octyl acetate; 2-nonanol; n-decyl acetate; benzyl acetate; benzyl alcohol; (2)-11-eicosen-1-ol	In bees: Cause alarm and loyal pheromones after evaporation from the surface of the sting alert, attract other bees to the marked target Affect physiological changes through the autonomous nervous system, inflammatory signaling, immune system changes and/or behavioral change.	4–8	Control of innate social behaviors and regulation of hormone levels.

and diluted BVs trigger a range of anti-inflammatory responses (61, 62), and have been deployed for management of diabetes, rheumatoid arthritis (RA), heart disease, obesity, asthma, skin diseases, and central nervous system-associated diseases, such as Alzheimer's disease, Parkinson's disease, and sclerosis (61–64). At low doses, BV can suppress inflammatory cytokines such as interleukin-6 (IL-6), IL-8, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α). A decrease in the signaling pathways responsible for the activation of inflammatory cytokines, such as nuclear factor-kappa B (NF-κB), extracellular signal-regulated kinases (ERK1/2) and protein kinase Akt, and porphyromonas gingivalis lipopolysaccharide (PgLPS)-treated human keratinocytes has been associated with treatments involving BV (65).

BV has been used as an anti-inflammatory agent by combining compounds in BV, i.e., secretory phospholipase A2, with phosphatidylinositol-(3,4)-bisphosphate or cells, mainly dendritic cells (DCs), or combining BV with DCs (66). Conjugation of hormone receptors and gene therapy transporters to BV peptides as a useful novel targeted therapy to positively modulate immune responses has been applied in anticancer and anti-inflammatory therapy (67).

BV immune reactions are toxic at high doses but when controlled or diluted (controlled concentrations) these immune reactions can serve as immune modulators. Controlled allergic immunity can be advantageous for host defense against antigens and pathogens including RNA viruses. BV can stimulate type 2 immune responses, type 2 immunity is initiated by T-cell (T-helper type 2) and immunoglobulin (Ig) antibodies (IgE and IgG1) and the action of the innate immune system, such as the epithelium and white blood cells and serves as a barrier defenses to eliminate antigens (68). BV group III sPLA2 shows *in vitro* and *in vivo* effects on the immune system. Modulated immune reactions from BV can alleviate immunological illnesses such as rheumatoid arthritis, inflammatory illnesses, asthma, and Parkinson's disease (69). The innate immune system induces a defensive immune response against BV antigens through pattern-recognition receptors (PRRs), including Toll-like receptors found on pathogen-associated molecular patterns (PAMPs) (70). BV in therapeutic disease, is anti-inflammatory (44) decreasing numbers of infiltrated inflammatory cells, and the expression of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, inhibition Toll-like receptor (TLR)2 and CD14. BVs also suppress the binding potential of nuclear factor-κB (NF-κB) and activator protein (AP)-1 (71). Human IL-1 receptor (anakinra) also shows anti-inflammatory activity (72), however information linking this receptor and Bee venom remain sparse.

Bee venom phospholipase 2 (*bv*PLA2) is the main allergen in BV and stimulates the innate immune system by binding to pattern-recognition receptors (PRRs), e.g., Toll-like receptors that recognize pathogen-associated molecular patterns (PAMPs), triggering a type 2 immune response. *bv*PLA2 induces T-helper cell-type reactions and group 2 innate lymphoid cells (ILC2s) facilitated through the enzyme-aided cleavage of membrane phospholipids and secretion of IL-33. *bv*PLA2 also induces the production of IgE shown to be protective in mice from future allergic/immunologic reactions [in the case of a lethal dose

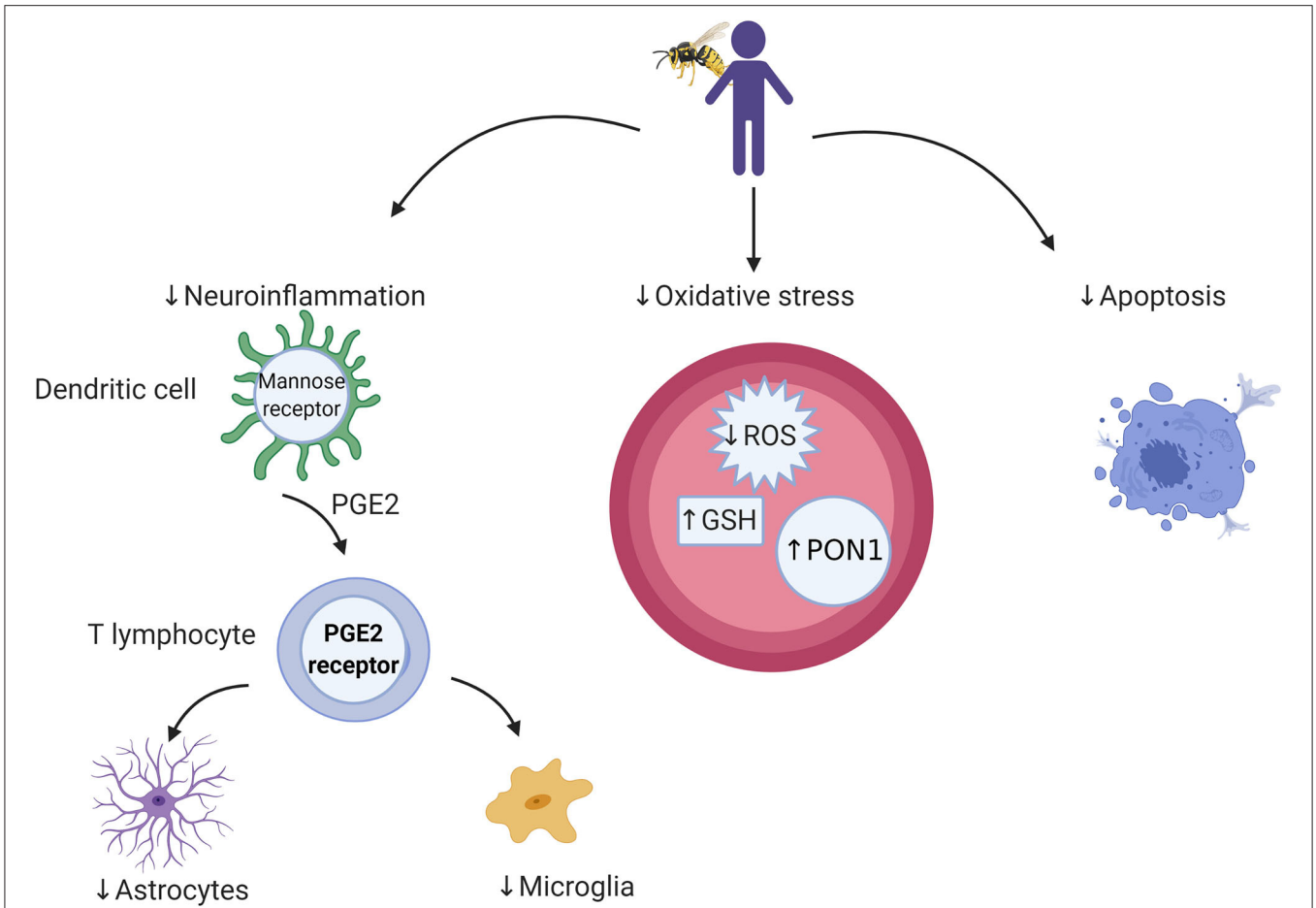

FIGURE 1 | Cellular and microbial targets relevant to bee venom components and targets for future research. Bee venom acts through dendritic cells to stimulate the immune system through which it activates cellular immunity. Its antioxidant activity is associated with a reduction in reactive oxygen species (ROS) and elevation in antioxidant enzymes (e.g., GSH and PON1), which leads to protection against cell death.

of BV (70)]. PLA2 plays a vital role in host defense in Th2 differentiation, ILC2 activation, immunoglobulin production, membrane remodeling, and anti-inflammatory reactions (44, 70).

BV shows positive immune-modulating roles; reducing the progression of tumors and activating the immune system by combining *bv*PLA2 with phosphatidylinositol-(3,4)-bisphosphate or cells, mainly dendritic cells (DC) (66). DCs prepared with BV *in vivo* have both anticancer and antiviral properties. DCs combined with antigens from a tumor or virus produce major histocompatibility complex (MHC) class I and II peptides epitopes to CD8 and CD4 T lymphocytes (**Figure 1**).

PLA_2 (bvPLA_2-H34Q) is membrane-binding and *in vivo* combines antigens with the human DC cell membrane, causing stimulation of CD8 T cells and antiviral and antitumor vaccines (DC vaccine) can be obtained from BV using DCs. These cell-based antiviral/antitumor vaccines are used during immunization against viruses including cytomegalovirus and for tumor suppression (73, 74). BV is a known adjuvant-potentiated antimicrobial and antitumor vaccine. Melittin, bvPLA2 and phosphatidylinositol-(3,4)-bisphosphate are effective adjuvants for anti-leishmania, anti-tumor and anti-cytomegalovirus vaccines (73–75). Conjugation of BV peptides with hormone receptors and gene therapy offer to positively modulate immune responses applied offer targeted anticancer and anti-inflammatory therapies (67).

BV can be used as an analgesic at controlled dose concentrations; inhibiting cyclooxygenase activity and blocking the prostaglandin synthetase system, leading to antipyretic, anti-inflammatory, and anti-nociceptive/analgesic cascades (76–78). In diluted form BV can induce anti-nociceptive effects via the α-adrenergic receptor (activation of the spinal α-adrenergic receptor) (61, 62). Conjugation of BV peptides to protein receptors such as hormones and genes transporting the peptides provides an innovative BV controlled anti-inflammatory, anti-nociceptive, and immune-modulatory therapy (67).

PHARMACODYNAMICS OF BEE VENOM CONSTITUENTS

Bee venom (BV) contains enzymes [phospholipase A2 (PLA_2), phospholipase B, hyaluronidases, acid

phosphatases, acid phosphomonesterases, α-D-glucosidases, and lysophospholipases]; peptides [lytic peptide melittin, apamin, mastocyte (mast cell) degranulating peptide, secapine, pamine, minimine, procamine A, B, protease inhibitor, tertiapine, cardiopep, and adolapin] (30–33); and amino acids include g-aminobutyric acid and a-amino acids. Non-peptide components include amines (dopamine, histamine, norepinephrine, neurotransmitters), carbohydrates (glucose, fructose), pheromones (iso-pentyl acetate; n-butyl acetate; iso-pentanol; n-hexyl acetate; n-octyl acetate; 2-nonanol; n-decyl acetate; benzyl acetate; benzyl alcohol; and (2)-11-eicosen-1-ol) (79, 80) (**Table 1**).

BV has been shown to have anti-inflammatory, antinociceptive, antioxidant, and anti-apoptotic properties and has been shown to alter gene expression and fibrosis (81–84). Side effects include proinflammation [higher doses of PLA2, mast cell degranulating peptides, hemolytic compounds (melittin)], allergic reactions to protease inhibitors and peptides, anaphylactic responses and death (76).

Multiple protein allergens in bee venom are responsible for the allergic response (85). Allergic reactions can take place in the respiratory system, gastrointestinal system, cardiovascular system, skin and stings and can result in severe anaphylactic shock, sometimes leading to cerebral or myocardial ischemia (86, 87). A non-immune-mediated mechanism of allergy to BV involves the production of bradykinin (BK) mediators, leading to anaphylaxis (88) from melittin activation of PLA2 (mimicking BKs).

BIOLOGICAL VARIABILITY OF BEE VENOM COMPOSITION AMONG BEE VARIANTS FOR BIOTOXIN ADMINISTRATION IN COMPLEMENTARY MEDICINE

Bees and wasps belong to the insect order Hymenoptera (89, 90). In bees, venom production is highest for queen bees on emergence. Hymenoptera venom causes toxic or allergic reactions mostly caused by biochemical compounds associated with local inflammatory action (91, 92). Stings defend the colony in all insects of the order Hymenoptera (93, 94). Melittin is the most prominent compound responsible for these allergic reactions (95, 96); although a combination of mastocytes with IgE invokes activity of leucotrienes, histamines and platelet activating factors during allergic reactions (93, 94, 97).

Hymenoptera venoms contain dopamine, adrenaline, hyaluronidase, noradrenaline, serotonin, histamine, phospholipases A and B (85) but only BV contains mast cell-degranulating peptide, melittin and apamin (57). Different bee species bees; *Apis mellifera mellifera and Apis mellifera ligustica* (in Europe) *and Apis mellifera scutellate (in Africa)* are responsible for human envenoming (57). The median lethal dose of BV ranges from 2.8 to 3.5 mg/kg body weight, and on average, 140–150 μg of BV is injected in a stinging episode (57). The chances of death from only a few bee stings is minimal in non-allergic persons (98) and the severity of the envenomation is duly influenced by the body weight, age and immune status of the victim (99, 100). Sting number and any previous sensitization to BV affect envenoming severity (99, 100).

BV is a clear, odorless, colorless watery liquid with a pH of 4.5–5.5 with a bitter taste and in some cases an ornamental pungent smell (101, 102). BV composition is affected by extraction methods due to its volatility (101). *Apis mellifera* venom is arguably the best characterized venom in Hymenoptera (103). Venom from all *Apis* species is similar in composition and quality. *A. florea*, a honey bee is smallest in its family, while *A. dorsata* is the largest (101). *Apis cerana* venom is twice as tocic as that of *Apis mellifera* (104). Differences in the composition of venom gland and venom sac secretion and the concentration of lipids, proteins, activity of acid phosphatase and hexokinase have been observed in the venom glands of *A. dorsata* > *A. cerana* > *A. mellifera* > *A. florea*. Lipid, protein, carbohydrate and alkaline phosphatase compositions have been found to be in the order of *A. cerana* > *A. mellifera* > *A. florea*. Glycogen was absent in both the venom gland and venom sac of Apis species (101).

Variability in bee venom composition is related to species, age, geographic localization and social condition (96). Young worker bees have lower concentrations of melittin and histamine and higher concentrations of apamin than older worker bees (57). Queen bees have low concentrations of melittin and apamin and high concentrations of histamine (57). APi M reaches its peak when the bee is ∼28 days old and declines with age (105). Levels of PLA2 reach a maximum at around day 10 of hatching (101). African bees release small quantities of venom in stinging episodes, with high concentrations of PLA2 and reduced concentrations of melittin and hyaluronidase (57). Seasonal variations in the composition of the BV have been reported (106); for example, during winter, APi M production increases but decreases during summer (107, 108).

CURRENT THERAPEUTIC ADVANCES OF BEE VENOM

Antiviral and Antibacterial Properties

Melittin and PLA2 exhibit antimicrobial activities and have been used as complementary antibacterial agents (103); inducing pore formation and destruction of bacteria (109). APi M shows antiviral properties against some enveloped viruses and non-enveloped viruses *in vitro* (110). Protection has been observed in mice following exposure to influenza A H1N1 virus but BV can also interact directly the viral surface (110) (**Figure 2**).

Management of Cancer

BV has been explored in cancer (111, 112); melittin is considered cytolytic but non-specific. Melittin can break down the membrane lipid bilayer and exhibits toxicity when injected intravenously (113). APi M has the ability to suppress tumor growth in breast, liver, prostate, and lung cancer cells (111, 112). *In vitro* and *in vivo* studies show that melittin can suppress growth of cancerous cells by inhibiting NF-κB signaling and activating caspase 3 and 9 pathways. Inhibition of hepatocellular carcinoma cell motility was observed *in vitro* and *in vivo* by suppression of Rac1-dependent pathways (114).

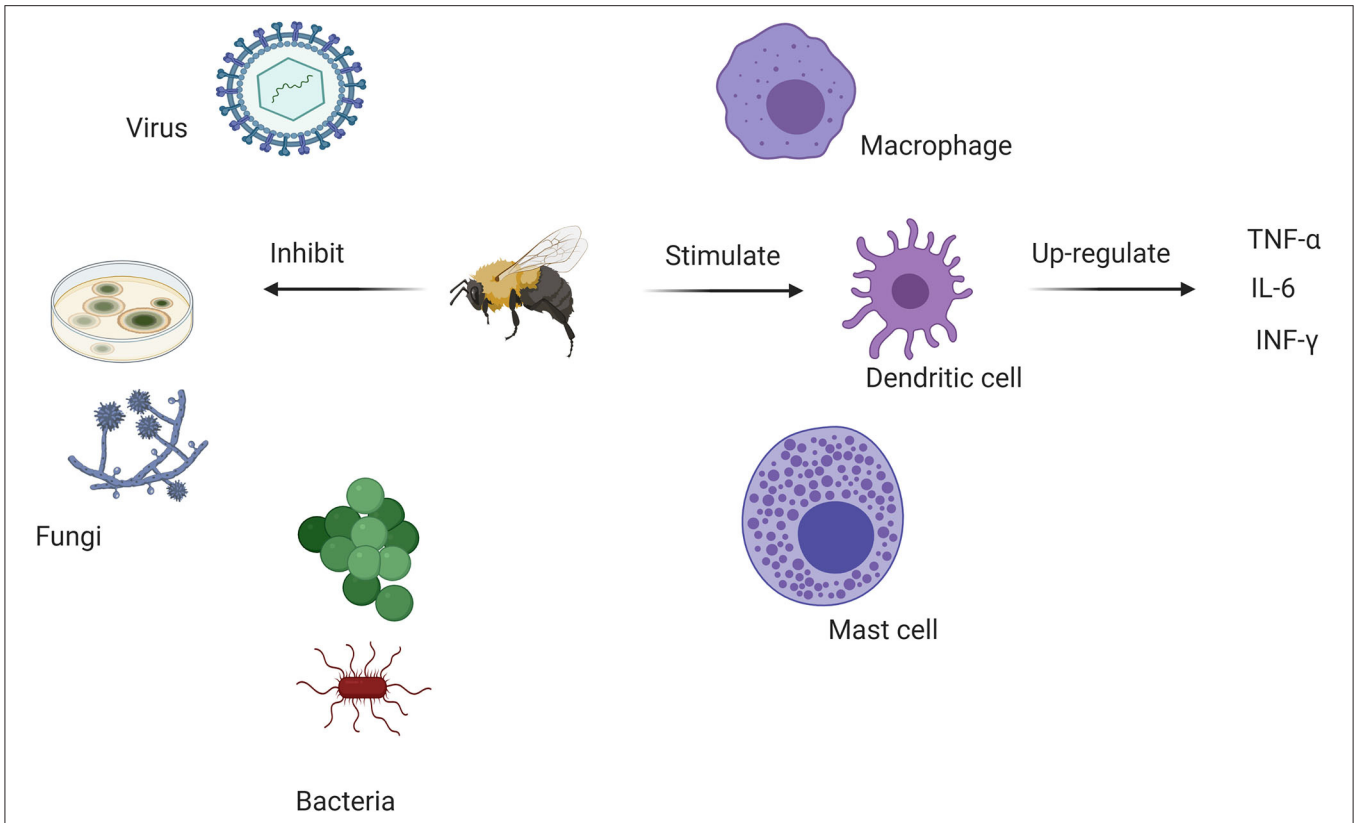

FIGURE 2 | Antimicrobial and immunomodulatory actions of various bee venom components. BV inhibits bacterial, antifungal and viral growth while stimulating dendritic cell activity through major anti-inflammatory cytokines. This offers a rationale for its use in complementary medicine to control the SARS-CoV-2 pandemic.

Anti-inflammatory Potential

Low doses of BV trigger a range of anti-inflammatory responses that have been explored in diabetes, rheumatoid arthritis (RA), heart diseases, obesity, asthma, skin diseases, and central nervous system-associated diseases (Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis) (63, 64). BV suppresses inflammatory cytokines, including interleukin-6 (IL-6), IL-8, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α). A decrease in the signaling pathways responsible for the activation of inflammatory cytokines, nuclear factor-kappa B (NF-κB), extracellular signal-regulated kinases (ERK1/2) and protein kinase Akt, and Porphyromonas gingivalis lipopolysaccharide (PgLPS)-treated human keratinocytes are associated with melittin treatment (65) (**Figure 2**).

HOST RESPONSES TO BEE VENOM

BV therapy can alleviate immune-related illnesses. Group III secretory phospholipase A_2 from BV (BV group III $sPLA_2$) shows *in vitro* and *in vivo* activity on the immune system and has been used to manage asthma, Parkinson's disease, and drug-induced organ inflammation (69). BV immune reactions can be dangerous when highly elevated, but when controlled, allergic immunity can be advantageous in host defense to stimulate type 2 immune responses. Type 2 immunity is mainly based on barrier defenses, and these responses are initiated by T helper type 2 (T_H2) cells, immunoglobulins E and G1 (IgE and IgG1) antibodies, and other components of the innate immune system (epithelial barriers, innate lymphoid cells-ILCs, eosinophils, mast cells, basophils, and activated macrophages) (68). The innate immune system senses components of venom, inducing a defensive immune response against antigens through pattern-recognition receptors (PRRs), e.g., Toll-like receptors found on pathogen-associated molecular patterns (PAMPs) (70). BV anti-inflammatory properties (44) may inhibit the activity of inflammatory antigens, reduce the number of infiltrated inflammatory cells, and inhibit the expression of (TNF)-α, IL-1β, Toll-like receptor (TLR)2 and CD14 expression, suppressing the binding activity of nuclear factor-κB (NF-κB) and activator protein (AP)-1 (71). The main Bet V 1 allergen, PLA2, stimulates the innate immune system, binding to PRRs, e.g., Toll-like receptors that recognize PAMPs, triggering a type 2 immune response in mice. PLA2 in BV induces T helper type 2 (Th2) cell-type reactions and group 2 innate lymphoid cell (ILC2) activation via the enzymatic cleavage of membrane phospholipids and secretion of IL-33. PLA2 induces the production of IgE, protecting mice from future allergic/immunologic reactions in the case of a lethal dose of BV (70); PLA2 plays a critical role in host defense by improving Th2 differentiation, ILC2 activation, immunoglobulin production, membrane remodeling, and anti-inflammatory reactions (44, 70).

BEE VENOM VACCINES

BV can suppress the progression of tumors and activate the immune system by combining secretory phospholipase A2 in BV with compounds including phosphatidylinositol-(3,4)-bisphosphate or dendritic cells (DCs) (66). DCs treated with BV *in vivo* show anticancer and antiviral properties. DCs combined with antigens from a tumor or virus can produce major histocompatibility complex (MHC) class I and class II peptide epitopes to CD8 and CD4 T lymphocytes, leading to a series of immune reactions in response to the antigens. BV phospholipase A_2 (bvPLA$_2$-H34Q) is membrane-binding and links antigens within the cell membrane of human DCs *in vivo*. This induces recognition by and activation of CD8 T cells with the implication that antiviral and antitumor vaccines may be derived from BV (DC vaccine). Vaccines from BV and DCs (cell-based antiviral/antitumor vaccines) are used for immunization against viruses such as cytomegalovirus and for suppression of tumors (73, 74). BV can be used as a potent adjuvant-potentiated antimicrobial and antitumor vaccine and shows potential in vaccines where melittin. sPLA2 and phosphatidylinositol-(3,4)-bisphosphate are effective adjuvants (anti-leishmania, antitumor and anti-cytomegalovirus vaccines) (73–75).

A leading adjuvant of SARS-CoV-2 therapies currently being promoted is aluminum hydroxide due to its slow release and increased interaction with antigen presenting cells (115). Bee venom offers a candidate for control SARS-CoV-2 infections and could offer advantages against COVID-19. PLA_2 has been associated with a level of success against SARS-CoV-2 infections (116, 117). Conjugation of BV peptides could offer a new approach in the development of the BV vaccine.

POTENTIAL RELATIONSHIP BETWEEN BEE VENOM PROTEINS AND COVID-19 PROTEINS

SARS-CoV-2 belongs to the β-coronavirus genus. SARS-CoV-2 has four obvious structural proteins: membrane, spike, nucleocapsid proteins, and envelope. The structural integrity of the SARS-CoV-2 virus is maintained by structural proteins and forms a protective coat around its RNA. The coronavirus membrane contains 3 or 4 viral proteins (118, 119), the membrane glycoprotein is the most abundant structural protein and spans the membrane bilayer three times, with a long COOH terminus inside the virion and a short NH2-terminal domain outside the virus (120). The SARS-CoV-2 genome encodes several reading frames (ORFs); ORF1a/b codes for 16 non-structural proteins and translates two polyproteins (pp1a and pp1ab) accounting for up to 2/3 of the viral RNA. The remaining ORFs code for structural proteins (spike glycoprotein, matrix protein, nucleocapsid protein, and small envelope protein) (118, 119). SARS-CoV-2 has accessory proteins that interfere with the innate immune response of the host (118).

The spike protein is usually a Type I membrane glycoprotein and constitutes the peplomers, known for involvement in antibody interaction. The membrane plays a significant role in the intracellular formation of virus particles independent of the viral spike. Coronaviruses grow and produce spikeless forms in the presence of tunicamycin, thereby resulting in the production of non-infectious virions that contain membranes but without spikes (118).

Melittin can puncture the protective membrane envelopes surrounding viruses, including human immunodeficiency virus (HIV) (119). Many viruses, including SARS-CoV-2, rely on their protective envelope and may be vulnerable in melittin-guided bee venom therapy (**Table 1**).

The phospholipase A2 components of bee venom have the potential for antiviral activities (121). Melittin-loaded nanoparticles delivered a significant amount of melittin intravenously, targeting and killing precancerous lesions in K14-HPV16 mice with squamous dysplasia and carcinoma containing human papillomavirus (HPV) transgenic elements (E6 and E7 oncogenes) (122, 123).

In Hubei Province, the epicenter of the SARS-CoV-2 outbreak in China, the local beekeeper association surveyed 5,115 beekeepers between 23rd February and 8th March (including 723 in Wuhan) and showed that none developed any symptoms observed for COVID-19 patients (124). Five apitherapists in Wuhan and 121 of their patients who had received apitherapy between October and December 2019 were interviewed; two apitherapists were exposed to suspected and/or confirmed COVID-19 victims without protection. None of the apitherapists developed symptoms associated with SARS-CoV-2 and none of their 121 patients contracted COVID 19, despite 3 having been exposed to SARS-CoV-2-infected relatives (124).

Apitherapy employs honeybees and their products (BV, honey, royal jelly, pollen, propolis, beeswax). BV therapy uses venom to modulate the body's immune system and improve/facilitate healing and includes either the use of live bee stings or injectable venom for the management of arthritis, rheumatoid arthritis, multiple sclerosis (MS), lupus, sciatica, low back pain, and tennis elbow (125, 126). Hymenopteran products are potent accelerators of wound healing (127). Insect venoms are less complex and less variable in composition and physiological activity compared to snake venoms (125, 126). BV can be administered to induce allergic immune responses, stimulating the innate immune system of the host (68), due to the presence of allergens that promote the type 2 immune responses (44, 68–71). BV antiviral and antitumor action when BV secretory phospholipase A2 is mixed with other compounds, such as phosphatidylinositol-(3,4)-bisphosphate or dendritic cells, and/or bee proteins, such as melittin, is advantageous (66) and employed in the production of cell-based antiviral/antitumor vaccines (73–75). The immunological properties of BV are also found in natural products that mimic bee venom (127, 128), and further studies regarding the role of bee venom as a potential candidate for use in complementary medicine for the management of viruses such as SARS-CoV-2 could consider other natural products that mimick BV activity.

FUTURE RESEARCH ON BEE VENOM

The development of adjuvant therapies (using APi M and PLA2) to use against SARS-CoV-2 infections offers a unique approach to viral therapy. Bee venom vaccine development using DCs using APi M and *bv*PLA2 offers a new opportunity for complementary medicine interventions against SARS-CoV-2 infections. Studies to examine cellular signaling between BV proteins, Janus Kinase (JAK) and activator of transcription (JAK-STAT) would help strengthen its adoption in complementary medicine against SARS-CoV-2. Inhibitors of JAK are associated with improved prognosis in COVID-19 patients (72, 129) but studies are needed to elucidate the cellular mechanisms. Synergistic activity through combinations in alternative and complementary medicine would help combat side effects associated with current monotherapies for the management of SARS-CoV-2 infections. SARS-CoV-2 is a novel virus and novel therapies may be needed to support management over time and may be of value in supporting the immune response in patients suffering from so called long-COVID.

CONCLUSION

SARS-CoV-2 effects on the ACE2 receptors have been associated with severe inflammatory activity and a poor prognosis, depending on the co-morbities of the patient and other associated risk factors. Even if patient recover from initial infection, they may be faced with a long and complicated convalescence and/or so called, long-COVID. It is unlikey that there will be a magic bullet therapy for COVID-19 soon, and complimentary therapies should be explored that compliment conventional therapy and support healthy recovery. BV melittin and phospholipase A2 activity have strong anti-inflammatory action and could be deployed to support recovery. That BV has successfully been used to manage neurological and immunological diseases strengthens the case for exploration of its use in complimentary medicine for SARS-CoV-2 infections. BV is a potential adjuvant against COVID-19 which should be added to the list of major therapies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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HYPOTHESIS AND THEORY
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Humidified Warmed CO_2 Treatment Therapy Strategies Can Save Lives With Mitigation and Suppression of SARS-CoV-2 Infection: An Evidence Review

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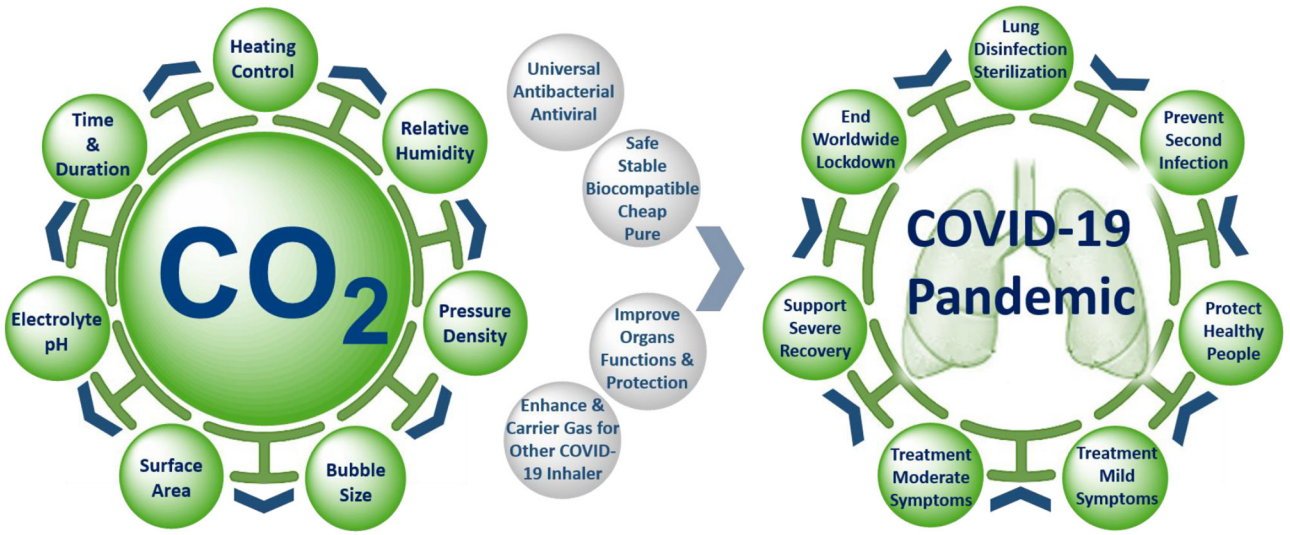
The coronavirus disease (COVID-19) outbreak has presented enormous challenges for healthcare, societal, and economic systems worldwide. There is an urgent global need for a universal vaccine to cover all SARS-CoV-2 mutant strains to stop the current COVID-19 pandemic and the threat of an inevitable second wave of coronavirus. Carbon dioxide is safe and superior antimicrobial, which suggests it should be effective against coronaviruses and mutants thereof. Depending on the therapeutic regime, CO_2 could also ameliorate other COVID-19 symptoms as it has also been reported to have antioxidant, anti-inflammation, anti-cytokine effects, and to stimulate the human immune system. Moreover, CO_2 has beneficial effects on respiratory physiology, cardiovascular health, and human nervous systems. This article reviews the rationale of early treatment by inhaling safe doses of warmed humidified CO_2 gas, either alone or as a carrier gas to deliver other inhaled drugs may help save lives by suppressing SARS-CoV-2 infections and excessive inflammatory responses. We suggest testing this somewhat counter-intuitive, but low tech and safe intervention for its suitability as a preventive measure and treatment against COVID-19. Overall, development and evaluation of this therapy now may provide a safe and economical tool for use not only during the current pandemic but also for any future outbreaks of respiratory diseases and related conditions.

Keywords: anti-COVID-19, antiviral, anti-cytokine storm, improve COVID-19 symptoms, carrier gas composition, enhancer antiviral, protect and improve organs function, suppression COVID-19 pandemic

BACKGROUND

The coronavirus disease (COVID-19) outbreak has presented enormous challenges for healthcare systems worldwide and caused terrible societal and economic impacts. There is also an urgent need to address health inequality in treating the current COVID-19 pandemic. Even now, scientists are racing to unravel sometimes conflicting information to understand the source, diagnose, and find effective treatments for SARS-CoV-2, and to conduct clinical trials of antiviral drugs and vaccines. Other COVID-19 mysteries include the appearance of new symptoms, the relation of silent hypoxia and sudden deaths, spikes insignificant vessel blockages, and increased risks of clotting (1). The



GRAPHICAL ABSTRACT | Precise control of the unique properties and intervention parameters of warmed humidified CO_2 gas make it a promising anti-COVID-19 therapy for mitigation and suppression of SARS-CoV-2 infection.

virus is now known to be able to target a wide variety of cells throughout the human body through ACE2 and TMPRSS2 receptors (2) and is believed to have caused a spike in a rare syndrome: "multi-system inflammatory state requiring intensive care" in children. Furthermore, the mode of transmission and the extent of environmental contamination is yet unknown. While the virus may not technically be airborne, it is definitely borne in the air as aerosols (3).

One of the most critical unanswered questions is why some COVID-19 patients develop severe disease, while others do not? Does the answer hidden in the origin and continuing evolution of SARS-CoV-2 virus mutation into mild and wild different strains (4)? Alternatively, does the answer depend on the two phases of the individual human body immune responses; a protective phase and a damaging phase due to inflammation-cytokine storms (5)? Other questions include whether bacterial co-infections such as bacterial pneumonia and sepsis with antibiotic resistance lead to increased COVID-19 disease severity and mortality (6) and how long it will take to create an effective vaccine. Potential SARS-CoV-2 vaccines have a variety of approaches that depend on viral life cycles (7), and it is estimated that a vaccine will either arrive in 1 or 2 years or will never arrive at all. Even if the vaccine trials are successful, will the new vaccine cover all SARS-CoV-2 mutant strains, and give full immunity to everyone with no issues when translation to clinical practice? Can we produce enough, how much will it cost and who will pay (a particularly important issue in developing countries)? Can the new vaccine stop the threat of a second inevitable wave of coronavirus, or other pandemic viruses emerging to produce a similar situation in the future?

Gas therapy is a highly effective viral inactivation strategy. Carbon monoxide (CO) gas is very flammable and highly poisonous and referred to as the "Silent Killer," because it binds to the parts of human blood that carry oxygen molecules, so it chemically blocks the body and organs from getting the needed oxygen. However, CO gas has also been shown to have antimicrobial and antiviral activities against infected cells (8), and two clinical trials (NCT02425579, NCT03799874) have demonstrated that the administration of low concentrations of CO is well-tolerated and safe in patients with sepsis-induced ARDS (9, 10). Similarly, while high concentrations of inhaled ozone (O_3) can damage the lungs, cause chest pain, coughing, shortness of breath, throat irritation, and worsen chronic respiratory diseases such as asthma as well as compromise the ability of the body to fight respiratory infections (11), ozone gas therapy has been demonstrated to inactivate airborne viruses (12) and could inactivate the SARS-CoV-2 virus through oxidizing the sulfhydryl groups in cysteine of the virus-cell (13). There are also at least four ongoing clinical trials (NCT04290871 - NCT04306393 - NCT04305457 - NCT04290858) testing the use of inhaled nitric oxide (NO) gas for patients with COVID-19 (14), as increasing airway NO levels via gas inhalation or precursor molecules may improve oxygenation in COVID-19 subjects (15). As with the other gases, there is another side to NO, which can be harmful due to the formation of highly toxic and irritating nitrogen dioxide (NO_2) gas and methemoglobinemia (16).

THE HYPOTHESIS AND EVIDENCE

Carbon dioxide (CO_2) is a fundamental biological gas and has been used for medical purposes for over a century due to its unique properties (**Figure 1**). Carbon dioxide gas is natural, biocompatible, chemically stable, and safer than any other medical gases (NO, O_3, or CO). It has been shown to possess antioxidant and anti-inflammatory properties, to improve blood oxygenation and enhance oxygen delivery to organs, to protect and improve lung function, to function as a carrier, or enhancer gas for drug delivery by rapid and direct open airway inhalation with easy administration in home, GP, emergency unit, and

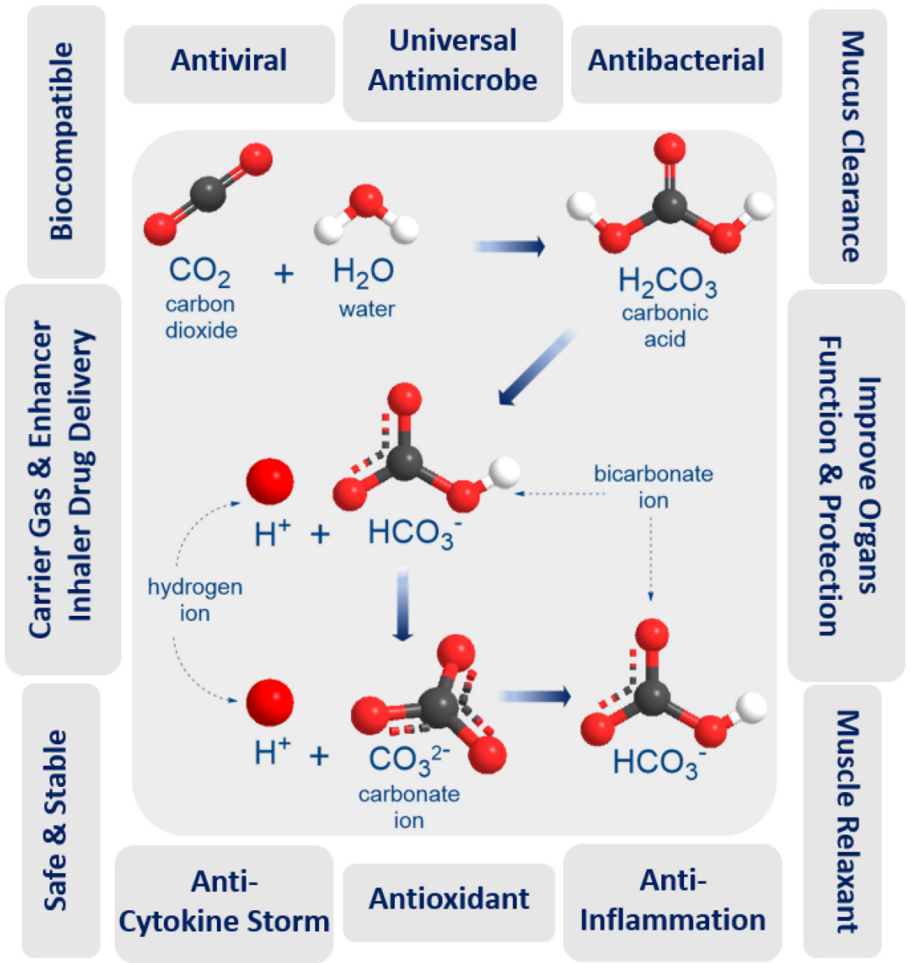


FIGURE 1 | Exceptional physical, biological, and medical properties of warmed humidified CO_2 gas.

ICU settings. These unique biological, physical, and medical properties of CO_2 make it a promising anti-COVID-19 therapy for mitigation and suppression of SARS-CoV-2 infection. Our hypothesis depends on inhaling precise doses of humidified and warmed CO_2 medical gas, either alone or as a composite carrier gas with other COVID-19 inhaler medications (bronchodilators, antivirals, antibiotics, or anti-cytokine agents), to disinfect the SARS-CoV-2 virus inside the infected human lung, as a preventative measure to stop coronavirus infection spreading, and to improve the treatment of mild, moderate, and severe COVID-19 symptoms. The following benefits and evidence of using medical carbon dioxide gas support the hypothesis.

Universal Virucidal and Antimicrobial Activity

Direct inactivation technologies have several limitations against the current virus. Moist, warm CO_2 gas could become a competitive disinfection technology. Carbon dioxide gas is an antiviral, antibacterial, and anti-infection agent effective not only on solid surfaces but also in aqueous solutions and water treatment settings (17). Heated, un-pressurized carbon dioxide bubbled through wastewater or aqueous media effectively destroys both waterborne bacteria and viruses (18). Moreover, supercritical CO_2 can in-activate and eliminate coronaviruses from an animal, human tissues and solid surfaces (19–21). Supercritical CO_2 offers a novel, user-friendly process to sterilize acellular tissue, such as lung matrices, for use in tissue and organ engineering (22). CO_2 can also enhance the effect of some other antibacterial agents, further improving the protection imparted (17). When breathing is impaired, CO_2-levels in the human body drop, which creates a favorable environment for bacterial growth and a higher risk of infection. Pure CO_2 significantly decreased the growth rate of most viruses and bacteria at body temperature; this inhibitory effect of CO_2 increased exponentially with time (23). This phenomenon could be attributed to unravels the secret of structure and function of the Endothelial Surface Layer (ESL) (24–27). As the venous ESL is probably comprised of nanobubbles of CO_2, generated from tissue metabolism, that presumably kills the viruses and bacteria exiting to the blood flow on the way to leaving via the lungs (27). Even though

the mechanism of inactivation of microorganisms by CO_2 is not yet resolved, there are a number of hypotheses that have been proposed to explain the unique disinfection action of CO_2 gas (28).

CO_2 gas is far superior to other similar gases, with much higher viral inactivation rates at lower temperatures (18–100°C) without the need for pressurization (18, 29). CO_2 interacts with water moisture to generate carbonic acid (pH 4.18), a reduced pH could affect virus and microbial cell inactivation, as lipid membrane stability is disrupted and permeability to carbon dioxide increases (30, 31). However, a reduction in the pH of the medium is not sufficient to account for the antimicrobial action of CO_2, since it shows a specific inhibitory effect which is greater than that of the other acids used to lower the pH of media (hydrochloric acid, phosphoric acid, etc.) (32). These acids do not penetrate the microbial cells as easily as carbon dioxide (33). Cheng et al. believe that CO_2 molecules could enter virus capsids much more easily than H^+ and inactivate the virus (34). CO_2-protein binding could also damage the capsid, inactivating the virus. Both mechanisms may be active during dense phase carbon dioxide treatment (DPCD) which has also been shown to effectively inactivate viruses (31). The warm atmospheric pressure CO_2 gas during DPCD is suggested to have high viral inactivation effect by penetrating the virus capsid due to the high density of CO_2 with a high interfacial area (α) produced by the continuous CO_2-moist contact surface area (29). Following this; CO_2 can bind inside the capsid proteins through acid/base interactions (35), producing the high virus inactivation rates (18). Also, when compared with other gases (Air, O_2, N_2, and Argon), CO_2 gas has the highest inactivated viruses and bacteria rates in different NaCl solutions, even at ambient temperatures and normal atmospheric pressure (18). Recently, Edwards et al. demonstrate the effectiveness of aerosol administration of nasal saline comprising calcium and sodium salts diminishes exhaled particles and acts as a new natural defense against airborne pathogens in the human airways (36). Moreover, Zare and his teamwork report that spraying micron-sized water droplets can act as an effective disinfectant by causing inactivation of over 98% of the bacteria. They propose that the combined action of reactive oxygen species present in micron-size water droplets (but not in bulk water) along with the droplet surface charge is responsible for the observed bactericidal activity (37). The efficiency of CO_2 technology will require adjustment and control of the mechanical and dynamic behavior of moist CO_2 bubbles and properties such as temperature, flow and density rates, pressure, electrolyte pH, bubble size and thickness, surfaces area, and duration. All of these factors contribute to the observed fast microbial death (38).

Safe and Tolerance for Human Clinical Trials and Treatment

Carbon dioxide (CO_2) gas is natural, inexpensive, non-toxic at low concentrations (5,000 ppm), non-flammable, and readily available in high purity from a variety of sources. When CO_2 gas dissolves in water, it exists in chemical equilibrium with carbonic acid (pH = 4.18) which plays an essential role in the bicarbonate buffer system used to maintain acid-base homeostasis in the human body. The duration and concentration of carbon dioxide inhalation may be the key to the effective and protective role of CO_2 gas therapy. A recent study investigated that pre-treatment by CO_2 inhalation for 10 min, but not for 60 min, could improve lipopolysaccharide LPS-induced lung injury (39). A pre-clinical sheep model used perflubron combined with 12% CO_2 to re-open constricted airways treatment for severe acute asthma (40). As a reference, OSHA has set a CO_2 permissible exposure limit (PEL) of 5,000 ppm over 8 h and 30,000 ppm over 10 min. This compares favorably to CO gas at 50 ppm, NO gas at 25 ppm, and O_3 gas at 0.10 ppm for 8 h. Humans can tolerate up to 10% CO_2 before severe adverse effects are encountered (41) although CO_2 tolerance decreases with age ($p < 0.0001$) (42). Two clinical trials (NCT02616770 & NCT02334553) showed that perflubron carried in gas with ascending doses of carbon dioxide (4, 8, and 12% CO_2) administered to healthy subjects was safe and effective in subjects with mild asthma (43, 44), while other ongoing clinical trials (NCT03903913) are testing the safety and tolerability the same formulation in subjects with cystic fibrosis. Moreover, CO_2 concentrations of up to 35% have been applied in other clinical trial study used "CO_2 inhalation challenge model" through a protected inhalation system to measure the anxiolytic and panicolytic effects of new test compounds (45, 46).

Suppressing Cytokine Storm

Evidence is accumulating inferring that a subcategory of patients with acute COVID-19 might experience cytokine storm syndrome (47). CO_2 gas is one of the potential treatment strategies to dampen an overactive immune system and to quell a cytokine storm (48, 49). Many researchers have reported that the presence of CO_2 reduces the production of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukins 1 and 6 (IL-1 and IL-6, respectively), suggesting that the gas temporarily inhibits macrophage activity via a mechanism that could be associated with the reduction of the local or systemic pH (50–54). Carbon dioxide gas can also affect the production of pro-and anti-inflammatory cytokines in endotoxin-stimulated human whole blood cultures under hypercapnic, normocapnic, and hypocapnic conditions (55). In another study, CO_2 was shown to differentially affect the cytokine release of macrophage subpopulations exclusively via alteration of extracellular pH. Decreasing the extracellular pH to 6.5 mimicked the effects of CO_2 and a decrease to 5.5 suppressed IL-6 release in cell lines (53).

Inhaled Carrier Gas Delivery System

CO_2 gas has unique safety, chemical stability, biocompatibility, and properties as well as a higher density than oxygen, high solubility in tissue and blood and high tolerance in vascular system (56). CO_2 itself is a respiratory stimuli, enhances mucus clearance, and seems to be a bronchodilator by general induction of smooth muscle relaxation (57). Additionally, warmed and humidified CO_2 insufflation leads to an improved body core temperature (BCT) maintenance, a reduction of the inflammatory and cytokine responses (58, 59) and improved quality of postoperative course, compared

with standard insufflation (60, 61). Also, it can reduce intraoperative hypothermia, coagulation dysfunction, early postoperative cough pain, days to first flatus and solid food intake, and the length of hospital stays (62). In recent years, CO_2-based technologies have accordingly gained considerable interest in the pharmaceutical industry. CO_2 bubble-generating carrier systems can be used to locally accumulate a drug at diseased tissue, reducing side effects on the healthy tissue and improving their therapeutic effectiveness (63). CO_2 may also be used as an enhancer and carrier gas for delivery of effective medical agents into a surgical wound (64) or respiratory diseases such as severe acute asthma and cystic fibrosis (40, 43, 44).

Clinical Usage and Medical Purposes

Medical carbon dioxide has been used as a pure gas or in specialized mixtures with other gases in anesthesia, as an insufflation gas for minimally invasive surgery (65), and in carboxytherapy (66). It can be used in the expansion of blood vessels to increase carbon dioxide level after rapid breathing, and to stimulate breathing after a period of non-breathing (67). Transdermal carbon dioxide gas therapy is widespread and uses carbon dioxide gas at high humidity, to increase tissue blood flow. Tissue oxygenation generates new blood vessels, and well-oxygenated tissues improve the effectiveness of antibiotic therapy. This is complemented by the antioxidant effect of CO_2 itself, which reduces oxidative stress in open surgery (68), and improves wound healing (69).

Benefits of Hypercapnic Therapy

Hypercapnic therapy (elevated CO_2 levels) has beneficial effects on the physiology of the respiratory, cardiovascular, and nervous system. In human critical care, hypercapnic acidosis (HCA) is frequently acceptable and improves innate immune function, resistance to infection, and protects and improves lung functions in patients with advanced lung disease. However, all these benefits require careful consideration of when and for how long hypercapnia will be applied. Hypercapnic acidosis, but not buffered hypercapnia, was reported to reduce the severity of sepsis-induced lung injury (70). Recent studies suggest that HCA is protective in the earlier phases of bacterial pneumonia-induced sepsis, just as HCA is protective in preclinical models of early and prolonged systemic sepsis (71). Also, CO_2 gas in therapeutic hypercapnia and other forms of acidosis techniques is an excellent antioxidant and anti-inflammatory agent (72). Hypercapnic acidosis was associated with benefits on lung and distant organs in several disease models, apart from the reduction of ventilation parameters

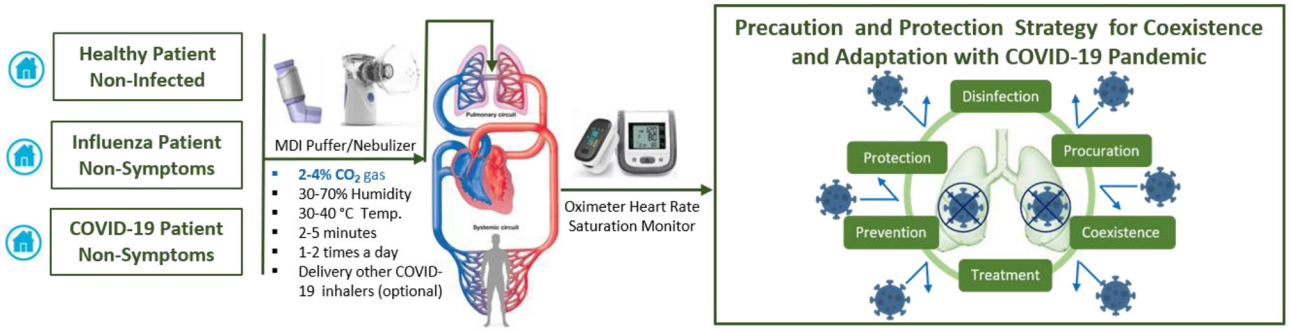


FIGURE 2 | Suggested protocol for early and daily inhaling CO_2 gas (2–4%) itself or composite with other COVID-19 inhaler medications could help in precaution and protection strategy to coexistence and adaptation with COVID-19 pandemic.

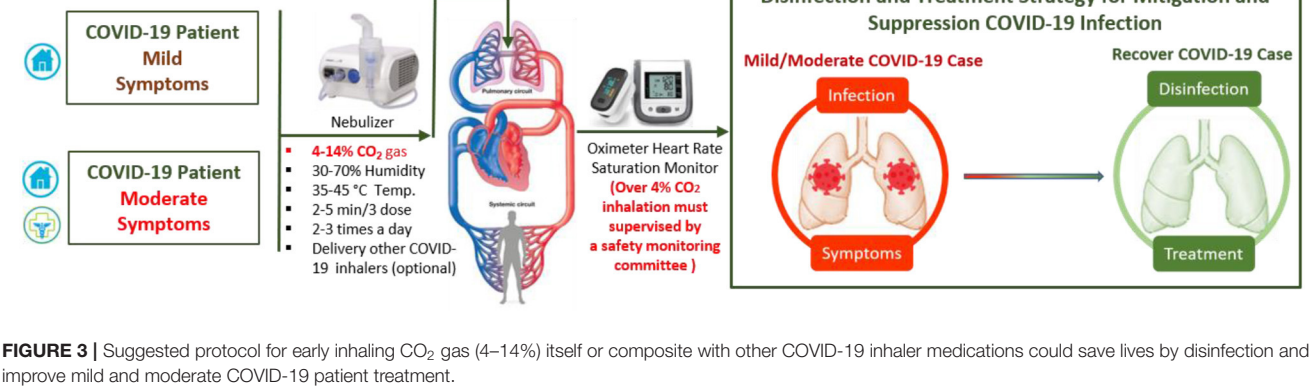


FIGURE 3 | Suggested protocol for early inhaling CO_2 gas (4–14%) itself or composite with other COVID-19 inhaler medications could save lives by disinfection and improve mild and moderate COVID-19 patient treatment.

such as ventilator-induced lung injury (73), acute respiratory distress syndrome (ARDS) (74), ischemia-reperfusion injury (75) and sepsis (76), therapeutic hypercapnia through inspired carbon dioxide attenuated lung injury, as measured by gas exchange, reduced cytokine release, lung oedema formation, and histological lung injury. Hypercapnic acidosis improves ventilation-perfusion matching that also improves gas exchange (77), prevents oedema formation (78), clears the alveolar fluid in pulmonary oedema (79), maintains the integrity of the blood-brain-barrier and reduces neurologic deficits after trauma (80). HCA also reduces the oxidative stress that contributes to pathologic thick mucus gel formation in the lung (81, 82). It is hoped that hypercapnia therapy may offer real benefits, but well-planned and executed clinical studies will be required.

Recent COVID-19 Contradictory Studies

The partial pressure of CO_2 in the atmosphere varies between 0.03 and 0.06% (83) but forms a high proportion (12.5–13.5%) with water vapor (1.3%) of the mainstream cigarette smoke (84). Recent studies have discovered the unusually low prevalence of current smoking was observed among hospitalized COVID-19 patients compared to the expected prevalence based on smoking prevalence in China. This preliminary analysis does not support the argument that current smoking is a risk factor for hospitalization for COVID-19, and might even suggest a protective role (85). Other cross-sectional studies in both COVID-19 out- and in-patients strongly suggests that daily smokers have a very much lower probability of developing symptomatic or severe SARS-CoV-2 infection as compared to the general population (86, 87). However, on the other hand, researchers at Baylor College of Medicine, the University of South Carolina and other institutions have identified tobacco smoking as a potential risk factor for infection of the COVID-19 virus, due to increasing the expression of ACE2, the receptor of SARS-CoV-2, in the lungs (88, 89). These two contradictory studies support our hypothesis of moist warm CO_2 gas resulted from cigarettes smoking could kill the SARS-CoV-2 viruses inside the infected lungs of smoker patients, and that leads to decreasing the infected COVID-19 patient from the smoker, not the nicotinic or other outcomes of mainstream cigarette.

TESTING THE HYPOTHESIS (A): PRECLINICAL STUDY AND INACTIVATION MECHANISMS

Herein, we recommend preclinical studies to optimize the relation between disinfection efficacy and toxicity level of warm humidified CO_2 gas while considering other related parameters to discover the possible mechanism of action of disinfection by CO_2 gas. The temperature inside healthy lungs is around 37°C, the pH is between 7.38 and 7.42, and the relative humidity ranges from 30 to 70%. It is essential to keep humidity stable as too high humidity provides optimal conditions for microbial growth, and low humidity and dry air can dry mucous membranes and make them more susceptible to infection (90). The SARS-CoV-2 virus is highly stable at 4°C, but it is very sensitive to heat. It is remarkably stable in a wide range of pH values (pH 3–10) at room temperature (22°C) (91, 92). However, the stability of SARS-CoV-2 under different environmental conditions of temperature, pressure, relative humidity, and pH with biological tissue and barriers require further investigation.

TESTING THE HYPOTHESIS (B): CLINICAL EVALUATION AND IMPLICATIONS

Whilst the properties and clinical applications of CO_2 have been known for many decades; parameters must be systematically studied before it can be used in a new clinical setting.

(I) Healthy, Non-symptomatic, Mild, and Moderate Care Levels

Optimizing the balance between disinfection efficacy and toxicity of humidified warmed CO_2 gas considering other parameters (temperature, relative humidity, pressure flow and density rates, electrolyte pH, bubble size and thickness, surfaces area, and duration) will be key. Different regimes will be needed to protect healthy and non-symptomatic patients and improve the condition of those suffering mild and moderate COVD-19 symptoms. Multiple-ascending dose studies in which subjects with mild to moderate COVID-19 will be enrolled [CO_2 max 14%, tolerance decreases with age ($p < 0.0001$)] (42). The suggested study could consist of a screening period, a run-in, dosing and evaluation periods, and a follow-up period. The dosing and evaluation period of the study could divide into three connected components. *First, a dose-escalation study*—This segment of the treatment period is designed to assess the safety and tolerability of escalating doses of medical CO_2 gas (2–4%) in a healthy volunteer (**Figure 2**), and (4, 8, 12, and 14%) in those with mild-moderate COVID-19 symptoms (**Figure 3**). *Second, a daily dosing study* - This segment of the treatment period is designed to assess the short term (5 days) safety and tolerability of 1–2 times daily administrations of a fixed dose of medical CO_2 gas in healthy volunteers, and 2–3 times daily administration of a fixed dose of medical CO_2 gas in patients with mild-moderate COVID-19. *Third, a drug delivery study* - This segment of the treatment period is designed to assess the safety, efficacy, enhancing, and tolerability of humidified warmed CO_2 gas (2–14%) composed with other inhaled medication such as an antiviral (Remdesivir or IFN-β SNG001), short-acting bronchodilator, antibiotic, anti-inflammation. The recommended clinical trial study may well-include placebo-control, humidified warmed CO_2 gas (2–14%), and humidified warmed CO_2 gas (2–14%) composed with other inhaled medication. Administration can be achieved through using simple comprised cartridge MDI puffer, portable nebulizer, or circularize II high-efficiency aerosol drug delivery system nebulizer in a negative pressure environment. Direct air/oxygen inhalation for a few minutes can be used to recover patients to

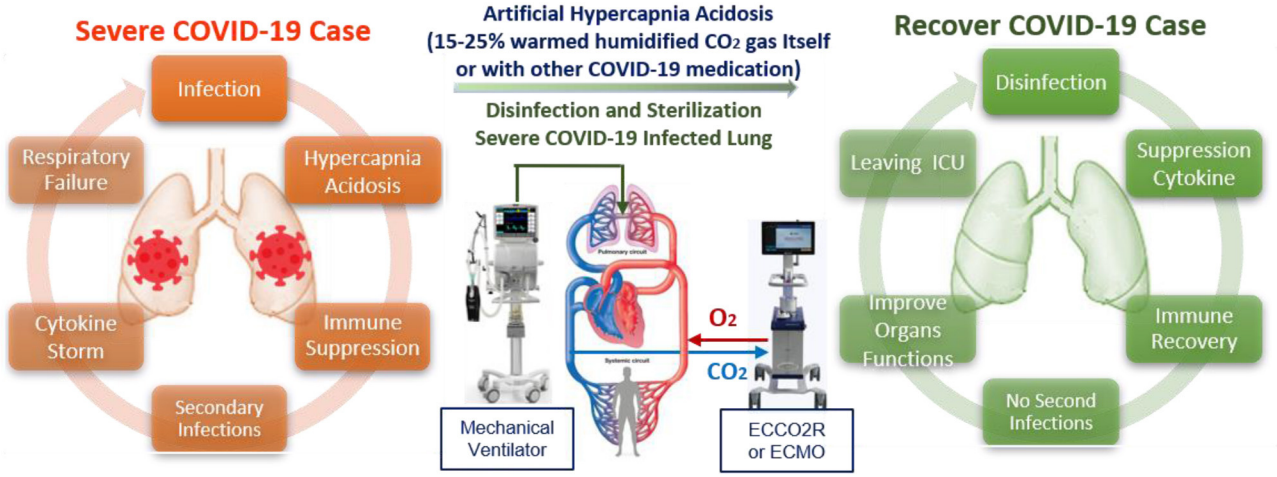


FIGURE 4 | Suggested supporting treatment protocol depending on time and duration of limited and convenient artificial hypercapnia acidosis (15–25%) that could help in saving more lives of severe COVID-19 patients.

baseline carbon dioxide levels. A safety monitoring committee must also review the results from each cohort before deciding continuation of the study at the next prescribed dose level, based on consideration of the clinical significance of safety and tolerability parameters.

(II) Severe Care Level

The damage mechanisms of SARS-CoV-2 are still unclear, with severe COVID-19 cases are complicated by high mortality rates due to compromised immune function and a high probability of antibiotic-resistant secondary infections. Most severe COVID-19 cases are associated with respiratory failure, with many already suffering from internal high hypercapnia acidosis (with humidity levels near 100%) that disrupt not only cardiac and neurological functions but also immune system function by suppressing both innate and adaptive immune responses to viral and bacterial proliferation and infection (54, 93–96). This dysfunction of the immune system with increasing SARS-CoV-2 infection can lead to an overreaction of the immune system (cytokine storm), during which white blood cells are misdirected to attack and inflame even healthy tissue, leading to failure of the lungs, heart, liver, intestines, kidneys, and genitals (Multiple Organ Dysfunction Syndrome, MODS). This may, in turn, lead to the lungs shutting down (Acute Respiratory Distress Syndrome, ARDS), which makes absorption of oxygen difficult. Most deaths due to COVID-19 are due to respiratory failure. To save the lives of severing COVID-19 patients, we must first stop the causes of SARS-CoV-2 infection and preventing secondary infections. However, due to the absence of a specific COVID-19 antiviral treatment, most severe COVID-19 patients be admitted to the intensive care unit to fight the symptoms, aiming to lower the mortality rate through intensive monitoring and supportive organ function treatments by anti-cytokine medications with artificial blood purification system machines (97). Herein, we cautiously suggest that external artificial hypercapnia acidosis (warmed humidified CO_2 15–25%) could be applied to disinfect and stabilize the lungs of SARS-CoV-2 infected patients and prevent secondary infections (**Figure 4**). However, it should only be considered for severely affected patients if they are already is connected to life support and artificial blood purification through mechanical means, and a controlled gas mixture consisting of 25% CO_2 and 75% O_2 is delivered through a protected inhalation system while monitoring a wide range of physiological parameters, and administering supportive organ function treatments.

CONCLUSION AND EXPECTING OUTCOMES

There is an urgent global need for a universal vaccine to cover all SARS-CoV-2 mutant strains to stop the threat of an inevitable second wave of coronavirus. Currently, there are hundreds of clinical trials, but not yet any approved antiviral drugs specific for the treatment of COVID-19. The physical, biological, and medical properties of CO_2 gas suggest that humified warmed CO_2 gas possesses multiple bioactivities and offer a new concept to SARS-CoV-2 viral disinfection and COVID-19 treatment. This inexpensive and broadly applicable therapy could lead to a massive reduction in the global number of infected, especially when used as a carrier for delivery of other inhaled drugs and creates new possibilities for mitigation and suppression of any COVID-19 second wave, or indeed any new future respiratory viral pandemic. In the future, more bioactive properties of CO_2 could be identified, and their mechanisms of action investigated. We believe well-designed clinical trials of CO_2 and its various bioactive properties are warranted to examine its efficacy against these diseases in human beings. It is hoped that

this hypothesis will serve as a stimulus for further investigation into this issue.

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

All authors contributed to the article and approved the submitted version. AE-B: conceptualization, methodology, writing—original draft, and writing—review and editing. EB: writing—review and editing. MG and KH: conceptualization and writing—review and editing.

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frontiers in Public Health

BRIEF RESEARCH REPORT
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Comparison of Deaths Rates for COVID-19 across Europe During the First Wave of the COVID-19 Pandemic

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Background: Europe overall suffered greatly in the early stages of the COVID-19 pandemic but the impact of different countries varied. Italy was in the forefront, but there too there were differences, with the Lombardy region the epicentre of the pandemic.

Methods: We report Crude Mortality Rates (CMRs) from deaths reported as due to COVID-19 and, in five countries where age-specific data are available, Standardized Mortality Rates (SMRs) in the European Union and United Kingdom.

Results: As of 30th August 2020, Belgium was the country with the highest cumulative CMR (86.3/100,000), but the Lombardy region reached almost double this figure (167.6/100,000), far ahead of the corresponding figure for the rest of Italy at 37.0/100,000. SMRs could be calculated for five countries (Italy, Portugal, Sweden, Germany, and Netherlands). Among them, Sweden had the highest SMR (61.6/100,000). The corresponding figures for Italy, Netherlands, Portugal and Germany were 50.2, 41.4, 15.9, and 10.1 per 100,000, respectively.

Conclusion: It is clear that countries within Europe have performed very differently in their responses to the COVID-19 pandemic, but the many limitations in the available data must be addressed before a definitive assessment of the reasons for these differences can be made.

Keywords: COVID-19, death rates, standardized mortality rate, epidemics, pandemics

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BACKGROUND

Europe was the continent worst affected in the initial phase of the SARS-CoV-2 pandemic. The first cases in Europe were in Italy and deaths were soon rising rapidly in several of its northern regions, especially Lombardy (1). As they watched graphic scenes of Italian hospitals struggling to cope, European governments adopted a series of unprecedented measures to contain the spread of the virus, although with differing speed and intensity. These included restrictions on movement outside the home, rules on physical distancing, mandatory face covering in closed public settings, and introduction of elements of find, test, trace, isolate, and support systems. Even where restrictions were minimal, as in Sweden, or delayed, as in the United Kingdom, many people changed their

behaviour in ways that reduced risks (2). Unlike the situation in Africa and the Americas, the initial peak of infection in Europe is now subsiding, and while some countries are seeing a resurgence associated with loosening of restrictions, it is timely to take stock of how Europe has fared in terms of deaths.

The impact of the pandemic can be measured several ways, with the two main outcomes reported being incident infection and mortality, both of which can be expressed in different ways, including trends over time and cumulatively. Both are sensitive to case definitions, which in turn are influenced by the extent of testing. Mortality rates are also affected by how the data are collected, with several countries operating separate systems collecting information from hospitals and/or long term care facilities to provide rapid information on emerging trends alongside their existing vital registration systems that allow for greater scrutiny of causes of death; definitions can vary, even within countries, in how a death from COVID-19 is defined, such as whether it is a death in someone who ever had a positive test, had one within a defined period before death, or did not have a test but had symptoms consistent with COVID-19 (3). As a consequence, excess all-cause mortality is widely viewed as the gold standard, with a recent study providing a detailed examination of 21 industrialised countries (4). It has benefits and drawbacks, as it includes deaths indirectly related to SARS-CoV-2, such as those resulting from overstretched health facilities, but it will also underestimate SARS-CoV-2 related deaths as there may be reductions in deaths from, for example, road traffic injuries.

In practice, most media and political attention has focused on reports of deaths attributed to COVID-19 in official reports. Yet their presentation often demonstrates a lack of even basic epidemiological understanding, for example as they are presented as numbers and not rates, and even less often as age-standardised rates. Given their widespread use, but recognizing their limitations, we have brought together the available data for EU countries plus the United Kingdom (UK), calculating where possible age standardized mortality rates (SMRs), and examining the situation now and cumulatively.

METHODS

We conducted an observational ecological study, comparing crude mortality rates (CMRs) and (SMRs) among EU countries and the UK. We focused on these two indicators as they best capture the trajectories of the pandemic and the impact of responses of different countries. We also examine the particular situation in Lombardy, the Italian region that was the first to report COVID-19 cases in Europe.

We obtained the absolute number of COVID-19 deaths in each EU country plus the UK as of August, 30th from the European Centre for Disease Prevention and Control (ECDC) (5). We calculated (CMRs) for COVID-19 using the daily number of deaths/100,000 resident population. We were only able to calculate SMRs for countries reporting identical age ranges (0–9, 10–19, 20–29, 30–39, 40–49, 50–59, 60–69, 70–79, >80) of COVID-19 deaths (Italy, Germany, Netherlands, Sweden, and Portugal), which we obtained from national data sources (6–10). To capture the overall burden of mortality officially attributed to COVID-19 we calculated CMRs based on cumulative deaths from 22nd February until 30th August, as reported to the ECDC and, for the five countries with age-specific data in national data sources, the age standardized cumulative figures. In the latter case, age-stratified data were available only between March 11th up to August 16th. When computing the crude mortality rates, we undertook two analyses, one including and one excluding the Lombardy region (10 million inhabitants), which was the epicentre of the Italian COVID-19 epidemics. As we were unable to use indirect standardization to compare all countries due to data limitations, we calculated the SMR by dividing the number of observed deaths in each country by the expected number of deaths. The expected deaths were estimated by multiplying the age-specific population in each country by the age-specific mortality rate of the standard population. The standard population was the total population of the EU (11). We were unable to calculate the standardized death rates in Lombardy alone, as data on age at death from COVID-19 were not publicly available.

RESULTS

As of 30th August, the CMR for COVID-19 varied greatly across EU countries, with Belgium reporting the highest value (86.3/100,000), followed by the UK (68.5/100,000) and Spain (62.1/100,000), while Slovakia had the lowest (0.6/100,000) (**Figure 1A**). When considering Lombardy region on its own, the CMR was almost twice that of Belgium, with 167.6/100,000 in Lombardy vs. 37.0/100,000 for the rest of the country (**Figure 1A**). Among the five countries where we could estimate age-standardised rates, Sweden reported the highest, with a SMR of 61.6/100,000, followed by Italy (50.3/100,000), Netherlands (41.4/100,000), Portugal (15.9/100,000), and Germany (10.1/100,000) (**Figure 1B**).

Turning to mortality trends, Lombardy region experienced the earliest steep increase in Europe, with death rates increasing from 0.2/100,000 on 1st March to 82.6/100,000 on 1st April. The worst affected of the remaining EU countries and the UK only increased steep increases in CMRs from the beginning of April until the beginning of May, with Belgium experiencing the highest increase among the 28 countries (from 12.0/100,000 to 68.7/100,000) in this period, followed by UK (from 3.7/100,000 to 39.9/100,000) and Spain (from 17.4/100,000 to 52.9/100,000). The CMR in Sweden showed a consistent increase from the beginning of April until the end of July (from 2.8/100,000 to 56.4/100,000) and it plateaued only in the second half of August (56.9/100,000) (**Figure 2A**).

When looking at cumulative SMRs, the trends were similar for Italy and the Netherlands (0.9/100,000 and 0.0/100,000 on 11th March, 40.9/100,000 and 36.4/100,000 on 11th May, 48.2/100,000, 41.0/100,000 on 21st July, 50.3/100 000 and 41.4/100,000 on 16th August, respectively) where the plateau was

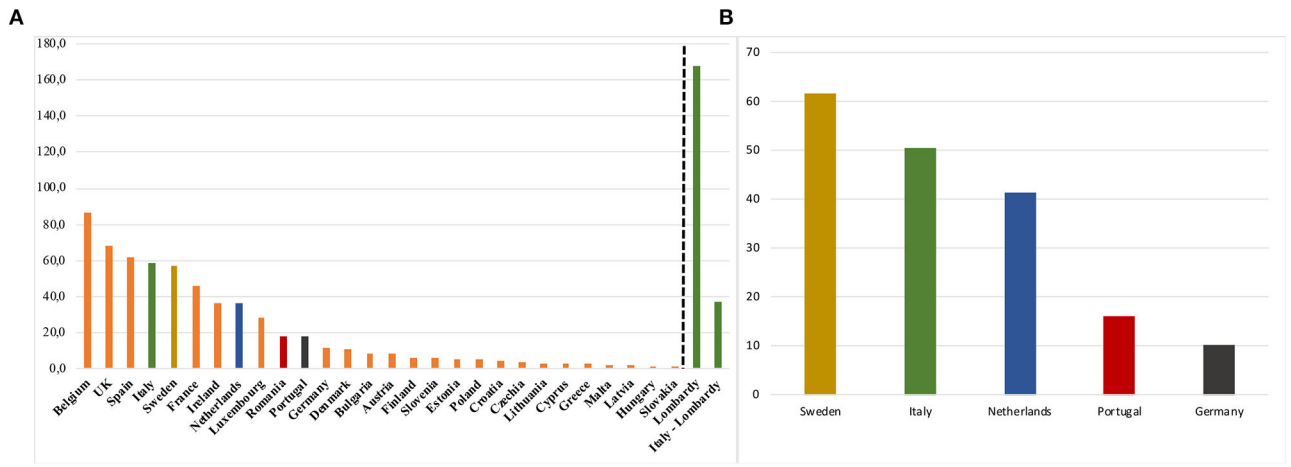

FIGURE 1 | Crude Mortality Rates **(A)** for COVID-19 in 27 EU Countries plus UK, and Standardized Mortality Rates **(B)** (×100,000 inhabitants) at August, 2020.

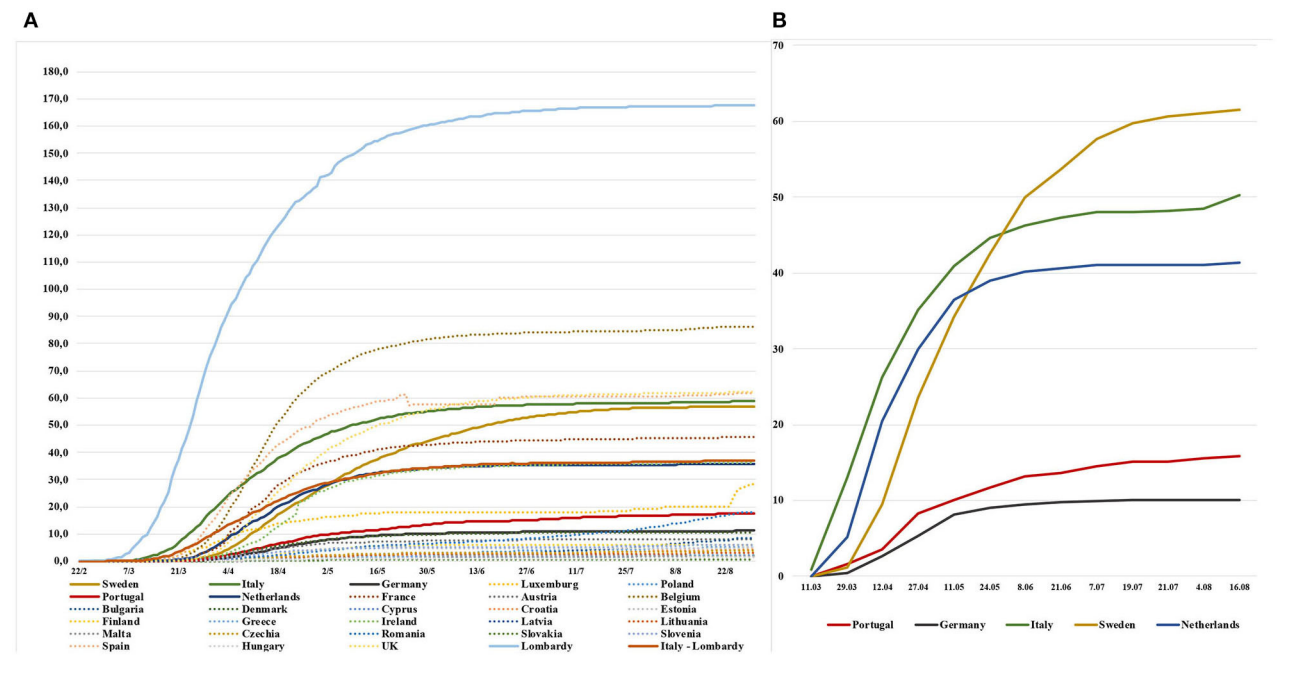

FIGURE 2 | Cumulative Crude Mortality Rates (×100,000 inhabitants) for COVID-19 in 27 EU Countries plus UK **(A)**, and Cumulative Standardized Mortality Rates (×100,000 inhabitants) for selected EU Countries **(B)**.

reached at the beginning of June (**Figure 2B**). Similar trends, although with lower values, were also observed for Germany and Portugal (both 0.0/100,000 on 11th March, 8.1/100,000 and 10.1/100,000 on 11th May, 10.0/100,000 and 15.1/100,000 on 21st July, 10.1/100,000 and 15.9/100,000 on 16th August, respectively) with the plateau reached in the second half of May in Germany, and in the first half of June in Portugal. Reflecting the trends mentioned above, as of 16th August, Sweden has not yet reached a plateau, experiencing a constant increase (0.0/100,000 on 11th March, 32.4/100,000 on 11th May, 60.7/100,000 on 21st July and 61.6/100,000 on 16th August).

DISCUSSION

Before discussing our findings, it is necessary to note some limitations, not least because they have implications for policy. It seems remarkable that, in the face of a common threat that has had an enormous impact on the burden of disease in Europe, the routine hospital services (12) and the economy, governments have been unable to develop a shared understanding of what is being measured or to ensure that there are systems in place to measure it accurately and report it in a timely way. The ECDC has performed remarkably in collating and presenting

the available data but it is constrained by what is collected by national and regional governments. Given that this will not be the last pandemic, this is something that should be addressed as a priority.

Our analysis does, however, have some important strengths. First, it does adjust for the age distribution of populations in some countries, rendering them more comparable, although even where we had age-specific data, the early reports from some countries had around 5–10% of missing values for age. Second, by waiting until the initial peaks had subsided, it is possible to compare the overall impact. This is a function of both the height of the peak and the time that the rate remained elevated. The importance of this can be illustrated by the situation in Lombardy. Initially there was some debate about how it had fared. Thus, despite the scenes of struggling hospitals, its death rate 30 days after the onset of the epidemic was well-below the corresponding figures in the Community of Madrid and in Brussels (41.4/100,000 in Lombardy vs. 77.1 and 48.6/100,000, respectively) (13). Yet it can now be seen that Lombardy has experienced overall the highest COVID-19 mortality rates in Europe (14). There are several possible reasons: it was the first region to be affected in Europe, at a time when there was little understanding how to manage this new illness. Lombardy adopted a hospital-centred approach, in contrast to neighbouring regions (45% of COVID-19 patients hospitalized versus 22% of other Italian regions) (15), its intensive care units were overwhelmed (16), and its nursing homes accommodated many elderly frail patients (17). The first COVID-19 clusters in the Netherlands, Germany, and Portugal started between one and two-weeks later than in Italy, by which time they had seen what was happening in Lombardy. Germany stands out from other countries. A plausible explanation relates to its much greater ICU capacity, with 29.2 beds/100,000 population in Germany vs. 8.4/100,000 in Italy, 4.2/100,000 in Portugal, and 6.4/100,000 in the Netherlands at the onset of the epidemics (16, 18). Sweden also stands out. Although it had made some recommendations about interpersonal distancing, it rejected many of the restrictions imposed elsewhere. At the time, advocates of the Swedish approach suggested that this would lead to a degree of immunity that would protect the country against subsequent waves but it is now clear that this was not the case (19).

The limitations of the data available for this analysis point to the need for future work by researchers and others. European governments and international agencies, including EUROSTAT and the WHO must find ways to collate and rapidly publish data on age at death for major causes. It is clear that the lethality of this disease increases with increasing age. Yet there is little information about whether this increase is the same everywhere. This is important information that could offer insights to inform policy but the data are lacking. More contentious, but as important, is the almost complete lack of data on mortality by ethnicity (the UK is a rare exception), so once again it is impossible to understand the scale and nature of inequalities within countries (20). Without this information, the scope for cross national learning is limited.

The COVID-19 pandemic is far from over. Already, it is clear that some countries have responded better than others. It is beyond the scope of this paper to determine why and as several countries are already experiencing a resurgence of cases, any definitive assessment would be premature. However, answers are likely to lie in three areas, political decision making, scientific advice, and health system and public health capacity (21). For now, in order to face the second wave of COVID-19, there is an urgent need to put in place systems that can provide timely, complete, and internationally comparable data (22).

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

Material preparation and data collection were performed by LV and SB. LV and FC performed the statistical analysis. The first draft of the manuscript was written by SB and LV and MM commented on the latest version of the manuscript. WR, MM, and SB supervised the study. All authors contributed to the study conception and design. All authors read and approved the final manuscript.

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ORIGINAL RESEARCH
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Underreporting of Death by COVID-19 in Brazil's Second Most Populous State

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The COVID-19 pandemic brings to light the reality of the Brazilian health system. The underreporting of COVID-19 deaths in the state of Minas Gerais (MG), where the second largest population of the country is concentrated, reveals government unpreparedness, as there is a low capacity of testing in the population, which prevents the real understanding of the general panorama of SARS-CoV-2 dissemination. The goals of this research are to analyze the causes of deaths in different Brazilian government databases (Civil Registry Transparency Portal and InfoGripe) and to assess whether there are sub-records showing an unexpected increase in the frequency of deaths from causes clinically similar to COVID-19. A descriptive and quantitative analysis of the number of deaths by COVID-19 and similar causes was performed in different databases. Our results demonstrate that different official sources had a discrepancy of 109.45% between these data referring to the same period. There was also a 758.57% increase in SARI deaths in 2020, when compared to the average of previous years. Finally, it was shown that there was an increase in the rate of pneumonia and respiratory insufficiency (RI) by 6.34 and 6.25%, respectively. In conclusion, there is an underreporting of COVID-19 deaths in MG due to the unexplained excess of deaths caused by SARI, respiratory insufficiency, and pneumonia compared to previous years.

Keywords: COVID-19 deaths, underreporting, SARI deaths, Minas Gerais, Brazil

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INTRODUCTION

Coronavirus 2 (CoV-2) is a new beta-coronavirus related to severe acute respiratory syndrome (SARS) that emerged in December 2019 in China and became a pandemic in March 2020 due to its high infection and mortality rates (1–3). COVID-19 was the official name given to the disease caused by the new coronavirus of 2019 (SARS-CoV-2) by the World Health Organization (WHO) (1).

The first epicenter of COVID-19 was observed in Wuhan, the capital of Hubei, China, in December 2019, based on several notifications of pneumonia cases (4).

Since then, COVID-19 has rapidly spread around the world and, as of May 12, 2020, more than 4.4 million cases of the disease have been confirmed, causing over 299,000 deaths worldwide (5). Of this total, Brazil registered its first case on February 26 and in May totaled more than 188,000 cases and more than 13,000 deaths (6, 7).

COVID-19 is classified according to symptom severity. Patients with the mild form (80% of the cases) present fever, dry cough, chills, malaise, muscle pain, and sore throat. Patients with the moderate form present fever, respiratory symptoms, and radiographic characteristics. Severe patients (5% of the cases) manifest dyspnea (> 30 bpm), low oxygen saturation (<93%), and a low PaO2/FiO2 ratio (<300 mmHg), which may evolve into respiratory failure, septic shock, and multiple organ failure (8–10).

Furthermore, increased age and the presence of comorbidities, such as hypertension, diabetes, and coronary disease, are associated with mortality in COVID-19 patients (11, 12).

The accurate diagnosis of COVID-19 is carried out by searching for the genetic material of the virus and, in a complementary way, by imaging methods. Computed tomography and radiographs can identify lesions in the lungs due to viral multiplication (13, 14).

Laboratory confirmation is essential for the timely management of cases to avoid the spread of transmission. However, the government of Brazil was unprepared (7, 15, 16), and is far below the ideal number of tests for COVID-19 (17) as there are not enough laboratory inputs to understand the overall panorama of the spread of the virus. Furthermore, confirmatory molecular tests depend on the availability of imported reagents, which are globally scarce, and on government investments that prioritize this strategy. This scenario has led to a delay in Brazil in the confirmation of the number of COVID-19 cases and deaths (18). These aspects become more aggravated when a patient dies, because effective testing for these cases is even more difficult. Considering the studies done to date, the recommendation is to collect blood and sputum to perform the culture, since these samples have a higher viral load (19).

The difficulty of death registration has also been presented in the state of Minas Gerais, which, by the end of April 2020, had 584 suspected death notifications, of which 81 (13%) had not yet been confirmed or discarded (20). Thus, it is possible to state that there is a disparity between the real number of COVID-19 deaths and the numbers that are reported in different Brazilian sources of information, since not all deaths have been confirmed or excluded and are potentially being caused by factors other than COVID-19.

The present study aims to analyze the causes of death in the notary records and in the Brazilian national disease notification system records, and thus evaluate the sub-registries and the possible increase in the frequency of deaths with causes clinically comparable with COVID-19 in the Minas Gerais territory.

METHODS

Data Preparation

The state of Minas Gerais (MG) has an estimated population of 21,168,791 people in a territory of 586,521.121 km², has the second largest population, and is the fourth largest state in the country (21). Its human development index (HDI) is 0.731, with a population composed of ∼22.25% aged 0–15 years, 69.31% aged 15 to 64 years, and 8.12% aged over 65 years (22).

For this study, the death records from the Civil Registry Transparency Portal database ("https://transparencia.registrocivil.org.br/inicio") (23) were analyzed from January to the first week of June 2020 (epidemiological weeks - EWs 1–23) in the state of MG according to their death cause. Additionally, to assess severe acute respiratory infection (SARI) excess deaths and COVID-19 deaths in these same EWs, information from the InfoGripe database ("http://info.gripe.fiocruz.br/") (24) was accessed referring to the range of years 2017 to 2020.

The Civil Registry Transparency Portal is a free access platform developed to provide information about births, marriages, and deaths. Due to the COVID-19 pandemic, these data are being grouped in the special section "COVID Registral Panel" ("https://transparencia.registrocivil.org.br/especial-covid") (25). The information presented here (accessed on 06/18/2020) is based on death certificates (DCs), presenting only one cause of death for each certificate (23). On DCs, the causes of death follow a specific order of completion and are named according to CID-10. Thus, the cause mentioned in the last line of the DC is considered the reason for the death to which it refers. With this data source, we also evaluated the excess deaths from causes that present clinical compatibility with COVID-19, according to the following etiology: pneumonia; respiratory insufficiency (RI), septicemia (sepsis/septic shock), and undetermined (causes of deaths linked to respiratory diseases, but not conclusive) (23).

The second data source, "InfoGripe database," is a platform of the Oswaldo Cruz Foundation (Fiocruz) (24) that aims to monitor and present alert levels for reported cases of SARI. On this platform, the records of SARI and COVID-19 were selected on 06/18/2020 according to the first and 23rd epidemiological weeks (EWs) from 2017 to 2020 for the state of Minas Gerais.

It is important to note that obtaining the data from the Civil Registry Transparency Portal takes up to 14 days, from the notification from the family to the update of the platform. The data analyzed here were obtained through those already in the system, without considering future updates (25). Similarly, the approximate period between notification in SINAN and the data presented on the InfoGripe website is 22 days (26).

The data were collected and analyzed in a spreadsheet by descriptive statistics and presented in raw numbers, relative frequency, and central tendency measures. The mortality rate was calculated using population data per 100,000 inhabitants from the state of Minas Gerais. To assess the excess SARI deaths per EW, the average, minimum, and maximum values of deaths from the years 2017 to 2019 were calculated and compared with the pattern of distribution in the same period of 2020. To assess the deaths from causes clinically comparable with COVID-19 in 2020 the data from each set were normalized to create a heatmap. All graphs were prepared using the *GraphPadPrism 7* software (GraphPad Software, Inc. San Diego, CA).

Statistics

Student's *t* test for independent samples was used to compare the average SARI deaths from 2017 to 2019 with the SARI deaths from 2020 during epidemiological weeks 1–23, using the *GraphPad Prism 7* software (GraphPad Software, Inc. San Diego,

CA). A p value < 0.05 was considered statistically significant. Furthermore, it was not possible to obtain the data from the Civil Registry Transparency Portal for the years 2017 and 2018 for deaths by other respiratory causes clinically comparable with COVID-19, a fact that made the statistical test for that case impossible.

RESULTS

Number of COVID-19 Deaths in the Databases

A total of 731 COVID-19 deaths were identified on the Civil Registry Transparency Portal, this number was 109.45% greater than the 349 total deaths registered in the InfoGripe database during the same period. While a total of 1,540 SARI deaths were identified on the InfoGripe database, this number was 539% greater than the 241 total deaths registered in the Civil Registry Transparency Portal during the same period (**Table 1**).

Excess Deaths From Causes Clinically Comparable With COVID-19 in 2020

The evaluation of the causes of death on the Civil Registry Transparency Portal showed an increase in the frequency of SARI deaths in 2020 in relation to the number of deaths from the same cause in 2019 and regarding the rates of the other diseases within the same period, no increase was observed (**Figure 1**).

The increase of SARI deaths in 2020 was in the order of 312.98% compared to 2019 (**Supplementary Tables 1, 2**).

Excess SARI Deaths in 2020

The analysis of the excess SARI deaths in 2020, according to epidemiological weeks 1–23, showed a significant increase in SARI deaths (66.95 ± 59.46) compared to the average of previous years 2017–2019 (7.79 ± 6.26) ($p < 0.0001$). As shown in **Figure 2**, the increase in SARI deaths was approximately 758.57%. Such a high rise in SARI deaths was observed in epidemiological week number 10 while the records of COVID-19 deaths in the state of MG were only reported from epidemiological week number 12 (**Supplementary Tables 3–6**).

DISCUSSION

The causes of death from respiratory diseases in Minas Gerais registered in the Civil Registry Transparency Portal and in the InfoGripe database were evaluated as proposed in our objective; however, we observed divergences between the different databases used. When analyzing deaths from causes clinically comparable with COVID-19, there was a significant increase in deaths from SARI in 2020 when compared to the averages of 2017, 2018, and 2019. As this increase occurred weeks before the registration of deaths by COVID-19 and it was an increase that exceeded the upper average of the previous 3 years, it is believed that deaths due to COVID-19 were underreported, since deaths from this cause may have been being registered as SARI.

The COVID-19 situation is particularly challenging because, besides being a new and unprecedented disease, it is also capable of triggering other conditions, such as pneumonia and SARI, which can be characterized as the main cause of death. In other words, COVID-19 may be the underlying cause; that is, it may not be the direct cause of death that has been registered. In this perspective, there is a subjectivity bias, since physicians can neither confirm nor deny that a death was caused by COVID-19 according to their clinical knowledge without the need of laboratory tests (27). This finding corroborates data from Hubei, China and Northern Italy, where mortality calculations were adjusted for the biases of preferential verification, symptomatic and severe cases, and the delay in death records. An increase in the mortality rate was found, which confirmed the underreporting of COVID-19 deaths in those regions (28). The context of the similarity of signs and symptoms between COVID-19 and SARI and of the subjectivity bias is real in Minas Gerais. This is reflected in the significant increase in deaths from SARI compared to the average of previous years at a time when COVID-19 occurred.

As this increase in SARI deaths in 2020 significantly exceeded the average number of deaths from this cause in 2017, 2018, and 2019 and due the current scenario of the COVID-19 pandemic, we believe this increase was, in fact, cases of COVID-19 registered as SARI, which would represent an underreporting of deaths by COVID-19. But still, in relation to underreporting in Brazil, the Ministry of Health (MH) reports that the number

TABLE 1 | Number of deaths by COVID-19 and SARI according to the information on the Civil Registry Transparency Portal and the InfoGripe database.

	January		February		March		April		May		June[a]		Total	Mean (SD)	Deaths per 100,000 inhabitants
COVID-19 deaths	***N***	**%**	***N***	**%**	***N***	**%**	***N***	**%**	***N***	**%**	***N***	**%**	***N***		
Civil Registry Transparency Portal	0	0	0	0	49	6.7	194	26.54	353	48.29	135	18.47	731	121.83 (136.98)	3.45
Infogripe database	0	0	1	0.29	48	13.75	94	26.94	163	46.7	43	12.32	349	58.17 (62.06)	1.65
SARI deaths															
Civil Registry Transparency Portal	8	3.32	6	2.49	35	14.52	84	34.85	88	36.51	20	8.30	241	40.17 (37.0)	1.14
Infogripe database	19	1.23	30	1.95	405	26.30	459	29.81	509	33.05	118	7.66	1540	256.67 (225.26)	7.27

[a] *Corresponding to the first week of June.*

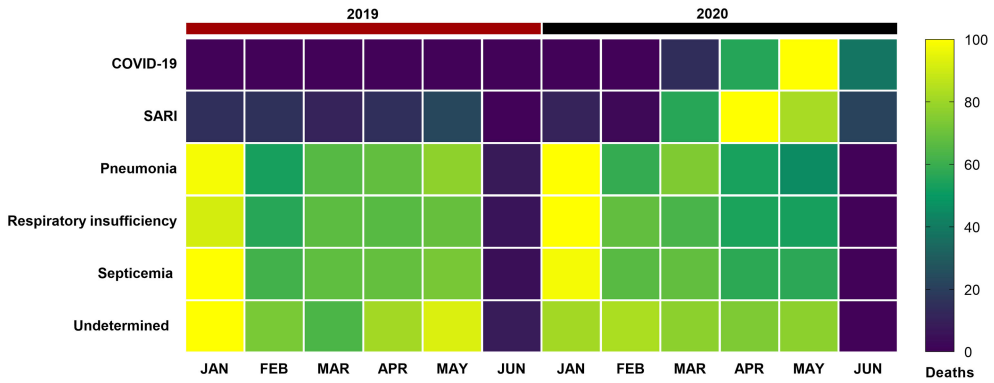

FIGURE 1 | Distribution of deaths by COVID-19, severe acute respiratory infection (SARI), pneumonia, respiratory failure (RSI), sepsis, and undetermined causes (deaths related to respiratory diseases, but not conclusive), according to the Civil Registry Transparency Portal, from January to the first week of June 2019 and 2020 in the state of Minas Gerais, Brazil. Each rectangle represents the percentage of deaths by disease colored by its normalized intensity scale from blue (fewer deaths) to yellow (more deaths).

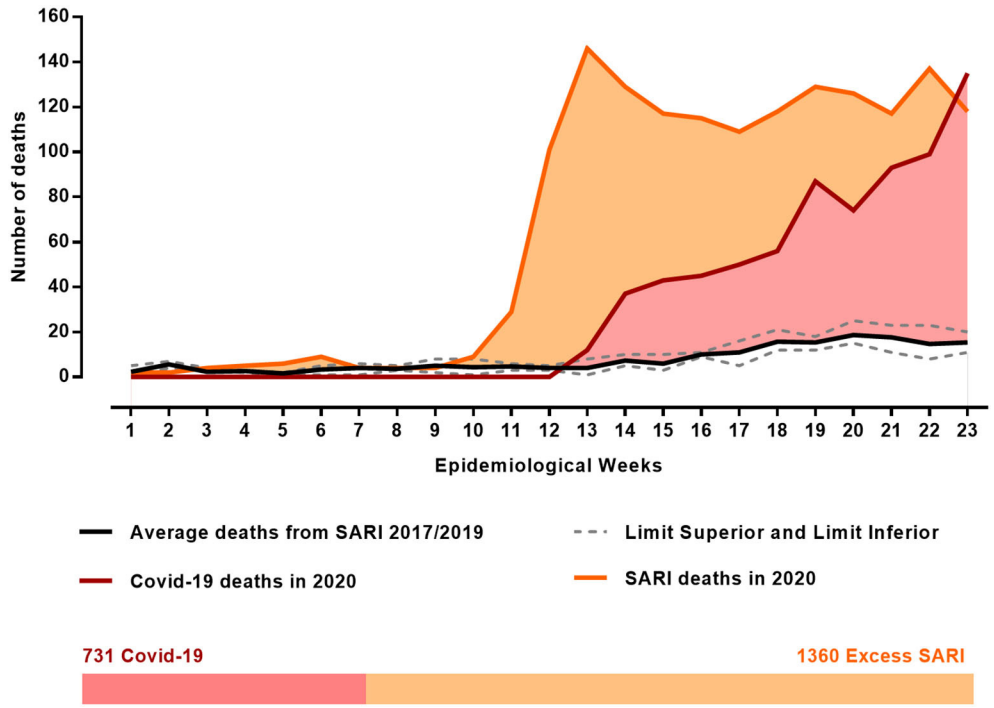

FIGURE 2 | Evaluation of excess deaths from SARI in 2020, during epidemiological weeks 1–23 compared with the average deaths in the same weeks of the previous years (2017–2019), according to the InfoGripe database. Data on deaths by COVID-19 during the same epidemiological weeks of 2020 were obtained on the Civil Registry Transparency Portal database in the state of Minas Gerais, Brazil.

of underreported deaths is low according to the Mortality Information System (MIS), because states and municipalities are advised to include deaths from COVID-19, either confirmed cases or only suspected cases, in the system as a priority in order to advance analysis of these cases (29).

Another issue that should be analyzed is that, although the Civil Registry Transparency Portal takes into consideration both confirmed and suspected deaths, the MH only discloses laboratory-proven COVID-19 deaths in its reports (29). However, suspected deaths need to be considered in the count, even though it is noted that they have not been confirmed. This is stated because it is known that many of these deaths will not be able to be analyzed, given the difficulties in collecting, transporting, and wrapping the post-mortem samples. Thus, if they are not mentioned, there will be an underestimation of the real situation in Brazil and, consequently, in the state of MG.

The Brazilian Ministry of Health also highlights that on the same death certificate more than one cause of death can be

described, so that the registration of COVID-19 can be associated with other diseases. However, the Civil Registry Transparency Portal presents these causes separately, even those included or registered on the same death certificate. Thus, for MH, it is not possible to add only the deaths made available on the portal by different diseases, as they would generate false overreporting. The solution would be a complete investigation that considers each death and the causes mentioned on the death certificate (29). However, according to the hierarchical criteria exposed in the Transparency Portal of the Civil Registry, only one cause of death is selected to make the count, and not all causes are presented on the same death certificate (25), which validates the exposed data on that platform and the information presented here.

It is worth mentioning that the different government death registration systems, such as in the various municipalities and states, are not fully connected and that several of them depend on manual work for registration. This can cause discrepancies and delays in data traffic and, consequently, in the production of timely and reliable information. This disconnect between the different systems was observed in our study, by demonstrating the divergence between the Civil Registry Transparency Portal and the InfoGripe.

InfoGripe is a system powered by data from the Influenza Surveillance Information System (SIVEP-Gripe) of the MH and SINAN system for the notification and investigation of cases of diseases and conditions that appear in the National List of Compulsory Notification Diseases (30). COVID-19 was added to this list on February 17, 2020 (31) and, according to the guidelines of the MH, all deaths due to severe acute respiratory syndrome associated with coronavirus (SARS-CoV-2), regardless of hospitalization, must be notified in SIVEP-Gripe, including those occurring in municipalities that do not have a SIVEP-flu record, as they do not have a hospital unit (32). Thus, this notification system is not restricted to the hospital environment and considers deaths at home or in the community. However, it is worth mentioning that, despite this system considering both environments, health and community, in Minas Gerais the pandemic generated an increase in deaths at home due to respiratory causes very similar to COVID-19, such as pneumonia, respiratory failure, sepsis, and SARI, when comparing the months from January to June of the years 2019 and 2020. Thus, given the difficulties in diagnosing and testing the disease, such as performing necropsies and collecting biological materials, it is possible that there would be a rise in underreported deaths by COVID-19 (33).

Furthermore, COVID-19 is closely related to high population density, since it is a contagious disease. In 2012, Minas Gerais occupied the 9th position in the national ranking of urbanization, which corroborates the state's greater vulnerability to the disease. However, in addition to extension, other factors influence vulnerability to the disease, such as education, sanitation, and development indicators (34). Thus, the state of MG has one of the best rates of education, economy, work, and income when compared to other states in the country (22) and that contributes to reduce the state's vulnerability to COVID-19. On the other hand, like most developing countries, Brazil has tested little when compared to developed countries and for the state of MG this reality is no different. The state has the third lowest number of tests per 100,000 inhabitants, a fact that contributed to the failure in the identification of potential transmitters and in counting the number of reported cases, leading to underreporting of the disease, especially when adding possible underreporting for different causes (34).

WHO has been advising countries on the need to expand laboratory testing capacity as a strategy to overcome the pandemic (35). This action will enable the collection of correct information regarding a population's immunity, providing reliable statistics for a better understanding of the circulation of the disease. Consequently, strategies to control the pandemic and even the relaxation of non-pharmacological measures, such as social isolation and quarantine, may be proposed. In Brazil, a network formed by referenced laboratories was established to help fight COVID-19 (19). However, the country is far below the optimal number of tests for COVID-19, as there are not enough tests to display an accurate account of the real number of cases and deaths. This scenario leads to a delay in compiling the records of COVID-19 in Brazil.

It is important to consider that this study can be influenced by limitations related mainly to the source databases consulted. These limitations are inherent to data processing, such as collection, recording, and punctuality, and can cause instability, leaving them subject to change. As a relevant limitation, this article highlights the lack of integration between the two sources consulted, the Civil Registry Transparency Portal and InfoGripe, and the different data collection systems used by them, which could influence the discrepancy between the data presented. The lack of integration between different existing information systems makes it impossible to integrate information from different sources (36). For this reason, several statistical models are proposed to correct this delay, which shows a possible justification for the discrepancy in data observed between the two systems used in the article (37). Therefore, it is necessary to address the methodology used in each database. The Civil Registry Transparency Portal, maintained by ARPEN, has a 14-day delay from death to the updating of its system and InfoGripe monitors the notification data for severe acute respiratory infection (SARI) in Brazil using the SINAN system (24, 38) and the approximate period between notification in SINAN and the data presented on the InfoGripe website is 22 days (39). However, due to the emergency of the pandemic, a technical standard was instituted, and hospitalized SARI cases were immediately notified in SIVEP-Gripe (40). This probably streamlined the process and shortened the deadline, since the registration could be streamlined by eliminating the need for communication between the State Health Secretariats and the SVS. Regarding the system methodology, InfoGripe uses a statistical model that estimates the most recent data, such as the use of the delay pattern between the first symptoms and the date of an update to estimate the cases that have not yet been added. In addition, the model also corrects possible false positives, which could explain the lower SRAG rates presented by the system (38).

Therefore, our results reveal that deaths due to COVID-19 in the state of Minas Gerais may be higher than the official statistics

presented. In view of these aspects, it is necessary to expand the diagnostic capacity of Brazil, which will allow us to recognize the real number of deaths and cases of COVID-19 in Minas Gerais. It is also worth mentioning that, although the spatial profile of this study is the state of MG, these observations and analysis can represent the general scenario experienced throughout the country. That is why the data presented here are important, as they reveal the need for other studies to be carried out in order to analyze the real situation of COVID-19 deaths in different Brazilian states; thus, we are able to improve the strategies to contain the COVID-19 pandemic in Brazil.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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 from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SO, TA, and TS contributed to the conception of the study. TA contributed to the acquisition, analysis, interpretation of data, and creation of tables. TS contributed to the statistical analysis, interpretation of data, and creation of tables and figures. NR and SS participated in revising the manuscript critically for important intellectual content within the discussion topic. All the authors co-wrote the paper and give final approval to the version submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.578645/full#supplementary-material

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Medical Liability in Cancer Care During COVID-19 Pandemic: Heroes or Guilty?

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Background: The COVID-19 outbreak rapidly became a public health emergency affecting particularly the frail category as cancer patients. This led oncologists to radical changes in patient management, facing the unprecedent issue whether treatments in oncology could be postponed without compromising their efficacy.

Purpose: To discuss legal implications in oncology practice during the COVID-19 pandemic.

Perspective: Treatment delay is not always feasible in oncology where the timing often plays a key role and may impact significantly in prognosis. During the COVID-19 pandemic, the oncologists were found between the anvil and the hammer, on the one hand the need to treat cancer patients aiming to improve clinical benefits, and on the other hand the goal to reduce the risk of COVID-19 infection avoiding or delaying immunosuppressive treatments and hospital exposure. Therefore, two rising scenarios with possible implications in both criminal and civil law are emerging. Firstly, oncologists may be "accused" of having delayed or omitted the diagnosis and/or treatments with consequent worsening of patients' outcome. Secondly, oncologists can be blamed for having exposed patients to hospital environment considered at risk for COVID-19 transmission.

Conclusions: During the COVID-19 pandemic, clinical decision making should be well-balanced through a careful examination between clinical performance status, age, comorbidities, aim of the treatment, and the potential risk of COVID-19 infection in order to avoid the risk of suboptimal cancer care with potential legal repercussion. Moreover, all cases should be discussed in the oncology team or in the tumor board in order to share the best strategy to adopt case by case.

Keywords: COVID-19, SARS-CoV-2, medical liability, pandemic, cancers

INTRODUCTION

The outbreak of coronavirus disease 2019 (COVID-19) became a public health emergency, since the World Health Organization declared the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) a pandemic on 11th March 2020 (1). Although the severity of this disease and the risk of death seem to be associated with old age and preexisting comorbidities such as cardiovascular disease, diabetes, and chronic obstructive pulmonary disease, cancer patients and cancer survivors could represent additional high-risk categories due to anticancer agent-related immunosuppression (2). Although the additional attributable risk to cancer is still unknown, there are some evidences showing a significant risk of COVID-19 infection among cancer patients over the age of 60 and concomitant lung comorbidities (3). This led clinicians to radical changes in patient management having the hard task of restructuring health systems to effectively manage the pandemic and at the same time provide the continuum of care (4). Therefore, following the Chinese model, globally many institutions were forced to adopt emergency measures such as workforce redeployment and reduction in capacity of oncology unit members due to staff shortages and to promptly adopt containment measures such as cancellation of scheduled surgical procedures and switching standard follow-up visits into phone follow-up visits or using other means of telemedicine in order to reduce hospital exposure (5).

This article describes the challenges handled by oncologists in providing cancer treatment during the COVID-19 pandemic, in particular the difficult task of balancing the expected benefits of treatment vs. the risk of exposing patients to SARS-CoV-2 infection and potential complications. Delayed treatment delivery and changes in treatment regimens can have a potential detrimental effect on prognosis and may expose treating oncologists to legal action against them.

MEDICO-LEGAL IMPLICATION IN CANCER CARE

This public health emergency forced clinicians to make difficult decisions concerning the timing of care (immediate vs. deferred) and to establish which treatments were essential for a relevant impact on prognosis (3). Therefore, during COVID-19 clinicians were called to find a compromising between the benefit achieved by immediate treatment and the possible odds of infection.

In this regard, oncologists were particularly under pressure given the growing concern for patients' vulnerability and often faced the unprecedent issue whether effective treatments could be postponed without compromising their efficacy (4).

Obviously, the delay of treatments is not always feasible in the oncology where often the timing of diagnosis and treatment may play a crucial role for the prognosis (6). Hence, in many cases the oncologists were found between the anvil and the hammer, on the one hand the need to treat cancer patients aiming to improve clinical benefits, and on the other hand the goal to reduce the risk of COVID-19 infection avoiding or deferring immunosuppressive treatments and hospital exposure.

Certainly, the pandemic has important medico-legal implications (7, 8). Unfortunately, despite the severity of the pandemic and the initial choral praise of the population for the utmost efforts of health personnel, this led to important repercussions in the field of legal medicine and numerous episodes of actions were undertaken against the legal liability of doctors (9). Therefore, several cases against medical malpractice or, more generally, regarding the responsibility of medical administrators for the inadequate measures of infectious risk control emerged, complaining on the drastic increase in deaths in nursing homes for elderly patients, but also against the inadequate medical care in non-COVID-19 emergencies (10).

Therefore, two rising scenarios with implications in both criminal and civil law are possible in oncology. Firstly, oncologists may be "accused" of having delayed or omitted the diagnosis and/or treatments with consequent worsening of patients' outcome. Secondly, oncologists can be blamed for having exposed patients to a hospital environment considered at risk for COVID-19 transmission. However, clinicians should be cautious and warned for forensic implications.

From a medico-legal point of view, in the first scenario the clinician may be "accused" of having delayed (or omitted) the diagnosis and/or treatment. The correlation of events between delay in diagnosis/treatment and disease progression could be investigated through a forensic study, analyzing tumor growth and progression over time with subsequent change in prognosis.

This change in prognosis and the reduction in life expectancy could be a relevant reason for medical malpractice.

On the other hand, oncologists can be liable for having exposed the patient (having to go to the hospital for treatment) to the infection. In these cases, it is very complex to distinguish whether COVID-19 infection occurred due to immune-suppression treatment related to any social contact inside and/or outside the hospital. Therefore, understanding whether the infection is related to the treatments or is independent is a complex task.

Only a close monitoring of all patients' contacts may give useful information for tracing those possibly responsible for the COVID-19 transmission.

Clinical decision making should be well-balanced through a careful examination between clinical performance status, age, comorbidities, aim of the treatment (cure vs. palliation), and the potential risk of COVID-19 infection in order to avoid the risk of suboptimal cancer care with potential legal repercussion (2).

Although avoiding or deferring effecting treatment in oncology during the COVID-19 pandemic is still a matter of debate (2, 11), in our opinion this concern involved only limited cases in daily clinical practice.

Immediate treatment should be promptly considered for those tumors at high risk of early mortality and highly sensible to chemotherapy (i.e., acute leukemia, aggressive lymphomas, metastatic germ cell tumors) where the cancer-related prognosis is poorer than COVID-19-related mortality. In the midst of the pandemic, an international survey among experts belonging to three cooperative groups (Italian germ cell tumors, European

G3 domain, genitourinary medical oncologists of Canada) posed the question whether the delay of treatment would be acceptable for a highly curable cancer as germ cell tumors (GCT) (12). Although there was a large consensus among experts in treatment discontinuation or delay for COVID-19-positive patients, management strategies of COVID-19-free GCT patients remained intact reflecting the priority to guarantee a high standard of care for GCT patients, as shown by the low rate of elective surgical delay as well as the management of poor-risk patients (12). Moreover, an immediate local treatment should be always offered in patients with localized disease where surgery or radiotherapy may play a curative role (13, 14). Suboptimal delivery of radiotherapy or surgery has been demonstrated to compromise both local control and survival (13, 14). For example, delaying the initiation of adjuvant radiotherapy >8 weeks after surgery doubles the risk of local recurrence in patients with breast cancer (15). Similarly, delaying the initiation of surgery in patients with stage II or III colon cancer negatively impacted overall survival (14).

Therefore, many institutions showed that radiotherapy has been safely delivered during the COVID-19 pandemic especially when used with curative intent, and in some clinical scenarios it could replace surgery maintaining similar outcomes avoiding intensive care unit occupation (i.e., radical radiotherapy on the prostate instead of radical prostatectomy in high-risk localized prostate cancer, concomitant chemotherapy, and radiotherapy for cervical cancer instead of surgery). Furthermore, many centers increased the use of hypofractionated regimens, which minimize the number of visits to hospitals while also avoiding potentially detrimental delays in the delivery of cancer care (13).

However, treatment delay may be taken into account in tumors slowly progressing and low early cancer mortality (i.e., basal cell carcinomas or low-risk prostate cancer) where the lethality due to COVID-19 infection is likely to be higher than cancer-specific mortality. In these cases, it is likely that treatment delay does not change the prognosis. Moreover, in these circumstances standard follow-up should be replaced with telematic evaluations (6). The most difficult task of choice is limited to other neoplasms (i.e., bladder cancer, breast cancer, colorectal cancer, lung cancer, melanoma, etc.) in which a diagnosis (through screening) or punctual therapy could change the prognosis. An Italian survey conducted among members of Italian association of cancers and the Italian breast cancer study group showed some potentially alarming signals of undertreatment (16). In the neoadjuvant setting, fewer oncologists compared with those before the emergency adopted weekly paclitaxel (68.5 vs. 93.9%) and a dose of anthracycline-based chemotherapy. Similarly, in metastatic settings fewer oncologists compared with those before the emergency adopted weekly paclitaxel upfront for Her-2-positive disease (41.8 vs. 53.9%) or CDK 4/6 inhibitors for ER-positive HER2-negative metastatic breast cancers with less-aggressive features (55.8 vs. 80%) (16).

Similarly, delays in chemotherapy for colorectal cancer is associated with lower survival. Furthermore, there is a 16% increase in the risk of death for every month of delay in radiation therapy for patients with head and neck cancer (6, 17–19). Moreover, given the uncertainty of an interference between immune checkpoint inhibitors and Sars-COV-2 pathogenesis, a survey conducted among Italian physicians involved in the administration of immune checkpoint inhibitors in oncology explored their perception about SARS-CoV-2-related risks in patients with solid tumors receiving these therapies, and the attitudes toward their management during the COVID-19 outbreak (20). Almost 47% of oncologists supported the hypothesis of a synergism between the mechanism of action of immune checkpoint inhibitors and the pathogenesis of SARS-CoV-2 infections and were concerned about the potential higher risks of COVID-19-related complications in cancer patients. Nevertheless, it was reassuring that 97.1% of respondents would not deny immune checkpoint inhibitors as a treatment option at the time of the COVID-19 outbreak only based on the possible risks of infection by SARS-CoV-2, considering the lack of evidence of a detrimental effect of their administration (20).

In this context, the clinicians are at risk of important legal consequences. For example, in Italy the doctor risks a conviction for manslaughter or personal injury (or impairment of health) from a criminal law point of view, whereas from the civil law perspective, the clinician risks to compensate (through insurance) a large sum of money (compensation for damage).

In these cases, the costs of the medico-legal dispute can increase the insurance charges. Therefore, economic resources are allocated to compensation for damage and are subtracted from the resources destined to improve the health service for the needs of patients.

No judgments have been delivered in this area yet, so we do not know the jurisprudential orientation. In our opinion, the delay in diagnosis/therapy of neoplasms (with poor prognosis if not treated immediately) could be justified (or in part partially "forgiven") in geographical areas with a very high incidence of infections (for example Lombardy), whereas an excessively prudent health attitude would not have enough justifications in areas with low incidence of COVID-19.

Furthermore, the reduction of the risk of transmission of SARS-CoV-2 through hospitals should be limited by an adequate triage and oncologists should provide complete information regarding drawbacks and benefits that are treatment-related as well as treatment plans which should be shared and accepted by the patients signing a written informed consent. Moreover, all cases should be discussed in the oncology team or in the tumor board in order to share the best strategy. At least, several efforts have been made by national and international scientific societies to offer guidelines for the delivery of anticancer treatment for standardized cancer care among different institutions, thus limiting the risk of medical malpractice and medico-legal implications (21).

CONCLUSIONS

COVID-19 has overwhelmed the capacity of the health system. Postponing cancer treatment is associated with certain risks. The latter should be balanced by benefits

yielded by anticancer agents, and clinical decision making should be discussed in the tumor board following international guidelines on the management of cancer patients' during the COVID-19 pandemic (21). The oncologists must do everything possible to avoid the risk of suboptimal care.

Abdul-Rahman Jazieh et al. (22) developed a detailed plan to help oncology services during a major coronavirus outbreak. The main objective was the prevention of new infections in the oncology service, managing currently infected patients and providing timely treatment of cancer for the entire patient population. The plan analyzed the management of infected patients, preventing new infections in patients or healthcare personnel, ensuring the continuity of cancer care, and incorporating measures to support these interventions up to the post-epidemic period. On the basis of this study, in our opinion patients should be divided into 3 general categories:

- Urgent: where surgical treatment, chemotherapy, or radiotherapy should not be postponed because of the high risk of worsening the prognosis.
- Intermediate: all cases should be discussed within the tumor board. Surgery may be rescheduled after a short delay, and the feasibility of chemotherapy and radiotherapy should be discussed case by case balancing risks and benefits.
- Postponable: the postponement of interventions does not change the prognosis. Therefore, if the risk of infection is high, we recommend postponing active treatment.

In any case, it is always necessary to test in-patients with nasopharyngeal swab at hospital admission and all out-patients before starting every cycle of systemic therapy. Similarly, healthcare personnel must be tested for SARS-CoV-2 periodically in order to avoid clusters of COVID-19 transmission within the Oncology Unit.

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

RB and FV: conceptualization. CM: methodology. CC: software. FV, AB, and CC: validation. RB and AB: formal analysis. RB: investigation, resources, and writing—original draft preparation. CM: data curation, writing—review and editing, and visualization. FV: supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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The Need for Antiviral Drugs for Pandemic Coronaviruses From a Global Health Perspective

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Respiratory failure due to SARS-CoV-2 has caused widespread mortality, creating an urgent need for effective treatments and a long-term need for antivirals for future emergent coronaviruses. Pharmacotherapy for respiratory viruses has largely been unsuccessful with the exception of early treatment of influenza viruses, which shortens symptom duration and prevents infection in close contacts. Under the rapidly evolving circumstances of the COVID-19 pandemic, most clinical trials of experimental treatments in the United States have focused on later stages of the disease process. Worldwide, the clinical studies of the most impactful drugs, remdesivir and dexamethasone in ACTT-1, RECOVERY, and Solidarity, have studied hospitalized patients. Less than half of clinical trials in the U.S. have investigated oral agents, and the majority have taken place in hospitals at a disease stage where the viral load is already decreasing. The limited success of treatments for respiratory viruses and the viral dynamics of COVID-19 suggest that an antiviral therapy with the greatest impact against pandemic coronaviruses would be orally administered, well-tolerated, target a highly conserved viral protein or host-coronavirus interaction and could be used effectively throughout the world, including resource-poor settings. We examine the treatment of respiratory viral infections and current clinical trials for COVID-19 to provide a framework for effective antiviral therapy and prevention of future emergent coronaviruses and call attention to the need for continued preclinical drug discovery.

Keywords: drug discovery, global health, viral pneumonia, antiviral drugs, outbreak preparedness, COVID-19, coronavirus, target product profile

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INTRODUCTION

The emergence of SARS-CoV-2 has caused a devastating pandemic that has crippled healthcare systems, destroyed economies, and killed more than one million people. SARS-CoV-2 has eclipsed previous emergent coronaviruses in its global reach, and though the extent of the pandemic remains to be seen, models have predicted that significant transmission will occur through 2022 and resurgences will be possible through 2024 (1). Whether SARS-CoV-2 transmission persists or not, other emergent coronaviruses remain an ongoing threat to global health. Outbreaks of SARS-CoV, SARS-CoV-2, and MERS-CoV have demonstrated the high pathogenicity and mortality of zoonotic coronaviruses when they infect humans. By comparison, the SARS-CoV outbreak resulted in close to 8,000 cases with a 9.6% case-fatality rate and MERS-CoV has resulted in multiple clusters since 2012 with a case fatality rate of up to 40%, while the SARS-CoV-2 case fatality rate is estimated

to be 2.3% (2, 3). Moreover, multiple zoonotic coronaviruses are capable of infecting humans and could potentially lead to future pandemics (4).

An effective antiviral that has broad activity against coronaviruses would decrease the impact of future emerging coronaviruses by preventing deaths and slowing viral transmission while public health measures are put into place and vaccines are developed. Large scale efforts are underway to find drugs that can be repurposed to treat COVID-19. Clinical trials and high throughput screens of repurposed drugs may reveal a safe and effective drug that coincidentally treats COVID-19; however, drugs that are discovered by this approach will likely need further structural optimization to increase antiviral efficacy against coronaviruses or decrease side effects. Clearly defining the essential characteristics of an effective anti-coronavirus treatment during these early stages is important to ensure that investments in drug development are allocated strategically, and that the search does not end prematurely or fail due to waning interest from public funding agencies and the pharmaceutical industry.

ANTIVIRAL DRUGS FOR VIRAL LOWER RESPIRATORY TRACT INFECTION

Viral respiratory disease mortality is remarkably difficult to reduce with antiviral medications. For most respiratory viral diseases, antiviral treatment is limited to severe cases in vulnerable populations due to a lack of effective therapies. For example, the only antiviral therapy currently utilized for measles is ribavirin. Based on a single 2011 randomized trial of 100 children that showed ribavirin decreased duration of fever and symptoms (5) and a limited number of case series, ribavirin is utilized for people with measles who are profoundly immunocompromised or who have severe pneumonia, to unclear benefit. Limited drug development for more common respiratory viruses, such as adenovirus, is likely related to the low incidence of severe lower respiratory tract disease. Cidofovir is the only consistently utilized antiviral for severe adenovirus based on case series showing clinical improvement in hematopoietic stem cell transplant recipients with severe adenovirus disease (6, 7). However, cidofovir has not been studied in randomized controlled trials (RCT) and has a high rate of severe adverse effects. Similarly, though RSV is common, supportive measures rather than antivirals are the mainstay of treatment. Studies of ribavirin are contradictory and have not consistently shown clinical benefit in RSV lower respiratory tract infection (8). Like cidofovir for adenovirus, the evidence of benefit from ribavirin for RSV in the treatment of adult stem cell transplant recipients is limited to observational studies (9). Despite limited efficacy in treating RSV lower respiratory tract infection, prophylaxis using palivizumab, a monoclonal antibody that targets the RSV fusion glycoprotein, has been found to reduce the incidence of severe lower respiratory tract infection among children with chronic lung disease, congenital heart disease or a history of premature birth (10). The absence of effective treatment for clinically significant lower respiratory tract viral infection may reflect both the lack of resources devoted to drug discovery and the inherent limitations of antivirals for viral respiratory infections.

Antivirals often do not change outcomes because most respiratory viral infections are self-limited, viral replication is often waning at the time that symptoms develop and antivirals are administered too late. Moreover, severe disease manifestations, such as acute respiratory distress syndrome, are primarily driven by host-mediated inflammation rather than ongoing viral replication. The possible efficacy of antivirals in immunocompromised patients and as prophylaxis suggests that antivirals alter the progression of disease during active viral replication and tissue spread when viral replication is not already inhibited by the early host immune response. That said, the lack of antiviral efficacy against the above-mentioned infections may also be due to the limited intrinsic potency of the antivirals that were repurposed to treat these infections.

Influenza treatment is a notable exception, for which oral antivirals decrease symptoms, are well-tolerated, and are effective as prophylaxis. Neuraminidase inhibitors (NAIs), such as oseltamivir, have been mainstays of influenza treatment. Inhibition of viral neuraminidase prevents cleavage of host cell membrane glycoproteins and release of influenza virions. More recently, baloxavir marboxil, a cap-dependent endonuclease inhibitor, has also proven effective against influenza. The success of antivirals with varied mechanisms of action against the influenza viruses indicate that influenza does not have a unique Achilles' heel that results in susceptibility to antivirals. Accordingly, coronavirus inhibitors targeting essential stages of viral proliferation would be expected to decrease the severity of disease if administered early enough to reduce the viral burden.

Both preclinical animal studies and clinical studies have shown that influenza treatment with oseltamivir within 36 h of symptoms shortens the duration of symptoms compared to placebo (11–14). Given the incubation period of 24–48 h for influenza (15), these patients were likely treated 60–84 h post-infection. Observational studies highlight the real-world challenges of initiating influenza treatment within 36 h. The majority of patients present 72 h or more after developing symptoms (16, 17). The role for antivirals early in the disease is well-demonstrated but mortality benefit later in the disease course is less clear (18, 19). In addition to treatment, oseltamivir and baloxavir have demonstrated efficacy as prophylactic agents for household contacts of people with influenza (20, 21). Compared to the influenza viruses, the value of prophylaxis is greater for highly pathogenic coronaviruses due to the higher mortality, immunologically naïve population and the prolonged incubation period and transmission during the asymptomatic phase (22). An effective, well-tolerated, orally administered prophylactic drug could both prevent progression to severe disease and play a crucial role in limiting SARS-CoV-2 transmission alongside aggressive testing strategies.

COVID-19 TREATMENT EFFICACY IN CLINICAL TRIALS

The understanding of SARS-CoV-2 viral dynamics is rapidly evolving, but two quantitative PCR studies showed the highest

viral loads at or just after symptom onset, with a subsequent gradual decline (23, 24). These data suggest that the viral dynamics are similar to influenza viruses, in which viral load peaks on the day of symptom onset (15). This indicates that starting antiviral therapy as close to symptom onset as possible, or after a high-risk exposure, has the greatest chance to reduce the viral burden of disease and pathology. That said, antiviral treatment of COVID-19 at a median of 9 days of symptoms has led to more rapid resolution of symptoms in certain patients, indicating that window for antiviral intervention may be longer for COVID-19 than it is for influenza (25).

The timing of antiviral therapy initiation in clinical trials of COVID-19 therapies has varied widely but has generally been later in the disease course after patients are hospitalized. The median time from symptom onset to randomization has been as long as 30 days but, for the most part, studies have enrolled patients with a median time from illness onset to randomization of 9–13 days (10, 26–29).

The results of RCTs that have shown clinical benefit suggest that antivirals are less effective in advanced disease. The ACTT-1 remdesivir trial reported a median of 9 days from symptom onset to randomization (25). Whereas, there was no significant difference between groups that received remdesivir before or after 10 days of symptoms, patients receiving mechanical ventilation or extracorporeal membrane oxygenation did not benefit from remdesivir like patients with less severe disease. Subsequent results from the Solidarity trial did not show a clear benefit from remdesivir; however, the duration of symptoms prior to treatment was not reported (30). In RCTs that evaluated earlier time points, favipiravir plus interferon-α reported clinical improvement in subjects with symptoms $\leq$7 days, and in a randomized open-label trial of lopinavir-ritonavir, interferon beta-1b, and ribavirin compared to lopinavir-ritonavir alone, *post-hoc* subgroup analysis showed a shorter time to negative PCR and clinical improvement in subjects treated within 7 days of symptom onset, but no benefit if treated later (31, 32). Subsequent trials of interferon have shown mixed results (30, 33, 34). More recently, monoclonal antibodies targeting the SARS-CoV-2 spike protein are reported to possibly reduce hospitalizations when given within a median of 4 days of symptoms, whereas, monoclonal antibody therapy was not effective in hospitalized patients (35).

Conversely, evidence from clinical trials suggests treatment of COVID-19 with immunosuppressive therapies is effective after patients require supplemental oxygen and may be harmful if given too early. For example, while the dexamethasone arm of the RECOVERY trial showed a reduction in 28-day mortality in hospitalized patients with COVID-19 requiring supplemental oxygen or mechanical ventilation, there was no mortality benefit for patients not receiving supplemental oxygen or with <7 days of symptoms (36). This is notable given that 27% of oral drugs in clinical trials are immunomodulators (**Figure 1**). That said, fluvoxamine, which is presumed to modulate the host response to COVID-19 by interacting with the human sigma-1 receptor, was recently found to decrease clinical deterioration in a small trial (37). This finding, if replicated in larger trials, would support targeted immunomodulation early in disease. Immunosuppression has played a key role in reducing mortality in hospitalized COVID-19 patients; however, this approach would not limit the spread of infection as post- or pre-exposure prophylaxis and may not be applicable to future emerging coronaviruses.

CURRENT CLINICAL TRIALS OF DRUGS FOR COVID-19 IN THE UNITED STATES

A comprehensive registry of COVID-19 trials listed 414 clinical trials of pharmacotherapeutic interventions in the United States as of November 1st, 2020 (38). Seventy-two trials studying vaccines, devices, oxygenation strategies, or other non-pharmacologic strategies were excluded. Of the 414 drug studies, 44% are of intravenous medications (**Figure 1**). The focus of clinical trials on hospitalized patients reflects efforts to treat severe disease. While this is an appropriate focus during a global health crisis, prior experience with respiratory viruses demonstrates marginal benefits from antivirals at this stage of disease. Ultimately, a drug administered early in infection to decrease viral transmission and to prevent progression from mild to severe disease will have the greatest impact. Only 44% of the current U.S. drug trials listed are studying oral therapies, and 31% of those studies involve hydroxychloroquine. Outpatient intravenous therapies such as remdesivir and the monoclonal antibodies, LY-CoV555 (bamlanivimab) and REGN-COV2, may limit disease progression in high-risk populations in resource-rich countries, but the cost, scale of production, and infrastructure required for intravenous administration prohibit their use as prophylaxis or treatment on a global scale (35). The prolonged time required to develop monoclonal antibodies for a novel virus also precludes them from being an initial response to an emerging pandemic virus.

Only 7% of clinical trials evaluating pharmacologic treatments for COVID-19 in the US are evaluating oral drugs with proposed antiviral mechanisms. Of the RCTs of outpatient oral treatment, 11 drugs have *in vitro* evidence of SARS-CoV-2 inhibition: AT-527, camostat mesylate, dipyridamole, ebselen, EIDD-2801, favipiravir, ivermectin, niclosamide, nitazoxanide, oleandrin, and toremifene (39–49). Of these drugs, only favipiravir has prior results from prospective clinical trials, which have had conflicting results (32, 50, 51). RCTs of favipiravir are ongoing in both the inpatient and outpatient setting. *In vitro* results for several of these drugs suggest that clinical efficacy is unlikely. Plasma concentrations achieved by current doses of ivermectin are far below the concentrations that are predicted to be required based on its inhibitory activity against SARS-CoV-2 in cell culture (41). The low selectivity index of toremifene of 2.8 indicates that *in vitro* activity may be related to host cell toxicity rather than efficacy, and toremifene would not be tolerated by patients at antiviral doses (46). Similarly, the therapeutic index of oleandrin is likely very narrow and the risk of overdose is high given that effective *in vitro* concentrations against SARS-CoV-2 overlap with plasma concentrations that have resulted in toxicity (49, 52, 53). Two oral prodrug compounds in particular, AT-527 and EIDD-2801, have shown promising broad active

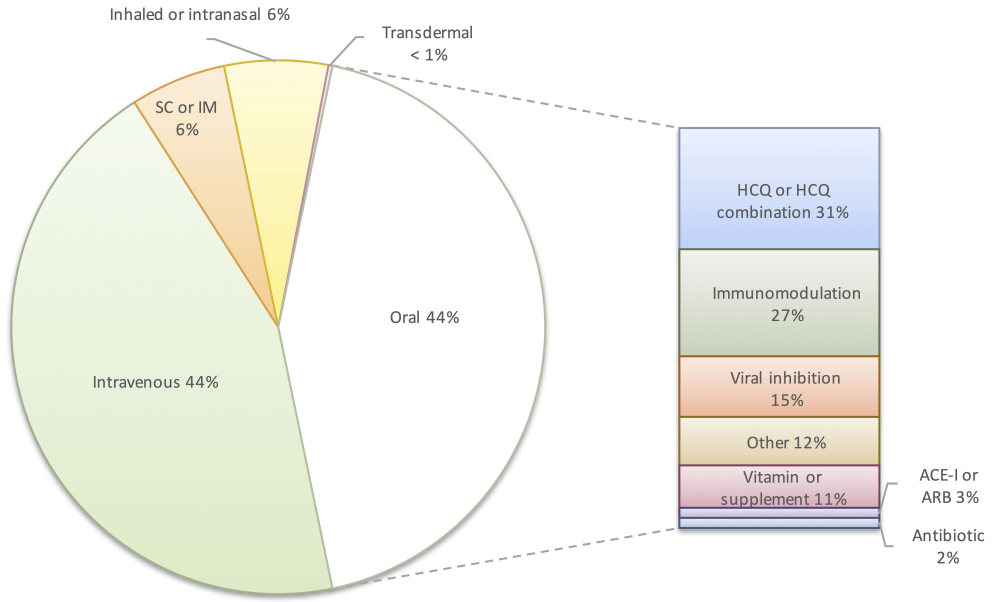

FIGURE 1 | Distribution of route of administration and types of oral pharmacologic intervention in clinical trials for COVID-19 ($n = 414$). SC, subcutaneous; IM, intramuscular; HCQ, hydroxychloroquine; ACE-I, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker.

against multiple coronaviruses. EIDD-2801, a ribonucleoside analog, inhibits 50% of SARS-CoV-2 replication in cell culture at concentrations $<0.1\,\mu M$, was active against SARS-CoV, MERS and several bat coronavirus strains, and reduced lung viral loads and improved pulmonary function in a mouse model of SARS-CoV and MERS (40). AT-527, a guanosine nucleotide analog previously studied in patients with hepatitis C, inhibited 90% of SARS-CoV-2 replication at a concentrations close to $0.5\,\mu M$, and was active against SARS-CoV and human coronaviruses, HCoV-229E and HCoV-OC43 (47). While encouraging that a few drugs in clinical trials could be repurposed to treat COVID-19 and emerging coronaviruses, this small number reveals a large unmet need for preclinical coronavirus drug development.

A TARGET PROFILE FOR A GLOBAL ANTI-CORONAVIRUS DRUG

Target product profiles are often constructed by industry, regulatory agencies, or public health organizations to strategically identify attributes required for drugs to meet essential needs. In the case of highly pathogenic coronaviruses, a target profile serves to define the minimal targets that should be met before drug discovery efforts cease rather than exclude drugs that currently offer incremental improvements (**Table 1**). After a successful COVID-19 vaccine is in widespread use, the economic incentives to discover drugs to treat COVID-19 and to prevent future coronavirus pandemics will be diminished. Defining benchmarks now will help set goals for drugs that could be stock-piled or ready for production and clinical testing in the event of another novel coronavirus outbreak and a consensus around preclinical development that will likely rely on funding from public agencies.

The target population for a coronavirus antiviral should be as broad as possible and include children and pregnant women. Mortality due to SARS-CoV-2 is increased in older adults, but young adults and children have served to spread infection, and future coronaviruses may have higher mortality in younger populations. An oral formulation is necessary for a drug to be available on a global scale and in infrastructure-limited regions. Given that zoonotic coronaviruses are globally distributed, the next pandemic could emerge in a resource-poor setting. Ideally, an additional parenteral or rectal formulation would allow for treatment of patients who are too ill to take oral medications.

A key part of defining a desired antiviral profile is setting targets for efficacy of treatment and prophylaxis. Given the limited success of treating respiratory viral infections, the goal of a 10% reduction in mortality when given within 72 h is ambitious; however, the trend toward improved mortality with remdesivir in hospitalized patients and the possible protective effect of anti-SARS-CoV-2 monoclonal antibodies given with a median of 4 days of symptoms suggest that this target is possible for COVID-19 (25, 35). In considering efficacy as prophylaxis, high SARS-CoV-2 household transmission rates of up to 53% suggest that partial efficacy would provide significant benefit (54). Decreased transmission rates to $\leq$50% of the natural transmission rates is a modest goal compared to influenza prophylaxis with NAIs or baloxavir, but would have a tremendous impact given the high transmission rates of SARS-CoV-2 in a non-immune population when there is no vaccine (20, 21).

TABLE 1 | Suggested drug profile.

Parameter to be demonstrated	Minimum essential	Ideal
Indication for use	• Post-exposure prophylaxis in close contacts of COVID-19 infected patients • Symptomatic with influenza-like illness for ≤72 h	• Post-exposure prophylaxis in close contacts of COVID-19 infected patients • Symptomatic with influenza-like illness for ≤96 h • Hospitalized patients with an oxygen saturation ≤94%
Target population	• Adults, including the elderly • Children • Pregnant women	• Adults, including the elderly • Children • Pregnant women
Formulation and route	• Oral	• Oral and intravenous, intramuscular or rectal
Clinical efficacy	• Decreased transmission in close contacts ≤50% of the naturally occurring rate • Decreased risk of hospitalization when administered within 72 h of symptom onset • Decreased risk of death ≥10% compared to no treatment in patients with confirmed infection when administered within 72 h of symptom onset	• Decreased transmission in close contacts ≤80% of naturally occurring rate • Decreased risk of hospitalization when administered within 96 h of symptom onset • Decreased risk of death ≥50% compared to no treatment in patients with confirmed infection when administered within 72 h of symptom onset, ≥20% at 96 h • Decreased risk of death ≥10% in patients with an oxygen saturation ≤94%
Susceptibility to resistance	• No immediate high-level resistance due to single point mutation after serial passage in culture without profound decrease in fitness	• No significant resistance after serial passage in culture without profound decrease in fitness • Drug target that not highly mutable or placed under selective pressure by drug
Spectrum of activity	• Active against betacoronaviruses	• Active against all alpha and betacoronaviruses and pandemic influenza viruses
Drug-drug interactions	• No unmanageable risk accounting for poly-pharmacy in elderly populations and critically ill patients.	• No identified risk accounting for poly-pharmacy in elderly populations and critically ill patients.
Safety and tolerability	• Few and manageable adverse events • No severe adverse events • No monitoring required	• Low incidence of mild adverse events • No severe adverse events • No monitoring required
Stability	• Stable for 3 years under controlled storage conditions • Stable for 6 months at 30 ± 2°C and 65 ± 5% relative humidity	• Stable for 5 years under controlled storage conditions • Stable for 12 months at 30 ± 2°C and 65 ± 5% relative humidity
Cost of production	• Amenable to rapid large-scale synthesis and global distribution	• Amenable to rapid large-scale synthesis and global distribution
Dosing regimen	• Three times daily	• Once daily

Recommended characteristics of a drug for SARS-CoV-2 and future potential pandemic coronaviruses.

Safety, tolerability and a lack of drug-drug interactions are an essential quality of a broadly used drug. Based on experience with the COVID-19 pandemic, the more than 3 months that were required to create and distribute an accurate test for SARS-CoV-2 necessitated a symptom-based approach to identifying and managing cases. A symptom-based approach to treatment and prophylaxis of an influenza-like-illness would result in many more people being treated than are infected. Moreover, the higher incidence of severe disease in older populations underscores the increased risk of side effects and polypharmacy and the importance of limiting drug-drug interactions.

Each viral disease is unique; however, the early days of the COVID-19 pandemic and the 2009 H1N1 influenza pandemic revealed obstacles that should be anticipated. The cost and scalability of production as well as the capacity to stockpile drug is equally important. Consequently, a drug must have long-term stability under heat and humidity, not require a cold chain, and if stockpiled as bulk powder, such as was the case with oseltamivir for H1N1, the capacity to rapidly reconstitute and distribute the drug must be in place (55). Finally, a drug that targets conserved coronavirus proteins or host-pathogen interactions and is broadly active against identified human and bat coronaviruses will have the greatest chance of being active against emerging coronaviruses. Creating a desired drug profile for an evolving pandemic or an anticipated coronavirus pandemic is challenging. In fact, the degree of impact of oseltamivir on the H1N1 pandemic remains a subject of debate (56). That said, we have suggested long term aims in hopes that drug development efforts for the next coronavirus pandemic will not end prematurely with a drug that only benefits specific populations in resource-rich countries.

CONCLUSION

Drug discovery efforts for respiratory viral illnesses have resulted in few effective treatments. For many of these illnesses, drug development is not a matter of urgency given the relatively

rare occurrence of severe pneumonia; however, the significant global mortality of lower respiratory tract infections from RSV in children and influenza reveals an unmet need for therapeutic interventions and the challenges of developing a respiratory antiviral drug that prevents severe disease. The mortality and societal costs of the highly pathogenic coronaviruses, SARS-CoV, MERS, and SARS-CoV-2 clearly show the immeasurable value of a drug to prevent the spread of a pathogenic coronavirus or prevent clinical progression to severe disease. Based on similarity in viral kinetics between influenza and SARS-CoV-2, examples of effective treatments for influenza, and preliminary evidence from COVID-19 clinical studies, medicines that can be administered early after symptom onset or as prophylaxis should be a primary target of coronavirus drug development. To accomplish this aim, drugs should have the standard characteristics of being well-tolerated, limited drug interactions, adequate tissue concentrations and a high degree of potency. In addition to standard characteristics, a drug to combat a global pandemic should be suitable for a range of global conditions and circumstances. Most importantly, the drug should target a viral protein or host cell pathway that is highly conserved among coronaviruses. The small number of repurposed drugs that currently meet these criteria indicate the need for robust preclinical drug discovery.

Alongside the first wave of clinical trials aimed at exploring off-the-shelf COVID-19 drugs, a parallel effort has searched for hits using phenotypic high throughput drug screens, *in silico* modeling of small molecule inhibitors with SARS-CoV-2 proteins and identification of host cell drug targets that are needed for viral proliferation (46, 57–59). These preclinical efforts have identified compounds that have low nanomolar IC_{50}s against SARS-CoV-2 (57, 60). Drugs and preclinical compounds identified up to this point may have limited impact on COVID-19 prior to widespread immunization, but they identify mechanisms and pharmacophores that serve as starting points for continued drug development. A deep pipeline of preclinical coronavirus drug candidates will be required to prepare for the next pandemic. Establishing long term research benchmarks to discover drugs with broad-spectrum activity against coronaviruses that will stop the next pandemic will be well worth the investment after the traditional financial incentives for drug development fade.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found at: https://www.covid-trials.org.

AUTHOR CONTRIBUTIONS

AV and SG contributed equally in conceiving and writing the manuscript. JL contributed expertise and edited the manuscript. JD contributed to the conception, writing, and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Case Report: Famotidine for Neuropsychiatric Symptoms in COVID-19

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Famotidine is of interest as a possible treatment for COVID-19, with effects on disease-related symptoms and survival reported in observational and retrospective studies, as well as *in silico* predictions of binding to potential SARS-CoV-2 drug targets. Published studies of famotidine for COVID-19 have focused on acute illness, and none have reported on neuropsychiatric symptoms. This case study reports on an 18-year-old man who sought psychiatric treatment for depression and anxiety, disruptive interpersonal conflicts, and impairments in attention and motivation following mildly symptomatic illness with COVID-19. The neuropsychiatric symptoms, which had been present for 16 weeks at the time of the initial evaluation represented a significant departure from the patient's previous behavioral baseline. The patient had no prior psychiatric history preceding his illness with COVID-19, and no history of any prior treatment with psychopharmacological medications. Famotidine 20 mg twice daily administered orally was begun without any additional medications. At 1-week follow-up the patient was much improved. Improvement was sustained through 12 weeks of follow-up during which the patient continued to take famotidine without apparent side effects. With progression of the COVID-19 pandemic it has become evident that persistent disease-related symptoms may follow acute COVID-19 and may include neuropsychiatric symptoms. Controlled clinical research on famotidine for COVID-19 should follow, as well as the development of valid and reliable research diagnostic criteria to define and operationalize the features of a putative COVID-19 neuropsychiatric residual.

Keywords: COVID-19, SARS-CoV-2, famotidine (FAM), depression, anxiety, psychiatry, cognitive, neuroinflammation

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INTRODUCTION

Famotidine, a histamine H2 receptor antagonist with labeled indications for heartburn and gastric reflux, has been suggested as a possible treatment for COVID-19 on the basis of observational and retrospective study evidence. In Wuhan, China, in January of this year, retrospective analysis of data of hospitalized patients indicated increased survival in patients with COVID-19 who had been taking famotidine at the time of admission to the hospital. The difference in mortality rates, 14 vs. 27% favoring those who had taken famotidine, did not reach statistical significance (1). Nonetheless, a physician familiar with these data observed an apparent treatment effect within 24 h after his sister, ill with COVID-19, took famotidine.

A published case series reports on 10 outpatients who self-treated with famotidine following the onset of COVID-19 symptoms (2). All of the patients in the series reported marked improvement in disease-related symptoms, with significant improvement in a group mean symptom score evident within 1 day of starting famotidine. Two retrospective cohort studies of patients hospitalized for COVID-19 compared patients who had taken famotidine to those who had not (3, 4). Both studies found a significant reduction of the primary endpoints of death and endotracheal intubation among patients who had taken famotidine, presumably for its labeled indications, within 24 h of hospital admission (3) or within 7 days of COVID-19 screening and/or hospital admission (4). An additional physician-sponsored cohort study of famotidine combined with cetirizine in hospitalized patients found rates of mortality that were said to compare favorably to published inpatient fatality rates from other regions (5).

Published studies of famotidine for the treatment of COVID-19 to date have focused on acute illness, and all but the case series (2) involved inpatients. None of the above studies reported on neuropsychiatric symptoms. With progression of the pandemic, it is increasingly evident that disease-related symptoms, including neuropsychiatric symptoms, may persist after acute illness with COVID-19 (6, 7). This case report describes treatment with famotidine for persistent neuropsychiatric symptoms following acute illness with COVID-19.

CASE DESCRIPTION

An 18-year-old man presented for psychiatric evaluation with complaints of "*I've been anxious, irritated and sad most of the time…*" and "*…inability to get motivated/concentrate and retain information.*" The patient was also seeking a second opinion after a psychotherapist had diagnosed him with Bipolar II Disorder (8).

The patient's presenting psychiatric symptoms were of relatively recent onset, an estimated 16 weeks before the initial evaluation, and represented a distinct change from the patient's previous behavioral baseline. In addition to depressed and anxious mood, he experienced disruptive behavioral episodes with increased emotional reactivity and somatic anxiety symptoms. He described these episodes as "*…break down in tears/hyperventilation... I'd blow up over something insignificant it would turn into a 5-hour argument.*" These episodes occurred in the context of but were not confined to interpersonal interactions, "*I also suffered them when thinking about career/future prospects*." Diagnostically, these events had features in common with panic attacks including prominent somatic anxiety with a paroxysmal onset, as well as features evident in bipolar mixed states, including heightened reactivity of mood and irritability. The patient's cognitive complaints included diminished motivation and sustained attention, as well as difficulty recalling memories from the previous days to weeks.

On initial evaluation, the patient scored 16 on the Beck Depression Inventory (BDI) (9) and 17 on the Beck Anxiety Inventory (BAI) (10). He denied any prior history of mood or anxiety symptoms or behavioral changes similar to those that had led him to seek treatment. He had never taken psychiatric medications apart from melatonin for sleep at dosages up to 10 mg per night starting 6 weeks prior to the initial psychiatric evaluation. His entire history of prior psychiatric treatment was limited to three visits with a psychotherapist over the month before the evaluation, which were not regarded to have been of benefit. The patient's family psychiatric history was limited to a paternal half-sibling who the patient viewed as possibly depressed but had never sought treatment. He denied any history suggestive of a substance use disorder and had no history or evidence of psychotic symptoms in the diagnostic interview.

TABLE 1 | Timeline of clinical case history, time is referenced to the initiation of treatment with famotidine (week = 0).

Event	Week
Probable COVID-19 exposure	−20
Onset of fatigue and cough	−19
Onset of neuropsychiatric symptoms	−16
Positive test for SARS-CoV-2 antibody	−5
Start famotidine	0
Follow-up (weeks +1, 3, 8, 9, 12)	+1–12

Approximately 19 weeks prior to the initial psychiatric evaluation, in the third week of February 2020, the patient experienced the onset of fatigue and cough (see **Table 1**). The fatigue persisted for approximately a week and the cough persisted for 3 weeks. The patient did not monitor temperature for 12 days following the onset of the fatigue and cough, as he explained, "*I suspected I just had seasonal allergies/ a cold. When more information about COVID began, including heightened temperature, that is when I began monitoring my temperature,*" and at this point, he was afebrile. At about 16 weeks prior to the evaluation, the fatigue recurred, with the onset at that time of the behavioral changes that would eventually lead the patient to seek psychiatric evaluation.

The week before the onset of cough and fatigue, the patient, who was attending college outside of New York State, went to an indoor conference in New York City that included approximately 100 attendees. He also visited his family at this time, including his father, a public transit worker in New York City with occupational exposure to the subway system. Both the patient and his father experienced the onset of cough and fatigue the week following their contact with one another, with the father experiencing relatively more severe symptoms. Nether the patient nor his father were tested for SARS-CoV-2 antigen, which was not systematically available in New York at that time. Five weeks prior to the initial psychiatric evaluation, the patient tested positive for SARS-CoV-2 antibody, following his father's positive antibody test result.

The working DSM-5 psychiatric diagnoses were Other Specified Mental Disorder and Mild Neurocognitive Disorder both due to COVID-19, according to a clinical hypothesis that the patient's presenting neuropsychiatric symptoms were related

to prior illness with COVID-19 (8). "Other Specified Mental Disorder" indicates that the symptoms did not meet criteria for any specific major mood or anxiety disorder, for example, the patient's disruptive behavioral episodes having some features of both mood and anxiety disorders but not fully meeting the criteria for either. "Mild Neurocognitive Disorder" subsumes symptoms in the domain of executive functions.

The patient was begun on oral famotidine 20 mg twice daily as per the drug labeling. No psychopharmacological or other medications were prescribed, melatonin was discontinued, and no behavioral interventions or lifestyle changes were made. On follow-up a week later, the patient said he felt "much better" and noted substantial improvement regarding his symptoms of heightened emotional reactivity and diminished motivation and sustained attention. He estimated the time interval between starting famotidine and symptomatic improvement at 4 days and described his state on the fourth day as "clear-headed," *"…I woke up and got out of bed without feeling awful…."* He reported no side effects. At 3-week follow-up, the patient's BDI and BAI scores were 1 and 2, respectively. A friend familiar with the patient's prior behavioral baseline described the patient's behavioral change following presumed COVID-19 illness and prior to treatment with famotidine as *"…more irritable or quiet…exhausted."* At 4 weeks following the initiation of famotidine, the friend described, *"… he seemed much more conversational as well as productive…very focused…."* The patient continues to report he is doing well at the time of this writing, 12 weeks following the initiation of treatment with famotidine, which he continues to take at the initially prescribed dosage of 20 mg twice daily.

DISCUSSION

This case suggests a possible treatment effect of famotidine for persistent neuropsychiatric symptoms following acute illness with COVID-19. It appears generally consistent with observational and retrospective study evidence for an apparent treatment effect of famotidine on disease-related symptoms and survival in COVID-19 (2–5).

The interval of 16 weeks from the initial onset of neuropsychiatric symptoms following apparent illness with COVID-19 to the initiation of treatment with famotidine is a distinctive aspect of this case. Individuals with persistent symptoms following acute illness with COVID-19, the "long haulers," are an increasing and arguably a presently relatively underserved population. A CDC study found that 35% of adult outpatients who were symptomatic at the time they tested positive for SARS-CoV-2 antigen had not returned to their usual state of health at 2–3 weeks following testing (7). Clinical investigation of famotidine for COVID-19 should include patients with disease-related symptoms at relatively extended time intervals following acute illness.

To date, the author has treated eight other patients in his general psychiatric practice with famotidine for persistent neuropsychiatric symptoms following acute illness with COVID-19. In contrast to the present case, these other patients were already receiving psychopharmacological treatment at the time of onset of COVID-19 and subsequent treatment with famotidine. Within the limits of uncertainty due to the intermingling of factors including psychiatric baseline, emotional responses to the pandemic, and variability in length of treatment with famotidine, most patients appear to have received some benefit. The most frequent domain of symptomatic improvement appears to be "brain fog," a term applied to a set of symptomatic features suggestive of problems with executive functions, including sustained attention/working memory and motivation, as well as word-finding and short-term memory. Patients have utilized the term "clearer" in their description of a famotidine effect.

Other symptomatic features of a putative COVID-19 neuropsychiatric residual relate to mood, anxiety, and emotional reactivity. Some patients with a prior history of depression describe mood changes following COVID-19 as distinct in quality from their previous depression. The term "despair" has been used, apparently connoting qualities of intensity and hopelessness, which may be of significance regarding suicidal risk. Irritability and highly reactive mood may be evident as interpersonal conflict. The expression of mood and anxiety symptoms may be episodic and paroxysmal. Development of valid and reliable research diagnostic criteria for a putative syndrome of COVID-19 neuropsychiatric residual would provide a basis for defining patient groups for clinical trials and measures of illness severity.

Neuroinflammation plays an increasingly appreciated role in psychiatric disorders (11). SARS-CoV-2 is neuroinvasive and neuroinflammatory (12). Baseline inflammatory markers predicted subsequent anxiety and depression 30 days after discharge from the emergency room or hospital in a cohort study of patients with COVID-19 pneumonia presenting for emergency evaluation (6).

SARS-CoV-2 viral persistence may mediate disease-related symptoms following acute COVID-19 illness. Clinical trial data indicate a potential for persistence of SARs-CoV-2; 41.5% of the subjects in a study of lopinavir–ritonavir still had a viral load detectable by oropharyngeal swab at 28 days following randomization (13), with an additional interval of 13 days between symptom onset and randomization. Further, SARS-CoV-2 may persist in anatomical regions inaccessible to nasal–oropharyngeal swab, such as the gut. SARS-CoV-2 RNA was detectable in fecal samples from 51.8% of COVID-19 patients in a recent meta-analysis (14) and has been reported to persist up to 70 days after symptom onset in individual cases (15). SARS-CoV-2 RNA may continue to be detectable in fecal samples from patients with a negative nasal–oropharyngeal swab and is associated with a longer interval from symptom onset to viral clearance (14, 16–18). Angiotensin-converting enzyme 2, which acts as a host receptor protein to bind coronavirus spikes and enable subsequent viral-host cell membrane fusion and viral entry, is expressed relatively strongly by intestinal epithelial cells (19–22). Future research should investigate a possible association of fecal SARS-CoV-2 RNA with persistent COVID-19 disease-related symptoms.

The SARS-CoV-2 proteins most studied as potential drug targets are the SARS-CoV-2 chymotrypsin-like protease

(3CLpro), also known as main protease (Mpro), and SARS-CoV-2 papain-like protease (PLpro). Both of these proteases are critical for viral replication, and PLpro additionally has effects on ubiquitination and interferon that may dysregulate host innate immunity. *In silico* methods, including virtual ligand screening of SARS-CoV-2 proteins against libraries of compounds, predict the binding of famotidine to Mpro (23), PLpro (24, 25), or both (26). These *in silico* predictions await laboratory target validation.

If famotidine is indeed effective for the treatment of COVID-19, it might be hypothesized to act as a virustatic protease inhibitor, possibly on the basis of an interaction with proteins involved in viral replication, such as but not limited to Mpro or PLpro. This may suggest a general analogy to virustatic protease inhibitors such as those used to treat HIV or hepatitis C (27). An alternative hypothesis suggests that the therapeutic effect of famotidine may be due to its action as an H2 antagonist against inflammatory effects mediated by H2-related signaling in the presence of a highly inflammatory pathogen (5).

The effect of famotidine appears rapid in its onset; in this present case, the patient reported significant improvement at 4 days. In the case series of 10 outpatients who self-treated acute COVID-19 illness with famotidine (2), group mean symptom scores separated significantly from pretreatment baseline by day 1 of treatment. These patients who self-treated with famotidine utilized dosages ranging from 60 to 240 mg daily for a median duration of 11 days. In the two studies that compared groups of hospitalized patients on the basis of famotidine use, the respective median values for the total cumulative dose received are 136 mg and 80 mg, and 5.8 and 4 days for duration of treatment (3, 4). Treatment in this present case is ongoing at 12 weeks. The risk–benefit calculus would appear to favor caution in lowering and discontinuing famotidine. Factors that favor continuing famotidine are its safety and the possibility that if the drug is indeed effective, it could be providing extended suppressive therapy in a setting of months of previous symptomatic illness. Discussion with the patient regarding when to initiate a gradual taper of famotidine is ongoing as of this writing.

A limitation of a single case report is its unknown reproducibility and the need for confirmation by controlled clinical investigation. Even if famotidine has indeed had a treatment effect in this case, its generalizability may be limited in view of the patient's relatively young age, which may have been a factor in his apparently favorable response and may not be representative of older people with COVID-19. The patient's use of melatonin might be considered a possible confound in view of the suggestion that its antioxidant actions might have beneficial effects on pulmonary inflammation (28). However, the patient commenced and stopped the use of melatonin without a change in clinical status, in contrast to the close temporal correspondence of treatment with famotidine and clinical improvement.

The premise that the patient's behavioral symptoms are etiologically related to COVID-19 requires their occurrence to have been subsequent to, and not prior to COVID-19. Testing for SARS-CoV-2 antigen was not obtained in this case. However, in New York City in late February of 2020, a diagnosis of COVID-19 would have been clinically likely for two individuals with new onset fatigue and cough a week following their contact with one another, both of whom subsequently tested positive for SAR-CoV-2 antibody. The patient's presenting neuropsychiatric symptoms began ∼3 weeks following the apparent onset of COVID-19 (see **Table 1**).

The possibility of a placebo effect potentially confounds the attribution of clinical improvement to famotidine. Attribution of the apparent clinical response to a placebo effect in this case would imply suggestion as the basis for the presenting neuropsychiatric symptoms, and weigh against mediation by biological effects of SARS-CoV-2 viral infection. There does not appear to be a compelling psychological explanation for the appearance of neuropsychiatric symptoms that departed markedly from the patient's prior behavioral baseline. Nonetheless, the possibility of a placebo effect is structural to any individual psychiatric case report due to reliance on behavioral features and lack of biological markers in present psychiatric diagnosis.

It is possible that this case report and other observational and retrospective evidence for an effect of famotidine on disease-related symptoms in COVID-19 might represent a collective type 1 statistical error, a false positive. The cost of this type of error would be the expense of a controlled clinical research effort that fails to confirm the hypothesized treatment effect. However, such a clinical research effort may be justified. A type 2 error, a false negative, is potentially more costly. Famotidine is inexpensive, and relatively safe. If it is indeed effective as an antiviral against SARS-CoV-2, famotidine may provide a novel mechanism of action for potential polytherapy synergies or offsetting antiviral resistance, as well as a scaffold for drug design involving rational pharmaceutical synthesis of structural analogs informed by structure activity relationships.

CONCLUSION

This case report is generally consistent with observational and retrospective evidence for an apparent effect of famotidine on disease-related symptoms and survival in COVID-19 (2–5). It should be followed by controlled clinical investigation. There is a need to develop treatment approaches for residual disease-related symptoms, including neuropsychiatric symptoms weeks to months following acute illness with COVID-19. Clinical research on famotidine for COVID-19 should include assessment of neuropsychiatric symptoms and more extended intervals of follow-up. This work would be optimally enabled by the development of valid and reliable research diagnostic criteria to define and operationalize the features of a putative syndrome of COVID-19 neuropsychiatric residual.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. 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

KA evaluated and treated the patient as described in this case report and authored the manuscript.

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BRIEF RESEARCH REPORT
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Knowledge, Attitudes, and Practices Toward COVID-19 Among Construction Industry Practitioners in China

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The COVID-19 pandemic has put labor-intensive industries at risk, among which the construction industry is a typical one. Practitioners in the construction industry are facing high probabilities of COVID-19 transmission, while their knowledge, attitudes, and practices (KAP) are critical to the prevention of virus spread. This study seeks to investigate the KAP of construction industry practitioners in China through an online questionnaire survey conducted from 15 to 30 June 2020. A total of 702 effective responses were received and analyzed. The results revealed that: (1) although an overwhelming percentage of respondents had the correct knowledge about COVID-19, there were significant respondents (15% of all) who were unsure or wrong about the human-to-human transmission of the virus; (2) practitioners generally showed an optimistic attitude about winning the battle against the COVID-19 pandemic and were satisfied with the governments' contingency measures; (3) practitioners tended to actively take preventive measures, although checking body temperature, wearing face masks, and keeping safe social distance still needs to be reinforced. This research is among the first to identify the KAP of construction industry practitioners toward the COVID-19 pandemic in China. Results presented here have implications for enhancing strategies to reduce and prevent COVID-19 spread in the construction industry.

Keywords: COVID-19, knowledge, attitude, practice, construction industry practitioner

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INTRODUCTION

Starting from December 2019, a novel coronavirus disease (COVID-19) emerged in the global community. Up to 17 August 2020, there have been a total of 21,598,893 confirmed cases with 773,934 deaths (1). The pandemic has also seriously affected many industries, and the construction industry is no exception. Many construction projects in Wuhan, the epicenter of the COVID-19 outbreak in China, have been suspended because of the city-wide shutdown of all non-essential work and restriction of public transportation that led to a shortage of essential materials and skilled practitioners (2). Although regions are able to continue their construction projects, the inherent labor-intensive nature of construction project causes additional challenges due to the onsite necessity of construction task delivery and the constraints on the feasibility of social distancing on an active jobsite.

When dealing with the COVID-19 pandemic, the health and safety of employees supersedes other priorities. Different countries have developed a series of guidelines and suggestions for infection prevention. For instance, the Center for Disease Control and Prevention (CDCP) of the U.S. suggested that temporary, mobile handwashing stations should be installed if hand sanitizer and running water is not available on the construction site (3). The Chinese government suggested that frequently touched surfaces, such as shared tools and other equipment, should be cleaned and disinfected (4). Following these guidelines and suggestions, individuals might need to change their behaviors in daily operations. For example, they should wear face masks and keep social distancing in the workplace in order to interrupt the human-to-human transmission chain.

Knowledge, attitudes, and practices (KAP) theory suggested that the changes in human behavior can be divided into three successive processes: knowledge acquisition, attitude generation, and behavior formation (5). Based on this theory, people's adherence to COVID-19 control measures could be affected by their knowledge and attitudes. Several recent studies have reported the KAP of residents toward COVID-19 during the rapid rise period of the virus outbreak in China, Malaysia, the Philippines, etc. (6–9). Intensive research efforts have also identified the KAP status of medical professionals who work on the frontline to prevent the spread of COVID-19 (10–13). However, less attention has been paid to the construction industry where the practitioners are at considerable risk for severe illness from COVID-19. Therefore, at this critical moment, there is an urgent need to assess the KAP toward COVID-19 among construction industry practitioners. Such an investigation will not only identify knowledge gaps that could enhance the understanding for COVID-19 control efforts, but also set priorities to address the most common problems in protecting practitioners from being infected with the COVID-19 virus.

This study conducted a KAP survey to investigate the KAP toward COVID-19 among construction industry practitioners in China. Three specific research objectives will be attained:

(1) To assess the knowledge of construction industry practitioners regarding the epidemiological features of COVID-19 and the prevention of infection;
(2) To evaluate the attitudes of construction industry practitioners toward the control of the COVID-19 pandemic;
(3) To identify the practices taken by construction industry practitioners regarding infection prevention.

In the next section, the protocol and process of the KAP survey are described. Section **Results** reports the main findings of the KAP assessment, and discussions of the findings are presented in Section **Discussion**. The last section concludes this study.

RESEARCH METHODS

Survey Platform and Sampling

In this study, the KAP survey was conducted following the recommendations of the World Health Organization and existing studies on individuals' KAP toward COVID-19. The survey was conducted between 15 and 30 June 2020, and the questionnaire was distributed through the Tencent platform (https://wj.qq.com/). Considering the target population of this survey focused on practitioners in the construction industry, this study did not adopt a convenience sampling strategy, but the authors approached the survey participants based on their personal networks. Through the Tencent survey platform, the participants were first provided with a brief introduction of the survey, including the survey objectives, procedures, voluntary nature of participation, and declarations of confidentiality, before they decided whether or not to take this survey.

Questionnaire

The main body of the questionnaire contained two sections. The first section collected socio-demographic information including gender, age, years of work experience, stakeholder, and the type and location of their engaged projects. Gender, age, years of work experience, and stakeholder were recorded as reported by the respondent. The type of project was classified as residential, commercial, industrial, infrastructure, and others; the location was classified as Wuhan, other cities in Hubei province, and other provinces. The second section—the KAP section—was further divided into the following three parts.

(1) The knowledge part consisted of fourteen questions that attempted to test the COVID-19 knowledge of the survey

TABLE 1 | Characteristics of survey participants (N = 702).

Characteristics		Number	Percentage (%)
Gender	Male	588	83.76%
	Female	114	16.24%
Age	<25	78	11.11%
	25–30	274	39.03%
	31–40	273	38.89%
	41–50	65	9.26%
	>50	12	1.71%
Years of work experience	≤5	259	36.89%
	6–10	243	34.62%
	11–15	100	14.24%
	16–20	49	6.98%
	≥21	51	7.27%
Stakeholder	Developer	49	6.98%
	Designer	103	14.67%
	Main contractor	504	71.80%
	Sub-contractor	30	4.27%
	Others	16	2.28%
Location of the project	Wuhan	94	13.39%
	Other cities in Hubei Province	26	3.70%
	Other provinces	582	82.91%
Type of the project	Residential	294	41.88%
	Commercial	185	26.35%
	Industrial	45	6.41%
	Infrastructure	167	23.79%
	Others	11	1.57%

participants. These questions were designed based on the second edition of the Health Education Manual that was published by the National Institute of Health Education of the National Health Commission in China; there were nine questions on epidemiological knowledge of COVID-19, and five regarding the prevention of COVID-19 virus infection. The knowledge questions were represented in a statement form, i.e., "COVID-19 can spread through person-to-person transmission," and the participants were asked to choose right or wrong on these questions. An additional "do not know" option was also provided. One point was assigned to a correct response, and 0 points was assigned to "do not know" or wrong responses. The total score thus ranged from 0 to 14.

(2) The attitude part had nine questions that assessed the attitudes of industry practitioners toward COVID-19; four questions were about their level of confidence regarding the

TABLE 2 | Knowledge of respondents toward COVID-19.

Questions	Yes (%)	No (%)	Don't know (%)
Humans are universally susceptible to COVID-19.	595 (84.76%)	71 (10.11%)	36 (5.13%)
COVID-19 can spread through person-to-person transmission.	694 (98.86%)	5 (0.71%)	3 (0.43%)
The general observation period of COVID-19 is 14 days.	661 (94.16%)	15 (2.14%)	26 (3.70%)
The transmission modes of COVID-19 include droplet transmission, contact transmission, and aerosol transmission.	681 (97.01%)	12 (1.71%)	9 (1.28%)
Not all people with COVID-2019 will develop to severe cases.	680 (96.87%)	12 (1.71%)	10 (1.42%)
Asymptomatic infection is contagious.	596 (84.90%)	33 (4.70%)	73 (10.40%)
Cured patients are still at risk of reinfection.	604 (86.04%)	35 (4.99%)	63 (8.97%)
The main clinical symptoms of COVID-19 are fever, fatigue, and dry cough.	696 (99.15%)	1 (0.14%)	5 (0.71%)
Until June 2020, there is no effective cure for COVID-19.	668 (95.16%)	8 (1.14%)	26 (3.70%)
People can wear medical masks to prevent infection.	671 (95.58%)	17 (2.42%)	14 (1.99%)
Masks should be replaced after contamination or moisture.	689 (98.15%)	6 (0.85%)	7 (1.00%)
Used masks should be discarded as hazardous waste.	678 (96.58%)	19 (2.71%)	5 (0.71%)
75% alcohol and chlorine-containing disinfectants can effectively eliminate the virus.	631 (89.89%)	33 (4.70%)	38 (5.41%)
Vinegar cannot effectively eliminate the virus.	642 (91.45%)	22 (3.13%)	38 (5.41%)

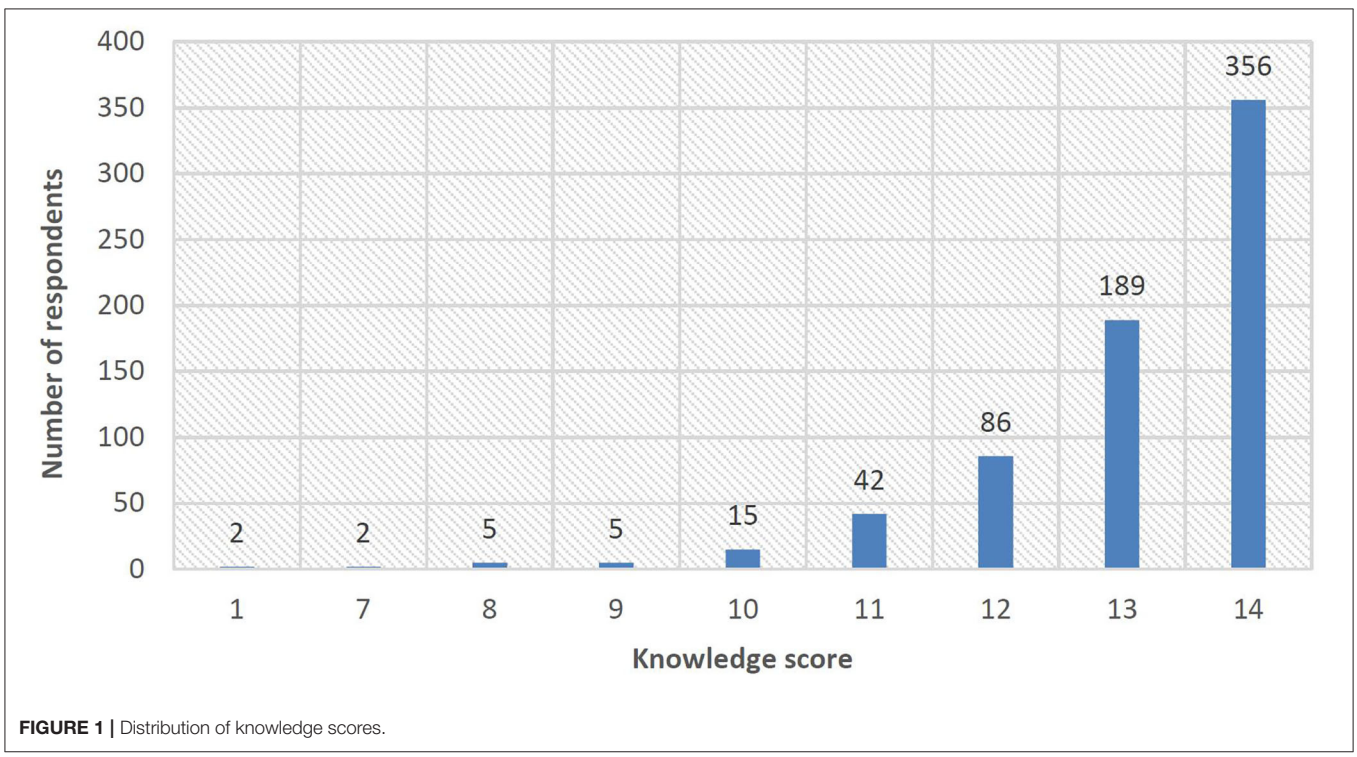

FIGURE 1 | Distribution of knowledge scores.

successful control of the pandemic, and five were about their level of satisfaction toward the control measures taken by the government and their companies. The participants were asked to reflect their attitude on a five-point Likert scale, in which 1 represented "very low" and 5 represented "very high."

(3) The practice part contained eight questions on preventive measures, for instance, whether they wore an appropriate mask during their work. The participants were asked to report the behaviors taken by themselves to prevent infections on a five-point Likert scale, in which 1 represented "never do that" and 5 represented "always do that."

All these KAP questions were reviewed by subject matter experts to enhance the adequacy and appropriateness, and a redundancy question was designed in the attitude part to help reduce social desirability bias in the responses.

Data Collection and Analysis

A total of 785 responses were collected initially. After removing the incomplete and invalid (i.e., providing a different answer for redundancy question) ones, 702 responses were finally obtained. The data collection conformed to the ethics guidelines of Huazhong University of Science and Technology, and the confidentiality of the participants' data was protected by hiding the identity of the respondents. Then, statistical analysis was performed by using SPSS v.22.0. The normality of data was assessed by Shapiro-Wilk tests. Additionally, Pearson Chi-Square and Kruskal-Wallis tests were adopted to assess whether the KAP levels were statistically different across different demographic characteristics of respondents.

RESULTS

Demographic Information of the Respondents

Of the 702 respondents, 588 (83.8%) were males and 114 (16.2%) were females; 94 were in Wuhan, 26 were in other cities in Hubei province, and the remaining 582 were in other provinces. Other demographic characteristics of the samples are summarized in **Table 1**.

Knowledge of the Respondents

Table 2 and **Figure 1** depict the knowledge of respondents toward COVID-19. The average knowledge score was 13.09 (SD = 1.36), and eight questions had an accuracy rate of over 95%. Such results indicated good knowledge of industry practitioners toward COVID-19. However, two knowledge questions deserved attention, i.e., "Humans are universally susceptible to COVID-19" and "Asymptomatic infection is contagious," since over 15% of the respondents reported wrong or unsure answers.

Differences in the knowledge scores across various demographic characteristics were evaluated by using Pearson Chi-Square and Kruskal-Wallis tests. As shown in **Table 3**, the knowledge scores were significantly different across ages, stakeholders, and project locations. However, no significant differences in the knowledge scores across genders, years of work experience, and project types were identified.

TABLE 3 | Knowledge score of COVID-19 by demographic characteristics.[#]

Characteristics		Mean (SD)	X^2	P
Gender	Male	13.08 (1.41)	7.902	0.443
	Female	13.10 (1.11)		
Age	<25	12.90 (1.28)	10.472	0.033*
	25–30	13.26 (1.02)		
	31–40	13.08 (1.56)		
	41–50	12.72 (1.64)		
	>50	12.42 (1.93)		
Years of work experience	≤5	13.14 (1.09)	5.427	0.246
	6–10	13.16 (1.38)		
	11–15	13.01 (1.80)		
	16–20	13.06 (1.11)		
	≥21	12.63 (1.73)		
Stakeholder	Developer	13.02 (1.09)	11.258	0.024*
	Designer	12.92 (1.20)		
	Main contractor	13.14 (1.42)		
	Sub-contractor	12.90 (1.16)		
	Others	13.00 (1.55)		
Location of the project	Wuhan	12.81 (1.20)	13.437	0.001*
	Other cities in Hubei Province	12.96 (1.18)		
	Other provinces	13.14 (1.39)		
Type of the project	Residential	13.05 (1.44)	1.606	0.808
	Commercial	13.11 (1.25)		
	Industrial	13.02 (1.10)		
	Infrastructure	13.13 (1.45)		
	Others	13.27 (0.79)		

[#]*X^2 for gender shows Pearson Chi-Square value and for other demographic characteristics shows Kruskal-Wallis H value.*

**P ≤ 0.05 indicates significance.*

Attitudes of the Respondents

Table 4 and **Figure 2** reveal the respondents' attitudes toward COVID-19. The average scores on confidence in overcoming the COVID-19 pandemic and satisfaction with the control measures were 11.52 (SD = 4.26) and 22.70 (SD = 3.68), respectively, indicating an overall positive attitude that the pandemic would be successfully addressed. It is also encouraging to see the majority of the surveyed industry practitioners can effectively continue their work during the COVID-19 outbreak, and 619 out of all respondents had high-level satisfaction with the measures taken by the government and their companies in controlling the virus spread.

Significant differences in the respondents' attitudes across their demographic characteristics were identified. As shown in **Table 5**, the attitude significantly differed across years of work experience. In addition, the respondents who worked in Wuhan had significantly lower satisfaction with the control measures than that of respondents in other cities. The satisfaction with the control measures also varied significantly across stakeholders.

TABLE 4 | Attitude of respondents toward COVID-19.

Questions		1 (%)	2 (%)	3 (%)	4 (%)	5 (%)
Confidence in overcoming the COVID-19 pandemic	I will not be infected with COVID-19.	160 (22.79%)	116 (16.52%)	242 (34.47%)	81 (11.54%)	103 (14.67%)
	My colleagues will not be infected with COVID-19.	168 (23.93%)	143 (20.37%)	228 (32.48%)	77 (10.97%)	86 (12.25%)
	I have no worry of going to work during the COVID-19 outbreak.	127 (18.09%)	145 (20.66%)	239 (34.05%)	103 (14.67%)	88 (12.54%)
	I do not feel tired at work during the COVID-19 outbreak.	74 (10.54%)	107 (15.24)	243 (34.62%)	136 (19.37%)	142 (20.23%)
Satisfaction with the control measures	I am satisfied with my company's requirements for wearing masks and temperature measurement.	15 (2.14%)	1 (0.14%)	20 (2.85%)	96 (13.68%)	570 (81.20%)
	I am satisfied with my company's regular disinfection.	15 (2.14%)	6 (0.85%)	44 (6.27%)	142 (20.23%)	495 (70.51%)
	I am satisfied with my company's preparation of anti-epidemic resources.	24 (3.42%)	12 (1.71%)	53 (7.55%)	150 (21.37%)	463 (65.95%)
	I think the government has timely publicized relevant information on COVID-19.	14 (1.99%)	11 (1.57%)	51 (7.26%)	168 (23.93%)	458 (65.24%)
	I think the control measures taken by the government are effective.	17 (2.42%)	7 (1.00%)	54 (7.69%)	163 (23.22%)	461 (65.67%)

Practices of the Respondents

The correlation coefficient between knowledge and practices is 0.91 ($P = 0.016$), revealing the significant association between them, i.e., those who have a better knowledge of COVID-19 have taken more preventive measures. Overall, 93.73% of the survey participants always attended the health education sessions organized by their companies. 89.03% of the respondents reported that they kept their work environment clean and ventilated; 74.79% washed their hands frequently during work hours. However, there were still some practices that were not widely adopted by industry practitioners. 32.91% of the respondents reflected that they did not conduct a frequent self-check of their body temperature, and 32.48% did not research the latest information about COVID-19. In addition, 25.21 and 16.52% of the respondents did not wear a face mask and keep sufficient social distance respectively, which potentially increased the chance of being exposed to the virus. Moreover, **Table 6** shows that females maintained safety practices better than males, and the respondents in Wuhan performed better virus preventive practices than those in other cities in China.

DISCUSSION

The emergence of COVID-19 continues to threaten public health in the global community. In response to this crisis, the construction industry has had a vital role in building hospitals and essential infrastructure that helped society to recover from the pandemic. However, the industry itself has been more seriously affected than many other economic sectors. Direct impacts caused by the COVID-19 crisis ranged from a slowdown of resource supply to terminations of entire projects. A recent report published by McKinsey & Company suggested that a fast return to pre-pandemic levels seems unlikely for the construction industry, and the industry must adapt to a "next normal" (14). This situation became much more severe in China since China has the world's largest construction market. Facing the sustained business downturn, the industry needs to assess its preparedness and arrange proper prevention and control measures, which call for the collation of industry practitioner's KAP toward COVID-19.

To the best of our knowledge, the present study is the first to investigate the KAP toward COVID-19 among construction industry practitioners in China. The differences in knowledge, attitudes, and practices were observed across various demographic characteristics, and the gaps were also identified so that essential health precautions can be enhanced to protect the practitioners from infection.

A high correct rate of COVID-19 knowledge was unsurprising because the survey was conducted in the middle stage of the COVID-19 outbreak. From December 2019 to June 2020, the government continuously provided the most up-to-date information of COVID-19 to the public through several social media channels. Nevertheless, respondents still showed a lack of understanding of who is susceptible to COVID-19 and whether asymptomatic infection is contagious. Such important knowledge gaps were also reported in Al-Hanawi et al. (15), Hayat et al. (16), and many other studies that targeted different groups of people in other parts of the world. Considering the world is still threatened by the COVID-19 pandemic, knowledge transmission strategies should be further explored to consolidate the knowledge of the public. On the other hand, since over 65% of the survey participants said they would search for information related to COVID-19, it is necessary to reduce the widespread levels of misinformation (17).

In a survey conducted by Zhong et al. (9), 90.8% of the surveyed Chinese residents believed that COVID-19 would be successfully controlled. Yue et al. (18) also reported that Chinese urban and rural residents had a positive attitude toward the pandemic. Such optimistic attitudes were in agreement with our findings. Additionally, the high-level satisfaction with

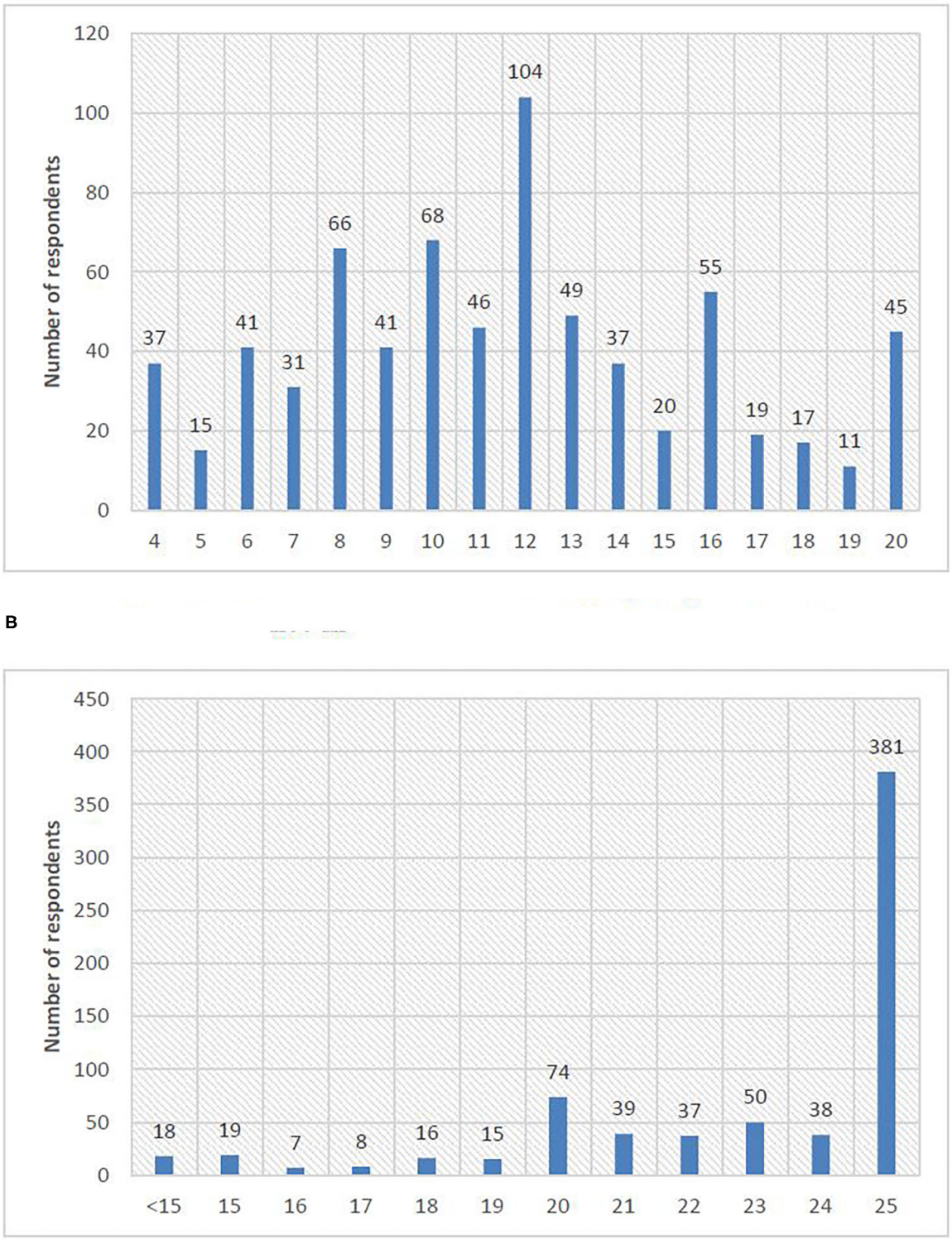

FIGURE 2 | Distribution of attitude scores. **(A)** Confidence in overcoming the COVID-19 pandemic. **(B)** Satisfaction with the control measures.

TABLE 5 | Attitude score by demographic characteristics.

Characteristics		Confidence in overcoming the COVID-19 pandemic		Satisfaction with the control measures	
		Mean (SD)	X^2 (P)	Mean (SD)	X^2 (P)
Gender	Male	11.563 (4.320)	0.178 (0.673)	22.759 (3.621)	0.934 (0.334)
	Female	11.325 (3.925)		22.404 (4.002)	
Age	<25	11.756 (4.316)	10.880 (0.028)*	22.603 (4.069)	3.556 (0.469)
	25–30	10.974 (4.259)		22.693 (3.428)	
	31–40	11.733 (4.232)		22.725 (3.770)	
	41–50	12.462 (4.280)		23.092 (3.390)	
	>50	12.750 (2.989)		20.833 (5.875)	
Years of work experience	≤5	11.100 (4.168)	10.378 (0.035)*	22.827 (3.219)	10.735 (0.030)*
	6–10	11.362 (4.270)		22.193 (4.302)	
	11–15	12.140 (4.459)		23.550 (2.904)	
	16–20	12.490 (4.416)		23.163 (2.889)	
	≥21	12.314 (3.834)		22.373 (4.418)	
Stakeholder	Developer	10.531 (3.836)	3.386 (0.495)	22.796 (3.014)	11.323 (0.023)*
	Designer	11.272 (4.282)		21.971 (3.932)	
	Main contractor	11.637 (4.293)		22.885 (3.643)	
	Sub-contractor	11.933 (3.973)		22.367 (3.378)	
	Others	11.875 (4.689)		21.938 (5.234)	
Location of the project	Wuhan	11.894 (3.650)	1.373 (0.503)	21.926 (3.699)	11.279 (0.004)*
	Other cities in Hubei Province	11.192 (3.720)		22.154 (4.370)	
	Other provinces	11.479 (4.371)		22.851 (3.638)	
Type of the project	Residential	11.320 (4.338)	1.960 (0.743)	22.616 (3.969)	0.958 (0.916)
	Commercial	11.838 (4.358)		22.514 (3.887)	
	Industrial	11.356 (3.199)		22.644 (3.791)	
	Infrastructure	11.587 (4.200)		23.012 (2.912)	
	Others	11.455 (5.298)		23.636 (2.157)	

$P \leq 0.05$ indicates significance.

government efforts can be attributed to the fact that the Chinese government has taken an active role in fighting against the COVID-19 pandemic. Several measures, such as imposing a strict lockdown in Wuhan and the development of Fangcang Hospital, have been considered effective in helping to control the virus spread as much as possible (19, 20). China has made strategic achievements in overcoming the COVID-19 disruption and has gradually resumed social and economic activities. Nevertheless, this study found that the construction industry practitioners in Wuhan have a lower satisfaction level than those in other cities. More investigations need to be conducted before reaching an in-depth understanding of this issue.

The majority of the surveyed industry practitioners took different preventive measures to prevent possible infection, and it is especially delighting to see that a large proportion of the respondents actively attended the health education training that has been acknowledged as a common but important activity for COVID-19 prevention (21). Overall, our findings on respondents' practices align with the findings of Li et al. (22) who reported better virus prevention practices of respondents living in Hubei province than those living in other provinces. However, it is notable that a handful of survey participants omitted the importance of checking body temperature and wearing face masks. The shortage of qualified resources (such as face masks and thermometers) during the COVID-19 outbreak could be one of the main reasons for such gaps in practices. Another serious issue is that over 16% of the respondents failed in maintaining safe social distance. Previous studies conducted in different countries found that keeping social distance was among the main preventive measures for the general population (23, 24). The unique characteristics of construction project delivery require that the practitioners not only work in independent offices but have to collaborate with each other on the construction site. Therefore, it is difficult for them to maintain a safe social distance and avoid face-to-face contact throughout the project. All these exposed practice gaps have implications for both enhanced short-term and long-term control measures in the construction industry. Where work continues, health and safety risk assessments need to be frequently conducted in order to block the virus transmission route and provide a safe working environment for employees.

TABLE 6 | Practice score by demographic characteristics.[#]

Characteristics		Mean (SD)	X^2	*P*
Gender	Male	34.357 (5.093)	37.614	0.050*
	Female	35.289 (4.367)		
Age	<25	34.808 (3.871)	5.348	0.253
	25–30	34.128 (4.928)		
	31–40	34.740 (5.352)		
	41–50	34.692 (4.740)		
	>50	35.000 (5.924)		
Years of work experience	≤5	34.073 (4.633)	9.773	0.044*
	6–10	35.021 (4.717)		
	11–15	34.190 (6.345)		
	16–20	34.429 (4.770)		
	≥21	34.980 (5.159)		
Stakeholder	Developer	33.714 (5.955)	2.506	0.644
	Designer	34.874 (4.614)		
	Main contractor	34.538 (5.039)		
	Sub-contractor	34.033 (4.106)		
	Others	34.563 (4.305)		
Location of the project	Wuhan	35.926 (4.615)	13.808	0.001*
	Other cities in Hubei Province	35.385 (3.900)		
	Other provinces	34.241 (5.056)		
Type of the project	Residential	34.374 (4.971)	1.707	0.789
	Commercial	34.492 (4.970)		
	Industrial	34.889 (5.314)		
	Infrastructure	34.611 (5.014)		
	Others	35.273 (4.941)		

[#]X^2 for gender shows Pearson Chi-Square value and for other demographic characteristics shows Kruskal-Wallis H value.
**P ≤ 0.05 indicates significance.*

This study enriched the data on the KAP of a specific group of people, i.e., construction industry practitioners in China, toward COVID-19. However, this study was prone to two limitations. First, due to the limited sample representativeness, caution should be taken when generalizing the findings to the construction industries in other regions. Second, it was unavoidable that some respondents would give socially desirable responses that did not reflect the actual situation. This limitation can be reduced by triangulation with on-site observation, and other data collection methods. However, the authors found it was difficult to conduct site visits since many companies showed reluctance to let people outside their projects enter the jobsite.

CONCLUSIONS

This study contributes to the global research effort in helping the construction industry to fight against the COVID-19 pandemic. It enriched the understanding of the KAP of construction industry practitioners toward COVID-19 through a comprehensive survey investigation. The findings indicated that most of the respondents have a good level of knowledge and are generally positive about the eradication of the pandemic. The respondents have taken precautious roles to protect themselves from infection. The identification of current KAP status also highlighted the gaps in respondents' knowledge and practices, which should be addressed to reduce COVID-19 spread in the construction industry.

Since the construction industry is vulnerable to the COVID-19 crisis, further studies could be conducted to assess the impacts of COVID-19 on the productivity of the construction industry and explore strategies to help the industry to deal with the disruptions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available from the corresponding author by request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the approval for this research was given by the Huazhong University of Science and Technology, China. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

KC and LM: conceptualization and resources. KC: methodology, writing—review and editing, and project administration. LZ: formal analysis, investigation, writing—original draft preparation, and visualization. All authors have read and agreed to the published version of the manuscript.

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Decreased CO_2 Levels as Indicators of Possible Mechanical Ventilation-Induced Hyperventilation in COVID-19 Patients: A Retrospective Analysis

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Background: Six months since the outbreak of coronavirus disease (COVID-19), the pandemic continues to grow worldwide, although the outbreak in Wuhan, the worst-hit area, has been controlled. Thus, based on the clinical experience in Wuhan, we hypothesized that there is a relationship between the patient's CO_2 levels and prognosis.

Methods: COVID-19 patients' information was retrospectively collected from medical records at the Leishenshan Hospital, Wuhan. Logistic and Cox regression analyses were conducted to determine the correlation between decreased CO_2 levels and disease severity or mortality risk. The Kaplan-Meier curve analysis was coupled with the log-rank test to understand COVID-19 progression in patients with decreased CO_2 levels. Curve fitting was used to confirm the correlation between computed tomography scores and CO_2 levels.

Results: Cox regression analysis showed that the mortality risk of COVID-19 patients correlated with decreased CO_2 levels. The adjusted hazard ratios for decreased CO_2 levels in COVID-19 patients were 8.710 [95% confidence interval (CI): 2.773–27.365, $P < 0.001$], and 4.754 (95% CI: 1.380–16.370, $P = 0.013$). The adjusted odds ratio was 0.950 (95% CI: 0.431–2.094, $P = 0.900$). The Kaplan-Meier survival curves demonstrated that patients with decreased CO_2 levels had a higher risk of mortality.

Conclusions: Decreased CO_2 levels increased the mortality risk of COVID-19 patients, which might be caused by hyperventilation during mechanical ventilation. This finding provides important insights for clinical treatment recommendations.

Keywords: infectious disease, pneumonia, COVID-19, CO_2, mechanical ventilation

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BACKGROUND

In December 2019, an outbreak of pneumonia of unknown etiology was reported in Wuhan, China, which then rapidly evolved into a pandemic (1). By January 7, 2020, Chinese scientists had rapidly isolated the novel coronavirus, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), with an incubation period of 2–14 days, and a potential asymptomatic human-to-human transmission; it is known to cause the coronavirus disease (COVID-19) (2–4). COVID-19 has been controlled in China, although the global number of infections continues to grow rapidly and has led to more than five million infections and 630,000 deaths (5).

In COVID-19 patients, fever and cough are the most common symptoms. There may also be uncommon symptoms, such as diarrhea (6). Thus, researchers have found that SARS-CoV-2 affects multiple organs in addition to the patients' lungs, based on the understanding garnered from COVID-19 studies. This explains the pathological changes identified from the minimal autopsies of three patients who died of COVID-19 in Chongqin, China (7–9). Studies have shown that the main targeted organs of SARS-CoV-2 are the lungs and airways. Furthermore, damage to other organs significantly increases the mortality rate of COVID-19 patients (10).

The measurement of carbon dioxide (CO_2) level in blood is vital not only for the early detection of respiratory depression and airway disorders but also for airway management (11). Hypoxemia and hypercapnia predicted poor prognosis for COVID-19 patients in a previous study (4). Hence, this study aimed to investigate whether decreased CO_2 levels would influence the prognosis of COVID-19 patients.

METHODS

Study Design and Participants

In this retrospective study, we collected data from 1,880 patients, who were clinically diagnosed with COVID-19 between February 8, 2020, and March 19, 2020, at Wuhan Leishenshan Hospital. Exclusion criteria included missing data on mortality and CO_2 level, pregnancy, death on admission, embolization, and transfer to any other hospital; thus, 1,776 patients were included finally. Data about demographics, medical history, treatment, laboratory findings, and imaging data were collected from the patients' original medical records. Two physicians independently reviewed these data.

This study was approved by the Research Ethics Commission of the Zhongnan Hospital of Wuhan University (approval number: 2020074). The need for patient consent was waived by the ethics committee because of the urgent need for insights into this rapidly evolving infectious disease.

Abbreviations: COVID-19, coronavirus disease; CO_2, carbon dioxide; CI, confidence interval; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; CT, computed tomography; GGO, ground-glass opacities; SD, standard deviation; IQR, median and interquartile range; WBC, white blood cell; PLT, platelet; MV, mechanical ventilation; ARDS, acute respiratory distress syndrome.

Primary Outcomes in This Study

In this study, the survival and illness severity of COVID-19 patients during hospitalization and images obtained from computed tomography (CT) scan were used to evaluate the patients' primary outcomes. However, survival was the most significant indicator. According to the Seventh Interim Guidance of Diagnosis and Treatment of COVID-19 published by the Chinese National Health Commission, one patient was staged into mild COVID-19 in this study. Thus, the severity of COVID-19 was categorized into three degrees: mild/common, severe, and critical.

Furthermore, after fulfilling the common standard criteria, all chest CT images were inspected and independently categorized by two experienced radiologists using the following scoring system according to previous studies and the characteristics of COVID-19. Score 1 included ground-glass opacities (GGO) characteristics, reticulation or cord change, consolidation, and pleural effusion, in which each feature was assigned one point, and Score 1 was the sum of these features. Score 2 (from 0 to 4 points) was generated depending on the area of involvement of the lung lobes as follows: no involvement, 0; $<$ 25% involvement, 1; 26–50% involvement, 2; 51–75% involvement, 3; 76–100% involvement, 4; the total score was the sum of scores 1 and 2.

Statistical Analyses

Continuous variables with normal distribution are presented as mean $\pm$ standard deviation (SD) or median and interquartile range (IQR). A CO_2 level $\leq$23 mmol/L was considered a decreased level (normal CO_2 range: 23–31 mmol/L). Furthermore, differences in continuous variables between the groups (decreased and non-decreased levels of CO_2), were determined using independent group *t*-test or the Mann-Whitney *U*-test. Categorical variables are presented as frequencies and percentages. For the proportions of categorical variables, the chi-square test was used to compare participants with decreased and non-decreased CO_2 levels. When parameters were expected to have a count $\leq$5, the Fisher exact test was used.

To determine whether the decreased CO_2 levels would influence the prognosis of COVID-19 patients, we used Cox regression analysis, after adjusting for age, history of cardiovascular disease, erythrocyte count, hemoglobin, leucocyte count, platelet count, lymphocyte count, and oxygen support. Furthermore, Kaplan-Meier analyses with log-rank tests were used to analyze the survival trends of patients.

All statistical analyses were performed using SPSS (version 23.0 for Windows) and EmpowerStats (version 2.0). A two-sided $P \leq 0.05$ was considered statistically significant.

RESULTS

Demographics, Clinical Information, and Laboratory Findings

The demographic characteristics and symptoms of this study cohort of 1,776 patients are presented in **Table 1**. The ratio of female to male patients was approximately one. The IQR value of age in this study population was 59 (48–68) years,

TABLE 1 | Demographic characteristics and symptoms of 1,776 patients with COVID-19.

Covariates	Levels	All patients (n = 1,776), n (%)	Non-declined CO_2 (n = 1,343), n (%)	Declined CO_2 (n = 433), n (%)	P-value
Gender					0.800
	Female	934 (52.59)	704 (75.37)	230 (24.63)	
	Male	842 (47.41)	639 (75.89)	203 (24.11)	
Age, median (IQR)		59 (48–68)	59 (49–68)	58 (47–67)	<0.001
Any comorbidity					
	Cardiovascular diseases	352 (19.82)	249 (70.74)	103 (29.26)	0.017
	Pulmonary diseases	89 (5.01)	62 (69.66)	27 (30.34)	0.179
	Endocrine diseases	135 (7.60)	104 (77.04)	31 (22.96)	0.690
	Malignancy	64 (3.60)	46 (71.88)	18 (28.13)	0.477
	Digest system diseases	45 (2.53)	35 (77.78)	10 (22.22)	0.733
	Neurological diseases	55 (3.10)	39 (70.91)	16 (20.09)	0.409
Initial symptoms, n (%)					
	Fever or fatigue	615 (34.50)	456 (74.10)	274 (34.60)	0.293
	Respiratory symptoms	626 (35.25)	465 (74.28)	161 (25.72)	0.333
	Digestive symptoms	82 (4.62)	52 (63.41)	30 (36.59)	0.008
	Neurological symptoms	26 (1.46)	20 (76.92)	6 (23.08)	0.876
	Other	26 (1.46)	19 (73.08)	7 (26.92)	0.761

with no apparent differences in the groups with decreased and non-decreased CO_2 levels.

In patients with cardiovascular comorbidity, a significant difference was observed between decreased and non-decreased CO_2 levels. However, there were no significant differences in other comorbidities, including pulmonary disease, endocrine disease, malignancy, and neurological disorders. Furthermore, among COVID-19 patients with decreased or non-decreased CO_2 levels, those with gastrointestinal disorders showed a significant difference. However, concerning fever, fatigue, or respiratory and neurological symptoms, there were no significant intergroup differences (**Table 1**).

We analyzed the laboratory results and the blood coagulation tests of patients in two groups (**Table 2**), and most of the laboratory indicators showed significant differences. The results of the blood coagulation test, except fibrinogen and thrombin time, showed significant intergroup differences among COVID-19 patients. The clinical treatment and outcomes are presented in **Table 2**. Anticoagulants and types of oxygen support significantly differed among patients in the two groups. However, the use of antiviral drugs, corticosteroids, and traditional Chinese medicine showed no significant differences between the groups. Concerning outcomes, disease progression showed a significant difference, with no significant difference in other outcome parameters.

Analysis of Patient Prognosis

Table 3 shows the mortality risk of COVID-19 patients with decreased and non-decreased CO_2 levels. Both unadjusted and adjusted Cox regression analyses showed that decreased CO_2 levels were associated, with poor prognosis compared to non-decreased CO_2 levels. After adjustment for age, history of cardiovascular disease, WBC, PLT, oxygen support, and lymphocyte count, the odds ratio for decreased CO_2 levels in COVID-19 patients were 4.754 [95% confidence interval (CI): 1.380–16.370, $P = 0.013$]. The hazard ratio for decreased CO_2 levels in COVID-19 patients was 8.710 (95% CI: 2.773–27.365, $P < 0.001$), and 4.754 (95% CI: 1.380–16.370, $P = 0.013$) after adjustment. Furthermore, the Kaplan-Meier curves illustrated that patients with decreased CO_2 levels faced higher mortality risks (**Figure 1**). With the fitted curves, though, in **Figure 2A**, the curves of patients with non-decreased CO_2 levels showed a slight downward trend, the CO_2 levels of most patients were increased (**Figures 2B–F**).

DISCUSSION

In this latest outbreak of pneumonia due to COVID-19, patients initially presented with fever with or without respiratory symptoms, although various degrees of pulmonary abnormalities developed later in all patients (1, 12). Furthermore, Tian et al. reported the early phase of the lung pathology of COVID-19 pneumonia in a lung cancer excision, which exhibited edema, proteinaceous exudate, focal reactive hyperplasia of pneumocytes with patchy inflammatory cellular infiltration, and multinucleated giant cells. However, hyaline membranes were not prominent (13). A report demonstrated that the rate of critical illnesses among COVID-19 patients was ∼26%, and critically ill patients had 61.5% mortality (12, 14). In another study from Wuhan, the 28-day mortality of COVID-19 patients who received

TABLE 2 | Laboratory and blood coagulation test results, clinical treatment, and outcomes of 1,776 patients with COVID-19.

Covariate	All patients (n = 1,776), Median (IQR)/n (%)	Non-declined CO_2 (n = 1,343), Median (IQR)/n (%)	Declined CO_2 (n = 433), Median (IQR)/n (%)	P-value
Laboratory test				
Leucocyte count, $\times10^9$/L				0.035
3.5–9.5	1,585 (89.35)	1,211 (76.40)	374 (23.60)	
<3.5	104 (5.86)	75 (72.12)	29 (27.88)	
>9.5	85 (4.79)	55 (64.71)	30 (35.29)	
Neutrophil count, $\times10^9$/L				0.029
1.8–6.3	1,553 (87.54)	1,185 (76.30)	368 (23.70)	
<1.8	116 (6.54)	88 (75.86)	28 (24.14)	
>6.3	105 (5.92)	68 (64.76)	37 (35.24)	
Lymphocyte count, $\times10^9$/L				0.848
1.1–3.2	1,457 (82.13)	1,103 (75.70)	354 (24.30)	
<1.1	291 (16.40)	216 (74.23)	75 (25.77)	
>3.2	26 (1.47)	22 (84.62)	4 (15.38)	
Erythrocyte count, $\times10^{12}$/L				0.820
4.3–5.8	636 (35.85)	477 (75.00)	159 (25.00)	
<4.3	1,127 (63.53)	855 (75.87)	272 (24.13)	
>5.8	11 (0.63)	9 (81.82)	2 (18.18)	
Monocyte count, $\times10^9$/L				0.012
0.1–0.6	1,251 (70.52)	968 (77.38)	283 (22.62)	
<0.1	6 (0.34)	3 (50.00)	3 (50.00)	
>0.6	517 (29.14)	370 (71.57)	147 (28.43)	
Hemoglobin, g/L				0.664
130.0–175.0	712 (40.14)	535 (75.14)	177 (24.86)	
<130.0	1,057 (59.58)	803 (75.97)	254 (24.03)	
>175.0	5 (0.28)	3 (60.00)	2 (40.00)	
Platelet count, $\times10^9$/L				0.011
125.0–350.0	1,546 (87.15)	1,185 (76.65)	361 (23.35)	
<125.0	76 (4.28)	48 (63.16)	28 (36.84)	
>350.0	152 (8.57)	108 (71.05)	44 (28.95)	
Albumin, g/L				0.921
40–55	449 (25.35)	340 (75.72)	109 (24.28)	
<40	1,322 (74.65)	998 (75.49)	324 (24.51)	
Alanine aminotransferase, U/L				0.918
9–50	1,421 (80.24)	1,076 (75.72)	345 (24.28)	
<9	96 (5.42)	71 (73.96)	25 (26.04)	
>50	254 (14.34)	191 (75.20)	63 (24.80)	
Aspartate aminotransferase, U/L				0.175
15–40	1,304 (73.63)	991 (76.00)	313 (24.00)	
<15	317 (17.90)	243 (76.66)	74 (23.34)	
>40	150 (8.47)	104 (69.33)	46 (30.67)	
Total bilirubin, μmol/L				0.099
5.0–21.0	1,582 (89.33)	1,207 (76.30)	375 (23.70)	
<5.0	120 (6.78)	82 (68.33)	38 (31.67)	
>21.0	69 (3.90)	49 (71.01)	20 (28.99)	

(Continued)

TABLE 2 | Continued

Covariate	All patients (n = 1,776), Median (IQR)/n (%)	Non-declined CO_2 (n = 1,343), Median (IQR)/n (%)	Declined CO_2 (n = 433), Median (IQR)/n (%)	P-value
Creatinine, μmol/L				<0.001
64.0–104.0	812 (45.72)	627 (77.22)	185 (22.78)	
<64.0	877 (49.38)	674 (76.85)	203 (23.15)	
>104.0	87 (4.90)	42 (48.82)	45 (51.72)	
Procalcitonin, ng/mL				0.002
<0.05	999 (66.42)	770 (77.08)	229 (22.92)	
> =0.05	505 (33.58)	352 (69.70)	153 (30.30)	
Interleukin-6, pg/mL				0.247
0–7.0	602 (83.96)	445 (73.92)	157 (26.08)	
>7.0	115 (16.04)	79 (68.70)	36 (31.30)	
SARS-CoV-19 IgM				0.598
No	387 (64.61)	303 (78.29)	84 (21.71)	
Yes	212 (35.39)	162 (76.42)	50 (23.58)	
SARS-CoV-19 IgG				0.772
No	49 (8.67)	37 (75.51)	12 (24.49)	
Yes	516 (91.33)	399 (77.33)	117 (22.67)	
Blood coagulation test				
Prothrombin time, s				<0.001
9.4–12.5	1,461 (92.41)	1,126 (77.07)	335 (22.93)	
<9.4	1 (0.06)	1 (100.00)	0 (0)	
>12.5	119 (7.53)	64 (53.78)	55 (46.22)	
International Normalized Ratio				0.004
0.8–1.3	1,504 (85.13)	1,144 (76.06)	360 (23.94)	
<0.8	19 (1.20)	14 (73.68)	5 (26.32)	
>1.3	58 (3.67)	33 (56.90)	25 (43.10)	
Activated partial thromboplastin time, s				0.012
25.1–36.5	1,038 (65.65)	785 (75.63)	253 (24.37)	
<25.1	462 (29.22)	356 (77.06)	106 (22.94)	
>36.5	81 (5.12)	50 (61.73)	31 (38.27)	
Fibrinogen, (g/L)				0.291
2.38–4.98	1,178 (74.51)	883 (74.96)	295 (25.04)	
<2.38	307 (19.42)	240 (78.18)	67 (21.82)	
>4.98	96 (6.07)	68 (70.83)	28 (29.17)	
Thrombin time, s				0.930
<=16.6	237 (14.99)	178 (75.11)	59 (24.89)	
>16.6	1,344 (85.01)	1,013 (75.37)	331 (24.63)	
D-dimer, g/L	0.38 (0.21–0.89)	0.37 (0.20–0.86)	0.41 (0.23–1.05)	<0.001
Clinical treatment				
Drugs				
Antibiotic	515 (29.00)	377 (73.20)	138 (26.80)	0.130
Antiviral drugs	858 (48.31)	655 (76.34)	203 (23.66)	0.494
Antimalarial drugs	139 (7.83)	108 (77.70)	31 (22.30)	0.552
Anticoagulants	119 (6.70)	79 (66.39)	40 (33.61)	0.015
Corticosteroid	104 (5.86)	72 (69.23)	32 (30.77)	0.118
Vitamin C	246 (13.85)	187 (76.02)	59 (23.98)	0.876
Traditional Chinese medicine	1,523 (85.75)	1,159 (76.10)	364 (23.90)	0.247

(Continued)

TABLE 2 | Continued

Covariate	All patients (n = 1,776), Median (IQR)/n (%)	Non-declined CO_2 (n = 1,343), Median (IQR)/n (%)	Declined CO_2 (n = 433), Median (IQR)/n (%)	P-value
Oxygen support				
Low–flow nasal cannula	269 (15.15)	226 (84.01)	43 (15.99)	<0.001
Positive pressure nasal cannula	34 (1.91)	27 (79.41)	7 (20.59)	0.603
High-flow nasal cannula	16 (0.90)	13 (81.25)	3 (18.75)	0.598
Invasive mechanical ventilation	5 (0.30)	1 (20.00)	4 (80.00)	0.004
ECMO	1 (0.06)	0 (0)	1 (100.00)	0.078
Outcomes				
CT scores				0.416
1–4	74 (39.57)	57 (77.03)	17 (22.97)	
5–7	113 (60.43)	81 (71.68)	32 (28.32)	
Disease progression				<0.001
Stableness/hospitalization	1 (0.06)	1 (100.00)	0 (0)	
Improvement/recover	1,738 (99.09)	1,323 (76.12)	415 (23.88)	
Death	15 (0.86)	4 (26.67)	11 (73.33)	
Days in hospital, median (IQR)	18 (13–24)	18 (13–24)	18 (12–23)	<0.001
ICU care	29 (90.63)	18 (62.07)	11 (37.93)	0.188
Severity on admission				0.359
Mild/common	1,473 (82.94)	1,123 (76.24)	350 (23.76)	
Severe	281 (15.82)	205 (72.95)	76 (27.05)	
Critical	22 (1.24)	15 (68.18)	7 (31.82)	
Severity at worst				0.226
Mild/common	928 (52.40)	718 (77.40)	210 (22.60)	
Severe	800 (45.20)	592 (74.00)	208 (26.00)	
Critical	43 (2.40)	31 (72.10)	12 (27.90)	

TABLE 3 | The hazards ratio and odds ratio associated with decreased CO_2 of patients with COVID-19 mortality/severity.

	Group	COX regression analysis			Logistic regression analysis		
		HRs	95 % CI	P-value	ORs	95 % CI	P-value
Univariate analysis	Non-declined	Ref			Ref		
	Declined	8.710	2.773–27.365	<0.001	1.213	0.617–2.384	0.575
Multivariate Analysis*	Non-declined	Ref			Ref		
	Declined	4.754	1.380–16.370	0.013	0.950	0.431–2.094	0.900

**Adjust for Age, History of cardiovascular disease, Erythrocyte count, Hemoglobin, Leucocyte count, Platelet count, Lymphocyte count, Oxygen support.*

mechanical ventilation (MV) was 81%, and patients with the acute respiratory distress syndrome (ARDS) had a mortality rate of nearly 50% (14, 15). Thus, it is undisputed that the lungs and airways are the target organs of this coronavirus infection.

In this study, we first proposed the correlation of patient prognosis with decreased CO_2 levels. According to the adjusted logistic regression, Cox regression analyses, and Kaplan-Meier curves, decreased CO_2 levels influenced the mortality of patients with COVID-19, but not disease severity. Furthermore, this influence on mortality did not differ by sex. However, decreased CO_2 levels in patients with comorbidity of cardiovascular disease or older age indicated poorer prognosis. Moreover, blood coagulation parameters, such as prothrombin time, international normalized ratio, active partial thromboplastin time, and D-dimer level, showed significant differences between COVID-19 patients with decreased and non-decreased CO_2 levels; however, decreased CO_2 levels showed no significant differences in other laboratory parameters.

The measurement of the CO_2 level is vital in airway management. Capnography is an effective method for the early detection of impaired airway function to identify early respiratory depression and airway disorders (16–18). For example, capnography presented results range 5–240 s earlier than dose pulse oximetry, and in many cases with sedation-induced apnea, doctors at the bedside did not recognize the apnea, whereas capnography could identify it (19, 20). Furthermore, capnography reduces serious complications by early diagnosis (16) and plays a critical role in detecting the CO_2 level of COVID-19 patients, in whom the target organs are the lungs and airways.

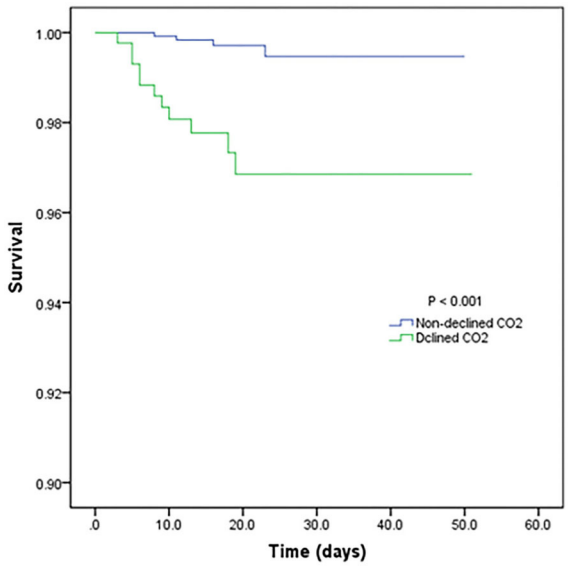

FIGURE 1 | Kaplan-Meier survival curves for patients with declined and non-declined levels of CO_2.

Elevated CO_2 levels and hypoxemia were associated with a poor prognosis in COVID-19 patients. For example, in the study conducted by Nuckton et al. (21), elevated CO_2 level likely reflected ARDS severity and an increased dead space fraction. Similarly, Yang et al. reported that most COVID-19 patients usually develop severe pneumonia and are at a high risk factor of ARDS (22). Furthermore, Buchner et al. directly identified that patients with more severe CO_2 retention might have a poor prognosis (23). Thus, most pneumonia patients with high CO_2 levels had poor prognosis.

In our study cohort, we found that decreased CO_2 levels increased mortality but had no significant effect on the disease severity. According to previous studies, the causes of decreased CO_2 levels are as follows: shortness of breath, reduction of pulmonary perfusion and increased alveolar dead space, and MV hyperventilation (11, 24, 25). Because most COVID-19

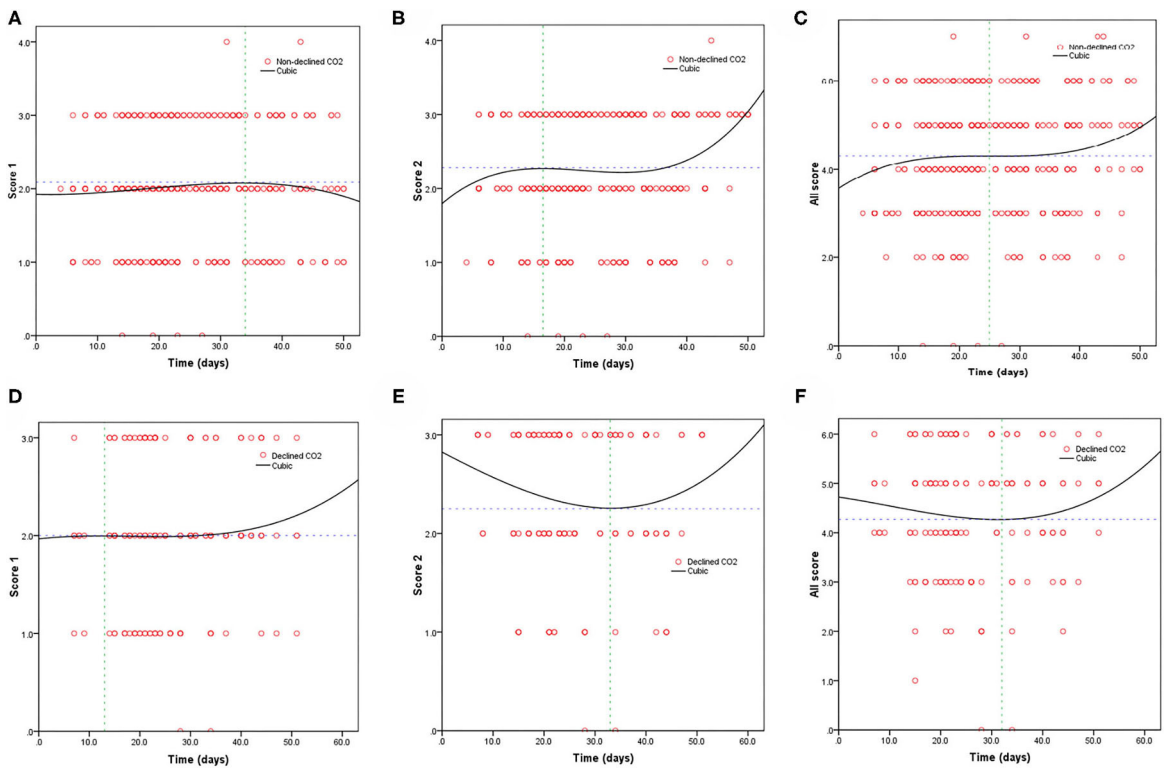

FIGURE 2 | Fitting curves of patients with COVID-19 divided by declined/non-declined levels of CO_2 based on CT score. Dynamic changes in patients with **(A)** CT score 1 and non-declined CO_2; **(B)** CT score 2 and non-declined CO_2; **(C)** total CT score and non-declined CO_2; **(D)** CT score 1 and declined CO_2; **(E)** CT score 2 and declined CO_2; and **(F)** total CT score and declined CO_2.

patients require various forms of oxygen support, among other treatments, we thought that clinicians should focus their attention on MV hyperventilation (26), which is an effective and practical measure to improve patients' survival. Furthermore, according to the fitted curves, compared with pneumonia patients with non-decreased CO_2 levels, the other study groups' trend showed an initial decrease and subsequent increase in CO_2 levels. This indicates that the oxygen flow was adjusted to meet the patients' requirements to treat pneumonia and prevent a decrease in the CO_2 levels due to hyperventilation.

This study has several limitations. Because the Leishenshan hospital was rapidly built as a designated hospital for COVID-19, it was difficult to share laboratory testing data with other hospitals. Thus, the data may be biased. For example, according to our study, there was no correlation between decreased CO_2 levels and illness severity in COVID-19 patients. Furthermore, the mechanism of how oxygen support influences CO_2 levels and thus affects patients' prognoses requires laboratory verification. However, this study makes a significant scientific contribution by providing evidence indicating that clinicians should pay attention to decreased CO_2 levels in pneumonia patients with COVID-19, and so to prevent hypocapnia and maintain homeostasis.

In this study, we demonstrated that decreased CO_2 levels increased the mortality risk of COVID-19 patients, but showed no significant impact on the severity of pneumonia. Furthermore, our study serves as evidence for clinicians to pay greater attention to the oxygen flow in COVID-19 patients who receive oxygen support to avoid treatment-related injuries. With these changes, the complications of COVID-19 can be further reduced, thereby improving the prognosis of COVID-19 patients with pneumonia.

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 authors.

AUTHOR CONTRIBUTIONS

SC, JH, YH, ML, and WL undertook the research. SW and QL performed the analyses and interpretation of data. DH, JL, and RG wrote the main manuscript text and prepared figures. ZL, LG, and XW revised the article critically for important intellectual content and final approval of the version to be submitted. All authors contributed to the design of the study, writing of the manuscript, reviewed, and approved the manuscript.

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Population-Level Preparedness About Preventive Practices Against Coronavirus Disease 2019: A Cross-Sectional Study Among Adults in Bangladesh

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This study assessed the preparedness regarding the preventive practices toward the coronavirus disease 2019 (COVID-19) among the adult population in Bangladesh. Data were collected through an online survey with a sample size of 1,056. We constructed four variables (individual, household, economic, and community and social distancing) related to preparedness based on the principal component analysis of eight items. We employed descriptive statistics and multiple linear regression analysis. The results showed that the accuracy rate of the overall preparedness scale was 68.9%. The preparedness level related to economic, individual, household, and community and social distancing was 64.9, 77.1, 50.4, and 83.2%, respectively. However, the economic preparedness significantly varied by sex, education, occupation, attitude, and worries related to COVID-19. Individual preparedness was significantly associated with education, residence, and attitudes. The household preparedness significantly varied by education, residence, and worries, while the respondent's community and social distancing-related preparedness significantly varied by sex, region, residence, and attitude. This study implies the necessity of the coverage of financial schemes for the vulnerable group. Increased coverage of health education regarding personal hygiene targeting the less educated and rural population should be ensured.

Keywords: Bangladesh, practices, prevention, preparedness, COVID-19, population-level

INTRODUCTION

The pandemic of novel coronavirus disease 2019 (COVID-19) is spreading rapidly across Bangladesh. The first case of COVID-19 in Bangladesh was confirmed on 8 March 2020. Bangladesh was having a slow and steady increase in the overall COVID-19 attack rate (AR) in the first 2 months.

However, the transmission of the virus is increasing very rapidly since the beginning of the third month. The average AR was only 1.0 per million population for the first month (7 April), which increased to 73.6 in the second month (7 May), followed by 389.5 (7 June) and 998.8 (7 July) in the third and fourth months, respectively (1). The total number of COVID-19 positive cases was 4,28,965 as of 13 November 2020 (1). Against these confirmed cases, reported death rates have reached 1.3% of the infected persons.

The increased numbers of COVID-19 positive persons and death rates have created massive pressure on the already fragile health systems of Bangladesh. Thus, the country has adopted several non-therapeutic measures in the absence of vaccine and treatment to flatten the curve of the infection and death rates, which included (a) declaring mass lockdown and public holiday (started from 26 March and ended on 31 June); (b) risk zone-based lockdowns (started from 9 June); (c) limited working hours (started from 31 May); and (d) maintaining social isolation protocol and restricting population movement through travel bans (started from 26 March and ended on 31 May) (2, 3). The primary aim of these non-therapeutic measures was to adopt preventive measures against the COVID-19. However, these state-level initiatives in Bangladesh were not effective enough to ensure preventive practices among the mass population because of their socioeconomic structure and controversies surrounding some specific policy decisions (4, 5). The Chinese experience shows that the adoption of strict preventive practices against COVID-19, such as avoiding crowded places and the mandatory wearing of masks, is dependent on the risk perception, knowledge regarding COVID-19, and the implementation of stringent prevention and control measures by the local governments (6). The studies conducted elsewhere on non-COVID-19-related diseases, and natural disasters show that the socioeconomic situation of the mass population also determined the individual level preparedness, which ultimately influenced them to adopt preventive practices (7–11).

Pandemic preparedness, be it related to the health system, individual, or household level, is one of the critical concerns across the countries for reducing the risk of COVID-19 (12). Thus, research on preparedness and preventive practices related to COVID-19 have significant public health policy implications, as preparedness is the key to navigating any public health crisis (13). A study conducted in Bangladesh shows that the country severely lacked the pandemic preparedness in its health and governance system. This study reported that lack of preparedness due to the "absence of planning and coordination, disproportionate resource allocations, challenged infrastructure, adherence to bureaucratic delay, lack of synchronized risk communication, failing leadership of concerned authorities, and incoherent decision-making" (14) had increased the country's epidemiologic vulnerability. However, no study was conducted to assess preparedness against the COVID-19 in Bangladesh at the individual and household levels, though research conducted elsewhere found that preparedness plays a significant role in adopting preventive practices (15). On the other hand, in Bangladesh, few studies have been conducted to explore the practices toward COVID-19. The findings of these studies show that different precarious practices such as not adopting protective measures and hygiene protocols, not wearing face masks in public places, and not maintaining social distance are prominent among mass population in Bangladesh (3, 16). Thus, the current study aimed to assess the preparedness regarding the preventive practices toward the COVID-19 among the adult population in Bangladesh using an online survey.

MATERIALS AND METHODS

Data Source

We conducted the survey using a cross-sectional research design. Population aged 18 years and above, living in Bangladesh, and who can read and write and use the internet were the criteria for selecting respondents. In Bangladesh, 74.7% of people aged 15 years and above can read and write a short, simple statement about their everyday life (17). On the other hand, as of March 2020, about 61% of the population are internet users in Bangladesh (18). We developed the study questionnaire based on the guidelines for conducting the behavioral insights on COVID-19 by the Regional Office for Europe of the World Health Organization (WHO) (19). The tool was adapted and customized for the Bangladesh country context. The tool was then translated into Bengali (local language) and pretested. The WHO (19) recommended having a sample size of 1,000 adult population. The data for this study were collected from 10 to 16 May 2020. The country was partially locked down during this period, and the government declared a general holiday. It was not possible to conduct face-to-face interviews for data collection during this period, as the population movement was restricted. Thus, the data were collected through the online survey portal, Google Forms, using Bengali as a language. A link to the form was then created and sent to the prospective participants, by e-mail, WhatsApp, or Facebook. All the participants to whom the survey link was sent were requested to share the link in their network to reach more people. The research team members circulated the survey link in their respective professional and social networks through the snowball process. As recommended by the WHO (19), the online data collection portal was active for 7 days. The respondents took an average of 20 min to complete the questionnaire. Though the initial decision was to reach a sample size of 1,000, a total of 1,059 respondents submitted their responses during these 7 days. However, three respondents did not consent to participate in this survey, and the final sample size was 1,056.

Outcome Variables

Preparedness Toward Coronavirus Disease 2019

Preparedness is the state of readiness to prevent the spread of the COVID-19 (20). We assessed the preparedness toward COVID-19 by using eight Likert items (**Table 1**), and the response options for these items were "strongly disagree = 5," "disagree = 4," "neither agree nor disagree = 3," "agree = 2," and "strongly agree = 1." We conducted a principal component analysis (PCA) by using these eight items. The PCA had an acceptable level of Kaiser–Meyer–Olkin (KMO) measure of sampling adequacy (KMO = 0.637). The varimax rotation with an eigenvalue higher

TABLE 1 | Rotated component matrix of principal component analysis of preparedness items against COVID-19 in Bangladesh.

Items	Components				Responses, *n* (%)		
	1	2	3	4	Disagree[a]	Neither agree nor disagree	Agree[b]
Individual preparedness (α = 0.809)							
Washing my hands often with water and soap for 20 s each time is inconvenient for me.		0.900			133 (12.6)	33 (3.1)	890 (84.3)
Disinfecting mobile phones, shoes, and clothes each time I return home is inconvenient for me.		0.919			257 (24.3)	54 (5.1)	745 (70.5)
Household preparedness (α = 0.617)							
Keeping distance with family members will be difficult if they/I show COVID-19-related symptoms.			0.837		264 (25.0)	56 (5.3)	736 (69.7)
Keeping the older people in the house is challenging.			0.838		328 (31.1)	77 (7.3)	651 (61.6)
Economic preparedness (α = 0.868)							
Due to the economic condition, I had to go to work, though I am aware of the risk of COVID-19.	0.923				431 (40.8)	69 (6.5)	556 (52.7)
I had to go out to save my job.	0.930				540 (51.1)	130 (12.3)	386 (36.6)
Community and social distancing-related preparedness (α = 0.618)							
Most of the people of my locality do not follow lockdown rules, so I also go out.				0.831	513 (48.6)	160 (15.2)	383 (36.3)
Go out for refreshment, as I feel bored for staying at home for a few days.				0.857	901 (85.3)	59 (5.6)	96 (9.1)

COVID-19, coronavirus disease 2019.
[a] *"Disagree" includes both "disagree" and "strongly disagree."*
[b] *"Agree" includes both "agree" and "strongly agree."*

than 1 was used as a selection criterion of components. The PCA produced four components that had an eigenvalue higher than 1. The eigenvalue of the first, second, third, and fourth components was 2.31, 1.75, 1.21, and 1.08, which explained 28.9, 21.9, 15.1, and 13.6% of the variance in the total items, respectively. These four components altogether explained 79.5% of the total items. The result of PCA indicates that the first two items, "washing my hands often with water and soap for 20 s each time is inconvenient for me" (19) and "disinfecting mobile phones, shoes, clothes each time I return home is inconvenient for me" (21, 22) (α = 0.809), were included under component 2, which was named "individual preparedness" (23). The third and fourth items, "keeping distance with family members will be difficult if they/I show COVID-19 related symptoms" and "keeping the older people in the house is challenging" (24) (α = 0.617), were included under component 3, which was named "household preparedness" (23). The fifth and sixth items, "due to the economic condition, I had to go to work though I am aware of the risk of COVID-19" and "I had to go out to save my job" (α = 0.868), were included under component 1, which was named "economic preparedness" (23, 25). The last two items, "most of the people of my locality do not maintain lockdown, so I also go out" and "go out for refreshment as I feel bored for staying at home for a few days" (α = 0.618), were included under component 4, which was named "community and social distancing-related preparedness" (26). We summed up the items of each component to create a continuous score for the preparedness scale about preventive practices against COVID-19, which ranges from 1 to 10, where the higher value indicated a higher level of preparedness.

Independent Variables

There were limited independent variables in the study instrument, as the survey was conducted online. We included the following independent variables: age, sex, educational attainment, occupation, region, place of residence, marital status, knowing someone as COVID-19 positive among the respondent's immediate social environment, and respondent's COVID-19 status. We also used the knowledge (**Supplementary Table 1**), attitudes (**Supplementary Table 2**), and worriedness (**Supplementary Table 3**) scales related to COVID-19 as covariates. We assessed the knowledge related to COVID-19, using a total of 25 items. The response options of these items were "yes," "no," or "not sure/do not know."

We assigned 1 point to a correct response, while an incorrect response was assigned 0 points. The total score of these 25 items ranged between 0 and 25, with a higher score indicating better knowledge about COVID-19. The reliability analysis was performed to check the internal consistency of these 25 items and found an acceptable level of Cronbach alpha ($\alpha = 0.689$). Attitudes are the way of feeling or thinking, while worriedness is the state of being worried or tensed. Attitudes toward COVID-19 ($\alpha = 0.671$) and worriedness during COVID-19 ($\alpha = 0.813$) were assessed using 8 and 10 Likert-type items, respectively. The response options for attitudes items were "strongly disagree = 1," "disagree = 2," "neither agree nor disagree = 3," "agree = 4," and "strongly agree = 5." The scores of attitudes toward COVID-19 ranged between 8 and 40, where a higher score of these scales indicates higher negative attitudes. On the other hand, the response options for worriedness items were "do not worry at all = 1," "worry sometimes = 2," "worry often = 3," and "worry all the time = 4." The scores of the worriedness scale also ranged between 8 and 40, and a higher score of these scales indicates a higher worriedness during the period of COVID-19.

Statistical Analysis

We first utilized univariate descriptive statistics [percentage, mean, and standard deviation (SD)] along with the accuracy test of each scale, where we divided the mean score of each scale by the total score of the respective scale. The independent sample *t*-test (if the independent variables had two categories), one-way ANOVA (if the independent variables had more than two categories), Pearson's product-moment correlation (if the independent variables were interval level), and Spearman's rank-order correlation (if the independent variables were ordinal) were used to produce the bivariate level statistics. We entered the statistically significant ($p \leq 0.05$) variables at the bivariate level into the multiple linear regression model after checking the assumptions and multicollinearity. We used the Statistical Product and Service Solutions (SPSS) software, version 26, to analyze the data.

Ethical Approval

The Bangladesh Medical Research Council approved the study (Registration Number: 302 1 1 05 2020). Participation in this online-based survey was entirely voluntary, and no incentives were provided to the participants. The respondents were informed about the aims, objectives, potential scopes, and implications of the findings of this study and were requested to participate voluntarily. As the data were collected through an online survey, the participants could only start filling up the questionnaire once they provided their consent to participate voluntarily.

RESULTS

Background Characteristics

The average age of the respondents was 32 years, with an SD of 10.56 (**Table 2**). Most of the respondents were from the age group of 18–30 years (58.3%). About two thirds (65.2%) of the respondents were men, while the majority of the respondents (50.4%) had an education level up to a Master's degree. One in five respondents (20.5%) were professionals, while 38.5% of the respondents were students and unemployed. Nearly two thirds (63.9%) of the respondents lived in the Dhaka division, while 73.4% of the respondents were from the middle region of Bangladesh, and 66.9% of the respondents were from the city corporation area. The proportion of unmarried respondents was slightly higher (52.2%) than the married respondents. One third (32.8%) of the respondents knew someone as COVID-19 positive in their immediate social environment. However, none of the respondents were COVID-19 positive, though 2.2% felt that they might be carrying the coronavirus infection but did not get tested. Moreover, the average score for knowledge, attitudes, and worriedness related to COVID-19 was 17.1, 13.7, and 25.5, respectively.

Prevalence of Preparedness Related to Coronavirus Disease 2019

Table 1 presents the distribution of the statements used to assess the preparedness of the respondents about preventive practices against the COVID-19. The mean score of the total preparedness scale was 27.6, with an SD of 4.7, and an overall preparedness level was 68.9% (27.55/40 * 100). Nearly a quarter of the respondents agreed that disinfecting daily-use commodities such as mobile phones, shoes, and clothes each time they return home was inconvenient for them, while 61.6% respondents agreed that it was challenging for them to keep the older people in the house as part of the prevention of COVID-19. Nearly half of the respondents (52.7%) agreed that they had to go to work due to their economic condition, and 36.6% of the respondents reported that they went out of their home, as most of the people of their locality did not follow the lockdown rules.

Differentials and Associates of Preparedness Related to Coronavirus Disease 2019

Table 2 shows that the mean score related to economic preparedness, individual preparedness, household preparedness, and community and social distancing-related preparedness was 6.49, 7.71, 5.04, and 8.32, respectively. The economic preparedness score was statistically significantly varied by sex, education, occupation, marital status, attitudes, and worriedness of COVID-19. The individual preparedness score was statistically significantly varied by education, occupation, place of residence, and knowing someone infected with COVID-19 in the respondent's immediate social environment. The household preparedness score was statistically significantly varied by education, place of residence, and worriedness of COVID-19. On the other hand, community and social distancing-related preparedness scores were statistically significantly varied by sex, region, place of residence, and knowing someone infected with the COVID-19 in the respondent's immediate social environment. Besides, higher knowledge related to symptoms and transmission of COVID-19 was statistically significantly correlated with higher individual preparedness, while higher negative attitudes toward COVID-19 was significantly negatively correlated with economic, individual, and community and social

TABLE 2 | Background characteristics (%) and mean distribution of different types of preparedness against COVID-19 in Bangladesh.

Background	*n* (%)	Economic	Individual	Household	Community and social distancing
		Mean (SE)	Mean (SE)	Mean (SE)	Mean (SE)
Age		$p = 0.410$	$p = 0.206$	$p = 0.627$	$p = 0.292$
18–24	341 (32.3)	6.70 (0.13)	7.61 (0.12)	5.07 (0.11)	8.20 (0.10)
25–30	275 (26.0)	6.42 (0.16)	7.57 (0.13)	4.87 (0.13)	8.47 (0.11)
31–39	184 (17.4)	6.31 (0.20)	7.73 (0.16)	5.11 (0.15)	8.33 (0.13)
40–49	178 (16.9)	6.33 (0.21)	7.93 (0.16)	5.07 (0.17)	8.42 (0.13)
50+	78 (7.4)	6.58 (0.29)	8.08 (0.22)	5.23 (0.28)	8.14 (0.17)
Mean (SD)	31.56 (10.56)	$r = -0.02$, $p = 0.470$	$r = 0.06$, $p = 0.055$	$r = 0.02$, $p = 0.444$	$r = 0.01$, $p = 0.865$
Sex		$p \leq 0.001$	$p = 0.123$	$p = 0.720$	$p = 0.001$
Male	688 (65.2)	6.28 (0.10)	7.64 (0.09)	5.06 (0.08)	8.19 (0.07)
Female	368 (34.8)	6.87 (0.13)	7.85 (0.10)	5.01 (5.01)	8.57 (0.08)
Education		$p = 0.050$	$p = 0.001$	$p = 0.013$	$p = 0.522$
Up to higher secondary	82 (7.8)	6.76 (0.27)	7.24 (0.29)	5.78 (0.26)	8.26 (0.17)
Graduate	352 (33.3)	6.50 (0.13)	7.66 (0.11)	5.00 (0.11)	8.22 (0.10)
Masters	532 (50.4)	6.33 (0.11)	7.68 (0.09)	4.97 (0.09)	8.38 (0.08)
MPhil/PhD	90 (8.5)	7.09 (0.28)	8.53 (0.19)	4.91 (0.24)	8.44 (0.19)
Occupation		$p \leq 0.001$	$p = 0.004$	$p = 0.154$	$p = 0.278$
Government and private sector job	181 (17.1)	5.71 (0.21)	7.39 (0.17)	5.25 (0.16)	8.44 (0.12)
Professional	211 (20.5)	6.82 (0.18)	7.95 (0.14)	4.93 (0.15)	8.41 (0.12)
NGO worker	173 (16.4)	6.53 (0.20)	8.13 (0.15)	4.90 (0.15)	8.46 (0.13)
Students and unemployed	407 (38.5)	6.7 (0.12)	7.57 (0.11)	4.97 (0.10)	8.20 (0.09)
Others	84 (8.0)	6.19 (0.26)	7.64 (0.25)	5.46 (0.24)	8.14 (0.18)
Region		$p = 0.130$	$p = 0.098$	$p = 0.649$	$p = 0.001$
Eastern (Sylhet and Chattaogram)	126 (11.9)	6.1 (0.22)	7.60 (0.20)	5.10 (0.18)	7.84 (0.16)
Middle (Dhaka, Barisal, and Mymensingh)	775 (73.4)	6.57 (0.09)	7.79 (0.08)	5.00 (0.08)	8.43 (0.06)
Western (Khulna, Rajshahi, and Rangpur)	155 (14.7)	6.37 (0.21)	7.40 (0.17)	5.17 (0.17)	8.15 (0.14)
Place of residence		$p = 0.120$	$p \leq 0.001$	$p = 0.031$	$p \leq 0.001$
Rural area	180 (17.0)	6.23 (0.18)	6.82 (0.19)	5.42 (0.18)	7.74 (0.15)
Urban (other than city corporation)	170 (16.1)	6.28 (0.20)	7.72 (0.16)	4.95 (0.16)	7.99 (0.14)
City corporation	706 (66.9)	6.6 (0.10)	7.94 (0.07)	4.96 (0.08)	8.55 (0.06)
Marital status		$p = 0.030$	$p = 0.476$	$p = 0.608$	$p = 0.487$
Married	505 (47.8)	6.31 (0.12)	7.76 (0.10)	5.07 (0.10)	8.36 (0.08)
Unmarried	551 (52.2)	6.65 (0.10)	7.67 (0.09)	5.01 (0.09)	8.29 (0.08)
Know someone as infected with the COVID-19 in their immediate social environment		$p = 0.230$	$p = 0.028$	$p = 0.560$	$p = 0.009$
No	710 (67.2)	6.42 (0.10)	7.61 (0.08)	5.06 (0.08)	8.22 (0.07)
Yes	346 (62.8)	6.62 (0.14)	7.92 (0.11)	4.98 (0.11)	8.53 (0.09)
Knowledge related to symptoms and transmission of COVID-19	16.92 (3.29)	$r = 0.03$, $p = 0.300$	$r = 0.06$, $p = 0.038$	$r = -0.02$, $p = 0.622$	$r = 0.05$, $p = 0.146$
Attitudes toward COVID-19	13.72 (3.69)	$r = -0.11$, $p \leq 0.001$	$r = -0.14$, $p \leq 0.001$	$r = 0.05$, $p = 0.096$	$r = -0.18$, $p \leq 0.001$
Worriedness about COVID-19	25.46 (5.420	$r = -0.10$, $p \leq 0.001$	$r = 0.03$, $p = 0.375$	$r = -0.16$, $p \leq 0.001$	$r = -0.05$, $p = 0.147$
Total	1,056 (100.0)	6.49 (0.08)	7.71 (0.07)	5.04 (0.07)	8.32 (0.05)

COVID-19, coronavirus disease 2019; NGO, non-governmental organization.

distancing-related preparedness. Finally, a higher level of worry about COVID-19 was statistically significantly correlated with lower levels of economic and household-related preparedness.

We entered the significant variables at the bivariate levels into the multiple linear regression models after checking the assumptions and multicollinearity. The age of the respondent was highly correlated with education ($r = 0.693$, $p \leq 0.001$) and marital status ($r = 0.761$, $p \leq 0.001$); thus, age was excluded from multiple regression analyses. **Table 3** presents the standardized beta coefficients of multiple linear regression with their statistical significance. It shows that women had mean 0.091-unit higher economic preparedness than men ($\beta = 0.091$, $p = 0.003$); other variables held constant. The respondents who worked in the government and private sectors had significant mean 0.113-unit lower economic preparedness ($\beta = -0.113$, $p = 0.008$) than the students and unemployed. The 1-unit increase of negative attitudes toward COVID-19 ($\beta = -0.083$, $p = 0.008$) and worriedness during COVID-19 ($\beta = -0.103$, $p = 0.001$) were decreasing the mean economic preparedness by 0.083 and 0.103 units, respectively. These predictors explained a 5.5% variation of the total model.

Table 3 also shows that the mean individual preparedness was 0.120 units higher among the respondents who had an MPhil/PhD level of education ($\beta = 0.120$, $p = 0.009$) than the respondents who had up to higher secondary level of education. Similarly, other things held constant, the urban respondents ($\beta = 0.128$, $p = 0.001$) and the city corporation area respondents ($\beta = 0.220$, $p \leq 0.001$) had 0.128- and 0.220-unit higher mean individual preparedness than the rural respondents. Besides, the negative attitudes toward COVID-19 had a negative influence ($\beta = -0.081$, $p = 0.010$) on individual preparedness. The independent variables of this model explained 6.5% of the variation of this model.

The mean household preparedness was 0.157, 0.171, and 0.101 units lower among the respondents with undergraduate ($\beta = -0.157$, $p = 0.006$), postgraduate ($\beta = -0.171$, $p = 0.004$), and MPhil/PhD ($\beta = -0.101$, $p = 0.018$) levels of education than that of higher secondary level. The respondents living in the urban areas ($\beta = -0.081$, $p = 0.039$) and the city corporation areas ($\beta = -0.083$, $p = 0.041$) had lower mean household preparedness than the respondents living in rural areas. It was also observed that the 1-unit increase of the worriedness related to COVID-19 decreased the mean household preparedness by 0.167 units ($\beta = -0.167$, $p \leq 0.001$). These predictors explained a 4.5% variation of the total model.

The mean community and social distancing-related preparedness was 0.077 units higher among women than men ($\beta = 0.077$, $p = 0.013$). The respondents living in the western part of Bangladesh had 0.088 units higher mean community and social distancing-related preparedness ($\beta = 0.088$, $p = 0.033$) than those in the eastern part. Similarly, compared with the respondents living in rural areas, the respondents living in the city corporation areas ($\beta = 0.161$, $p \leq 0.000$) had 0.161 units higher preparedness. In contrast, 1-unit increment of the attitudes toward COVID-19 had negatively influenced ($\beta = -0.144$, $p \leq 0.001$) the community and social distancing-related preparedness by 0.144 units. These regressors explained around 6.9% of the total variation of the model.

DISCUSSION

The study sought to explore the preparedness regarding preventive practices against COVID-19 in Bangladesh. The study found that the overall preparedness level was 68.9% (27.56/40 * 100).

Individual Preparedness

The level of individual preparedness for preventing practices against COVID-19 was 77.1% (7.71/10 * 100). The findings show that 12–24% of respondents reported their inconvenience regarding proper handwashing practices and disinfecting items of personal use after each time they return home. This inconvenience related to personal hygiene could be attributed to factors like the availability of handwashing commodities, price, facilities, and knowledge and attitudes toward handwashing (27–30).

This study found that individual preparedness was higher among the respondents who had MPhil/PhD level of education, which is similar to the studies conducted elsewhere (23, 31). The relation between education and individual preparedness creates health communication scope among the mass population with the utmost importance (32, 33). Findings regarding other recent infectious disease outbreaks in Bangladesh (dengue, chikungunya, Nipah virus, etc.) also indicate that mass population's knowledge level and preventive practices amidst disease outbreaks are significantly associated (34). The findings of this study showed that the respondents living in the urban and the city corporation areas had higher individual preparedness than the respondents living in rural areas. The urban populations are in an advantageous position because they are more likely to afford and have access to personal hygiene-related amenities (32).

The negative attitudes toward COVID-19 were producing less individual preparedness. This finding is consistent with the Chinese study (6). Our study measured negative attitudes toward COVID-19 by using items like COVID-19 is a punishment from the creator and we (respondents) can be safe if we pray to Allah/God/Creator regularly. These attitudes possibly reduced the risk perception (35) of the respondents, which push them to be less prepared (36).

Household Preparedness

The level of household preparedness for preventing practices against COVID-19 was 50.4% (5.04/10 * 100). Around two thirds of the respondents reported their inconvenience in keeping older persons in the house and maintaining social distance with family members showing symptoms related to COVID-19. Maintaining social distancing with family members, especially with older persons within the home setting, was also challenging in other studies (37–40).

The household preparedness was found lower among the respondents with undergraduate, postgraduate, and MPhil/PhD levels of education than higher secondary levels. This finding needs to be interpreted with the fact that more respondents

TABLE 3 | Association between background characteristics and different types of preparedness against COVID-19 in Bangladesh.

Background characteristics	Economic	Individual	Household	Community and social distancing
Sex (Ref: Male)				
Female	0.091**			0.077*
Educational attainment (Ref: Up to higher secondary)				
Undergraduate	−0.065	0.072	−0.157**	
Post-graduate (Masters)	−0.036	0.044	−0.171**	
MPhil/PhD	0.038	0.120**	−0.101*	
Occupation (Ref: Students and unemployed)				
Government and private sector	−0.113**	−0.063		
Professional	0.012	−0.011		
NGO worker	−0.023	0.038		
Others	−0.053	0.002		
Region (Ref: Eastern)				
The middle part of Bangladesh				0.070
The western part of Bangladesh				0.088*
Place of residence (Ref: Rural)				
Urban (other than city corporation)		0.128**	−0.081*	0.023
City corporation		0.220**	−0.083*	0.161**
Marital status (Ref: Married)				
Unmarried	0.053			
Know someone as COVID-19 positive within their immediate social environment				
Yes (Ref: No)		0.028		0.047
Knowledge related to COVID-19		0.016		
Attitudes toward COVID-19	−0.083**	−0.081**		−0.144**
Worriedness related to COVID-19	−0.103**		−0.167**	
Constant[a]	7.830**	7.232**	7.341**	8.057**
Model summary				
N	1,056	1,056	1,056	1,056
R	0.235	0.254	0.208	0.262
R^2	0.055	0.065	0.043	0.069
Adjusted R^2	0.045	0.052	0.037	0.062

COVID-19, coronavirus disease 2019; NGO, non-governmental organization.
*$p \leq 0.05$.
**$p \leq 0.01$.
[a]*Unstandardized beta.*

with higher secondary level education were living in rural areas while higher educated respondents were living in urban and city corporation areas. The housing pattern of the rural and urban areas is structurally different (41), and urban housing in Bangladesh lacks the comfortability for the older people. This finding is supported by the findings that the respondents living in urban areas had lower household preparedness. It was challenging for many city dwellers to maintain social distancing at home in the densely populated cities and the congested housing system (2, 42, 43). It was also observed in the current study that the higher the worriedness related to COVID-19, the lower the household preparedness would be. The adverse impacts of COVID-19 imposed social isolation, be it physical or psychological, may lead people to be less willing to isolate family members (44, 45), including older persons, even if they show related symptoms.

Economic Preparedness

The level of economic preparedness for preventing practices against COVID-19 was 64.9% (6.49/10 * 100). More than half of the respondents reported economic consideration as their motive to go outside of the home, whereas more than one third of the respondents reported saving jobs as their prioritized concern even in the lockdown period. Financial fears have also been reported in other studies as the main motive for going outside in the present context (46). Working-class people were less likely to comply with the lockdown protocols because of their economic urgency and drive to save jobs (47, 48).

The findings of this study showed a more secure economic position and higher preparedness among women and students, and the unemployed. The economic reliance of these subgroups on men and employed family members contributed to their

better-secured position and better economic preparedness. The financial fear and perceived insecure position among employed respondents were reported in other studies, too (23, 49). The effect of negative attitudes toward COVID-19 and worriedness negatively influenced economic preparedness in this study.

Community and Social Distancing-Related Preparedness

The level of preparedness related to community and social distancing was 83.2% (8.32/10 * 100). In this regard, respondents who were women and living in the city corporation areas were found to be more prepared. Women's higher perception and better compliance regarding community and social distancing-related preparedness were also found in other studies (50, 51). The rural respondents were also found to be showing poor social distancing patterns, which has been supported in another study as well (52). The negative attitudes toward COVID-19 were found to be having a negative influence on the social distancing-related preparedness, as negative attitudes possibly reduced the risk perception of the respondents, which push them to be less prepared (53, 54).

Conclusions and Implications

In a context where a better preparation level and evidence-based preventive practices can make things more comfortable, this study found an overall preparedness level of 68.9%, which significantly implies scopes of priority-based interventions. Specific preparedness levels concerning economic (64.9), individual (77.1), household (50.4), community, and social distance (83.2) aspects also are supporting the necessity of the above-mentioned implications. Inconvenience regarding ensuring personal hygiene-related practices was reported, which reflected the lack of individual-level preparedness. Maintaining social distance was very challenging, which was significantly influenced by the presence of negative attitudes toward COVID-19. Protection of the older population who are "the most at-risk population" by successfully making them stay within the house faces challenges too because of their particular contexts. Financial urgency, including the drive to save jobs, was seen to triggering mass population's tendency to not follow the rules of lockdown and social distancing. The findings of this study implicate the necessity of taking comprehensive efforts to ensure the coverage and receipt of the different social protection schemes, especially for older persons, to release them from the financial fears and urgency to go outside amidst the coronavirus period. A fixed amount of financial compensation, especially toward economically vulnerable groups who are found to be not following lockdown rules for the drive to save job, can also be considered in this regard to provide them with temporary support and also enable them to sustain their daily lives under financial protection. Policy interventions to increase individual awareness have been observed to be effective in creating preventive behaviors and preventing infectious diseases in incidents of other outbreaks in the context of Bangladesh (55). Thus, the findings of this study can be applied to the broader context of infectious disease-related disaster preparedness, such as dengue, chikungunya, and Nipah virus. The current study also implicates the necessity of ensuring the broader coverage of health education related to personal hygiene practices to increase the level of awareness through appropriate channels, particularly in the rural areas where the level of individual preparedness was lower.

Strength and Limitations

This study provided efforts to explore the preparedness regarding the preventive practices of the mass population against COVID-19 with a broad geographical coverage within a short period. Such rapid snapshots with robust statistical analyses can provide food for thought for the policy planners. However, a rapid assessment survey to understand the preparedness regarding the preventive practice of the mass population in Bangladesh regarding COVID-19 clearly has certain limitations. First, this was an online survey, and it covered somewhat a homogenous population in terms of knowledge and skills, and level of awareness regarding health issues. Thus, these findings have certain limitations in establishing generalizability. Second, as it was a rapid assessment online-based survey, the study team had to take the time issue (required minutes to fill up the questionnaire) of the respondents into consideration, and it left scopes for reaching depth with potential items to use to assess the preparedness level and preventive practice in a better way. Third, the sample size used in this study varied greatly across different divisions. The imbalanced sample size may cause bias in the study findings. Besides, some essential covariates were not included in the questionnaire due to the online nature of the survey. Finally, this study leaves ample room for further exploration of the population level preparedness and its relevance with the recurrent infectious disease outbreaks in the context of Bangladesh.

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 Bangladesh Medical Research Council. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MBH, MA, MI, SS, and MF conceptualized the study. MBH, MA, MI, SS, MF, SR, MM, MAH, SK, AM, HM, and SSS designed the study and collected the data. MA, MI, and SS analyzed the data with guidance from MBH. MA, SS, and MBH interpreted the data and drafted the manuscript.

MI, MF, SR, MM, MAH, SK, AM, HM, and SSS revised the manuscript critically for valuable intellectual content and approved the final version to be published. All authors remain in agreement to be accountable for all aspects of the work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.582701/full#supplementary-material

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Correlation Between Local Air Temperature and the COVID-19 Pandemic in Hubei, China

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Objective: To clarify the correlation between temperature and the COVID-19 pandemic in Hubei.

Methods: We collected daily newly confirmed COVID-19 cases and daily temperature for six cities in Hubei Province, assessed their correlations, and established regression models.

Results: For temperatures ranging from −3.9 to 16.5°C, daily newly confirmed cases were positively correlated with the maximum temperature ∼0–4 days prior or the minimum temperature ∼11–14 days prior to the diagnosis in almost all selected cities. An increase in the maximum temperature 4 days prior by 1°C was associated with an increase in the daily newly confirmed cases (∼129) in Wuhan. The influence of temperature on the daily newly confirmed cases in Wuhan was much more significant than in other cities.

Conclusion: Government departments in areas where temperatures range between −3.9 and 16.5°C and rise gradually must take more active measures to address the COVID-19 pandemic.

Keywords: COVID-19, infectious disease, weather-outbreak correlation, climate and health, temperature, daily new confirmed infections

INTRODUCTION

Coronavirus disease 2019 (COVID-19), a severe acute respiratory syndrome caused by coronavirus-2 (SARS-CoV-2), broke out in the city of Wuhan, China, in the early winter of 2019. Since then, it has had a substantial effect on global health, economics, and lifestyles, prompting world governments to take various measures to reduce the damages caused by the outbreak. The pandemic has attracted worldwide attention (1) and many recent studies have focused on the relationships between temperature and COVID-19 (2–4). If such relationships could be determined, corresponding measures could be taken to reduce viral morbidity.

The SARS-CoV-2 is primarily transmitted by fomites or respiratory droplets (5). Environmental factors, such as temperature, have an impact on the survival and spread of viruses transmitted through the respiratory tract (6–8). Therefore, temperature are assumed to have an impact on the spread of COVID-19. In the past, severe acute respiratory syndrome (SARS), the infectious disease caused by another respiratory-borne coronavirus, broke out in November of 2002 in China and spread rapidly throughout Southeast Asia. In previous studies on SARS, a negative correlation was found between local air temperatures and daily new cases of SARS (9). This supports our conjecture that temperature may affect the spread of COVID-19.

Until now, the relationship between temperature and the spread of the COVID-19 has not been clarified. Limited studies have shown that temperature have impacts on the spread of the COVID-19 (10–12). Tosepu et al. (13) found a positive correlation between temperature and the COVID-19 pandemic. Conversely, Prata et al. (14) found that daily cumulative confirmed cases were negatively correlated with temperature. The results of most of the previous studies are not entirely consistent, and the relationships between temperature and the spread of COVID-19 remain controversial (15). Our study is mainly focused on analyzing the impacts of temperature on the spread of COVID-19, with the aim of guiding the prevention and management of COVID-19 transmission in the real world based on empirical data.

Hubei Province is the region in China that has been the most severely affected by COVID-19. It is, therefore, extremely valuable to study the impact of temperature on the spread of the virus in Hubei. In this study, six cities in Hubei Province were selected due to the greater severity of the COVID-19 outbreak in these cities. We collected the daily new confirmed cases (DNCC) and daily local temperature (maximum and minimum) data to analyse the relationship between temperature and the spread of COVID-19. Considering the incubation period from the date of a patient's infection to the onset of symptoms and the time from the onset of symptoms to a clear diagnosis, the date of diagnosis must necessarily follow the date of infection (16). Therefore, the impact of temperature on the spread of the virus can only be manifested after a period of time (including the incubation period and the time from the onset of symptoms to a clear diagnosis). As the incubation period of COVID-19 is typically 1–14 days, the impact of temperature on DNCC would inevitably display a certain lag between infection and diagnosis (17). To clarify the influence of temperature on the COVID-19 pandemic, we analyzed the correlations between the DNCC and the temperature 0–14 days before diagnosis and established regression models to understand trends in these relationships.

MATERIALS AND METHODS

The study area included six cities across Hubei Province in China, namely Wuhan, Xiaogan, Huanggang, Suizhou, Jingzhou, and Huangshi. Among all cities in this province, the numbers of patients infected with COVID-19 were the highest in these selected cities. The DNCC were collected between 25 January and 11 February of 2020 from the government websites of each city (Wuhan: http://www.wuhan.gov.cn/; Xiaogan: http://www.xiaogan.gov.cn/; Huanggang: http://www.hg.gov.cn/; Suizhou: http://www.suizhou.gov.cn/; Jingzhou: http://www.jingzhou.gov.cn/; Huangshi: http://www.huangshi.gov.cn/). Temperature factors included two indicators: daily maximum and minimum temperatures (°C). Considering that COVID-19 has an incubation period of 1–14 days, we collected the daily temperature from 11 January to 11 February of 2020. The temperatures of each city were obtained from local weather stations.

In order to clarify any correlations that might exist between temperature and the COVID-19 pandemic in Hubei, we analyzed the correlations between the DNCC and the daily maximum and minimum temperatures from 0 to 14 days before a diagnosis was confirmed. Statistical analyses were performed using SPSS software v. 22.0 (IBM Corp., USA). First, considering the small sample size, we conducted a Shapiro–Wilk normality test to analyse the daily maximum and daily minimum temperature data, as well as the DNCC for the six studied cities (**Supplementary Table 1**). We found that the daily maximum and daily minimum temperature data for these cities were normally distributed, as were the DNCC data from Wuhan, Huanggang, Jingzhou, and Huangshi. We, then, employed Pearson correlation analyses to determine the correlations between the DNCC and the daily maximum temperature (**Table 1**) and daily minimum temperature (**Table 2**) from 0 to 14 days prior to the a confirmed diagnosis of COVID-19. As the DNCC data from Xiaogan and Suizhou were not normally distributed, we instead used Spearman rank correlations to analyse these data. Through correlation analyses, we were able to identify the days prior to the diagnosis wherein local temperatures were the most strongly correlated with DNCC.

After correlation analyses, we determined the temperatures corresponding to the days for which there was a statistical significance ($p < 0.05$) in the correlation coefficient between the DNCC and the temperatures. We used DNCC as the dependent variable and the temperature corresponding to the selected days as the independent variable to perform stepwise multiple linear regressions. Finally, we established a multiple linear regression model to analyse the relationship between temperature and the COVID-19 pandemic in Hubei and used relevant parameters to evaluate the reliability of our model.

RESULTS

DNCC and Daily Temperatures in Selected Regions

As shown in **Figure 1**, the trends in the DNCC differed slightly among the six cities investigated. Beginning on 25 January, the DNCC increased in all cities. In Wuhan, Xiaogan, Huanggang, Suizhou, Jingzhou, and Huangshi, the DNCC peaked at $n =$ 1,985, 424, 276, 183, 166, and 104 on the 7th, 5th, 1st, 3rd, 2nd,

TABLE 1 | The correlations between the DNCC and daily maximum temperature 0–14 days prior to COVID-19 diagnoses in Hubei.

Day	Wuhan		Xiaogan		Huanggang		Suizhou		Jingzhou		Huangshi	
	CC	*P*	CC	*P*	CC	*P*	CC	*P*	CC	*P*	CC	*P*
0	0.312	0.207	0.548	0.019	0.655	0.003	0.383	0.117	0.313	0.207	0.592	0.010
1	0.370	0.131	0.621	0.006	0.510	0.031	0.711	0.001	0.399	0.101	0.612	0.007
2	0.467	0.051	0.610	0.007	0.396	0.104	0.558	0.016	0.501	0.034	0.665	0.003
3	0.607	0.008	0.543	0.020	0.364	0.137	0.430	0.075	0.582	0.011	0.696	0.001
4	0.761	<0.001	0.473	0.047	0.225	0.370	0.243	0.332	0.538	0.021	0.585	0.011
5	0.668	0.002	0.285	0.251	−0.197	0.434	0.127	0.615	0.217	0.386	0.276	0.268
6	0.531	0.023	0.126	0.620	−0.512	0.030	−0.203	0.420	−0.143	0.571	−0.034	0.895
7	0.443	0.065	−0.022	0.930	−0.610	0.007	−0.387	0.112	−0.276	0.261	−0.238	0.342
8	0.370	0.130	−0.125	0.547	−0.317	0.200	−0.256	0.304	−0.310	0.210	−0.162	0.520
9	0.289	0.246	−0.073	0.772	0.035	0.889	−0.251	0.316	−0.247	0.323	−0.095	0.706
10	0.175	0.487	0.036	0.887	−0.064	0.800	−0.041	0.871	−0.187	0.458	0.049	0.848
11	0.035	0.889	−0.171	0.497	−0.018	0.942	0.215	0.392	−0.140	0.580	−0.002	0.993
12	−0.063	0.803	−0.229	0.361	0.063	0.805	0.252	0.313	−0.111	0.660	−0.173	0.491
13	−0.073	0.773	−0.115	0.650	0.156	0.536	0.304	0.221	0.077	0.760	0.076	0.763
14	0.026	0.920	0.151	0.548	0.240	0.338	0.352	0.152	0.356	0.147	0.292	0.239

CC, correlation coefficient.

TABLE 2 | The correlations between the DNCC and daily minimum temperature 0–14 days prior to COVID-19 diagnoses in Hubei.

Day	Wuhan		Xiaogan		Huanggang		Suizhou		Jingzhou		Huangshi	
	CC	*P*	CC	*P*	CC	*P*	CC	*P*	CC	*P*	CC	*P*
0	0.168	0.504	0.081	0.750	0.129	0.611	−0.028	0.911	0.206	0.413	0.319	0.196
1	0.037	0.883	−0.035	0.892	−0.017	0.945	−0.308	0.214	0.372	0.129	0.190	0.451
2	0.059	0.817	−0.140	0.579	−0.282	0.256	−0.438	0.069	0.106	0.676	−0.070	0.782
3	−0.165	0.513	−0.159	0.529	−0.625	0.006	−0.209	0.405	−0.157	0.534	−0.445	0.064
4	−0.348	0.157	−0.255	0.307	−0.674	0.002	−0.353	0.151	−0.345	0.161	−0.649	0.004
5	−0.235	0.347	−0.169	0.502	−0.466	0.051	−0.451	0.060	−0.536	0.022	−0.475	0.047
6	−0.344	0.162	−0.263	0.291	−0.264	0.289	−0.141	0.578	−0.423	0.080	−0.320	0.196
7	−0.112	0.659	−0.234	0.351	0.149	0.555	0.107	0.674	−0.213	0.396	−0.150	0.552
8	−0.066	0.795	−0.043	0.866	0.352	0.152	0.260	0.297	−0.016	0.950	−0.063	0.803
9	0.016	0.950	0.148	0.557	0.317	0.200	0.538	0.021	0.154	0.541	0.190	0.450
10	0.168	0.506	0.291	0.241	0.574	0.013	0.499	0.035	0.442	0.066	0.425	0.079
11	0.354	0.149	0.454	0.059	0.650	0.004	0.382	0.118	0.584	0.011	0.617	0.006
12	0.492	0.038	0.524	0.026	0.486	0.041	0.228	0.364	0.597	0.009	0.653	0.003
13	0.735	0.001	0.695	0.001	0.216	0.390	−0.014	0.956	0.117	0.643	0.608	0.007
14	0.618	0.006	0.582	0.011	0.051	0.841	−0.228	0.363	−0.104	0.682	0.498	0.035

CC, correlation coefficient.

and 4th of February, respectively. After reaching these peaks, the overall trends in the DNCC declined across all cities, although there were some fluctuations.

Figures 2, **3** show the daily maximum and minimum temperatures from 11 January to 11 February of 2020. The lowest maximum temperature was 2.4°C and the highest was 16.5°C. The lowest minimum temperature was −3.9°C and the highest was 9°C.

Correlation Between DNCC and Daily Temperature

Tables 1, **2** present the results of Pearson correlation and Spearman rank correlation analyses, depending on the normality of the underlying data. We, first, evaluated the correlations between the DNCC and daily temperature 0–14 days prior to COVID-19 diagnoses in each city and found that the correlations differed among the cities. In Wuhan, the DNCC were positively

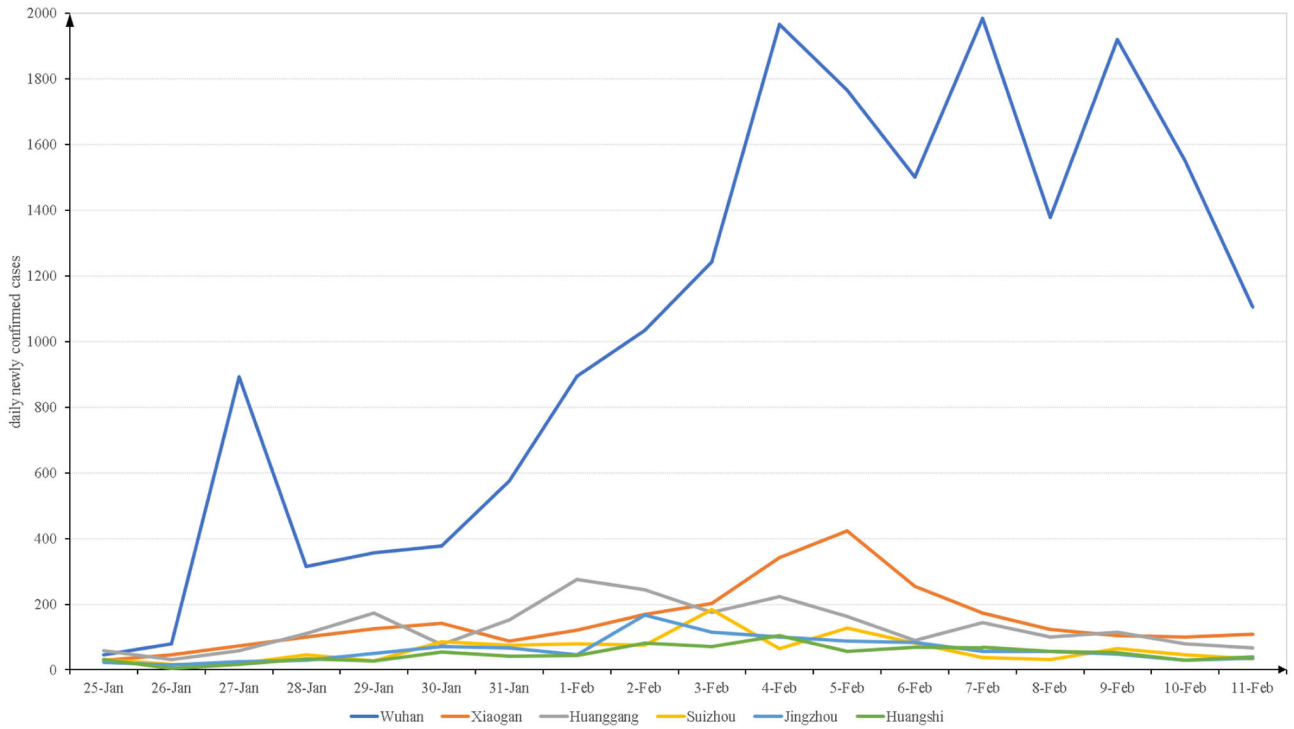

FIGURE 1 | The daily newly confirmed cases from 2020-1-25 to 2020-2-11 in Hubei.

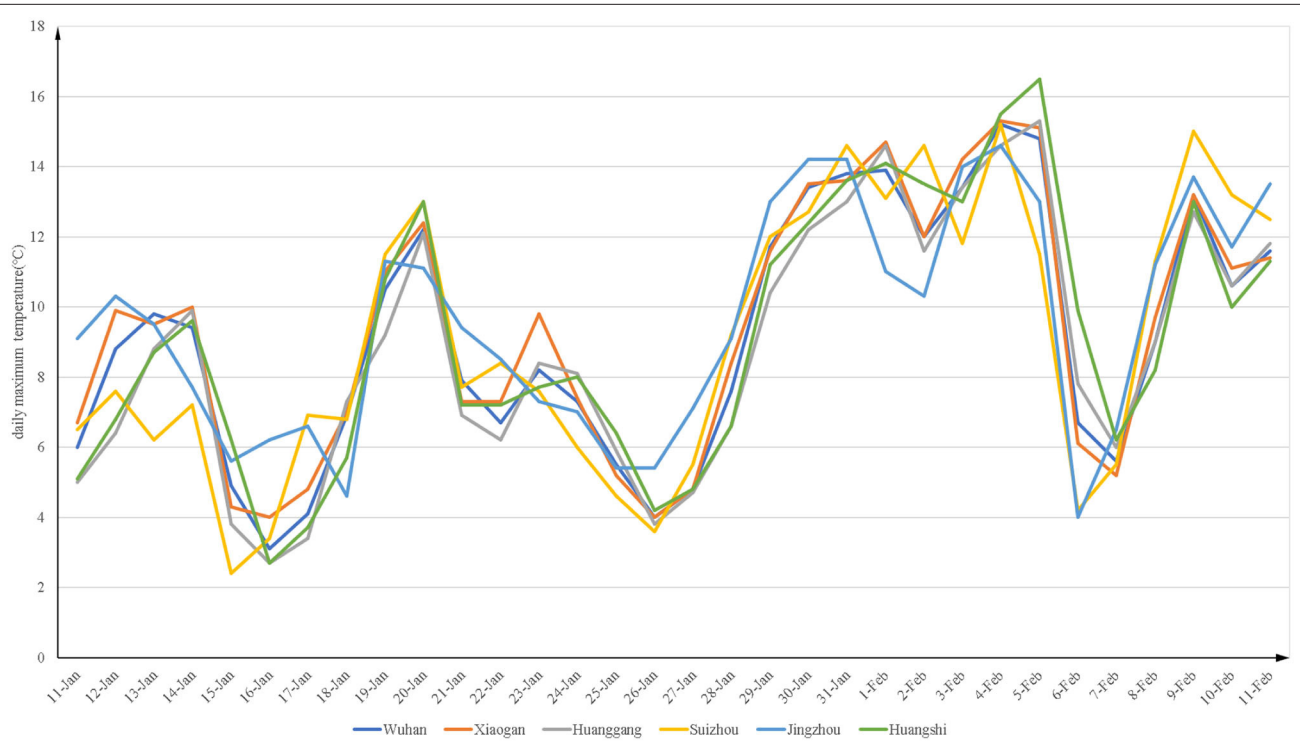

FIGURE 2 | The daily maximum temperature (°C) from 2020-1-11 to 2020-2-11 in Hubei.

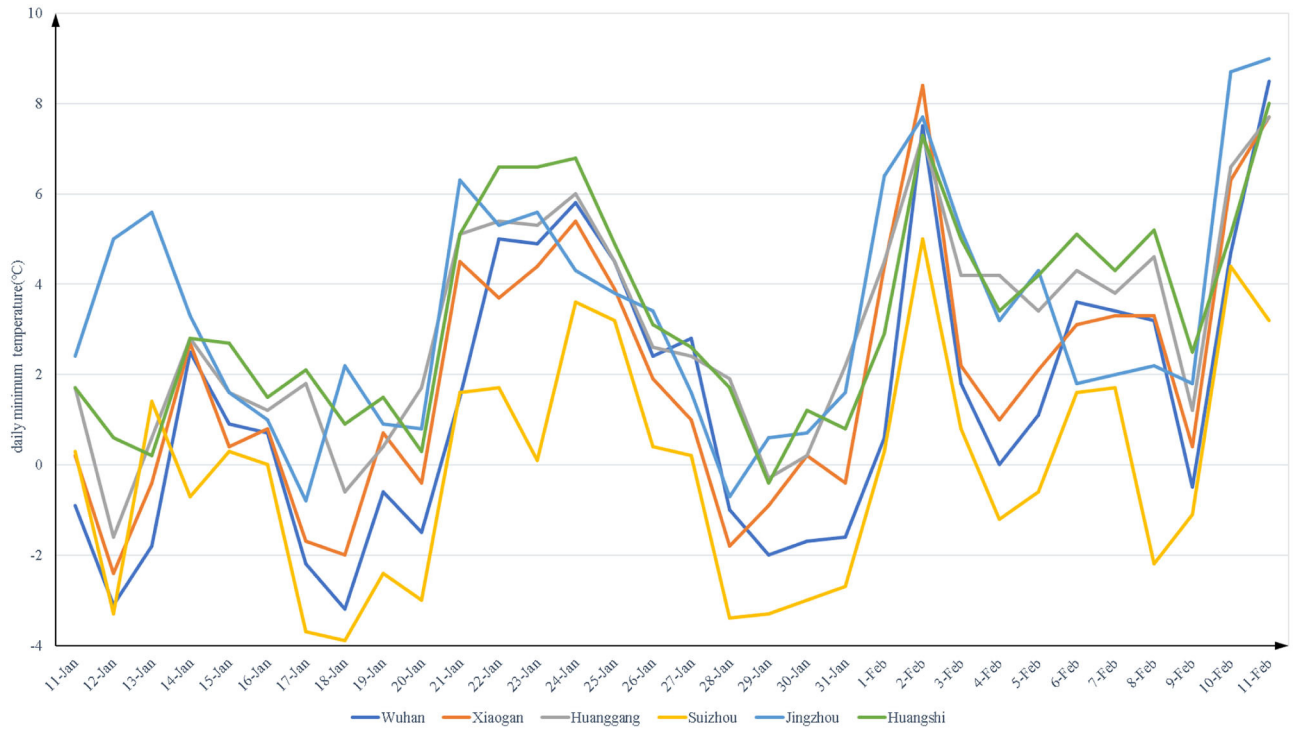

FIGURE 3 | The daily minimum temperature (°C) from 2020-1-11 to 2020-2-11 in Hubei.

correlated with the daily maximum and minimum temperatures 3–6 and 12–14 days prior to diagnosis ($p < 0.05$), respectively. In Xiaogan, the DNCC were positively correlated with the daily maximum temperature 0–4 days prior and to the daily minimum temperature 12–14 days prior to diagnosis ($p < 0.05$). In Huanggang, the DNCC were positively correlated with the daily maximum temperature 0–1 days prior and the daily minimum temperature 10–12 days prior to diagnosis ($p < 0.05$), while they were negatively correlated with the daily maximum and minimum temperatures 6–7 and 3–4 days prior to diagnosis ($p < 0.05$), respectively. In Suizhou, the DNCC were positively correlated with the daily maximum temperature 1–2 days preceding diagnosis and to the daily minimum temperature 9–10 days prior ($p < 0.05$). In Jingzhou, the DNCC were positively correlated with the daily maximum and minimum temperatures 2–4 and 11–12 days prior to diagnosis ($p < 0.05$), respectively; meanwhile, they were negatively correlated with the minimum temperature 5 days prior to diagnosis ($p < 0.05$). Finally, in Huangshi, the DNCC were positively correlated with the daily maximum and minimum temperatures 0–4 and 11–14 days preceding diagnosis ($p < 0.05$), respectively, while they were negatively correlated with the daily minimum temperature 4–5 days prior to diagnosis ($p < 0.05$).

Model Fitting

To further assess the quantitative relationship between DNCC and daily temperatures, stepwise multiple linear regression was used to screen the temperature factors. All temperature data for which $p < 0.05$ in **Tables 1**, **2** were included in the regression model for further analysis. **Table 3** shows the statistical data for the linear regression equation for each city. As shown in **Table 3**, a one unit increase in the maximum temperature 4 days before the patient was diagnosed positive caused an increase of 129.449 standard deviations of the DNCC in Wuhan. However, an increase of 1°C in the maximum temperature 1 day prior to the diagnosis by was associated with an increase of ∼7 in the DNCC in Suizhou. The impact of temperature on the DNCC in Wuhan was much greater than that in other cities. In most cities, an increase in temperature led to an increase in the DNCC, except in Huanggang, where a one unit increase in the minimum temperature 4 days prior to the diagnosis caused a decrease of 16.432 standard deviations in the DNCC.

The linear regression models in our study differed among the cities. In Wuhan, the DNCC were positively correlated with the maximum temperature 4 days preceding the diagnosis, whereas in Xiaogan, the DNCC were positively correlated with the maximum temperature on the day when the patient was confirmed and with the minimum temperature 12 and 14 days prior to the diagnosis. In Huanggang, the DNCC were positively correlated with the minimum temperature 11 days prior to diagnosis and in Suizhou, they were positively correlated with the maximum temperature just 1 day prior to the diagnosis. In Jingzhou, the DNCC were positively correlated with the minimum temperature 12 days prior and in Huangshi, they were positively correlated with the maximum temperature on the day of diagnosis and 3 days prior. Overall, the DNCC in all cities were

TABLE 3 | Statistical data of the linear regression equation in Hubei.

Region	Model formula
Wuhan	$Y_{DNCC} = -185.716 + 129.449X_{max-d4}$
Xiaogan	$Y_{DNCC} = -24.925 + 12.120\,X_{max-d0} + 22.606X_{min-d12} + 17.963X_{min-d14}$
Huanggang	$Y_{DNCC} = 156.597 - 16.432X_{min-d4} + 14.031X_{min-d11}$
Suizhou	$Y_{DNCC} = -7.314 + 6.839X_{max-d1}$
Jingzhou	$Y_{DNCC} = 35.330 + 10.199X_{min-d12}$
Huangshi	$Y_{DNCC} = -21.574 + 3.075X_{max-d0} + 3.820X_{max-d3}$

DNCC, daily newly confirmed cases; max-d0, daily maximum temperature on the day when the patient was confirmed; max-d1(3/4), daily maximum temperature 1(3/4) days prior to the diagnosis; min-d4(11/12/14), daily minimum temperature 4(11/12/14) days prior to the diagnosis.

positively correlated with the maximum temperature ∼0–4 days prior to diagnosis or with the minimum temperature ∼11–14 days prior.

Model Evaluation

As is well-known, the following four conditions must be met when constructing a linear regression model (18, 19): ① there must be a linear relationship between the independent and dependent variables; ② the residuals must be normally distributed; ③ the residuals must be independent; ④ the residual must exhibit homoscedasticity. In this study, the independent and dependent variables, first, underwent Pearson correlation or Spearman rank correlation analysis, so that they were linearly related. Second, a histogram of the regression-standardized residuals of the dependent variable (**Figure 4**) showed that the residuals were normally distributed, and the normal P–P plot of the regression-standardized residuals of the dependent variable (**Supplementary Figure 1**) further demonstrated the normality of the residuals. We, then, found that the residuals were independent by the Durbin–Watson (DW) test because DW $\approx$ 2 (**Table 4**), which indicates the absence of autocorrelation (20). Finally, from the scatter plot of regression-standardized predicted values and residuals (**Figure 5**), we observed that the residuals were randomly distributed and did not increase or decrease as the predicted value increased, indicating that the variance of the residuals was homogeneous and, thus, that our model was reliable.

To evaluate our model further, we conducted *F*-tests, for which the results were <0.05 for all cities, suggesting that our model was successfully constructed. As shown in **Table 4**, the minimum adjusted coefficient of determination (R^2) was 0.316 and the maximum value was 0.824. This indicates that 31.6–82.4% of all factors affecting the DNCC were included in the multiple linear regression models. Therefore, our model was appropriate and reliable. Additionally, we tested the significance of the partial regression coefficients of the independent variables in all models and found that they were all statistically significant. Considering that there were more than one independent variable in the models for Xiaogan, Huanggang, and Huangshi, collinearity diagnostics for independent variables in these models were adopted. The models displayed collinearity when the variance inflation factor was >5 (21, 22). After the analysis, we found no collinearity in our models.

Verifying Our Results in Other Cities

According to the results of our research in Hubei, the DNCC were positively correlated with the maximum temperature ∼0–4 days or the minimum temperature ∼11–14 days prior to a confirmed diagnosis of COVID-19. In order to determine whether or not this relationship was universal, we included other cities in the study. Among the cities with higher morbidities near Hubei Province, Shaoyang in Hunan Province, and Xinyang in Henan Province were randomly selected for inclusion in our study. **Supplementary Figure 2** shows the trends of the DNCC and temperatures in Shaoyang and Xinyang.

Using Shapiro–Wilk normality tests, we found that the daily minimum temperature in Xinyang was not normal, while other data were normally distributed. Therefore, we used Spearman rank correlation analyses to analyse the correlations between the DNCC and the daily minimum temperatures in Xinyang, and Pearson correlation analyses to assess the correlations between the DNCC and daily temperatures (maximum and minimum) in Shaoyang and daily maximum temperatures in Xinyang. Through these analyses, we evaluated the correlations between the DNCC and daily temperature 0–14 days before a diagnosis of COVID-19 was confirmed.

In Shaoyang, the DNCC were positively correlated with the maximum temperatures on the day of the diagnosis and with the minimum temperature 14 days prior to the diagnosis (**Supplementary Table 2**). In Xinyang, the DNCC were positively correlated with the maximum temperature 3–5 days and minimum temperature 11–12 days preceding diagnosis, respectively. After including these DNCC and temperature data in the multiple linear regression analysis, we observed that the DNCC in Shaoyang and Xinyang were positively correlated with the minimum temperatures 14 and 12 days prior to the diagnosis, respectively (**Table 5**).

The histogram and normal P–P plot (**Supplementary Figure 3**) of the regression-standardized residuals and Durbin–Watson tests for Shaoyang and Xinyang suggest that the residuals were normal and independent. From the scatter plot of regression-standardized predicted values and residuals, we observed that the variance in the residuals was homogeneous. Finally, the results of p(F), p(X), and adjusted-R^2 showed that our model was reliable. Considering the models for Shaoyang and Xinyang, the conclusions drawn from the original six cities studied appear to be universal. In most cases, the DNCC were positively correlated with the maximum temperature ∼0–4 days or the minimum temperature ∼11–14 days prior to the diagnosis.

DISCUSSION AND CONCLUSIONS

Over the temperature range of −3.9–16.5°C, our results showed that the DNCC were positively correlated with the maximum temperature ∼0–4 days or the minimum temperature ∼11–14 days prior to the diagnosis in nearly all selected cities, except for Huanggang. However, Prata et al. (14) found that

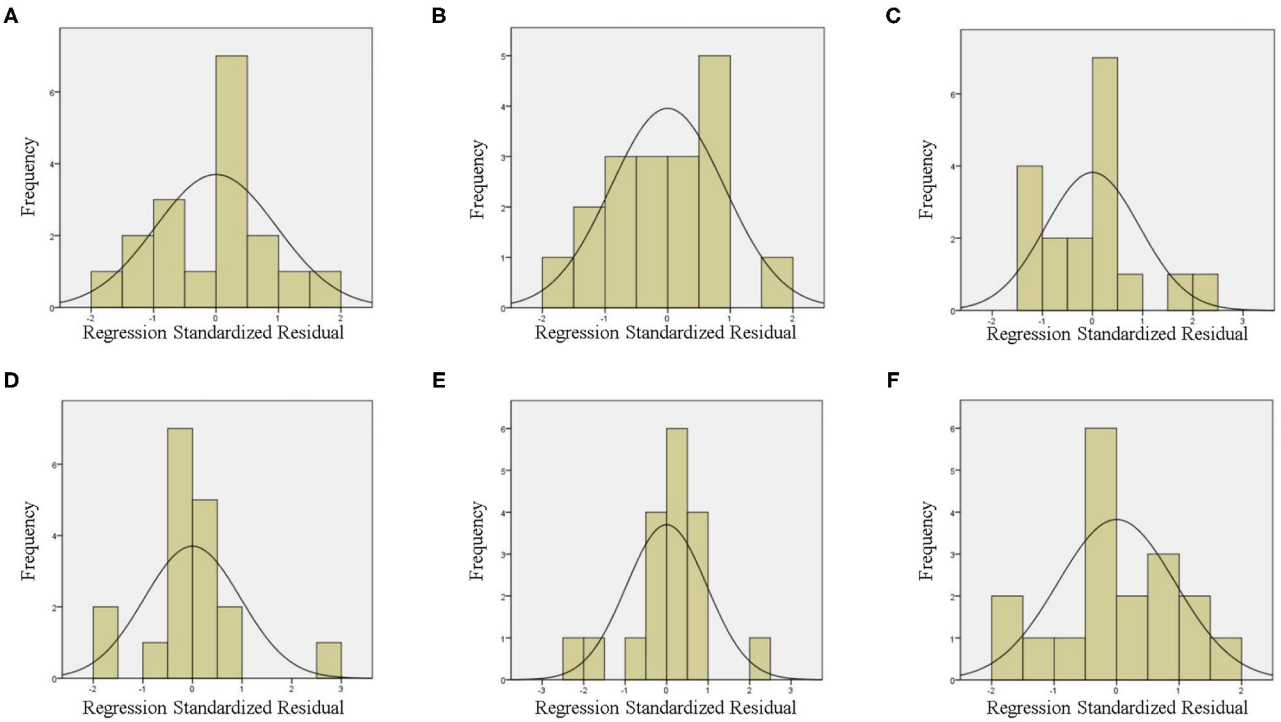

FIGURE 4 | Histogram of the regression standardized residuals of the dependent variable in Wuhan **(A)**, Xiaogan **(B)**, Huanggang **(C)**, Suizhou **(D)**, Jingzhou **(E)**, and Huangshi **(F)**.

TABLE 4 | The results of model evaluation in Hubei.

	DW	p(F)	X	p(X)	VIF	R^2	Adjusted R^2
Wuhan	1.017	<0.001	max-d4	<0.001		0.579	0.553
Xiaogan	1.846	<0.001	max-d0	<0.001	1.007	0.855	0.824
			min-d12	<0.001	1.148		
			min-d14	0.001	1.149		
Huanggang	1.636	0.001	min-d4	0.030	1.346	0.582	0.526
			min-d11	0.049	1.346		
Suizhou	1.754	0.002	max-d1	0.002		0.462	0.429
Jingzhou	0.711	0.009	min-d12	0.009		0.356	0.316
Huangshi	2.719	<0.001	max-d0	0.005	1.042	0.698	0.657
			max-d3	0.001	1.042		

DW, Durbin-Watson test; F, F test of multiple linear regression model; p(F), the significance of F test; X, independent variable; p(X), the significance of the partial regression coefficient of the corresponding independent variable; VIF, variance inflation factor; R^2, coefficient of determination.

daily cumulative confirmed cases were negatively correlated with temperature between 16.8 and 27.4°C. In addition, Chen et al. (23) pointed out that the transmissibility of COVID-19 could be lower when the local temperature rised. These results suggest that there might be a temperature range that is optimal for the transmission of COVID-19. If temperatures fall below this range, DNCC and temperature would be positively correlated, whereas if temperatures exceed this range, DNCC and temperature would be negatively correlated.

The influence of temperature on the DNCC differed slightly among the studied cities. In our linear regression model, the influence of temperature on the DNCC in Wuhan was much more significant than in other cities. We considered that the following factors were responsible for the differences in our model results for different areas: geo-social diversity and prevention and control measures implemented by the government. According to local government websites, traffic control and city blockade measures were implemented on 26 January, 30 January, 31 January, 25 January, 2 February, and 3 February of 2020 in Wuhan, Xiaogan, Huanggang, Suizhou, Jingzhou, and Huangshi, respectively. Although the times at which local governments adopted traffic control and

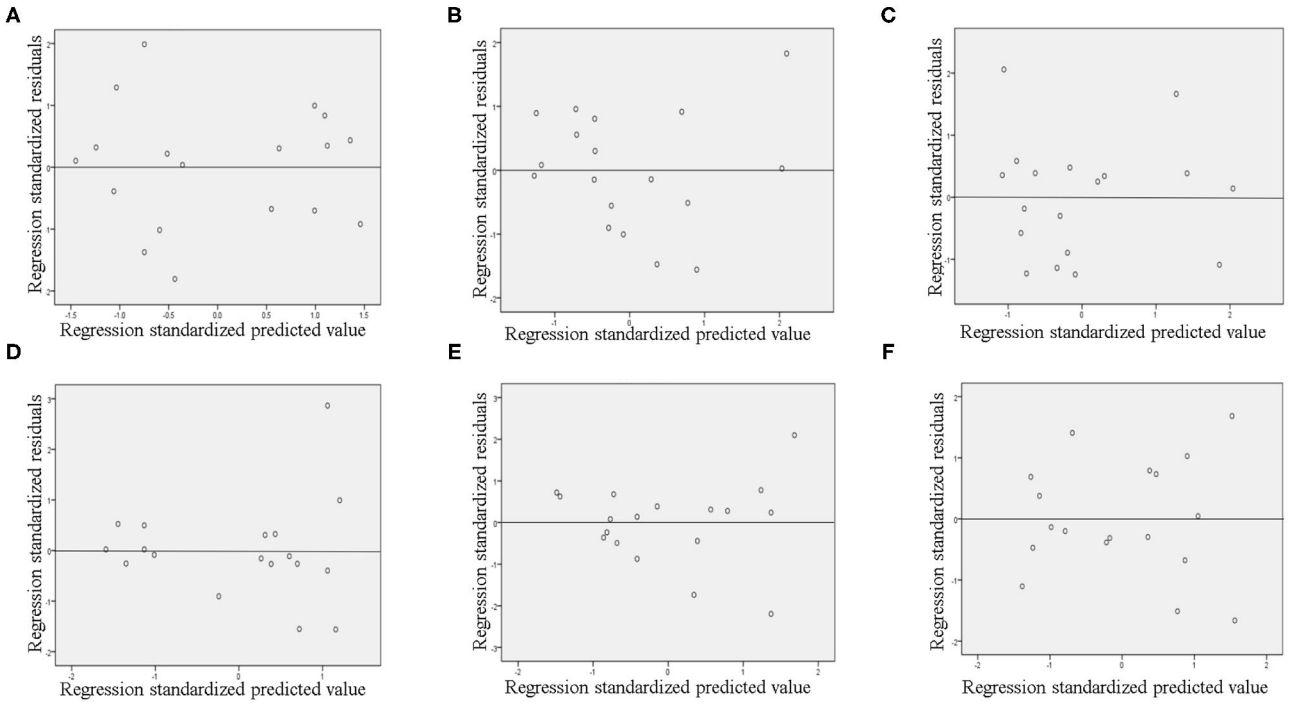

FIGURE 5 | The scatter plot of regression standardized predicted values and regression standardized residuals in Wuhan **(A)**, Xiaogan **(B)**, Huanggang **(C)**, Suizhou **(D)**, Jingzhou **(E)**, and Huangshi **(F)**.

city blockade measures were similar, their slight differences may have caused differences in the correlations between the temperature and DNCC, causing our model results to differ. Furthermore, in the early stages of the COVID-19 pandemic in China, the diagnosis of patients was limited by the availability of SARS-CoV-2 nucleic acid detection kit, thereby not meeting the scale of the medical need. Considering that patients were diagnosed using nucleic acid detection kits, the differences in the numbers of these kits allocated to different cities would have affected the DNCC, thereby affecting the results of our model. Other environmental factors, such as humidity and wind speed, may be confounding factors in this study. As our analyses were focused on the impacts of temperature on the DNCC, we did not include these variables. According to previous reports, humidity and wind speed may affect the DNCC; however, these results remain controversial. Behnood et al. (24) found that an increase in relative humidity could increase infection rates. However, Meo et al. (25) suggested that an increase in humidity reduced the DNCC in world's top ten hottest countries. Another previous study showed that higher wind speeds 14 days preceding diagnoses resulted in higher DNCC (26); however, Rendana et al. (27) claimed that lower wind speeds could increase the cases of COVID-19. Therefore, the influences of humidity and wind speed on DNCC require further exploration.

Based on the results of model fitting, the adjusted-R^2 in our study ranged from 0.316 to 0.824. Zhu et al. (15) constructed a multiple linear regression model for which adjusted-R^2 = 0.096–0.639, which is much less than the range determined here. Additionally, they did not analyse the residuals nor the collinearity of the independent variables in their model, which may have limited the effectiveness of their model.

TABLE 5 | Statistical data of the multiple linear regression equation in Shaoyang and Xinyang.

Statistical data	Shaoyang	Xinyang
Model	$Y_{DNCC} = 2.576 + 0.805X_{min-d14}$	$Y_{DNCC} = 8.639 + 3.658X_{min-d12}$
Durbin-Watson test	1.931	1.506
p(F)	0.031	0.001
X	min-d14	min-d12
p(X)	0.031	0.001
R^2	0.259	0.518
Adjusted R^2	0.213	0.488

DNCC, daily newly confirmed cases; min-d14, minimum temperature 14 days prior to the diagnosis; min-d12, minimum temperature 12 minimum temperature 14 days prior to the diagnosis; p(F), the significance of F test; X, independent variable; p(X), the significance of the partial regression coefficient of the corresponding independent variable.

This study has several limitations. First, some environmental factors that might affect the DNCC were not included, such as the wind speed and humidity. Second, socioeconomic

status, medical resources, and social policies could also affect the spread of COVID-19; hence, these confounding factors should also be included in future studies. Nevertheless, as limited information is currently available on the relationship between environmental conditions and viral transmission, based on our model results, government departments in areas where temperature ranges between −3.9 and 16.5°C and where temperatures are gradually rising should take more active measures to address the COVID-19 pandemic.

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

C-yH and L-sX designed the research study, analyzed the data, and wrote the paper. H-bZ analyzed the data and revised the manuscript. LL and HZ designed the research study and analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.604870/full#supplementary-material

Supplementary Figure 1 | The normal P-P plot of the regression standardized residuals of the dependent variable in Wuhan **(A)**, Xiaogan **(B)**, Huanggang **(C)**, Suizhou **(D)**, Jingzhou **(E)**, and Huangshi **(F)**.

Supplementary Figure 2 | The daily temperature and the DNCC in Shaoyang and Xinyang.

Supplementary Figure 3 | Histogram of the regression standardized residuals of the dependent variable in Shaoyang **(A)** and Xinyang **(D)**, the normal P-P plot of the regression standardized residuals of the dependent variable in Shaoyang **(B)** and Xinyang **(E)**, and the scatter plot of regression standardized predicted values and regression standardized residuals in Shaoyang **(C)** and Xinyang **(F)**.

Supplementary Table 1 | The normality test of the data in Hubei.

Supplementary Table 2 | The correlations between the DNCC and the daily temperature in Shaoyang and Xinyang.

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Controlling COVID-19 Pandemic: A Mass Screening Experience in Saudi Arabia

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A highly accelerating number of people around the world have been infected with novel Coronavirus disease 2019 (COVID-19). Mass screening programs were suggested by the World Health Organization (WHO) as an effective precautionary measure to contain the spread of the virus. On 16 April 2020, a COVID-19 mass screening program was initiated in Saudi Arabia in multiple phases. This study aims to analyze the number of detected COVID-19 cases, their demographic data, and regions most affected in the initial two phases of these mass screening programs. A retrospective cross-sectional study was conducted among the high-risk population as part of the COVID-19 mass screening program across all regions in Saudi Arabia during April and May 2020. A Chi-square-test was used to determine the associations between positive cases and various demographic variables. Out of 71,854 screened individuals, 13.50% ($n = 9701$) were COVID-19 positive, of which 83.27% ($n = 59,835$) were males. Among positive cases, in the 30–39 years age group, 6.36% were in the active phase, and 2.19% were in the community phase. Based on our experience, launching mass screening programs is crucial for early case detection, isolation, and pattern recognition for immediate public interventions.

Keywords: COVID-19, screening, mass testing, pandemic, Saudi Arabia

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INTRODUCTION

The emergence of the novel Coronavirus disease 2019 (COVID-19), which was first detected in December 2019 in Wuhan, China, led to the infection of an accelerating number of individuals, causing a local epidemic (1). Shortly after, COVID-19 was a global threat and declared a pandemic by the World Health Organization (WHO) on 11 March 2020 (2). Moreover, the virus can also spread through asymptomatic patients, thus increasing the number of infected patients with an

estimated basic reproduction number (R0) that ranges from 2.0 to 3.5 (3, 4). Common symptoms displayed by infected patients include fever, cough, and sore throat. COVID-19 can also lead to serious complications, such as acute respiratory distress syndrome (ARDS), mostly seen in patients with associated comorbidities (5, 6). Although the diagnosis of COVID-19 depends on the epidemiological linkage and clinical presentation, the most reliable method for virus detection is by analyzing respiratory discharges through real-time quantitative polymerase chain reaction (RT-qPCR) (7). Other analyses can be used, including nucleic acid detection, computerized tomography (CT) scan, immune identification of IgM/IgG, enzyme-linked immunosorbent assay (ELISA), blood culture, and a reverse transcriptase PCR (RT-PCR) point of care test, however, with less reliability (8).

Infection with COVID-19 spreads rapidly, with an exponentially growing number infected daily. This has prompted governments to introduce radical measures to control the spread of the virus. Different efforts have been made by various countries to mass test their citizens, to detect new cases, and to evaluate potential solutions (9). The WHO has referred to a fair number of extensive test results, which is between 3–12% of the total number of positive cases (10). The number of people screened in each country has varied depending on several factors, including demographic characteristics, resource availability, and the precautionary measures adopted (11).

The first detected case of COVID-19 in Saudi Arabia was reported by the Ministry of Health (MoH) on 2 March 2020. The patient was immediately quarantined, as well as all his traced contacts (12, 13). By the 12th of September 2020, a total of 325,050 positive COVI-19 cases were detected, 301,836 recovered, and 4,240 cases died (14). This had led to a very low case fatality rate of 1.3% in the country compared with the international case fatality rate of 3.2% (15). Saudi Arabia responded to the pandemic rapidly and imposed several measures to reduce the spread of the infection, including enforced partial curfew hours in multiple cities, as well as suspending events, schools, social gatherings, Umrah, mosques prayers, and business. At one point, a general lockdown was enforced (16–18). In addition to these measures, a national campaign of mass screening was initiated. In the first phase of the campaign, both symptomatic and asymptomatic suspected COVID-19 cases were screened with their close contacts (19). This first phase is also known as the active screening phase involved field teams from MoH targeting intensely populated neighborhoods and labor residential buildings in several cities. Although increasing the number of positive cases detected, this phase helped to contain and locate local outbreaks areas (20). Accordingly, escalated measures were enforced to limit the spread of COVID-19 from these heavily infected areas; an intense lockdown was imposed soon after.

Due to the risk of transmitting the disease from asymptomatic individuals, a second mass screening campaign was initiated. This second phase also known as the community screening phase; targeted low-to-intermediate-risk groups based on their epidemiological risk profile. Risk groups were determined with the aid of the electronic application "Mawid" screening tool (21). Professional health care workers (HCWs) then collected the samples of the targeted population through scheduled appointments in primary care centers. Given the success of these first two phases, the third phase of the mass testing campaign involved screening asymptomatic individuals after applying for electronic appointments through specialized drive through (Takkad) centers, serving more than 2 million beneficiaries from its launch by the end of May 2020 until August 2020 and is still ongoing as planned to continue until the pandemic is eradicated (22).

In this study, our goal was to determine the effectiveness of mass screening programs in Saudi Arabia in the two initial phases by analyzing the number of detected COVID-19 cases, their demographic data, and most regions affected.

MATERIALS AND METHODS

The present retrospective cross-sectional study was conducted among COVID-19 screened individuals across all regions in Saudi Arabia. Data from the first two phases of the mass screening program, between 16 April 2020 and 19 May 2020 were included. All screened individuals were included, and there were no exclusion criteria.

Phase one was defined as an active screening phase performed by the MoH HCWs to screen random individuals in dense districts, between 16 April 2020 and 3 May 2020. Phase two was defined as a community screening phase performed in primary care centers between 4 May 2020 and 19 May 2020. Phase two selected cases based on their epidemiological risk profile through filling self-assessment electronic forms available in the Central Appointment System (Mawid) which is an electronic service originally provided by the MoH to enable patients to book, cancel, or reschedule their appointments at designated primary health care centers. These forms were based on a scoring system with questions about recent travel, contact with confirmed COVID-19 cases, and the presence or absence of specific COVID-19 symptoms. The targeted population in this phase were those with a score of 0-2 (low risk) and 3-4 (intermediate risk) as per MoH screening guidelines (**Figure 1**) (21, 23).

The confirmatory detecting test in the mass screening was performed by RT-qPCR from Nasopharyngeal swabs following the WHO standardized protocol (24).

The Health Electronic Surveillance Network (HESN) database contains clinical, demographic data, and regions of all screened individuals entered by the HCWs. Positive COVID-19 results, demographic data, and regions of all screened individuals were retrieved from HESN and independently entered by two data collectors into electronic sheets, in which any discrepancies were reviewed and resolved by an assigned investigator. All steps were taken to safeguard data confidentiality and privacy. Ethical approval was obtained from the Institutional Review Board of the Central Committee of the Ministry of Health, KSA, with approval Letter Number 20-115M.

Statistical Analysis

The data were imported into the most recent version of R, 3.6.3, on the RStudio (1.2.5033) (25). Data were analyzed using the Chi-square goodness-of-fit test to determine the impact of

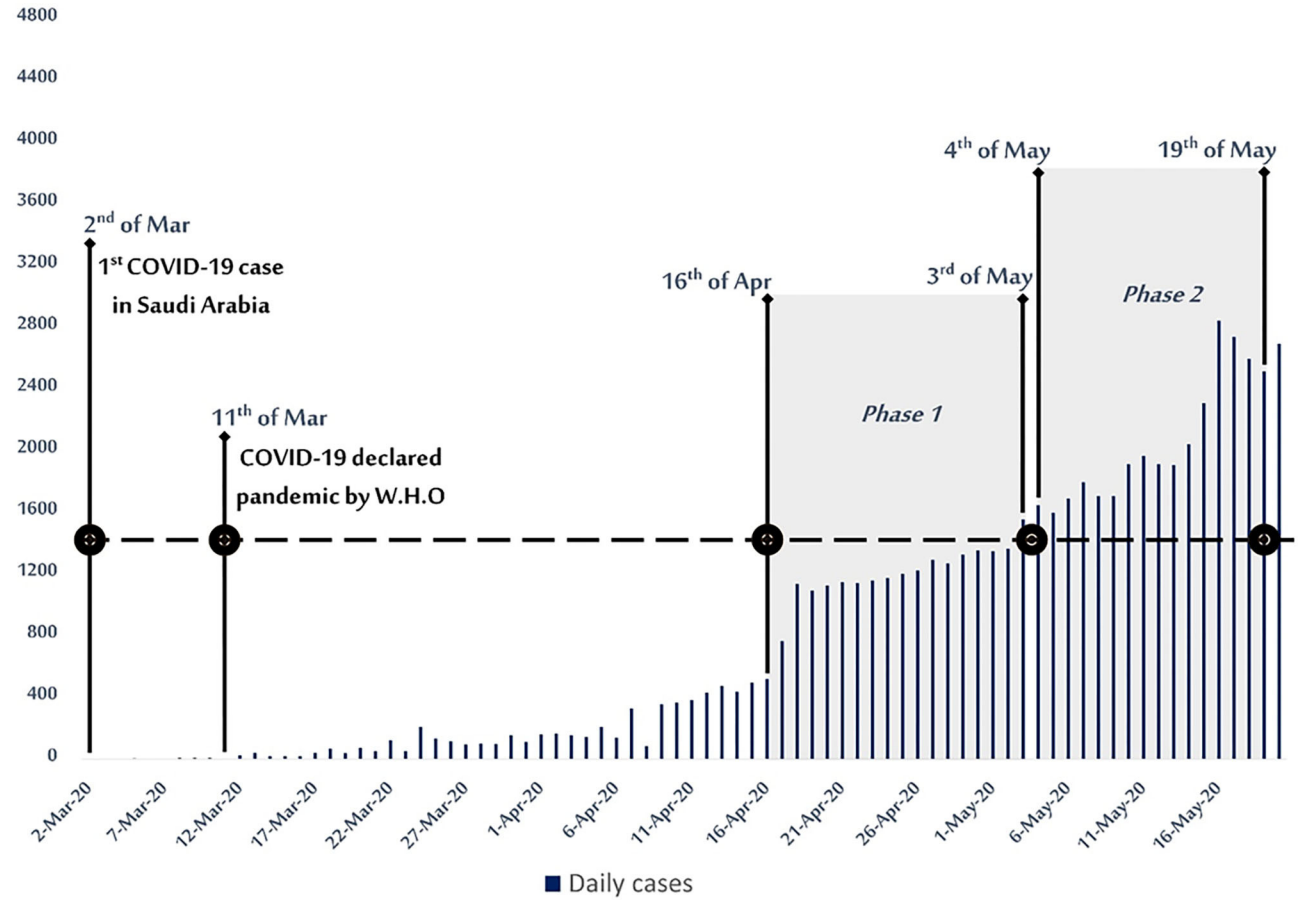

FIGURE 1 | COVID-19 epidemiological curve with active and community mass screening phases between 16 April- 19 May 2020 in Saudi Arabia.

categorical variables, like Saudis/Non-Saudis, gender, age group, and region on test positivity. Follow-up, pairwise comparisons, and Chi-square testing with Bonferroni correction were made to identify which pairs are different from one another concerning COVID-19 test positivity. In the present study, p-value < 0.05 was considered significant.

RESULTS

Phase 1 (Active Phase)

In the active phase of mass screening, a total of (42,765) individuals were screened. Positive cases accounted for 18.18% (n = 7,776), where 84.13% (n = 35,979) of those tested were males (**Table 1**). Approximately 34.95% (n = 15,822) of active phase screened cases were from the aged 30–39 years, representing the highest proportion of positive cases (6.36%, n = 2,717), followed by the 40–49 year old and 25–29 year old age groups with 3.61% (n = 1,543) and 2.96% (n = 1,267), respectively (**Figure 2**).

The regions with the most positive cases among the active phase screened were the Eastern region, Al-Madinah, and Riyadh, with 7.81% (n = 3,339), 3.48% (n = 1,490), and 1.85% (n = 791), respectively (**Figure 3**).

Our Chi-square test shows a significant association between the number of positive COVID-19 tests and Saudis/non-Saudis (p = 0.0). It also shows a significant correlation between males and females with test positivity ($p < 0.05$). Moreover, different age groups showed significant correlation with positive COVID-19 tests (p = 0) (**Table 1**). For regions and age groups with the number of positive COVID-19 cases, the Chi-square shows a significant association between regions, age groups, and the number of positive COVID-19 test cases (p = 0.0002) (**Figures 2**, **3**).

Phase 2 (Community Phase)

In the community phase screening, a total of (29,089) individuals were screened, of which positive cases accounted for 6.62% (n = 1,925) (**Table 1**). Males accounted for 82.01% (n = 23,856) of those screened, of which 78.13% (n = 1,504) were positive (**Table 1**). The most positive cases among the community phase screened individuals were in the 30–39 years old group (2.19%, n = 638), followed by the 40–49 years old and 25–29 years old groups: 1.17%, (n = 339) and 1.1% (n = 319), respectively (**Figure 2**).

TABLE 1 | Demographic variables for COVID-19 positive cases during active and community phases for mass screening.

Phase 1		**Screened cases** ***N* = 42,765**	**Positive cases** ***N* = 7,776 (18.18%)**	***P*-value**
	Nationality			=0
	Saudi (%)	11,724 (27.41%)	1,847 (23.75%)	
	Non-Saudi (%)	31,041(72.59%)	5,929 (76.25%)	
	Gender			=0.0002
	Male (%)	35,979(84.13%)	6,649 (85.51%)	
	Female (%)	6,786 (15.87%)	1,127 (14.49%)	
	Age (years)			=0
	<1	230 (0. 54%)	47 (0.6%)	
	01–14	2,345 (5.48%)	603 (7.75%)	
	14–19	942 (2.2%)	226 (2.91%)	
	20–24	3,541 (8.28%)	509 (6.55%)	
	25–29	7,904 (18.48%)	1,267 (16.29%)	
	30–39	15,822 (36.95%)	2,717 (34.94%)	
	40–49	8,051 (18.83%)	1,543 (19.84%)	
	50–59	3,108 (7.27%)	675 (8.68%)	
	60–69	635 (1.48%)	155 (1.99%)	
	70+	172 (0.4%)	33 (0.42%)	
Phase 2		**Screened Cases** ***N* = 29, 089**	**Positive Cases** ***N* = 1,925 (6.62%)**	
	Nationality			=0
	Saudi (%)	20,368 (70.02%)	1,018 (52,88%)	
	Non-Saudi (%)	8,721 (29.98%)	907 (47.12%)	
	Gender			=0
	Male (%)	23,856 (82.01%)	1,504 (78.13%)	
	Female (%)	5,233 (17.99%)	421 (21.87%)	
	Age (years)			=0
	<1	99 (0.34%)	9 (0.47%)	
	01–14	1,305 (4.49%)	150 (7.79%)	
	14–19	1,023 (3.52%)	63 (3.27%)	
	20–24	2,954 (10.16%)	176 (9.14%)	
	25–29	5,213 (17.92%)	319 (16.57%)	
	30–39	11,178 (38.43%)	638 (33.14%)	
	40–49	4,807 (16.53%)	339 (17.61%)	
	50–59	1,803 (6.20%)	174 (9.04%)	
	60–69	524 (1.8%)	43 (2.23%)	
	70+	177 (0.61%)	13 (0.68%)	

The regions with the most positive cases were Jeddah, Tabouk, and the Eastern region, with 1.61% ($n = 496$), 1.31% ($n = 381$), and 1.21% ($n = 352$), respectively (**Figure 3**). The Chi-square-test shows a significant association between the number of COVID-19 test-positive cases and Saudi/non-Saudi, gender, age groups, and regions with a p-value = 0 (**Table 1** and **Figures 2**, **3**).

DISCUSSION

The COVID-19 pandemic condition continues to devastate most countries around the world. The WHO warned that the pandemic is far from over, and it recognizes the risk of COVID-19 spreading between and within countries and regions. Thus, strategies for detecting and responding to COVID-19 and resource allocation will vary according to national risk assessments. It is recommended by WHO that all suspected cases are tested according to the organization's case definition (24).

Mass screening strategies were adopted by several countries around the world to contain the spread of the virus and to ease lockdown measures. Some of these countries performed massive mass testing programs in which they screened a large number of people daily. Others were moderate in doing mass testing, while others manage to do very few or did not pursue mass testing at all. A model to control the infection was submitted by a group of scientists in the UK, which involved mass screening for the whole population (26). However, this model is difficult to apply due to the massive labor and costs entailed. According to the Economic Cooperation and Development (OECD) report on 30 April 2020, Iceland is considered one of the countries that managed to test a high proportion of its population: one in eight of its population has been tested (9). Like Saudi Arabia, Iceland performed its mass screening in phases; the first mass screening program was followed by another testing campaign to reach about 12% of the population, with a success rate of 93% (9). A high percentage was also achieved in Saudi Arabia; with 45,000 tests been performed daily between May and June 2020, which was then accelerated to 70,000 daily tests in July 2020 (27, 28).

Countries such as Luxembourg and Estonia have achieved a higher rate of mass screening as well. However, these countries are considered sparsely populated nations, which might have helped them to increase the capacity of their mass screening programs (29). Mass screening in highly populated countries can be challenging, even in countries with robust health care systems such as Japan and the UK. However, other highly populated countries have managed to act with great professionalism and efficiency to screen more, such as South Korea and Singapore. South Korea initiated a mass screening program at the end of February 2020, with about 20,000 tested daily. These tests were conducted via mobile examination, drive-through testing, and walk-through testing (30). Singapore managed to test confirmed cases more than once and imposed a whole-nation mass testing campaign, like Saudi Arabia (31). New York state was massively infected with COVID-19, where it adopted precautionary measures that included mass screening tests. A large number were tested daily, which unexpectedly resulted in increasing anxiety among the public. Many were flooding into hospitals and testing centers and queued to be screened, which accelerated the spread of infection (32). Also, the north part of Italy, the crowded tourist region, was heavily infected with the virus. In the early days of the epidemic, the government planned to screen only symptomatic patients. Later on, when the infection had spread to the rest of the country, and the north had contained the spread of COVID-19, they applied population-wide testing for both symptomatic and asymptomatic persons (33, 34). On the other hand, Saudi Arabia has adopted a different approach, as the targeted population in the initial phase of mass screening included both symptomatic and asymptomatic cases which helped extensively to allocate heavily infected areas, thus

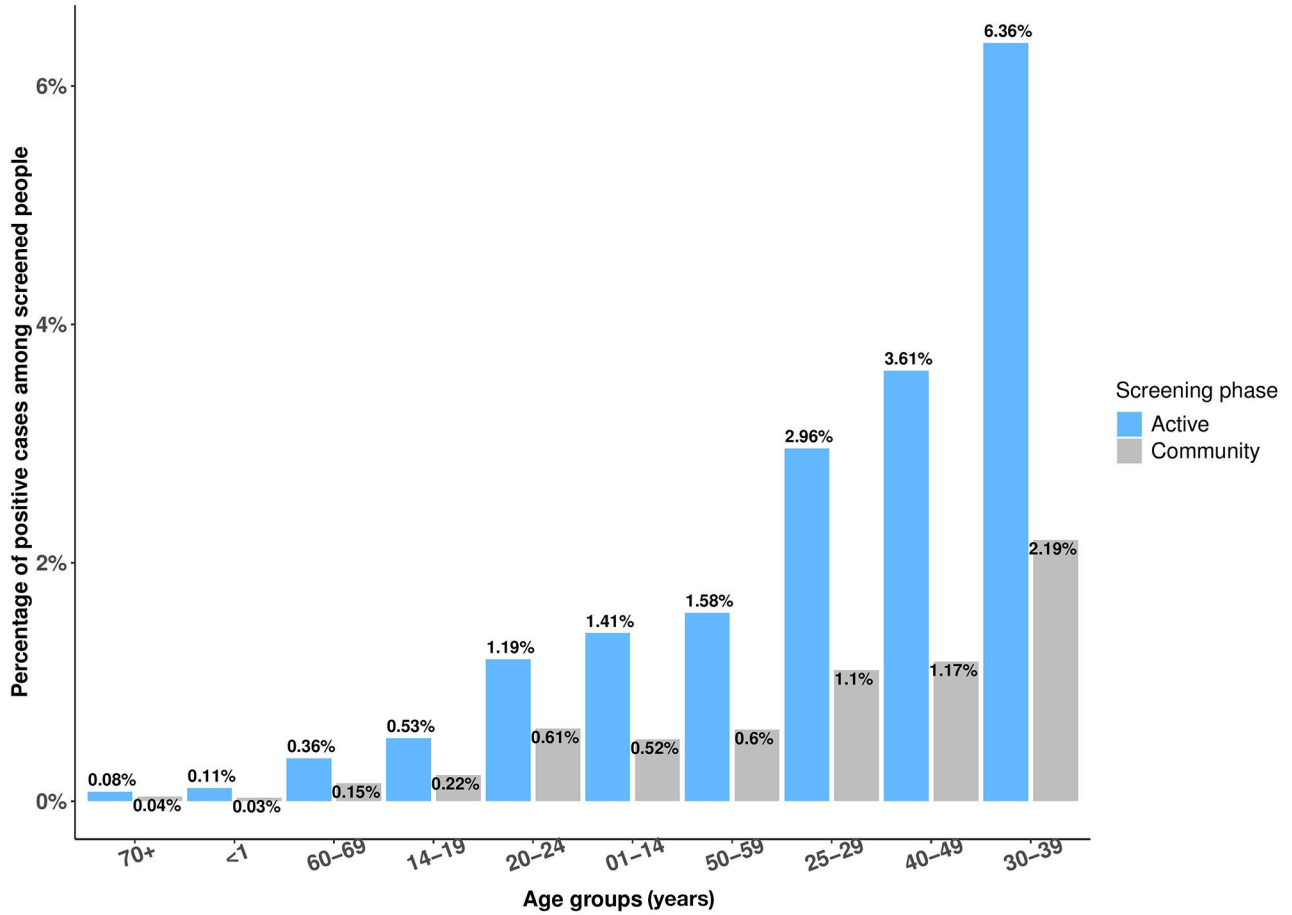

FIGURE 2 | The percentage of positive COVID-19 cases among all screened individuals in Saudi Arabia between 16 April−19 May 2020 in active and community phases in terms of age groups.

appropriate measures were applied accordingly with to reduce the infection rates.

Saudi Arabia has carried out a series of ongoing precautionary measures to control the spread of COVID-19 infection and to provide early detection strategies of the disease. When the mass screening program was initiated, the number of infected cases had already exceeded 11,000 cases. The number of cases accelerated after the start of the mass screening program, indicating that positive cases were better detected and allocated (35). Analysis of the mass screening data in the screening phases showed that positive cases in males were significantly exceeding the number of positive cases in females. Similarly, males were more infected than females in Italy (82%), the USA (61.3%), and China (54.3%) (36–38). This can be explained by several factors including that more men are involved in the workforce than women and that men are more susceptible to be infected with the virus than women (39, 40).

The non-Saudi cases were higher in the active phase compared with the community phase. This is likely because most of the screened people in the active phase were from densely populated districts and worker housing (20). However, the community phase was targeting individuals based on their low to intermediate epidemiological risk profile after filling electronic screening tools. Thus, as expected, most of the screened were Saudi (60.7%) (21, 41).

In terms of age, our data showed that the 30–39 years old group was the most screened in both phases, which has also been observed in other countries, such as the USA and China (42–44). It was expected that this age group would be the most infected in both phases, considering that workers and most of the Saudi population are within this age group.

Regarding the overall coverage of the mass screening program across regions in Saudi Arabia, most were screened in both phases. Regions such as Eastern and Riyadh had a larger number of screened individuals compared to Makkah and Al-Madinah, despite that mass screening centers were equally distributed in most Saudi regions. These variabilities can be attributed to several factors, including peoples' lack of awareness, being afraid of the test, as well as being worried about positive test results (45, 46). Thus, it is essential to improve risk communication and community engagement regarding COVID-19 pandemic (47). The 1st reported COVID-19 case entered through the Saudis

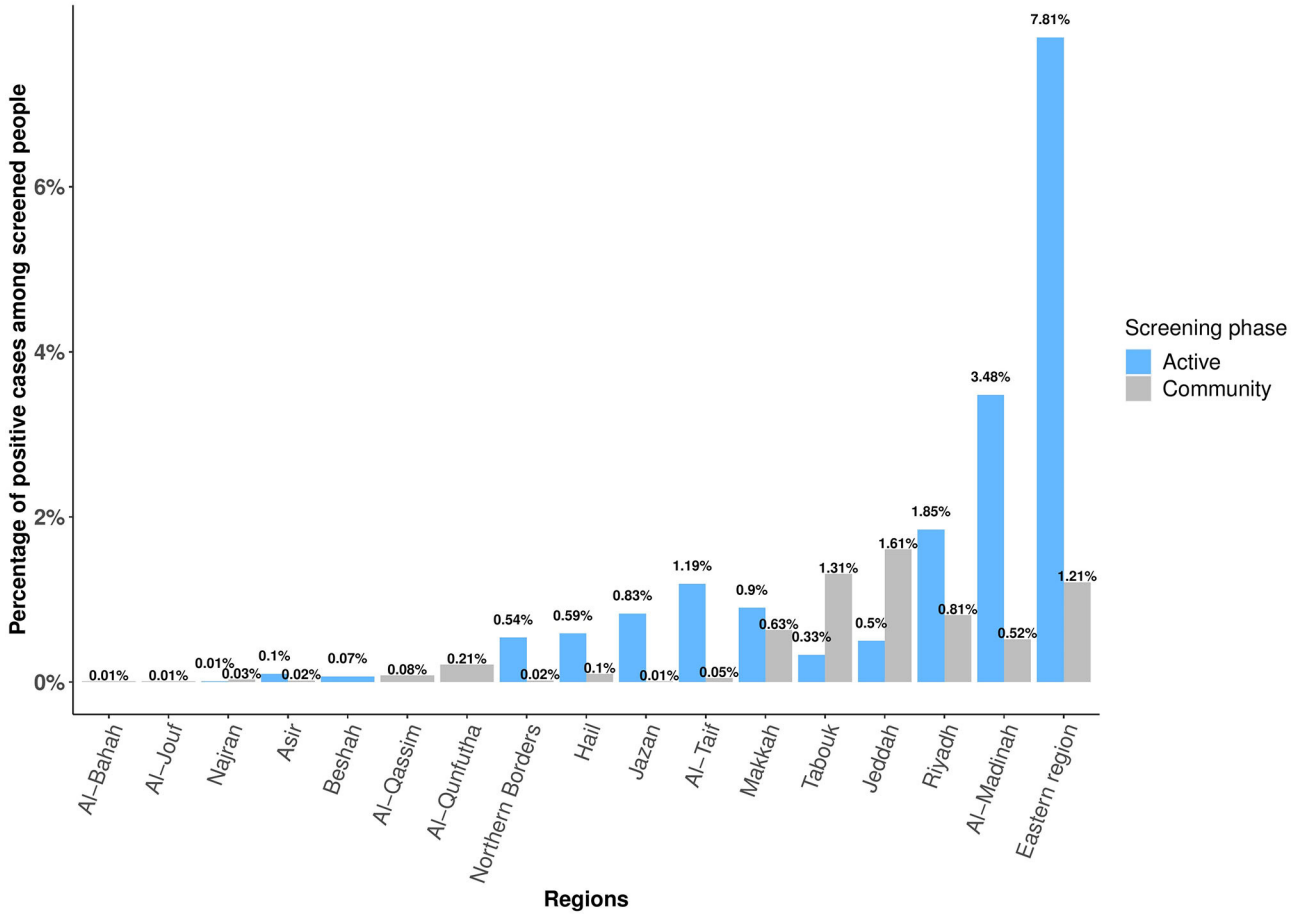

FIGURE 3 | The percentage of positive COVID-19 cases in different regions in Saudi Arabia between 16 April and 19 May 2020 among active and community phases of mass screenings.

Eastern region port of entry leading to the lockdown of a number of its Governorate in the early phases of the pandemic (5, 48). This would explain the high positivity rate of cases in the Eastern region in the initial active phase, which was eventually reduced in the community phase because of effective curfew measures. Additionally, a high number of positive cases in phase one in both Al-Madinah and Riyadh regions was observed, which could be attributed to the high number of dense districts easing the spread of the virus. This led to enforce lockdown on these dense areas in the early phases of the pandemic to reduce the number of positive cases in Al- Madinah and Riyadh (49, 50). Despite more people were being aware about screening services at designated primary care centers in phase two, nevertheless Jeddah and Tabouk regions were the highest in the number of positive cases, which can be explained by unstable epidemiological situation in both regions and lower level of awareness about precautionary measures. This increase in the number of cases led to impose further curfew measures to curb the spread of the disease (14, 49, 51).

Based on the COVID-19 mass screening experience in Saudi Arabia, 13.50% of all screened individuals (71,848) in the initial two phases were positive. This percentage nearly falls in the recommended percentage of an adequate number of tests as suggested by the WHO; 3%-12% of positive cases of the total screened people. Despite the number of tests in these two phases can be considered small, still, these phases helped to locate heavily infected areas and introduced appropriate measures to control the spread of the infection. Due to the finding that most of the positive cases were from densely populated areas and within the 30–39 years old group, it is crucial to focus on increasing the level of community awareness, especially among those targeted populations (47, 52).

To our knowledge, this is one of the initial studies to address mass screening in Saudi Arabia. However, this study has some limitations. First, some variables were missing in the electronic database (HESN), such as clinical characteristics, patients' disposition, and disease outcomes. Further analysis of these variables could have been achieved to describe detailed demographic data of cases screened, map the disease severity, and guide targeted areas for mass screening. Second, the community screening phase included those with a pre-defined epidemiological risk profile, excluding

many other cases that could have an added value to our results. Third, the mass screening program will continue, with additional phases, but this study addressed only the first two phases limiting the generalizability of the results in this study.

CONCLUSION

Launching Saudi Arabia's mass screening program during the early phases of the pandemic was a helpful epidemiological surveillance tool, which was based on accumulative experiences with previous outbreaks such as MERS CoV. Phase 1 showed high COVID-19 positive cases in densely populated areas, males, and age groups between 30–39 years. Effective awareness campaigns for these groups are critical to contain the infection. A high number of COVID-19 cases in phase 2, in Jeddah, Tabouk, and the Eastern region, was expected due to their unstable epidemiological situation at that specific time interval. Following screening phases of the mass screening program should address gaps from earlier phases which include screening more individuals, using rapid screening techniques, and providing more reliable results in less time to limit the spread of COVID-19.

DATA AVAILABILITY STATEMENT

The original contributions generated in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The central IRB-MoH, No: 20-115M, GCMGM-MoH. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AK, HA, RMA, and YA: worked on concepts. HA, YA, HS, and AK: design. HA, RHA, HS, and YA: literature search, writing, and manuscript preparation. SMA, SSA, AA, and HJ: data acquisition. RMA and HA: statistical analysis. HA, AK, FA, and RA: manuscript editing. AK, HJ, AA, and SMA: manuscript review. AK: guarantor. All authors: contributed to the article and approved the submitted version.

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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 MM declared a shared affiliation with several of the authors YA, SSA, AA, and HJ to the handling editor at time of review.

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Prognostic Value of a Clinical Biochemistry-Based Nomogram for Coronavirus Disease 2019

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Background: This study aimed to explore the predictive value of a clinical biochemistry-based nomogram in COVID-19.

Methods: The plasma or serum concentrations/levels of carcinoembryonic antigen (CEA) and other biomarkers, e.g., C-reactive protein (CRP), white blood cell (WBC), interleukin-6 (IL-6), ferritin (Fer), procalcitonin (PCT), lymphocyte percentage (L%), D-dimer (D2), and neutrophils percentage (Neu%), were assessed in 314 hospitalized patients with confirmed COVID-19. The area under the curve was used to estimate the diagnostic and prognostic value for COVID-19. Cox and logistic regression analyses were used to estimate the independent prognostic risk factors for the survival of patients with COVID-19.

Results: Receiver operating characteristic (ROC) curves were used to determine the area under the curve (AUC) values for CEA, IL-6, CRP, PCT, Fer, D-dimer levels and L%, Neu%, and WBC to assess disease classification. The critical values for these markers to predict severe disease type were then determined. The hazard ratio of prognosis for risk of COVID-19 identified CEA, WBC, CRP, PCT, Fer, D-dimer, Neu%, and L% as independent prognostic factors. For the nomogram of overall survival (OS), the C-index was 0.84, demonstrating a good discriminative performance.

Conclusions: An OS nomogram for the clinical diagnosis and treatment of COVID-19 was constructed using biomarkers. These data will be useful for the diagnosis, management, and therapy of COVID-19.

Keywords: Coronavirus disease 2019, inflammatory markers, carcinoembryonic antigen, hazard ratio, prognosis

HIGHLIGHTS

- We constructed an OS nomogram to diagnose and treat COVID-19, with a good C-index.
- CEA, WBC, CRP, PCT, Fer, D-dimer, Neu%, and L% were independent prognostic factors.
- The prognostic risk score identified high risk populations for OS.
- According to the hazard ratio for prognosis, we identified high risk factors for patient OS.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) has become a worldwide threat to human health. It is caused by infection with a virus known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1). Intensive efforts are being made to prevent and treat this disease. According to the seventh edition of the diagnostic and treatment guidelines for the novel coronavirus, the diagnosis of this disease has been linked to epidemiological history, typical chest computed tomography imaging features of COVID-19, and other etiological investigations (2). The levels of certain inflammatory biomarkers, such as C-reactive protein (CRP), lymphocyte (L) percentage, neutrophils percentage (Neu%), interleukin-6 (IL-6), procalcitonin (PCT), ferritin (Fer), D-dimer (D2), and the white blood cell (WBC) count, have been used to assess disease progression (3–5). Our previous study noted that the carcinoembryonic antigen (CEA) level is an independent prognostic marker for COVID-19 (6). In the present study, we aimed to explore the value of all the above markers to diagnose and predict the prognosis of COVID-19. In addition, we aimed to use these factors to construct and validate a nomogram to predict the overall survival (OS) of patients with COVID-19.

METHODS AND MATERIALS

Patient Cohort

From January 24 to April 26, 2020, 314 patients infected with SARS-CoV-2 at Wuhan Jinyintan Hospital agreed to be included in this study. COVID-19 was confirmed in these patients based on characteristic manifestations on chest computed tomography (CT), etiological evidence, and epidemiological history (not including the presence of tumors). According to the seventh edition of the diagnosis and treatment plan for COVID-19 in China, the clinical conditions of patients with COVID-19 may be classified into four types: mildly affected, moderately affected, severely affected, and critically severely affected (2, 7). At the time of admission, the classification of the 314 patients was as follows: 83 cases had moderate symptoms with fever, CT manifestations, and respiratory distress; 155 cases showed severe symptoms; and 76 cases were critically severely affected, with acute respiratory distress syndrome. Throat swabs were collected from enrolled patients to detect SARS-CoV-2 RNA using real-time PCR with a Nucleic Acid Extraction Kit (8) (Zhijiang Orient Gene Biotechnology Company, Shanghai, Chins) and a 2019-nCoV ORFlab and N genes target detection kit (Zhijiang Orient Gene Biotechnology). The ethics committee of Jinyintan Hospital approved the study (Ethical approval number: KY-2020-69.01). The study was carried out in accordance with the current revision of the Declaration of Helsinki.

Detection of CEA and Inflammatory Biomarkers

The serum levels of CEA and Fer were detected using a chemiluminescence immunoassay (Abbott Laboratories, Chicago, IL, USA) and their associated reagents, while the levels of CRP were detected using a biochemical analyzer (Abbott Laboratories). Blood counts were performed using a

TABLE 1 | The clinical characteristics of 314 patients with COVID-19.

Group	*N*	IL-6 (pg/ml)	WBC ($\times 10^9$/L)	L%	N%	CRP (mg/L)	PCT (ng/ml)	D2 (μg/ml)	CEA (ng/ml)	Fer (ng/ml)
Sex										
Male	181	19.78 ± 2.95	9.86 ± 0.48	10.99 ± 0.71	82.19 ± 1.17	78.56 ± 5.33	0.99 ± 0.25	12.84 ± 2.36	13.63 ± 1.05	1192 ± 59.91
Female	133	12.49 ± 1.41	10.38 ± 0.57	13.19 ± 0.96	80.87 ± 1.22	68.49 ± 6.60	0.60 ± 0.25	11.35 ± 2.47	15.04 ± 1.16	742.7 ± 65.52
P-value		0.054	0.483	0.061	0.438	0.231	0.283	0.685	0.368	<0.001
Age										
≥65	166	18.64 ± 2.82	10.54 ± 0.51	10.55 ± 0.70	84.15 ± 0.95	79.06 ± 5.62	0.88 ± 0.25	15.71 ± 2.65	16.02 ± 1.20	1062 ± 62.59
< 65	148	14.3 ± 2.07	9.52 ± 0.53	13.72 ± 0.95	78.31 ± 1.43	68.18 ± 6.19	0.74 ± 0.27	7.46 ± 1.75	12.12 ± 0.89	931.5 ± 70.43
P-value		0.251	0.171	0.007	0.001	0.194	0.698	0.020	0.012	0.166
The admission classification										
Moderate	83	16.81 ± 3.49	7.78 ± 0.49	17.5 ± 1.14	75.94 ± 1.35	48.34 ± 6.64	0.16 ± 0.06	2.99 ± 1.29	12.11 ± 1.21	704.8 ± 75.05
Severe	155	16.33 ± 2.55	10.2 ± 0.50	11.13 ± 0.75	82.18 ± 1.24	75.21 ± 5.55	0.83 ± 0.23	9.16 ± 1.71	14.78 ± 1.13	1088 ± 60.89
Critical severe	76	18.43 ± 4.47	12.91 ± 0.93	7.01 ± 0.89	88.27 ± 1.33	111.7 ± 9.84	1.97 ± 0.76	10.97 ± 2.65	16.01 ± 1.92	1342 ± 109.1
P-value		0.913*, 0.775$^{\&}$, 0.685$^{\#}$	0.002*, <0.001$^{\&}$, 0.008$^{\#}$	<0.001*, <0.001$^{\&}$, 0.003$^{\#}$	0.002*, <0.001$^{\&}$, <0.001$^{\#}$,	0.003*, <0.001$^{\&}$, 0.001$^{\#}$	0.037*, 0.002$^{\&}$, 0.056$^{\#}$	0.017*, 0.004$^{\&}$, 0.585$^{\#}$	0.127*, 0.073$^{\&}$, 0.569$^{\#}$	<0.001*, <0.001$^{\&}$, 0.041$^{\#}$

*The * symbol represents the comparison of the moderately affected patients vs. the severely affected patients; the & symbol represents the comparison of the moderately affected patients vs. critically severely affected patients; the # symbol represents the comparison of the severely affected patients vs. the critically severely affected patient.*

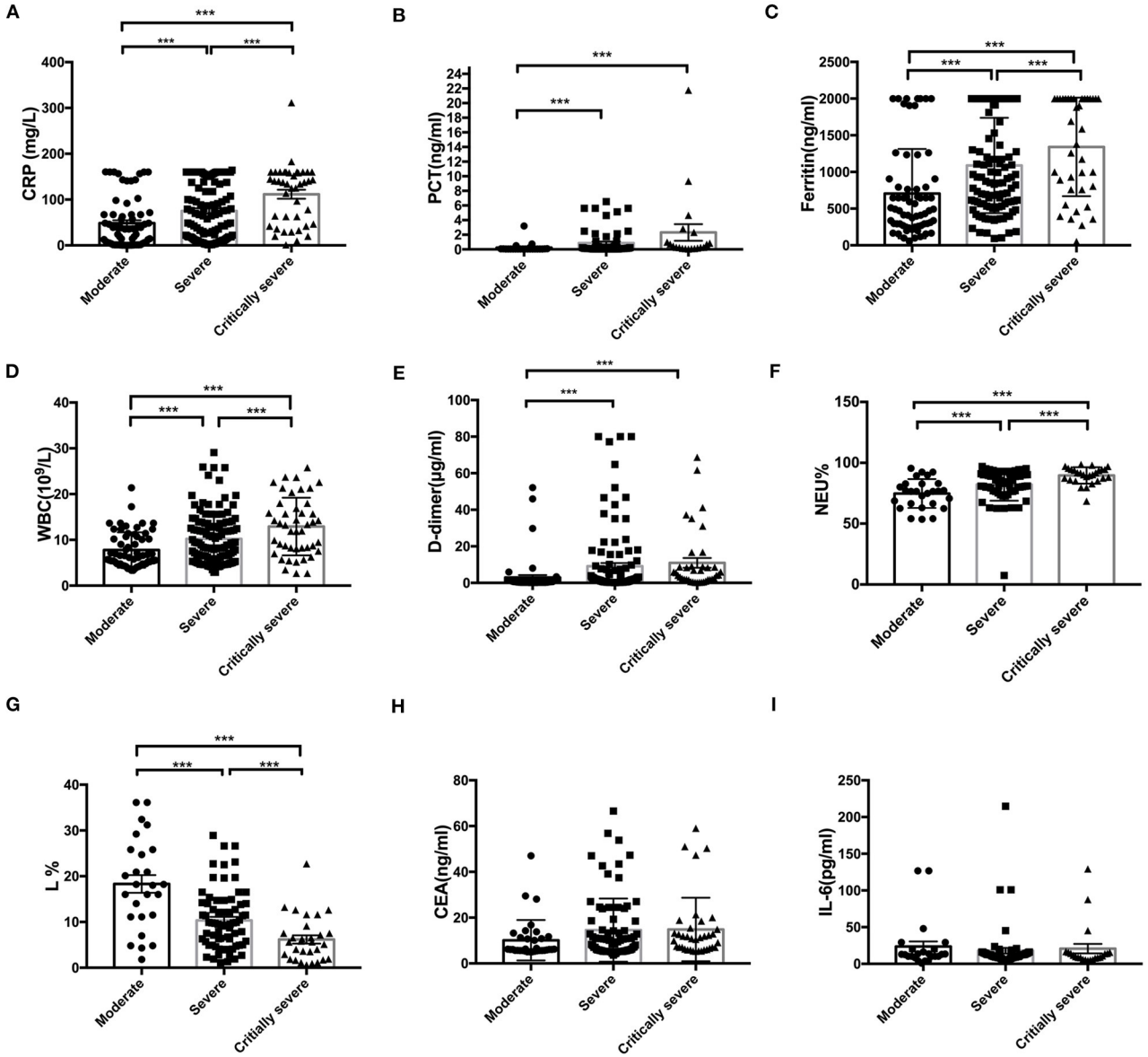

FIGURE 1 | Correlation between the initial levels of CRP, PCT, Fer, WBC, D2, Neu%, L%, CEA, and IL-6 and clinical classification. **(A–F)** The levels of CRP, PCT, Fer, D2, WBC counts, and Neu percentage were significantly higher in the critically severe patients ($n = 76$) and severe patients ($n = 155$) than in the moderate patients ($n = 83$) ($P < 0.05$). **(G)** The L percentage was significantly lower in severely and critically severely affected patients than in moderately affected patients ($P < 0.001$). **(H,I)** No significant differences in the levels of CEA and IL-6 between the critically severe or severe patients and moderate patients were observed from the time of admission. CRP, C-reactive protein; PCT, procalcitonin; Fer, ferritin; WBC, white blood cell; D2, D-dimer; Neu%, neutrophils percentage; L%, lymphocyte percentage; CEA, Carcinoembryonic antigen; IL-6, interleukin 6. ***$P < 0.05$.

Mindray BC-6900 blood hematology analyzer (Mindray medical international limited, Shenzhen, China) and its associated reagents. The levels of IL-6 were detected using a Roche automatic electrochemiluminescence immunoassay and its associated reagents (Roche diagnostic Company limited, Basel, Switzerland). The PCT levels were assessed using a mini-Vidas immunofluorescence analyzer (BioMerieus Company, Craponne, France), The D-dimer level was assessed using a Stago automatic coagulometer (Stago diagnostic Company limited, Paris, France).

Clinical Classification

All patients were clinically classified as follows (1, 9–11): (1) Mild: patients' clinical symptoms were mild, with no signs of pneumonia on CT scans; (2) Moderate: the patient has fever, respiratory tract symptoms, and signs of pneumonia on CT scans; (3) Severe: the patient met any of these criteria: shortness of breath, return rate (RR) over 30 times per min; an at-rest oxygen saturation (SpO2) level lower than 93%; partial pressure of arterial oxygen (PaO_2)/the fraction of inspired oxygen (Fi02) lower than 300 mmHg (1 mmHg = 0.133 kpa); chest CT scans

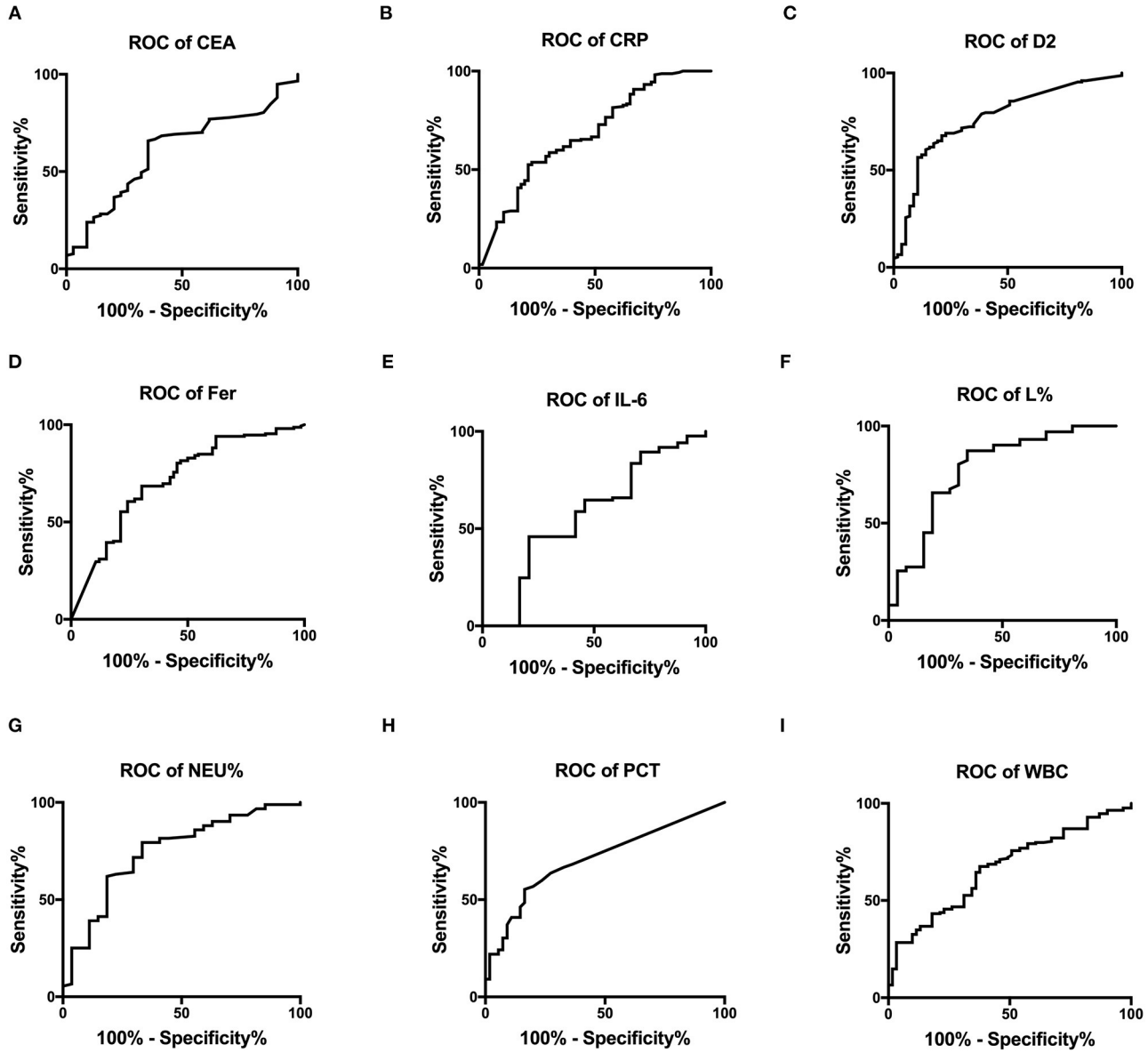

FIGURE 2 | The ROC curves for CEA, CRP, D2, Fer, IL-6, L%, Neu%, PCT, and WBC were analyzed to assess disease classification. **(A–I)** ROC, receiver operating characteristic; AUC, area under the curve; CRP, C reactive protein; PCT, procalcitonin; Fer, ferritin; WBC, white blood cell; D2, D-dimer; Neu%, neutrophils percentage; L%, lymphocyte percentage; CEA, Carcinoembryonic antigen; IL-6, interleukin 6.

showing significant disease progression within 1 to 2 days; and (4) Critically severe: the patient met any of these criteria: respiratory failure requiring mechanical ventilation; shock; and complications related to organ failure that required ICU stay.

Statistical Analysis

Statistical analysis was performed using SPSS version 20.0 (IBM Corp., Armonk, NY, USA). To analyze the differences in the levels of CEA, CRP, and other biomarkers among patients with COVID-19, the chi-square test and Kruskal-Wallis *H*-test were used. Univariate analysis and multivariate Cox regression were used to identify independent prognostic factors. The R software package (Version 3.4.4) was used to analyze the constructed nomograms for OS probability. To evaluate the specificity and sensitivity of the indicator levels to predict the severity of pneumonia, receiver operating characteristic (ROC) curves were used. Spearman's rank correlation significance test was used to analyze the association between individual patient variables. Statistical significance was accepted at $p < 0.05$.

RESULTS

Demographic Characteristics of the Patients

Table 1 details the clinical characteristics of the included patients. Of the 314 patients, 83 showed moderate symptoms, 155 had

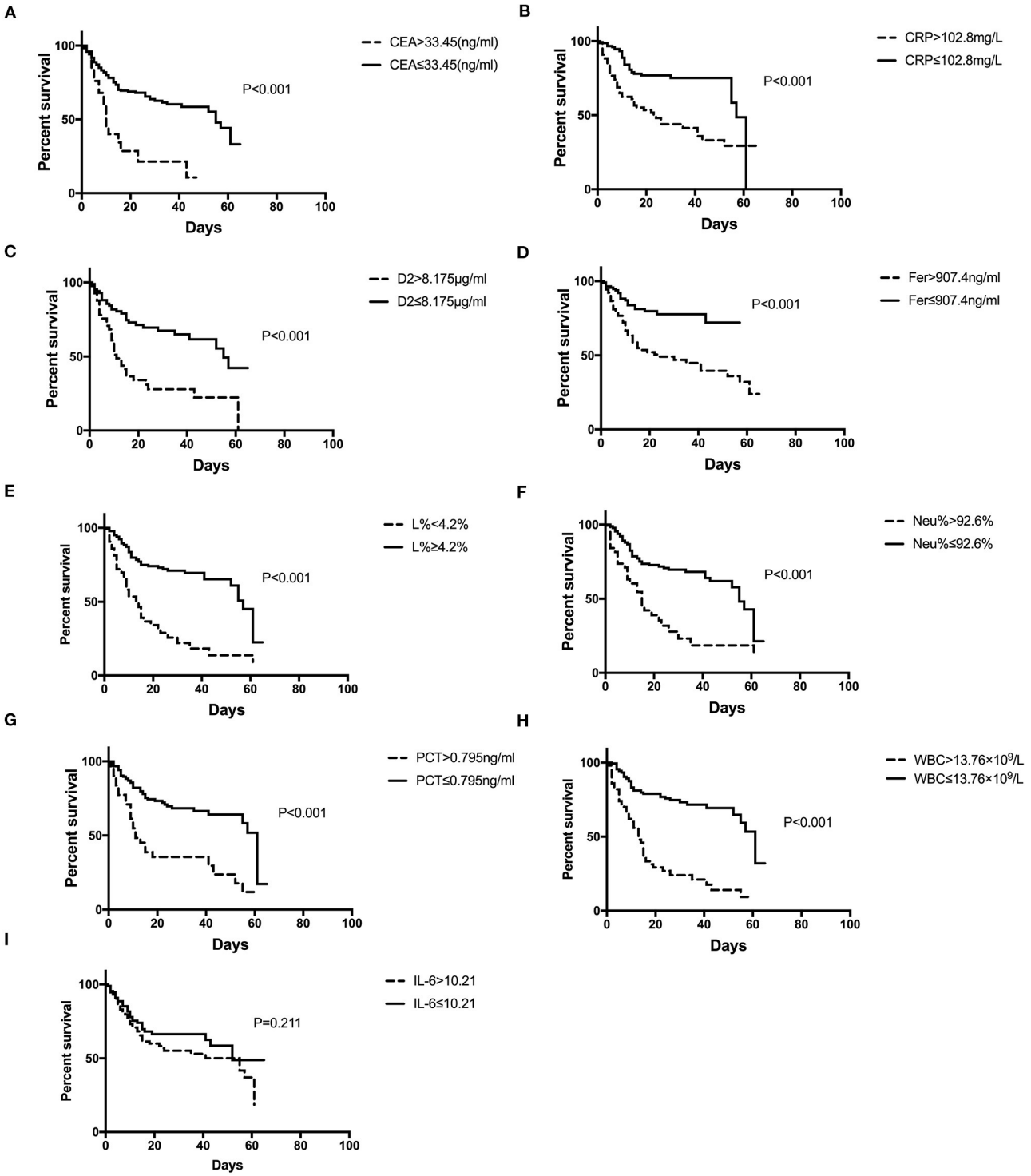

FIGURE 3 | Survival curves constructed for the different initial levels of CEA, CRP, D2, Fer, IL-6, L%, Neu%, PCT, and WBC among the patients. **(A–D, F–H)** Patients with COVID-19 with initial CEA levels >33.45 ng/mL, CRP over 102.8 mg/L, D2 over 8.18 μg/ml, Fer over 907.4 ng/ml, Neu% over 92.6%, PCT levels >0.795 ng/ml, and WBC counts over 13.76 × 10^9/L had poorer outcomes than those with lower levels, while patients with L% <4.2% had poorer outcomes **(E)**. **(I)** Patients with IL-6 levels higher or lower than 10.21 pg/ml showed no difference in outcome. COVID-19, Coronavirus disease 2019; CRP, C-reactive protein; PCT, procalcitonin; Fer, ferritin; WBC, white blood cell; D2, D-dimer; Neu%, neutrophils percentage; L%, lymphocyte percentage; CEA, Carcinoembryonic antigen; IL-6, interleukin 6.

severe symptoms, and 76 displayed critically severe symptoms at the time of admission. Of the 314 patients, 133 were female, and 181 were male. The patients' ages ranged from 35 to 91 years old, with a mean age of 64.65 years old. Around 52.87% (166) of the patients were over 65 years old. In our study, no significant differences in IL-6, CRP, PCT, or WBC counts by sex or age were observed ($P > 0.05$). However, the levels of CEA, D2, L%, and Neu% were higher in patients over 65 years old ($P < 0.05$), while have no significant differences in sex ($P > 0.05$).

Correlations Between CEA, IL-6, CRP, PCT, Fer, D-Dimer Levels, L%, Neu%, WBC, and Clinical Classification

The correlations between the CRP level, WBC count, L count, and clinical classification are shown in **Figure 1**. In the critically severely affected patients ($n = 76$), CRP levels were significantly higher compared with those in moderately affected patients ($n = 83$) ($P < 0.001$) and severely affected patients ($n = 155$) ($P = 0.001$). The levels of PCT in severely and critically severely affected patients were significantly higher compared with those in moderately affected patients ($P = 0.037$, $P = 0.002$, respectively). The levels of Fer and the WBC counts in critically severely affected patients were significantly higher compared with those in moderately affected patients ($P < 0.001$). The levels of D2 in severely and critically severely affected patients were higher than those in moderately affected patients ($P = 0.017$, $P = 0.004$, respectively). The L% values in severely and critically severely affected patients were lower compared with those in moderately affected patients ($P < 0.001$). The Neu% values in severely and critically severely affected patients were higher ($P = 0.002$, $P < 0.001$, respectively). CEA and IL-6 levels were not associated with the clinical classification of COVID-19: no significant differences were seen between the three types of patients. These results suggested that the levels of CRP, PCT, Fer, D2, WBC counts, Neu%, and L% correlated closely with disease classification.

The Critical Values of CEA, IL-6, CRP, PCT, Fer, D-Dimer Levels, L%, Neu%, and WBC to Assess COVID-19 Classification

Figures 2A–I show the ROC curves for CEA, IL-6, CRP, PCT, Fer, D-dimer levels, L%, Neu%, and WBC, which were used to evaluate disease classification. For these markers, the area under the curve (AUC) values were determined as (from high to low): L% (0.776 ± 0.057) > D2 (0.766 ± 0.037) > Neu% (0.746 ± 0.055) > Fer (0.716 ± 0.039) > PCT (0.709 ± 0.039) > CRP (0.680 ± 0.04) > WBC (0.665 ± 0.038) > CEA (0.607 ± 0.053) > IL-6 (0.573 ± 0.072). The critical values for these markers to predict severe disease type were L% < 4.2%, Neu% > 92.6%, PCT > 0.795 ng/ml, D2 > 8.18 μg/ml, WBC > 13.76×10^9/L, Fer > 907.4 ng/ml, CEA > 33.45 ng/ml, CRP > 102.8 mg/L, IL-6 > 10.21 pg/ml. According to the ROC curve analysis, we regarded the moderate type as negative and regarded severe and critically severe as positive.

TABLE 2 | Univariate and multivariate Cox proportional hazards regression analysis for overall survival (OS).

Variables	Univariate			Multivariate		
	HR	95 CI	*P*-values	HR	95 CI	*P*-values
Gender	1.49	1.06–2.10	0.03			
F	Ref					
Age	1.83	1.30–2.58	<0.001	2.63	1.14–6.08	0.006
<65	Ref					
Admission type	8.99	6.10–13.26	<0.001	2.29	1.27–4.14	0.024
Moderate	Ref					
Fer	2.80	1.77–4.45	<0.001	2.70	1.61–4.42	0.001
≤907.4 ng/ml	Ref					
IL-6	1.33	0.85–2.10	0.21			
≤10.21 pg/ml	Ref					
WBC	4.08	2.36–7.06	<0.001	2.19	1.08–4.44	0.003
≤13.76×10^9/L	Ref					
Neu%	2.65	1.48–4.75	<0.001	2.53	1.60–4.03	0.001
≤92.6%	Ref					
L%	3.27	1.84–5.60	<0.001			
≥4.2%	Ref					
PCT	2.74	1.45–5.19	<0.001			
≤0.795 ng/ml	Ref					
D2	2.85	1.62–5.04	<0.001	2.22	1.13–4.35	0.021
≤8.175 μg/ml	Ref					
CRP	2.57	1.61–4.08	<0.001			
≤102.8 mg/L	Ref					
CEA	3.07	1.43–6.59	<0.001	2.00	1.19–3.35	0.009
≤33.45 ng/ml	Ref					

Correlations Between CEA, IL-6, CRP, PCT, Fer, D-Dimer, L%, Neu%, and WBC Levels and COVID-19 Prognosis

Figure 3 shows the survival curves for patients with COVID-19 with varying CEA, IL-6, CRP, PCT, Fer, D-dimer, L%, Neu% levels, and WBC counts at admission. Patients with initial CEA levels in excess of 33.45 ng/mL, WBC counts in excess of 13.76×10^9/L, Neu% in excess of 92.6%, PCT levels in excess of 0.795 ng/ml, CRP levels in excess of 102.8 mg/L, Fer levels in excess of 907.4 ng/mL, and D2 levels in excess of 8.175 μg/ml displayed poorer prognosis compared with that of patients with lower amounts of these markers (**Figures 3A–G**). While patients with an initial L% <4.2% had worse outcomes (**Figure 3H**). However, there were no differences in the prognosis of patients with IL-6 levels over 10.21 pg/mL (**Figure 3I**). **Table 2** shows the effects of these markers on OS, as assessed using univariate and multivariate Cox regression analysis. The Forest plots of these markers and other factors (age, sex, and admission type) are shown in **Figure 4**. The hazard ratio and 95% confidence interval (CI) of the variables (Fer > 907.4 ng/ml, IL-6 > 10.21 pg/ml, WBC > 13.76×10^9/L, Neu% > 92.6%, L% < 4.2%, PCT > 0.795 ng/ml, D2 > 8.18 μg/ml, CRP > 102.8 mg/L, and CEA > 33.45 ng/ml, along with the admission type, age, and sex) were 2.80 (1.77–4.45),

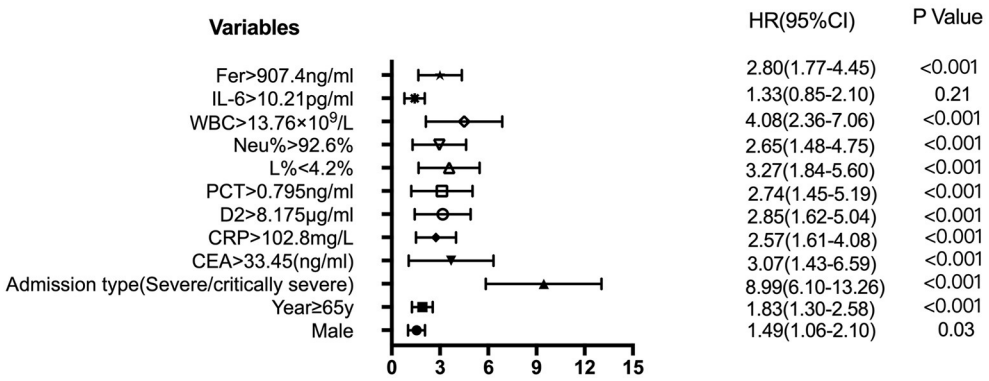

FIGURE 4 | Forest plots of CEA, CRP, D2, Fer, IL-6, L%, Neu%, PCT, and WBC levels with other factors to assess the HR of the prognosis of COVID-19. The variables shown on the left of the axis, while the *P*-values are shown to the right of the HR. CI, confidence interval; HR hazard ratio; COVID-19, Coronavirus disease 2019; CRP, C-reactive protein; PCT, procalcitonin; Fer, ferritin; WBC, white blood cell; D2, D-dimer; Neu%, neutrophils percentage; L%, lymphocyte percentage; CEA, Carcinoembryonic antigen; IL-6, interleukin 6.

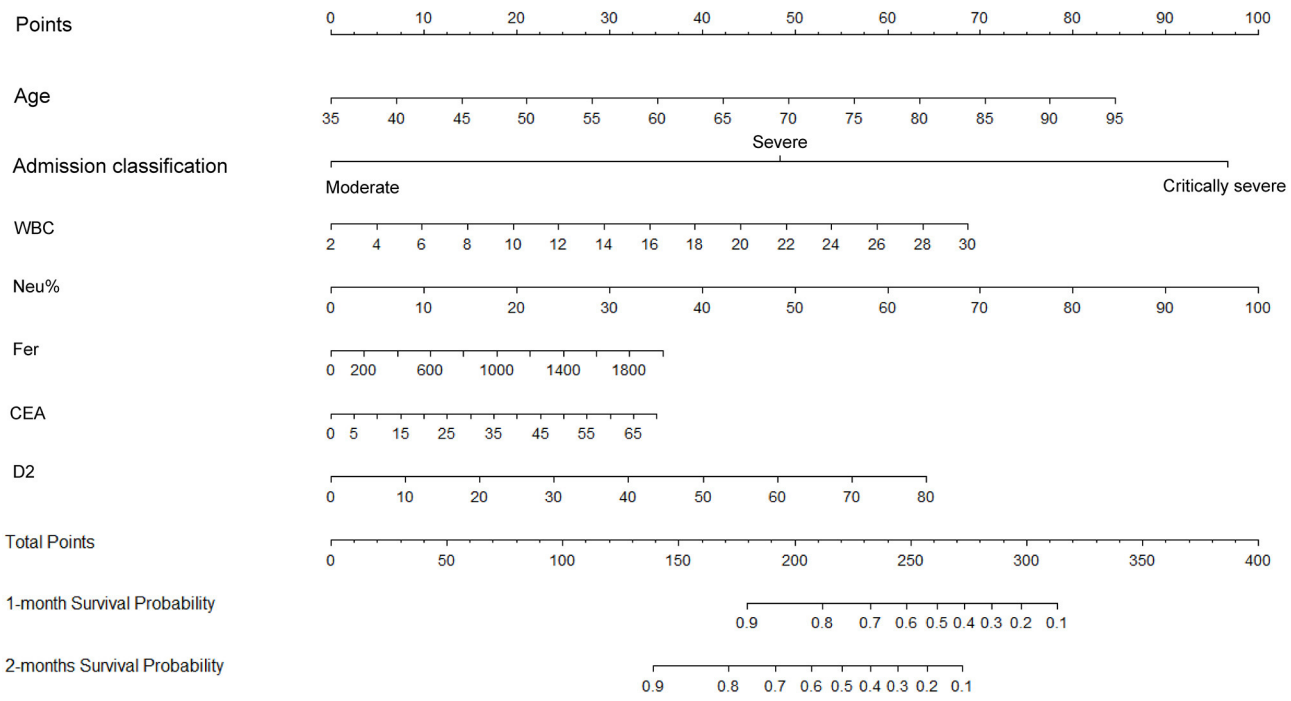

FIGURE 5 | Construction of a nomogram to predict the overall survival of patients with COVID-19 comprising CEA levels and other significant indicators. The points total are located on the Total Point axis, and a vertical line is traced downward to the survival axes to predict the likelihood of an OS of 1 or 2 months. COVID-19, Coronavirus disease 2019; CEA, Carcinoembryonic antigen.

1.33 (0.85–2.1), 4.08 (2.36–7.06), 2.65 (1.48–4.75), 3.27 (1.84–5.6), 2.74 (1.45–5.19), 2.85 (1.62–5.04), 2.57 (1.61–4.08), 3.07 (1.43–6.59), 8.99 (6.10–13.26), 1.83 (1.3–2.58), and 1.49 (1.06-2.1), respectively. Most variables showed significant differences ($P < 0.05$), except for IL-6 > 10.21 pg/ml ($P = 0.21$). Thus, for the OS of patients with COVID-19, the independent prognostic risk factors comprised CEA, WBC, CRP, PCT, Fer, D-dimer, Neu%, and L%.

The Prognostic Nomogram for OS

The independent indicators from the multivariate analysis were used to construct the prognostic nomogram for OS of patients with COVID-19 (**Figure 5**). Compared with that of the other variables, for the outcome in patients with COVID-19, the prognostic value of Neu% was more significant ($P < 0.001$). In order of importance, the remaining factors were Fer ($P = 0.000$), CEA ($P = 0.000$), D2 ($P = 0.000$), WBC ($P = 0.000$), CRP (P

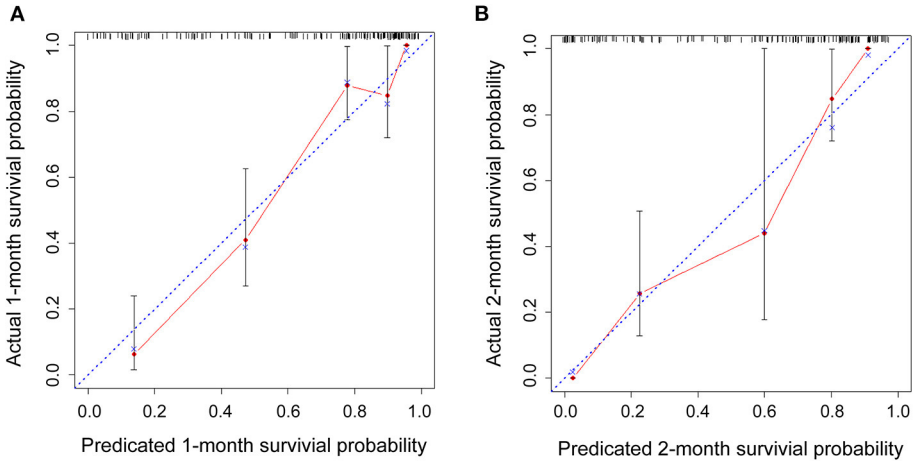

FIGURE 6 | Internal cross-validation calibration curves at 1 and 2 months. **(A,B)** Internal cross-validation calibration plots at 1 and 2 months.

= 0.000), and PCT (P = 0.000), while the nomogram model was not affected significantly by IL-6 (P = 0.21; **Table 2**). In the nomogram, each predictor was given a score (top scale), the sum of which indicated the probability of OS for 1 or 2 months (bottom scale). For OS, the nomogram had a C-index of 0.84 (95% CI, 0.79–0.88), demonstrating that the model had a good discriminative ability (admission classification + WBC + Neu% + Fer + CEA + D2, **Figure 5**).

The OS Nomogram Model Calibration Curves

Figure 6 displays the calibration curves for internal validation at 1 and 2 months. For the internal cross-validation, the calibration plots for 1 and 2 months closely approximated to the observed estimates (**Figures 6A,B**). For OS for 1 and 2 months, the AUC values were 0.87 (95% CI, 0.81–0.94) and 0.83 (95% CI, 0.76–0.89), respectively.

DISCUSSION

Since the COVID-19 outbreak, SARS-CoV-2 infection has resulted in more than 40 million infections and over 1 million deaths worldwide. The infected patients may develop acute respiratory distress syndrome and die rapidly from a series of complications, including acute inflammation, coagulation dysfunction, septic shock, and multiple organ failure, which is especially the case for elderly patients with underlying diseases (5, 12). The severe disease-related complications and diverse clinical characteristics mean that early diagnosis and treatment can improve prognosis and reduce mortality in patients with COVID-19 (1, 13).

COVID-19 severity is associated with the levels of CEA, IL-6, CRP, PCT, Fer, D-dimer, L%, Neu%, and WBC. Here, we found that the critical values for those indicators were: L% < 4.2%, Neu% > 92.6%, PCT > 0.795 ng/ml, D2 > 8.18 μg/ml, WBC > 13.76 × 10^9/L, Fer > 907.4 ng/ml, CEA > 33.45 ng/ml, CRP > 102.8 mg/L, IL-6 > 10.21 pg/ml, respectively. The AUC values for these markers (from ROC curve analysis) from high to low were L% (0.776 ± 0.057) > D2 (0.766 ± 0.037) > Neu% (0.746 ± 0.055) > Fer (0.716 ± 0.039) > PCT (0.709 ± 0.039) > CRP (0.680 ± 0.04) > WBC (0.665 ± 0.038) > CEA (0.607 ± 0.053) > IL-6 (0.573 ± 0.072). Thus, clinicians should monitor changes in these indicators during patient treatment. Increased CEA, Fer, PCT, D2, CRP levels, Neu%, and WBC counts indicate severe pneumonia, while decreased levels indicate treatment effectiveness and disease improvement. However, an increased L% indicates disease improvement, while decreased ratios indicate disease progression. Furthermore, our data show that CEA levels decreased below 5 ng/mL in well-recovered patients. CRP, WBC count, L%, Neu%, PCT, IL-6, and Fer are inflammatory markers commonly used to evaluate the inflammatory state of patients. D-dimer is a marker of thromboembolism (13–15). Studies have demonstrated that an increased level of D2 indicates a high risk for venous thromboembolism in patients with COVID-19. The levels of CRP, Fer, PCT, and IL-6, an acute phase protein, increase in the body immediately in response to infection or tissue damage (16, 17). This results in the activation of the complement system and strengthening of the phagocytic cell-mediated defense against invading microorganisms. WBCs and Ls are the major immune cells that rapidly initiate immune responses when the body is infected with a virus (18).

The serum CEA level has been identified as a prognostic marker for HIV-related pneumocystis carinii pneumonia (PCP) (19), in which patients with PCP and acute respiratory distress have increased CEA levels. Moreover, fatal outcomes were only associated with high concentrations of CEA (> 20 ng/mL) in patients with a PaO_2 value lower than 50 mmHg (19, 20). The results of the present study also showed that patient outcome in COVID-19 is associated with preliminary CEA levels.

In our study, we constructed an OS nomogram for the clinical diagnosis and treatment of COVID-19 with the models (Admission classification + WBC + Neu% + Fer + CEA + D2), and the nomogram of OS had a C-index of 0.84 (95% CI, 0.79–0.88). The model could be used to assess the clinical risk factors to predict the OS of patients with COVID-19. Furthermore, the calibration plots for the internally cross-validated cohort closely approximated to the observed estimates.

From the prognostic risk score, we could identify the populations of patients at high risk of shorter OS and provide effective treatment for a better outcome. According to the hazard ratio for the prognosis of risk variables for COVID-19, the admission classification (severe or critically severe), age over 65 years old, levels of Fer over 907.4 ng/ml, PCT over 0.795 ng/ml, D2 over 8.175 μg/ml, CRP over 102.8 mg/L, CEA over 33.45 ng/ml (excluding tumors), a WBC count over 13.76 $\times$ 10^9/L, Neu% over 92.6%, and L% below 4.2% were higher risk factors for poor patient OS. However, our data showed no significant difference in the HR between different levels of IL-6. In conclusion, our study provided a nomogram model comprising clinical biomarkers, such as Fer, PCT, CRP, D-dimer, and CEA. These data will provide useful information for the diagnosis, management, and therapy of COVID-19.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JY, LN, and XZ had access to all the clinical data generated by the study, took responsibility for data integrity, accuracy of the data analysis, concept, and design. DW and JC: acquisition, analysis, or interpretation of data. JY and LN: manuscript preparation. ZY and LZ: statistical analysis. DL: supervision. All authors contributed to the article and approved the submitted version.

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Vigilance on New-Onset Atherosclerosis Following SARS-CoV-2 Infection

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The pandemic of coronavirus disease 2019 (COVID-19), caused by SARS-CoV-2, has become a global challenge to public health. While its typical clinical manifestations are respiratory disorders, emerging evidence of cardiovascular complications indicates the adverse interaction between SARS-CoV-2 infection and cardiovascular outcomes. Given that viral infection has emerged as an additional risk factor for atherosclerosis, in this paper, we attempt to clarify the susceptibility to new-onset atherosclerosis in individuals infected with SARS-CoV-2. Mechanistically, serving as functional receptors for SARS-CoV-2, angiotensin-converting enzyme 2 (ACE2) mediates SARS-CoV-2 infection of endothelial cells (ECs) directly, leading to endothelial dysfunction and dysregulation of the renin-angiotensin system (RAS). In addition, high expression of CD147, an alternative receptor, and activation of the NLRP3 inflammasome may also contribute to atherosclerosis in the context of COVID-19. More importantly, SARS-CoV-2 attacks the immune system, which results in excessive inflammation and perpetuates a vicious cycle of deteriorated endothelial dysfunction that further promotes inflammation. The alterations in the blood lipid profile induced by COVID-19 should not be ignored in assessing the predisposition toward atherosclerosis in victims of COVID-19. A better understanding of the underlying mechanisms of SARS-CoV-2 infection and the long-term monitoring of inflammatory factors and endothelial function should be considered in the follow-up of patients who have recovered from COVID-19 for early detection and prevention of atherosclerosis.

Keywords: COVID-19, SARS-CoV-2, atherosclerosis, endothelial dysfunction, inflammation, angiotensin-converting enzyme 2

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) caused by a novel coronavirus, namely severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become a severe public health emergency worldwide. Although its typical clinical manifestations are respiratory dysfunctions, intriguingly, some patients suffering from COVID-19 show cardiovascular symptoms, even as the first symptom (1, 2). Furthermore, patients with prior cardiovascular diseases, such as hypertension and coronary heart disease, tend to have an increased risk of death, highlighting the adverse interaction between SARS-CoV-2 infection and cardiovascular outcomes (3).

Atherosclerosis remains the leading cause of various cardiovascular disorders, including myocardial infarction, stroke, and disabling peripheral artery disease. Although multiple studies have depicted the possible role of viral infection and atherosclerosis since the 1970s (4–6), with less than a year after the outbreak, it certainly appears to still be too early to determine the atherosclerotic risk of COVID-19 victims, which may evolve silently over many years until clinical features occur. However, it is of great concern to note that more than 90% of the confirmed cases will recover because SARS-CoV-2 possesses a lower mortality rate than severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS), although with more substantial transmission properties (7–9), implying that we are very likely going to face a heavier cardiovascular burden related to atherosclerosis in the future. Consequently, it is necessary to evaluate the risk of atherosclerosis in COVID-19 survivors and to alert people to its complications early. In this paper, we will attempt to clarify the susceptibility to new-onset atherosclerosis in people recovered from COVID-19 as well as pursue the underlying mechanisms.

VIRAL INFECTION AND ATHEROSCLEROSIS

Established risk factors for atherosclerosis, such as hyperlipidemia, hypertension, and smoking, have been efficaciously reduced, however, the occurrence of atherosclerotic disease is still high. In addition, 30–50% of patients actually lack these traditional risk factors, suggesting that other factors are involved in atherosclerotic pathogenesis (10). Clinical data have shown a higher prevalence of subclinical atherosclerosis in human immunodeficiency virus-infected patients (HIV^+) than in HIV^- subjects, independent of the traditional atherosclerosis risks (11, 12). Furthermore, vulnerable plaque characteristics are more common among HIV^+ patients than among control individuals (13). Clinical observations have indicated that the atherosclerosis risk in patients with hepatitis C is approximately double and the severity is higher (14). A prospective cohort study performed in Japan revealed that human T-cell leukemia virus-1 (HTLAV-1) infection could emerge as an independent predictor of increased carotid intima–media thickness (CIMT) (15). In addition, cytomegalovirus (CMV), Epstein-Barr virus (EBV), influenza viruses, herpes simplex virus-1 (HSV-1), and HSV-2 have also been demonstrated to be closely related to atherogenesis or atherosclerosis-related events in human and animal models (16–18). It is therefore speculated that viral infection has a potential implication in atherosclerosis. Several studies has proposed "direct" mechanisms due to the presence of viral pathogens within atherosclerotic lesions but not within normal blood vessels (19, 20). The virus can enter, lay dormant or replicate in cells and then exert local pro-atherosclerotic effects, including endothelial dysfunction, leukocytes transmigration, vascular smooth muscle cell proliferation, thrombosis and plaque rupture, along with a chronic inflammatory environment in the vessel wall. Regardless of whether viral pathogens are detected *in situ* in the plaque, indirect effects of non-vascular infections leading to systemic inflammation have been related to atherosclerosis. The imbalanced immune response, elevates oxidative stress and disturbs autophagy, which can contribute to the production of plasma inflammatory factors (21, 22). However, mechanistic experimental studies regarding virus-associated atherosclerosis are very limited.

DIRECT INFLUENCES OF SARS-CoV-2 ON ATHEROSCLEROSIS

To better determine the susceptibility to atherosclerosis in COVID-19 survivors, it is vital to learn about SARS-CoV-2 and understand how virus-host interactions manifest as risk factors. Accordingly, the risk factors can delineate regulatory programs that mediate atherosclerotic occurrence, provide valuable clues about disease determinants, and help establish appropriate public health measures.

SARS-CoV-2, ACE2 and Atherosclerosis

ACE2-Mediated Endothelial Dysfunction

Coronaviruses are enveloped viruses, consisting of a set of structural proteins that include spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. Among these proteins, the S protein can bind to the membrane receptor on host cells, thus gaining entry into cells and replicating potential in human cells. Similar to SARS-CoV, SARS-CoV-2 also utilizes angiotensin-converting enzyme 2 (ACE2) for cell attachment and infection through the S protein (23). Host transmembrane protease serine 2 (TMPRSS2) cleaves spike protein, which is a necessary step for virus fusion to cellular membranes and entry into the cell (24). SARS-CoV-2 has a higher affinity for binding to ACE2 than SARS-CoV, and binding involves more substantial numbers of interaction sites (25). ACE2 is widely expressed in cardiovascular tissue, including endothelial cells (ECs), in support of a possible mechanism of direct viral injury (26). Notably, circulating endothelial cells are elevated in patients admitted to the hospital with COVID-19 (27). Varga et al. provided microscopic evidence of SARS-CoV-2 viral particles in ECs and diffuse endothelial inflammation (28). *In vitro,* SARS-CoV-2 has been proven to infect engineered human blood vessel organoids directly (29). The plasma levels of Von Willebrand factor (VWF), angiopoietin-2, Fms-related tyrosine kinase 3 ligand (FLT-3L), and plasminogen activator inhibitor type (PAI)-1 are significantly elevated in patients with COVID-19, further supporting the hypothesis of SARS-CoV-2-induced endothelial dysfunction or damage (30, 31). In addition, researchers in Italy and the UK found a significant increase in the incidence of Kawasaki-like disease among children who tested positive for SARS-CoV-2 or were potentially exposed to SARS-CoV-2, highlighting the importance of SARS-CoV-2 infection in coronary artery abnormalities (32, 33). Taken together, these studies point to endothelial SARS-CoV-2 infection as a possible direct trigger of endothelial adverse effects (**Figure 1**). Endothelial dysfunction is an initial step in the

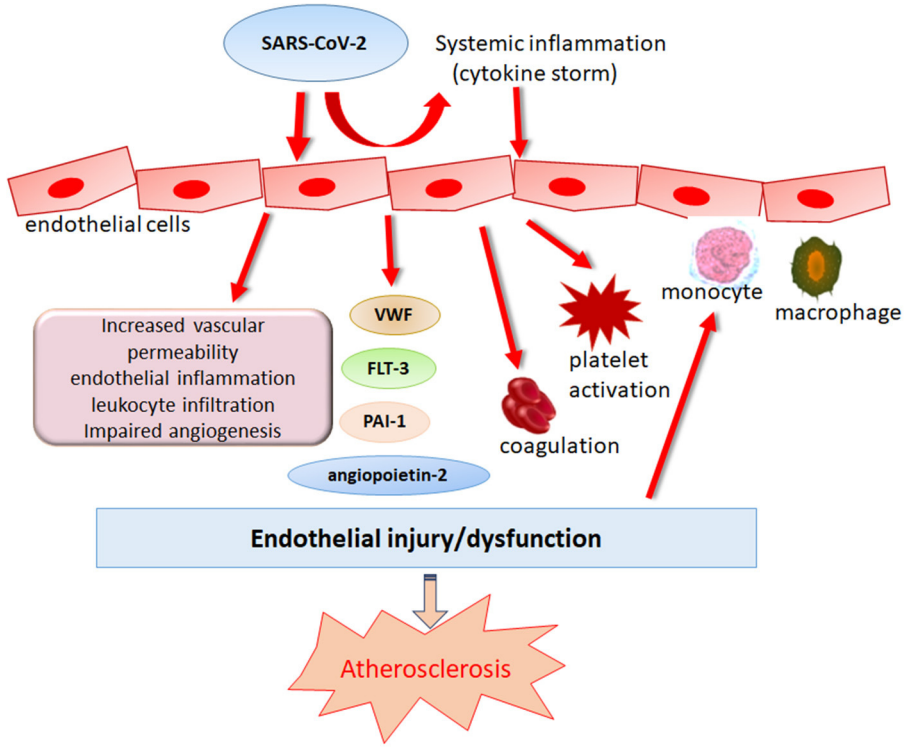

FIGURE 1 | Role of SARS-CoV-2 in endothelial dysregulation. Endothelial dysfunction is an initial step in the development of atherosclerosis that precedes clinical symptoms. SARS-CoV-2 can induce endothelial damage directly or indirectly by eliciting immune dysregulation which causes cytokine storm, leading to the deteriorations of endothelial damage.

development of atherosclerosis that precedes clinical symptoms and has prognostic value for future cardiovascular events (34, 35). Furthermore, endothelial dysfunction emerges as one of the essential mechanisms corresponding to the enhanced atherosclerotic risk among HIV, HCV and other viral infected people (14, 36, 37). Therefore, endothelial dysfunction induced by SARS-CoV-2 infection indeed becomes a strong contributor to upcoming atherosclerosis in subjects who have recovered from COVID-19.

Dysregulation of RAS

Well-known as a negative regulator of the renin–angiotensin system (RAS) with the ability to cleave angiotensin-II (Ang-II) into the vasodilator Ang-(1-7), ACE2 has been documented to have pleiotropic beneficial actions in the process of atherosclerosis. Ang-(1-7) appears particularly important in the antiatherosclerotic effects of ACE2. Sahara et al. revealed that the deletion of ACE2 promotes the development of Ang-II-mediated vascular inflammation and atherosclerosis in apolipoprotein E knockout mice (38). Overexpression of ACE2 could reduce atherosclerotic lesion size and increase the collagen content of plaques (39). Alternatively, Ang-(1-7) treatment was also shown to prevent early atherosclerosis and enhance plaque stability (40, 41). However, in the context of SARS-CoV-2 infection, binding via ACE2 results in downregulation of membrane-bound ACE2 and the concurrent loss of catalytic activity of ACE2 in the RAS system, which leads to a decrease in the level of Ang-(1-7) and an increase in Ang-II concentration. In contrast to ACE2/Ang-(1-7), Ang-II can promote proliferation, inflammation and oxidative stress, contributing to atherosclerosis development (42, 43). Thus, SARS-CoV-2 entry is expected to shift the RAS balance from the protective ACE2-Ang-(1-7) arms to the detrimental ACE-Ang-II axis, implying that the inhibition of atherosclerosis from ACE2/Ang-(1-7) is weakened, however, acceleration of atherosclerosis from Ang-II is enforced. Notably, dysregulation of RAS seems to be independent of ongoing SARS-CoV-2 infection.

Accumulating evidence has shown that angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) exert numerous beneficial actions on cardiac and vascular structure and function beyond their blood pressure-lowering effects (44, 45). In principle, the use of ACEIs and ARBs that produce endothelial protective effects could alleviate COVID-19 symptoms and potentially reduce the severity of the disease (46). However, concerns have been raised regarding whether individuals on ACEIs/ARBs are at a greater risk of SARS-CoV-2 infection and COVID-19 exacerbation, as this class of drugs is suspected to be a risk factor for ASRS-CoV-2 infection by upregulating ACE2 (47, 48). Remarkably, a large consecutive cohort study of 1,200 patients in the UK and a multicenter retrospective study in China both support the beneficial effects of RAS inhibitors in patients with COVID-19

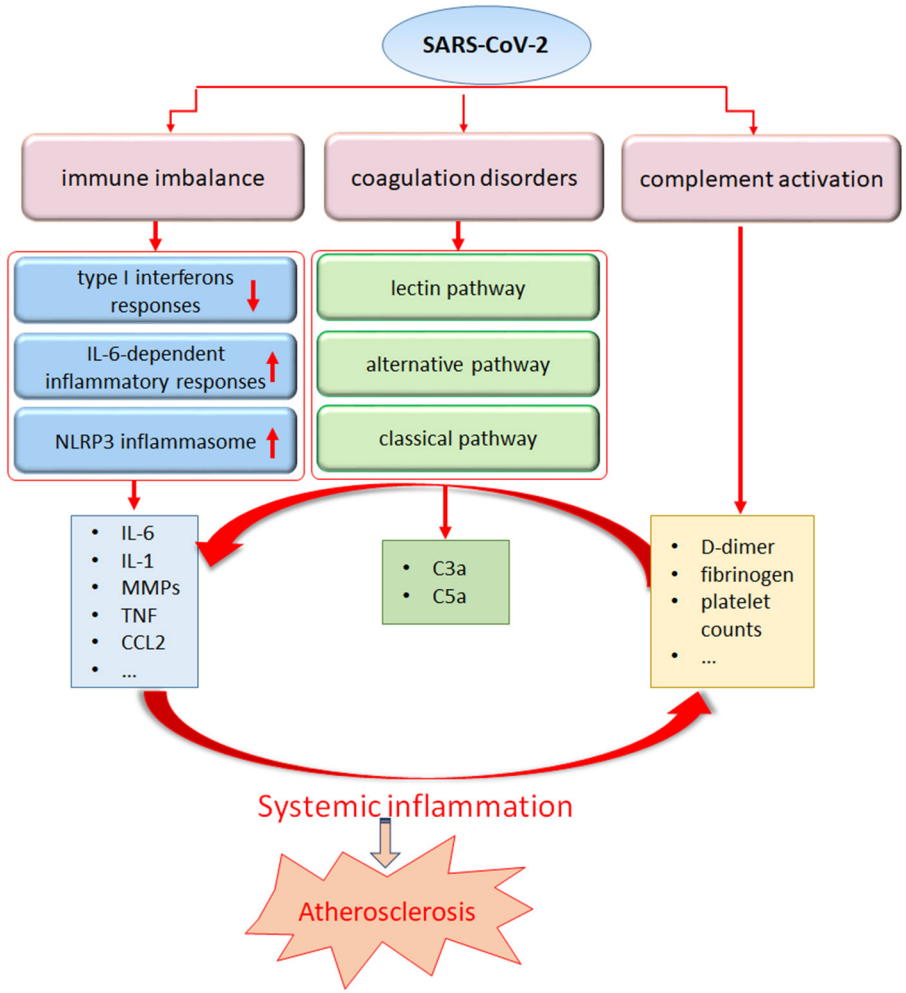

FIGURE 2 | The systemic inflammatory response from SARS-Cov-2 may trigger atherosclerosis. SARS-CoV-2 infection can stimulate a pronounced immune response including dysregulation of interferon and IL-6 pathway, NLRP3 inflammasome activation, leading to an uncontrolled production of proinflammatory mediators. In addition, coagulation disorders and complement abnormalities induced by SARS-CoV-2 infection may contribute to the maladaptive inflammatory response.

and so far, there is no evidence for the potential adverse effect of these agents in patients with COVID-19 (49, 50). However, whether treatment with ACEIs/ARBs can decrease the incidence of atherosclerosis in COVID-19 survivors needs long-term follow-up research.

SARS-CoV-2, CD147, and Atherosclerosis

A current study elegantly found that CD147 can potentially bind to SARS-CoV-2, providing an additional infection route (51). CD147 is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily and expressed at varying levels in many cell types in its different glycoforms (52). Similar to SARS-CoV-2-induced pulmonary damage, CD147 levels are increased in patients with chronic obstructive pulmonary disease (53). Meplazumab, a humanized anti-CD147 antibody, has been shown to inhibit SARS-CoV-2 replication *in vitro* (51). Recently, an open label, concurrent controlled add-on clinical trial in China revealed that the percentage of improvement in patients with severe COVID-19 presentations seems to be higher in patients receiving weekly treatment with meplazumab than in patients receiving conventional treatment. In addition to viral clearance, meplazumab is likely to facilitate restoration of normal lymphocyte counts and decrease C-reactive protein (CRP) levels (54). SARS-CoV-2 has been found to efficiently infect immune cells expressing low ACE2, such as macrophages and T lymphocytes, through CD147-mediated viral entry (55). Therefore, CD147 is upregulated and possibly participates in hyperinflammation induced by SARS-CoV-2. Accumulating studies have highlighted the potential proatherosclerotic effects of CD147 in atherosclerosis (56). Furthermore, statins achieve antiatherosclerotic roles that partly rely on downregulation of CD147 (57). Of note, statins have been recommended to serve as add-on or coadjuvant therapy against COVID-19 (58), strongly suggesting that SARS-CoV-2 infection and atherosclerosis tend to both experience similar pathological processes related to CD147.

SARS-CoV-2 and the NLRP3 Inflammasome

Following an RNA viral infection, the host cell response involves the activation of the Nod-like receptor family pyrin domain-containing three (NLRP3) inflammasome, leading to secretion of the proinflammatory cytokines interleukin (IL)-1β and IL-18 (59). Accumulating evidence has indicated that NLRP3 recognizes RNA viruses by sensing the cellular distress induced by viroporins (60–62). Viroporins are small virus-encoded proteins that are able to permeabilize membranes for ions by forming membrane channels (63, 64). It has been shown that the E protein of SARS-CoV can form Ca^{2+} permeable ion channels, thereby activating the NLRP3 inflammasome (63). SARS-CoV-2 shares many biological features with SARS-CoV owing to the 79.6% genomic sequence identity (65), which implies that SARS-CoV-2 also has the ability to activate the NLRP3 inflammasome. A subsequent study found another viroporin in SARS-CoV, namely 3a protein, which is responsible for activation of the NLRP3 inflammasome (66). The 3a protein is also present in the SARS-CoV-2 genome, raising the possibility that SARS-CoV-2 enables direct activation of the NLRP3 inflammasome (67). In COVID-19, dysregulation of the NLRP3 inflammasome in monocytes and macrophages seems to be involved in a hyperinflammatory state contributing to severe tissue damage (68, 69). The first clinical study of an NLRP3 inflammasome inhibitor (tranilast) to treat COVID-19 is ongoing in China. The activated NLRP3 inflammasome has been widely linked to a large number of diseases, and several experimental studies highlighted that atherosclerosis may not be intrinsically caused by the NLRP3 inflammasome, but is closely linked to and often aggravated by NLRP3 inflammasome activation (70). Thus, the NLRP3 inflammasome probably fuels inflammation in the context of COVID-19 to promote the progression of atherosclerosis.

COVID-19 AND SYSTEMIC INFLAMMATION

In addition to the lungs, immune organs are the second most attacked system by SARS-CoV-2. An excessive inflammatory response to SARS-CoV-2, referred to as a cytokine storm, has been implicated in COVID-19 severity and death, as evidenced by the increased levels of CRP, IL-6, IL-7, tumor necrosis factor (TNF) and inflammatory chemokines, including CC-chemokine ligand 2 (CCL2), CCL3 and CXC- chemokine ligand 10 (CXCL10), as well as IL-2 receptor. Higher levels of IL-6 in the serum have been linked to a worse prognosis in patients suffering from SARS-CoV-2 infection (1, 3). Accompanied by the uncontrolled cytokine response, the presence of a global T cell lymphopenia serves as a common feature in patients with COVID-19 and is particularly prominent in severe patients (71). Furthermore, the T cell numbers appear to be negative correlated with the serum levels of TNF, IL-6 and IL-10 (72). In addition to immune factors such as type I interferons and dysregulation of IL-6-dependent inflammatory responses (73), several retrospective observational studies of patients have shown that SARS-CoV-2 engages robust activation of complement and coagulation cascades (74–76). Elevated levels of D-dimer and fibrinogen, with minor abnormalities in prothrombin time, activated partial thromboplastin time, and increased platelet counts, have been detected in the initial stage of SARS-CoV-2 infection (77). A case series from New York reported large-vessel ischemic stroke in five patients infected with SARS-CoV-2 (78). Furthermore, acute limb ischemia was also described in 20 infected patients in a case series from Italy. All 20 patients were diagnosed with COVID-19- related pneumonia before acute limb ischemia was detected (79). At present, anticoagulation treatment has been linked to survival in patients hospitalized with COVID-19, and a wide range of clinical trials are evaluating the use of low-molecular-weight heparin to treat patients with COVID-19 (80–82). While encountering systemic inflammation, exposure of the endothelium to an array of proinflammatory cytokines may also act as a key source of inflammatory cytokines, leading to aggravated endothelial damage and amplified vascular and systemic inflammation accompanied by an imbalance of pro- and anticoagulant pathways (**Figure 2**).

It is highly acknowledged that atherosclerosis is, in fact, an inflammatory process with innate and adaptive immune activation that plays a part in the entire disease. Our previous studies have suggested that systemic inflammation induced by zymosan could accelerate the progress of atherosclerosis in high fat diet-treated rabbits and rats, and the imbalance of the cytokine network was responsible for deteriorated lipid disorders and advanced atherosclerotic plaques (83, 84). Indeed, diseases with a proinflammatory state, such as rheumatoid arthritis and systemic lupus erythematosus, entail an elevated risk of atherosclerosis (85, 86). Canakinumab, a fully humanized monoclonal antibody that targets IL-1β, has received approval in many countries as an orphan drug to treat rare heritable chronic inflammatory diseases (87). Currently, several clinical trials concerning canakinumab are in progress to test the inflammatory hypothesis of atherosclerosis (88). Of note, clinical trials to assess cytokine blockade, including canakinumab and tocilizumab (targeting IL-6) in patients with COVID-19 are ongoing. Accordingly, inflammation engendered by SARS-CoV-2 infection represents one such state that shares the common pathophysiological milieu of atherosclerosis.

DYSLIPIDEMIA AND COVID-19

To date, the study of the blood lipid profile related to COVID-19 is in its infancy. Two retrospective studies were performed to underline a sharp decrease in high-density lipoprotein (HDL) levels in patients with severe COVID-19. However, there is no consensus regarding the value of serum total cholesterol (TC), low-density lipoproteins (LDL) and triglyceride (TG) (89, 90). A 12-year follow-up study based on 25 SARS survivors demonstrated that 68% of victims had significant alterations in lipid metabolism, which correlated with hyperlipidemia, cardiovascular abnormalities, and abnormal glucose metabolism (91). Altered serum lipid concentrations have been documented to appear during viral infection, including HIV and HCV (12, 14, 92). In addition, high-dose pulses of methylprednisolone,

antiviral drugs including liponovir/ritonavir, and tocilizumab have all been reported to be associated with disturbed lipid metabolism (93–95). Multiple lines of incontrovertible evidence have proven a causal role for high-serum LDL in atherosclerosis. In general, LDL activates intracellular pathways to increase local and systemic inflammation, monocyte adhesion, endothelial cell dysfunction and apoptosis, and smooth muscle cell proliferation, resulting in foam cell formation and the genesis of atherosclerotic plaques. In contrast, HDL is capable of preventing or attenuating atherosclerosis (96, 97). Although the blood lipid profile requires long-term monitoring, the direct participation of hyperlipidemia should not be discarded in assessing the risk of atherosclerosis in COVID-19 survivors.

CONCLUSION

Accumulating evidence has indicated that EC dysfunction is a central feature of COVID-19, accordingly, the major link to SARS-CoV-2-induced atherosclerosis may be centered on endothelial cells. It is proposed that the endothelial dysfunction and injury occurring in COVID-19 reflects direct infection of ECs by SARS-CoV-2 in receptor-dependent and independent manners. The indirect bystander injury resulting from systemic inflammation further amplifies endothelial dysfunction, perpetuating a vicious cycle of endothelial dysfunction that promotes inflammation. It has been appreciated that there is not a specific virus or pathogen that initiates atherosclerosis but rather the inflammatory level and its chronicity and intensity. To date, our knowledge and understanding of COVID-19-associated atherosclerosis is limited by what is known about traditional atherosclerosis because current knowledge has been gained almost exclusively through clinical studies. There is a pressing need to experimentally unravel the missing link between SARS-CoV-2 and atherosclerosis.

AUTHOR CONTRIBUTIONS

YL wrote and drafted of the article. H-GZ revised the manuscript critically. All authors contributed to the article and approved the submitted version.

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HYPOTHESIS AND THEORY
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Can GPR4 Be a Potential Therapeutic Target for COVID-19?

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Coronavirus disease 19 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), first emerged in late 2019 and has since rapidly become a global pandemic. SARS-CoV-2 infection causes damages to the lung and other organs. The clinical manifestations of COVID-19 range widely from asymptomatic infection, mild respiratory illness to severe pneumonia with respiratory failure and death. Autopsy studies demonstrate that diffuse alveolar damage, inflammatory cell infiltration, edema, proteinaceous exudates, and vascular thromboembolism in the lung as well as extrapulmonary injuries in other organs represent key pathological findings. Herein, we hypothesize that GPR4 plays an integral role in COVID-19 pathophysiology and is a potential therapeutic target for the treatment of COVID-19. GPR4 is a pro-inflammatory G protein-coupled receptor (GPCR) highly expressed in vascular endothelial cells and serves as a "gatekeeper" to regulate endothelium-blood cell interaction and leukocyte infiltration. GPR4 also regulates vascular permeability and tissue edema under inflammatory conditions. Therefore, we hypothesize that GPR4 antagonism can potentially be exploited to mitigate the hyper-inflammatory response, vessel hyper-permeability, pulmonary edema, exudate formation, vascular thromboembolism and tissue injury associated with COVID-19.

Keywords: COVID-19, GPR4, inflammation, endothelial cell, vascular permeability, thromboembolism

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INTRODUCTION

COVID-19 first emerged in Wuhan, China in December 2019 and has rapidly become a global pandemic with confirmed cases in more than 200 countries and regions. By November 5, 2020, nearly 48 million confirmed cases and over 1.2 million deaths have been reported around the world (1). The pandemic is continuing to spread and more confirmed cases and COVID-related deaths are reported every day. In addition to the staggering number of human casualties and as a global effort to stop the pandemic, social distancing, stay-at-home orders, and closure of schools and businesses have caused enormous societal burdens and economic losses. Development of effective vaccines and therapeutics is critical to curb the pandemic and save the lives of patients afflicted by COVID-19.

For COVID-19 patients, disease severities span from asymptomatic infection, mild respiratory illness to severe pneumonia with respiratory failure and death (2–4). In a study of 44,415 cases in China, 81% of patients had mild symptoms, 14% had severe symptoms and 5% had critical disease

manifestations (5). The worldwide mortality rate is approximately 2.5% among the confirmed cases (1,221,781/47,930,397 as of November 5, 2020) (1), with a higher mortality rate in elderly patients and those with underlying conditions such as hypertension, diabetes, and cardiovascular disease.

The pathophysiology of COVID-19 is not completely understood. SARS-CoV-2 infects a wide range of cells, including type II pneumocytes, vascular endothelial cells, pericytes, macrophages, T cells, cardiomyocytes, enterocytes, kidney epithelial cells and podocytes, all of which express the SARS-CoV-2 receptor ACE2 (angiotensin converting enzyme 2) (4, 6). Airway epithelial cells infected by SARS-CoV-2 trigger an inflammatory response, with production of increased levels of cytokines and chemokines that stimulate the infiltration of neutrophils, monocytes, and lymphocytes into the lung and other target organs (4, 6). Autopsy studies of patients succumbing to COVID-19 have revealed some key pathological findings, such as diffuse alveolar damage, inflammatory cell infiltration, pulmonary edema, proteinaceous exudates, and vascular thromboembolism in the lung, which potentially contribute to disease severity, acute respiratory distress syndrome (ARDS) and respiratory failure in the patients (7–9). In addition to lung injuries, COVID-19 complications also include impaired function of the liver, kidney, heart, brain, and coagulation system (4, 6, 10).

HYPOTHESIS

We propose that GPR4 is involved in COVID-19 pathophysiology and can be exploited as a potential therapeutic target for COVID-19. GPR4 is a pro-inflammatory GPCR that regulates endothelial cell adhesion, leukocyte infiltration, blood vessel permeability, and angiogenesis (11–20). GPR4 is expressed in various tissues, with high expression in the lung, heart, and kidney (21, 22). The cell types predominantly expressing GPR4 are vascular endothelial cells and GPR4 gene expression is also found in other cell types such as neurons, kidney epithelial cells, osteoblasts, and chondrocytes (12, 14, 23–26). Biochemically, GPR4 can be activated by extracellular protons (acidosis), with acidotic conditions commonly existing in inflamed tissues due to hypoxia and glycolytic cell metabolism (27, 28). Importantly, genetic and pharmacological inhibition of GPR4 alleviates inflammatory responses, reduces leukocyte infiltration, and decreases tissue edema in several animal models of inflammatory disorders including arthritis, inflammatory bowel disease, chronic obstructive pulmonary disease (COPD), and ischemic diseases (14, 15, 17–20, 29). Many of the GPR4-regulated inflammatory processes described above share cardinal pathological features observed in COVID-19 patients (7–9). Therefore, we hypothesize that GPR4 plays a role in COVID-19 pathophysiology and GPR4 antagonism is a potential therapeutic approach to mitigate COVID-19 complications.

Abbreviations: ACE2, angiotensin converting enzyme 2; ARDS, acute respiratory distress syndrome; ATF3, activating transcription factor 3; CCL2 (MCP-1), C-C motif chemokine ligand 2 (monocyte chemoattractant protein-1); CCL3 (MIP-1α), C-C motif chemokine ligand 3 (macrophage inflammatory protein-1α); CHOP, C/EBP homologous protein; COPD, chronic obstructive pulmonary disease; COVID-19, coronavirus disease 19; COX2, cyclooxygenase-2; CXCL10 (IP-10), C-X-C motif chemokine ligand 10 (interferon gamma-induced protein 10); DSS, dextran sulfate sodium; ER, endoplasmic reticulum; FDA, Food and Drug Administration; GPCR, G protein-coupled receptor; GPR4, G protein-coupled receptor 4; IBD, inflammatory bowel disease; ICAM-1, Intercellular Adhesion Molecule 1; ICU, intensive care unit; IgG, immunoglobulin G; IL, interleukin; iNOS, inducible nitric oxide synthase; KO, knockout; MMP, matrix metalloproteinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PGE2, prostaglandin E2; pH, potential of hydrogen; RAAS, renin-angiotensin-aldosterone system; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor.

EVALUATION OF THE HYPOTHESIS

Does GPR4 Play a Role in the Pathophysiology of COVID-19?

Pathophysiology of Inflammatory Responses in COVID-19

COVID-19 is caused by the infection of SARS-CoV-2, a novel β-coronavirus sharing ∼88% and ∼80% sequence homology with the bat derived SARS-like coronaviruses and SARS-CoV, respectively (30). Similar to SARS-CoV, the spike glycoprotein (S protein) of SARS-CoV-2 binds to cell surface ACE2 receptors to gain entry into cells (4, 6). In the early stage of disease, SARS-CoV-2 targets the respiratory system, infecting nasal and bronchial epithelial cells and lung pneumocytes and stimulating inflammatory responses in these cells. Consequently, the infected cells produce increased levels of cytokines and chemokines, such as interleukin-6 (IL-6), IL-1, TNF-α, CXCL10 (IP-10), CCL2 (MCP-1), and CCL3 (MIP-1α). The cytokines and chemokines, in turn, induce massive infiltration of leukocytes into the lung. The accumulation of neutrophils, monocytes, and macrophages in the lung further increases the production of cytokines and chemokines, generating a vicious cycle of inflammation. Excessive production of cytokines can lead to "cytokine storm," acute respiratory distress syndrome (ARDS), tissue injury, multi-organ failure, and death in critically ill COVID-19 patients (4, 6).

Besides airway epithelial cells, SARS-CoV-2 also infects many other types of cells expressing the ACE2 receptor, such as vascular endothelial cells and pericytes (4, 6, 10). SARS-CoV-2 infection of lung microvascular endothelial cells aggravates the inflammatory response. Endothelial cells function as a physiological interface to interact with leukocytes and platelets. Pulmonary endothelial and epithelial barriers are critical for the regulation of gas exchange and immune cell recruitment in the lung. SARS-CoV-2 infection of alveolar epithelial cells and pulmonary endothelial cells can cause cell death and stimulate inflammatory responses. Consequently, disruption of pulmonary endothelial and epithelial barriers leads to excessive leukocyte infiltration into the lung, plasma fluid flooding into interstitial and alveolar spaces (i.e., permeability edema), shortness of breath, hypoxemia, pneumonia, and ARDS in COVID-19 patients.

In addition to the hyper-inflammation and damage to pulmonary epithelial and endothelial barriers, vascular thromboembolism in the lung and other organs is also a

common complication observed in severe COVID-19 patients that is associated with fatal outcomes. In a study of 184 patients admitted to the intensive care unit (ICU), thrombotic complications were observed in 31% of the patients (31). Autopsy studies of the lungs from seven patients who died from COVID-19 showed widespread thrombosis in pulmonary vessels and microthrombi in alveolar capillaries (32). Increased new blood vessel growth (angiogenesis) was also observed in the lungs of these patients (32). Vascular thromboembolism is closely associated with mortality in COVID-19 patients. Predisposition to thromboembolism is believed to be due to excessive inflammation and coagulopathy in COVID-19 patients (31, 32).

Extrapulmonary complications of COVID-19 have been observed in multiple organ systems, such as the cardiovascular, hematological, gastrointestinal, renal, endocrine, dermatological and neurological systems (4, 6, 10). While COVID-19 is caused by SARS-CoV-2 infection, the host inflammatory response also plays critical roles in the pathophysiology of the disease. Overall, SARS-CoV-2 infection-induced direct cytotoxicity, hyper-inflammation, endothelial cell dysfunction, thromboembolism, cytokine-release syndrome, and dysregulation of the renin-angiotensin-aldosterone system (RAAS) are considered as the major mechanisms responsible for systemic COVID-19 complications in multiple organ systems (4, 6, 10).

How Is GPR4 Potentially Involved in the Pathophysiology of COVID-19?

As described above, hyper-inflammatory responses in patients with increased levels of cytokines, chemokines, leukocyte infiltration, vascular permeability, tissue edema, endothelialitis, and thromboembolism represent some key pathophysiological features in COVID-19 (4, 6). Herein, we evaluate the potential involvement of GPR4 in the pathophysiology of COVID-19.

GPR4 is highly expressed in vascular endothelial cells and has emerged as a key regulator of inflammatory responses (11–20, 27). As a proton-sensing GPCR, GPR4 can be activated by acidosis which is a microenvironment hallmark of numerous pathological conditions such as inflammation, ischemia, and tumors (27, 28). Activation of GPR4 by acidosis stimulates the expression of inflammatory chemokines, cytokines, adhesion molecules and the NF-κB pathway in endothelial cells, increases endothelium-leukocyte adhesion, and facilitates leukocyte infiltration (11–16, 20). Moreover, activation of GPR4 by acidosis promotes the endoplasmic reticulum (ER) stress response and apoptosis of endothelial cells (12, 13, 33).

Multiple lines of evidence support a pro-inflammatory role of GPR4 in various pathological conditions (11–20, 27, 29). Using GPR4 knockout (KO) mice, studies demonstrated that GPR4 deletion reduces inflammation in mouse colitis models (14, 19). In the dextran sulfate sodium (DSS)-induced acute colitis mouse model, GPR4 deletion ameliorates intestinal inflammation (14). The indicators of disease severity, such as body weight loss, mesenteric lymph node expansion, colon shortening, fecal diarrhea score, and intestinal histopathology, are alleviated in the GPR4 KO mice compared to wild-type mice. GPR4 deletion reduces the expression of endothelial adhesion molecules E-selectin and VCAM-1 in the colon of the DSS-induced colitis mice (14). Another study also demonstrated that GPR4 deletion alleviates intestinal inflammation in the DSS-induced colitis and the IL10-/- spontaneous colitis mouse models (19). Interestingly, GPR4 mRNA is over-expressed by approximately 5-fold in the inflamed intestinal lesions of inflammatory bowel disease (IBD) patients when compared to normal intestinal tissues (14). Furthermore, in the tourniquet cuff-induced hindlimb ischemia-reperfusion mouse model, GPR4 deletion reduces inflammatory response, leukocyte infiltration, vascular permeability, tissue edema and proteinaceous exudate formation in the limb tissue (20).

Based on its biological functions, GPR4 can potentially regulate multiple aspects of COVID-19 pathophysiology (**Figure 1**). SARS-CoV-2 infection of lung epithelial cells and endothelial cells induces inflammatory responses in these cells with increased expression of cytokines and chemokines (4, 6). As the disease severity of COVID-19 progresses, alveolar epithelial and endothelial barriers become disrupted, oxygen/carbon dioxide exchange is impaired, and the lung tissues become hypoxic. The pH of inflammatory and hypoxic tissues is acidic due to reduced oxygen levels, glycolytic cell metabolism, and proton accumulation (27, 28, 34–36). In addition to acidotic pH in inflamed and hypoxic tissues, respiratory and metabolic acidosis is a common complication observed in COVID-19 patients, especially in patients with severe disease (37). Also, COVID-19 may aggravate ketoacidosis in diabetes patients and cause kidney injuries, leading to metabolic acidosis in patients (38–40). As a proton-sensing GPCR, GPR4 is optimally activated under acidic extracellular pH (6.4–6.9) and partially activated at physiological pH 7.4 (41, 42). As described above, activation of GPR4 increases the expression of inflammatory adhesion molecules, chemokines, and cytokines in vascular endothelial cells, which can in turn enhance leukocyte infiltration (11, 12, 14, 16). Increased inflammation and adhesiveness of endothelial cells can be prothrombotic and stimulate the adhesion and aggregation of platelets and leukocytes (43). Moreover, activation of GPR4 augments paracellular gap formation and permeability of endothelial cells, which can lead to fluid accumulation and edema in the tissues (17, 18, 20). All these biological functions of GPR4 are highly relevant to COVID-19 patient pathophysiology, including the hyper-inflammatory response, leukocyte infiltration, blood vessel leakage, pulmonary edema, and vascular thromboembolism (4, 6). Moreover, GPR4 gene expression is up-regulated in COVID-19 patient samples. A recent study used RNA sequencing (RNA-Seq) to identify differentially expressed genes (DEGs) in lung and colon samples from patients succumbing to COVID-19 (44). Compared to normal lung and colon samples, GPR4 mRNA levels were increased by 2.3-fold ($p = 3.04E-06$) and 3.9-fold ($p = 0.0074$), respectively, in COVID-19 patient lung and colon samples. The up-regulation of GPR4 gene expression in COVID-19 patient tissues may further aggravate the GPR4-mediated pro-inflammatory effects and contribute to COVID-19 pathophysiology.

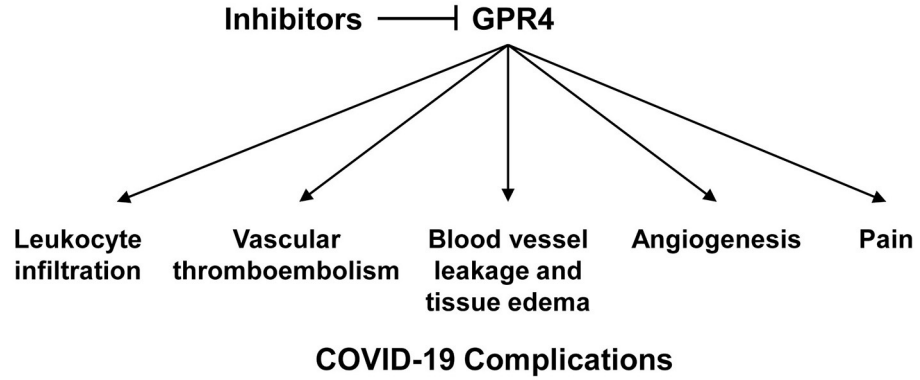

FIGURE 1 | A hypothetical model to depict the roles of GPR4 in COVID-19 pathophysiology and the inhibition of GPR4 as a potential approach to mitigate COVID-19 complications.

Can GPR4 Antagonism Provide Therapeutic Benefits for the Treatment of COVID-19?

Current Treatment Approaches for COVID-19

Current therapeutic modalities for COVID-19 mainly include anti-viral drugs, antibodies, and anti-inflammatory agents. A randomized clinical trial demonstrated that the treatment with the antiviral drug remdesivir accelerates the recovery of hospitalized COVID-19 patients (11 vs. 15 days, compared to placebo, $P < 0.001$) and also shows a trend of survival benefit (a mortality rate by 14 days of 7.1 vs. 11.9%, compared to placebo, $P > 0.05$) (45). However, another randomized clinical trial failed to demonstrate significant therapeutic benefits of remdesivir in patients with severe COVID-19 (46). Transfusion with convalescent plasma containing antiviral neutralizing antibodies demonstrates signs of improvement in critically ill COVID-19 patients. In 35,322 severely ill COVID-19 patients (52.3% in the ICU and 27.5% receiving mechanical ventilation) transfused with convalescent plasma on the Expanded Access Program, a decrease of 30-day mortality was observed in patients transfused within 3 days of COVID-19 diagnosis compared to 4 or more days after diagnosis (21.6 vs. 26.7%, $p < 0.0001$), suggesting a potential benefit of early intervention. Also, the mortality of the patients receiving high IgG plasma was lower than that of the patients receiving low IgG plasma (47). However, a phase II randomized trial with hospitalized, moderately ill COVID-19 patients (235 in the intervention arm and 229 in the control arm) failed to demonstrate any reduction in mortality with convalescent plasma treatment (14.5% in the intervention arm vs. 13.5% in the control arm) (48). In a phase II randomized trial involving outpatients with mild or moderate COVID-19, the SARS-CoV-2 neutralizing antibody LY-CoV555 was shown to decrease viral load and reduce the percentage of patients who had a COVID-19 related hospitalization or emergency department visit (1.6 vs. 6.3%, compared to placebo) (49). In addition to antiviral drugs, convalescent plasma and neutralizing antibodies, studies have evaluated the therapeutic effects of anti-inflammatory agents in COVID-19 because hyper-inflammatory responses (cytokine storm) are observed in some COVID-19 patients. The Recovery trial showed that dexamethasone reduces mortality rate in hospitalized COVID-19 patients within 28 days (22.9% in the dexamethasone group vs. 25.7% in the usual care group, $P < 0.001$), with a trend of more benefits in patients receiving invasive mechanical ventilation or oxygen (50). Tocilizumab, a monoclonal antibody targeting the interleukin-6 receptor (IL-6R), demonstrated clinical benefits in some COVID-19 patients by reducing the hyper-inflammatory responses; however, it was also observed that some patients were refractory to tocilizumab treatment (51, 52). This is likely because a multitude of inflammatory cytokines and molecules are involved in the hyper-inflammatory response. In a recent open-label cohort study of severe COVID-19 patients with systemic hyper-inflammation, blockade of IL-6R with sarilumab did not significantly improve overall clinical outcomes or reduce mortality compared to standard of care, but sarilumab treatment was associated with faster recovery in a subset of patients (53). Overall, current therapeutic approaches for COVID-19 patients with severe disease are not particularly effective. It is critical to fully understand the pathophysiology of COVID-19 and develop more effective therapeutics to significantly reduce COVID-19 mortality and morbidity.

Can GPR4 Antagonism Mitigate COVID-19 Complications?

Recently, GPR4 specific inhibitors have been developed and their biological effects have been characterized (**Table 1**). Derivatives of imidazo-pyridine and pyrazolopyrimidine compounds have been identified as novel GPR4 antagonists (16–18, 54). Studies have shown that GPR4 antagonists reduce inflammation in the antigen-induced arthritis rat model, the DSS-induced acute colitis mouse model, and the short-term emphysema-exacerbation COPD mouse model as well as alleviate inflammatory pain in the complete Freund's adjuvant-induced hyperalgesia rat model (15, 17, 18, 29). In the DSS-induced acute colitis model, GPR4 antagonist 13 (NE-52-QQ57) ameliorates intestinal inflammation and decreases the

TABLE 1 | Biological effects of GPR4 antagonists *in vitro* and *in vivo*.

Experimental models	GPR4 antagonist effects	References
Endothelial cell culture (*in vitro*): microarray, qRT-PCR, and Western blot analyses; endothelial cell adhesion, gap formation, and permeability assays	Inhibiting the expression of inflammatory chemokines, cytokines, adhesion molecules, COX2, NF-κB pathway genes, and ER stress genes in endothelial cells in response to acidosis; Reducing endothelial cell-leukocyte adhesion; Decreasing endothelial paracellular gap formation and permeability.	(12, 13, 16, 20)
Chondrocyte culture (*in vitro*): SW1353 chondrocyte cell line; RT-PCR, Western blot, and ELISA analyses; nitric oxide assay; NF-κB reporter assay	Inhibiting the advanced glycation end products (AGEs)-induced expression of inflammatory molecules such as TNF-α, IL-1β, IL-6, iNOS, nitric oxide (NO), COX2, and PGE2; Inhibiting the expression of matrix metalloproteinase (MMP)-3 and MMP-13; Suppressing the NF-κB pathway.	(24)
Mouse myocardial infarction model (*in vivo*): Kaplan–Meier survival analysis	Prolonging mouse survival in the myocardial infarction model with permanent left anterior descending coronary artery ligation.	(54)
Rat antigen induced arthritis model (*in vivo*): rats sensitized with methylated bovine serum albumin (mBSA)/complete Freund's adjuvant (CFA)	Reducing knee swelling, inflammatory cell infiltration, joint damage, and proteoglycan loss, comparable to the effects of dexamethasone.	(17, 18)
Rat hyperalgesia model (*in vivo*): inflammatory pain induced by CFA	Demonstrating antinociceptive effects, comparable to diclofenac, in the complete Freund's adjuvant (CFA) induced hyperalgesia rat model.	(17, 18)
Mouse angiogenesis model (*in vivo*): porous tissue chambers filled with VEGF	Inhibiting VEGF-induced angiogenesis.	(17, 18)
Mouse and rat cardiorespiratory models (*in vivo*): evaluation of the GPR4 antagonist NE 52-QQ57 on cardiorespiratory effects in rodents	Having no effects on hemodynamics, cerebral blood flow, and blood oxygen level dependent responses in anesthetized rats; Causing a small reduction in the ventilatory response to 5 and 10% CO_2 in awake but not in anesthetized mice and rats; Having no serious adverse effects on cardiovascular and respiratory systems in rodents.	(23)
Mouse colitis model (*in vivo*): DSS-induced colitis model studied by gene expression and histopathologic analyses	Alleviating intestinal inflammation in the DSS-induced colitis mouse model; Attenuating leukocyte infiltration in the colon; Reducing mesenteric lymph node enlargement; Decreasing the expression of VCAM-1, E-selectin, and TNF-α in colon blood vessels.	(15)
Mouse hindlimb ischemia-reperfusion model (*in vivo*): evaluated by gene expression and histopathologic analyses	Suppressing the inflammatory response in mouse hindlimb post the tourniquet-induced ischemia-reperfusion; Reducing tissue edema, inflammatory exudate formation, and leukocyte infiltration; Decreasing the expression of VCAM-1, and E-selectin in the hindlimb tissue post ischemia-reperfusion.	(20)
Mouse COPD model (*in vivo*): porcine pancreatic elastase and lipopolysaccharide induced emphysema-exacerbation model	Attenuating inflammation in the short-term emphysema-exacerbation COPD mouse model; Reducing lung edema and permeability; Decreasing leukocyte infiltration, inflammatory cytokine expression, mucin production, and protease (MMP9 and MMP12) expression in the lung.	(29)

expression of TNF-α in the inflamed mouse colon tissues (15). In the COPD mouse model, treatment with GPR4 antagonist reduces leukocyte infiltration, inflammatory cytokine expression, mucin production, and protease expression in the lung (29). Like GPR4 genetic knockout mice, GPR4 antagonists exhibit anti-angiogenic effects and attenuate inflammatory responses, tissue edema and exudate formation (17, 18, 20). Additionally, GPR4 antagonists reduce the expression of inflammatory chemokines, cytokines, adhesion molecules, NF-κB pathway genes, and stress responsive genes, such as IL-1, IL-8, CXCL1, CXCL2, CCL2, CCL7, VCAM-1, ICAM-1, E-selectin, RELB, COX2, ATF3, and CHOP, in cultured endothelial cells (12, 13, 16). Treatment with the GPR4 antagonist NE-52-QQ57 inhibits the expression of inflammatory molecules including TNF-α, IL-1β, IL-6, iNOS, nitric oxide (NO), COX2, and PGE2 in cultured chondrocytes (24). Furthermore, studies have demonstrated that genetic knockout and pharmacological inhibition of GPR4 protect mice from ischemic injury in the myocardial infarction, renal ischemia-reperfusion, and hindlimb ischemia-reperfusion mouse models (20, 54, 55).

Based on the effects of GPR4 antagonists in other disease models, inhibition of GPR4 can be explored as a novel approach to mitigate COVID-19 complications. GPR4 antagonists potentially target several key aspects of COVID-19 pathophysiology (**Figure 1**). *First*, GPR4 antagonists may inhibit inflammatory responses and leukocyte infiltration in the lung and other affected organs of COVID-19 patients. Hyper-inflammatory responses and massive leukocyte infiltration are observed in COVID-19 patients exhibiting severe disease symptoms (4, 6). Inhibition of GPR4 can suppress the expression of inflammatory adhesion molecules, chemokines, and cytokines in vascular endothelial cells and subsequently decrease leukocyte-endothelium adhesion, extravasation and inflammatory responses (11–20, 29). *Second*, GPR4 antagonists may reduce vascular leakage, tissue edema and inflammatory exudate formation in COVID-19. Increased vascular permeability and disruption of epithelial and endothelial barriers in COVID-19 patients result in fluid accumulation and exudate formation in the lung, with impaired gas exchange and hypoxemia (4, 6). As shown in the hindlimb ischemia-reperfusion, arthritis, and COPD animal models, inhibition of GPR4 can reduce vessel permeability and tissue edema (17, 18, 20, 29). *Third*, GPR4 antagonists may attenuate vascular thromboembolism in COVID-19. Due to coagulopathy, endothelial dysfunction and hyper-inflammatory responses, vascular thromboembolism is a common complication in severely ill COVID-19 patients (31, 32).

Activation of GPR4 increases endothelial cell adhesiveness and blood cell-endothelium interactions (11, 12, 16). Inhibition of GPR4 may lessen inflammatory response, blood cell-endothelium adhesion and aggregation, and thromboembolism (43). *Fourth*, GPR4 antagonists may decrease angiogenesis in COVID-19. While the pathophysiological significance is still unclear, angiogenesis is increased in the lung of COVID-19 patients (32). Inhibition of GPR4 hinders blood vessel formation by modulating the VEGF pathway (17, 18, 56). GPR4 antagonists can potentially curtail angiogenesis in COVID-19. *Fifth*, GPR4 antagonists may alleviate pain associated with COVID-19. Muscle aches, sore throat, headache, and chest pain are common symptoms of COVID-19. GPR4 is expressed in nociceptors such as dorsal root ganglion neurons and consequently aggravates inflammatory pain (17, 18, 57). Inhibition of GPR4 can potentially mitigate inflammatory pain in COVID-19 patients.

DISCUSSION

SARS-CoV-2 infection in the lung and other organs cause cellular injury and inflammatory responses in COVID-19 patients (4, 6, 10). Clinical manifestations of COVID-19 range widely from asymptomatic carriers to severe disease and death. Based on the current incomplete understanding of COVID-19 pathophysiology, therapeutic strategies have been directed toward anti-viral, anti-inflammatory, and anti-coagulatory agents. The applications of remdesivir, convalescent plasma, dexamethasone, tocilizumab, and low molecular weight heparin have achieved limited success in severely ill COVID-19 patients (4, 6). Because various factors are involved in COVID-19 pathophysiology, combination therapy targeting both the SARS-CoV-2 virus and the host inflammatory response may be required to achieve optimal treatment outcomes. A better understanding of COVID-19 pathophysiology will help develop novel therapeutic approaches.

We hypothesize that GPR4 plays an integral role in COVID-19 pathophysiology and inhibition of GPR4 can be explored as a novel approach to mitigate COVID-19 complications. G protein-coupled receptors (GPCRs) are the largest family of cell surface receptors that serve as pharmacological targets of ∼34% of all FDA approved drugs (58). GPR4 antagonists have recently been developed and characterized. Consistent with its pro-inflammatory function, GPR4 inhibition by its antagonists alleviates inflammation, edema, and pain in preclinical disease models (15, 17, 18, 20, 29). To evaluate the potential therapeutic effects of GPR4 antagonists in COVID-19, the inhibitors can first be tested in preclinical animal models predisposed to infection with SARS-CoV-2, such as the human angiotensin-converting enzyme 2 (hACE2) transgenic mice and hamsters (59, 60). In addition to inflammatory responses, the effects of GPR4 antagonists on other COVID-19 complications such as blood vessel permeability, lung edema, vascular thromboembolism, and pain can also be evaluated in these preclinical animal models.

With regard to the safety profile and adverse effects of the GPR4 antagonists, an oral dose of 30–100 mg/kg (b.i.d.) is well tolerated in preclinical animal models without overt adverse effects (15, 17, 18, 20, 23, 29). The optimized GPR4 antagonist 13 (NE 52-QQ57) has no documented serious adverse effects on the cardiovascular and respiratory systems in mouse and rat models (23). Specifically, the GPR4 antagonist 13 (NE 52-QQ57) is selective for the GPR4 receptor and has no or minimal effects on other proton-sensing GPCRs or the common off-targets such as the H3 receptor and hERG channel (17). GPR4 antagonist 13 (NE 52-QQ57) does not affect hemodynamics, blood oxygen level dependent responses, or cerebral blood flow in rodents (23). It causes a slight reduction in the ventilatory response to 5 and 10% CO_2 in non-anesthetized but not in anesthetized mice and rats (23). Moreover, phenotypic observations from GPR4 knockout mice indicate several facets of GPR4 functions. A small percentage of GPR4-null mice exhibit perinatal complications (61). Upon acid overload, GPR4-null mice have slightly decreased renal acid excretion (26). GPR4 is also involved in carbon dioxide chemosensing (62). Deletion of GPR4 is associated with lower blood pressure, lower binding to angiotensin II receptor, and increased insulin sensitivity (63, 64); these aspects are of particular interest as hypertension and diabetes are risk factors associated with COVID-19 mortality (4). The functional characteristics from knockout studies should be closely monitored when GPR4 antagonists are applied *in vivo*, although the biological effects from genetic knockout are not necessarily identical to pharmacological inhibition. Overall, the GPR4 antagonists exhibit a good pharmacological profile and oral bioavailability in preclinical animal models, providing a foundation for therapeutic evaluation in COVID-19 disease models.

Due to the complex pathophysiology of COVID-19, combination therapy is likely needed to achieve optimal treatment outcomes in COVID-19 patients with severe disease. In this respect, there are several strategies to apply GPR4 antagonists in combination with other therapeutic agents. One strategy is to combine GPR4 antagonists with anti-viral agents such as remdesivir to target both SARS-CoV-2 replication and the host hyper-inflammatory responses. Another strategy is to combine GPR4 antagonists with other anti-inflammatory agents such as dexamethasone, of which GPR4 antagonists target the endothelium-leukocyte interactions and dexamethasone targets immune cells. These strategies can be assessed in preclinical COVID-19 animal models and eventually patients. In summary, our central hypothesis is that GPR4 is a pro-inflammatory receptor involved in COVID-19 pathophysiology and GPR4 antagonists, whether as a single therapeutic agent or in combination with other agents, can be explored as a potential therapeutic approach to mitigate COVID-19 complications and may also find applications in other related diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

LY conceived the project and drafted the manuscript. KO, MT, MM, SN, and JM contributed to valuable intellectual discussions and manuscript revision. All authors contributed to the article and approved the submitted version.

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COVID-19 and Italian Healthcare Workers From the Initial Sacrifice to the mRNA Vaccine: Pandemic Chrono-History, Epidemiological Data, Ethical Dilemmas, and Future Challenges

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On March 11, 2020, the World Health Organization (WHO) declared the coronavirus disease 2019 (COVID-19) outbreak a pandemic. Simultaneously, in Italy, in which the first case had occurred on February 18, the rigid phase of the lockdown began. The country has attracted worldwide attention, becoming at the same time a field of study both concerning the spread of the pandemic and advanced assessments of the effectiveness of political, public health, and therapeutic measures. The protagonists of the Italian crisis were the healthcare workers (HCWs) who were exposed to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) without having any perception of what they were facing, courageously contributing to the containment of the epidemic to be defined by the media as "heroes." However, in the first phase of the pandemic (March–May 2020), the price that the Italian Public Health System had to pay both in terms of the number of positive virus cases and deaths among the HCWs was beyond and represented a peculiarity compared to what happened in other countries. In the current study, after a summary of the evolution of the pandemic in Italy, we offer an analysis of the statistical data concerning contagions and deaths among healthcare workers (physicians in particular). In conclusion, we describe the critical issues that still need to be resolved and the future challenges facing healthcare workers and the general population.

Keywords: COVID-19, COVID-19 healthcare workers, COVID-19: specialties of dead doctors, COVID-19 future challenges, COVID-19 mRNA vaccine, COVID-19 Italian physician's positivities and deaths, COVID-19 ethical dilemmas, COVID-19 HCWs deaths

INTRODUCTION: EPIDEMIC CHRONO-HISTORY AND THE EVOLUTION OF THE ITALIAN SCENARIO

The first domestic case of COVID-19 was detected on February 21 in a 38-year-old man from Lombardy (1). Thereafter, the local epidemic expanded rapidly to the neighboring areas with an estimated basic reproduction number (R0) of between 2.43 and 3.10 (2). A difference in terms of incidence began to emerge between the Northern and the Southern regions of Italy.

Different hypotheses have been proposed to explain this inhomogeneous distribution of cases from a demographic, geographic, and genetic perspective (3–5). Although the Italian Government-mandated containment restriction extended to all national territories on March 11, on March 19, Italy overtook China in the number of deaths due to coronavirus disease 2019 (COVID-19) (3,405) and was (temporarily) the country with the most deaths due to the disease.

Early epidemic phases in Italy were characterized by widespread unpreparedness of the National Healthcare System (NHS) for a similar large-scale event [such as ICU beds, personal protective equipment, and healthcare workers (HCW)]. These NHS shortcomings led HCWs to apply a very selective triage procedure to patients requiring invasive respiratory support to decide who to "treat" with the best available means and who to "palliate" based on the highest probability to survive. In the attempt to unburden attending physicians of the weight of their ethical and deontological decisions, the more prominent Italian Scientific Society in the Intensive Care context (SIAARTI) drew up a recommendation addressing the fair allocation of scarce medical resources (6, 7).

Likely, a profound and irreparable health crisis was avoided by the lockdown, the advent of new therapies, and the widespread distribution of PPE among staff. In contrast, later phases were initiated with a progressive increase in daily recovering people and appeared in conjunction with better knowledge about viral features and an increase in the availability of medical resources.

The progressive containment of the pandemic has been achieved through the establishment of a rigid lockdown (March 9–May 3, 2020; Italian Phase I) followed by a phase of mitigation of the measures (May 4–June 14; Italian Phase II), and finally, from 15 June, the phase of coexistence with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). However, the increase in the number of cases led to new restrictive measures in November 2020 (**Figure 1**).

Pandemic and restrictive measures have led to important economic and social changes in the country (8–10).

The report released on 25 November 2020 describes 1,454,529 confirmed cases, 49,931 deaths, and 66,618 cases for COVID-19 in healthcare workers (HCWs) (Istituto Superiore di Sanita Epidemia COVID-19).

EPIDEMIOLOGICAL DATA: THE SACRIFICE OF ITALIAN FAMILY DOCTORS

Description of the Data Source

An important factor to consider for understanding the impact of COVID-19 on the health system is the percentage of COVID-infected HCWs. The dimension of the phenomenon regarding the number of affected and deceased health professionals can be obtained by consulting various data sources. The general data concerning the Italian population's data in general as regards the number of positive individuals, the number of deaths, and the number of positive health workers were obtained through the data provided by the "Istituto Superiore di Sanità" (ISS) (11). The data concerning the work subcategories were obtained by analyzing the data of the "National Institute for Accident Insurance" (INAIL), the Italian Insurance Institute that awards workers in the event of accidents and occupational diseases. As far as health is concerned, employee workers in public or private structures are protected by the Institute (12). Unfortunately, some figures relevant to public health, such as general practitioners, are not covered by the institution.

The data concerning the deaths of doctors are instead obtained from the archive of the "Federazione Nazionale degli Ordini dei Medici Chirurghi e degli Odontoiatri" (FNOMCeO). This archive appears to be the most complete and reliable because anyone practicing the profession in Italy must register. As of January 1st, 2020, there were 403,454 members. During the pandemic, the FNEOMCEO reported the name of every Italian doctor that has died, whether employed or freelance, and the data related to the type of specialization (13).

COVID-19 and HCWs in Italy: The Report by INAIL

Data from ISS daily reports say HCWs made up 12% of positive patients in July (14). The percentage value will change if we consider the post-lockdown period (June–September period), during which healthcare professionals made up 4.5% of positive patients nationwide (11). The INAIL report of 30 September 2020 showed that out of a total of 54,128 complaints about COVID-19, 70.3% (around, 38,052 cases) concerned the "Health and Social Assistance" sector. The subanalysis of the data showed that the most affected professionals were "health technicians" (nurses, midwives, podiatrists, physiotherapists, speech therapists, orthopedists–ophthalmology assistants, neuro- and psychomotor therapists of developmental age, psychiatric rehabilitation technicians, professional educators, occupational therapists) with 39.2% of the total cases followed by qualified professions in health and social services (social health workers) (20%), doctors (10.1%), and unskilled personnel (auxiliaries, stretcher-bearers) (4.7%). Among the remaining categories, social assistance operators (careers) stand out, accounting for 8.9% of cases.

The data set showed a peculiar temporal and geographical trend (15, 16). Most of the positive cases and deaths for all sectors occurred between February and May. Similarly, the cases of COVID mainly affected the regions of the northwest (55.1%), followed by the northeast (24.4%), the center (11.9%), the south (6.2%), and from the islands (2.4%). The subanalysis carried out on the category of physicians showed slightly different data, with 67% of cases concerning the northern regions, 20% concerning the center, 9% the south, and 4% the islands. The positivity among the category of doctors concerning the global computation of INAIL complaints went from 10.3% (March–May) to 5.7% (June–September).

Another aspect investigated is that of mortality: in fact, the report describes 319 fatal cases due to COVID-19 (about one-third of the deaths reported since the beginning of the year and one incidence of 0.9% compared to the total of national deaths from COVID-19 communicated by the ISS as of September 30); of these, 35.7% died in March, 54.5% in April, 6.0% in May, 1.6%

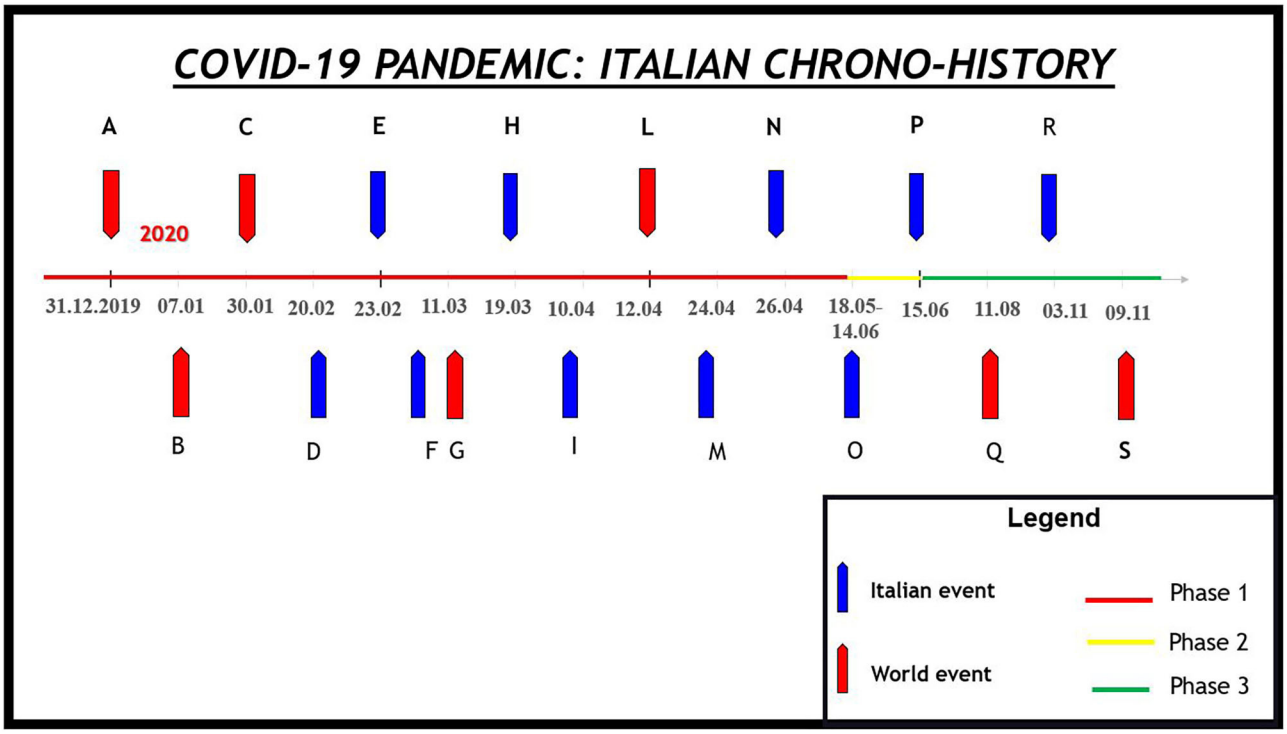

FIGURE 1 | The coronavirus disease 2019 (COVID-19) pandemic Italian chrono-history. **(A)** The Chinese Health authority informs the WHO about 41 patients with mysterious pneumonia. **(B)** Chinese authorities identify a new type of coronavirus. **(C)** The WHO declares a global public health emergency. **(D)** The first case of COVID-19 in Italy. **(E)** The Italian government extends restrictions on mobility and assembly of the person in restricted regions of the country. **(F)** The WHO declares COVID-19 a pandemic. **(G)** The Italian government extends restrictions on mobility and assembly of persons across the whole nation: the Italian lockdown began. **(H)** Italy becomes the world leader in COVID-19 deaths. **(I)** The Italian lockdown is extended until May 4, 2020. **(L)** For the first time in history, Easter Mass is celebrated without worshipers to prevent contagion. The mass was shared on various communications routes (to avoid what happened in the Black Plague). **(M)** For the first time, the number of recovered patients was higher than the number of new cases. **(N)** The Italian government announces the end of the first phase of the pandemic and the beginning of a second deal from May 4, 2020. **(O)** Italian Phase II. Easing of restrictive measures. **(P)** Italian Phase III. Period of coexistence with the SARS-CoV2. **(Q)** Russia became the first country to approve a vaccine against severe acute respiratory syndrome coronavirus 2. **(R)** Announcement of the effectiveness of a vaccine produced by an American pharmaceutical company. **(S)** New restrictive measures (including national night curfew) differentiated according to the situation in the different regions.

in June, 1.9% in July, 0.3% in August, and no cases reported in September.

The analysis by geographic origin shows a distribution of deaths of 56.7% in the northwest (Lombardy, 41.7%), 13.8% in the northeast (Emilia Romagna, 9.7%), by 11.6% in the center (Lazio, 4.7%), by 16.0% in the south (Campania, 7.2%), and of 1.9% in the islands (Sicily, 1.9%). The provinces with the most deaths are Bergamo (11.6%), Milan (8.2%), Brescia (7.8%), and Naples (6.0%). The analysis by profession of the injured person shows that about one-third of deaths concerns health and social assistance personnel. In detail, the more categories affected by the deaths are those of health technicians (58% are nurses), with 9.5% of codified cases and doctors with 6.9%, followed by socio-health workers with 5.1%, non-qualified personnel in health services (auxiliary, porters, stretcher-bearers) with 3.6%, and social welfare workers with 3.3%, and finally the specialists in the life sciences (toxicologists and pharmacologists) with 2.2%.

A very recent study considered the number of deaths from COVID-19 on the entire population of HCWs in 37 countries. The number of deaths in Italy was 0.35 per 100,000, second only to Mexico (0.9/100,000) and Azerbaijan (0.44/100,000) (17, 18). At present, it has not yet been investigated why the ratio of deaths to total workers regarding Italy is among the highest in the world (17).

However, the variables for explaining this difference can be divided into two main categories: (1) those that occurred when exposure to the SARS-CoV-2 among healthcare professionals had not yet been described and (2) those that emerged after the state of emergency became clear (18).

About physicians, in a first pre-emergency phase (during which there was a total unawareness of the importance of COVID-19 outbreak on public health), some medical fields were more penalized than others (e.g., those with a high number of contacts or those requiring the execution of procedures involving the formation of aerosols).

According to EUROSTAT statistical data, it appears that Italian doctors hold the European record with regards to age, with an average age of 55 years. A further reflection is possible if we

compare the data with states such as Germany (GE) or Austria (AT), in which the average age of the population is equal or higher than the Italian population. According to the report, in Italy, the percentage of those over 65 years old was 18.1%, between 55 and 64 years old was 37.7%, while that of over 35 years old was 8.6%. In Germany and Austria, the over 65-year-olds accounted for 6.4 and 6.1%, those in the 55–64-year-old age group 38.5 and 25.4%, while the under 35-year-olds for 20.7 and 18.7%, respectively. The health policies of the last decade, characterized by the lengthening of the retirement age and the hiring freeze, have resulted in the average age of doctors in the national health service moving from 50.8 years in 2010 to 52.9 years in 2017 (19).

At the onset of the epidemic, the disease's high transmissibility was underestimated, and therefore, the use of suitable PPE was not strongly recommended. Simultaneously, due to the lack of knowledge on transmission routes, the need for specific recommendations made it necessary to apply guidelines for previous coronaviruses, such as the Middle East respiratory syndrome coronavirus (MERS-CoV) and SARS-CoV-2, which have different characteristics (20, 21).

Although these indications have proven to be useful for COVID-19, measures should be updated in accordance with recent data. Indeed, unlike other coronaviruses, SARS-CoV-2 can be transmitted from asymptomatic patients (22).

Another problem was the initial lack of knowledge of the transmission routes of SARS-CoV-2. SARS-CoV-2 can probably remain in an aerosol suspension for up to 16 h (23). Moreover, fecal–oral (24) and ocular (25) routes might also be crucial in limiting the diffusion of the ongoing pandemic even though they are not fully understood. In the next phase, when the state of emergency became evident, the numerous previously observed variables were combined with others, such as the initially limited availability of PPE, the low rate of staff turnover (due to the shortage of collaborators), and the failure to adapt medical liability to the moment of emergency to facilitate the use of emerging therapies (26, 27).

COVID-19 and HCWs in Italy: The FNOMCeO Report: Differences Between Public and Private Physicians

A recent document from the Federazione Nazionale degli Ordini dei Medici Chirurghi e degli Odontoiatri (FNOMCeO), the national federation of Italian medical doctors and dentists, provided data on the deaths during the epidemic with data on the specialization of each deceased physician (13, 28). These data are only apparently in contradiction with those of INAIL previously provided; for many Italian doctors (for example, general practitioners, freelancers), they are not protected by this Institute or continue to work privately after retiring as public employees (**Figure 2**).

The most affected active categories were those of general practitioners (GPs) and dental practitioners (DPs). Regarding GPs, it is possible to postulate that these figures are due to the high number of accesses. Especially in February–March, the ordinary PPE supplies were insufficient to deal with SARS-CoV-2.

Specifically, for GPs, the scarce use (due to shortages of supplies) of individual protection devices and intensive exposure to biological hazards might have played a role. In the case of dentists, the production of aerosols during the procedures carried out, and the lack of interventions for environmental sanitation between one intervention and another, could have played a role.

DISCUSSION AND FUTURE CHALLENGES

Although the situation has improved at present, the near future presents several challenges for Italian HCWs. The first challenge is returning to pre-emergency activities; even though the number of infections has decreased, it has not yet reached zero. A return to activities as before could lead to a spread of the virus. For this reason, it is essential to strive for greater knowledge of the virus that would allow the application of adequate preventive and sanitization techniques on which there is still no shared strategy. However, there have been interesting and valid proposals (29).

A second critical point is represented by the possibility of pharmacologically preventing the disease, especially in the event of a new epidemic wave. Unfortunately, due to the decrease in the number of infected people on which to test a vaccine, an effective vaccine has not yet been put in place nor has it been ascertained that immunization is possible. Even the trials on chemoprophylaxis and the results of the application of this strategy were not encouraging. However, it is necessary to consider that HCWs exposed to high biological risks can represent this virus's source (30–32). One of the most topical issues concerns the use of vaccines produced up to now. From August to today, there have been announcements on the discoveries of various vaccines, some proteins (Gam-COVID-Vac), and others for the first time in the history of "genetic" type to mRNA (MRNA-1273 and BNT162b). Especially for the latter category, no long-term safety data are currently available. This point raises ethical and moral questions, especially if we consider that this category of vaccine is being used for the first time and that HCWs—as a high-risk category and potential source of the outbreak—could be required to have compulsory vaccination for access to work (33–35).

Until effective prophylactic protocols are elaborated, the continuous adaptation of the guidelines based on the knowledge of the virus's characteristics is essential to minimize the biological risks (36–38).

The spread of the pandemic has given rise to important ethical and medicolegal dilemmas (38). In fact, in the first phase of the pandemic, due to the high biological risk, no or limited autopsies were carried out. This has contributed to slowing down the accumulation of knowledge on the effects of the disease and the therapeutic management of patients. Knowledge of the pathogenesis and its consequences will also be important to evaluate any permanent damage reported by the HCWs in carrying out their work.

Another aspect concerns informed consent and visits. In current clinical practice, consent is extended to each patient who accesses a visit with questions about possible contacts and symptoms attributable to COVID-19. Another aspect concerns

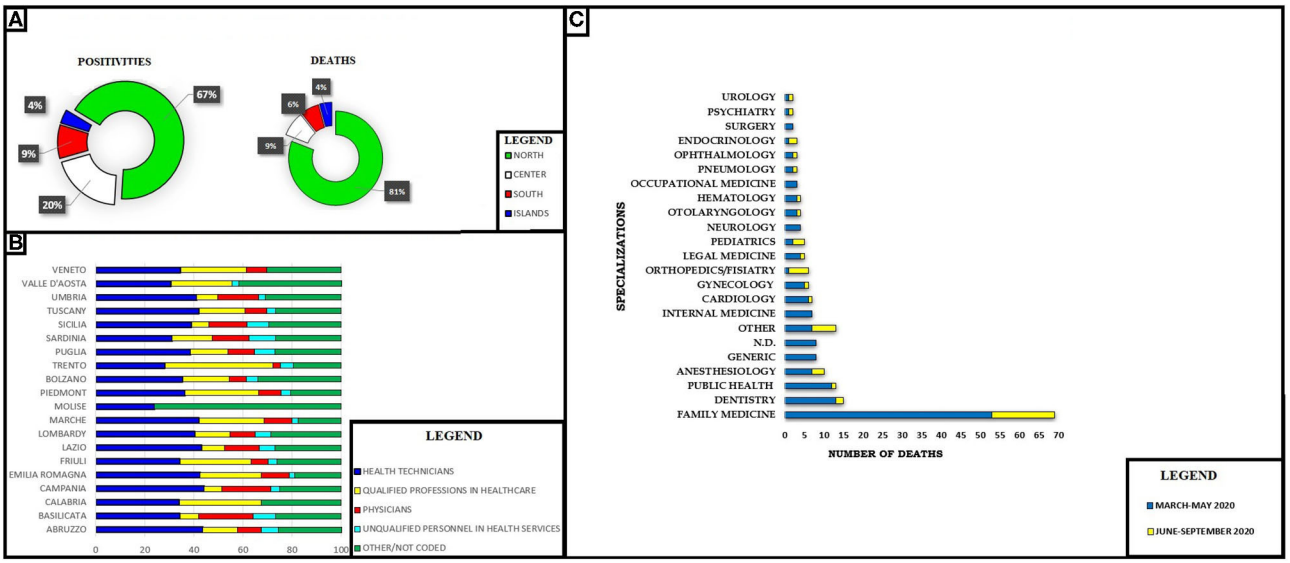

FIGURE 2 | (A) Coronavirus disease 2019 (COVID-19) and Italian physician's positivities and deaths. North: Valle d' Aosta, Emilia Romagna, Friuli Venezia Giulia, Liguria, Lombardy, Piedmont, Veneto, Trento, Bolzano. Center: Abruzzo, Lazio, Marche. South: Puglia, Basilicata, Calabria, Campania, Tuscany, Umbria Islands: Sardinia, Sicily. **(B)** COVID-19 positivities and occupational sector in Italian regions. **(C)** Number of deaths for specialization. N.D., not declared; Other, thermal medicine, radiology, penitentiary medicine, homeopathy, geriatrics, bioethics.

the development of telemedicine for which remote evaluations have been developed, the carrying out of which was unthinkable until 2019 (39).

A last but very important problem for Italian HCWs is a professional responsibility. Until July 2020, Italy remained one of the few countries in the world not to have provided a criminal shield for those who provided healthcare during the epidemic, especially in the first period (40–42).

Class action suits against doctors, healthcare facilities, and Italian HCWs have been taken and advertised, and this battle is on two fronts: (1) the one against SARS-CoV-2 not yet finished and (2) the one in court that will probably start soon. In particular, in the current medicolegal practice, requests for evaluations are frequent, not so much for fatal cases linked to COVID-19 but rather for delays and omissions due to the "state of emergency."

CONCLUSION

The battle between the Italian Healthcare Workers and COVID-19 has been characterized by highly criticality moments and has resulted in a high number of infections and deaths. The emergency, which underlines the fragility of a state-of-the-art health system, such as the Italian system, cannot be considered complete despite the great progress in the number of infected people, intensive care patients, and deaths. Among the critical points highlighted are the need to acquire further knowledge about the virus, of validating shared sanitation techniques for the resumption of daily health activities, and of developing prevention techniques.

An Italian peculiarity is represented by the need to approve a penal shield, which is also present in other countries and would allow HCWs to work with peace and security regarding medical liability, even in times of crisis.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://portale.fnomceo.it/elenco-dei-medici-caduti-nel-corso-dellepidemia-di-covid-19/; https://www.inail.it/cs/internet/docs/alg-scheda-tecnica-contagi-covid-30-settembre-2020.pdf.

AUTHOR CONTRIBUTIONS

MN, Ed'A, and PN conceived of the presented idea. MF and JL developed the theory. MN wrote the manuscript in consultation with Ed'A, PN, JL, and MF. All authors contributed to the article and approved the submitted version.

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OPINION
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Vitamin D Deficiency as an Important Biomarker for the Increased Risk of Coronavirus (COVID-19) in People From Black and Asian Ethnic Minority Groups

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INTRODUCTION

Ever since the new 2019 coronavirus disease (COVID-19) was first identified in Wuhan, Hubei province of China its spread has become a global pandemic affecting almost every country worldwide. World Health Organization (WHO) has confirmed more than 71 million cases of COVID-19 and over 1,620,000 deaths globally (until 16 December 2020) (1), and the numbers are increasing rapidly.

There is growing evidence to suggest that people from Black (mostly African) and Asian (mostly South Asians/South East Asians) ethnic groups are disproportionately affected by COVID-19, leading to poorer outcomes (higher mortality and morbidity) compared to White British or Americans (2–4). Public Health England (in August 2020) reported that Black people are 2–3 times more likely to be infected with COVID-19 compared to White people after adjusting for age (5). A study from 260 hospitals across England, Scotland and Wales found that people from Black and South Asian backgrounds were, 36 and 28% respectively, more likely to be admitted for critical care, after adjusting for age, gender and deprivation of area lived (6). Data from the intensive care units showed that people from Black and Asian ethnic groups accounted for more than 25% of all COVID-19 admissions (until end of July 2020) (7), despite comprising only about 11% of the total population of the UK. COVID-19 related deaths within Black and Asian ethnic groups working in the health care settings in the UK was even higher (63%) (8, 9).

In Chicago, USA, more than 50% of the total COVID-19 cases and nearly 70% of COVID-19 related deaths were reported in Black people, although they comprised only about 30% of Chicago's total population (10). Centers for Disease Control and Prevention reported that the rate of coronavirus (COVID-19) infection was 2.6 times higher, hospitalization 4.7 times higher and deaths 2.1 times higher in Black/African Americans compared to White (Non-Hispanic) Americans (11). In Asians, the rate of coronavirus infection reported was 1.1 times higher and hospitalization 1.3 times higher compared to White (Non-Hispanic) Americans.

Various reasons have been offered to explain why people from Black and Asian ethnic minority groups are more at risk of coronavirus infection and mortality. These include socio-demographic factors, underlying heath issues, overcrowded households, living in deprived areas, difficulty in health care access due to language barriers, unhealthy lifestyles, and performing "higher-risk" frontline healthcare or essential work (9). However, research suggests that even after adjusting for age, gender, lifestyles, socio-economic factors, language barriers, self-reported health/disability conditions, people from Black and Asian ethnic groups were still more likely to be infected and die from COVID-19 than White people (12, 13). In the UK, data show that COVID-19 related

deaths were 1.9 times higher in Black people and 1.6–1.8 times higher in Asians compared to White people, after adjusting or age, socio-economic characteristics and self-reported health/disability measures (13).

In exploring these health and social determinants of inequality in ethnic minorities, differences in other factors such as low levels of Vitamin D have not been addressed adequately. Vitamin D deficiency poses a potential risk factor for COVID-19. Vitamin D deficiency is identified as a risk factor in older age, diabetes, obesity, and hypertension (14, 15) which are significantly associated with COVID-19 (16). Recent studies showed negative correlations between mean vitamin D levels and COVID-19 cases across different European countries including Spain, Italy, and Switzerland and in the US (17, 18). Although it is appreciated that correlations do not suggest causality, these findings cannot be discounted. There are number of limitations and methodological differences in these studies. Ile et al. (17) is an ecological study reporting only crude associations, and findings may be limited by the fact that the number of positive cases are directly affected by the proportion of COVID-19 tests performed, which may vary between countries. In Kaufman et al. (18) study, vitamin D data were obtained within the preceding 12 months and hence may not all be up-to-date. Also, it is likely that findings from the study may not be representative of the general population as participants who took part belonged to certain priority groups such as those who had symptoms, had come in contact with people who had tested positive or who belonged to the "high-risk" categories for COVID-19 infections.

Significant ethnic variations in the gene GC that encodes Vitamin D binding protein (DBP) (protein that circulates Vitamin D/metabolites in the blood) have been reported. Black people and Asians are more likely to carry the GC1F variant of this (GC) gene, which has been associated with low DBP levels, and lower synthesis and metabolism of Vitamin D (19). On the other hand, white people are more likely to carry the GC1S variant in whom higher DBP levels are generally observed (20).

It is known that darker skin in Black people and Asians can lead to a lower concentration of vitamin D in the blood as the increased melanin in their skin reduces the absorption of sunlight needed to produce vitamin D (21, 22). It is likely that lower exposure to sunlight, for example, with cultural attire, may also contribute to reduced vitamin D concentration as would more time spent indoors during lockdown.

Serum 25-hydroxyvitamin D level of <50 nmol/L (20 ng/mL) is classified as Vitamin D deficiency in adults (23). In Europe, people from the dark-skinned ethnic background were found to be more at risk of vitamin D deficiency compared to white counterparts (22). Vitamin D deficiency has also been reported in infants, adults and pregnant women of Asian families living in the UK (24–26).

Vitamin D supplementation could reduce the risk of influenza and COVID-19 infections and mortality (27) by reducing the viral replication rates and expression of pro-inflammatory cytokines which injure the lining of the lungs, leading to pneumonia, thereby providing a protection against COVID-19 (28). Therefore, it is important vitamin D deficiency should not be overlooked as an important risk factor for COVID-19 in Black and Asian ethnic groups in whom vitamin D deficiency is more prevalent.

It is important to acknowledge that the effect of vitamin D deficiency on COVID-19 and its outcomes can be confounded by obesity, that in itself poses additional risk for viral infections, their progression and recovery. Vitamin D deficiency has been shown to be higher in obese individuals. A systematic review and meta-analysis, published in 2015, shows a 35% higher prevalence of vitamin D deficiency in obese individuals (29). In addition, obesity has been shown as an additional risk for other viral infections such as from H1N1 and influenza A with delayed recovery time (30, 31).

The affected immune system in COVID-19 is thought to play an important role in obesity-induced adipose tissue inflammation and metabolic dysfunctions such as diabetes, hypertension, and cardiovascular disease (32). These underlying conditions, known to be significant risk factors for COVID-19 complications, are more prevalent in people from Asian countries including India, Pakistan, and Bangladesh (33). The role played by body mass index (BMI) in COVID-19 was shown by data from China (34), in which 88% of people who did not survive had a higher BMI (>25 kg/m^2) compared to 19% who survived, suggesting obesity poses a significant additional risk for COVID-19 infection and its progression.

Vitamin D has been stipulated as a risk factor in other virus infections in people from ethnic minorities. A cross-sectional study of vitamin D levels in 200 HIV-infected patients in south-central US (Houston, Texas) found that nearly two-thirds (64%) of patients were vitamin D deficient, and that African-American (in whom HIV infection was more prevalent) were over three times (odds ratio = 3.53) more likely to have vitamin D deficiency compared to White Americans (35). In the UK, data from 1077 HIV patients, showed that 73.5% of patients had vitamin D deficiency, with Black patients 3 times more likely to be deficient (36). In Spain, a hospital-based study showed that HIV patients from non-Caucasian background were 3.18 times more likely to have vitamin D deficiency than from Caucasian background (37).

Ethnic differences in vitamin D levels are also reported in patients infected with Hepatitis B virus (HBV) and Hepatitis C Virus (HCV). A study on African American and White Americans infected with HCV found that vitamin D deficiency was significantly greater in African Americans (44%) compared to White Americans (15%) (38). A recent systematic review and meta-analysis (39) of seven studies reported significantly reduced vitamin D levels in patients infected with HBV than in healthy controls, with the highest reduction in Indian patients (40).

One study by Hastie et al. (41), reported lack of evidence on the potential link between vitamin D levels and the risk of COVID-19 infection in people from Black and Asian ethnic groups. The baseline data on vitamin D levels, ethnicity, underlying health conditions, socioeconomic status, etc. of participants enrolled in the UK Biobank (between 2006 and 2010) were examined against those participants who tested positive for COVID-19 in 2020. However, a number of limitations in the study need to be taken into account. The data on Vitamin D and health status were obtained a decade ago and these were then examined for participants who tested positive in 2020. It is likely

that these might have changed significantly over the course of 10 years. In addition, their analysis is based on only 32 Black people, 19 south Asians and 13 people from other ethnicities who were hospitalized with COVID-19 over a month (between 16 March and 17 April 2020, short time frame). We argue that this is not representative of the general population and more research on larger numbers is needed.

CONCLUSION

Evidence suggests vitamin D deficiency plays an important role in the high rate of infection and mortality of COVID-19 in Black and Asian ethnic minority groups, but more research is needed to confirm this. This should be a priority for future research including large clinical trials in order to better understand the vulnerability of these ethnic groups and ascertain the effectiveness of using vitamin D supplements to reduce the risk of COVID-19 infection, severity and mortality. A number of trials have led the way and examined the role of vitamin D or its analogs/metabolites [e.g., calcitriol, calcifediol, 1,25(OH)2D3] in preventing and treating COVID-19 (42–44). Findings suggest that calcitriol exhibits significant potent activity against the coronavirus infection (42), and a high dose of Calcifediol/25-hydroxyvitamin D reduces the need for intensive care treatment (43). Another trial showed that patients who received vitamin D had improved clinical recovery as evidenced by shorter lengths of hospital stay, lower oxygen requirements, and reduced inflammatory markers (44). Whilst promising, there are a number of limitations to these findings including small sample sizes and selected cohorts such as people who were hospitalized. It is also not clear whether vitamin D analogs/metabolites would benefit people at an earlier stage of the disease. The possible role of obesity was not considered (43) and the effects on ethnicity has not been examined in detail on a larger sample of people (44). While these results are encouraging, larger trials are needed to draw firmer conclusions. Obviously, other health and social determinants influencing the high risk of COVID-19 facing Black people and Asians should not be overlooked.

AUTHOR CONTRIBUTIONS

SP and RS drafted the manuscript. SP, RS, and LS revised the manuscript. All authors contributed to the article and approved the submitted version.

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Using Health Insurance Network Provider Data and Public Data Sets to Identify SARS-CoV-2 Vaccinators in the USA

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Objective: Mass vaccination planning is occurring at all levels of government in advance of regulatory approval and manufacture of a SARS-CoV-2 vaccine for distribution sometime in 2021. We outline a methodology in which both health insurance provider network data and publicly available data sources can be used to identify and plan for SARS-CoV-2 vaccinator capacity at the county level.

Methods: Sendero Health Plans, Inc. provider network data, Texas State Board of Pharmacy data, US Census Bureau data, and H1N1 monovalent vaccine data were utilized to identify providers with demonstrated capacity to vaccinate the population in Travis County, Texas to achieve an estimated SARS-CoV-2 herd immunity target of 67%.

Results: Within the Sendero network, 2,356 non-pharmacy providers were identified with 788 (33.4%) practicing in primary care and 1,569 (66.6%) practicing as specialists. Of the total, 686 (29.1%) provided at least one immunization between January 1, 2019 and September 30, 2020. There are 300 pharmacies with active licenses in Travis County with 161 (53.7%) classified as community pharmacies. We estimate that 1,707,098 doses of a 2-dose SARS-CoV-2 vaccine series will need to be administered within Travis County, Texas to achieve the estimated 67% herd immunity threshold to disrupt person-to-person transmission of the SARS-CoV-2 virus based on 2020 census data.

Conclusion: A community-based health insurance plan can use data from its provider network and public data sources to support the CDC call to action to identify SARS-CoV-2 vaccinators in the community, including physicians, nurse practitioners, physician assistants, and pharmacies in order to provide macro level estimates of SARS-CoV-2 administration and throughput.

Keywords: SARS-CoV-2, COVID-19, vaccination, mass vaccination, Sendero Health Plans, vaccinators

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INTRODUCTION

The US Centers for Disease Control and Prevention (CDC) released guidance on August 31, 2020 outlining the nationwide process for distributing and administering the SARS-CoV-2 vaccine (1). On September 16, 2020 the CDC released an interim "playbook" to guide jurisdictional operations on vaccine distribution and administration (2).

The timeline for implementing this guidance is aggressive and reflects the need to prepare for and enable both logistical and operational components of vaccine distribution in advance of regulatory approval and vaccine manufacture. The logistical and operational components of vaccine distribution are well-established for childhood vaccines; however, these processes have not been applied to vaccine distribution on the scale, magnitude, and timeframe needed to achieve vaccine-induced herd immunity envisioned for the COVID-19 pandemic. Indeed, the closest comparison for an expedited large-scale vaccine distribution network to providers was during the novel H1N1 influenza pandemic of 2009 when 80.1 million doses of the monovalent H1N1 vaccine were distributed nationally, representing a nationwide monovalent vaccine coverage rate of 27.0% (3, 4).

The challenges related to vaccine distribution and administration of the SARS-CoV-2 vaccine should not be underestimated. Among the many challenges are: (1) distribution of vaccine quantities on a scale never attempted in the United States; (2) the likely need for two-dose administration of the vaccine; (3) an estimated coverage rate of 67.0% needed to achieve vaccine-induced herd immunity (which is nearly 2.5 times higher than the monovalent H1N1 vaccine coverage achieved in 2009) (3); and (4) inherent limitations on provider enrollment and capacity. (There are other logistical and operational challenges related to cold chain management and storage, particularly requirements related to ultracold storage at −70°C for the CDC labeled "Vaccine A"; however, cold chain management and storage is not within the scope of this paper.)

This policy paper reviews the challenges related to vaccine distribution and administration with a focus on identifying SARS-CoV-2 vaccinators in the community. Further, it outlines how managed care provider network data from a community-based health insurance plan can be used to assist public health officials to identify existing community providers with a demonstrated capacity to support SARS-CoV-2 mass vaccination activities. Concepts identified in this policy paper will be illustrated using Sendero Health Plans, Inc. (Sendero) provider network data, Texas State Board of Pharmacy data, US Census Bureau data, and H1N1 monovalent vaccine data.

BACKGROUND

Implementing a mass vaccination strategy is a complicated process. Currently, state and local health departments across the United States are considering using a combination of open and closed Points of Distribution (POD) sites, mobile immunization teams for vulnerable populations, and private clinics and pharmacies to support mass vaccination activities. With regard to the latter, CDC guidance advises jurisdictions to identify providers in the community who can provide vaccination services when expanded quantities of vaccine are available beyond that required for critical workforce populations (5). Centers for Disease Control and Prevention guidance notes that jurisdictions should establish and build upon existing relationships, including with "health insurance issuers" to identify SARS-CoV-2 vaccinators (2).

Abbreviations: CDC, Centers for Disease Control and Prevention, US; COVID-19, Corona Virus Disease, 2019; CPT, Current Procedural Terminology; H1N1, Hemagglutinin Type 1 and Neuraminidase Type 1 influenza straing; NDC, National Drug Code; POD, Point of Distribution; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2.

METHODOLOGY

A five step methodology is outlined to estimate quantities of vaccine that a jurisdiction can be expected to provide to achieve herd immunity and to identify potential SARS-CoV-2 vaccinators in the community (see **Figure 1**). These steps include:

1. Estimating herd immunity
2. Estimating vaccine coverage rates
3. Implementing the SARS-CoV-2 vaccine schedule
4. Identifying providers with demonstrated capacity to administer the SARS-CoV-2 vaccine
5. Estimating vaccine throughput

Estimating Herd Immunity

Herd immunity is the level of protection against a pathogen that must be attained in the population to disrupt person-to-person transmission of a virus (6). It is a population health metric that is often associated with success or failure of a vaccination campaign. Reaching the herd immunity threshold therefore allows for protection against the virus at the population level, even for those who are unable to be immunized (e.g., because of medical contraindications or because they are immunocompromised). One estimate of herd immunity for the SARS-CoV-2 virus is 67.0% (6).

From a technical perspective herd immunity is the sum of naturally acquired immunity and vaccine induced immunity in a given population. The level of vaccine-induced immunity required for a particular population is therefore dependent on the level of naturally acquired immunity achieved during community transmission of the virus. For this paper we will assume that herd immunity and vaccine coverage for SARS-CoV-2 are equal because, while rigorous in its estimation, a precise herd immunity threshold cannot be calculated until the basic reproduction number (R_0) is confirmed.

Estimating Vaccine Coverage Rates

Estimating vaccine coverage is a function of both past experience and the likely expectations and assumptions for the future. The only comparison example of a recent large scale, nationwide, mass vaccination campaign using the private provider network occurred during the 2009/10 H1N1 pandemic. As such, coverage rates during that incident can provide a baseline expectation of SARS-CoV-2 vaccine coverage. Additionally, assumptions about the current pandemic can provide a guide for expected coverage. For example, the demand for the SARS-CoV-2 vaccine will likely be high because of the reported morbidity and mortality associated with COVID-19 and because elected leaders and health officials have noted that herd immunity is

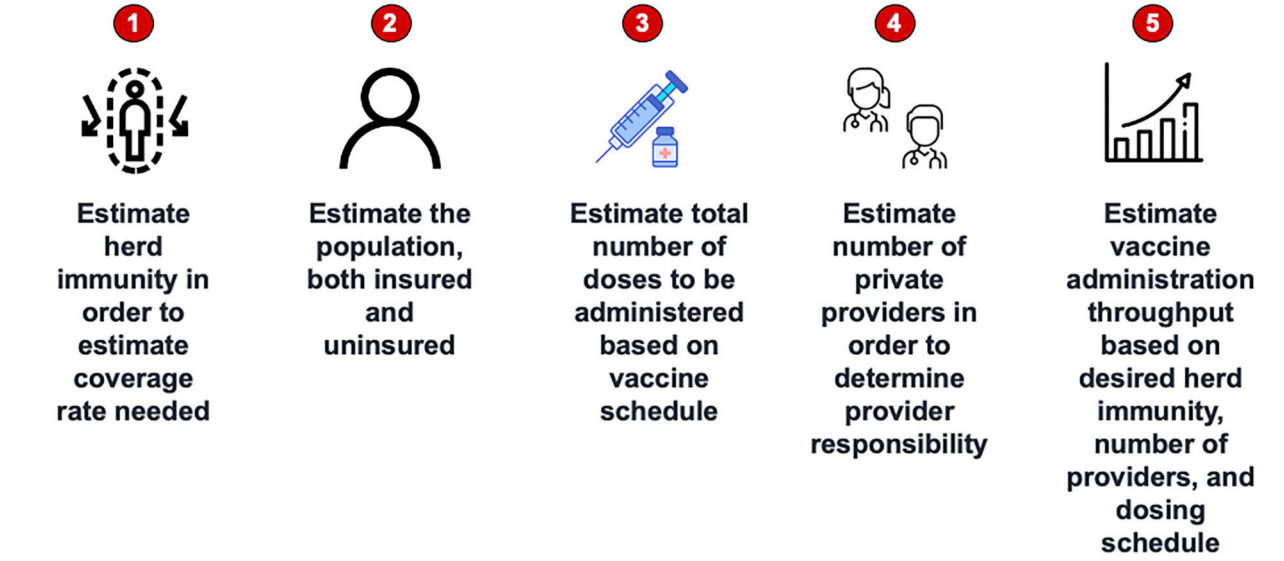

FIGURE 1 | Model to estimate vaccine quantity and providers.

necessary before economic and social activities can return to pre-COVID-19 levels.

Nationally, the CDC estimated that 80.1 million (27.0%) persons aged 6 months and older received the monovalent H1N1 vaccine in 2009/10 (3). Coverage varied across age and risk factor cohorts with persons aged 25–64 years having the lowest rate of vaccine coverage at 16.7% (17.8 million doses) and children aged 6 months−17 years having the highest rate of vaccine coverage at 40.2% (29.1 million doses). At the county level, the Texas Department of State Health Services distributed 326,095 doses of the monovalent H1N1 vaccine to providers in Travis County, Texas through August 3, 2010 (7). Based on the 2010 Travis County population of 1,024,266 (8) the estimated monovalent H1N1 vaccine coverage rate for provider administered vaccine was 31.8%. An additional 32,300 doses were distributed to the local health department in Austin, Texas (9). Vaccine distribution data provided by the Texas Department of State Health Services does not account for unused, spoiled, expired, or wasted H1N1 vaccine. Therefore, the numerator is more accurately a measure of distributed vaccine, not administered vaccine, and may serve to overestimate vaccine coverage in the population when distribution data is used as a proxy for vaccine administration.

Implementing the SARS-CoV-2 Vaccine Schedule

Current CDC planning guidance outlines two potential vaccine scenarios. Both scenarios involve vaccines that have a 2-dose schedule (10). Vaccine Scenario A denotes a 2-dose series to be administered 21 days apart using an ultracold vaccine that requires storage at −70°C, thawing, and reconstitution before administration. Vaccine Scenario B denotes a 2-dose series to be administered 28 days apart with refrigeration between 2 and 8°C, no on-site mixing, and administration within 7–14 days of refrigeration. No 1-dose scenario has been outlined by the CDC. Distribution and administration of Vaccine A will likely be limited to healthcare professionals, essential workers, and long-term care facility staff and residents (10).

From a practical perspective, this means that each person will receive two-doses of the SARS-CoV-2 vaccine. This doubles the volume of vaccine that must be distributed, stored, and administered when compared to the H1N1 single dose monovalent vaccine. The increased quantity of SARS-CoV-2 vaccine will have storage implications at the provider level and will require appropriate staffing levels to maintain vaccine administration throughput. In addition, the primary and secondary dose must be matched by vaccine manufacturer, presenting additional logistical requirements for ordering and storage.

Identifying COVID-19 Vaccination Providers

Jurisdictions are advised to develop a network of trained, competent, and accessible providers as part of the overall mass vaccination strategy. Centers for Disease Control and Prevention guidance advises jurisdictions to recruit—among others—doctor's offices, pharmacies, and occupational health settings of large employers (to develop closed PODs). This is largely a renewal of the successful strategy used during H1N1 to expand the number and locations of providers to vaccinate as many people as possible. Explicit in this guidance is that jurisdictions should build upon relationships with existing partners, including health insurance companies, to identify potential SARS-CoV-2 vaccinators. To assist in this call to action,

Sendero used its provider network to identify potential SARS-CoV-2 vaccinators within Travis County, Texas. Providers were identified as follows:

1. We identified all providers and pharmacies in the Sendero provider network who are eligible to submit a claim for vaccine administration.
   a. Providers included doctors of medicine, doctors of osteopath, nurse practitioners, and physician assistants.
   b. Providers were further classified as either a specialist or a primary care provider based on pre-identified designations.
2. Of the providers identified in (1) we queried the Sendero claims database to determine how may of these providers administered at least one vaccine based on CPT codes 90460, 90461, 90471, 90472, 90473, 90474.
3. Pharmacies were identified based on Texas State Board of Pharmacy Data. Pharmacies with active licenses were reviewed to determine if they are open to the public for retail services and prescriptions (i.e., community pharmacies). Establishments identified as a community pharmacy were queried in the Sendero claims database to determine if they administered at least one vaccine based on a National Drug Code (NDC) for any vaccine.
4. The date of service for all Sendero data queries was from January 1, 2019 through September 30, 2020.

For data reporting, some data are stratified by individuals with health insurance and without health insurance. Stratifying by health insurance coverage status is designed to more accurately represent likely throughput by provider type. Indiviudals with health insurance are more likely to have a medical home and to use procedures associated with their medical home to obtain the SARS-CoV-2 vaccine when available. Those without health insurance typically do not have a medical home and are likely to utilize public health services to obtain the SARS-CoV-2 vaccine. The SARS-CoV-2 vaccine will be free for consumers at the point of service regardless of health insurance status or place of service. Providers in Texas will be allowed to charge an administration fee based on the state Medicaid schedule.

A total of 2,356 non-pharmacy providers were identified in the Sendero network (see **Table 1**) Primary care providers represented 787 (33.4%) of those identified and specialists represented 1,569 (66.6%). Among the primary care providers, 538 (68.4%) were designated as vaccinators based on submitting at least one claim for any type of immunization during the study period. Among specialists 148 (9.4%) were similarly designated as vaccinators. In total, 686 (29.1%) Sendero providers are classified as vaccinators while 1,670 (70.9%) are classified as non-vaccinators. **Table 2** shows the number of primary care providers who provided at least one immunization during the study period stratified by degree type.

The Texas State Board of Pharmacy reports 300 active, licensed pharmacies in Travis County (11). A review of this data indicates that slightly more than half ($n = 161$; 53.7%) of these licensed pharmacies are open to the public for the purposes of dispensing medicaitons and providing pharmaceutical care. The remaining 139 pharmacies include specialty, compounding, hospital, and government pharmacies, all of whom do not dispense medications directly to the public. Of the 161 community pharmacies in Travis County, 93 (57.8%) administered at least one vaccine to a Sendero member during the observation period, with 87 of these pharmacies classified as a chain community pharmacy (i.e., CVS/pharmacy, Costco, HEB, Randall's, Walgreen's, Walmart, and Sam's Club). Six independent community pharmacies in Travis County dispensed at least one medication to a Sendero member during the study period.

TABLE 1 | Sendero provider network of physicians, nurse practitioners, and physician assistants stratified by either specialist or primary care provider type and whether the provider administered at least one vaccine from January 1, 2019 to September 30, 2020.

Provider type	Immunizer	Non-immunizer	Total
Primary care provider*	538 (68.4%)	249 (31.6%)	787
Specialist	148 (9.4%)	1,421 (90.6%)	1,569
Total	686 (29.1%)	1,670 (70.9%)	2,356

**Primary care provider include doctors of medicine, doctors of osteopath, nurse practitioners, and physician's assistants.*

TABLE 2 | Sendero provider network for physicians, nurse practitioners, and physician assistants stratified by degree type and whether the provider administered at least one vaccine from January 1, 2019 to September 30, 2020.

Degree	Immunizer	Non-immunizer	Total
DO	51	19	70
MD	420	96	516
NP	52	106	158
PA	16	28	44
Total	539*	249	788

DO, Doctor of Osteopath; MD, Medical Doctor; NP, Nurse Practitioner; PA, Physician's Assistant.

The primary care provider immunizer total in **Table 1 ($n = 538$) does not match the primary care provider immunizer total in **Table 2** ($n = 539$) because one provider reports two degrees (NP and PA) in the Sendero Network Master Provider List.*

Estimating Vaccine Throughput

Using the methodology outlined above vaccine throughput by potential SARS-CoV-2 vaccinators can be estimated. The CDC Vaccine Scenario B is used in these estimates because it more accurately represents the technical specifications of the vaccine likely to be distributed to providers and pharmacies. **Table 3** outlines throughput using the 686 providers identified by Sendero data using different levels of immunity within the community for those with health insurance. **Tables 4** and **5** outline throughput using public health operations (i.e., PODs or mobile vaccination teams) for those without health insurance. Throughput for pharmacies is not included as we do not have enough data to make meaningful estimates; however, it is likely that community pharmacies, particularly chain community pharmacies, will have capacity and will participate as SARS-CoV-2 vaccinators.

TABLE 3 | Estimated number of doses of the SARS-CoV-2 vaccine to be administered to persons with health insurance and the mean number of doses to be administered over a 4 week period by providers who have a demonstrated capacity to provide vaccines based on data analysis of the Sendero provider network for five different levels of immunity that could be achieved in the community.

	Level of immunity to be achieved in the community				
Variable	**40%**	**50%**	**60%**	**67%***	**70%**
A. 2020 estimated population for Travis County Texas	1,273,954	1,273,954	1,273,954	1,273,954	1,273,954
B. Proportion of people without health insurance	14.8%	14.8%	14.8%	14.8%	14.8%
C. Number of people without health insurance	188,545	188,545	188,545	188,545	188,545
D. Number of people with health insurance	1,085,409	1,085,409	1,085,409	1,085,409	1,085,409
E. Proportion of people to obtain vaccine based on desired level of herd immunity	434,164	542,704	651,245	727,224	759,786
F. Vaccine schedule (doses)	2	2	2	2	2
G. Total number of doses to be administered to people with health insurance	868,327	1,085,409	1,302,491	1,454,448	1,519,572
H. Number of vaccinators (physician, nurse practitioner, physician's assistant)	686	686	686	686	686
I. Mean number of doses to be administered by each vaccinator to meet desired level of herd immunity for people with health insurance	1,266	1,582	1,899	2,120	2,215
J. Mean number of doses to be administered in Round 1	633	791	949	1,060	1,108
K. Mean number of doses to be administered in Round 2	633	791	949	1,060	1,108
L. Mean number of doses administered per hour in each round	4	5	6	7	7

**67% is the estimated herd immunity level for the SARS-CoV-2 virus.*

A. Based on US Census Data; B. Based on US Census Data; C. Calculated as the product of (A) times (B); D. Calculated as the difference of (A) minus (C); E. Calculated as the product of (D) times the desired level of herd immunity to be achieved in the community; F. Based on CDC guidance; G. Calculated as the product of (E) times (F); H. Calculated based on the number of doctors, nurse practitioners, and physician assistants who administered at least one vaccine to Sendero members between January 1, 2019 and September 30, 2020; I. Calculated as the quotient of (G) divided by (H); J. The mean number of doses to be administered in Round 1 is the quotient of (I) divided by two; K. The mean number of doses to be administered in Round 2 is the quotient of (I) divided by two; L. Each round is allocated 160 h (four weeks) for vaccine administration. The mean number of doses administered per hour is the quotient of (J) or (K) divided by 160.

TABLE 4 | Estimated number of doses of the SARS-CoV-2 vaccine to be administered to persons without health insurance for five different levels of immunity that could be achieved in the community.

	Level of immunity to be achieved in the community				
Variable	**40%**	**50%**	**60%**	**67%***	**70%**
A. Number of people without health insurance	188,545	188,545	188,545	188,545	188,545
B. Proportion of people to obtain vaccine based on desired level of herd immunity	75,418	94,273	113,127	126,325	131,982
C. Vaccine schedule (doses)	2	2	2	2	2
D. Total number of doses to be administered to people without health insurance	150,836	188,545	226,254	252,650	263,963

**67% is the estimated herd immunity level for the SARS-CoV-2 virus.*

*A. Calculated from **Table 3** line (C); B. Calculated at the product of (A) times the desired level of herd immunity to be achieved in the community; C. Based on CDC guidance; D. Calculated as the product of (B) times (C).*

DISCUSSION

One or more SARS-CoV-2 vaccines are expected to be approved by regulatory authorities with subsequent manufacturing and distribution in 2021. Centers for Disease Control and Prevention guidance lays out a process for these activities. Guidance documents note that successful implementation of a mass vaccination campaign will be highly dependent on identifying SARS-CoV-2 vaccinator capacity in the community and that "if a jurisdiction has a good understanding of its (SARS-CoV-2) vaccination providers and locations and their vaccine administration capacities, then planners can generate rough estimates of (SARS-CoV-2) vaccination capacity in their jurisdiction (2)."

Partnering with a health insurance company that provides coverage within the jurisdiction of interest can help identify vaccinator capacity. In the methodology outlined above, knowing the types of providers in the community and being able to validate that they have demonstrated capacity based on claims data can assist local officials in understanding who within the community is likely to contribute to mass vaccination activities.

Having access to data from a past pandemic response can also provide a guide to community capacity. In 2009/10 326,095 doses of the monovalent H1N1 vaccine were distributed to providers in Travis County, Texas achieving a vaccine coverage

TABLE 5 | Estimated number of doses of the SARS-CoV-2 vaccine to be administered to persons without health insurance and the mean number of doses to be administered over a 4 week period by Points of Distribution sites ($N = 3$) for five different levels of immunity that could be achieved in the community.

Variable	Level of immunity to be achieved in the community				
	40%	50%	60%	67%*	70%
A. Total number of doses to be administered to people without health insurance	150,836	188,545	226,254	252,650	263,963
B. Number of POD	3	3	3	3	3
C. Mean number of doses to be administered by each POD to meet desired level of herd immunity for people without health insurance	50,279	62,848	75,418	84,217	87,988
D. Mean number of doses to be administered in Round 1	25,139	31,424	37,709	42,108	43,994
E. Mean number of doses to be administered in Round 2	25,139	31,424	37,709	42,108	43,994
F. Mean number of doses administered per hour in each POD	157	196	236	263	275

**67% is the estimated herd immunity level for the SARS-CoV-2 virus.*

*A. Calculated from **Table 4** line (D); B. Estimated number of PODs that could be established; C. Calculated as the quotient of (A) divided by (B); D. The mean number of doses in to be administered in Round 1 is the quotient of (C) divided by two; E. The mean number of doses to be administered in Round 2 is the quotient of (C) divided by two; F. Each round is allocated 160 h (four weeks) for vaccine administration. The mean number of doses administered per hour is the quotient of (D) or (E) divided by 160 h.*

rate of 31.8%. For the current pandemic, 1,454,448 doses for those with health insurance and 252,650 doses for those without health insurance are estimated to be administered to achieve a SARS-CoV-2 herd immunity rate of 67% using a 2-does vaccine schedule. For those with health insurance, this represents nearly four times the quantity of vaccine distributed a decade ago. This is an enormous increase in volume and represents a seismic shift in daily operations for private providers. While the overall vaccination period will continue for many months, once the first dose is provided to a group of individuals, the second dose will need to be provided 28-days apart, representing a minimum 8 week period from start to finish for any vaccine cohort. Indeed, the increase in volume represents over 1 million additional doses to be administered via private providers in Travis County, Texas as compared to 10 years ago.

Some of these doses can also be administered by pharmacies, of which there are 161 commmunity pharmacies in Travis County, Texas. The remaining 139 include compounding, specialty, hospital, and governmental pharmacies. Sendero members preferentially used chain community pharmacies to obtain vaccines during the study period ($n = 87$; 93.5%). Such utilization patterns may reflect ease of access, geographical preference, population density, and general changes in the marketplace that tend to favor chain community pharmacies over independent community pharmacies (12). A detailed analysis of Sendero member pharmacy preference to obtain a vaccine has not been conducted. However, there is general recognition that both independent and chain community pharmacies play a role in supporting public health services like immunizations (12).

It is also important to remember that providers with demonstrated capacity are those who have evidence of vaccine administration, are able to manage the cold-chain storage of vaccines in their clinic or pharmacy, and have the administrative processes and staff in place to record vaccination activity in state immunization registries. State level guidance that suggests vaccinator surge capacity is simply a magnitude of order increase above current vaccine for children providers is naïve and should be viewed with caution as such estimates do not account for cold chain storage capacity or demonstrated experience in vaccine administration. Realistic guidance will, at a minimum, consider the five steps outlined above.

In addition, many potential vaccinators are already at capacity for conducting routine business. While most will naturally want to do their part, it is not possible to estimate the extent of surge capacity for individual clinical practices or pharmacies. The questions then must be asked: (1) how and where will additional staff be recruited to support surge capacity for SARS-CoV-2 vaccination?; (2) what are the plans to train allied health providers to serve as SARS-CoV-2 vaccinators beyond the already established local community capacity?; and (3) what plans and support are available to provide increased vaccine storage capacity at individual clinics or pharmacies to accommodate increased vaccine volume?

Now is the time to prepare the infrastructure and operational activities for SARS-CoV-2 vaccination. The methodology outlined above provides a macro level estimate of vaccinator capacity, the number of doses to be administred, and throughput based on different delivery methods. At the macro level, the stakes are clear—over one million doses of vaccine will need to be administerd in Travis County, Texas in a short period of time in order to disrupt person-to-person transmission of this virus.

LIMITATIONS

This study has several limitations. Firstly, while our provider network data indicate which provider has administered a vaccine between January 1, 2019 and September 30, 2020, we do not know provider vaccine storage capacity, workforce capacity to administer the vaccine, or administrative staff

capacity to record vaccine data in state immunization databases as required by the CDC; any limitations in these variables will likely reduce throughput. Secondly, the Sendero provider network does not represent all physicians within Travis County, so it is possible that there is additional SARS-CoV-2 vaccination capacity unaccounted for in this study; however, it would be naïve to think that all currently licensed providers will become SARS-CoV-2 vaccinators, particularly those who do not typically provide vaccines. Thirdly, the data in this paper estimates capacity based on assumed business practices. However, such capacity is subject to change as has been demonstrated by medical office closures for non-emergent procedures during the pandemic. Finally, calculations are based on the analysis using structures applied to a specific country and local jurisdiction within that country.

This study does not address vaccine efficacy. Such information continues to be released as vaccine candidates progress through clinical trial and regulatory processes. Furthermore, this model does not consider infection-induced herd immunity, due to surveillance variability at the national, regional, and local level. Therefore, it is possible that persons who experience asymptomatic disease may choose to receive the vaccine. That said, infection-induced immunity will contribute, along with vaccine-induced immunity, to overall herd immunity within the community.

This study also does not address vulnerability characteristics, social dynamics, and inequity associated with vaccine uptake. Our focus was to create a method to identify likely sources of vaccinator capacity with demonstrated experience to vaccinate the population of a large urban center in the United States. Future work is needed to better understand the dynamics associated with vaccine uptake across the population spectrum within a large urban center in the United States.

CONCLUSION

A community-based health insurance plan can use data from its provider network and public data sources to support the CDC call to identify SARS-CoV-2 vaccinator capacity in the community, including physicians, nurse practitioners, physician assistants, and pharmacies. A model is proposed that can be used to develop population estimates of expected quantities of the SARS-CoV-2 vaccine to be received, distributed, and administered at the macro level to achieve different levels of vaccine-induced immunity. This model illustrates the importance of data on the operational and logistical components in preparation of the SARS-CoV-2 mass vaccination campaign.

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 project is exempt from Institutional Review Board approval because it does not report on personal health information of Sendero members.

AUTHOR CONTRIBUTIONS

JL, RT, NT, and WD substantial contribution to study conception. JL, RT, and NT design of the work, data acquisition or analysis, and interpretation of data. JL and RT drafted work or substantially revised it. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: WD is employed by Sendero Health Plans. The remaining authors are contractors with Sendero Health Plans.

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SYSTEMATIC REVIEW
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Serum Prealbumin Concentrations, COVID-19 Severity, and Mortality: A Systematic Review and Meta-Analysis

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Excessive inflammation and malnutrition are associated with coronavirus disease 2019 (COVID-19) severity and mortality. Combined biomarkers of malnutrition and inflammation, such as serum prealbumin, might be particularly attractive for early risk stratification. We conducted a systematic review and meta-analysis of studies reporting serum prealbumin in patients with COVID-19. We searched PubMed, Web of Science and Scopus, between January and November 2020, for studies reporting data on serum prealbumin, COVID-19 severity, defined as severe illness, prolonged viral load, receiving mechanical ventilation or admitted to intensive care unit (ICU), and mortality. Nineteen studies in 4,616 COVID-19 patients were included in the meta-analysis. Pooled results showed that serum prealbumin concentrations were significantly lower in patients with severe disease and non-survivors (standard mean difference, SMD, −0.92, 95% CI, −1.10 to −0.74, $P < 0.001$). Extreme heterogeneity was observed ($I^2 = 77.9\%$; $P < 0.001$). In sensitivity analysis, the effect size was not significantly affected when each study was in turn removed (range between −0.86 and −0.95). The Begg's ($P = 0.06$) and Egger's t-tests ($P = 0.26$) did not show publication bias. Pooled SMD values were significantly and negatively associated with age ($t = -2.18$, $P = 0.045$) and C-reactive protein ($t = -3.85$, $P = 0.002$). In our meta-analysis, lower serum prealbumin concentrations were significantly associated with COVID-19 severity and mortality. This combined marker of malnutrition and inflammation might assist with early risk stratification and management in this group.

Keywords: prealbumin, COVID-19, disease severity, mortality, biomarker

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INTRODUCTION

Coronavirus disease 2019 (COVID-19), the condition responsible for the current global pandemic, is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). COVID-19 severity and adverse clinical outcomes are positively associated with the presence of excessive systemic inflammation and immune response, and consequently with oxidative stress, coagulation disorders, and multiorgan failure (1–3). There is also increasing evidence that patients with more severe forms of COVID-19 are at risk of malnutrition, and that malnutrition itself is associated with adverse clinical outcomes (4). Pending the development of effective vaccines and in the absence of effective pharmacological treatments, with the exception of the glucocorticoid dexamethasone

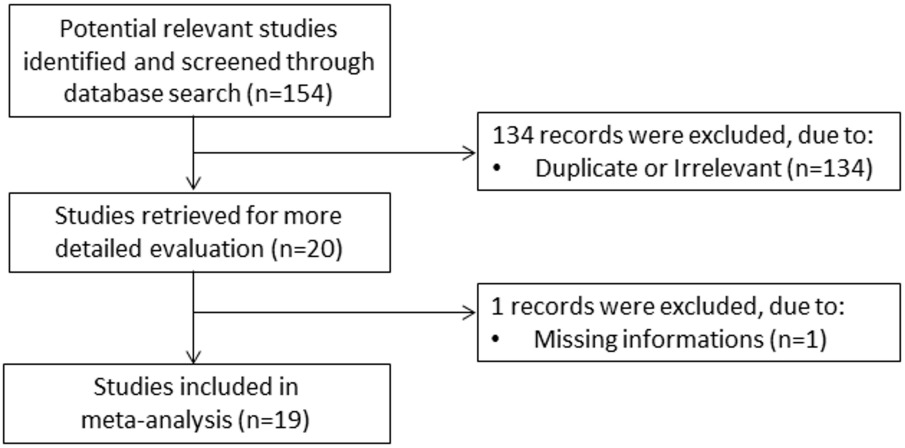

FIGURE 1 | Flow chart of study selection.

(5, 6), the identification of early markers of disease severity would assist with the appropriate selection of COVID-19 patients that benefit from intensive treatment and monitoring. This would also streamline specific care pathways, with positive effects on resource management and health care costs. Given the pathophysiological role of inflammation and nutrition in patients with COVID-19, markers that provide a combined assessment of these processes might be particularly useful in terms of predictive capacity and appropriate clinical decisions. The protein prealbumin, also known as transthyretin, is a negative acute phase-reactant produced in the liver that acts as a transport protein for thyroxine and is used as a marker of nutrition. Compared to albumin, prealbumin has a relatively short half-life, between 2 and 3 days vs. 20 days, is catabolized in the kidney and is not significantly affected by the presence of protein-losing enteropathy (7–9). Serum prealbumin concentrations <10 mg/dL have been shown to be associated with malnutrition, hospital length of stay, and mortality in other disease states (10–12). Given the capacity of low serum prealbumin concentrations to indicate the presence of a systemic inflammatory state and/or malnutrition, we conducted a systematic review and meta-analysis of the available evidence on the clinical implications of this protein specifically in patients with COVID-19.

METHODS

Search Strategy, Eligibility Criteria, and Study Selection

We searched, using the terms "prealbumin" or "transthyretin" and "coronavirus disease 19" or "COVID-19," the electronic databases PubMed, Web of Science, Scopus, and Google Scholar, between January and November 2020, to identify peer-reviewed studies reporting serum prealbumin concentrations, measures of COVID-19 severity, specifically the presence of clinically severe illness, prolonged viral load, need for mechanical ventilation or admission to intensive care unit (ICU), and mortality. The references of the articles identified were also searched for additional studies. Eligibility criteria were as follows: a) reporting continuous data regarding serum prealbumin concentrations in COVID-19, (b) investigating COVID-19 patients with different disease severity or clinical outcomes, particularly mortality, (c) investigating adult patients, (d) written in English, and (e) full-text available. Two investigators independently screened the abstracts. If relevant, they independently reviewed the full articles. The Newcastle-Ottawa scale was used to assess study quality by evaluating the cohort selection, cohort comparability on the basis of the design or analysis, how the exposure was determined and how the outcomes of interest were evaluated. Studies with a score of ≥6 indicated high quality (13).

Statistical Analysis

Standardized mean differences (SMD) and 95% confidence intervals (CIs) were calculated to build forest plots of continuous data and to evaluate differences in serum prealbumin concentrations between COVID-19 patients with low vs. high disease severity or survivor vs. non-survivor status. A $P < 0.05$ was considered statistically significant. If concentrations were reported as median and interquartile range (IQR), the corresponding mean and standard deviation were calculated (14). Between-study heterogeneity in SMD values was assessed using the Q-statistic ($P < 0.10$ indicated significance). Inconsistency across studies was assessed using the I^2 statistic ($I^2 < 25\%$ indicated no heterogeneity; I^2 between 25 and 50%, moderate heterogeneity; I^2 between 50 and 75%, large heterogeneity; and $I^2 > 75\%$, extreme heterogeneity) (15, 16). A random-effects model was used, in the presence of high heterogeneity, to calculate the pooled SMD values and 95% confidence intervals. The influence of each study on the overall effect size estimate was investigated using sensitivity analysis, by sequentially excluding one study at a time (17). The associations between study size and magnitude of effect were analyzed using the Begg's adjusted rank correlation test and the Egger's regression asymmetry test, at the $P < 0.05$ level of significance, to assess the presence of potential publication bias (18, 19). The Duval and Tweedie "trim and fill" procedure was used to further test the effect of publication bias (20). This method recalculates a pooled SMD

TABLE 1 | Characteristics of the selected studies in COVID-19 patients, according to disease severity and survival status.

References	Study design	Outcome	NOS (stars)	Milder disease or survival				Severe disease or death			
				n	Age (Years)	Gender (M/F)	Prealbumin mg/dL (Mean ± SD)	*n*	Age (Years)	Gender (M/F)	Prealbumin mg/dL (Mean ± SD)
Chen Z et al. (22)	R	Severe Non-severe	6	615	54	282/333	19.2 ± 10.3	221	63	129/92	10.9 ± 6.5
Duan J et al. (23)	R	Severe Non-severe	6	328	44	170/158	22.7 ± 7.7	20	58	14/6	14.8 ± 5.4
Feng X et al. (24)	P	Good outcome Pooroutcome*	7	94	63	58/36	12.0 ± 4.5	20	69	13/7	11.9 ± 7.3
Fu HY et al. (25)	R	Severe Non-severe	3	33	40	NR	23.9 ± 7.4	4	66	NR	15.2 ± 6.9
Gao C et al. (26)	R	Prolonged load Non-prolongedload**	3	63	59	26/37	13.7 ± 8.7	49	68	25/24	10.1 ± 4.7
Guo J et al. (27)	R	Survivor Non-survivor	6	43	60	22/21	17.8 ± 9.8	31	68	21/10	9.2 ± 5.0
Ji M et al. (28)	R	Severe Non-severe	6	70	NR	NR	15.3 ± 6.9	51	NR	NR	12.1 ± 55
Li G et al. (29)	R	ICU Non-ICU	6	312	49	131/181	18.3 ± 7.3	211	62	119/92	14.7 ± 6.2
Li L et al. (30)	P	Severe Non-severe	6	60	51	NR	18.3 ± 5.4	12	45	NR	10.8 ± 3.2
Li T et al. (31)	R	Survivor Non-survivor	7	66	NR	NR	21.3 ± 5.2	9	NR	NR	11.4 ± 6.0
Luo Y et al. (32)	NR	Survivor Non-survivor	6	986	59	476/510	21.9 ± 7.7	129	70	87/42	13.7 ± 4.9
Sun L et al. (33)	R	Severe Non-severe	7	40	40	23/17	21.0 ± 3.9	15	67	8/7	13.0 ± 6.4
Wu C et al. (34)	R	ARDS NoARDS	7	117	48	68/49	13.5 ± 5.2	84	59	60/24	10.2 ± 3.8
Xue G et al. (35)	NR	Severe Non-severe	4	56	61	30/26	17.5 ± 9.2	58	64	34/24	9.8 ± 6.2
Xue J et al. (36)	P	Prolonged load Non-prolongedload**	6	35	42	23/12	19.7 ± 6.5	13	61	6/7	16.4 ± 8.3
Yang P et al. (37)	R	Severe Non-severe	4	65	41	32/33	21.8 ± 5.7	68	60	40/28	7.0 ± 10.4
Zhang XY et al. (38)	R	Severe Non-severe	6	89	66	35/54	13.8 ± 5.5	21	71	17/4	8.3 ± 3.6
Zhang Y et al. (39)	R	Severe Non-severe	6	84	44	29/55	20.4 ± 8.2	31	65	20/11	12.2 ± 7.4
Zhao X et al. (40)	R	Severe Non-severe	6	346	59	175/171	15.6 ± 8.1	67	65	37/30	10.4 ± 5.4

ARDS, acute respiratory distress syndrome; ICU, intensive care unit; Non-severe, patients with mild or moderate disease; NOS, Newcastle-Ottawa quality assessment scale; NR, not reported; P, prospective; R, retrospective; Severe: patients with severe or criticaldisease.

**Patients that were discharged, those with non-severe condition, and those not requiring mechanical ventilation were considered to have a good outcome. Patients requiring mechanical ventilation and those who died were considered to have a pooroutcome.*

***Viral clearance.*

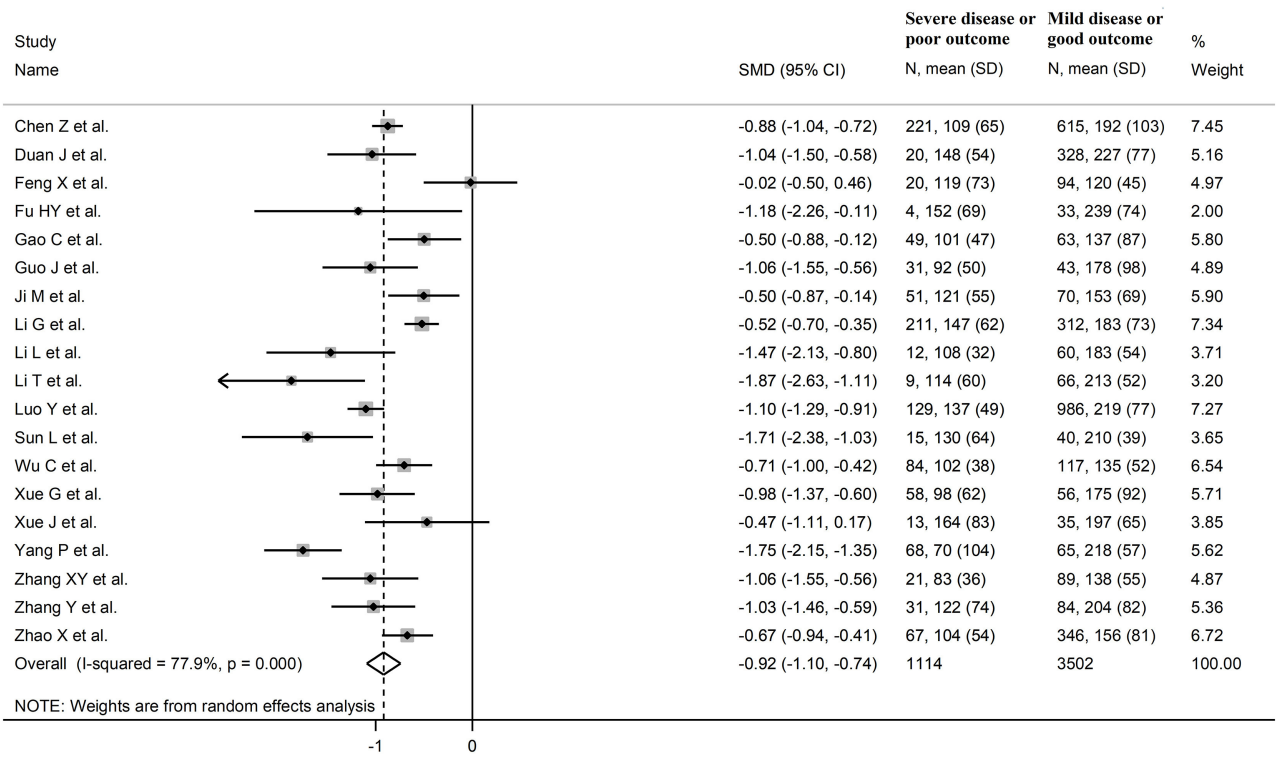

FIGURE 2 | Forest plot of studies examining serum prealbumin concentrations in patients with COVID-19.

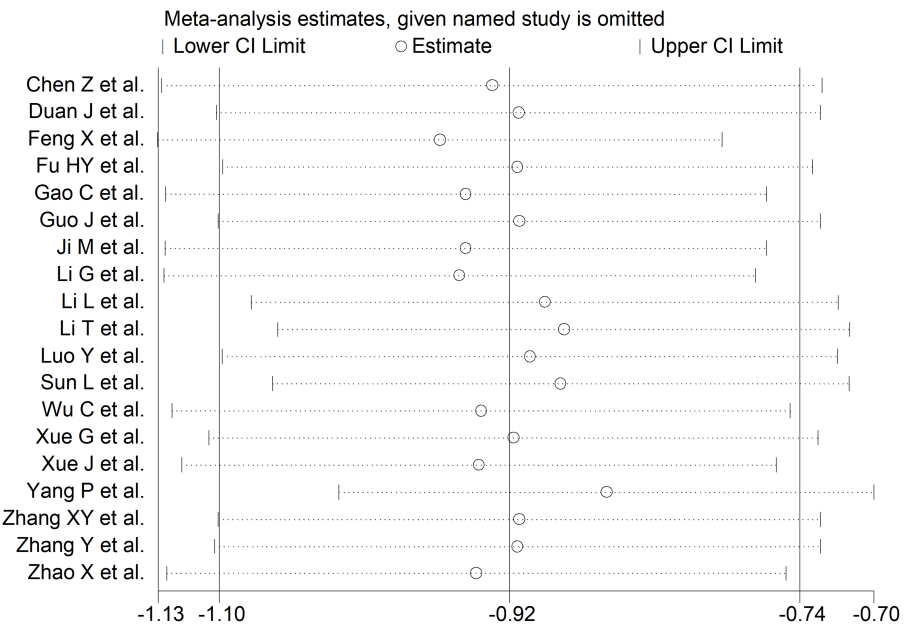

FIGURE 3 | Sensitivity analysis of the association between serum prealbumin and COVID-19. The influence of individual studies on the overall standardized mean difference (SMD) is shown. The middle vertical axis indicates the overall SMD and the two vertical axes indicate the 95% confidence intervals (CI). Hollow circles represent the pooled SMD when the remaining study is omitted from the meta-analysis. The two ends of each broken line represent the 95% CIs.

by incorporating the hypothetical missing studies as though they actually existed, to augment the observed data so that the funnel plot is more symmetric. Statistical analyses were performed using Stata 14 (STATA Corp., College Station, TX, USA). The study was fully compliant with the PRISMA statement on the reporting of systematic reviews and meta-analyses (21).

RESULTS

Literature Search and Study Selection

A flow chart describing the study screening and selection is presented in **Figure 1**. From a total of 154 studies initially identified, 134 were excluded because they were either duplicates or not relevant. After a full-text review of the remaining 20 articles, one study was further excluded because of missing data, leaving 19 studies for analysis (22–40). The characteristics of these studies, all conducted in China, are described in **Table 1**. Overall, they included 4,616 COVID-19 patients, 3,502 (48% males, mean age 52 years) with low severity or who survived and 1,114 (61% males, mean age 64 years) with high severity or who died. Three studies were prospective (24, 30, 36),14 were retrospective (22, 23, 25–29, 31, 33, 34, 37–40), whereas the remaining two did not explicitly state the study design (32, 35). Disease severity, based on current clinical guidelines, was assessed in 11 studies (22, 23, 25, 28, 30, 33, 35, 37–40), prolonged viral clearance in two (26, 36), transfer to ICU in one (29), survival in three (27, 31, 32), presence of acute respiratory distress syndrome in one (34), and multiple end points in one (24).

Meta-Analysis

The overall SMD values in patients with mild vs. severe disease or survivor vs. non-survivor status in the 19 studies are described in **Figure 2**. In all studies, patients with severe disease or non-survivor status showed lower serum prealbumin concentrations when compared to those with milder disease or survivor status (mean difference range, −1.87 to −0.02). However, in two of these studies the difference was not statistically significant (24, 36). The pooled results showed that serum prealbumin concentrations were significantly lower in COVID-19 patients with severe disease or non-survivor status (SMD −0.92, 95% CI −1.10 to −0.74, $P < 0.001$) (**Figure 2**). There was extreme heterogeneity between studies ($I^2 = 77.9\%$; $P < 0.001$). In sensitivity analysis, the effect size was not affected when each study was in turn removed (effect size range, between −0.86 and −0.95) (**Figure 3**). The Begg's ($P = 0.06$) and Egger's t-tests ($P = 0.26$) showed no evidence of publication bias. The trim-and-fill method did not identify any study that was missing or should be added (**Figure 4**).

We investigated possible factors contributing to the observed between-study variance, particularly the effect of age, gender, specific end points, study design (retrospective vs. prospective), and the inflammation biomarker C-reactive protein, on SMD by univariate meta-regression analysis. Both age ($t = -2.18$, $P = 0.045$) and CRP ($t = -3.85$, $P = 0.002$) were significantly and negatively associated with the pooled SMD (**Figure 5**). By contrast, there were no significant correlations between SMD and gender ($t = -0.83$, $P = 0.42$). The pooled SMD value in studies assessing survival (−1.21, 95% CI −0.87 to −1.56, $P < 0.001$; $I^2 = 47.5\%$, $P = 0.15$) was lower than that observed in studies assessing disease severity (−1.06, 95% CI −0.83 to −1.28, $P < 0.001$; $I^2 = 70.5\%$, $P < 0.001$) and viral clearance (−0.49, 95% CI −0.16 to

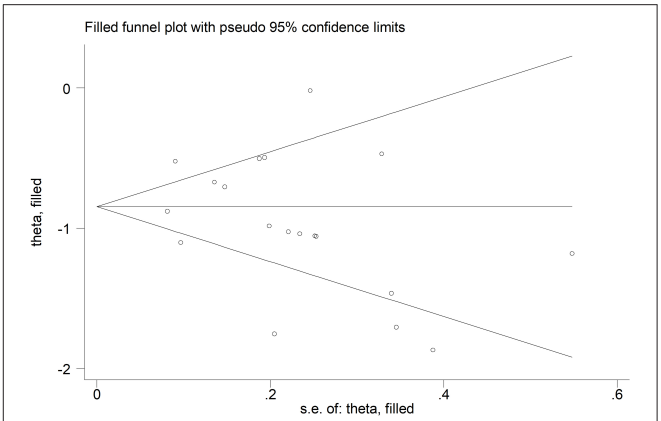

FIGURE 4 | Funnel plot of studies investigating low vs. high severity or survivor vs. non-survivor status after trimming and filling. Dummy studies and genuine studies are represented by enclosed circles and free circles, respectively.

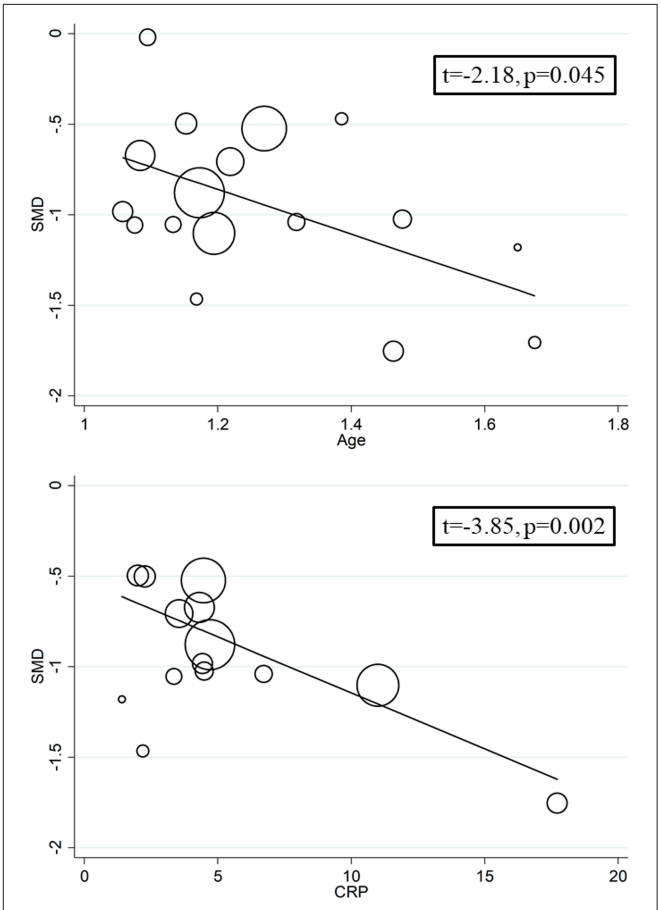

FIGURE 5 | Univariate meta-regression analysis between effect size, age and C-reactive protein.

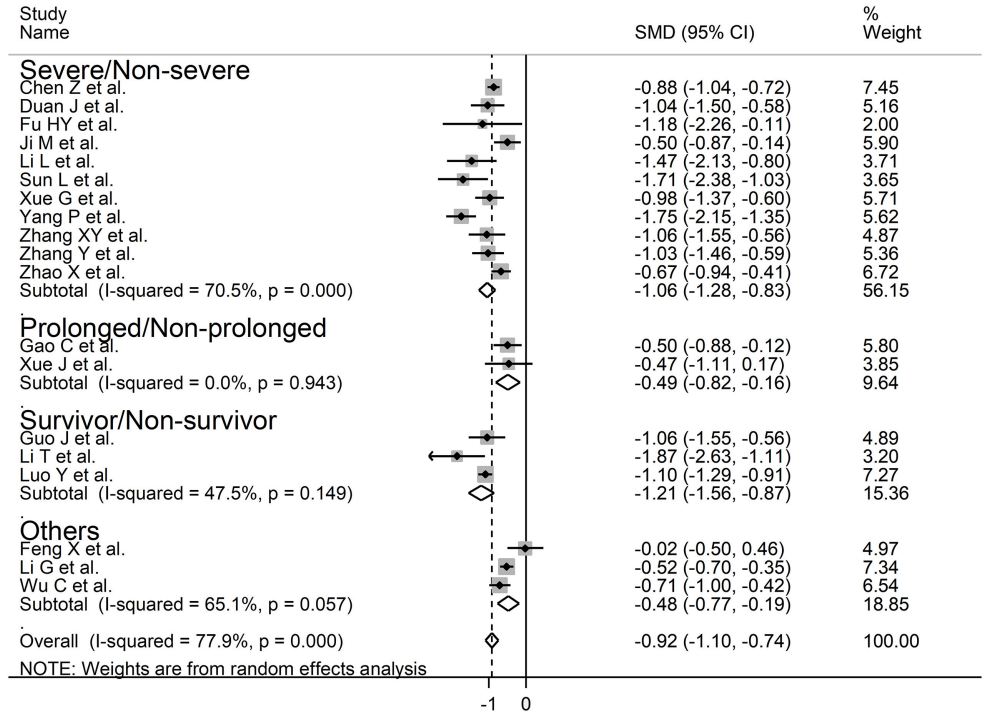

FIGURE 6 | Forest plot of studies examining serum prealbumin concentrations and COVID-19 according to disease severity or outcome.

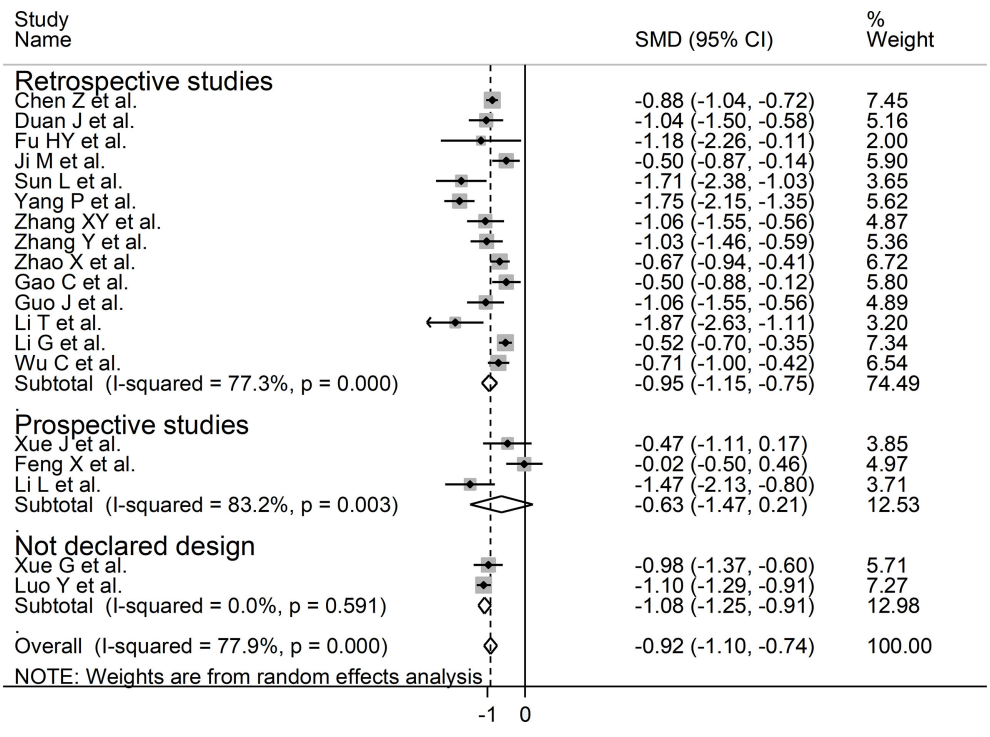

FIGURE 7 | Forest plot of studies examining serum prealbumin concentrations in patients with COVID-19 according to study design (prospective vs. retrospective).

-0.82, $P = 0.003$; $I^2 = 0.0\%$, $P = 0.94$) however the difference was not statistically significant in meta-regression analysis ($t = 1.69$, $P = 0.11$, **Figure 6**). A relatively low heterogeneity was observed in studies assessing survival, $I^2 = 47.5\%$, and viral clearance, $I^2 = 0.0\%$, compared to that in studies assessing severity, $I^2 = 70.5\%$. No significant differences ($t = 0.21$, $P = 0.84$) were observed between pooled SMD values in retrospective (-0.95, 95% CI -0.75 to -1.15, $P < 0.001$; $I^2 = 77.3\%$, $P < 0.001$) and prospective studies (-0.63, 95% CI 0.21 to -1.47, $P = 0.14$; $I^2 = 83.2\%$, $P = 0.003$, **Figure 7**).

DISCUSSION

Our systematic review and meta-analysis showed that serum prealbumin concentrations were significantly lower in COVID-19 patients with severe disease, i.e., with clinically severe illness, prolonged viral load, receiving mechanical ventilation or admitted to ICU, or those that succumbed to the disease when compared to patients with milder forms of the disease or who survived. The observed SMD value, -0.92, suggests a large, biologically and clinically relevant, effect size (41). Despite the extreme between-study heterogeneity in sensitivity analysis the overall effect size was not significantly affected when individual studies were removed. Furthermore, there was no evidence of publication bias. Notably, the SMD was significantly and negatively associated with age and CRP concentrations but not with gender, type of end point studied (disease severity, viral load clearance, need for mechanical ventilation, admission to ICU, or survival), or study design (retrospective vs. prospective).

A reduction in serum prealbumin concentrations typically indicates the presence of acute inflammation and/or malnutrition, unlike other biomarkers such as CRP and procalcitonin which predominantly reflect the inflammatory burden (42–44). Its relatively short half-life makes it a suitable marker to assess and monitor rapid changes inflammatory burden and nutritional state. Normal serum prealbumin concentrations range between 16 and 35 mg/dL. Concentrations <10 mg/dL have been associated with malnutrition and adverse outcomes in non-COVID-19 patient groups (10–12). In particular, studies have reported that serum prealbumin can predict adverse outcomes in patients with burn injuries, respiratory disease, cardiac surgery, and systemic sclerosis (45–48). The results of our systematic review and meta-analysis expand the potential clinical applications of serum prealbumin as the early assessment of this parameter might assist with management decisions in hospitalized patients with COVID-19. This is particularly important given that SARS-CoV-2 is a relatively new virus and, consequently, the models of care for COVID-19 undergo regular review and update following the emergence of novel disease biomarkers and/or therapeutic strategies. Low serum albumin concentrations on admission might help to identify, together with other clinical and demographic characteristics, those patients that are more at risk of severe disease and/or transfer to ICU. Furthermore, unlike CRP and procalcitonin, they could guide nutritional intervention strategies as an important element of care (49).

The exact mechanisms responsible for the lower serum prealbumin concentrations observed in high-risk COVID-19 patients are unclear however they are likely related to the excess inflammation and cytokine release commonly observed in this group (50, 51). Prealbumin is a well-known negative acute-phase reactant, therefore its serum concentrations typically decrease during acute inflammatory processes (52). The significant negative associations observed between the SMD values and CRP concentrations in univariate meta-regression analysis support this hypothesis. There is also emerging evidence that malnutrition is a negative prognostic factor in COVID-19. For example, in a study of 348 hospitalized COVID-19 patients 139 (40%) had moderate-severe malnutrition. The latter group was characterized by older age, higher male prevalence, and higher CRP concentrations and had an increased risk of acute cardiac injury and mortality when compared to patients with mild malnutrition. In multivariate regression analysis, the controlling nutritional status score independently predicted mortality (odds ratio 1.41, 95% CI 1.09–1.82, $P = 0.009$) (53).

While the extreme between-study heterogeneity represents a potential limitation the overall effect size was not significantly affected in sensitivity analysis. Furthermore, no evidence of publication bias was observed. Notably, unlike age and CRP concentrations, the SMD values of serum prealbumin concentrations were not significantly associated with specific study end points (disease severity, viral load clearance, need for mechanical ventilation, admission to ICU, and survival) or design (prospective vs. retrospective). However, the relatively low heterogeneity observed in studies assessing survival and viral clearance suggests that the selection of specific end points may, at least partially, contribute to the observed between-study variance. It is also possible that other, unreported, factors might have contributed to the observed heterogeneity. Another potential limitation is that all selected studies were conducted in China. Therefore, additional studies in other ethnic groups and geographical locations are required to support the generalizability of the results. In conclusion, our systematic review and meta-analysis has shown that lower serum prealbumin concentrations are significantly associated with high disease severity and mortality in patients with SARS-CoV-2 infection. The measurement of serum prealbumin, singly or in combination with other clinical and demographic parameters, might represent a relatively inexpensive and easy to derive biomarker to guide clinical decisions in hospitalized patients with COVID-19.

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

AZ: initial idea and data collection and analysis. AZ and AM: data interpretation and writing—review & editing. AM: writing—first draft. All authors contributed to the article and approved the submitted version.

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Analysis of COVID-19-Related RT-qPCR Test Results in Hungary: Epidemiology, Diagnostics, and Clinical Outcome

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Background: Effective testing is an essential tool for controlling COVID-19. We aimed to analyse the data from first-wave PCR test results in Hungary's Southern Transdanubian region to improve testing strategies.

Methods: We performed a retrospective analysis of all suspected COVID-19 cases between 17 March and 8 May 2020, collecting epidemiological, demographic, clinical and outcome data (ICU admission and mortality) with RT-qPCR test results. Descriptive and comparative statistical analyses were conducted.

Results: Eighty-six infections were confirmed among 3,657 tested patients. There was no difference between the positive and negative cases in age and sex distribution; however, ICU admission (8.1 vs. 3.1%, $p = 0.006$) and in-hospital mortality (4.7 vs. 1.6%, $p = 0.062$) were more frequent among positive cases. Importantly, none of the initially asymptomatic patients ($n = 20$) required ICU admission, and all survived. In almost all cases, if the first test was negative, second and third tests were performed with a 48-h delay for careful monitoring of disease development. However, the positive hit rate decreased dramatically with the second and third tests compared to the first (0.3 vs. 2.1%, OR = 0.155 [0.053–0.350]). Higher E-gene copy numbers were associated with a longer period of PCR positivity.

Conclusion: In our immunologically naïve suspected COVID-19 population, coronavirus infection increased the need for intensive care and mortality by 3–4 times. In the event of the exponential phase of the pandemic involving a bottleneck in testing capacity, a second or third test should be reconsidered to diagnose more coronavirus infections.

Keywords: COVID-19, SARS-CoV-2, PCR diagnostics, testing, epidemiology, surveillance

INTRODUCTION

In December 2019, a new strain of human coronavirus, the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), emerged, causing the Coronavirus Disease 2019 (COVID-19) (1). From the beginning of the pandemic to 1 November 2020, more than 46 million individuals have been infected and more than 1.2 million have died from the infection in 216 affected countries (2). Incidence is still rising on a global scale, and countries are well into the second or third wave of the pandemic.

Due to the possibility of rapid human-to-human transmission and lack of specific therapy, fast, and reliable diagnostic tests are essential. Timely and rapid testing prevents the spread of the virus and optimizes infection control measures. It enables early case identification, isolation of cases and comprehensive contact tracing (3). By the end of the first wave, it became clear that countries that test more have lower mortality rates (4–6).

To identify active cases, nucleic acid amplification tests (NAAT), such as the quantitative reverse-transcription polymerase chain reaction (RT-qPCR) test, are the gold standard (7, 8). During the pandemic, many countries have faced difficulties in maintaining effective screening due to limited access to laboratory equipment and commercial consumables for PCR. Although these tests are now more widely available, rapid increase in testing requirements in different loci throughout the pandemic can interfere with testing capacity (9).

If transmission intensity exceeds testing capacity, countries need to prioritize who will be tested. There are international recommendations on the prioritization of testing among new suspected cases. As regards retesting, protocols are not evidence-based, and they differ among countries and hospitals (10). Retesting of initially negative cases and follow-up testing of positive cases need to be rationalized.

In addition to rationalizing the testing strategy, the allocation of scarce resources among COVID-19 cases is vital. Timely shifting of resources to higher risk groups is an option. Therefore, identification of early prognostic factors can help clinicians' decisions. Some risk factors, such as older age or obesity and the presence of comorbidities, have already been identified (11, 12). However, the role of several other factors is still unclear (13). For instance, our understanding of differences between symptomatic and asymptomatic cases is limited.

We aimed to analyse the data of first-wave PCR test results in Hungary's Southern Transdanubian region to aid in decision-making on the necessity and timing of testing and retesting, especially when testing capacity is limited.

MATERIALS AND METHODS

We performed a retrospective analysis of all suspected COVID-19 cases between 17 March and 8 May 2020. We used the definition of case according to the WHO interim guidance (14). Diagnostic PCR tests were performed by the accredited Department of Laboratory Medicine of the Medical School at the University of Pécs. This center is responsible for PCR testing of all samples in Hungary's Southern Transdanubian region.

Patients Involved in the Analyses

Subjects enrolled in our epidemiological analysis were identified for SARS-CoV-2 PCR testing by the regional offices of Hungary's National Public Health and Medical Officer Service, 29 health care providers, including hospital and clinical sites, and 258 general practices in the Southern Transdanubian region (including four counties: Somogy, Tolna, Baranya, and Zala). All included cases were tested with PCR, and COVID-19 was diagnosed based on WHO interim guidance.

Testing criteria covered epidemiological and/or clinical indication (presence of symptoms listed on a questionnaire provided by our laboratory). Health care personnel who were contacts of confirmed COVID-19 patients were also enrolled.

Epidemiological indication for testing was defined as (1) close contact with a confirmed COVID-19 case and (2) travel from a COVID-19-affected area within 14 days to symptom onset. Epidemiological risk assessment and contact tracing were carried out by regional and national public health officers who did interviews about exposure, travel history and symptoms, identified contacts of confirmed COVID-19 patients, ordered isolation and monitored symptom development.

Clinical indication for testing included (1) presence of fever and/or upper and lower respiratory symptoms, (2) cough, (3) chest discomfort or pain, (4) shortness of breath or breathing difficulties, and (5) gastrointestinal symptoms, including abdominal discomfort, nausea, vomiting, and diarrhea. General practitioners and clinicians indicated testing of symptomatic patients.

The algorithm for SARS-CoV-2 PCR testing is summarized in **Supplementary Figure 1**.

Outcomes and Data Collections

Participants' medical records were analyzed, and the following epidemiological, demographic, clinical, and outcome data were collected: reason for testing, date of sampling, age, gender, presence of symptoms, viral excretion, ICU admission, and mortality. Mortality data refer to the hospitalized period (in-hospital mortality). An assessment of viral excretion is detailed below and in **Supplementary Document 1**.

Sampling

Samples were taken by health care professionals at the National Emergency Service or local health care providers. Specimens were collected from the lower respiratory tract (with a tracheal sputum) among hospitalized and ventilated cases and from the upper respiratory tract (with a nasopharyngeal or oropharyngeal swab) in all other participants. All specimens were labeled with the patient (or contact) name, date of birth, specimen type, and date, and time of collection.

Sample collection tubes were individually packaged in a sterile double wall plastic bag and transferred to the laboratory at 4°C for nucleic acid extraction. Nucleic acid was extracted from 200 μl specimens either manually or with the MagNaPure 96 automated nucleic acid extraction system (Roche, Mannheim, Germany). PCR amplification was carried out in LightCycler 480 and Cobas Z 480 PCR systems. Fluorescence data were converted into concentrations using a standard curve and

analyzed accordingly. The test was considered positive if the sample was positive for at least two genes in the fortieth PCR cycle (cycle threshold/Ct value = 40) (15). Sample processing and PCR amplification are detailed in **Supplementary Document 1**.

Statistical Analyses

All of the statistical analyses were performed using the R statistical environment, R Core Team v3.6.1 (16). A p-value <0.05 was considered as statistically significant. For age, we calculated the median and the range. The Wilcoxon Rank Sum and Signed Rank Tests were used to compare the age between the negative and positive cases. For sex, the presence of symptoms, ICU admission and mortality odds ratios (OR) with the corresponding 95% confidence interval (CI) were calculated with the odds ratio function from the epitools package for R (17). The Wilcoxon Rank Sum and Signed Rank Tests were used for viral excretion (copy number of the E-gene).

Ethical Issues

In this study, data were collected retrospectively and analyzed in compliance with ethical requirements. Ethical approval was granted by the National Centre for Public Health (20800-6/2020/EÜIG).

RESULTS

Between 17 March and 8 May 2020, 3,657 people with suspected SARS-CoV-2 infection were tested in Hungary's Southern Transdanubian region, which represents 41.67 per thousand of the resident population living there (18). Among individuals with suspected COVID-19, a total of 5,463 tests were performed for 3,657 people, and 86 infections (2.35% of all participants and 1.57% of the total tests) were confirmed positive. The number of tests performed showed a steady increase in the first week and then relative stability until the end of the observation period. The median age of the individuals tested was found to be 52 years (range 0–98), and the proportion of male participants was 47.2%. During the study period, the mean age and sex distribution also showed relative constancy (**Figure 1**).

Dynamics of the COVID-19 Pandemic

The first case confirmed by PCR was found in Pécs on 18 March 2020, along with some symptomatic cases and contacts during the following 5 days. The last new positive case was diagnosed on 2 May. PCR-confirmed SARS-CoV-2-infected cases are plotted on the map of Hungary's Southern Transdanubian region. The increase of incident cases representing the dynamics of the pandemic spreading can be followed by 5-day intervals (**Figure 2**). The mapping methodology is specified in **Supplementary Document 1**. Most confirmed cases were identified in large cities in the region, e.g., Pécs, while only a few cases were found in rural areas there.

Clinical Characteristics of Coronavirus Infection

Comparative analyses of the SARS-CoV-2 PCR test-positive and test-negative subpopulations were performed for age, sex, presence of symptoms, ICU admission and mortality (**Figure 3**). There was no statistical difference between positive and negative cases in terms of age at diagnosis (50.0 ± 17.9 vs. 51.3 ± 21.8, respectively; W = 162,302, $p = 0.353$), but a slightly lower proportion of women was observed among the confirmed cases (46.5 vs. 53.4%, OR = 0.787 [0.510–1.209], $p = 0.274$). Among suspected COVID-19 cases, the proportion of symptomatic patients was higher in those with a positive test (76.7 vs. 60%, OR = 2.146 [1.319–3.652], $p = 0.001$). ICU admission was significantly more frequent in PCR positive cases compared to negative ones (8.1 vs. 2.6%, OR = 3.379 [1.374–7.048], $p = 0.010$). As regards ICU admitted cases, a higher proportion of male participants was found in the confirmed group (85.7 vs. 57.0%, OR = 0.248 [0.009–1.592], $p = 0.158$). Crude mortality among the confirmed COVID-19 cases was marginally higher than of the PCR negative group (4.7 vs. 1.6%, OR = 3.287 [0.957–8.291], $p = 0.057$).

All patients had symptoms at the time of the first testing among the PCR-positive participants, who later developed severe outcomes and were admitted to the ICU ($n = 7$). As regards deceased patients ($n = 4$), the proportion of initially symptomatic cases was also 100%. We found that among initially asymptomatic patients, no ICU admission or death occurred.

The mean age of participants admitted to the ICU was higher compared to those who did not require intensive care. 85.7% of ICU-admitted patients were male. The mean age of deceased participants was higher compared to those who survived. Three of the four deceased patients were male (**Figure 3**).

Testing Results of the General Population and Health Care Providers

As the indication of RT-qPCR testing differs between the general population and health care providers, we also analyzed these two populations separately. During the observation period, we identified 70 and 16 SARS-CoV-2-infected individuals in the general population and among health care providers, respectively (**Figure 4**; **Supplementary Figure 2**).

In the general population, the proportion of symptomatic COVID-19 cases was 82.9%. Symptomatic COVID-19 cases had higher viral excretion (copy number of the E-gene) compared to those with asymptomatic infection, although the difference was not statistically significant (328,694 ± 1,198,275 [$n = 58$] vs. 40,234 ± 110,046 [$n = 12$]; W = 257, $p = 0.159$). The rate of cases that tested positive decreased with subsequent testing (2.1, 0.3, and 0.5% during the first, second, and third test, respectively). 92.9% of the positive participants tested positive on their first PCR test. Only 7.1% of the RT-qPCR positive cases ($n = 5$) were identified after a negative first test. Four infections (5.7%) were confirmed with a second test, and only one (1.4%) was confirmed with a third. The viral excretion of the infections identified later was significantly lower compared to those with positive first tests (230 ± 353 vs. 300,707 ± 1,134,581; W = 249, $p = 0.0499$).

Among health care providers, the proportion of symptomatic COVID-19 cases was lower (50.0%). We did not find a difference between symptomatic and asymptomatic health care providers as regards viral excretion (8,901 ± 18,103 vs. 11,637 ± 22,711,

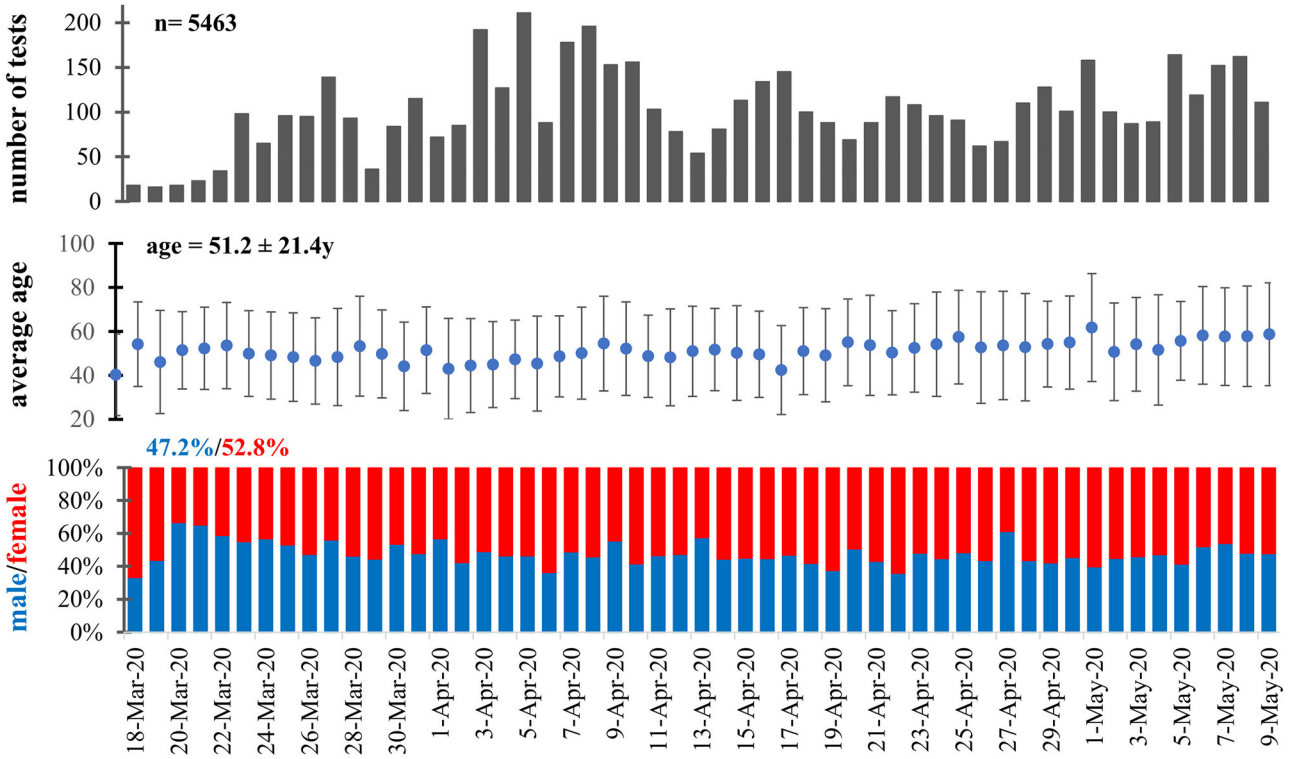

FIGURE 1 | Age and sex distribution of the study population during the observation period.

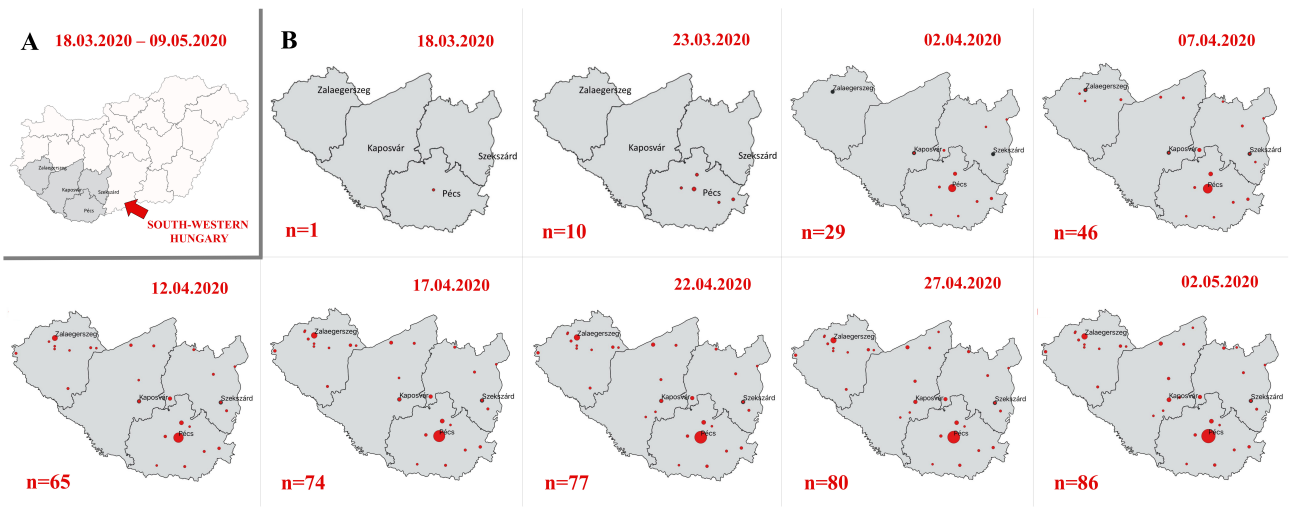

FIGURE 2 | Incident cases representing the dynamics of the pandemic spreading in 5-day intervals. **(A)** Hungary and **(B)** south-western Hungary.

respectively; W = 10, $p = 1$). 56.3% of the participants tested positive with the first RT-qPCR test among health care professionals with confirmed infection. Contrary to the general population, the viral excretion of infections identified later was not different from those with positive first tests (10,117 ± 18,957 vs. 9,437 ± 23,326; W = 33, $p = 0.916$) among health care providers.

Absolute quantification of the SARS-CoV-2 genetic segments also allowed us to characterize the individual disease progression with copy number changes. E-gene copy

			Sars-CoV-2			
			POSITIVE		NEGATIVE	
ALL		Number of tests	131		5332	
		Number of patients	**86**	**100,0%**	**3571**	**100%**
	SEX	male	47	54,7%	1696	47,5%
		female	39	45,3%	1875	52,5%
	AGE	average age	**50,0**		**51,3**	
		SD	17,9		21,8	
	SYMP	YES	66	76,7%	2157	60,4%
		NO	20	23,3%	1411	39,5%
SEVERITY		**Mild**	**79**	**91,9%**	**3462**	**97%**
	SEX	male	41	51,9%	1633	47,2%
		female	38	48,1%	1829	52,8%
	AGE	average age	**48,3**		**51**	
		SD	17,5		21,8	
	SYMP	YES	59	74,7%	2075	59,9%
		NO	20	25,3%	1384	40,0%
		Severe (critical)	**7**	**8,1%**	**109**	**3,1%**
	SEX	male	6	85,7%	63	57,8%
		female	1	14,3%	46	42,2%
	AGE	average age	**69,0**		**60,8**	
		SD	8,7		20,4	
	SYMP	YES	7	100,0%	82	75,2%
		NO	0	0,0%	27	24,8%
		Mortality	**4**	**4,7%**	**54**	**1,6%**
	SEX	male	3	75,0%	31	57,4%
		female	1	25,0%	23	42,6%
	AGE	average age	**71,00**		**69,7**	
		SD	11,6		13,2	
	SYMP	YES	4	100,0%	47	87,0%
		NO	0	0,0%	7	13,0%

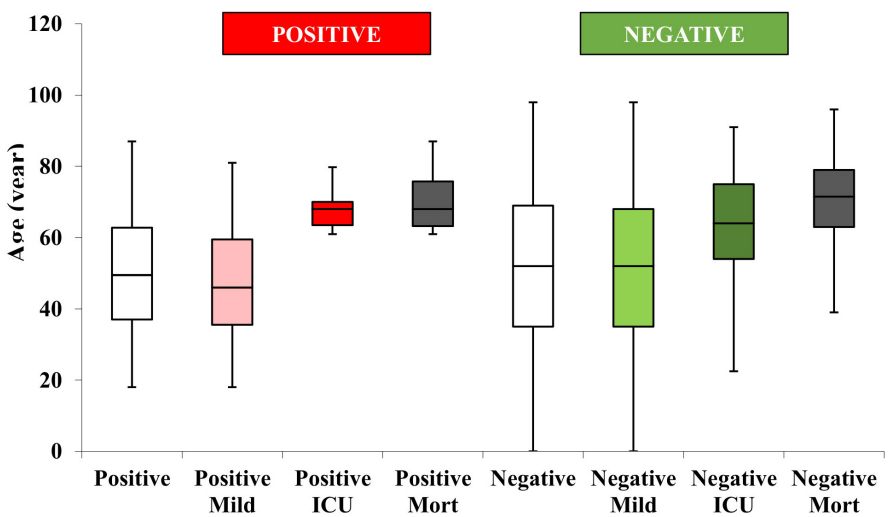

FIGURE 3 | Epidemiology and clinical outcome of the study population and comparison of negative and positive cases confirmed by polymerase chain reaction (PCR). SYMP, symptoms; SD, standard deviation; ICU, intensive care unit; Mort, mortality.

A GENERAL POPULATION

	n	%
First test positivity	65	**92.9%**
Second test positivity	4	**5.7%**
Third test positivity	1	**1.4%**
Intermittent	9	**12.9%**

	POSITIVE	NEGATIVE	% of POS
First test chance for positivity	65	3048	**2.1%**
Second test chance for positivity	4	1372	**0.3%**
Third test chance for positivity	1	183	**0.5%**

	n	Age	Symptoms	E-gene copy/ul
First three positivity (followed by a negative one)	4	**48.0** ± 24.4	**100.0%**	**1718998** ± 3380763
First two test positivity (followed by a negative one)	9	**44.1** ± 16.7	**100.0%**	**491147** ± 1385188
First test positivity (followed by a negative one)	25	**48.1** ± 16.1	**64.0%**	**184000** ± 788811
First test negativity (followed by a positive one)	4	**50.0** ± 22.3	**75.0%**	**283** ± 383
First two test negativity (followed by a positive one)	1	**22.0**	**100.0%**	**18**

B HEALTHCARE WORKERS

	n	%
First test positivity	9	**52.9%**
Second test positivity	5	**29.4%**
Third test positivity	1	**5.9%**
Fourth test positivity	1	**5.9%**
Intermittent	1	**5.9%**

	POSITIVE	NEGATIVE	% of POS
First test chance for positivity	9	527	**1.7%**
Second test chance for positivity	5	255	**1.9%**
Third test chance for positivity	1	79	**1.3%**
Fourth test chance for positivity	1	34	**2.9%**

	n	Age	Symptoms	E-gene copy/ul
First test positivity (followed by a negative one)	6	**42.8** ± 13.6	**50.0%**	**8150** ± 18436
First test negativity (followed by a positive one)	5	**39.8** ± 12.8	**40.0%**	**12861** ± 27649
First two tests negativity (followed by a positive one)	1	**39.0**	**0.0%**	**1750**
First three tests negativity (followed by a positive one)	1	**31.0**	**100.0%**	**6**

FIGURE 4 | Links between polymerase chain reaction (PCR) positivity with first and subsequent testing and viral excretion among the **(A)** general population and **(B)** health care providers.

numbers intraindividually showed a decreasing tendency parallel with the relief of clinical symptoms. Patients with higher viral excretion tend to have a longer period of RT-qPCR positivity (**Supplementary Figure 3**). Remission of the first wave of the local outbreak was observed in close conjunction with a decreasing frequency of the laboratory identification of individuals with high E-gene copy number.

DISCUSSION

The coronavirus pandemic is spreading progressively. The number of new cases diagnosed daily is still continuously increasing worldwide. There are countries that have engaged in an ongoing fight from the start, and there are countries over the second wave of the pandemic. Even local interception of the fast transmission has a significant impact on the economic and health care burden. Analysis and interpretation of the early results of different local outbreaks should not be delayed because analytical learning is essential to developing effective prevention.

Importantly, if testing capacity becomes insufficient, countries might need to prioritize who is tested (19). Under these circumstances, the WHO recommends that tests be provided for patients at higher risk for developing a more severe disease, for first symptomatic patients in closed communities and for

healthcare workers (14). However, we have no evidence on the indication for retesting. Considering this question, our study suggests other factors that would call for further research and recommends their inclusion be considered in future guidelines.

In our study, the rate of cases tested positive decreased with subsequent (second and third) testing; therefore, the number of unnecessary repeat tests was high. This implies that if the number of tests is limited, instead of retesting, resources should be devoted to screening for other suspected COVID-19 cases where the chances of test positivity are higher (20, 21). For example, the presence of COVID-specific symptoms is an important factor to consider (21). This is consistent with our results, as the proportion of PCR-positive cases was higher among participants with symptoms.

There is also no consensus on the timing of the follow-up test. Local protocols mainly determine it by the time of symptom onset and resolution (12). However, the duration of symptoms and viral shedding is not always in synchrony; other factors might therefore also be considered. In our study, higher initial viral loads (E-gene copy numbers) were associated with longer test positivity. This phenomenon has been described by Wolfel et al. (22). They found that higher E-gene copy numbers were associated with a more severe disease course, and the viral load persisted longer compared to those who had lower copy numbers (80 vs. 11 days). Therefore, retesting of patients with high viral loads might be delayed, and the number of unnecessary tests performed too early can be reduced among patients with a high viral load.

In our study, cases with lower initial viral loads turned negative earlier. The isolation and hospitalization could thus end sooner for these patients, leading to economic benefits for both individuals and societies. Lifting the quarantine sooner for these patients can lead to decreased loss of daily wage earnings and reduced isolation costs. In summary, in the case of a PCR test, it is worth considering not only the fact of positivity, but also the degree of viral load.

In addition to obvious infection control aspects, the importance of identifying new cases is supported by the threefold increase of ICU admission and mortality rates among the COVID-19 positive cases in our study.

These results are likely to be independent of demographic features, since they were similar among positive and negative groups, except for the slightly higher rate of male participants among the positive cases. A gender difference favoring men has been observed in previous studies and suggests that the virus is more likely to infect men (23, 24).

Among our limited number of positive cases, elderly, men and symptomatic patients were more likely to be admitted to the ICU or to die in our study, a finding which is consistent with previous results (25).

Previous studies have implied that the vast majority of asymptomatic patients at the time of the first positive test recover spontaneously with a mild disease course (26, 27). We came to the same conclusion since we found that no ICU admission or death occurred among initially asymptomatic patients. These patients thus do not require close observation, and the number of follow-up examinations could therefore be minimized.

The literature suggests that asymptomatic patients can also transmit the disease and viral excretion may be associated with symptomatic patients (28, 29). In contrast, we found much lower E-gene copy numbers in the asymptomatic cases emerging from the general population compared to the symptomatic ones.

It is possible that some of these patients were pre-symptomatic at the time of testing and developed symptoms later. A study comparing truly asymptomatic and pre-symptomatic cases found that the virus could be detected for a longer period in pre-symptomatic cases (26, 30). If we add this to our findings of gene copy number and duration of test positivity, truly asymptomatic patients may be candidates for earlier retesting and released from isolation as soon as possible.

Healthcare personnel were analyzed separately because the PCR test indication was fundamentally different in their case, and this population has different demographic characteristics. In their case, our previous findings are not necessarily correct. Nevertheless, it is difficult to draw a definite conclusion because of the small sample size. We hypothesize that these individuals may have been identified at an earlier stage of the disease. Based on a previous study, we can also assume that they were exposed to a lower viral load due to the use of protective equipment, resulting in lower viral gene concentration in their samples (31).

Strength and Limitations

This is the first multi-center study in Hungary that reports on the links between PCR testing and viral excretion, along with demographic and clinical data. The observation period covers the entire first wave of the region under pandemic surveillance of the COVID-19 outbreak, and we included every sample of suspected cases analyzed in the primary testing center Hungary's Southern Transdanubian region (32). Nevertheless, virus isolation was mostly performed manually, which allows the detection of lower virus copy numbers.

This study has some limitations. The first is the retrospective nature of the data collection. Secondly, despite the large number of tests performed, our conclusions may be limited by the relatively low number of confirmed cases and its influence on the power of the performed statistical analyses. Lastly, some deviations occurred in distant areas following the strict screening protocol in some cases, which resulted in missing data.

Although, most of our results are in line with exiting published data, these new data from the specified Hungarian population contribute to the knowledge and understanding of this global pandemic.

Implication for Practice

- To avoid diagnostic insufficiency, when testing capacity reaches its limits in the future, focusing on testing new cases instead of repeated screening could be feasible.
- We recommend considering the viral copy number when choosing the timing for retesting positive cases (follow-up tests). Our results support earlier follow-up testing with lower gene copy numbers and delayed follow-up testing with higher copy numbers.
- Quantitative detection of viral excretion and different segments of the viral genome which help to determine a

potential infectious state may be useful for clinicians to plan patient management, placement in the proper health care ward and translocation.
- We would like to draw clinicians' attention to an important finding: mortality and ICU admission were three times more common among confirmed cases compared to "only" suspected cases; however, further analyses are required with larger datasets, as the difference was not significant due to the low positive case numbers.
- Lack of symptoms at the time of the first test indicates a good outcome.

Implications for Research

Additional studies are warranted to confirm our recommendations. A particularly important area of research is the relation between viral load and disease duration. Further studies need to identify factors that can narrow the range of testing indication in the case of insufficient testing capacity.

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 National Center for Public Health (20800-6/2020/EÜIG). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

KG, PH, and AS: conceptualization. KG, LG, DC, KF, TN, and BS: data collection and curation. KG, RH, and AS: formal analysis. LG, DC, and PH: funding acquisition. KG, AG, TN, AM, PH, and AS: methodology. AG, AM, and PH: resources. AG, PH, and AS: supervision. RH and AS: visualization. KG, MF, SK, and PH: writing – original draft. KG, MF, SK, RH, AG, LG, DC, KF, TN, AM, BS, PH, and AS: writing – review & editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.625673/full#supplementary-material

Supplementary Figure 1 | The algorithm for SARS-CoV-2 polymerase chain reaction (PCR) testing.

Supplementary Figure 2 | Polymerase chain reaction (PCR) results, viral excretion and presence of symptoms with first test among the **(A)** general population and **(B)** health care providers. SP, symptomatic positive; NSP, non-symptomatic positive; SN, symptomatic negative; NSN, non-symptomatic negative.

Supplementary Figure 3 | Results of individual disease progression with E-gene copy number changes.

Supplementary Document 1 | Details of the assessment of viral excretion, sample processing, PCR amplification, and mapping methodology.

Supplementary Table 1 | (A) Median, quartiles, minimum and maximum values in the negative and positive severity and mortality groups. **(B)** Sex distribution, symptoms, severity and mortality among positive cases in the normal population and healthcare personnel groups. **(C)** Sex distribution, symptoms, severity and mortality in the negative and positive groups. **(D)** First, second and third test positivity in the normal population.

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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
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Geographical and Epidemiological Characteristics of 3,487 Confirmed Cases With COVID-19 Among Healthcare Workers in China

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As the first area to report the outbreak, China used to be the front line of the battle against the novel coronavirus SARS-CoV-2. The present descriptive analysis of 3,487 COVID-19-confirmed cases with health workers reported through April 30, 2020 offers important new information to the international community on the epidemic in China. These data showed that Chinese measures including the high-grade protective gear used, mask wearing, and social distancing, are effective in reducing transmission in hospitals.

Keywords: severe acute respiratory syndrome coronavirus 2, health workers, COVID-19 disease, human-to-human transmission, scientific protective measures

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INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a previously unknown virus that was first reported to cause severe respiratory infections in Wuhan, China, in late December 2019 (1–4). As the hardest-hit city in the outbreak, Wuhan city was shut down in January 23, 2020 to curb the rapid spread of the virus across China. Despite the local government's heightened disease control and prevention efforts, the highly contagious SARS-CoV-2 continued to spread, and some areas in Hubei province saw a spike in the number of infected cases.

Because of person-to-person transmission of the novel coronavirus SARS-CoV-2 (1, 5–12), healthcare workers were at an increased risk of infection as they were in close contact with patients infected by the coronavirus disease, COVID-19 (3). Some healthcare workers had contracted the COVID-19 on the front line of the battle against the new coronavirus. As of April 30, 2020, there were 3,487 confirmed COVID-19 cases of medical staff in China, including 2,961 (84.9%) in Wuhan city, one of the major epicenters of SARS-CoV-2 infections of medical staff in China. In this article, we reported geographical and epidemiological findings of 3,487 confirmed cases among healthcare workers in hospitals.

METHODS

We collected publicly available data from the Zi Jie Tiao Dong Humanitarian funding for medical workers infected by SARS-CoV-2, Red Cross Society of China, including batch 1–63 big data (https://it.gmw.cn/2020-03/30/content_33699059.htm?s=gmwreco2) and batch 64–70 between March 30 and May 8, 2020 (https://www.redcross.org.cn/html/2020-05/71073.html). All confirmed cases with COVID-19 were detected using viral nucleic acid testing. Analyses included the geo-temporal analysis, examination of age distributions and sex ratios, and department distributions of confirmed cases with healthcare workers.

RESULTS

Epidemiological Curve of Confirmed Cases With Health Workers in Hospitals

As of April 30, 2020, a total of 3,487 confirmed cases of healthcare workers were reported (**Figure 1A**). Among them, 1,853 cases (53.1%) were diagnosed before January 31, 2020 (**Figure 1B**); 1,564 cases (44.9%) were confirmed from February 1 to 29, 2020 (**Figure 1C**); 70 cases (2.0%) were infected from March 1 to April 30, 2020 (**Figures 1D,E**).

The COVID-19 epidemic curve with number of cases plotted by date of confirmed diagnosis of health workers from January 1, 2020 to April 30, 2020 is shown in **Figure 2**. Confirmed cases based on positive viral nucleic acid test were stacked to show the total daily cases. The inset showed that the peak number of confirmed cases for all cases overall occurred on January 23, 2020. Since February 2, 2020, confirmed cases of health workers had declined.

Age Distribution and Sex Ratio

The age distribution of cases in China overall is presented in **Figure 3A**. The proportion of confirmed cases 25–59 years of age at baseline (i.e., date of confirmed diagnosis) was 90.6% for cases in China overall (which includes Hubei Province and 14 other provincial-level administrative divisions). The male-to-female ratio (male, $n = 1{,}026$; female, $n = 2{,}461$) was 0.42:1 in China overall (**Figure 3A**).

Post Distribution of Confirmed Healthcare Workers

Post distribution of confirmed healthcare workers is shown in **Figure 3B**. Among them, nursing staff (51.5%), doctors (33.5%), administrative staff (6.3%), medical technicians (3.8%), logistics management staff (3.1%), pharmacists (1.4%), and others (0.5%) were diagnosed; according to the number of confirmed cases with COVID-19, 7 posts were arranged in sequence from code 1 to 7.

Top 10 Distributions of Confirmed Healthcare Workers in Clinical Departments

Top 10 distributions of confirmed healthcare workers in clinical department are shown in **Figure 3C**. Among them, the Department of Respiratory Medicine (23.4%), Emergency (14.6%), Neurology (11.9%), Fever Clinic (8.4%), Gynecology and Obstetrics (8.3%), Gastroenterology (8.1%), Critical Medicine (7.0%), Orthopedics (6.8%), Oncology (6.0%), and Cardiology (5.5%) were identified; according to the number of confirmed cases with COVID-19, 10 clinical departments were arranged in sequence from code 1 to 10.

Geographical Epidemiological Characteristics of 10 Hospitals With Confirmed Cases in Wuhan

By April 30, 2020, 10 hospitals in Wuhan had reported over 1,361 confirmed cases among medical staff. Geographical distribution of these hospitals is shown in **Figure 4A**. According to the number of confirmed case with COVID-19, these hospitals were arranged in sequence as code 1–10. Hospital-1 and−9 are closer to the Huanan Seafood Wholesale Market than the rest, and reported 319 and 81 COVID-19-confirmed cases of medical staff, respectively (**Figure 4B**). Four hospitals farther from the Huanan Seafood Wholesale Market are located in the south region of the Yangtze River and reported 199, 113, 95, and 93 COVID-19-infected cases, respectively (**Figure 4B**).

Geographical and Epidemiological Characteristics of 17 Counties With Confirmed Healthcare Workers in Hubei Province

As of April 30, 2020, a total 3,487 confirmed cases were diagnosed from 15 provinces, autonomous regions, and municipalities, and Hubei Province (3,441 cases) accounted for 98.7%. Huanggang, Xiaogan, and Ezhou counties were closer to Wuhan city than the rest, and reported 213, 82, and 70 COVID-19-confirmed cases of medical staff, respectively (**Figures 4C,D**).

DISCUSSION

A main finding of this characterization analysis among healthcare workers infected with COVID-19 was that this novel coronavirus is highly contagious. Here we offered a first description of the 3,487 confirmed cases among health workers between first recognition of the outbreak of unknown pneumonia on December 31, 2019 to the end of the study period on April 30, 2020. Even with extreme response measures in Wuhan and another 15 cities including the complete shutdown and isolation of whole cities on January 23, 2020, cancellation of Chinese New Year celebrations, and prohibition of attendance at school and work, the coronavirus continued to spread rapidly, and some areas saw a spike in the number of infected healthcare workers.

A major contribution of the current study was a first description of the epidemic curves for COVID-19-confirmed cases with health workers. **Figures 1**, **2** showed the COVID-19 epidemic curve with the number of cases plotted by confirmed date from January 1, 2020 to April 30, 2020 for all cases among health workers nationwide. Our data showed that peak timing

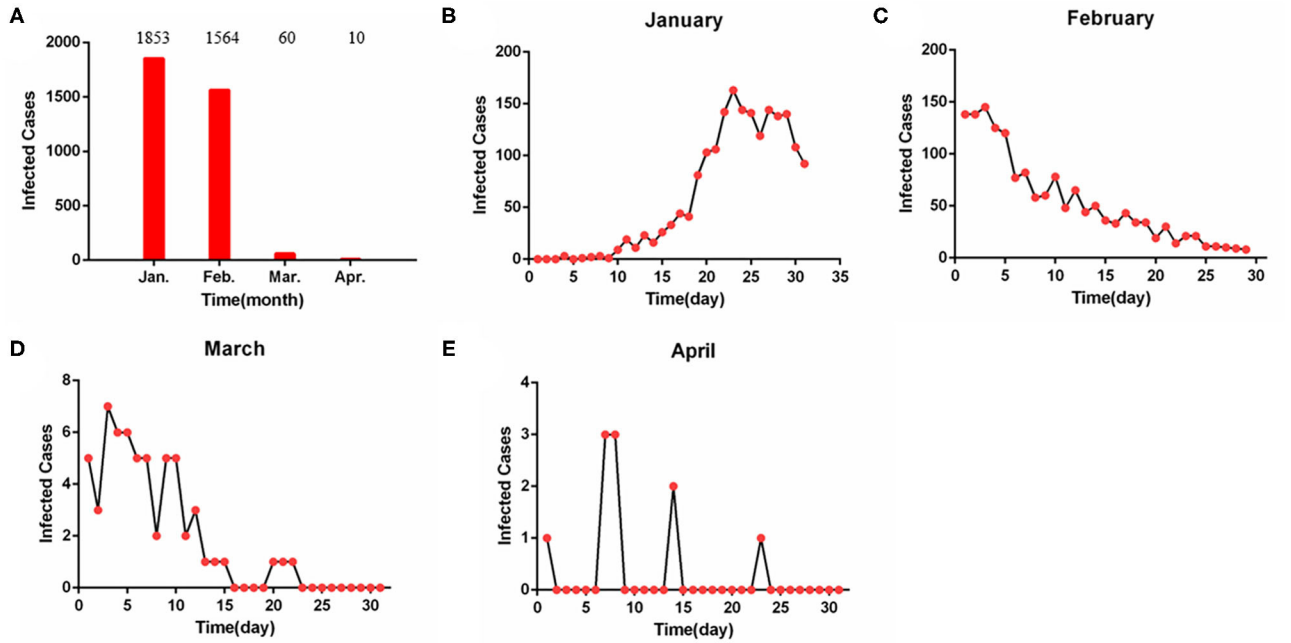

FIGURE 1 | The epidemic curve of confirmed cases in health workers. **(A)** The confirmed cases from January to April. **(B)** The confirmed cases from January 1 to 30. **(C)** The confirmed cases from February 1 to 29. **(D)** The confirmed cases from March 1 to 30. **(E)** The confirmed cases from April 1 to 30. The confirmed cases peaked on January 23, then began to decline leading from February 2, 2020.

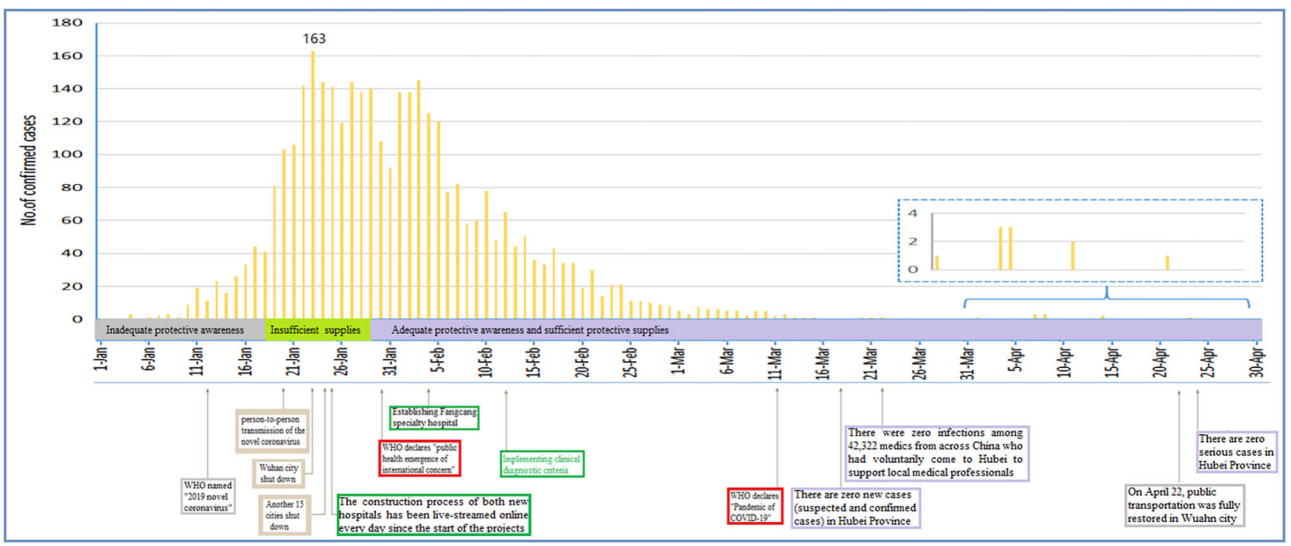

FIGURE 2 | The graph's x-axis (dates from January 1 to April 30, 2020) is used as a timeline of the key events and dynamic profile of confirmed healthcare workers during the COVID-19 outbreak. The profiles of confirmed healthcare workers are shown in the graph's y-axis. The key international events included the international public health emergency (January 30, 2020) and the declaration of COVID-19 as a pandemic (March 11, 2020) by the WHO. The key national events included that Wuhan city shut down (January 23, 2020) and public transportation restored in Wuhan city (April 22, 2020). The statuses of "insufficient supplies" were determined based on insufficient high-grade mask wearing, e.p., the N95 masks, and insufficient high-grade protective gear was used. The statuses of "adequate protective awareness and sufficient protective supplies" were determined based on broadcast of critical information (e.g., promoting hand washing, mask wearing, and care seeking) with high frequency through multiple channels and mobilization of a multi-sector rapid response teams including 42,322 medical staff from across China who voluntarily came to Hubei to support local medical healthcare professionals from January 25 to March 26, 2020.

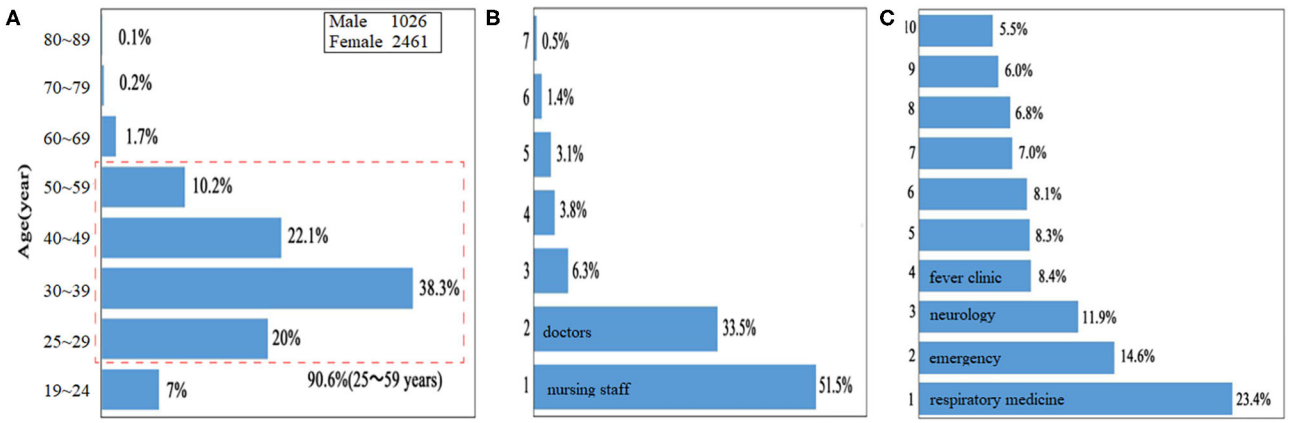

FIGURE 3 | Clinical characteristics of confirmed healthcare workers. **(A)** Characteristics of age distributions. **(B)** Post distribution of confirmed healthcare workers, including nursing staff (51.5%), doctors (33.5%), administrative staff (6.3%), medical technicians (3.8%), logistics management staff (3.1%), pharmacists (1.4%), and others (0.5%); according to the number of confirmed cases with COVID-19, seven posts were arranged in sequence from code 1 to 7. **(C)** Top 10 distributions of confirmed healthcare workers in clinical departments, including the Department of Respiratory Medicine (23.4%), Emergency (14.6%), Neurology (11.9%), Fever Clinic (8.4%), Gynecology and Obstetrics (8.3%), Gastroenterology (8.1%), Critical Medicine (7.0%), Orthopedics (6.8%), Oncology (6.0%), and Cardiology (5.5%); according to the number of confirmed cases with COVID-19, 10 clinical departments were arranged in sequence from code 1 to 10.

of confirmed date among infected cases occurred on January 23, 2020. In addition, confirmed cases with health workers had declined after February 3, 2020 (**Figure 2**). Data from The Novel Coronavirus Pneumonia Emergency Response Epidemiology Team reported subgroup analysis of all cases among health workers including confirmed vs. suspected, clinically diagnosed, and asymptomatic cases, and indicated that a total of 3,019 health workers had been infected (1,716 confirmed cases) in the 422 medical facilities serving COVID-19 patients (13).

The status "adequate protective awareness and sufficient protective supplies" means high-grade protection for the healthcare workers in a way in **Figure 2**. This status covered from January 30 to April 30. However, there were still a lot of infected cases after the adequate protective awareness and sufficient protective supplies, and a peak on February 2 after a decrease on January 30. The reasons for this were that (1) because of the incubation period (7–14 days) of the COVID-19, some healthcare workers were infected with SARS-CoV-2 from January 15 to 29, 2020 (period of insufficient protective supplies), and they were confirmed with SARS-CoV-2 after January 30, 2020 (period of sufficient protective supplies). (2) A large proportion of cases were not confirmed by nucleic acid testing from January 15 to January 29, 2020, since this process is slow, labor intensive, and requires specialized equipment and skilled technicians.

In light of this rapid spread, early assessment was that the virus might be from a still-unknown animal into humans at the Huanan Seafood Wholesale Market in Wuhan (2, 14, 15). A new cluster of 114 COVID-19 cases in Beijing on June 11–16, 2020 had been traced to the sprawling Xinfadi seafood market (16). The common characteristics of the two seafood markets are important to understand the origin of the novel coronavirus SARS-CoV-2 and links between cases.

Huang et al. (2) showed 41 patients with COVID-19 cases who had a history of exposure to the Huanan Seafood Wholesale Market. Thus, we described the geographical epidemiological characteristics for 10 hospitals with infected healthcare workers by using **Figure 4A**. On April 30, 2020, there were 319 and 122 COVID-19-confirmed cases of medical staff in Hospital-1 and−9, which were closer to the Huanan Seafood Wholesale Market, respectively. The number of cases in these two hospitals (Hospital-1 and−9) are largely different; the reason for this was that at the early days of the outbreak, the administrators of Hospital-1 did not encourage mask wearing and care seeking, e.g., using protective gear, resulting in a lot of infected cases for the healthcare workers including four dead cases, whereas the administrators of Hospital-9 promoted mask wearing and care seeking.

Social distancing is very important to curb the epidemic of the COVID-19 (17). In accordance with the principle of putting the safety of the masses and health first, the government authorities had adopted maximum effort and scientific measures to curb the spread of the outbreak. With the exception of the residents under quarantine indoors for 14 days, local authorities had stepped up disinfection, ventilation, and screening measures in public spaces, and got manufacturers of protective suits, surgical masks, safety goggles, negative pressure ambulances, and drugs back in full production as soon as possible. Beginning on January 23, 2019, health and public health personnel as well as military medical units were massively mobilized and voluntarily came to Wuhan city. As of March 22, 2020, a total of 42,322 medical staff from across China supported local medical healthcare professionals (**Figure 2**). Despite the extremely rapid spread of the novel coronavirus (18–22), there was zero infection among the 42,322

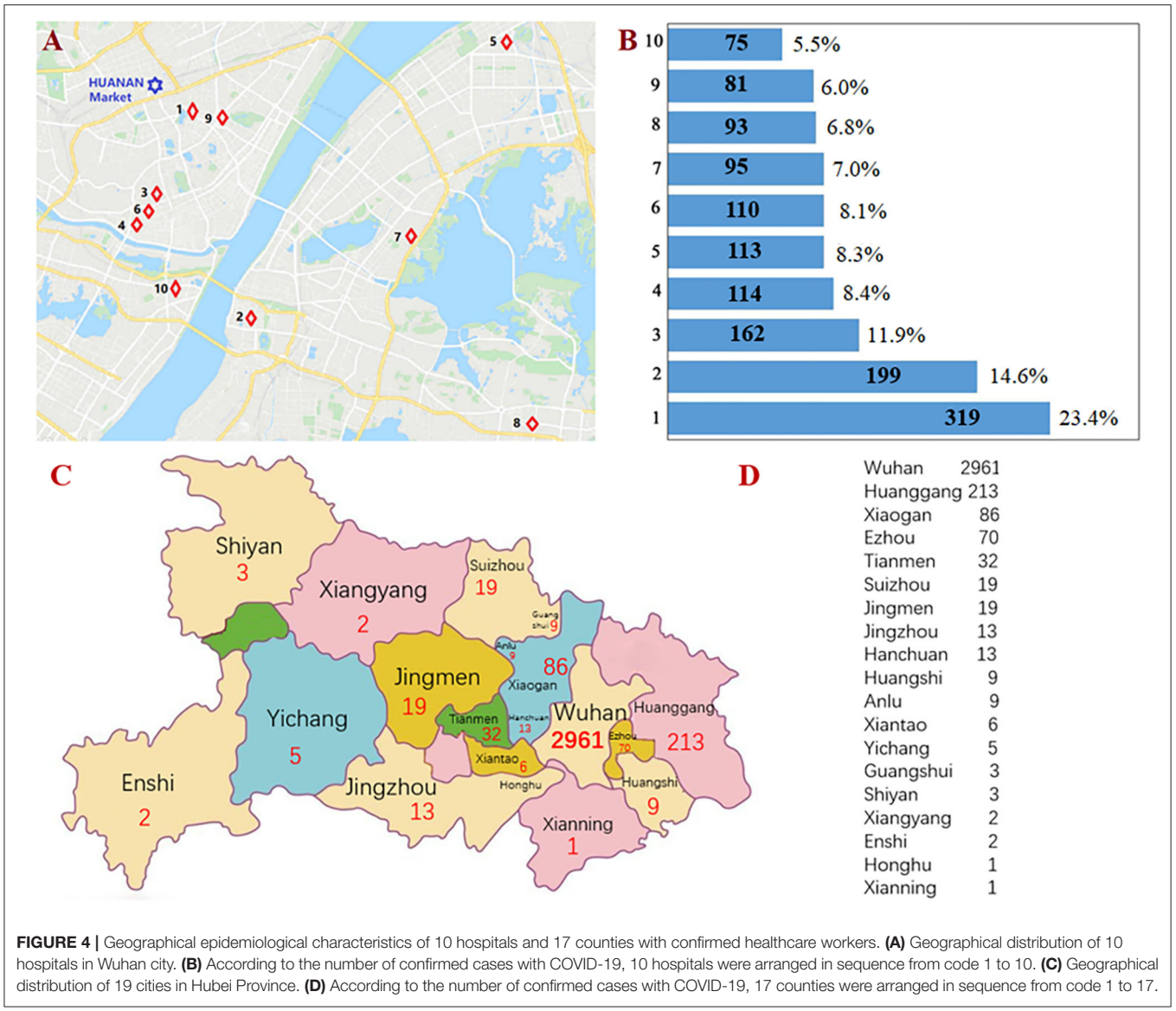

FIGURE 4 | Geographical epidemiological characteristics of 10 hospitals and 17 counties with confirmed healthcare workers. **(A)** Geographical distribution of 10 hospitals in Wuhan city. **(B)** According to the number of confirmed cases with COVID-19, 10 hospitals were arranged in sequence from code 1 to 10. **(C)** Geographical distribution of 19 cities in Hubei Province. **(D)** According to the number of confirmed cases with COVID-19, 17 counties were arranged in sequence from code 1 to 17.

health workers, suggesting that China's coronavirus response highlights the importance of implementing effective public health strategies.

In conclusion, the present descriptive analysis of 3,487 COVID-19-confirmed cases with health workers reported through April 30, 2020 offers important new information to the international community on the epidemic in China. Despite this analysis chronicles the extremely rapid spread of the novel coronavirus, Chinese scientific measures including high-grade protection and social distancing are crucial strategies to prevent human-to-human transmission in hospitals.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MF, ZL, and JX: data collection. WX and BX: data interpretation and writing. All authors contributed to the article and approved the submitted version.

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Association of Viral Load in SARS-CoV-2 Patients With Age and Gender

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Background: The coronavirus disease 2019 (COVID-19), caused by the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), is a global public health emergency. Age and sex are two important factors associated with risks and outcomes of various diseases. COVID-19 morbidity also seems to be affected by patient age and sex. It has been found that older age groups have more severe COVID-19 symptoms and higher fatality rates while children tend to have lower prevalence and milder symptoms than adults.

Methods: The study reviewed electronic medical records of COVID-19 patients from Madinah city, Saudi Arabia. The study included all cases who tested positive ($n = 3,006$) between March 20 and May 22, 2020. Data were obtained from the Health Electronic Surveillance Network (HESN) database.

Results: Approximately 80% of the study sample were males and half were in the 30–40-year-old age group. The Ct value of the whole sample ranged from 15.08 to 35, with a mean of 27.44 (SD: 5.23; 95% C.I. = 27.25–27.66). The means of Ct values varied between age groups from 27.05 to 27.82. Analysis of the mean differences between age groups using one-way ANOVA indicated no statistically significant difference among the groups ($F_{6,2999} = 1.63$; p-value = 0.135). A comparison of mean Ct values of males ($n = 2,422$) and females ($n = 584$) revealed that males had a statistically significant higher mean Ct value (27.61 ± 5.20) than females (26.72 ± 5.31). The difference between the means of the two groups was −0.89 (95% C.I. = −1.36 to −0.42; t-test −3.71; df = 3,004; p-value < 0.001).

Conclusion: The study found no statistically significant difference in viral loads between age groups. It showed that females had a higher SARS-CoV-2 viral load compared to males. The findings have implications for preventive strategies. Further studies are needed to correlate viral load with clinical symptoms and outcomes.

Keywords: SARS-CoV-2, age, gender, CT value, HESN

INTRODUCTION

The coronavirus disease 2019 (COVID-19), caused by the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), is a global public health emergency. The mortality and morbidity caused by the disease constitute a major challenge to healthcare authorities around the globe. The challenge has been aggravated by lack of knowledge of the epidemiological and clinical attributes of the emerging disease (1).

On the 2nd of March 2020, a Saudi man at the Eastern Province coming from Iran confirmed positive for COVID-19 (2). Since then, the Saudi Ministry of Health (MOH) took the case seriously and isolated the patient and all of the contacts. Spreading of the virus was dramatically and several cases were reported in the same region due to the same reason of transmitting the infection of the first case. Such a disease creates huge burden on health care providers and governments as that of MERS Co-V in 2012 (3). Additionally, the worries about re-infection is of a major concern, whoever, protection from reinfection has been reported (4).

Age and sex are two important factors associated with risks and outcomes of COVID-19 disease (5). COVID-19 morbidity also seems to be affected by patient age and sex. It has been found that older age groups have more severe COVID-19 symptoms and higher fatality rates while children tend to have lower prevalence and milder symptoms than adults (6). Nonetheless, the potential role of asymptomatic or mildly symptomatic children in transmitting infection cannot be disregarded and is still debated (7–9). Preliminary evidence also indicated that sex has a role in the disease epidemiology. For example, a study from China indicated that men are at higher risk of severe disease and mortality compared to women (10).

Studying viral dynamics and their variation among population subgroups may help in understanding the role of age, sex, and other factors in the disease's epidemiology. One uncertainty of the new disease is COVID-19's viral dynamics and how they relate to factors in the population. Prior studies revealed that viral load was associated with disease severity and the number of days since the beginning of symptoms (11, 12). However, evidence on the association between viral load and other factors, including age and sex, has not been conclusive. Some studies found that higher viral load in the respiratory system was associated with higher in-hospital mortality and morbidity (13), and a higher risk of transmission (14); other studies found no such relationship (11). Understanding viral load dynamics and covariates is critical for identifying protective measures for individuals and the general public. Therefore, this study investigates the association of viral load with the age and sex of COVID-19 patients.

METHODS

Study Design

The study reviewed electronic medical records of COVID-19 patients from Madinah city, Saudi Arabia. The study included all cases who tested positive between March 20 and May 22, 2020. Data were obtained from the Health Electronic Surveillance Network (HESN) database. HESN is a web-based platform run by the Ministry of Health to integrate public health programs in order to detect and control diseases, and monitor the population's health.

Setting

Al-Madinah region has a population of 2.13 million. The main city in the region is Al-Madinah city, a holy city and home to the Prophet's Mosque. It attracts year-round visits from religious pilgrims from all over the world.

Procedure Used by MOH in Specimens Collection of SARS-CoV-2 Patients

The MOH obligates all the health care workers who collect specimens to use appropriate personal protective equipment (PPE) such as, eye protection, surgical mask, while dealing with suspected Covid-19 patients. They must collect the respiratory specimen under aerosol generating procedure; personnel should wear a particulate proficient N95 respirator. Additionally, specimens should be placed for carriage in leak-proof specimen bags (secondary container) that have a detached sealable pocket for the specimen, with the patient's name tag on the specimen container (primary container). HESN printed lab requisitions must be sent with samples and national lab reception report and result values must be informed on HESN on their consistent time.

In terms of sources of sample testing, the most generally tested sources are nasopharynx and oropharynx (15). However, viral RNA in several biological specimens such as stool, tears and blood has been detected with variable positivity rates (16). Two types of samples are usually requested by the physicians be collected from the patients. First, lower respiratory tract samples, containing endotracheal aspirate, bronchoalveolar lavage fluid or sputum. Second, upper respiratory tract samples, nasopharyngeal swab (with or without oropharyngeal swab) in viral transport medium in a single tube. If initial testing is negative, repeat testing should be accomplished in case of there is a high index of suspicion. Finally, all results should be reported via HESN starting from registering of the case, for test requested select COVID-19, and select the designated regional laboratory. During shipment of samples they should be at 2–8°C and ship on ice pack to lab. Samples can be stored at 2–8°C for $\leq$48 h, if longer storage is required, samples should be stored at −70°C. If sample is frozen at −70°C, ship on dry ice https://www.moh.gov.sa/Ministry/MediaCenter/Publications/Documents/Coronavirus-Disease-2019-Guidelines-v1.2.pdf.

Participants

All positive cases from Al-Madinah region were included in this analysis. Laboratory results of cases in the region were reported to the HESN database by the Al-Madinah Regional Lab. Epidemiological information on cases and samples originated from hospitals and primary healthcare centers in Al-Madinah region. In addition, samples collected during contact tracing or active surveillance were included in the HESN database.

Variables

The outcome was the cycle threshold (Ct) value as measured by quantitative reverse transcriptase polymerase chain reaction

TABLE 1 | Baseline characteristics of the sample by age and sex.

	Total	Female		Male	
Age group		**No.**	**%**	**No.**	**%**
<10	212	97	45.75	115	54.25
10 – <20	269	118	43.87	151	56.13
20 – <30	533	96	18.01	437	81.99
30 – <40	1,504	195	12.97	1,309	87.03
40 – <50	374	53	14.17	321	85.83
50 – <70	27	1	3.7	26	96.3
≥70	87	24	27.59	63	72.41
Total	3,006	584	19.43	2,422	80.57

(RT-PCR) assay. Lower Ct values indicate higher viral load and vice versa. Positive cases were defined as cases having Ct value of <35 in their sample. Age was calculated based on the date of birth and the sample submission date. Age was divided into seven categories (under 10 years old, 10 – <20, 20 – <30, 30 – <40, 40 – <50, 50 – <70, and 70 years or older).

Statistical Analyses

Statistical analyses were performed using Stata 14.0 (StataCorp LLC, TX, USA). Data were presented as mean, standard deviation (SD), 95% confidence intervals (C.I.) or proportions as appropriate. One-way ANOVA was used to compare the differences of means between groups in a univariate analysis. Two-way ANOVA was used in a multivariable analysis to model the relationship between Ct values (outcome), and age group and sex (independent variables). Interaction was tested in the model. Equality of variances was assessed using Levene's Test.

Ethical Considerations

Data collection was required by MOH as a part of the public health surveillance system. This investigation was conducted according to international and national ethical guidelines and approved by the regional research ethics committee of the Madinah Health Directorate (**IRB** number H-03-M-084).

RESULTS

This study used data from the national HESN database. It included 3,006 positive COVID-19 cases reported in the Al-Madinah region from March 20 to May 22, 2020. Approximately 80% of the study sample were males and half were in the 30 to 40-year-old age group (**Table 1**). The Ct value of the whole sample ranged from 15.08 to 35, with a mean of 27.44 (SD: 5.23; 95% C.I. = 27.25–27.66; **Table 2**). The means of Ct values varied between age groups from 27.05 to 27.82.

Univariate Analysis

Analysis of the mean differences between age groups using one-way ANOVA indicated no statistically significant difference among the groups ($F_{6,2999} = 1.63$; p-value = 0.135).

A comparison of mean Ct values of males (n = 2,422) and females (n = 584) revealed that males had a statistically significant higher mean Ct value (27.61 ± 5.20) than females (26.72 ± 5.31). The difference between the means of the two groups was −0.89 (95% C.I. = −1.36 to −0.42; t-test −3.71; df = 3,004; p-value < 0.001).

TABLE 2 | Summary statistics for Ct values in the study sample by sex and age group.

	N	Mean	SD	SE	95% confidence interval		*p*-value*
Overall	3,006	27.44	5.233	0.095	27.25	27.62	
Sex							
Males	2,422	27.61	5.200	0.106	27.40	27.82	<0.001
Females	584	26.72	5.312	0.220	26.29	27.15	
Age group							
<10	212	27.07	5.431	0.373	26.34	27.80	0.135
10 – <20	269	26.64	5.383	0.328	26.00	27.28	
20 – <30	533	27.37	5.269	0.228	26.93	27.82	
30 – <40	1,504	27.59	5.211	0.134	27.33	27.85	
40 – <50	374	27.63	5.047	0.261	27.11	28.14	
50 – <70	27	27.05	5.319	1.024	25.05	29.06	
≥70	87	27.82	5.059	0.542	26.75	28.88	

**calculated by t-test for sex and one-way ANOVA for age groups.*

TABLE 3 | Two-way Analysis of Variance of Ct values with age groups and sex.

Source	Partial SS	df	Mean Square	*F*	*p*-value
Model	524.575	7	74.939	2.750	0.008
Age group	150.112	6	25.019	0.920	0.481
Sex	257.015	1	257.015	9.430	0.002
Residual	81,752.071	2,998	27.269		

Multivariable Analysis

Two-way ANOVA analysis was conducted to examine the effect of age and sex on Ct value. There was no significant interaction between age and sex and Ct value (F = 1.61, p-value = 0.139). The main effect showed that the statistically significant difference between males and females persisted after adjustment for age group (p-value = 0.002). The two-way ANOVA analysis also showed no statistically significant difference between age groups (**Table 3**).

DISCUSSION

This study compared COVID-19 viral load, as indicated by Ct value, across seven age groups, and between men and women. It found that viral load in patients did not differ by age group, but was higher among women than men. The argument in this study was that viral load is proportional to infectiousness of viral infections. The relationship between viral load and risk of transmission has been established in other viral diseases (17, 18); COVID-19 seemed likely to follow a similar pattern (19). Therefore, identifying factors related to viral load could aid prevention strategies and identification of groups contributing to higher transmission risk.

The distribution of viral load observed in this study is consistent with results from previous studies. In Switzerland, Jacot et al. (20) analyzed data on 4,172 positive patients and concluded there was no statistically significant difference between 5 year age groups. Another study from Switzerland compared 352 patients older than 16 years with 53 children under 16 years old and found a similar mean viral load between the two groups (21). Similarly, a study in the United States which included 4,428 patients with positive lab results found no variation in mean and median viral load values (22). Notably, other studies with smaller sample sizes had conflicting results regarding the relationship between SARS-CoV-2 viral load and age. One study of 23 patients concluded that older age groups had higher viral loads (23); another study (24) of 145 patients found that children under 5 years of age had higher viral loads.

This study found higher SARS-CoV-2 viral loads (lower Ct values) among females compared to males. Previous studies were not conclusive on the sex difference in viral load. Jacot et al. (20) and Kleiboeker et al. (22) reported comparable viral loads between males and females. Takahashi et al. (25) found the clinical status of patients was a modifier for the relationship between sex and viral load. Finding that sex effects viral load and immunological response to infectious disease is not surprising; it has been demonstrated in other diseases. This is thought to be related to a difference in immune response in which females develop a higher immune response to infectious agents, making them less susceptible to diseases (26). Gender differences in the response to hepatitis B virus were reported in humans as well (27). Similarly, sex difference seems to play a role in COVID-19 infection; various mechanisms have been suggested to explain this difference (28). Women mount stronger immune responses to infections as well as vaccinations and outlive men (29). As we do not have enough clinical data to investigate the disease severity and correlate that with age and gender, other study showed that men tended to get much sever cases than women. Additionally, older age was greater number in the deceased patients than in the patients who survived. However, several reports showed that there was no difference in terms of susceptibility to SARS-CoV-2 between women and men (10).

The present study provides evidence on age and sex differences in SARS-CoV-2 viral load in a large sample size. It also included a good number of young children who are often less represented in similar studies.

Limitations of the study are a lack of clinical data and the consequent inability to correlate laboratory values with illness stage or severity. Additionally, only respiratory tract specimens were considered and the study non-including alternative shedding routes and that could represent future developments of the study.

In conclusion, the study found no statistically significant difference in viral loads between age groups. It showed that females had a higher SARS-CoV-2 viral load compared to males. The findings have implications for preventive strategies. Further studies are needed to correlate viral load with clinical symptoms and outcomes.

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 Madinah Health Directorate (IRB number H-03-M-084). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AA-Z and WM conceived and designed the study, conducted the preliminary review of articles, wrote the initial and final drafts of the article, and provided logistic support. AA-Z, WM, and AA provided research scope, and collected and organized the extracted data. AA-Z, WM, AD, and HA analyzed and interpreted the data, have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript. All authors contributed to the article and approved the submitted version.

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Young Canadian e-Cigarette Users and the COVID-19 Pandemic: Examining Vaping Behaviors by Pandemic Onset and Gender

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The aim of this study was to test how youth and young adult e-cigarette users responded to the COVID-19 pandemic. The 2020 *Youth and Young Adult Vaping Survey* (N = 1,308) included 540 (44.7%) participants that reported differences in their vaping behaviors since the onset of the pandemic. Gender was the only relevant covariate that yielded a significant effect and/or interaction through a multivariate test. A two-way multivariate analysis of variance was used to test the effect of pandemic onset (pre- vs. during-pandemic), gender (males vs. females), and their interaction on vaping behaviors (days of vaping per week, episodes of vaping per day, and puffs per vaping episode). Respondents reported fewer days of vaping per week, episodes of vaping per day, and puffs per vaping episode during-pandemic than pre-pandemic [$F_{(3,533)} = 52.81$, $p < 0.001$, $\eta_p^2 = 0.229$]. The multivariate effect of gender on the three vaping outcomes was not statistically significant [$F_{(3,\ 533)} = 2.14$, $p = 0.095$, $\eta_p^2 = 0.012$], though the interaction between pandemic onset and gender was [$F_{(3,\ 533)} = 2.86$, $p = 0.036$, $\eta_p^2 = 0.016$]. Males reported fewer episodes of vaping per day [$t_{(262)} = 7.40$, $p < 0.001$, 95% CI: 5.19–8.97] and puffs per vaping episode [$t_{(263)} = 3.23$, $p = 0.001$, 95% CI:0.292–1.20] during-pandemic than pre-pandemic. Females reported fewer vaping episodes per day during-pandemic than pre-pandemic [$t_{(273)} = 5.14$, $p < 0.001$, 95% CI: 2.76–6.18]. Further, females reported more frequent puffs per vaping episode in comparison to males during-pandemic [$t_{(538)} = -2.38$, $p = 0.017$, 95% CI: −2.09–0.200]. The COVID-19 pandemic presents an opportunity to reduce vaping through health promotion messaging. Since females take more puffs per vaping episode overall, they may benefit the most from greater vaping cessation supports.

Keywords: electronic cigarette, coronavirus, teenager, vaper, substance use

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INTRODUCTION

The novel SARS-CoV-2 virus and the resulting declaration of the 2020 COVID-19 pandemic sparked discourse concerning vaping and smoking as risk factors for morbidity and mortality of COVID-19 (1–3). These concerns add to a plethora of research from recent years documenting the rise of vaping among youth and young adults (4). Almost without exception, this research is appended with the caution that the findings should be considered in the context that there is a

dearth of evidence on the harms of vaping, especially among young and non-smoking persons, and that more research is needed to investigate this issue. The COVID-19 pandemic adds a layer of complexity to this research. Specifically, a population which is at low risk of COVID-19 harms (i.e., youth and young adults) may become more vulnerable by way of vaping behaviors (i.e., hand-to-mouth virus transmission) and its associated respiratory harms (5).

The act of vaping requires repetitive physical contact between a person's hands, mouth, and e-cigarette (6). If an e-cigarette user is exposed to a person or surface with COVID-19, they would presumably be at higher risk of contracting the virus. In some instances, individuals may share their device with others, further increasing the risk of virus transmission (7). In the event that a person is wearing a mask in a public setting, as now recommended in several jurisdictions and by the World Health Organization, they would ultimately have to remove it to use an e-cigarette, which could increase both the risk of exposure and also transmission to others (8, 9). Recent evidence suggests that seeking a COVID-19 test and receiving a positive result was more likely among youth and young adult e-cigarette users compared to non-users, especially among dual cigarette and e-cigarette users (10).

While respiratory harms resulting from smoking have been well-established, such as tuberculosis, lung cancer, COPD, and asthma, evidence on the respiratory harms associated with vaping is scarcer, yet emerging (11). Short-term respiratory symptoms (e.g., cough, phlegm) are more frequently reported among young and adult e-cigarette users (12, 13). Further, many e-cigarette users use flavored products, which contain chemical additives, which may pose yet-to-be-established harms to the lungs (14, 15). Due to the novelty of e-cigarettes, it may require decades of research to establish the long-term biologic harms of vaping.

Emerging evidence suggests that nicotine exposure may exacerbate the pathobiology of COVID-19, namely through its interaction with Angiotensin-converting enzyme 2 (ACE2) (16). More specifically, cigarette and e-cigarette use can stimulate ACE2 receptors in the brain and lungs and put users of these products at higher risk for complications resulting from COVID-19 (17).

Pandemic Onset and Vaping

The unprecedented COVID-19 pandemic presents a unique opportunity to examine vaping under atypical conditions. Specifically, it allows us to hypothesize how youth and young adults are adapting their vaping behaviors in response to several aspects of daily life interrupted by the pandemic. Youth and young adults are not attending secondary and post-secondary schools and many young adults are working remotely from home. Early evidence suggests that Canadian high school students reduced vaping in the weeks following the recommendation for physical distancing [early April 2020; (18)].

Youth and young adults may use e-cigarettes less during the pandemic in comparison to pre-pandemic for a number of reasons. Vaping behaviors among youth are often hidden from parents and guardians, so youth spending more time at home than at school may limit their opportunity to use e-cigarettes without suspicion (19). Furthermore, youth who are underage and cannot access vaping products traditionally may not be able to meet with older peers or other social sources who purchase products on their behalf (20, 21). There is also the potential that regular users had reduced or no access to e-cigarettes from physical vape stores, which are the primary means of access to e-cigarettes in Canada (22). With respect to physical distancing guidelines, there are also fewer opportunities to meet with peers to socialize, an occasion that facilitates vaping among youth and young adults (23), which is especially true regarding the early months of the pandemic. Finally, vaping may reduce among the whole sample as a result of public health messaging on the risks of vaping during the pandemic (24, 25).

Evidence that concerns how e-cigarette users changed their vaping behaviors after pandemic onset is limited, especially among adolescent populations. One study of youth and young adult (aged 13–24 years) e-cigarette users in the United States reported that more than half (56.4%) of users reported different vaping behaviors since the pandemic onset, with 66.7% of those reporting different behaviors reducing use (26).

The Current Study

We administered the *2020 Youth and Young Adult Vaping Survey* to Canadian e-cigarette users aged 16–24 during April and May 2020. The first COVID-19 case in Canada was confirmed on February 20, 2020 and the World Health Organization declared a pandemic on March 11, 2020 (27). By the time that our survey was administered, the number of cases in Canada exceeded 20,000 (27). Thus, we had a unique opportunity to ask respondents to report their vaping behaviors prior to learning about the pandemic (retrospective), with the advantage of a limited recall period, and their vaping behaviors after the onset of the pandemic.

The aim of this study is to examine how youth and young adult e-cigarette users responded to the onset of the COVID-19 pandemic with respect to their vaping behaviors. Specifically, our goal is to test the effect of pandemic onset (pre- vs. during-pandemic) on three vaping behaviors: days of vaping per week, episodes of vaping per day, and puffs per vaping episode. The behaviors we chose measure vaping frequency, rather than prevalence. It is well-established that vaping is more prevalent among youth and young adults. However, the vaping behaviors among regular users that we chose are less evidenced and provide more insight into how youth and young adults engage in vaping behaviors rather than a dichotomous confirmation of past-30-days use. All of the aforementioned evidence suggests that youth and young adult e-cigarette users are likely to engage in vaping behaviors less during-pandemic relative to pre-pandemic.

Our study will make notable contributions to the vaping literature by adding evidence that examines multiple vaping behaviors both pre- and during-pandemic time periods. Additionally, we will present our findings in the context of three vaping behaviors, rather than overall use, which will identify which specific aspects of vaping (e.g., number of puffs) changed/did not change. We anticipate that our findings will inform prevention and policy strategies that target regular

e-cigarette users with respect to any differences identified through our analysis.

METHODS

Recruitment

We recruited youth (16–18 years old) and young adult (19–24 years old) e-cigarette users residing in five Canadian provinces (Alberta, British Columbia, Manitoba, Ontario, and Saskatchewan) for the *2020 Youth and Young Adult Vaping Survey*. The aim of this project was to gain insight into the perceptions and experiences of vaping among regular e-cigarette users (at least once/week). A youth and young adult sample was chosen because the prevalence of e-cigarette use in Canada is highest among these age groups compared to persons 25 and older (28).

Recruitment advertisements posted on Facebook and Instagram invited persons interested in the survey to a landing page on Qualtrics, an online survey platform. We enabled Qualtrics' "Prevent Ballot Box Stuffing" feature to limit fraudulent responses (i.e., taking the survey more than once). Participants viewed an online informed consent form and verified eligibility through a series of questions. We verified eligibility by asking if they are regular e-cigarette users (at least once a week over the last 3 months), reside in one of the five listed provinces, and are between the ages of 16 and 24. However, given the online nature of the study and in order to maintain anonymity, we were not able to confirm participants' age or e-cigarette use. An automated process invited eligible participants to complete the survey through Qualtrics. We entered eligible participants in a draw for 1 of 5 $100 gift cards and compensated those that completed the survey in full with $10. Email addresses could only be entered once to discourage participants from trying to take the survey more than once. Ethics approval was obtained from Saint Mary's Research Ethics Board (#19–105).

Survey

The 2020 Youth and Young Adult Survey was a cross-sectional survey that contained demographic questions and questions about the respondent's vaping behaviors, product preferences, and experiences. Respondents first completed screening questions to verify that they lived in Nova Scotia, were regular e-cigarette users ["Over the past 3 months, have you been vaping regularly (at least once a week)?], and met the age requirements "(What is your age? Please enter the number only)." Respondents selected their gender as "Male," "Female," or "Other (please specify)."

For the purpose of this study, respondents reported their vaping behaviors pre-pandemic by responding to three questions: "How many days per week do you vape?," "On the days you vape, how many times do you use it each day? Please enter a number only (e.g., 5)," and "When taking your vape out of your pocket/purse/backpack, how many puffs do you usually take in a single sitting before putting it away? Please enter a number only (e.g., 5)." Next, respondents reported their during-pandemic vaping behaviors by responding to the question "Since becoming aware of the novel COVID-19 (coronavirus) pandemic, I use my vape" with response options as "Less than before," "The same as before," or "More than before." Respondents who answered with more or less than before were prompted with the same three vaping outcome questions outlined above, but with the preface "Since becoming aware of the novel COVID-19 (coronavirus) pandemic. . . ."

Data Analysis

For the purpose of this study, we excluded e-cigarette users who used products not containing nicotine. Further, our primary analysis was limited to respondents who indicated different vaping behaviors pre- and during-pandemic. We produced descriptive statistics to report demographic and vaping characteristics among this sample. Gender was found to affect vaping behaviors during a multivariate test, while age category (youth vs. young adult)[1], employment status (yes or no)[2], and flavor preference (yes or no)[3] did not have an effect on the outcomes of the interest as a main effect or an interaction. Therefore, we conducted a two-way multivariate analyses of variance (MANOVA) to compare the group means of three vaping behaviors (days of vaping per week, number of vaping episodes per day, and number of puffs per vaping episode) by gender (males vs. females) and pandemic onset (pre- vs. during-pandemic). To establish pre- and during-pandemic behaviors, we asked respondents to report the three vaping behaviors prior to learning about the pandemic (pre-pandemic) and after learning about the pandemic (during-pandemic). For significant multivariate effects, we conducted univariate analyses to test the effect of pandemic onset, gender, and their interaction on each individual outcome. We then conducted paired *t*-tests for statistically significant univariate tests to test differences in vaping behaviors of each gender with respect to pandemic onset, and an independent samples *t*-test to test differences in vaping behaviors at each pandemic period (pre- vs. during-pandemic) between genders. A $p < 0.05$ indicated a significant effect. SPSS 26.0 was used for analysis.

RESULTS

Sample Characteristics

Though 1,308 respondents completed the survey, only 1,209 (92.4%) reported using a nicotine-containing e-cigarette and only these participants were analyzed. 44.7% of respondents ($n = 540$) reported different vaping behaviors pre- vs. during-pandemic and 55.3% ($n = 669$) reported unchanged behaviors. Of those who reported different vaping behaviors after pandemic onset, ($n = 540$), 51.1% ($n = 274$) were female, 55.9% ($n = 302$) were youth aged 16–18, 56.7% ($n = 306$) were employed, and 88.6% ($n = 453$) preferred using flavored vape/e-juice.

Respondents who reported unchanged vaping behaviors after pandemic onset reported different vaping behaviors overall than respondents who reported changed behaviors pre- and during-pandemic, $F_{(3,\ 1,205)} = 24.20$, $p < 0.001$, $\eta_p^2 = 0.057$. Compared

[1]Main effect: $F_{(3,533)} = 1.18$, $p = 0.318$; Interaction: $F_{(3,533)} = 1.45$, $p = 0.266$.
[2]Main effect: $F_{(3,533)} = 1.79$, $p = 0.147$; Interaction: $F_{(3,533)} = 1.04$, $p = 0.373$.
[3]Main effect: $F_{(3,504)} = 1.23$, $p = 0.300$; Interaction: $F_{(3,504)} = .106$, $p = 0.956$.

TABLE 1 | Univariate effects for pandemic onset, gender, and their interaction for the three vaping outcomes.

		F	*p*	η_p^2
Pandemic onset	Days of vaping/week	117.61	0.000*	0.180
	Episodes of vaping/day	80.04	0.000*	0.130
	Puffs/vaping episode	3.91	0.048*	0.007
Gender	Days of vaping/week	1.37	0.242	0.003
	Episodes of vaping/day	0.52	0.47	0.001
	Puffs/vaping episode	3.43	0.065	0.006
Pandemic onset x Gender	Days of vaping/week	0.94	0.334	0.002
	Episodes of vaping/day	4.10	0.043*	0.008
	Puffs/vaping episode	5.73	0.017*	0.011

**Indicates significant effect, $p < 0.05$.*

to respondents who reported changed behaviors, those reporting unchanged behaviors reported a lower number of days of vaping per week [$M = 6.51$, $SD = 1.38$; $F_{(1,\ 1,207)} = 41.18$, $p < 0.001$, $\eta_p^2 = 0.033$], number of vaping episodes per day [$M = 5.93$, $SD = 1.86$; $F_{(1,\ 1207)} = 49.58$, $p < 0.001$, $\eta_p^2 = 0.039$], but not number of puffs per vaping episode [$M = 6.13$, $SD = 4.79$; $F_{(1,\ 1207)} = 0.001$, $p = 0.979$]. Respondents reporting changed and unchanged behaviors were not different with respect to age [$t_{(1,\ 207)} = 0.796$, $p = 0.426$, 95% CI: −0.127−−0.30] or gender [X^2 (1, $N = 1{,}209$), $p = 0.079$].

Main Results

The within-subjects multivariate effect of pandemic onset was statistically significant, $F_{(3,\ 533)} = 52.81$, $p < 0.001$, $\eta_p^2 = 0.229$. Respondents reported a lower number of days of vaping per week, number of vaping episodes per day, and number of puffs per vaping episode. The between-subjects multivariate effect of gender on the three vaping outcomes was not statistically significant, $F_{(3,\ 533)} = 2.14$, $p = 0.095$, $\eta_p^2 = 0.012$. However, the multivariate interaction between pandemic onset and gender was significant, $F_{(3,\ 533)} = 2.86$, $p = 0.036$, $\eta_p^2 = 0.016$.

The effects at the univariate level are displayed in **Table 1**. There was a significant main effect of pandemic onset on all three vaping outcomes. With respect to the interaction of pandemic onset and gender, there was a significant main effect on number of vaping episodes per day and number of puffs per vaping episode, but not days of vaping per week (**Figure 1A**).

Vaping Behaviors pre- vs. During-Pandemic by Gender

A series of 2-tailed paired *t*-tests revealed that males and females responded differentially to pandemic onset (**Table 2**). Males reported fewer vaping episodes per day during-pandemic compared to pre-pandemic, $t_{(262)} = 7.40$, $p < 0.001$, 95% CI: 5.19–8.97 (**Figure 1B**). Males also reported fewer number of puffs per vaping episode during-pandemic than pre-pandemic, $t_{(263)} = 3.23$, $p = 0.001$, 95% CI:0.292–1.20 (**Figure 1C**). Females too reported fewer vaping episodes per day, albeit to a lesser extent than males, during-pandemic compared to pre-pandemic, $t_{(273)} = 5.14$, $p < 0.001$, 95% CI: 2.76–6.18 (**Figure 1B**). However, females did not reduce the number of puffs per vaping episode during-pandemic compared to pre-pandemic, $t_{(275)} = -0.17$, $p = 0.868$, 95% CI: −0.5.35−0.452 (**Figure 1C**).

Vaping Behaviors Pre-pandemic by Gender

Independent samples *t*-tests revealed that males and females were not different with respect to number of vaping episodes per day pre-pandemic, $t_{(538)} = 1.24$, $p = 2.15$, 95% CI: −1.76–7.82 (**Figure 1B**), or number of puffs per vaping episode pre-pandemic, $t_{(538)} = -0.86$, $p = 0.39$, 95% CI: −1.18−0.458 (**Figure 1C**).

Vaping Behaviors During-pandemic by Gender

The results of independent samples *t*-tests revealed that males and females were not different with respect to the number of vaping episodes per day during-pandemic, $t_{(535)} = 0.11$, $p = 0.88$, 95% CI: −4.21, 4.9 (**Figure 1B**). However, females reported more frequent puffs per vaping episode in comparison to males during-pandemic, $t_{(538)} = -2.38$, $p = 0.017$, 95% CI: −2.09-−0.200 (**Figure 1C**).[4]

DISCUSSION

The current study examined how youth and young adult e-cigarette users changed their vaping behaviors during the COVID-19 pandemic relative to the period preceding the pandemic. Further, the study findings shed light on gender differences in vaping behaviors in response to the pandemic. These findings are discussed in the context of the limited existing literature on this topic and the implications of the findings.

The main finding of the study is the reduced vaping behavior overall among participants who reported different vaping behaviors pre- and during-pandemic: lower days of vaping per week, lower episodes of vaping per day, and puffs per vaping episode. However, this finding should be considered alongside the fact that less than half of respondents indicated that they changed their vaping behaviors after learning about the pandemic. This is in line with the findings of another study that suggest that young (<21 years old) e-cigarette users in the United States who changed their vaping behaviors after pandemic onset were more likely to report decreased use than increased use, and also that roughly half of this sample reported different vaping behaviors after pandemic onset (26). There is both concern and promise in this finding. The concern is centered around the fact the findings depict small changes in vaping behaviors among youth and young adults during the COVID-19 pandemic, despite difficulties in accessing e-cigarettes (e.g., closed retail outlets, physical distancing, reduced social sourcing) (20–23). This suggests that efforts to reduce vaping, even in the midst of the pandemic, are crucial. Recent literature has identified potentially promising vaping cessation strategies for youth and young adults, such as traditional and mobile health counseling (29). Such strategies need to be urgently implemented. The promise in this finding, however, is related to visualizing the COVID-19 pandemic as an opportunity for effective health

[4]Equal variances not assumed, Levene's Test for Equality of Variances, $p = 0.001$.

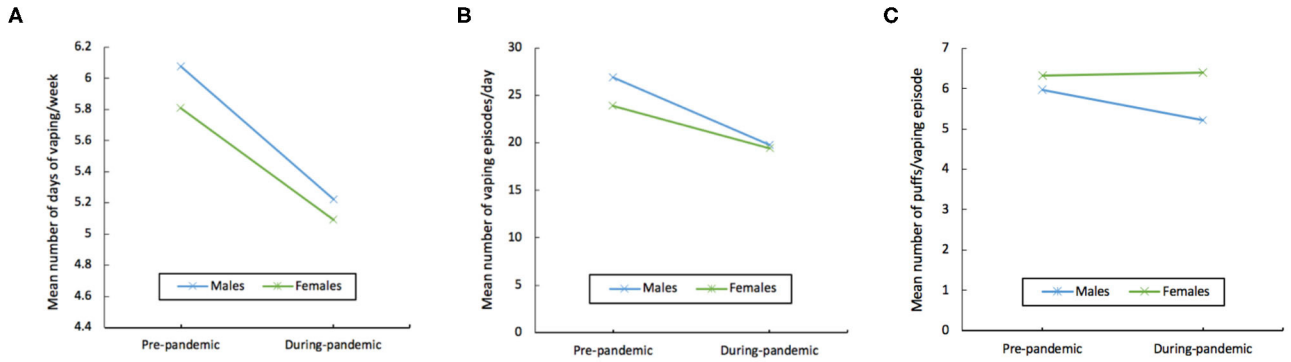

FIGURE 1 | Interaction graphs illustrating mean values segmented by pandemic onset and gender of **(A)** number of days of vaping per week, **(B)** number of vaping episodes per day, and **(C)** number of puffs per vaping episode.

TABLE 2 | Mean vaping behaviors segmented by pandemic onset and gender.

	Pre-pandemic (*M, SD*)			During-pandemic (*M, SD*)		
	Male	**Female**	**Total**	**Male**	**Female**	**Total**
Days of vaping/week	6.08 (1.82)	5.81 (1.87)	5.94 (1.85)	5.22 (2.42)	5.09 (2.38)	5.15 (2.40)
Episodes of vaping/day	26.87 (29.46)	23.90 (27.39)	25.35 (28.43)	19.79 (26.84)	19.43 (27.09)	19.61 (26.94)
Puffs/vaping episode	5.96 (4.74)	6.32 (4.94)	6.14 (4.85)	5.21 (5.21)	6.39 (5.97)	5.81 (5.64)

promotion messaging to youth and young adults that frames e-cigarette use as a preventable behavior that may increase risk of COVID-19 transmission and severity. Arguably, reduced vaping behaviors during the COVID-19 pandemic are at least partially attributed to the fear of potential complications from vaping if one were to contract the virus or simply the increased prospects of contracting the virus from vaping socially (e.g., sharing the devices). Recent research that demonstrated increased risk of COVID-19 among e-cigarette users reinforces this argument (10). Social marketing campaigns utilizing fear and self-efficacy messages may be an important approach to capitalize on in order to reduce vaping during the pandemic (30). The main finding of the study contradicts some findings on changes in substance use for other addictive products during the pandemic, namely the unchanged weekly consumption of alcohol, cannabis, and tobacco use among Canadians 15–34 years old (31). This difference may be explained by the increased cautionary messages from public health entities on the risks of vaping during the COVID-19 pandemic (24, 25). However, research conducted in other regions indicates that youth and young adults (>16 years old) in England increased alcohol use during the early months of the pandemic (32).

The second main finding is the differential response of females and males to the pandemic—in particular male, but not female, reductions in puffs per vaping episode during-pandemic. This is consistent with past research that demonstrated higher female receptivity to non-nicotinic elements of the vaping experience, including stress reduction (33). Further, stressors surrounding the pandemic, such as uncertainties in females' personal life [e.g., parenting; (34)], may keep female vaping consistent throughout the pandemic in comparison to pre-pandemic. The finding serves as an alert for the need of healthier coping mechanisms for females as recommended by prior research (33).

Two observations with respect to gender differences pre- and during-pandemic are worth noting. The first is the lack of differences between males and females in vaping behaviors pre-pandemic. This finding is consistent with the lack of gender differences in ever vaping (35). However, such studies tested differences in "ever use," which is a prevalence measure, while we used measures of vaping frequency, which indicates how much youth and young adults use e-cigarettes (35). In this sense, our findings extend the literature by indicating that, besides lack of differences in ever use of e-cigarettes, vaping frequency is also not different among genders. Second, both males and females reported the same number of vaping episodes per day during-pandemic, despite females taking more puffs per vaping episode during-pandemic. This finding highlights an urgency for selective vaping cessation for females as they are more vulnerable to taking more puffs from their e-cigarette when they have the opportunity to use it during the pandemic relative to males. This is partially in line with the findings of at least one study that found that females consume more alcohol during the pandemic (36). Altogether females seem to be more vulnerable to higher substance use relative to males during the pandemic.

Limitations

There are a number of limitations for the current study. First, we used a cross-sectional survey to examine vaping behaviors pre- and during-pandemic using retrospective measurement of past behaviors. This may lead to inaccuracies in self-reported behaviors by respondents. However, we conducted the survey within 2 months of the onset of the COVID-19 pandemic

which minimizes recall bias and serves as a good first step toward understanding pandemic effects on vaping behaviors. Nevertheless, longitudinal studies may capture more precise changes in vaping behaviors. The cross-sectional nature of our study also limits what we can infer about which aspects of the pandemic encouraged respondents to change their vaping behaviors, especially participant characteristics that were not collected in our survey (e.g., whether participants are students). Second, our sample consisted of regular (at least once/week over the last 3 months) Canadian e-cigarette users aged 16–24, which may limit the generalizability of the results to users in other geographic regions, older users, and experimental e-cigarette users. However, we examined a sample of Canadians who are the most prevalent e-cigarette users (youth and young adults) from five diverse provinces. Further, vaping characteristics have been found to be reasonably universal across different regions (37). Third, though we assessed cigarette use and other substance use (alcohol and cannabis) among our sample, we did not examine changes in cigarette or other substance use pre- vs. during-pandemic. Thus, we cannot infer whether decreases in vaping behavior may have influenced cigarette or other substance use and vice versa. Fourth, our findings are limited to the early months of the pandemic and should not be extrapolated to the present and future days of the pandemic without further investigation.

CONCLUSION

To conclude, the current study provides insight into how pandemic onset, alone and considered by gender, may have influenced vaping behaviors among Canadian youth and young adults. The still-concerning proportion of vaping behaviors among this demographic, even in the current pandemic, emphasize the need for immediate resources aimed at reducing or discontinuing use among youth and young adults, rather than prevention-focused efforts. As the pandemic continues to evolve, it is necessary to continue monitoring vaping behaviors among this population.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because ethics clearance was not obtained to provide a publicly available dataset. Requests to access the datasets should be directed to mohammed.al-hamdani@smu.ca.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Saint Mary's University Research Ethics Board. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MA-H and DBH: Conceptualization, methodology, software, validation, formal analysis, investigation, writing—review and editing, and project administration. N/A: resources. DBH: Data curation, writing—original draft preparation, and visualization. MA-H: supervision and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Humoral and Cellular Response of Frontline Health Care Workers Infected by SARS-CoV-2 in Nice, France: A Prospective Single-Center Cohort Study

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Frontline health care workers (HCWs) have been particularly exposed to Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) since the start of the pandemic but the clinical features and immune responses of those infected with SARS-CoV-2 have not been well described. In a prospective single center cohort study, we enrolled 196 frontline HCWs exposed to the SARS-Cov-2 and 60 patients with moderate and severe forms of the coronavirus disease 2019 (COVID-19). Serological tests and cytokines assay were performed to analyze SARS-CoV-2-specific humoral and cellular immunity. Of the 196 HCWs tested, 15% had specific antibodies against SARS-CoV-2 and 45% of seropositive HCWs were strictly asymptomatic. However, in comparison to moderate and severe forms, HCWs with mild or asymptomatic forms of COVID-19 showed lower specific IgA and IgG peaks, consistent with their mild symptoms, and a robust immune cellular response, illustrated by a high production of type I and II interferons. Further studies are needed to evaluate whether this interferon functional immune assay, routinely applicable, can be useful in predicting the risk of severe forms of COVID-19.

Keywords: health care workers, SARS-CoV-2, COVID-19, humoral response, cellular response, blood immune biomarkers

INTRODUCTION

Following the first descriptions of acute respiratory syndrome cases in Wuhan, Hubei province, China, at the end of December 2019, a novel beta coronavirus called Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) was identified (1). This virus, responsible for the new coronavirus disease (COVID-19), quickly spread to other regions of China and then outside the country. The pandemic stage was declared by the World Health Organization (WHO) on March 11, 2020.

The transmission of COVID-19 to health care workers (HCWs) is a serious concern as it puts potentially very vulnerable patient populations at risk. Nasopharyngeal swabs (NPSs) are being widely used as specimens for real-time reverse transcription (RT)-PCR to detect symptomatic HCWs (fever, cough, fatigue, muscle pain, diarrhea). This common practice helps to slow or stop the spread of infection and protect patients and other HCWs. However, a significant proportion of those infected were asymptomatic or pauci-symptomatic but still transmitted the virus (2–4).

As shown in previous studies, patients infected with SARS-CoV-2 develop an antibody response against the virus (5). Asymptomatic individuals, however, appear to reveal a weaker humoral immune response (6). Other studies, conducted in patients with moderate to severe forms of COVID-19, looked at the cellular immune response. They showed that lymphopenia (1, 7), and type I and II interferon (IFN) deficiency secreted by the remaining T cells (8–10) correlate with the severity of the disease. At present, this cellular immune response has not yet been studied in asymptomatic subjects.

To our knowledge, only few studies have been conducted characterizing both humoral and cellular immune response to SARS-CoV-2 infection (11), and no study investigated this global immune response in a specific population of frontline HCWs particularly exposed to SARS-CoV-2 during the pandemic. In this prospective single-centered cohort study, we first sought to assess the SARS-CoV-2 antibodies seroprevalence of asymptomatic and pauci-symptomatic SARS-CoV-2 infection in frontline health care workers, as well as compare their humoral and cellular response to patients with moderate and severe forms of COVID-19. In addition to improving knowledge on the immune response to this emerging disease, the identification of potential blood immune biomarkers predictive of the response to SARS-CoV-2 could allow us to better prevent the onset of severe forms of COVID-19, particularly in subjects highly exposed to the virus such as frontline HCWs.

MATERIALS AND METHODS

Study Design and Participants

We performed a prospective cohort study of subjects exposed to SARS-CoV-2 virus at Nice University Hospital, France. For this, we included volunteer frontline health care workers (HCWs) defined as those working in units providing care for patients with confirmed COVID-19, in Nice University Hospital from April 15 to May 26, 2020. After signing an informed consent, they completed a self-questionnaire and had their blood drawn to perform a serological test and a functional immune assay. Exclusion criteria were: (1) pregnancy or breastfeeding; (2) HCWs having received previous immunosuppressive therapy for COVID-19 treatment. We divided seropositive SARS-CoV-2 HCWs into four subgroups according to the symptoms that occurred in the 3 months preceding the blood test and that they had to declare in the questionnaire: (1) strictly asymptomatic; (2) mild symptoms if they had common symptoms of COVID-19, including fever, fatigue, cough, rhinorrhea, muscle pain, headache, diarrhea, anosmia or other flu-like symptoms (1, 7); (3) moderate form of COVID-19 if they were hospitalized in infectious diseases units due to clinical symptoms associated with dyspnea and radiologic findings consistent with a COVID-19 pneumonia on thoracic CT-scan; (4) severe form of COVID-19 if they were either hospitalized or transferred to the intensive care unit with respiratory failure requiring mechanical ventilation, or with multiple organ failure. Household members of the HCWs tested seropositive for SARS-CoV-2 infection were also invited to participate in the study.

We performed a second prospective cohort study made up of patients infected with SARS-CoV-2 followed at Nice University Hospital, France. The inclusion criteria were: (1) all adult patients hospitalized for COVID-19 in infectious diseases units (IDU) or in intensive care unit (ICU), in Nice University Hospital from March 13 to April 16, 2020; (2) ability to sign an informed consent. Exclusion criteria were: (1) age under 18; (2) patients under custody, in prison or with a mental illness; (3) pregnancy or breastfeeding; (4) patients having received previous immunosuppressive therapy for COVID-19 treatment. The patients were divided into two groups according to the severity of infection with SARS-CoV-2: moderate or severe forms of COVID-19 as above. All patients presented a COVID-19 symptomatology according to WHO recommendations (12) with a CT-scan characteristic of COVID-19 (13) or two consecutive positive RT-PCR tests for SARS-CoV-2 on upper and lower respiratory tract specimens (NPS or invasive respiratory sample).

Procedures

Data Collection

Epidemiological and clinical data were collected using the electronic medical records applications Clinicom® and ORBIS® for COVID-19 patients and the self-questionnaire for HCWs. This self-administered questionnaire collected information on demographic factors, medical history, previous or present treatments, hospital function, known risk factors for COVID-19, and symptoms that may have occurred in the 3 months preceding the blood sample. HCWs were also asked if they had already been tested for COVID-19 RT-PCR and what were the results. When available, the time delays (in days) between the onset of the first symptoms of COVID-19 and inclusion, i.e., the day of the first blood sampling, were recording. For asymptomatic IgA-positive HCWs without anti-SARS-CoV-2 IgG antibodies, we estimated this time to be between 7 and 10 days. We considered this data to be missing for asymptomatic HCWs who were IgG-positive with or without IgA antibodies.

Sampling Process

SARS-CoV-2 virological tests for patients followed the World Health Organization recommendations (12). NPSs were obtained by nurses or physicians using a standard technique and were immediately placed in a transport medium and delivered to our central laboratory to confirm COVID-19 by real-time reverse transcription-polymerase chain reaction (RT-PCR) methods. Blood samples were collected at day 0 of the admission and at several follow-up points up to 2 months after hospital admission for COVID-19 patients, and at inclusion for HCWs. For hospital staff tested positive for SARS-CoV-2 infection, a

second blood sample was taken 1 month after inclusion. Samples were immediately processed and then frozen and stored at −20°C until serological tests and functional immune assay (cellular response/cytokines assay) were performed. Freeze-thaw cycles were minimized to preserve the quality of the samples.

Laboratory Methods

Serological Test

Serological tests for anti-SARS-CoV-2 IgA and IgG isotypes antibodies were performed on serum using a commercially available enzyme-linked immunosorbent assays (ELISA) which used the S1-domain of the spike protein of SARS-CoV-2 as the antigen (Euroimmun AG, Lübeck, Germany, # EI 2606-9601 A and # EI 2606-9601 G). They were run on IF Sprinter IFT/ELISA (Euroimmun) according to the manufacturer's protocol. The results are evaluated by calculating the ratio between the optical density (OD) of the sample at 450 nm and the OD of the calibrator at 450 nm, according to the following formula:

$$\frac{OD\ of\ the\ sample}{OD\ of\ the\ calibrator} = OD\ ratio$$

According to the manufacturer's recommendations, the results were then interpreted as follows: OD ratio <0.8 = negative; ≥ 0.8 and <1.1 = indeterminate; ≥ 1.1 = positive (14).

Cellular Response/Cytokines Assay

One milliliter of whole blood was stimulated with immune ligands [anti-CD3 as T-cells stimulant, and R848 as Toll-like receptors 7/8 (TLR 7/8) agonist] on single lyophilized spheres (LyoSphere™, Qiagen) within 8 h from blood collection. Stimulated blood samples were incubated for 16–24 h at 37°C and then centrifuged at 2,000–3,000 × g for 15 min to harvest the stimulated supernatant. Levels of cytokines after non-specific stimulation were measured using IFN-γ ELISA microplates from QuantiFERON-Monitor test (Qiagen®) and Ella (ProteinSimple®) custom-designed cartridges for the detection of IFN-α, following the manufacturers' instructions.

Statistical Analyses

For descriptive statistics, data are presented as mean and standard deviation for quantitative variables with Gaussian distribution, as median and range for quantitative variables with non-Gaussian distribution, or as numbers and percentages for qualitative variables. The Shapiro-Wilk test was used to determine if a variable had a Gaussian distribution or not. Quantitative variables were compared by the unpaired *t*-test or one-way ANOVA if the values were normally distributed and by the Mann-Whitney test if they were not. Qualitative variables were compared using Chi-square test or Fisher's exact test as appropriate. A Wilcoxon matched pairs signed rank test was used to compare two measurements of a quantitative variable. Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA). Differences were considered significant when P value < 0.05.

Ethics and Consent

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was reviewed and approved by our local institutional review committee (NCT04355351). Written informed consent was obtained from participants prior to inclusion in the study. All collected data and samples were securely stored.

RESULTS

Participants' Characteristics

Between April 15 and May 26, 2020, we enrolled 196 frontline HCWs in Nice University Hospital. Twenty-nine (15%) were seropositive for anti-SARS-CoV-2 antibodies. Nine HCWs had a positive NPS: one with moderate symptoms of COVID-19 requiring hospitalization in infectious diseases unit (IDU), seven with mild symptoms and one asymptomatic subject but with close contact with a confirmed COVID-19 case. Twenty HCWs had no NPS but were found seropositive: eight had presented mild symptoms compatible with a COVID-19, and 12 were asymptomatic (**Figure 1**). Overall, 1/29 (3%) seropositive HCWs had moderate symptoms, 15/29 (52%) had mild symptoms of COVID-19, and 13/29 (45%) were strictly asymptomatic (**Figure 2B**).

Of these 29 infected HCWs, 12 presented only IgA antibodies and 17 had IgA and IgG seroconversion (**Figure 2A**). Among them, the nine infected HCWs who had a positive PCR had both IgA and IgG. The presence of IgA antibodies would indicate contamination more than 10 days ago with a sensitivity of 100% (14), while IgG detection would signify contamination more than 21 days ago with a sensitivity of 100% (14).

Twenty-one (72%) infected HCWs were women with a median (IQR) age of 38 (31–43) years, while 23 (38%) infected patients were women with a median age of 65 (54–74) years, reflecting the high proportion of young women in health care. Most HCWs were nursing assistants [six seropositive for SARS-CoV-2 out of 32 tested (15%)], physicians [7/41 (17%)], nurses [8/53 (15%)] and medicine residents [4/32 (12.5%)] (**Table 1**). The other HCWs in the cohort were dietitians, nursing students, physiotherapists, and psychologists (none seropositive for SARS-CoV-2 out of seven tested). HCWs working in COVID units but not directly in contact with patients were hospital engineers [two seropositive for SARS-CoV-2 out of three tested (67%)], laboratory technicians [1/3 (33%)], hospital service agents [1/11 (9%)], senior health managers (0/9), medical secretaries (0/4) and clinical research assistants (0/1). We did not find any significant difference in the rate of SARS-CoV-2 infection between HCWs directly exposed and those not directly exposed to infected patients ($p = 0.38$) (**Table 1**, **Figure 3**).

Between March 13 and April 16, 2020, we enrolled 60 patients with COVID-19 in Nice University Hospital, divided in two subgroups: moderate ($n = 30$) and severe cases of COVID-19 ($n = 30$). This cohort was compared to the frontline HCWs.

Demographic and baseline characteristics of the 29 HCWs and 60 patients with COVID-19, separated into three groups according to the severity of symptoms, are summarized in **Tables 2**, **3**. The cohort of infected HCWs included significantly

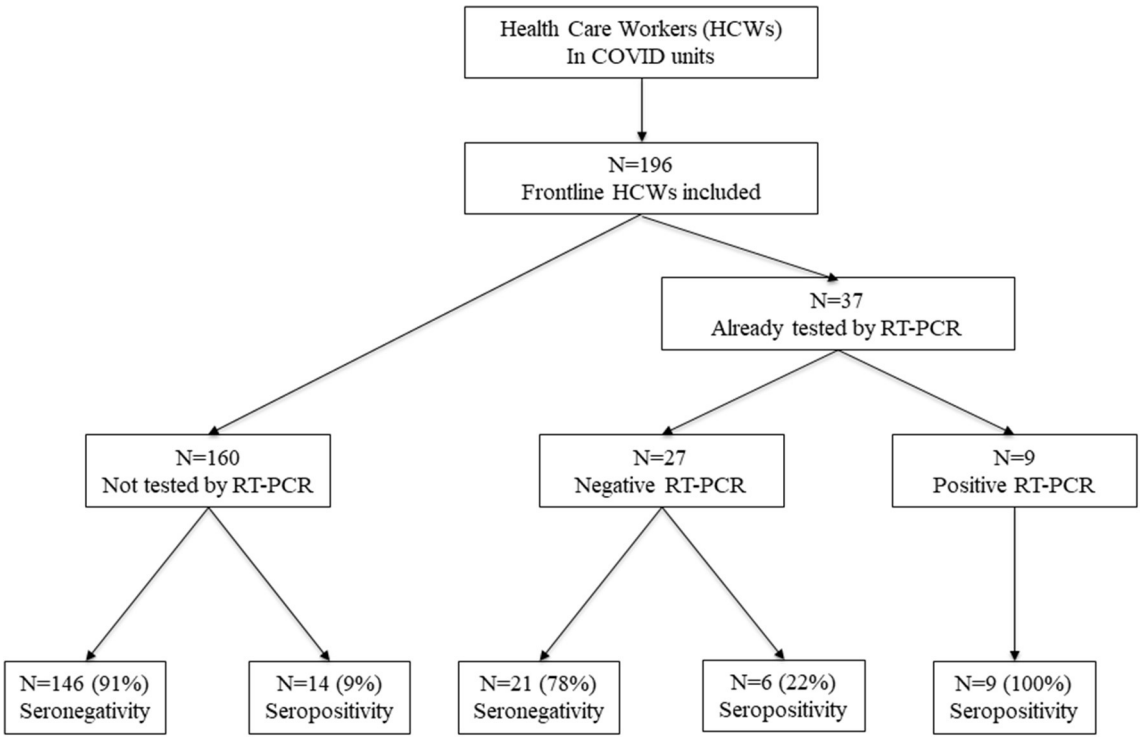

FIGURE 1 | Enrollment of HCWs and the subgroups formed according to SARS-CoV-2 infection. HCWs, health care workers.

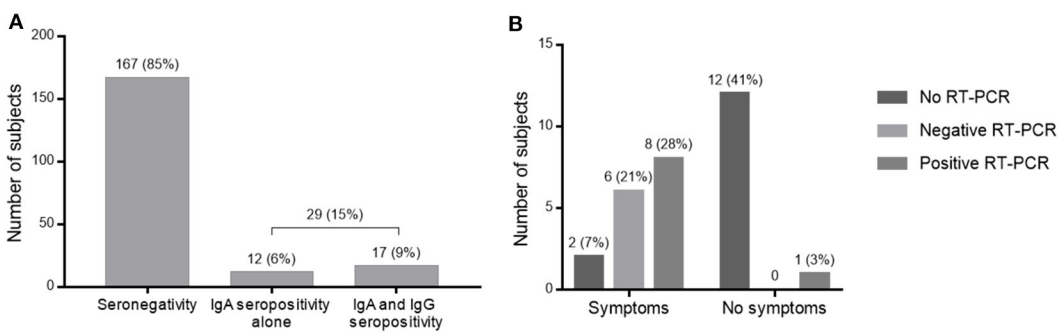

FIGURE 2 | Results of serological tests. **(A)** Serological screening test for SARS-CoV-2 among HCWs in COVID units. **(B)** RT-PCR results and symptomatology of HCWs seropositive for SARS-CoV-2.

fewer men (8/29, 28%) than that of patients (37/60, 62%). The HCWs were also younger [38 (31–43) years] than the patients [65 (54–74) years]. The rate of comorbidities in affected HCWs was 31% (9/29), which is significantly lower than patients whose rate of comorbidities was 82% (49/60). The most common comorbidities among HCWs with SARS-CoV-2 infection were asthma [3 (10%)], hypertension [2 (7%)] and cancer [2 (7%)] while those among patients were hypertension [28 (47%)], diabetes [16 (27%)] and cardiovascular disease [10 (17%)]. There was no difference in taking treatments known to cause severe COVID-19 symptoms in the two cohorts. As in previous studies, being overweight defined by a BMI $>$ 25 was found to be a risk factor for a severe form of COVID-19 (mean 22.76 in asymptomatic and mild cases, 25.31 in moderate cases and 27.02 in severe cases, global p value $= 0.0005$). In our study, there was the same rate of smokers in the three groups ($p = 0.1941$). Most of the infected HCWs were strictly asymptomatic [13 (45%)], but fever [9 (31%)], cough [7 (24%)], and headache [5 (17%)] were prevalent. In COVID-19 patients the three most common symptoms were dyspnea [44 (73%)], cough [38 (63%)], and fever [35 (58%)]. The median time from the onset of first symptoms of COVID-19 to inclusion, otherwise the date of first blood collection, was 7 (7–54) days for HCWs, 9 (5–14) days for patients with moderate COVID-19 infection and 8 (5–10) days for severe

cases. There was no difference in demographics, comorbidities, and symptoms between HCWs in COVID-dedicated units who were directly in contact with infected patients, from HCWs not in direct contact with patients (data not shown).

Humoral Immune Responses to SARS-CoV-2 in Health Care Workers and Patients

Kinetics of Specific Antibodies to SARS-CoV-2 in Severe COVID-19 Patients

We evaluated SARS-CoV-2 specific antibody responses in 13 severe cases who recovered from the infection using serum samples collected at day 0 of the admission and at several follow-up points up to 2 months after hospital admission. The proportion of patients with positive SARS-CoV-2-specific IgA and IgG at admission was 9/13 (69%) and 6/13 (46%), respectively, and reached 100% for the two isotypes after 15 days of hospitalization (**Figures 4A,B**). During the first 2 weeks after the admission for IgA and 4 weeks after the admission for IgG, titers for SARS-CoV-2-specific antibodies were generally increasing. The IgA level then decreased, although it was still positive even at 7 weeks, while that of IgG remained relatively stable over time.

Kinetics of Specific Antibodies to SARS-CoV-2 in Health Care Workers

For eight infected HCWs with only IgA at inclusion, a second serum sample was collected 1 month later to verify IgG seroconversion. The levels of IgA and IgG antibodies specific to SARS-CoV-2 increased significantly between the two time points but only two individuals achieved the level of IgG positivity and one exhibited an undetermined result (**Figures 4C,D**).

TABLE 1 | SARS-CoV-2 infection rate by function within the COVID unit.

Function within the COVID unit	Seropositivity rate/total number of agents tested, *n/N* (%)
Hospital workers directly in contact with SARS-CoV-2 infected patients (*n* = 165)	
Nursing assistants	6/32 (19%)
Physicians	7/41 (17%)
Nurses	8/53 (15%)
Medicine residents	4/32 (12.5%)
Dietitians	0/1 (0%)
Nursing students	0/3 (0%)
Physiotherapists	0/2 (0%)
Psychologists	0/1 (0%)
Hospital workers not directly in contact with SARS-CoV-2 infected patients (*n* = 31)	
Hospital engineers	2/3 (67%)
Laboratory technicians	1/3 (33%)
Hospital service agents	1/11 (9%)
Senior health managers	0/9 (0%)
Medical secretaries	0/4 (0%)
Clinical research assistants	0/1 (0%)

TABLE 2 | Baseline characteristics of health care workers and patients with COVID-19.

	HCWs (*n* = 29)	Patients (*n* = 60)	*P* value
Age, years	38 (31–43)	65 (54–74)	<0.0001
Males, *n* (%)	8 (28%)	27 (45%)	0.1150
Any comorbidity, *n* (%)	9 (31%)	49 (82%)	<0.0001
Diabetes, *n* (%)	0 (0%)	16 (27%)	0.0021
Hypertension, *n* (%)	2 (7%)	28 (47%)	0.0002
Cardiovascular disease, *n* (%)	0 (0%)	10 (17%)	0.0196
COPD, *n* (%)	0 (0%)	2 (3%)	0.3200
Asthma, *n* (%)	3 (10%)	4 (7%)	0.5457
Cancer, *n* (%)	2 (7%)	8 (14%)	0.3675
Previous treatment			
NSAIDs, *n* (%)	0 (0%)	2 (3%)	0.3200
Corticosteroids, *n* (%)	1 (3%)	3 (5%)	0.7405
Immunosuppressive therapy, *n* (%)	1 (3%)	5 (8%)	0.3890
BMI	22.09 (20.33–23.78)	25.40 (23.06–29.19)	0.0003
Smoking, *n* (%)	3 (10%)	2 (3%)	0.1782

BMI, body mass index; COPD, chronic obstructive pulmonary disease; HCWs, hospital care workers; NSAIDs, non-steroidal anti-inflammatory drugs.

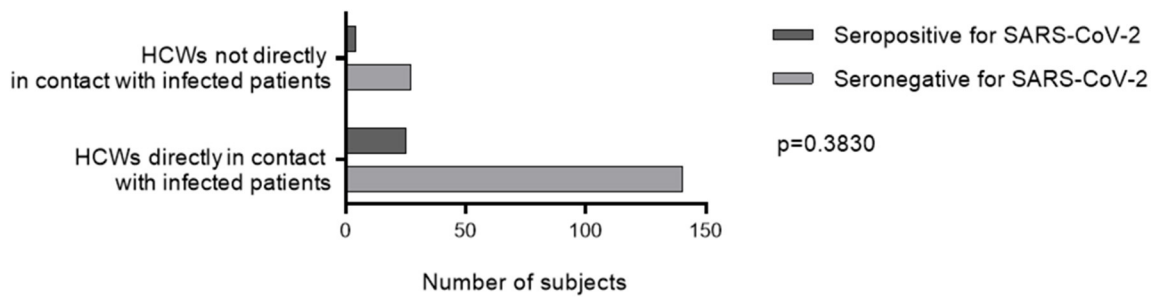

FIGURE 3 | SARS-CoV-2 seropositivity rate according to occupational exposure. A two-way ANOVA test was used to compare the seropositivity rate HCWs directly exposed and those not directly exposed to infected patients. HCWs, health care workers.

TABLE 3 | Demographic and baseline characteristics of health care workers and patients with COVID-19 according to the severity of symptoms.

	Asymptomatic and mild cases: HCWs after screening ($n = 28$)	Moderate cases: HCW, $n = 1$, and patients hospitalized in IDU, $n = 30$ ($n = 31$)	Severe cases: patients hospitalized in ICU ($n = 30$)	Global *P* value
Characteristics at baseline				
Age, years	38 (31–43)	64 (54–75)	65 (53–72)	<0.0001
Males, *n* (%)	7 (25%)	17 (55%)	21 (70%)	0.0024
Any comorbidity, *n* (%)	9 (32%)	27 (87%)	22 (73%)	<0.0001
Diabetes, *n* (%)	0 (0%)	7 (23%)	9 (30%)	0.0086
Hypertension, *n* (%)	2 (7%)	15 (48%)	12 (40%)	0.0019
Cardiovascular disease, *n* (%)	0 (0%)	4 (13%)	6 (20%)	0.0513
COPD, *n* (%)	0 (0%)	2 (6%)	0 (0%)	0.1475
Asthma, *n* (%)	3 (11%)	2 (6%)	2 (7%)	0.7951
Cancer, *n* (%)	2 (7%)	3 (10%)	5 (17%)	0.4885
Previous treatment				
NSAIDs, *n* (%)	0 (0%)	1 (3%)	1 (3%)	0.6250
Corticosteroids, *n* (%)	1 (4%)	1 (3%)	2 (7%)	0.7782
Immunosuppressive therapy, *n* (%)	1 (4%)	2 (6%)	3 (10%)	0.6193
BMI	22.76 ± 4.33	25.31 ± 4.03	27.02 ± 5.17	0.0005
Smoking, *n* (%)	3 (11%)	0 (0%)	2 (7%)	0.1941
Days after first signs of COVID-19	7 (7–54)[a]	9 (5–14)[b]	8 (5–10)[c]	0.1363
Signs and symptoms of COVID-19				
Fever, *n* (%)	8 (29%)	20 (65%)	16 (53%)	0.0195
Cough, *n* (%)	6 (21%)	23 (74%)	16 (53%)	0.0003
Headache, *n* (%)	5 (18%)	5 (16%)	3 (10%)	0.6686
Muscle pain, *n* (%)	4 (14%)	8 (26%)	2 (7%)	0.1178
Dyspnea, *n* (%)	3 (11%)	22 (71%)	23 (77%)	<0.0001
Anosmia, *n* (%)	4 (14%)	5 (16%)	4 (13%)	0.9517
Diarrhea, *n* (%)	3 (11%)	9 (29%)	7 (23%)	0.2180

BMI, body mass index; COPD, chronic obstructive pulmonary disease; HCWs, hospital care workers; ICU, intensive care unit; IDU, infectious disease unit; NSAIDs, non-steroidal anti-inflammatory drugs.

[a]*data was missing for eight patients,* [b]*data was missing for four patients,* [c]*data was missing for four patients.*

Levels of IgA and IgG in patients with severe COVID-19 were significantly higher than maximum levels obtained in infected HCWs [IgA: 9.59 (5.10–26.89) vs. 1.82 (1.37–3.29) respectively, $p < 0.0001$; IgG: 9.75 (8.05–10.75) vs. 1.12 (0.52–3.24) respectively, $p < 0.0001$] (**Figures 4E,F**).

Detection of Specific Antibodies to SARS-CoV-2 in Household Members of Infected HCWs

People sharing the same household as the 29 HCWs tested seropositive for SARS-CoV-2 were also included in the study. Demographic and baseline characteristics of the seven volunteers included are depicted in **Table 4**. Only two (29%) household members had specific antibodies against SARS-CoV-2. Both were the spouses of HCWs who had typical symptoms and a positive RT-PCR on NPS.

Nonspecific Cellular Immune Response and Production of Type I and II Interferon in Health Care Workers and Patients Diagnosed With COVID-19

To evaluate cellular immune responses of pauci- and asymptomatic HCWs, we stimulated whole blood samples from 29 HCWs and 60 patients (with moderate and severe symptoms) diagnosed with COVID-19 with immune ligands and analyzed levels of the cytokines IFN-α and IFN-γ secreted by innate and adaptive cells. When compared to COVID-19 patients with moderate or severe symptoms, innate and adaptive cells of infected HCWs, whether symptomatic or presenting mild symptoms, secreted significantly more IFN-α [infected HCWs: 602.00 (309.00–1335.00) pg/mL; patients in IDU: 7.76 (0.58–51.53) pg/mL; patients in ICU: 6.28 (1.06–74.30) pg/mL, $p < 0.0001$] and IFN-γ [infected HCWs: 537.00 (115.50–886.00) IU/mL; patients in IDU: 16.30 (7.45–50.50) IU/mL; patients

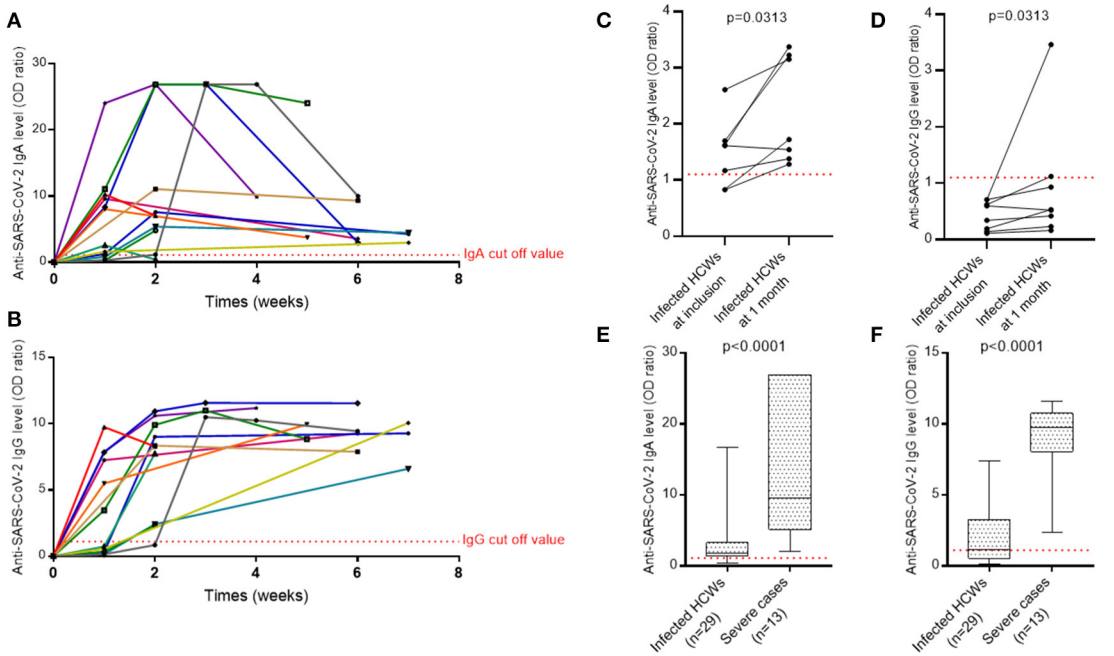

FIGURE 4 | The evolution of antibody response against SARS-CoV-2. **(A)** IgA antibody response over time against the spike protein of SARS-CoV-2 in ICU patients. **(B)** IgG antibody response over time against the spike protein of SARS-CoV-2 in ICU patients. **(C)** IgA antibody response against the spike protein of SARS-CoV-2 in pauci- or asymptomatic HCWs. **(D)** IgG antibody response against the spike protein of SARS-CoV-2 in pauci- or asymptomatic HCWs. A Wilcoxon matched pairs signed rank test was used to compare anti-SARS-CoV-2 IgA and IgG levels at screening and at 1 month. **(E)** IgA levels of infected HCWs vs. severe cases. **(F)** IgG levels of infected HCWs vs. severe cases. A non-parametric two-tailed test (Mann-Whitney) was used to compare the IgA and IgG levels of infected HCWs to severe cases. Quantitative results of IgA and IgG levels were expressed in arbitrary units by OD ratio obtained by calculating the ratio of the OD of the sample over the OD of the calibrator (as described in Methods). Each colored line in **(A,B)** represents a patient. HCWs, health care workers.

in ICU: 7.15 (1.33–48.25) IU/mL, $p < 0.0001$), which suggests impaired type I and II interferon response in patients with moderate or severe SARS-CoV-2 infection (**Figures 5A,B**). Using ROC-Curve we defined a threshold below 93.00 pg/ml for IFN-α and below 12.10 IU/mL for IFN-γ associated with hospitalization with a sensitivity of 84 and 51%, respectively, and a specificity of 96 and 96%, respectively, ($p < 0.0001$, AUC = 0.93 and $p < 0.0001$, AUC = 0.92, respectively, **Supplementary Figure 1**). No difference in IFN-γ secretion was found between infected and uninfected HCWs ($p = 0.4684$, data not shown). Because of a higher proportion of women and young subjects among the HCWs compared to the hospitalized patients (**Tables 2**, **3**), we matched the HCWs and hospitalized patients for age and gender using a 2:1 ratio. After matching, we found the same results as before: infected HCWs produced significantly more IFN-α and IFN-γ after nonspecific stimulation than patients with moderate or severe symptoms (**Figures 5C,D**). Moreover, immune stimulation with CD3 agonist during active infection could induce immune cells apoptosis and explain the IFN defect measured. To verify this hypothesis, we perform a cell count before and after stimulation in 3 patients with COVID-19 (2 severe and 1 moderate form). We did not observe any significant difference in the number of live and dead cells on anti-CD3 agonist stimulated blood compared to unstimulated blood ($p = 0.1732$, **Supplementary Figure 2**).

DISCUSSION

SARS-CoV-2 is an emerging virus responsible for the COVID-19 pandemic that has spread rapidly around the world. The clinical features and immune responses, both humoral and cellular, of frontline health care workers infected with SARS-CoV-2 have not yet been well described. To better understand the immune responses of this particularly exposed population, we compared the results to those obtained on a cohort of patients from the same hospital, and therefore from the same geographical location, and after matching on age and sex. As of May 26, 2020, of the 196 HCWs tested, 29 (15%) had specific antibodies against SARS-CoV-2 and 45% of these 29 seropositive HCWs have been strictly asymptomatic. These results are comparable to those obtained in other studies performed on frontline HCWs, at the same time and under the same conditions with IgG serology coupled with IgA and/or IgM serology (15–17). The significant proportion of asymptomatic infected subjects transmitting the SARS-CoV-2 (2–4) and the relatively high seroprevalence of SARS-CoV-2 infections among frontline HCWs (15–17) suggest that the use of screening strategies based on symptoms alone may not be effective in preventing the introduction and spread of SARS-CoV-2 in a hospital setting. However, in our study only the two HCWs who had typical COVID-19 symptoms with a positive RT-PCR on NPS transmitted the virus to their spouses,

TABLE 4 | Demographic and baseline characteristics of household members of infected HCWs.

	1	2	3	4	5	6	7
Infected HCWs characteristics							
COVID-19 symptoms	yes	yes	no	no	yes	yes	no
SARS-CoV-2 RT-PCR	negative	positive	ND	ND	negative	positive	ND
SARS-CoV-2 seropositivity	yes	yes	yes	yes	yes	yes	yes
Household member characteristics							
Relationship with HCW	parent	spouse	spouse	spouse	spouse	spouse	child
Age, years	70	61	47	39	38	28	20
Sex	F	M	M	M	F	F	M
Any comorbidity	yes	yes	no	no	yes	no	no
Diabetes	no	yes	no	no	no	no	no
Hypertension	no	no	no	no	yes	no	no
Cardiovascular disease	no	no	no	no	no	no	no
COPD	no	no	no	no	no	no	no
Asthma	no	yes	no	no	no	no	no
Cancer	no	no	no	no	no	no	no
Treatments: NSAIDs, corticosteroids or immunosuppressive therapy	no	no	no	no	no	no	no
BMI	22.03	31.14	23.51	20.45	31.23	20.18	24.34
Smoking, *n* (%)	no	no	yes	yes	no	no	no
COVID-19 symptoms	no	yes	no	no	no	no	no
SARS-CoV-2 RT-PCR	ND	positive	ND	ND	ND	negative	ND
SARS-CoV-2 seropositivity	no	yes	no	no	no	yes	no

BMI, body mass index; COPD, chronic obstructive pulmonary disease; HCWs, health care workers; ND, not done; NSAIDs, non-steroidal anti-inflammatory drugs; RT-PCR, real time reverse transcription polymerase chain reaction.

while five other infected HCWs, with no or negative NPS, did not transmit the SARS-CoV-2 to their household members. However, the small sample size prevented us from drawing statistically significant conclusions. A large study conducted in the United States showed that out of 498 members of confirmed COVID-19 case's households, 57% were infected with SARS-CoV-2 (18). Another study found, after analyzing viral spread among HCWs and residents of a nursing facility, a weak correlation between symptoms and viral shedding (viral titers from respiratory tract), despite difficulty of determining precise dates of symptoms onset, especially if the subjects were pauci-symptomatic or with atypical symptoms (19). These data strengthen current recommendations for expanded screening of HCWs and the universal use of face masks for all, especially in health care.

In our study, all HCWs included worked in units caring for COVID-19 patients, but there was no difference in the rate of SARS-CoV-2 seroprevalence between HCWs directly or indirectly in contact with infected patients. Indeed, although the contamination conditions have not been clearly identified in our cohort of HCWs (close contact with a COVID-19 patient or with another infected HCW during professional activity, or contamination outside the hospital), the seroprevalence of SARS-CoV-2 infection in frontline HCWs is higher than in HCWs from non-COVID units (1.47% on June 25, 2020 in our hospital) and is higher than the estimated seroprevalence in the general population [5.3% on May 11, 2020 in France (20)]. However, the serologies carried out by occupational medicine in our hospital for staff screening only included the determination of IgG and not IgA and IgM, responsible for a probable underestimation of the number of cases.

Knowing the strength and duration of immunity after SARS-CoV-2 infection would allow a better assessment of individual immune protection and aid in decision making on easing restrictions on physical distancing and wearing of a face mask. Several studies characterizing adaptive immune responses to SARS-CoV-2 infection have reported that most convalescents have detectable neutralizing antibodies, which correlate with the number of virus-specific T cells and decrease within 2 months after infection (5, 6, 11, 21). Confirming these previous studies, we have shown a proportion of seroconversion in COVID-19 patients of 100% after 15 days of hospitalization. We then observed a decrease in SARS-CoV-2-specific IgA antibodies titer from the 4th week, although it remained positive. The SARS-CoV-2-specific IgG antibodies titer remained stable during the

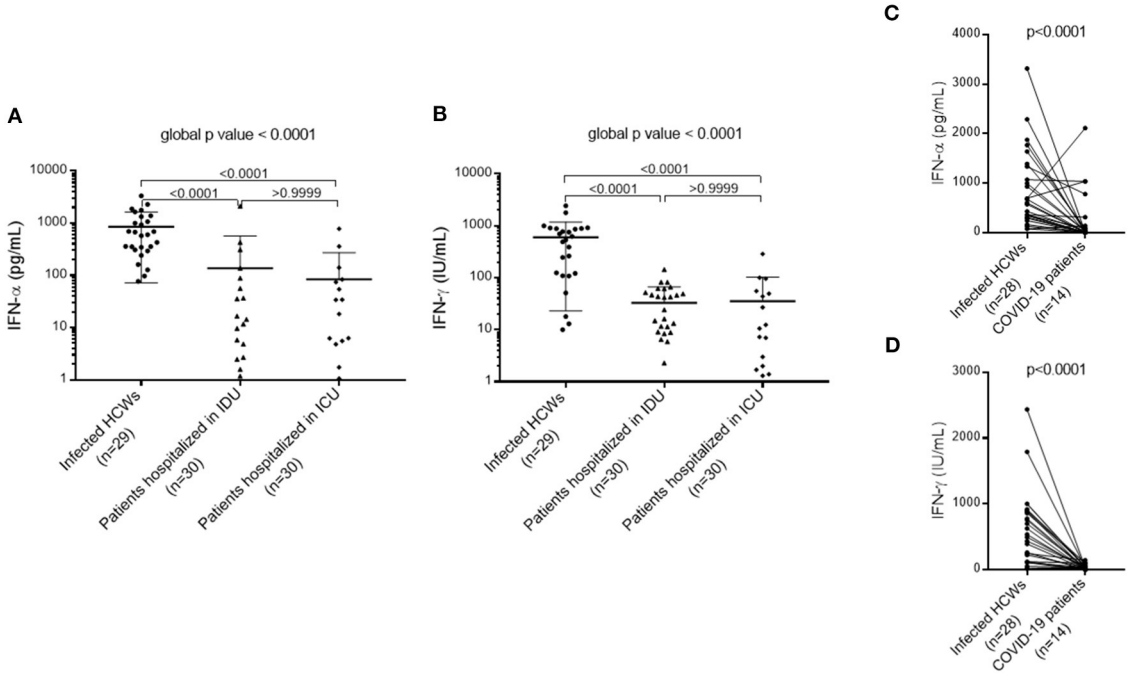

FIGURE 5 | Type I and II interferon response in patients with moderate or severe SARS-CoV-2 infection compared to infected HCWs. **(A)** Type I interferon (IFN-α) response in patients with moderate or severe COVID-19 compared to infected HCWs. **(B)** Type II interferon (IFN-γ) response in patients with moderate or severe COVID-19 compared to infected HCWs. A non-parametric Kruskal-Wallis test was used to compare the three subgroups and obtain a global *p* value. A Dunn's multiple comparisons test was used to compare the subgroups in pairs. **(C)** Type I interferon (IFN-α) response in severe COVID-19 patients after matching (1:2) for age and gender with infected HCWs. **(D)** Type II interferon (IFN-γ) response in severe COVID-19 patients after matching (1:2) on age and gender with infected HCWs. A Wilcoxon matched pairs signed rank test was used to compare IFN-α and IFN-γ levels in infected HCWs to COVID-19 patients. HCWs, health care workers; ICU, Intensive care unit; IDU, Infectious diseases unit.

7 weeks of follow-up. In comparison, the IgA and IgG peaks of HCWs were lower, which is consistent with their mild symptoms (6). IgA and IgG levels increased during HCWs follow-up, but most did not reach positivity for IgG levels (IgG OD ratio $\geq$ 1.1), as shown previously (6). During SARS-CoV-2 infection, the IgA response is earlier, stronger, and more persistent than the IgM response (22, 23), but its protective efficacy is still poorly understood, especially when this IgA response is isolated. It is well known that the IgA response is a crucial first-line defense in mucosal tissue, and SARS-CoV-2 infiltrates mainly mucosal tissues. Sterlin et al. also suggested that IgA-mediated mucosal immunity is an essential defense mechanism against SARS-CoV-2 that may reduce the contagion of human secretions and thus reduce viral transmission (24). Thus, some authors have suggested that vaccination against SARS-CoV-2 should trigger IgA responses (25). This explains why we chose to study the prevalence of SARS-CoV-2-specific IgA antibodies rather than IgM antibodies in our cohort. Additional serological surveys of more symptomatic and asymptomatic individuals and longer follow-up are needed to determine the duration of the antibody response. Moreover, the low IgG levels found, or even the absence of IgG, in asymptomatic individuals reinforce the need for a serological survey including a search for IgA antibodies to study the actual infection rate.

Our investigation showed impaired immune cellular responses, illustrated by a type I and II interferons deficiency, in patients with moderate and severe forms of COVID-19 compared to HCWs with mild or asymptomatic forms. It is already well known that immune responses are altered by aging (26), but these results remain significant after matching for age and sex. Our data confirm the results of the study by Hadjadj et al. (10) which suggests that a deficiency of type I interferon in the blood could be a characteristic of severe COVID-19 and could justify therapeutic approaches combining the administration of interferon and anti-inflammatory therapies. However, it is well known that inflammation leads to a secondary deficit of cellular immunity through the suppression of IL-12 expression. As a result, this lack of type I and II interferons could also be secondary to the infection. Other studies showing mutations in type I IFN-related genes (27) or the presence of neutralizing autoantibodies against type I IFN (28) in patients with severe COVID-19 support the hypothesis of a pre-existing immune deficiency predisposing to severe forms of COVID-19 as described in other context (29). Additional studies are needed to clarify this point. If the hypothesis of a pre-existing immune deficiency is confirmed, the deficiency of type I and II interferons revealed after *in vitro* immune stimulation could be a functional blood immune biomarker predicting the severity of the COVID-19. In addition, this immune assay is applicable for routine use.

Our study brings new data but has several limitations. First, difficulties in determining symptoms may have resulted

in misclassification of the severity of COVID-19 in some HCWs and patients. In fact, the collection of HCWs' symptoms was done using a self-questionnaire, which can lead to a memorization bias or on the contrary an overestimation of possible symptoms in this particular context of a pandemic. In addition, some patients were probably wrongly classified in the "moderate form" subgroup because they were hospitalized in IDU because of their advanced age, severe comorbidities, or social isolation and not because of the severity of their COVID-19 symptoms. Second, young women represent most health care professionals, a bias that we tried to cushion by performing age and gender matches with patients. Third, this investigation is single-center, carried out only in units caring for COVID-19 patients, resulting in a small sample size. More studies are needed to better understand the immune response of this population continuously exposed to SARS-CoV-2 infection since the start of the pandemic.

A longer clinical and serological follow-up is essential to investigate the efficacy of the protection induced by an isolated IgA response and study the persistence of the antibodies over time. Thus, HCWs included in this study will benefit from extended clinical and serological follow-up.

Defense against SARS-CoV-2 requires both humoral and cellular immune responses. The more detailed study of the immune response in HCWs, highly exposed to SARS-CoV-2 for a prolonged period of time, could provide a better understanding of the alteration of the immune system of patients with a severe form, and thus manage them better. This knowledge could also allow us to adapt the exposition of HCWs according to their immune profile and the treatment in case of infection preventing the evolution to a severe form of COVID-19 combining the administration of interferon and anti-inflammatory therapies.

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 Protection des Personnes Sud-Ouest et outre-mer 1. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BS-P and MC designed the study. MC and VB carried out experiments. MC, VB, KZ, CF, and CR collected data. MC and BS-P analyzed and interpreted the data. MC, BS-P, and VB drafted and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.608804/full#supplementary-material

Supplementary Figure 1 | IFN-α **(A)** and IFN-γ **(B)** ROC-Curve of infected HCWs vs. patients with severe COVID-19.

Supplementary Figure 2 | Cell count before and after *in vitro* stimulation by anti-CD3 agonist in three patients with COVID-19 (two severe and one moderate form).

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Functional Exhaustion of Type I and II Interferons Production in Severe COVID-19 Patients

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Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has emerged in Wuhan in December 2019 and has since spread across the world. Even though the majority of patients remain completely asymptomatic, some develop severe systemic complications. In this prospective study we compared the immunological profile of 101 COVID-19 patients with either mild, moderate or severe form of the disease according to the WHO classification, as well as of 50 healthy subjects, in order to identify functional immune factors independently associated with severe forms of COVID-19. Plasma cytokine levels, and cytokine levels upon *in vitro* non-specific stimulation of innate and adaptive immune cells, were measured at several time points during the course of the disease. As described previously, inflammatory cytokines IL1β, IL6, IL8, and TNFα associated with cytokine storm were significantly increased in the plasma of moderate and severe COVID-19 patients ($p < 0.0001$ for all cytokines). During follow-up, plasma IL6 levels decreased between the moment of admission to the hospital and at the last observation carried forward for patients with favorable outcome ($p = 0.02148$). After *in vitro* stimulation of immune cells from COVID-19 patients, reduced levels of both type I and type II interferons (IFNs) upon *in vitro* stimulation were correlated with increased disease severity [type I IFN (IFNα): $p > 0.0001$ mild vs. moderate and severe; type II IFN (IFNγ): $p = 0.0002$ mild vs. moderate and $p < 0.0001$ mild vs. severe] suggesting a functional exhaustion of IFNs production. Stimulated IFNα levels lower than 2.1 pg/ml and IFNγ levels lower than 15 IU/mL at admission to the hospital were associated with more complications during hospitalization ($p = 0.0098$ and $p = 0.0002$, respectively). A low IFNγ level was also confirmed by multivariable analysis [$p = 0.0349$ OR = 0.98 (0.962; 0.999)] as an independent factor of complications. *In vitro* treatment with type IFNα restored type IFNγ secretion in COVID-19 patients while the secretion of pro-inflammatory cytokines IL6 and IL1β remained stable or decreased, respectively. These results (a) demonstrate a

functional exhaustion of both innate and adaptive immune response in severe forms of COVID-19; (b) identify IFNα and IFNγ as new potential biomarkers of severity; and (c) highlight the importance of targeting IFNs when considering COVID-19 treatment in order to re-establish a normal balance between inflammatory and Th1 effector cytokines.

Keywords: immunology, infectious diseases, COVID-19, interferon, personalized medicine

INTRODUCTION

In the beginning of December 2019 the first cases of a viral pneumonia of unknown origin were identified in Wuhan, the capital of the Hubei province in China (1, 2). The virus responsible has been identified as a new beta coronavirus now called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from the same family as SARS-CoV responsible for the SARS outbreak in 2003. This coronavirus which causes the new coronavirus disease (COVID-19) has since spread across the world and caused a pandemic (3, 4).

Common symptoms in patients with COVID-19 include fever, dry cough, anosmia, shortness of breath and other flu-like symptoms (3–5). Even though the majority of patients may remain completely asymptomatic or may present with only mild symptoms, 10–20% of patients progress to severe disease characterized by severe pneumonia, acute respiratory distress and multiple organ failure, requiring immediate hospitalization in intensive care units, and often leading to death (3, 6, 7). Severe clinical symptoms such as diffuse alveolar damage, thrombosis, haemophagocytosis, and immune cell depletion have been described in the subset of patients with severe COVID-19 (8). Patients suffering from diabetes, cancer or other chronic diseases are most at risk of developing a severe form (9).

To better stratify patients who might be at risk for complications, numerous studies identified biological markers of worse prognosis, such as lymphopenia, and inflammatory markers such as C-reactive protein (CRP), lactate dehydrogenase (LDH), and cytokine levels (3, 10–15).

Several authors investigated the role of different cytokines in COVID-19 patients. As a part of the immunological response to a SARS-CoV-2 infection, patients often display an aggressive and uncontrolled inflammatory response with a secretion of large amounts of pro-inflammatory cytokines such as interleukin (IL) 6, IL10 and tumor necrosis factor α (TNFα), in an event known as cytokine storm (3, 16–22). Cytokine storm is directly correlated with lung injury, multiple organ failure, and unfavorable prognosis (19).

The interplay between the innate and adaptive immune response seems to be crucial in determining the patient's evolution, characterized by an imbalance of pro- and anti-inflammatory cytokines and the subsequent dysregulation of patient's immune response (23). Interferons (IFNs) act as a key link between the innate and the adaptive immune response. Type I IFNs (IFN-α/β) are secreted by plasmacytoid dendritic cells (pDCs), while type II IFNs (IFN-γ) are predominantly produced by natural killer cells and in minor proportion by T cells and macrophages (24–26). Both type I and type II IFNs have a plethora of antiviral effects such as inducing apoptosis of infected cells and activating macrophages, natural killer (NK) cells and T lymphocytes (24–26). In COVID-19 patients, several studies have shown a dysregulation of IFNs production (14, 27, 28).

In search of new and powerful biomarkers of unfavorable outcome in COVID-19 patients, we analyzed the capability of the immune system response by the means of *in vitro* stimulation of both adaptive and innate immune cells, thus effectively mimicking a viral infection. Indeed, *in vitro* stimulation of innate and adaptive immunity cells has previously been shown to be predictive of worse outcome in other immune-related diseases (29, 30), but has to our knowledge not yet been investigated in COVID-19 patients. In this prospective, single-center study we compared the function of innate and adaptive immune cells of COVID-19 patients with either mild, moderate or severe form of the disease, aiming to underline the mechanism responsible for the dysregulation of immune response.

MATERIALS AND METHODS

Study Design and Population

We performed a prospective cohort study at Nice University Hospital. The inclusion criteria were: (1) all adult patients admitted for COVID-19 in consultation unit (dermatology or infectious diseases unit), in infectious diseases units or in intensive care unit, in Nice University Hospital, from March to April 2020; (2) not having received immunosuppressive therapy in the 6 months prior to inclusion; (3) ability to sign an informed consent. Exclusion criteria were: (1) all patients under 18; (2) patients under custody, in prison or with a mental illness; (3) pregnant or breastfeeding; (4) with a known immunodeficiency or having received previous immunosuppressive therapy. Fifty healthy donors not infected with SARS-CoV-2 were also recruited (confirmed by negative serological test).

According to the severity of infection with SARS-CoV-2, the patients were divided in three groups: (a) patients with a severe form of COVID-19 were those hospitalized or transferred in the intensive care unit with respiratory distress or respiratory failure requiring mechanical ventilation or multiple organ failure; (b) patients with a moderate from of COVID-19 were patients hospitalized in the infectious diseases units, defined by clinical symptoms associated with dyspnea and radiological findings of pneumonia on thoracic CT scan; (c) COVID-19 patients with mild symptoms of COVID such as chilblains in fingers and toes or flu-like symptoms not requiring hospital supervision. All

patients presented a COVID-19 symptomatology according to WHO classification with a CT scan characteristic of COVID-19 (31) or chilblains (32) or two consecutive positive RT-PCR tests for SARS-CoV-2 on upper and lower respiratory tract specimens (nasopharyngeal swab or invasive respiratory sample) or positive serological test (Euroimmun® ELISA).

Epidemiological, biological and clinical data at day 0 (D0) are reported in **Table 1** and **Supplementary Table 1**. Treatment(s) received after D0 are summarized in **Supplementary Table 2**. Complications were defined as all adverse events such as admission in intensive care unit after worsening of the symptoms, mechanical ventilation, deep vein thrombosis, secondary bacterial infection, kidney failure, hepatitis, heart failure and death.

An informed consent was obtained for all patients. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the appropriate institutional review committee (NCT04355351).

Blood Collection and Cytokine Assay

Blood samples were collected at D0 and several follow-up time points up to 2 months after admission to the hospital. One milliliter of whole blood was stimulated with immune ligands (anti-CD3 as T-cells stimulant, and R848 as Toll-like receptors 7/8 (TLR 7/8) agonist) on single lyophilized spheres (LyoSphere™, Qiagen) within 8 h from blood collection. Stimulated blood samples were incubated for 16–24 h at 37°C and then centrifuged at 2,000 to 3,000 × g for 15 min to harvest the stimulated serum. Non-stimulated serum and plasma and stimulated serum were stored at −20°C until the analysis and freeze-thaw cycles were minimized to preserve the quality of the samples. Serum and plasma levels of cytokines with or without non-specific stimulation were measured using either QuantiFERON-Monitor test for the detection of IFN-γ, or custom-designed cartridges Ella (ProteinSimple) for the detection of IL-1β, IL-6, IL-8, IL-10, IL-17A, TNF-α, and IFN-α, following the manufacturers' instructions.

Studies *in vitro*

For 18 COVID-19 patients, one milliliter of whole blood taken at D0 was pretreated with different molecules for 6 h at 37°C, followed by stimulation with immune ligands on single lyophilized spheres (LyoSphere™, Qiagen), as described under Blood collection and cytokine assay. The molecules used were those commonly administered to COVID-19 patients (33–43): hydroxychloroquine (100 μM, Inresa), anti-IL6 Tocilizumab (100 μg/mL, RoActemra, Roche), methylprednisolone (20 μg/mL, Mylan), anti-TNFα Adalimumab (10 μg/mL, Humira, AbbVie), recombinant human IL-2 (6 ng/mL, Sigma), recombinant human IFN-alpha (100 ng/mL, Sigma) and Nivolumab (1 μg/mL, Opdivo, Bristol Myers Squibb).

Statistics

For descriptive statistics, data are presented as mean and standard deviation for continuous values with Gaussian distribution, as median and range for continuous values with non-Gaussian distribution, and as counts and percentages for categorical variables. The D'Agostino & Pearson normality test was used to determine if a variable had a Gaussian distribution or not. Groups of continuous values were compared by the Mann-Whitney test, one-way ANOVA (>2 groups), or Kruskal-Wallis test (>2 groups). Multiple comparison tests were performed with Kruskal-Wallis test using Dunn's *post hoc* test. Categorical variables were compared using Chi-square test. AUC (Area Under the Curve) ROC (Receiver Operating Characteristic) curve was used to define an IFN-γ threshold that best discriminates patients with or without complications. Log-rank test was used to compare survival data. A Wilcoxon matched pairs signed rank test was used to compare two measurements of a continuous variable performed on the same subjects (paired data). Logistic regression were performed to determine ODDS ratios and 95% confidence intervals (CI). In the mulitivariable model, we adjusted for age, sex and BMI.

Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA) or SAS 9.4. All comparisons were two-tailed, and the differences were considered significant when P-value < 0.05.

RESULTS

Study Cohort

A total of 101 patients with a symptomatology of COVID-19 infection (**Table 1**) were included and divided in three groups based on the severity of their symptoms into mild ($n = 41$), moderate ($n = 30$) and severe cases ($n = 30$), as described in Methods. Fifty healthy donors were also recruited. As described previously (1, 3, 4), there was a significant difference in age, gender, BMI and number of comorbidities among the three groups of patients ($p < 0.0001$, $p = 0.0002$, $p = 0.0003$, and $p = 0.0018$ respectively). Most common symptoms of COVID-19 infection included cough, dyspnea and fever in 52, 52, and 42% of patients, respectively (**Supplementary Table 1**).

Cytokine Levels in Non-stimulated Plasma

As expected, higher plasma levels of pro-inflammatory cytokines IL1β, IL6, IL8, and TNFα at admission and before specific treatment were positively correlated with the severity of COVID-19 symptoms ($p < 0.0001$ for all cytokines) (**Table 2**), confirming the results from previous studies (3, 16–18).

Cytokine Levels in Serum After *in vitro* Non-specific Stimulation of Innate and Adaptive Immunity Cells

While the current state of inflammatory response to SARS-CoV-2 infection, as evidenced by the plasma cytokine levels, reflects the ongoing interplay between innate and adaptive immunity, it tells us little about immune function. To this end, we stimulated innate cells and T lymphocytes of COVID-19 patients at admission and before specific treatment with Toll-like receptor 7/8 (TLR 7/8) agonist and anti-CD3, respectively, and we measured the cytokines secreted. TLR7 is predominantly expressed in plasmacytoid dendritic cells (pDC) (44) and TLR8 is more strongly expressed in myeloid dendritic cells, monocytes and to a lesser extent in pDC (45). Th17 cytokine

TABLE 1 | Demographics and baseline characteristics of healthy donors and of patients with COVID-19.

	All cases $n = 151$	Healthy donors $n = 50$	Mild cases $n = 41$	Moderate cases $n = 30$	Severe cases $n = 30$	*P*-value
Age (years)	51 (36; 62)	43 (36; 53)	31 (21; 49)	65 (53; 76)	66 (54; 72)	<0.0001
Sex ratio (M/F)	66/85	11/39	17/24	17/13	21/9	0.0002
Co-morbidities (Y/N)	72/23[a]	NA	23/17[b]	27/3	22/3[c]	0.0018
BMI	24.1 ± 4.6	NA	22.1 ± 3.5	25.2 ± 4.3	26.5 ± 4.9	0.0003
Days after first signs of COVID-19	11 (7; 17)	NA	15 (10; 24)	9 (5; 13)	9 (5; 12)	0.0001
Lymphocytes (count/mm^3)	1.5 (1.0; 1.9)	NA	1.8 (1.6; 1.3)	1.2 (0.8; 1.7)	1.0 (0.8; 1.2)	<0.0001
Monocytes (count/mm^3)	0.5 (0.4; 0.7)	NA	0.5 (0.4; 0.6)	0.5 (0.4; 0.8)	0.5 (0.3; 0.7)	0.2957

Data are presented as the median (IQR), average ± SD, or n. Global p-values comparing the differences between the groups are reported and are from one-way ANOVA, Chi-square and Kruskal-Wallis test, as appropriate. BMI, body mass index; NA, not available.
[a]*Data missing for six patients and 51 healthy donors.*
[b]*Data missing for one patient.*
[c]*Data missing for five patients.*

TABLE 2 | Non-stimulated plasma cytokine levels of healthy donors and of patients with COVID-19, at baseline.

	All cases $n = 151$	Healthy donors $n = 50$	Mild cases $n = 41$	Moderate cases $n = 30$	Severe cases $n = 30$	*P*-value
Plasma IL-1β (pg/mL)	0.1 (0.0; 0.2)	0.0 (0.0; 0.1)	0.1 (0.0; 0.1)	0.2 (0.1; 0.2)	0.3 (0.2; 1.0)	<0.0001
Plasma IL-6 (pg/mL)	1.5 (0.8; 12.6)	1.1 (0.7; 1.8)	0.8 (0.7; 1.3)	25.3 (4.3; 43.8)	53.7 (15.9; 74.3)	<0.0001
Plasma IL-8 (pg/mL)	3.1 (2.3; 8.7)	2.7 (2.2; 3.6)	2.4 (1.8; 2.9)	6.4 (4.0; 19.0)	13.6 (8.7; 17.9)	<0.0001
Plasma TNFα (pg/mL)	7.0 (5.7; 10.6)	6.2 (5.3; 7.3)	5.9 (5.2; 6.8)	11.9 (8.1; 15.5)	13.8 (10.6; 19.8)	<0.0001
Plasma IL17A (pg/mL)	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	>0.9999
Plasma IFNα (pg/mL)	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	>0.9999
Plasma IFNγ (IU/mL)	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	>0.9999

Data are presented as the median (IQR). Global p-values comparing the differences between the groups are from Kruskal-Wallis test. IL, interleukin; IFN, interferon; NA, not available; ND, not detected; TNF, tumor necrosis factor.

IL17A, as well as type I and type II IFN were not detectable in non-stimulated plasma of COVID-19 patients (**Table 2**). However, after *in vitro* stimulation of immune cells significant differences in cytokine levels emerged between patients with various severity of COVID-19 (**Table 3**, **Figure 1**), reflecting the fitness of their immune system. On the innate immunity side, DCs and NK cells of moderate and severe patients were functionally exhausted as illustrated by lower IFNα (and IFNγ from NK cells) levels upon *in vitro* stimulation ($p < 0.0001$) (**Figure 1A**), as previously suggested (46), and the differences remained significant after correction for monocyte count ($p < 0.0001$ mild vs. moderate and mild vs. severe) (**Figure 1B**). Levels of IL6, which is secreted by cells of both innate and adaptive immunity, remained unchanged between groups upon *in vitro* stimulation ($p = 0.1247$) (**Figure 1C**). On the adaptive immunity side, functional exhaustion was observed for Th17 lymphocytes producing IL17A in severe COVID-19 patients in comparison to mild forms and healthy subjects ($p = 0.0004$ and $p = 0.002$ respectively) (**Figure 1D**). Strikingly, lower secretion of IFNγ correlated with increased severity of COVID-19 ($p < 0.0001$) (**Figure 1E**). This lower production of IFNγ remained significant even when corrected for lymphocyte count ($p = 0.0183$ mild vs. moderate and $p = 0.0009$ mild vs. severe) (**Figure 1F**).

Correlation Between IFNs Production After *in vitro* Stimulation and COVID-19-Related Complications

The level of IFNα and IFNγ production upon *in vitro* stimulation of innate and adaptive immunity cells at admission and before specific treatment was predictive of the risk of complications ($p = 0.003$ and $p < 0.0001$, respectively) (**Figures 2A,C**). Indeed, patients with a level of IFNα and IFNγ lower than 2.1 pg/mL and 15 IU/mL, respectively, as defined by a ROC curve (data not shown), were more likely to develop complications during

TABLE 3 | Serum cytokine levels after non-specific stimulation of T lymphocytes and DCs, in healthy donors and in patients with COVID-19, at baseline.

	All cases $n = 151$	Healthy donors $n = 50$	Mild cases $n = 41$	Moderate cases $n = 30$	Severe cases $n = 30$	*P*-value
Stimulated IL1β (pg/mL)	2,850 (1,846; 4,701)	3,819 (2,820; 5,407)	3,226 (1,930; 4,723)	2,241 (1,254; 3,824)	1,918 (1,252; 3,208)	0.0001
Stimulated IL6 (pg/mL)	36,792 (26,906; 51,355)	35,922 (27,333; 43,741)	32,890 (25,031; 46,975)	48,567 (35,469; 55,791)	36,263 (17,096; 54,257)	0.1247
Stimulated IL8 (pg/mL)	34,869 (22,395; 65,475)	30,284 (22,371; 39,873)	28,386 (15,100; 55,727)	62,032 (33,230; 135,877)	69,042 (33,065; 133,094)	<0.0001
Stimulated TNFα (pg/mL)	6,537 (4,435; 10,538)	9,844 (6,222; 13,167)	7,461 (6,089; 13,202)	4,571 (2,372; 6,420)	3,003 (1,162; 8,262)	<0.0001
Stimulated IL17A (pg/mL)	97 (37; 299)	234 (72; 331)	192 (46; 346)	62 (42; 140)	28 (10; 76)	0.0002
Stimulated IFNα (pg/mL)	262 (13; 778)	544 (321; 1,109)	724 (241; 1,303)	6 (0; 37)	12 (1; 70)	<0.0001
Stimulated IFNγ (IU/mL)	82 (15; 230)	211 (93; 438)	98 (46; 245)	24 (8; 52)	7 (1; 36)	<0.0001

Data are presented as the median (IQR). Global p-values comparing the differences between the groups are from Kruskal-Wallis test. DC, dendritic cell; IL, interleukin; IFN, interferon; NA, not available; TNF, tumor necrosis factor.

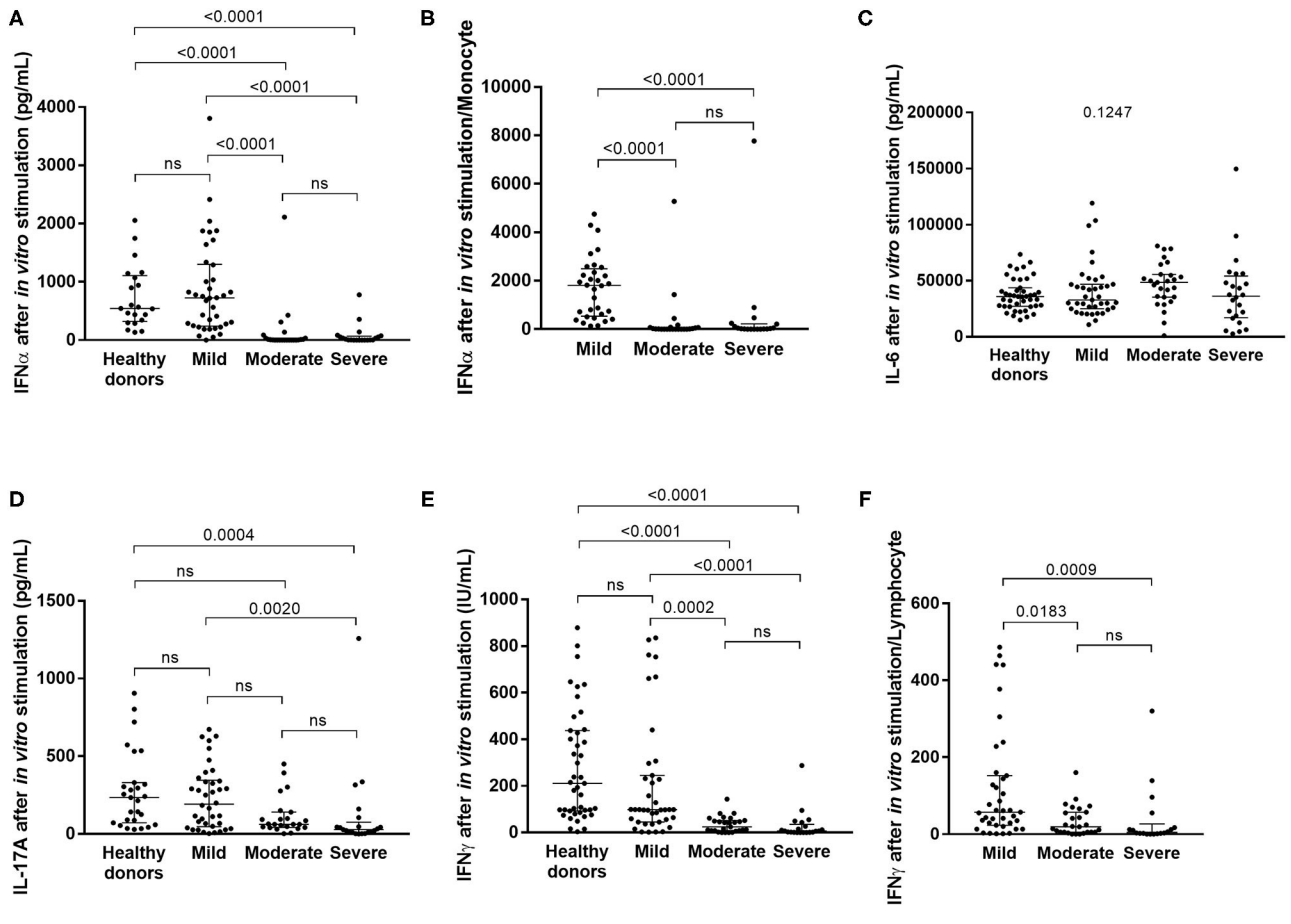

FIGURE 1 | Serum cytokine levels after *in vitro* stimulation of innate and adaptive immune cells in healthy subjects and in COVID-19 patients with mild, moderate or severe symptoms. The levels of IFNα (**A**: in mild COVID-19 3 points are missing, in moderate COVID-19 3 points are missing and in severe COVID-19 patients 6 points are missing), IL6 (**C**: 3, 3, and 6 points, respectively), IL17 (**D**; 3, 7, and 9 points, respectively) and IFNγ (**E**: 2, 3 and 9 points, respectively) were measured, and the levels of IFNα and IFNγ were corrected for monocyte **(B)** and lymphocyte **(F)** count, respectively. Differences between groups were compared with Kruskal-Wallis test using Dunn's *post hoc* test.

TABLE 4 | Multivariable analysis for the evaluation of the relationship between considered variables at baseline and complications.

	Odds ratio (95% CI)	*P*-value
Age (years)	1.041 (0.977–1.110)	0.2157
Gender (M/F)	4.119 (0.466–36.402)	0.2029
BMI	1.218 (0.966–1.536)	0.0954
Plasma IL6 (pg/mL)	1.072 (1.015–1.133)	0.0128
Stimulated IFNγ (pg/mL)	0.980 (0.962–0.999)	0.0349

BMI, body mass index; IL, interleukin; IFN, interferon.

hospitalization ($p = 0.0098$ and $p = 0.0002$, respectively) (**Figures 2B,D**). As confirmed also by multivariable analysis (**Table 4**), stimulated IFNγ levels are an independent predictor of complications in patients with COVID-19 [$p = 0.0349$ OR = 0.98 (0.962; 0.999)].

The Evolution of Cytokine Levels Depending on Clinical Outcome

We further assessed the evolution of cytokine production and IFN response during hospitalization in moderate and severe cases. During follow-up, non-stimulated plasma IL6 levels decreased between the moment of admission to the hospital and at the last observation carried forward for patients with favorable outcome ($p = 0.02148$) (**Figure 3A** and **Supplementary Figure 1A**), while they remained high in deceased patients ($p = 0.5625$) (**Figure 3B** and **Supplementary Figure 1B**). The level of IFNγ after *in vitro* stimulation, however, did not significantly differ between the time of admission to the hospital and the last observed time point (**Figures 3C,D**), which was likely due to a small number of patients per group. Two individual cases were chosen to better demonstrate the evolution of cytokine production during the course of the disease. The first case resulted in recovery with an increased stimulated IFNγ levels at the last point (**Supplementary Figure 1A**), while the second case resulted in death with stable low stimulated IFNγ levels throughout hospitalization (**Supplementary Figure 1B**).

Effect of *in vitro* Treatment With Therapeutic Molecules on the Restoration of Cytokine Balance

Several drugs commonly used to treat COVID-19 patients were tested for their potential to restore cytokine balance *in vitro*, notably to increase IFNγ production and decrease the production of inflammatory cytokines, while keeping the secretion of regulatory cytokines constant. Chloroquine and methylprednisolone proved efficient in reducing secretion of all cytokines (**Figure 4**) while Adalimumab reduced only IL6 and IL10 secretion. Interestingly, IFNα had a more balanced effect with a strong stimulation of IFNγ and a decrease of inflammatory cytokine IL1β, while the secretion of T regulatory cytokine IL10 and pro-inflammatory cytokine (IL6) remained unchanged. The individual results are detailed in **Supplementary Table 3**.

DISCUSSION

We report here a cohort of 101 patients with a symptomatology of COVID-19 infection. We aimed to reveal their specific immunological profiles and to correlate them to the evolution and the extent of symptoms in individual patients. Our results confirmed a functional exhaustion of type I (NK cells and DCs) and type II IFN (T cells) production in moderate and severe patients traducing an evasion of both innate and adaptive immune response and in accordance with recent studies (14, 16, 18, 21, 47–49).

It is well-known that the innate immune response is triggered by virally infected cells which can be recognized by host pattern-recognition receptors (PRRs) expressed by DCs that produce a variety of cytokines (50) such as type I IFN, which in turn recruit lymphocytes and monocytes to inflamed sites (51–54). Type I IFN primarily activates epithelial cells and reduces the mononuclear macrophage-mediated proinflammatory activity (55). Type II IFNs have different functions, eliciting T helper 1 (Th1)-driven immune responses, and also enabling induced regulatory T (Treg) cells to control and regulate immune responses (56). Consequently, SARS-CoV-2 has evolved several mechanisms to inhibit type I IFN induction and signaling (57). During SARS-CoV-2 infection, both innate and adaptive immune response are required for successful virus clearance and must be adequately controlled to minimize immunopathological damage (57). By assessing the response of immune cells of infected patients after stimulation, we demonstrate here a marked decrease in type I and type II IFN response from mild to severe patients. The molecular mechanism(s) of this IFN evasion remain to be confirmed, however, several studies have suggested different pathways that could contribute to the decreased amount of IFNs in severe COVID-19 patients, from concealed viral production invisible to PPARs to direct synthesis of structural and nonstructural viral proteins that antagonize IFN signaling (47–49). Indeed, SARS-CoV-2 induced an aberrant type-I IFN response in cultured cells, characterized by a delayed antiviral response which may provide a window for virus replication and an improper recruitment of inflammatory monocyte macrophage populations (21).

The originality of our work lies in the stimulation of TLR7 and TLR8 which reproduce *in vitro* a viral infection by the activation of innate immune system and produce type I IFN (58). On the other hand, the stimulation of T lymphocytes by an anti-CD3 allowed us to quantify the production of type II IFN and to evaluate the adaptive immune response. The innate immune recognition of virus infection triggers antiviral immune responses by residual genomic RNA recognized by PRR expressed mainly by DCs (59). In moderate and severe COVID-19 patients we observed that innate cells produce less type I IFN, and consequently NK cells produce less type II IFN. In accordance with previous studies (21, 60), our results

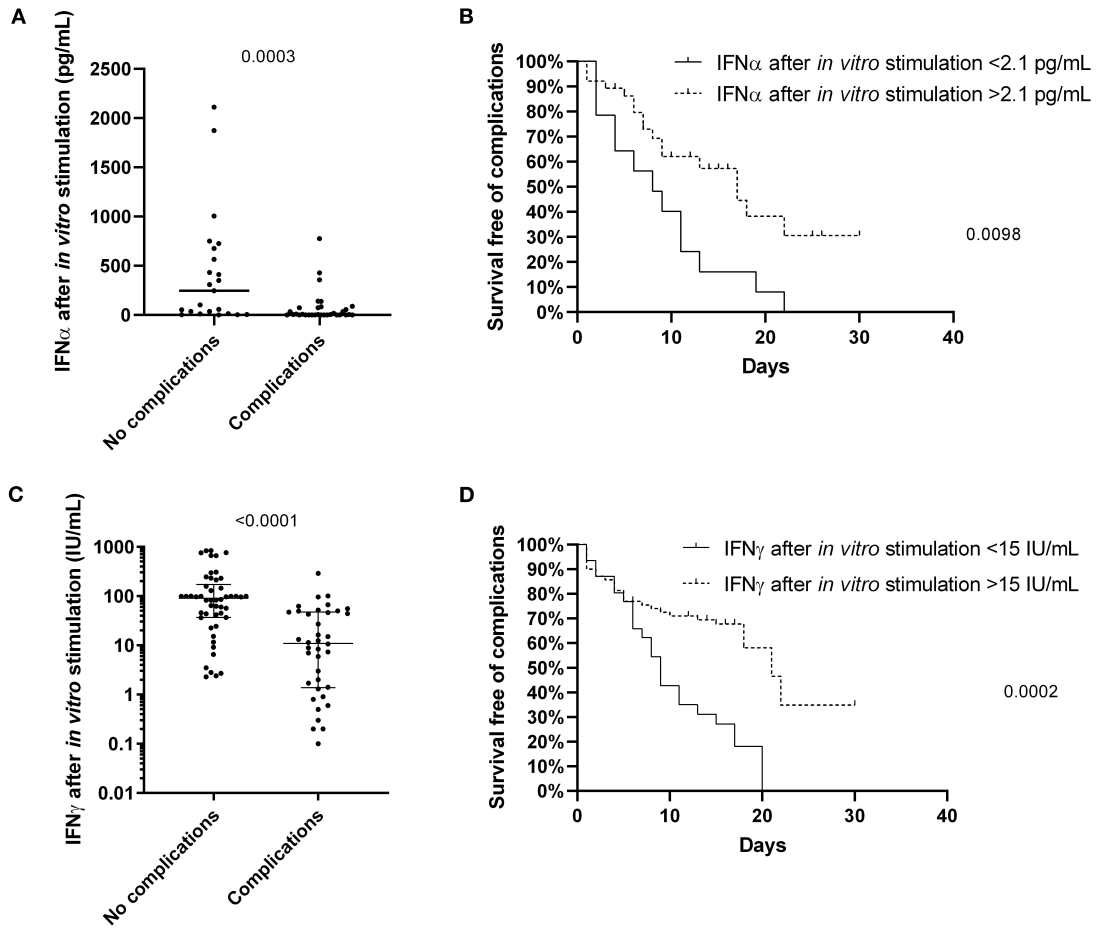

FIGURE 2 | The predictive value of IFNα and IFNγ after non-specific stimulation of innate and adaptive immune cells for the patients with COVID-19. The level of IFNα (**A**; $n = 55$) and IFNγ (**C**; $n = 89$) differs between the patients with and without complications, and predicts the survival free of complications for patients with COVID-19 **(B,D)**. Differences between groups were compared with Mann-Whitney test, and log-rank test was used to compare survival data.

suggest that an uncontrolled infection maintains monocyte and macrophagic activation, and that the regulatory T lymphocytes remain inactivated due to a weak production of type II IFN, thus reinforcing the cytokine storm and leading to severe complications in patients with COVID-19.

This longitudinal study allowed us to conclude that a functional analysis of IFN production at the beginning of the hospitalization is a powerful tool to predict the clinical evolution of patients infected with SARS-CoV-2. While previous studies demonstrated opposing results with either impaired (14, 28) or increased (61) type I IFN response in severe COVID-19 patients, our results tip the balance toward impaired IFN signaling. According to our study on stimulated IFN production as well as in other studies (14, 21), type I IFN plays a major role in the activation of type II IFN and represents a strategic target for early treatment of COVID-19 patients, in order to destroy the immune evasion caused by SARS-CoV-2 and to treat the specific immune dysfunction.

However, there is increasing evidence that patients with severe COVID-19 may have a robust type I IFN response, which contrasts the delayed, possibly suppressed, IFN response seen early in infection (27, 62). While limited by a small sample size of eight and seven patients, respectively, Zhou et al. and Wilk et al. demonstrated that many IFN-stimulated genes are overexpressed in COVID-19 patients (62, 63).

Since more studies are needed to further illuminate the role of IFNs in COVID-19, both from a clinical and a molecular perspective, IFN treatment remains controversial as well. Nevertheless, an *in vitro* study on cultured cells has shown a potential benefit of IFNβ treatment (48), as well as a recent clinical study NCT04276688 with favorable outcome for IFNβ (33), while others are still in progress. Two recent retrospective studies found that IFNα treatment may be beneficial for COVID-19 patients (37, 38), however it seems that adequate timing in IFN administrating is crucial for its efficacy since early administration decreased mortality, while late administration had an opposite effect (38).

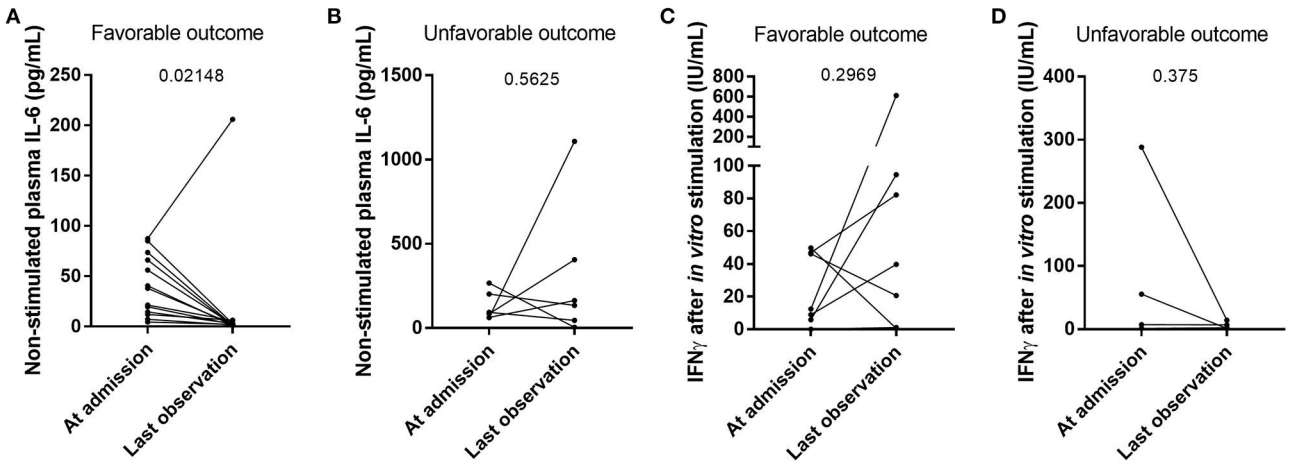

FIGURE 3 | The evolution of cytokine levels in individual patients following their clinical outcome. The levels of IL6 in non-stimulated plasma (**A,B**; $n = 13$ and $n = 6$, respectively) and the levels of IFNγ after *in vitro* non-specific stimulation of innate and adaptive immune cells (**C,D**; $n = 6$ and $n = 3$, respectively) were compared between the patients who recovered from their SARS-CoV-2 infection **(A,C)** and the deceased patients **(B,D)**. Differences between groups were compared with a Wilcoxon matched pairs signed rank test.

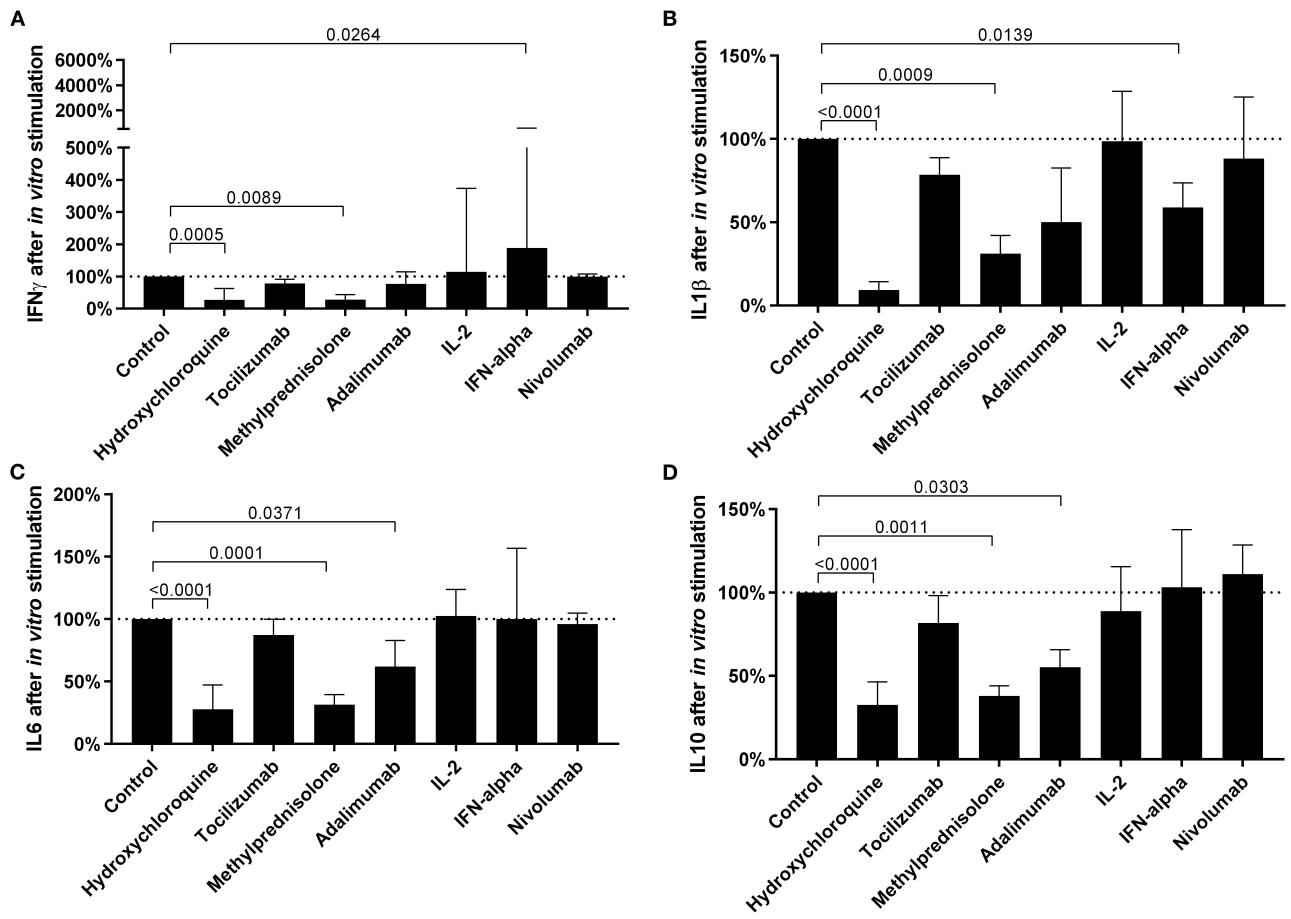

FIGURE 4 | The efficacy of *in vitro* treatment with different drugs commonly used in COVID-19 to modulate cytokine expression. The levels of IFNγ **(A)**, IL1β **(B)**, IL6 **(C)**, and IL10 **(D)** after *in vitro* pretreatment with drugs, followed by non-specific stimulation of innate and adaptive immune cells, in 18 COVID-19 patients. Differences between groups were compared with Kruskal-Wallis test using Dunn's *post hoc* test.

Our study presents several limitations. First, this is an observational study showing an association between IFN I and II levels and COVID-19 severity and outcome. Randomized clinical trials using functional interferon assays at admission to predict outcome are needed to clearly evaluate the efficacy and utility of IFN measurement in clinical practice. Second, while our *in vitro* tests and several recently published studies (33, 37, 38, 48) show the potential of IFNα treatment in order to restore the cytokine balance, the results nevertheless need to be confirmed in large controlled clinical trials. Third, this is a study on a relatively small number of patients that needs to be confirmed in larger cohorts. Notably, there were only six deaths in our cohort of COVID-19 patients, severely limiting the power of statistical analyses. Fourth, while male gender, older age and obesity have been shown to be strongly associated with increased mortality in COVID-19 patients (10, 11), low number of deaths prevented us from identifying these factors in our cohort. Instead, we tested the predictive power of age, gender and BMI on COVID-19-related complications, but apart from plasma IL6 levels and stimulated IFNγ levels the other variables remained non-significant at multivariable analysis.

The variability of symptomatology lies at the heart of our cells, among the immune responses involved in fighting infection by the COVID-19. IFNγ represents a predictive biomarker of the evolution of SARS-CoV-2 which can be safely and routinely measured in laboratory by QuantiFERON Monitor. It could allow clinicians to provide adjusted treatment and medical care in this epidemic context. Based on our results, our functional test could be an important tool to predict severe COVID-19 and guide personalized therapy targeting the immune restoration of NK and T-cells (inhibiting check-point inhibitor) and IFN production.

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 CPP SUD-OUEST et OUTRE-MER I. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CR, VB, MC, SB, and BS-P designed the study. VB and MC carried out experiments. KZ, CF, CR, and MC collected clinical data. BS-P, CR, VB, and MC analyzed and interpreted the data. BS-P, CR, ED, KR, JC, EC, CI, JD, and TP provided medical oversight. BS-P, CR, and VB drafted and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.603961/full#supplementary-material

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General Practitioners' Experiences During the First Phase of the COVID-19 Pandemic in Italy: A Critical Incident Technique Study

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Since February 2020, when coronavirus disease began to spread in Italy, general practitioners (GPs) were called to manage a growing number of health situations. The challenges experienced by Italian GPs remained unrevealed. This study aimed at exploring Italian GPs' care experiences and practices associated with critical incidents during the first wave of the pandemic. A qualitative study design involving the critical incident technique through an online survey was applied. Sociodemographic data and open-ended responses were collected. While participants' characteristics were analyzed through descriptive statistics, qualitative data were thematically analyzed employing the framework method. 149 GPs responded to the survey and 99 participants completed the survey (dropout rate = 33%). Eight themes emerged indicating factors related to the organization of the healthcare system and factors related to the clinical management of patients, that were perceived as impacting on the GPs' care provision. The analysis revealed difficulties in communicating with other local services. This, together with the lack of coordination among services, was reported as a major challenge. Primary care was perceived as having been undervalued and criticalities in the organization of GP courses, led in a bureaucratic fashion, posed at risk some trainees to be infected. The digital technologies adopted for remote patient consultations were seen as useful tools for daily practice helping the GPs to stay emotionally connected with their patients. Besides, the improvement in the GP–patient relationship in terms of solidarity between patients and doctors and compliance to rules, had a positive impact. Moreover, many respondents addressed the importance of professional collaboration and teamwork, in terms of both support in practical issues (to find PPE, diagnostics and guidelines) and emotional support. At the same time, the lack of resources (e.g., PPE, swabs) and of specific guidelines and protocols impacted on the care provision. Our findings suggest that GPs in Italy are at risk of being left behind within the epidemic

management. Communication and coordination among services are essential and should be substantially improved, and primary care research should be initiated to collect the context-specific evidence necessary to enhance the system's preparedness to public health emergencies and the quality of primary care services.

Keywords: pandemic, public health, doctor-patient relationship, health emergency, qualitative study, Italy, COVID−19, general practice

INTRODUCTION

Italy was the first European country affected by the coronavirus disease (COVID-19) pandemic. Since the first case of COVID-19 was identified in Codogno, in the northern region of Lombardy, on February 20, 2020, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) rapidly spread, mainly in northern Italy, partially sparing the southern regions of the country. To date, in Italy there have been more than 2 millions of confirmed cases, 71,620 deaths, and 23,571 people are currently hospitalized (1). Strict nationwide lockdown measures were adopted on March 9 and 11, 2020 (2, 3). During the lockdown, the Italian healthcare services were strongly challenged, especially regarding their capacity to deliver appropriate care to both COVID-19 patients and other patients. Outpatient secondary care services were closed to the public all over the country and planned patient consultations for non-life-threatening conditions were suspended. In this context, general practitioners (GPs), including out-of-hours doctors and doctors at prisons and nursing homes, were called to manage a growing number of health situations while reorganizing their services and altering how they provided care. Many GPs rapidly switched to remote consultations, though local, regional, and national evidence-based guidelines on COVID-19 management were lacking at that time. Services and care provision reorganization were left to the capacities of the individual GPs.

Rapidly moving to the frontline of COVID-19 management was demanding and put GPs in an unprecedented situation. Understanding the demands and challenges faced by frontline healthcare professionals and how they adjusted their efforts during the COVID-19 outbreak is essential (4). According to the World Health Organization (WHO), primary care services, in emergencies, should promote not only effective emergency responses but also a prepared system that can recover from emergencies (5). Primary care has been fundamental both for providing essential routine health services and for identifying/managing suspected COVID-19 patients (5–7).

Given the central role of primary care services during emergencies, we conducted a study collecting information on the experiences of Italian GPs during the COVID-19 pandemic. This study aimed at exploring Italian GPs' care experiences and practices associated with critical incidents during the first wave of the pandemic. In particular, this article reports on a qualitative analysis of "free text" (open-ended) survey data related to critical incidents experienced by Italian GPs. Findings reported in this article provide an insight on the GPs' experiences of positive as well as negative events arising from the crisis and on how GPs adapted to the changes of their activities. Obtaining a better understanding of the real difficulties and challenges faced by GPs could help to prevent them in the future. The research question which drove the study was: "what did help or hinder Italian GPs' activity during the first wave of the pandemic?"

TABLE 1 | Open-ended questions.

1.	In this period of emergency caused by SARS-CoV-2, thinking about your recent clinical practice, could you tell us one or more experiences that involved you personally and that surprised you positively?
2.	In this period of emergency caused by SARS-CoV-2, thinking about your recent clinical practice, could you tell us one or more experience that involved you personally and that surprised you negatively?
3.	Could you please tell us what you would change so that the facts you describe do not happen again in the future?

MATERIALS AND METHODS

We used a qualitative study design involving the critical incident technique (CIT), as it was particularly consistent with the study aim (8, 9). The CIT is a qualitative methodology (rather than only a method) that involves a flexible set of principles (10). It does not focus on opinions but analyses the context of events. The critical incidents (CIs) (11) in this study refer to situations perceived as relevant by GPs dealing with the COVID-19 pandemic. The concept of "technique" in the CIT entails critical reflection focused on analyzing the human behaviors and contextual factors underlying the phenomena in question (12, 13).

As described by Viergever (10) CIT has commonly five steps: (1) description of the aim of the activity to deepen; (2) specifications of the nature of critical incidents to report and participants' characteristics; (3) data collection in line with the research question; (4) data analysis which includes pooling critical incidents into themes or areas; (5) interpretation and results' report.

As to the aim of the activity and the nature of related CIs (steps 1 and 2) the researchers planned to include a broad interest on care provision and clinical practice during the pandemic (especially referring to March and April 2020) of Italian GPs. In this context, reporting CIs was used to understand experienced obstacles and proposed solutions to the faced practical problems. Researchers decided to let the participants free to report any significant or important event (see **Table 1**) applying a broad-ranging version of CIT.

Data Collection, Sampling, and Recruitment

As to step 3, we designed an online form for collecting data on what GPs perceived to be factors, events, behaviors or experiences which helped or hindered their care experience or clinical practice (using the SurveyMonkey© survey application), which was available from March 12 to April 17, 2020.

The data collection strategy involved gathering sociodemographic information (age, gender, workplace province and setting, and quarantine experience) and asking three open-ended questions (on the positive and negative CIs during the pandemic and the GPs' proposals to avoid the negative CIs). Although CIT usually requires researchers to collect qualitative data through interviews, for practical reasons (i.e., the need to capture data from a geographically widely distributed population and the need to collect data in a timely manner) researchers designed a questionnaire-based CIT study as suggested elsewhere (14), in line with a qualitative research approach (15). The data were collected through the online survey platform using a purposive sample of GPs, which was obtained using snowball sampling (16). The researchers invited potential participants (from among the doctors that they knew personally who they believed would be interested in the study) via phone calls in which they explained the study aims and addressed emerging questions. Following each phone call, a weblink to a brief explanation of the study, an informed consent form, and the survey itself was sent to the potential participant (via WhatsApp©, text message, or email). Each respondent who agreed to participate was asked to recruit other potential participants.

The participants were requested to reflect on and identify one or more specific CIs that they perceived to be positive and one or more CIs that they perceived to be negative regarding their care provision during the pandemic, and to detail any proposals that they may have regarding how to avoid the negative CIs in the future. The survey was piloted with a convenience sample of 10 participants. Thereafter, the three open-ended questions were reformulated to enhance comprehensibility and readability, as shown in **Table 1**.

Data Analysis

As to step 4, namely data analysis, before approaching the dataset, the analysts were given focused training on qualitative data analysis by a qualitative methodologist (LG). Thematic analysis (17) was used, which involved defining an analytic framework (18). This framework method is suitable for multidisciplinary teams to analyze large datasets (19, 20), as recently demonstrated (21).

The analysis involved the following steps:

- All authors extensively read all the responses to the open-ended questions.
- Four authors (Al.S., PKK, Ar.S., and MD) met for subsequent discussion sessions on the provisional themes and thematic areas.
- Based on the responses of the first 20 participants, an analytic framework was developed as follows: four authors (Al.S., PKK, Ar.S., and MD) independently labeled all the responses and then met to discuss the emerging framework, with any disagreement being resolved by another researcher (LG).
- Two authors (Al.S. and Ar.S.) applied the framework to the remaining responses to the open-ended questions.

TABLE 2 | Sociodemographic profile of respondents by geographical area.

		Northern regions	Central regions	Southern regions	Total
Age	26–35	48 (32.21%)	20 (13.42%)	31 (20.81%)	99 (66.44%)
	36–45	5 (3.36%)	4 (2.68%)	6 (4.03%)	15 (10.07%)
	46–55	5 (3.36%)	1 (0.67%)	4 (2.68%)	10 (6.71%)
	56–65	12 (8.05%)	2 (1.34%)	6 (4.03%)	20 (13.42%)
	66+	2 (1.34%)	2 (1.34%)	1 (0.67%)	5 (3.36%)
	Total	72 (48.32%)	29 (19.46%)	48 (32.21%)	149 (100.00%)
Gender	F	44 (29.53%)	18 (12.08%)	27 (18.12%)	89 (59.73%)
	M	27 (18.12%)	11 (7.38%)	20 (13.42%)	58 (38.93%)
	Other	1 (0.67%)	0	1 (0.67%)	2 (1.34%)
	Total	72 (48.32%)	29 (19.46%)	48 (32.21%)	149 (100.00%)
Work setting	General Practice	57 (38.26%)	20 (13.42%)	20 (13.42%)	97 (65.10%)
	Out of Hours	9 (6.04%)	7 (4.70%)	20 (13.42%)	36 (24.16%)
	GP in training	5 (3.36%)	1 (0.67%)	5 (3.36%)	11 (7.38%)
	Prison	0	0	1 (0.67%)	1 (0.67%)
	Other	1 (0.67%)	1 (0.67%)	2 (1.34%)	4 (2.68%)
	Total	72 (48.32%)	29 (19.46%)	48 (31.21%)	149 (100.00%)
Quarantined	Yes	11 (7.38%)	5 (3.36%)	10 (6.71%)	26 (17.45%)
	No	61 (40.94%)	24 (16.11%)	38 (25.5%)	123 (82.55%)
	Total	72 (48.32%)	29 (19.46%)	48 (31.21%)	149 (100.00%)

- Researchers renamed themes to highlight hindering and facilitating factors according to the research question.
- The last stage entailed grouping themes into two main thematic categories.

Finally, as to step 5, a report of the results was shared among the team and the final interpretation of data was specifically discussed in many team meetings.

The quantitative data, including sociodemographic variables, were analyzed using SAS® 9.4 (SAS Institute Inc., Cary, NC, USA).

RESULTS

There were 149 GPs who responded to the survey. Their demographic data are shown in **Table 2**. The majority (66.4%) were aged ≤35 years (26–≥66), and 38.9% were male; 26 participants declared that they were or had been quarantined. All 149 participants completed the sociodemographic form, but 50 did not answer any of the three open-ended questions (dropout rate = 33%). Among the remaining 99 respondents, all reported at least one negative CI, while three did not report any positive CIs.

Each theme signifies a factor perceived as particularly relevant for GPs and was classified according to whether it helped or hindered their care experiences (i.e., had a positive or negative impact) during the first wave of the COVID-19 pandemic. Two main thematic categories emerged: factors related to the organization of the healthcare system (HS) and factors related to the clinical management of patients, whose summary is shown in **Figure 1**.

Factors Related to the Organization of the HS

Participants reported many CIs related to the overall organization of the HS: a lack of communication, coordination, and leadership of the services in charge of the crisis' management as well as a lack of organization of primary care services and training of new GPs impacted negatively. GPs perceived huge difficulties in performing public health responsibilities that could contain the epidemic.

Communication, Coordination, and Leadership in the HS

The GPs reported that the poor coordination and communication among and within services was one of the leading causes of the system's inflexibility and inefficiency to the detriment of patient care and effectiveness regarding containing the epidemic.

"It became clear to me the impossibility of applying our job to reality (...): fragmentation, lack of sharing and collaboration, lack of communication, abandonment, inexperience, and incapacity, the non-evidence-based practice." (39)

"There was no coordination (...) we received directives that an hour later were denied by the other department." (52)

The respondents indicated a lack of leadership as a trigger for the inadequacy of the services that were coordinating the response to the pandemic.

"I am in the public health service (...), I have received consciously erroneous instructions from my superiors (. . .). They provided handwritten, unsigned, and unrecorded instructions in blatant contradiction to ministerial provisions (...) We work only with pen and paper, papers are repeatedly photocopied and distributed in different places until they are lost, so that mistakes of the individuals can hide the intention of the organization to cover up." (31)

"There is no clear organization of services; there is no leadership. The indications given are schizophrenic (...)" (37)

A significant system's fragmentation among regions and provinces emerged from the analysis of the respondents' experiences, which led to criticalities in patient care, disappointment, and confusion.

"I would like to point out the lack of coordination [. . .] of two neighboring health districts, which follow different working criteria. This generated in me—and in the patient—false expectations, confusion, and disappointment." (100)

The respondents reported that public health services struggled to take charge of and to manage suspected cases, which led to the spread of the infection and the overload of the healthcare services.

"There are no swabs. [. . .] in the initial chaos there was no adequate surveillance. Wards, Emergency Departments, hospitals. . . are collapsing." (67)

Organization of the Primary Care Services

Regarding the organization of the primary care service and its relationship with the rest of the National Health Service (NHS), participants reported that the importance of primary care was underestimated with a hospital-centric organization of care that contributed to the system's overload.

"The total lack of preparation to face the biggest emergency in the area since the Second World War with an excessively hospital-centered vision caused the wards to become saturated within a week." (27)

"Much more attention is needed on primary care, which in this emergency has been abandoned to give resources to the hospital." (73)

"I suffer every day when I see how the current organization of Primary Care is tragically inadequate and unable to face this challenge and all the other challenges that had been posed in recent decades [. . .]." (125)

In this context, many CIs revealed that a common experience among participants was a sense of isolation, uselessness, and lack of protection.

"No one protects us." (13)

"I would like to be more useful in this situation, but I feel alone, like a hamster running in a wheel. I would like to act and make sense of my actions; instead, it seems to me I'm not moving an inch forward." (107)

However, some respondents proposed that their professional roles could be developed during the pandemic, with the

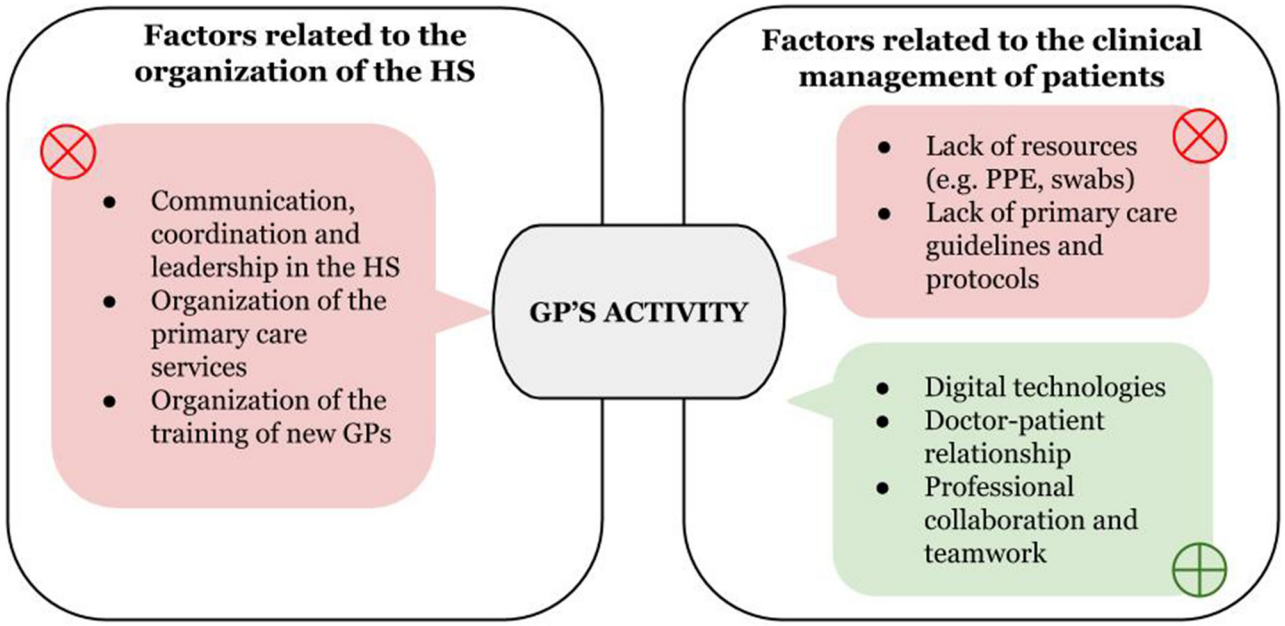

FIGURE 1 | Summary of findings–themes that impacted on GPs care experiences according to study participants. Figure shows the factors emerged from the analysis that helped (green) or hindered (red) the GPs' activity. These factors were related to two main domains: the organization of the healthcare system (HS) and the clinical management of patients. GPs, General Practitioners; PPE, personal protective equipment.

organizational advancement of the primary care service being fostered.

"GPs should have a stable, recognized, and integrated role in the organization of the NHS." (3)

Organization of the Training of New GPs

According to the participants, the pandemic also impacted the training of new GPs. The respondents perceived that the training programs were run by "bureaucratic" structures that were unable and uninterested in training them.

"Due to the SARS-Cov2 epidemic, my training was interrupted. We were basically left to ourselves by those who should have organized it: administrative staff, teaching coordinators, and regional representatives." (109)

"As a GP trainee, I found that course coordinators were completely unable to reschedule internships and reallocate the trainees [. . .] [During that time] I received information and updates exclusively through unofficial channels unrelated to the GP course." (56)

The reported lack of organization of GP courses emerged as a health risk for trainees: in some cases, trainees were told to begin internships without being provided with PPE.

"As a trainee, I was quarantined because I came into close contact with a colleague who tested positive." (10)

"I was told to begin a hospital internship [. . .]. I felt a constant risk related to generating gatherings; we had no PPE available." (56)

Some participants proposed possible solutions to overcome this lack of organization and to avoid negative experiences in the future.

"The GP course should become a real course with a defined role, and it should be recognized by institutions as a specialization course like any other." (66)

Factors Related to the Clinical Management of Patients

Several CIs showed how the lack of resources (e.g., PPE, swabs) and of clinical guidance affected their daily practice while digital technologies, the doctor-patient relationship, and professional collaboration helped to overcome difficulties in patients care.

Lack of Resources

Participants reported huge difficulties in the management of their patients due to the lack of resources. Many participants reported that they were not provided with PPE. This was perceived to be an obstacle to the delivery of appropriate care to both COVID-19 and non-COVID-19 patients.

"I regret not being able to give my full contribution due to the lack of adequate PPE." (115)

"Hearth attacks and strokes that probably have occurred and have remained unrecognized because we do not have PPE to protect ourselves..." (52)

The lack of PPE led to a sense of loneliness, vulnerability, and to psychological distress.

"During a shift in the GP out-of-hours (OoH) Service I had to visit a suspected Covid-19 patient without all the PPE (except for a surgical mask and a pair of gloves). This showed me all the vulnerability, anxiety, fear, the sense of loneliness and the lack of preparation (even psychological) for these events." (59)

Moreover, the scarcity of PPE generated conflicts over its allocation and the responsibility for its provision.

"A colleague was infected because the head physician denied him even surgical masks." (29)

" I was not provided with a mask with a filter or glasses/visor despite the fact that I had to visit the patient. Instead, the paramedics [. . .] were fully equipped with PPE." (63)

Alongside PPE, the lack of other diagnostic equipment (such as swabs), hospital beds, therapeutics (such as oxygen tanks) and even telephone lines were reported as a criticality that negatively impacted the possibility of respondents engaging in their daily work to assure continuity of care.

"77-year-old patient in a nursing home [. . .] he was not taken to the hospital and he died after 2 days... the patient was a suspected case [. . .] he was not even swabbed" (14)

"I cry when I think of the call to a lady just 50 years old: I am at home, they gave me oxygen, I ran out of oxygen [. . .] I struggle to breathe, I don't want to go to the emergency department, I'm afraid, I'm afraid to die because they have no beds." (67)

"We spent 10 days looking for oxygen tanks throughout the province of [OMISSIS] for emergency therapy while people saturated at 82%. A real nightmare." (52)

"In my office we have only one telephone line where patients can call (for a city of 150,000 inhabitants). It rings continuously." (106)

Lack of Primary Care Guidelines and Protocols

Another factor that was mentioned in many of the CIs as critical for care delivery to patients was the lack of guidelines and protocols for primary care drawn up by authoritative and respected sources.

"I had the impression that primary care was left without a leader—without a single authoritative voice from a scientific point of view." (20)

"I would try to strengthen the primary care service, outlining guidelines so that we know what to do." (76)

The absence of clear guidelines and authoritative sources of knowledge, in the view of some participants, led space to differences and divergencies in the health care professionals' behaviors and views, affected teamwork, and hindered access to care.

"The different ideas about work management—even more when clear guidelines were lacking and in such a delicate phase for everyone—is making teamwork unsustainable [. . .]." (8)

Digital Technologies

In this context of lack of resources and clear protocols, the respondents felt positively about the digital technologies adopted for patient consultations such as electronic prescriptions.

"I was also surprised by the rapid possibility of making electronic prescriptions accessible to patients directly from the pharmacy." (17)

Many other positive CIs regarding telephone-based care were reported to have occurred during the process of adaptation to the pandemic situation: the possibility of remotely taking care for patients and giving emotional support to those in isolation.

"The rediscovered importance of words, of a telephone conversation that becomes an essential connection, and which is able to concentrate all the possible humanity, closeness and help." (39)

"Every day I called them, I entered their homes, I saw their eyes, I evaluated their breathing. [. . .] I have been living with them for these 20 days." (42)

Doctor-Patient Relationship

Participants reported that relational aspects of their work (relationships with patients and colleagues or other healthcare professionals as well as the attitudes and behaviors of patients) had a role in their clinical practice.

Mainly, what facilitated them in facing their work in the context of the COVID-19 emergency was the doctor/patient relationship. They described events in which patients expressed gratitude, understanding and appreciation to their efforts.

"The relief of this person in being reassured after having been visited... She thanked me. This is to date one of the very few positive experiences of this period." (8)

"Another thing that surprised me positively was hearing a patient asking me: 'Before starting, doctor, first of all tell me how you are, because right now you are the people who most need to hear this asked. And maybe nobody does.'" (25)

According to some CIs, GPs' clinical practice was also supported by the behavior of their patients who accepted the access limitation to health services during the first phase of the emergency. The GPs reported a relatively high level of compliance to the rules.

"The only positive aspect that I can find right now is that patients [. . .] have understood and have used the service in an appropriate way." (10)

"[A positive experience has been] The understanding of my elderly homebound patients when I had to cancel the planned home visits and the collaboration of their families in helping them and providing medicines for them." (29)

Professional Collaboration and Teamwork

Also, relationships with GP colleagues, with doctors working in different outpatient settings, with those in hospitals, and with other healthcare professionals had a facilitating role for our participants' daily work.

In this context, professional collaboration served as emotional support and allowed GPs to overcome the sense of loneliness and the emotional burden caused by the epidemic.

"I observe mutual help (an attitude that was not always previously present). Despite the forced distance, we did not feel alone, or at least, it is true for me. [. . .]" (18)

"Another consideration concerns colleagues: I have discovered (or perhaps was confirmed) that some of them may be your strength, your constant mirror, your vent valve, the eyes that most offer you an understanding in such an emotionally and professionally heavy period." (8)

"The need to talk to colleagues more often, every day, even several times a day to share what is happening [. . .] the feeling of being alone amplifies distances. Colleagues, at this moment, save you more than anyone else." (114)

The teamwork with doctors and other healthcare professionals was experienced as a factor that helped to overcome practical

difficulties in daily work such as the lack of PPE, of official guidance and of diagnostics, and allowed for better patient management.

"We started communicating on group chats to support us and exchange information, to get masks, gowns, and oximeters. We adopted a common emergency management line." (144)

"I immediately got in touch with my colleague in the nearby ED and my radiologist colleague and I agreed on the most appropriate and fastest path for my patient." (25)

"If I only could have had the help of the nurse to go with PPEs to see the elderly homebound COVID-19 patient." (22)

"[a positive experience was] the post-discharge management of a patient I have been following since March 19. I collaborated with the cardiologist and with the ADI [integrated homecare service]" (53)

DISCUSSION

Summary of the Main Findings

As far as we know, this study is the first to systematically analyze GPs' experiences on the COVID-19 pandemic by employing the CIT. The surveyed GPs reported both positive and negative CIs occurring during the first peak of the outbreak.

The analysis identified factors that impacted their care experiences. The summary of findings (**Figure 1**) shows how these factors are related to two main domains: "organization of the HS" and "management of patients." With regards to the first domain, it is notable that we could not identify any factor in the data that could have favorably impacted the GPs' work. Conversely, the GPs in our study experienced a lack of organization within the NHS, a lack of interinstitutional cooperation, a lack of leadership, and a lack of clear communication at all levels of the emergency response system. They described a sense of abandonment and solitude and felt that they were not part of a system that could sustain them during the emergency. The lack of reliable clinical and organizational guidance was a major challenge as was the absence of commitment regarding the teaching and supervision of GP trainees. Concerning the second domain ("clinical management of patients"), digital technologies, and meaningful and empathic doctor/patient relationships–along with collaborations with doctors (specialists, GPs) and other healthcare professionals–were described as factors that helped the GPs to cope with the organizational and emotional challenges of being on the frontline. On the contrary, lack of resources (e.g., PPE, swabs) and of reliable guidance affected patient management.

Comparison to Existing Literature

Study participants reported a significant lack of structured coordination that resulted in communication problems between the different health services and in bureaucratic obstacles. The published literature shows that similar issues have been experienced in other healthcare systems. In the US, the fragmentation of primary care and its weak connection to the emergency response infrastructure has been an obstacle to an efficient response to the pandemic (22). In the UK, even though primary care is a cornerstone of the NHS, the links between primary care and Public Health England's broader preventive activities have been reported as unclear (7). This disconnection was also highlighted by several participants in our study and seems to be widespread. It is even more surprising in Italy because there is a well-established publicly funded surveillance network for influenza and influenza-like illnesses that involves GPs and primary care pediatricians since 1999 (the Italian Influenza Surveillance Network, InfluNet) (23). Despite the recognition that the structured involvement of GPs in infectious disease surveillance and control measures is an essential element of pandemic preparedness (24), this network only began to be involved in COVID-19 surveillance on October 14, 2020 (25).

Many GPs in our study reported that the role of primary care was underestimated—much more importance was given to hospital care—and they felt unprotected and isolated. In fact, most Italian GPs still work single-handedly in solo practices that are somewhat isolated from the rest of the NHS (26). Indeed, a recent study showed how Italian GPs represent 44.1% of the total COVID-19-related deaths among doctors, and organizational issues (i.e., working alone), along with the lack of PPE, were proposed as explanations for the high burden suffered by GPs (27). Moreover, in many regions of Italy such as Lombardy, regional policies fostered a strong hospital-centric organization while primary care has been underfinanced for many years (28). The autonomy of the Italian regions in organizing their healthcare systems led to different healthcare models across the country. To date, no published study has yet addressed the impact of these different care organizations (i.e., hospital vs. primary care centered) in Italy.

Regarding training of new GPs, courses were perceived as poorly organized and led in a bureaucratic fashion. Respondents reported that this negatively impacted their training and their safety during the first peak of the outbreak. These findings are consistent with previously published studies, which showed how GP trainees in Italy are enrolled in non-academic regional courses of questionable quality (29, 30). In fact, during the first months of the outbreak, many GP traineeship activities were stopped due to the lack of PPE, which was not available for trainees undertaking purely observational internships. Since then, trainees have been employed in GP out-of-hours services in the COVID-19 special units and to replace regular GPs; a government decree stated that the hours worked in these services would be recognized as part of the traineeships despite the fact that they occurred without clinical supervision (31). GP trainees therefore became part of the paid GP workforce. Further research on the quality of GP training in Italy is needed to overcome these critical issues (29, 30). This knowledge will explain how training has been impacted by COVID-19.

Difficulties in obtaining reliable information, guidelines, and protocols on patient management were described. This finding is consistent with influenza pandemic research, which highlighted that there were multiple information sources with conflicting recommendations and a lack of guidelines tailored to primary care providers (32). As stated elsewhere, primary care practice guidelines need to be underpinned by evidence collected in primary care settings (33, 34). There are no primary care departments in Italian Universities, and it is not possible for

Italian GPs to pursue a PhD in general practice. Moreover, local or national networks with accessible research databases are missing; the only Italian general practice research database is owned by a private company, and data are not accessible to independent epidemiological research (35). As a result, to date, the context-specific evidence needed to underpin guidelines relevant for Italian GPs is missing.

In addition, the GPs in our study reported events related to a lack of resources (such as PPE or swabs) allocated in the primary care setting. Scarcity and unequal distribution of PPE were reported previously in other countries (36–38) as well as in Italy (39). The lack of PPE was psychologically stressful for our study participants—a finding that is consistent with recent studies showing how the lack of PPE and proper safety procedures are associated with higher levels of anxiety and depression (40) while access to adequate PPE is associated with reduced psychological morbidity (41). Besides, other resources such as oxygen and swabs for COVID-19 testing were also reported as insufficiently available leading to difficulties in taking charge of patient needs. In this context, the issue of how primary care should be organized, financed, and staffed is considered one of the top ten international research priorities (42). Such studies are currently lacking in Italy and could inform policies on the most efficient allocation of resources within the NHS.

The rapid switch to remote assessment via telephone or video consultations has been perceived as generally positive. A study in the UK found that the rate of initial general practice consultations in the form of digital consultations dramatically increased between February and May 2020; the UK primary care service concurrently faced profound organizational challenges (43). No published studies have yet analyzed nor quantified these changes in Italy, and research is needed to evaluate the impact of COVID-19 on GP consultations, digital technology use, and remote assessment and on the related health outcomes of these procedures.

The GPs reported that they experienced an improvement in their relationships with their patients in terms of compliance, patient understanding, and solidarity. Similar findings in China have been reported despite the decline in doctor/patient relationships since the late 1970s (44). A possible explanation for this phenomenon is that an outbreak of the scale of COVID-19 could reduce the emotional distance between doctors and patients. In fact, the so-called hidden curriculum traditionally encourages detachment between emotions and clinical reasoning (45, 46) to preserve the objectivity of clinical judgement, i.e., avoiding the interference of the doctor's empathic concern that could affect clinical decision making (47). Doctors could have perceived increased solidarity from their patients as the doctors themselves felt closer to them because they were sharing the common concern of COVID-19. More research is needed to understand the impact of COVID-19 on empathy in medicine and, more broadly, on the doctor/patient relationship.

From the perspective of the GPs in our study, collaboration among professionals and teamwork seemed to be a valuable resource to cope with the clinical and emotional challenges that they have faced. In line with our findings, in the case of influenza outbreaks, teamwork and interprofessional collaboration were described as factors that can lead to a more adequate response (48). Healthy teams showed to be effective in preventing burnout among GPs (49), in improving professional motivation (50, 51), and patient and family-centered care (52). That said, interprofessional collaboration and teamwork is not well-established among Italian GPs (53), and further research is needed to address the impact of the working environment on mental health, safety, and care delivery of Italian GPs during the COVID-19 pandemic.

Strengths and Limitations

Our findings need to be interpreted in light of their explorative nature. Nevertheless, they do offer a direction for policymakers and for further studies. One limitation of this study is that the data collection was via an online survey. In the CIT methodology, even if survey/online data collection is allowed, interviews are recommended to collect in-depth information (14). The risk of survey-related poor data collection was balanced by capturing a wide range of experiences and perspectives in an unexplored area of research and in a large, diverse and geographically widely distributed population. Moreover, piloting helped to identify possible problems with the interpretation of the questionnaire and with the open-ended questions being modified based on the preliminary feedbacks. Moreover, due to the open-ended nature of the questions and the fact that no personal data were collected, motivated participants were free to disclose their experiences without fear of being judged and without manipulation introduced by an external interviewer (15, 54).

The second limitation relates to the findings' generalizability. Indeed, this study followed a qualitative approach in sampling and analysis (8, 55). Bearing in mind this approach and the explorative, rather than definitive, nature of the results, the generalizability to the entire population of Italian GPs was beyond the aims of this study (56). Nonetheless, the richness of the data allowed us to acquire a meaningful picture of GPs managing the outbreak through an analysis from which the themes in common across the participants could emerge (57). The majority of the participants were from northern Italy where the pandemic started and was more threatening. This could explain the wider participation of GPs located in these regions and probably contributed to their informative responses. In addition, most of the participants were aged $\leq$35 years, and it is likely that our findings match the perspectives of younger GPs. Some of the participants stated that they were enrolled in a GP specialization course. This should not be interpreted as meaning that their responses do not reflect the perceptions of actual GPs because GP specialization schools were suspended shortly after the beginning of the first outbreak, and GP trainees were asked to take part in the response to the healthcare emergency becoming part of the paid GP workforce.

Regarding the analysis, the principal investigators of this study were notably GPs. As such, they may have analyzed the CIs from an emic perspective, and interpretation could have been

limited. Nonetheless, every step of the analysis was performed by at least two researchers, and analytical decisions were made by reaching an agreement among an interdisciplinary team during each step. Additionally, public health experts (MFM and MGC) and GPs collaborated with two authors with no health-related background (i.e., LG, a qualitative methodologist, and Ar.S., who works as a lawyer in training with a special interest in medico-legal issues).

Implications for Policy

This study suggests that GPs in Italy are not part of a coherent strategy that prepares the Italian primary care service for epidemic outbreaks.

Several recommendations may be drawn. Communication and coordination between primary care and public health authorities are essential and should be substantially improved. Funding should be allocated for the integration of primary care and public health services, and structured teamwork should be enhanced through shared protocols and guidelines to contain the outbreak. Efforts should be made to adequately train GPs based on national guidelines on the management of COVID-19 and non-COVID-19 patients in their setting. To inform these guidelines in the longer term, primary care research is a necessity for the Italian NHS that, in the light of this pandemic, can no longer be postponed. Unfortunately, there is currently no publicly funded and institutional general practice research in Italy but it is urgently needed to produce context-specific evidence (33, 58) to help GPs in their daily practice and to effectively train the next generation of primary care doctors.

Implications for Further Research

Research is needed to ascertain how the Italian primary care service and, more broadly, European primary care services are coping with the pandemic. With Europe facing a second wave of COVID-19, a follow-up study could be useful to ascertain whether and how GPs' experiences change over time. To further enhance the credibility of our findings, themes that emerged in this study, such as the impact of COVID-19 on the doctor–patient relationship, should be more extensively explored, e.g., through semi-structured and in-depth interviews with doctors and patients. Moreover, quantitative studies should be performed to ascertain the generalizability of the results of the present study. Moreover, participatory methodologies, such as participatory action research, could be applied to develop an understanding of the collective experience of this pandemic and to enable healthcare professionals to cope effectively with the challenges they face during health emergencies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the corresponding author under reasonable request.

ETHICS STATEMENT

No detailed personal data or any identifying information (e.g., names) were collected during the study. Before data collection, the ethics committee of the Local Health Authority of Area Vasta Emilia Nord (AVEN) was approached, and the authors were advised that, according to Italian law, formal ethical approval was not necessary.

AUTHOR CONTRIBUTIONS

PKK, AlS, and LG conceptualized the study in several discussions that involved all the authors. MFM and MGC were consulted as experts in public health research issues. PKK, AlS, MD, and AM, with the collaboration of the Research Team of the Giotto Movement, performed data collection. PKK, AlS, ArS, and MD performed the qualitative data analysis under the supervision of LG while iteratively discussing the emerging themes with MFM, AM, and MGC. MFM and MGC performed the quantitative analyses of the dataset. PKK and LG drafted the manuscript, which was discussed among all the authors, who all made relevant amendments and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.623904/full#supplementary-material

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Preventing SARS-CoV-2 In-Hospital Infections in Cardiovascular Patients and Medical Staff: An Observational Study From the German Heart Center Berlin

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Objective: COVID-19 is a highly contagious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Preventing in-hospital infections is crucial to protect patients and hospital staff.

Methods: At the very beginning of the COVID-19 pandemic, the German Heart Center initiated obligatory wearing of surgical face masks for patients and employees, SARS-CoV-2 screening for all patients, and symptom-based testing for employees. In addition, access restriction, closure of outpatient departments, and postponing non-urgent procedures were implemented with community-initiated regulations.

Results: During the observation period (03/16/2020–04/27/2020), 1,128 SARS-CoV-2 tests were performed in 983 persons (1.1 tests/person; 589 in patients and 394 in hospital employees). Up to 60% of the clinical workforce was tested based on symptoms and risk (62.5% symptoms, 19.3% direct or indirect contact to known COVID-19, 4.5% returnee from risk area, 13.7% without specific reason). Patient testing for SARS-CoV-2 was obligatory (100% tested). The overall prevalence of positive tests during the observation period was 0.4% ($n = 5$ out of 1,128 tests performed). The incidence of new infections with SARS-CoV-2 was 0.5% ($n = 5$ out of 983 individuals; three healthcare workers, two patients). No nosocominal infections occurred, despite a mean number of 14.8 in-hospital contacts.

Conclusion: Comprehensive SARS-CoV-2 testing and surgical face masks for patients and hospital staff, in addition to others measures, are key factors for the early detection of COVID-19 and to prevent spreading in the vulnerable hospital population.

Keywords: COVID-19, SARS-CoV-2, prevention, health care worker, face mask, nosocomial infection, in-hospital transmission

INTRODUCTION

Clustering of a severe acute respiratory distress syndrome was first described in Wuhan, China, in December 2019, with the subsequent identification of the coronavirus SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) as the causal agent of a disease now termed COVID-19 (coronavirus disease 2019) (1). COVID-19 is a highly contagious lower respiratory tract infection mostly transmitted via droplets, but airborne transmission was also reported (2, 3). Cardiovascular risk factors and cardiovascular complications during the course of the infection are important disease modifiers, contributing to a higher mortality (4–6). As of November 17, the number of infected patients exceeds 55.4 million globally, causing a death toll of more than 1,300,000 (7). In Germany, the first COVID-19 patient was reported in the southern state of Bavaria on January 27, 2020 (8), whereas the first case in the northern state of Berlin was reported on March 1, 2020 (9). Thereafter, the number of infected patients increased rapidly, reaching 817,526 in Germany up to date with 12,833 deaths (7). In several countries, the COVID-19 pandemic has led to an overwhelming demand on intensive care beds and ventilator therapy.

Infectiousness in the early stage of the disease and transmissions in the presymptomatic state or from persons with an asymptomatic course of the disease is likely high (10–12). This has been shown to cause clusters in vulnerable population, such as residents of nursing homes, as well as hospitalized patients (13, 14). Likewise, caretakers and healthcare workers (HCWs) are at increased risk of SARS-CoV-2 infection (15, 16).

Based on initial reports, a concept of strict compartmentalization between designated COVID-19 and non-COVID-19 hospitals has been recommended to prevent in-hospital transmissions (17). The University Hospital Charité and the state senate of Berlin established a 3-level model to ensure the distribution and care of COVID-19 and non-COVID-19 patients ("SAVE-Berlin/Brandenburg@COVID-19") (18). Within this network, the University Hospital Charité is the level I center primarily responsible for the coordination and the treatment of severe cases. Additionally, there are 16 level II centers for COVID-19. In contrast, level III centers ($n = 20$) are designated to stay "COVID-19–free". The German Heart Center Berlin [Deutsches Herzzentrum Berlin (DHZB)] is a tertiary cardiovascular center and classified as level III. In addition to this allocation, all hospitals were required to postpone elective treatments and to increase the number of immediately available intensive care unit (ICU) beds.

As there is a lack of data on the prevention of in-hospital infections with SARS-CoV-2 in patients and HCWs, the purpose of this report is to describe the combined effect of hospital-initiated measures in addition to governmental regulations during the early phase of the COVID-19 pandemic in Berlin.

METHODS

The study was approved by the local ethics committee (no. EA2/092/20, PREV-SARS-CoV-2-DHZB) and was performed in accordance to the declaration of Helsinki. Human studies are presented. Informed consent was obtained from all participants orally and in writing.

The German Heart Center Berlin is a specialized hospital for the treatment of cardiovascular diseases (cardiothoracic surgery for adults and children, cardiology, pediatric cardiology, anesthesiology), which treated >8,300 inpatients and >25,500 outpatients in 2019 employing a staff of 1,404 people.

During the time of this study (03/16/2020–04/27/2020), several recommendations and rules were initiated by German and local government agencies to contain the spread of COVID-19. **Figure 1A** depicts the timeline of measures initiated by German/state authorities and the German Heart Center in relationship to the COVID-19 pandemic.

Measures Initiated by Government and State Agencies

By 03/12/2020, the German government and local authorities decided to cancel major events of more than 1,000 people, to postpone elective medical procedures and increase ICU capacities. On 03/13/2020, 14 of the 16 German federal states decided to close their schools and nurseries, including Berlin. Visits to nursing homes and hospitals were prohibited. Contact restrictions were expanded on 03/22/2020, with gatherings of more than two people banned and a required minimum physical distancing of 1.5 m in public (19).

Measures Initiated by the German Heart Center

The following measures were initiated: standard operating procedures focusing on patient admission/treatment and protection of patients/employees from SARS-CoV-2 infections were implemented. From 03/13/2020, twice daily temperature screening for HCWs was done. From 03/15/2020, all visitors were prohibited, except for pediatric patients <16 years of age (maximum one parent). From 03/16/2020, patient risk stratification/triage for planned procedures/operations was initiated based on disease, symptoms, and comorbidities, and non-emergent medical/surgical treatments were postponed. Upon hospital admission, patients underwent a questionnaire survey including symptoms, contacts to COVID-19, and travel history. Outpatient departments were closed for routine visits. Routine testing of all patients for SARS-CoV-2 infection was started on 03/19/2020. Universal in-hospital masking (surgical masks; employees; and patients) was obligatory from 03/23/2020 on hospital premises. From 03/16/2020, a voluntary testing was offered to all employees in case of a suspected SARS-CoV-2-infection (**Figure 1A**).

SARS-CoV-2 Testing

As shown in **Figure 1B**, 1,128 SARS-CoV-2 polymerase chain reaction (PCR) tests were performed in 983 individuals during the period of this study (589 tests in patients, with all patients tested and 539 tests in 394 employees). Indications for testing were different for patients and hospital employees. All patients admitted to the hospital were routinely tested for SARS-CoV-2. Whenever possible, the test was administered by the patient themselves as a swap from the posterior wall of the oropharynx,

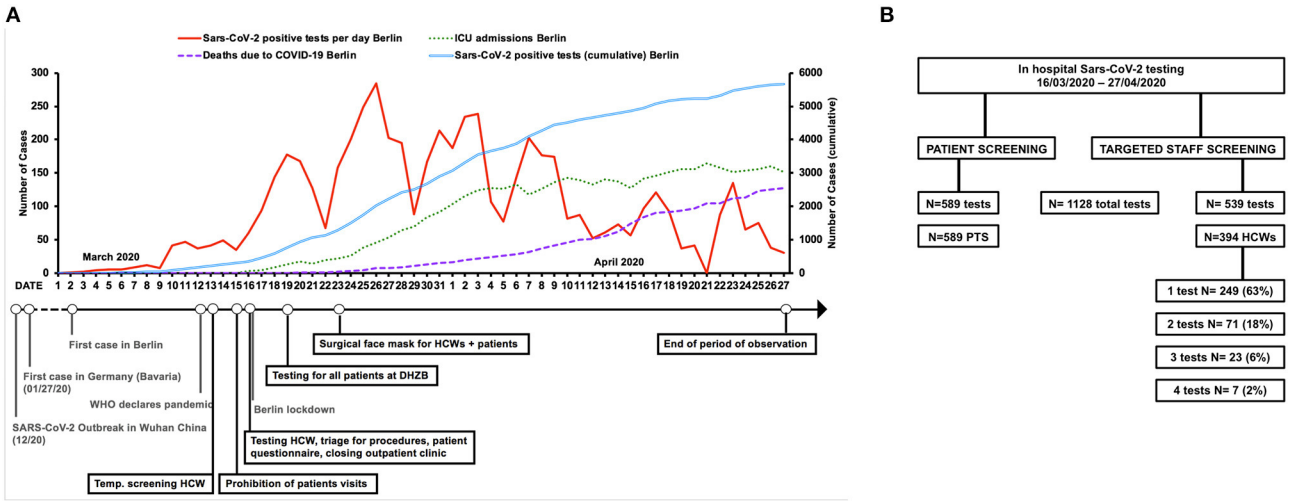

FIGURE 1 | Timeline of the COVID-19 pandemic in Berlin and preventive measures initiated by the German Heart Institute (DHZB). **(A)** Timeline of COVID-19 pandemic and in-hospital SARS-CoV-2 preventive measures. Upper part: positive SARS-CoV-2 tests per day (red line) and cumulative SARS-CoV-2 tests (blue line), cumulative number of ICU admissions (green dotted line), and deaths due to COVID-19 (dotted purple line) in Berlin. Lower part: timeline and key measures initiated during the SARS-CoV-2 pandemic (gray: national and worldwide actions, black: DHZB measures). **(B)** Flowchart of SARS-CoV-2 RT-PCR testing at the German Heart Institute Berlin (DHZB) during the period of 03/16/2020–04/27/2020 in healthcare workers (HCWs) and patients (PTS). HCWs, healthcare workers; DHZB, Deutsches Herzzentrum Berlin (German Heart Center Berlin); RT-PCR, real-time polymerase chain reaction; PTS, patients; Temp, temperature.

which was successfully done in >95% of cases. Patients were given standardized instructions from a nurse along with a visual aid for self-collection. If the patient needed assistance, the test was performed by an HCW using adequate personal protective equipment (PPE). In case of a positive test, the patient was isolated and transferred to a COVID-19–designated hospital, a contact list compiled, and reporting to health authorities done. Contacts were tested for SARS-CoV-2.

Voluntary testing was offered to 1,404 clinical and non-clinical employees including 199 physicians and 383 nurses (= clinical workforce). Testing was offered in case of illness, but also to asymptomatic employees who returned from risk areas, had contact to a SARS-CoV-2 positive person, or had close contact to a person who had contact to a COVID-19 patient. The test was administered by the employees as a swap from the posterior wall of the oropharynx with a visual aid provided. A questionnaire documented reason/motivation for testing (i.e., contact, symptoms, risk area travel). Symptoms were specified as follows: fever, dry cough, productive cough, fatigue, shortness of breath, jaw pain, sore throat, headache, chill, nausea, general malaise, myalgia, rhinitis, diarrhea, and stuffed nose. In case of a positive test result, quarantine was ordered, and a contact list done. Contacts were tested for SARS-CoV-2.

SARS-CoV-2 Real-Time PCR

Swab collections were performed with identical test material (flocked swab, transport tube with 2–3 mL of viral transport medium). Three different systems for SARS-CoV-2 RNA-detection were used, based on prioritization: tests on patients with highly urgent treatment indication were performed using the Xpert® Xpress SARS-CoV-2 (Cepheid, Sunnyvale US), a cartridge-based system that provides results for SARS-CoV-2-RNA detection in <1 h. Other testing was performed on the BD-MAX™ System (Becton Dickinson, Franklin Lakes, US) using VIASURE SARS-CoV-2 RT-PCR reagents (Certest Biotec, Zaragoza, Spain), with a test duration of 2.5 h. These two systems are available on-site. In case of insufficient capacity, tests are additionally performed at the Medizinische Infektiologiezentrum Berlin. In this off-site location, tests were done on a Seegene Inc. Nimbus IVD system using the Allplex™ 2019-nCoV Assay on a Bio-Rad CFX96 Real-Time-PCR cycler with a test duration of 4.5 h. Test performance of all systems was shown to be identical. All laboratory sites are accredited by the Deutsche Akkreditierungsstelle GmbH (DAKKs) for performing molecular testing on viral pathogens. All assays used are CE/IVD-marked, and test performance was evaluated using positive patient samples and samples from External Quality Assessment (EQA) Panels including successful participation in EQA trials with all used systems.

Statistical Analysis

We retrospectively analyzed data of a 6-week observational period from 03/16/2020 to 04/27/2020. Continuous variables are described by mean ± standard deviation or median (minimum–maximum or interquartile range), respectively. After testing for normal distribution by Shapiro–Wilk test, group comparisons were performed by using Student *t*-test or Mann–Whitney *U*-test. Categorical variables are presented in absolute numbers and relative frequencies, group comparisons were performed by using the Pearson χ^2-test. Odds ratios (ORs) and confidence interval (CI) were calculated by logistic regression. Throughout all calculations, a two-tailed probability $P < 0.05$ indicated

TABLE 1 | Baseline characteristics of hospital employees tested for SARS-CoV-2.

Variables	Overall ($n = 394$)
Median age, years (range)	42 (19–71)
Female	256 (65)
Physicians	107 (27)
Nurses	231 (59)
Others	56 (14)
No. of tests, mean (median)	1.35 (1)

Values are given as n (%), mean or median (range).

statistical significance. Statistical analysis was conducted using SPSS version 26 (SPSS Inc., Cary, NC, USA).

RESULTS

The DHZB has a total of 1,404 employees including 199 physicians and 383 nurses.

Figure 1A depicts the hospital-initiated measures in relationship to restrictions by German and local authorities, as well as their temporal correlation to the number of positive SARS-CoV-2 tests, COVID-19 ICU admissions, and COVID-19–related deaths in Berlin. Overall, 1,128 SARS-CoV-2-PCR tests were done in 983 individuals during the period of this study. Of these, 589 tests were done in patients, with all patients (100%) undergoing one single test. In contrast, 394 employees did 539 tests, with 37% receiving more than one test (mean, 1.37 test/employee). The majority of HCWs had one test (72%, $n = 286$), 20% had two tests ($n = 78$), 6% had 3 tests ($n = 23$), and 2% had 4 tests ($n = 7$) (**Figure 1B**). In total 28.1% of hospital employees were tested. With regard to the clinical staff, we tested 57% (mean: nursing staff 60%, doctors 54%).

Symptom-Based SARS-CoV-2 Testing in Hospital Employees

Characteristics of employees tested for SARS-CoV-2 are shown in **Table 1**. More females (65%, $n = 256$) than males (35%, $n = 138$) were tested. The median age was 42 years [range, 19–71 years; interquartile range (IQR), 42–53 years]. One hundred fifty-nine of the tests were done in physicians (29%), 319 in nurses (59%), and 61 (11%) in persons from other work areas (i.e., mechanics, administration). Accordingly, the rate of tests in individual employees done per profession was 54% in physicians ($n = 107$ of 199), 60% in nurses ($n = 231$ of 383), and 7% in other work areas ($n = 56$ of 822). The majority of tests were done during the first 2 weeks of the observation period (up to 44 tests/day on 03/19/2020).

Hospital employee's motivation for undergoing SARS-CoV2-testing is shown in **Figures 2A,B**. Most tests (62.5%, 337 of 539 tests) were done due to the development of symptoms ($P < 0.01$), whereas 202 of 539 tests (37.5%) were done in asymptomatic employees. Of tests done in the asymptomatic employees, 4.5% ($n = 24$) were in returnees from risk areas, 19.3% ($n = 104$) in employees reporting contact to a COVID-19 patient (direct or close indirect contact), and 13.7% ($n = 74$) in asymptomatic employees without any contact, symptoms, or risk-area stay. Symptoms as motivation for testing was significantly more often denoted by non-physicians and non-nursing staff as compared to nurses ($P = 0.015$; OR, 2.23; CI, 1.16–4.28) and physicians ($P = 0.003$; OR, 2.83; CI, 1.42–5.64). Contact to a confirmed case of COVID-19 was denoted significantly less often by non-HCWs as compared to nurses ($P = 0.028$; OR, 0.37; CI, 0.14–0.92) and physicians ($P = 0.018$; OR, 0.32; CI, 0.12–0.9; **Figure 2A**). Even if more than one test was done, symptoms remained the driving force (first test = 66%, $n = 259$; second test = 56%, $n = 60$; third test = 47%, $n = 14$; fourth test = 57%, $n = 4$), but the percentage of asymptomatic HCWs that requested testing due to contact with an (presumptively or confirmed) infected person increased (first test = 14%, second test = 32%, third test = 37%, fourth test = 43%). Contact to a confirmed case of COVID-19 was denoted significantly less often in the first compared to second test ($P < 0.001$; OR, 2.77; CI, 1.69–4.55), third test ($P = 0.001$; OR, 3.49; CI, 1.58–7.74), and fourth test ($P = 0.034$; OR, 4.53; CI, 0.99–20.77). Risk-area return was a rare initial reason (5%, $n = 21$; **Figure 2B**). Testing performed in employees was accompanied by a questionnaire (multiple symptoms possible). The four most common symptoms reported were general malaise (59%, n = 200), sore throat (52%, $n = 176$), cough (48%, $n = 163$), and nasal catarrh (45%, $n = 153$; **Figure 2C**). Myalgia was reported in 20% ($n = 68$), shortness of breath in 15% ($n = 52$), and gastrointestinal symptoms in 11% ($n = 38$). In contrast, fever was indicated in only 7% ($n = 25$). In 9% ($n = 30$), employees stated being symptomatic, but did not specify any other reason (**Figure 2C**).

Of the 539 tests performed in 394 employees, only 3 (0.8%) tested positive for SARS-CoV-2. Two positive results occurred in first-time participants. The characteristics of positive HCWs are outlined in **Table 2**. Two of them had no comorbidity; one reported hypertension and bronchial asthma. The suspected source of infection was community acquired in all cases (one returnee from a risk area, one indirect contact via children's school, and one at a medical conference). Two of the three HCWs made use of testing because of mild symptoms (**Table 2**). The third HCW was initially asymptomatic, but underwent testing because of travel return from Ischgl/Austria. Her initial test was negative, but her fiancé tested positive. Thus, SARS-CoV-2 testing was redone 4 days later and was positive. She reported anosmia, but none of the three staff members reported fever.

Obligatory SARS-CoV-2 Testing in Hospitalized Patient

All 589 inpatients (100%) admitted were tested for SARS-CoV-2 at admission. Patients were mostly male (69.1%, $n = 407$; female: 30.9%, $n = 182$), with a median age of 64 years (range, 0–90 years; IQR, 49–74 years). Of the 589 tests performed, 58% ($n = 342$) were done in the department of cardiothoracic surgery, 30.1% ($n = 177$) in the department of medicine/cardiology, and 11.9% ($n = 70$) in the department of pediatric cardiology.

In 2019, the DHZB treated 8,378 inpatients and 23,523 outpatients. During the observation period, the DHZB treated

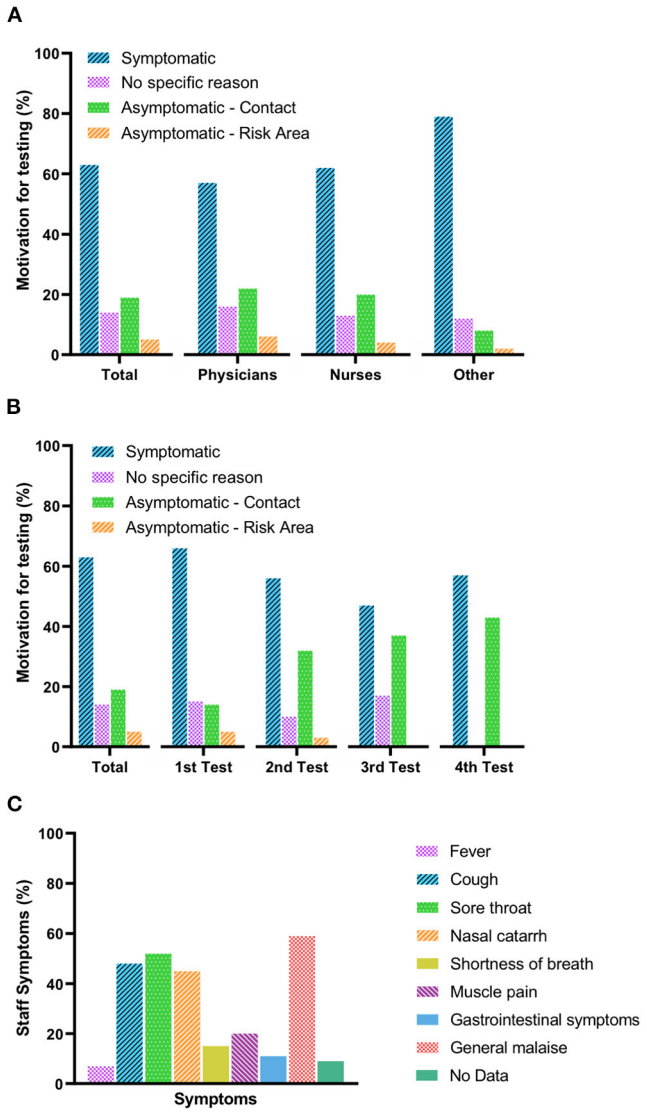

FIGURE 2 | Motivation for SARS-CoV-2 testing and symptoms reported by healthcare workers. **(A)** Motivations (percentage) for SARS-CoV-2 testing per professional group. **(B)** Motivations (percentage) for SARS-CoV-2 retesting in healthcare workers. **(C)** Symptoms reported by healthcare professionals. Fever is defined as body temperature over 38°C; cough includes dry and productive cough; nasal catarrh includes rhinitis and stuffed nose; muscle pain includes general and jaw muscle pain; gastrointestinal symptoms include diarrhea, nausea, and vomiting; and general malaise includes fatigue, headaches, chill, and general discomfort.

TABLE 2 | Characteristics of healthcare workers tested positive for SARS-CoV-2.

Variables	HCW 1 (physician)	HCW 2 (nurse)	HCW 3 (nurse)
Age (years)	40	30	50
Sex	Male	Female	Female
Comorbidities	None	None	Arterial hypertension Bronchial asthma
Symptoms	Myalgia Dry cough Headaches Dyspnea	Initially Asymptomatic Anosmia	Myalgia Anosmia Headaches
Suspected source of infection	Medical conference	Risk area (Ischgl/Austria)	Contact (indirect)
In-hospital contacts	12	7	25
Contacts tested	12 (100)	7 (100)	24 (96)
Tested positive for SARS-CoV-2	0	0	0

Values are given as n (%). HCW, healthcare worker.

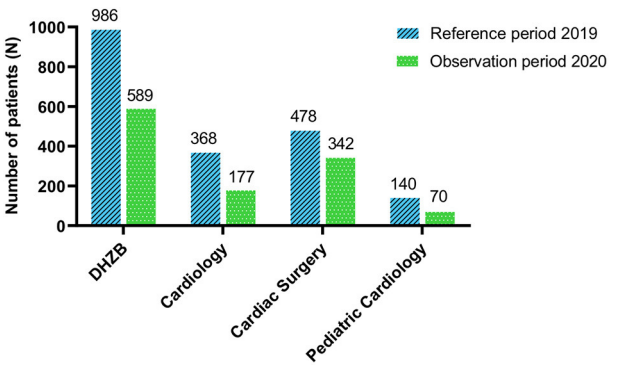

FIGURE 3 | Number of inpatients treated during the COVID-19 pandemic in comparison to the reference period in 2019. Total reduction of patients admitted during the reported period compared to the same time period in 2019 in different departments of the German Heart Center Berlin. DHZB, Deutsches Herzzentrum Berlin (German Heart Center Berlin).

40.3% less inpatients compared to the corresponding period in 2019 (2019 $n = 986$ patients vs. 2020 $n = 589$ patients; **Figure 3**). Patients treated in the department of medicine/cardiology ($n = 165$) were further analyzed with respect to their comorbidities and compared to patients in 2019. During the surge of COVID-19, patients admitted had significantly more cardiovascular risk factors (3.50 vs. 3.09, $P < 0.02$), significantly more heart failure (52.7 vs. 37.1%, $P < 0.001$), and a significant decrease in left ventricular systolic ejection fraction (48.2 vs. 52.7%, $P < 0.001$). In addition, valvular heart disease was significantly more present (41.2 vs. 29.7%, $P = 0.01$). Neither age, body mass index, nor the diagnosis of coronary artery disease, peripheral artery disease, or chronic obstructive lung disease was different (**Table 3**).

Of the 589 patients screened for SARS-CoV-2, only two patients tested positive (0.3%; one female and one male). One of the patients was asymptomatic with respect to COVID-19 and admitted for valvular heart surgery; the other one had pulmonary symptoms, fever, and diarrhea, and COVID-19 was suspected. As this patient was a heart transplant recipient and presented as emergency, he was admitted and isolated. None of the patient's contacts (mean $n = 15$) was infected. Detailed characteristics of the patients are shown in **Table 4**.

TABLE 3 | Baseline characteristics of patients in the department of medicine/cardiology during the reference period in 2019 and the study period.

Variables	Reference period (2019) ($n = 377$)	Study period (2020) ($n = 165$)	*P*-value
Mean age, years	70 ± 12.5	68 ± 12.6	0.68
Mean LVEF, %	52.7 ± 12	48.2 ± 13.8	<0.001
Mean BMI, kg/m²	28.09 ± 5.7	27.60 ± 5.2	0.10
Mean number of CVRF	3.09 ± 1.3	3.50 ± 1.5	0.02
Heart failure	140 (37.1)	87 (52.7)	<0.001
Coronary artery disease	243 (64.5)	102 (61.8)	0.56
Valvular heart disease	112 (29.7)	68 (41.2)	0.01
Peripheral artery disease	41 (10.9)	14 (8.5)	0.44
COPD	37 (9.8)	19 (11.5)	0.54

Values are given as n (%), mean ± standard deviation. Reference period: 03/16/2019–04/27/2019, study period: 03/16/2020–04/27/2020. LVEF, left ventricular ejection fraction; BMI, body mass index; CVRF, cardiovascular risk factors (arterial hypertension; hypolipoproteinemia, diabetes mellitus, smoking, family history of cardiovascular disease); COPD, chronic obstructive pulmonary disease.

TABLE 4 | Individual characteristics of the two patients tested positive for SARS-Cov-2.

Variables	Patient 1	Patient 2
Age (years)	72	53
Sex	Female	Male
Comorbidities	Hypertension CAD (CABG) Heart failure Valvular heart disease Chronic aortic dissection Thoracic aortic aneurysm Surgery	Heart transplant Hypertension DCM CKD (hemodialysis) Thoracic aortic aneurysm Surgery
Symptoms	Dyspnea (overlap to underlying disease)	Fever (38.8°C) Productive cough Rhinitis Dyspnea Diarrhea
Date of first positive test result	27/03/2020	20/04/2020
Days after first confirmed case in Germany/Berlin	60/25	84/49
Suspected source of infection	Unknown	Unknown
Contacts	8	22
Contacts tested positive for SARS-CoV-2	0	0

Values are given as n (%). CAD, coronary artery disease; CABG, coronary artery bypass grafting; DCM, dilative cardiomyopathy; CKD, chronic kidney disease.

DISCUSSION

We report interventions undertaken by a major cardiovascular center to prevent nosocomial patient and hospital employee SARS-CoV-2 infection, resulting in a low overall infection rate of 0.5%. Our data focus on the time span in which a number of restrictions were initiated (03/12/2020) by German and regional authorities (e.g., closure of schools, physical distancing) due to the exponential up rise of the COVID-19 pandemic and ends when these restrictions were partly lifted (e.g., reopening of schools and retail) due to lessening of the infection rates (19, 20). Restrictions were escorted by several hospital-initiated measures, including the review of scheduled visits for urgency and postponing elective operations, as well as closure of outpatient departments. However, in contrast to health authorities who did not recommend wearing a surgical face mask or screening for SARS-CoV-2 infection in patients and HCWs during that time, we initiated both at the very early beginning of the pandemic (21). Furthermore, symptom-based staff testing (03/16/2020) and mandatory patient testing (03/19/2020) were initiated early on.

During this observation period, the number of positive SARS-CoV-2 tests sharply increased in Germany and Berlin and was paralleled by an increase in COVID-19 ICU admissions and deaths. The reported positive tests/day rate for Germany was 6.8% at the beginning and declined to 3.9% at the end of our study period (22). However, we found only three HCWs (0.8%) and two patients (0.3%) infected with SARS-CoV-2, despite the fact that we screened 100% of patients and up to 60% of the clinical workforce (nurses and physicians).

Healthcare workers are at an increased risk of SARS-CoV-2 exposure, but may also be the source of nosocomial infections for patients and coworkers (15, 23). Early in the course of the pandemic, a single-center study from a large tertiary hospital in Wuhan, China (>7,000 beds), reported an infection rate of 0.5% in "first-line" HCWs, which was mostly hospital-acquired (15). Interestingly in this study, first-line HCWs working in close contact to COVID-19 patients had a lower infection rate than HCWs working in other clinical departments (1.6%), likely due to a better adherence to the use of PPE (15). More recently, a study from seven community hospitals in Texas reported the opposite, with 5.4% HCWs from COVID-19 units being SARS-CoV-2-positive, but only 0.6% from non-COVID-19 units (24). For the United States, the Centers of Disease Control and Prevention states that up to 55% of infected HCWs had contact with a COVID-19 patient solely in a healthcare environment, suggesting that work-related COVID-19 is common in HCWs (16). In contrast, in the Netherlands, SARS-CoV-2 infection among HCW was reported to be mostly community acquired (25). For Germany, data from a national survey reported a total of 495 COVID-19 outbreaks in hospitals/rehabilitation facilities across the country, resulting in 5,225 infections (26). At least 7% of SARS-CoV-2–infected persons were working in a medical setting in Germany during the first wave (27).

Here we describe the initiation of measures initiated at the same time, which may have worked in concert. First, this involves the designation of our hospital as "non–COVID-19" hospital, and like others, we postponed non-urgent cases, significantly reducing the number of patients by 40% (28, 29).

Second, with regard to SARS-CoV-2 testing, in this report, we investigated different hospital populations (patients vs. employees) by different modes (obligatory vs. symptom-based) of testing. Both groups likely differ by risk behavior, with

cardiovascular patients at older age presumptively practicing more physical distancing during the pandemic. In Germany and other countries, the pandemic is mostly driven by the younger/middle-age working population (30). Strikingly, the infection rate in this age group is low in our hospital. However, we did not screen all employees for SARS-CoV-2 and may have missed asymptomatic/presymptomatic infected. The viral load in asymptomatic and symptomatic patients is comparable, and transmission of SARS-CoV-2 by atypical/presymptomatic individuals has been shown to cause clusters of cases in defined sectors (8, 13, 31). Data on the numbers of asymptomatic infected persons vary significantly, ranging from 1% in early publications from China to more than 10% in a population-based study in individuals in Iceland (12, 32, 33). In contrast to our ubiquitous patient testing, we had to use a symptom-based approach for employee testing, because of limited resources. Symptoms mostly reported in our study included general malaise, sore throat, cough, and nasal catarrh. Still, a recent report demonstrated the limitation of symptom-based screening: when fever, cough, shortness of breath, or sore throat were asked, up to 17% of SARS-CoV-2–infected cases were missed, and even when expanding these criteria to include myalgias and chills, 10% were still missed (34). Thus, ubiquitous staff testing would have been desirable.

Indeed, a number of reports demonstrated that COVID-19 outbreaks can result from single index cases (13, 31, 35). A detailed epidemiological/phylogenetic study from South Africa showed that one SARS-CoV-2–infected person led to clusters in different hospital wards, leading to 39 infected patients and 80 infected staff members (35). Likewise, a recent report from a German teaching hospital demonstrated that only one index COVID-19 patient led to five infected staff members, subsequently resulting in more than 30% of infected hospitalized patients, emphasizing the need for a widespread SARS-CoV-2 testing and rapid isolation of positive cases (14). Thus, it is imperative to provide a safe hospital environment for patients and employees.

Third, in addition to widespread testing, studies now demonstrate that in contrast to early advice from health authorities, face masks are not a substitute, but significantly impact on SARS-CoV-2 transmission by protecting others from infected droplets (23, 36–39). A study performed in the largest healthcare system in Massachusetts (12 hospitals, >75,000 employees) demonstrated that prior to universal masking of HCWs and patients, new infections among HCWs sharply raised from 0 to 21.3% (39). Following mandatory face masking for patients and staff (among other restrictions), the positivity rate decreased linearly down to 11.46% (39). Another study done at Duke Health in North Carolina, US (>20,000 HCWs, including a tertiary care facility, community hospitals, primary care, and specialty practices) reported an analysis in which 70% of healthcare-associated SARS-CoV-2 infections were related to unmasked exposure to another HCW and only 30% secondary to direct care of SARS-CoV-2-positive patients (23).

Even though not randomized trials, these studies and our present report, in which we initiated surgical face masking for patients and HCWs at the very beginning, support that this simple intervention in combination with testing for SARS-CoV-2 is a key means to prevent COVID-19 in-hospitals outbreaks.

Study Limitations

We used self-administered oropharyngeal swaps instead of HCW-administered nasopharyngeal specimen collection. This lowers the risk of infection for the clinical staff and saves PPE resources. Indeed, studies demonstrated that swaps from different clinical specimens are comparable and that collection of patient samples for SARS-CoV-2 testing is accurate and valid (40, 41). Therefore, it is unlikely that this affected the results of our observation. Another limitation is that we could not provide universal screening to all employees because of limited testing resource. In addition, we report a single-center, non-interventional study that might not represent all healthcare systems/providers across Germany/Europe.

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 institutional ethics committee (Ethics Committee of the Charité - Universitätsmedizin Berlin; number: EA2/092/20; acronym: PREV-Sars-CoV-2-DHZB) and was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its most recent amendment 2013. Informed consent was obtained from all participants orally and in writing according to the Helsinki Declaration.

AUTHOR CONTRIBUTIONS

DS, KW, ME-M, MH, OM, FS, SH, and PS contributed to conception, design of the work, contributed to analysis, and interpretation of the data for the work. DS, KW, and PS drafted the manuscript. MH, OM, FS, SH, and BP critically revised the manuscript. All authors gave the final approval and agree to be accountable for all aspects of work ensuring integrity and accuracy.

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Active Surveillance of Asymptomatic, Presymptomatic, and Oligosymptomatic SARS-CoV-2-Infected Individuals in Communities Inhabiting Closed or Semi-closed Institutions

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Background: The high COVID-19 dissemination rate demands active surveillance to identify asymptomatic, presymptomatic, and oligosymptomatic (APO) SARS-CoV-2-infected individuals. This is of special importance in communities inhabiting closed or semi-closed institutions such as residential care homes, prisons, neuropsychiatric hospitals, etc., where risk people are in close contact. Thus, a pooling approach—where samples are mixed and tested as single pools—is an attractive strategy to rapidly detect APO-infected in these epidemiological scenarios.

Materials and Methods: This study was done at different pandemic periods between May 28 and August 31 2020 in 153 closed or semi-closed institutions in the Province of Buenos Aires (Argentina). We setup pooling strategy in two stages: first a pool-testing followed by selective individual-testing according to pool results. Samples included in negative pools were presumed as negative, while samples from positive pools were re-tested individually for positives identification.

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Results: Sensitivity in 5-sample or 10-sample pools was adequate since only 2 Ct values were increased with regard to single tests on average. Concordance between 5-sample or 10-sample pools and individual-testing was 100% in the Ct $\leq$ 36. We tested 4,936 APO clinical samples in 822 pools, requiring 86–50% fewer tests in low-to-moderate prevalence settings compared to individual testing.

Conclusions: By this strategy we detected three COVID-19 outbreaks at early stages in these institutions, helping to their containment and increasing the likelihood of saving lives in such places where risk groups are concentrated.

Keywords: SARS-CoV-2, COVID-19, coronavirus, pooling, RT-qPCR, asymptomatic

INTRODUCTION

COVID-19, caused by SARS-CoV-2, emerged on December 12, 2019 with 27 cases in Wuhan, China, and spread rapidly, surpassing 45 million infected people and one million deaths all over the world in October 2020. Its symptomatology was classified in six groups that might correlate with illness severity (1, 2). Elderly, and those with underlying medical conditions are at higher risk of developing serious illness. Meanwhile, others can become infected and develop moderate symptoms or even carry the infection asymptomatically. Such asymptomatic, presymptomatic, and oligosymptomatic (APO) people represent a great concern for health system since they may go unnoticed while contributing to SARS-CoV-2 circulation (3–5). In addition, APOs cannot be detected by passive surveillance, which diagnoses only suspicious cases.

Mitigating SARS-CoV-2 circulation necessitates continuous tracking, detection, and isolation of cases, for which active surveillance with massive and opportune APO detection methods is required. A possible strategy may be pooling individual samples for molecular diagnosis. This strategy, which was used successfully for syphilis, HIV, HBV, HCV, *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (6–13), consists of mixing several samples together and then test the pooled samples in one reaction. If the pool test is negative, it may be presumed that all patients are negative, while if it is positive, each sample is separately tested to find out which is responsible for that result. Thus, fewer tests are run overall, saving time and testing supplies, allowing faster return of results in most cases. As expected, when prevalence is low, pooling is usually cost-saving regarding testing samples individually. Using certain algorithms (i.e., dividing positive pools into halves, testing each of the two new smaller pools and continue subdividing positive pools, or 2 two-dimensional array with master pool testing etc.), 60–80% savings were calculated (14–16).

SARS-CoV-2 was isolated and described (17, 18) enabling molecular-diagnosis, which is performed mostly by retrotranscribed quantitative PCR (RT-qPCR) (19). With this technique, the pooling-strategy was assayed with different algorithms, particularly in asymptomatics, since low prevalence is expected there. By implementing the linear eight-sample Dorfman clustering to test 26,576 samples from asymptomatic individuals, 31 (0.12%) SARS-CoV-2 positives were identified, thus achieving a 7.3-fold increase in throughput (20). Moreover, by using a Shiny application (https://www.chrisbilder.com/shiny), efficiency of the pool size was assessed (21).

Special concern exists for SARS-CoV-2 dissemination in closed or semi-closed institutions such as residential care homes, neuropsychiatric hospitals, prison houses, police stations housing prisoners, etc. because they are inhabited by people in close contact that, in addition, have one or more risk factors. If the disease gets access to these vulnerable high-density communities, the demands for hospitalization, complex treatments, and assisted breathing could suddenly increase. To cope with this risk, in this study we implemented an active surveillance through a pooling-strategy aimed at early APOs detection in closed or semi-closed institutions in the Province of Buenos Aires, Argentina (population: 17.5 million, 38.5% of Argentina population) at different moments of the pandemic. The study is part of the active surveillance carried out by the Ministry of Health of the Province of Buenos Aires, and complements the passive surveillance that is being performed from the beginning of the pandemic.

MATERIALS AND METHODS

Sample Collection

Swabs (Britania or any rayon or dacron swab approved by the Argentine regulatory body) from both nostrils and the throat were collected by healthcare providers, and placed immediately into a sterile transport tube containing 2–3 ml of either viral transport medium, Amies transport medium, phosphate buffered saline, or sterile saline. For processing, all samples were properly labeled with the patient's filiation data and accompanied by their corresponding notification forms. Samples thus conditioned were shipped to the VacSal laboratory in refrigerated safety containers, and stored at 2–8°C for a maximum of 3 days, after which they were processed and analyzed.

RNA Extraction From Individual and Pooled Samples

Sample inactivation and RNA extraction were done using certified class-II biological safety cabinet. RNA was extracted from five-sample and 10-sample pools, as well as from individual samples, using the same RNA extraction kit (RNA Mini Kit Genaid RT300, Geneaid Biotech Ltd) following manufacturer's

instructions. Briefly, 200 µl of individual or pooled samples in viral transport media were used for RNA extraction. The individual samples, as well as the pools, were included in the same extraction batch, and the same aliquot was used. Negative pools with 3, 5, or 10 negative samples were included in the assays.

Retrotranscribed Quantitative PCR (RT-qPCR) for SARS-CoV-2 RdRp, E, ORF1ab, and N Genes

Single-step RT-qPCR for SARS-CoV-2 targeting the RdRp, E and N genes (GeneFinder™ COVID-19 PLUS RealAmp Kit) and ORF1ab and N genes (DisCoVery SARS-CoV-2RT-PCR Detection Kit Rox) was performed on the extracted RNA from individual and pooled samples immediately after RNA extraction.

To assess the sensitivity of the pooling strategy, we arbitrarily chose positive RNA samples with different Ct previously quantified, to prepare *ad hoc* diluted mixes with negative RNA samples. RT-qPCR was performed according to the procedure for individual samples in the clinical laboratory, with identical thermocycler and program (Applied Biosystems® 7500 fast), and with reagents used at the VacSal and Facultad de Ciencias Exactas y Naturales labs. Reaction mixtures using GeneFinder kit were heated to 50°C 20 min for reverse transcription, denatured at 95°C 10 min, and then 50 cycles of amplification were carried out at 95°C 15 s and 58°C 60 s. Fluorescence was measured using the FAM (for RdRp gene), Texas Red (for E gene), JOE (ABI)/VIC (CFX96) (for N gene), and Cy5 (for internal control) channels. Reaction mixtures using DisCoVery kit were heated to 50°C 10 min for reverse transcription, denatured at 95°C 30 s, and then 45 cycles of amplification were carried out at 95°C 5 s and 58°C 34 s. Fluorescence was measured using the FAM (for ORF1ab gene), VIC (CFX96) (for N gene), and Rox (for internal control) channels.

Concordance between individual and pooled sample testings was calculated, and expressed in percentages.

Determination of the Limit of Detection

The limit of detection for the pooling method was assessed following the protocol already described (22). The test material was RNA obtained from anonymous SARS-CoV-2 negatives and positives, which were collected at Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (INBIRS). First four independent positive RNA extracts with Ct ranging 31–34 were analyzed in 10 replicates of 1:20 pools. Then, one positive RNA sample (Ct 31.7) was analyzed in 20 replicates of different pool sizes (1, 10, 20, 40, 80, and 160). Negative RNAs were used for dilution. RT-qPCRs were performed as described above. The limit of detection for RT-qPCR methods was estimated from analysis of replicate standard curves.

Surveillance in Semi-closed Institutions

This strategy was implemented since the end of May 2020 as part of surveillance activities coordinated by the Ministry

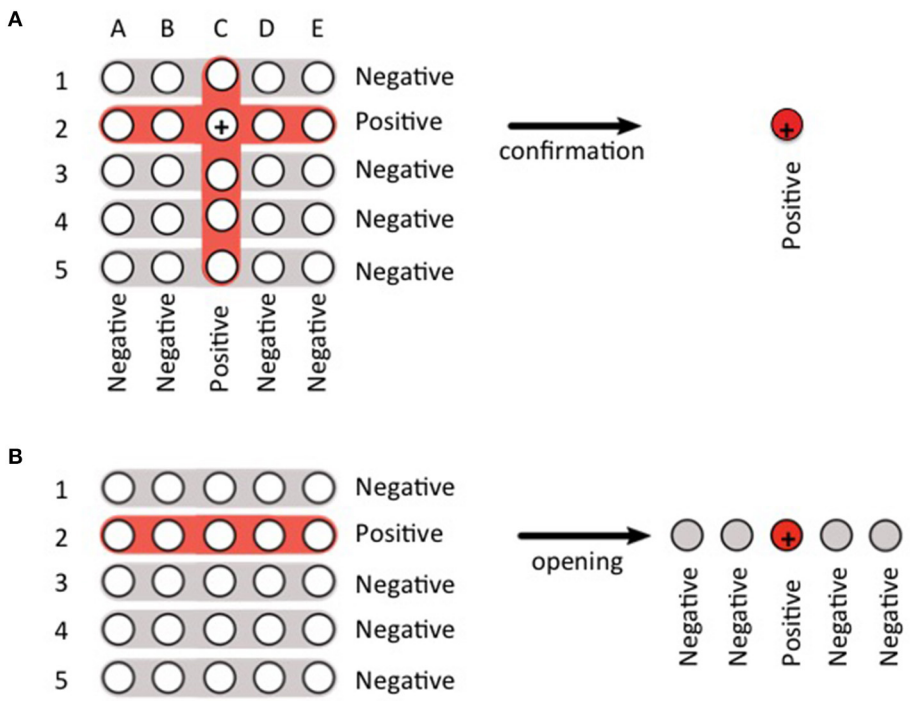

FIGURE 1 | Scheme of the two pooling strategies used in this work. **(A)** square matrix array. Here the samples are grouped in pools 1–5 representing rows, and in pools A–E representing columns. If pools 2 and C are positive, it is concluded that the third sample of pool 2, which is also the second sample of pool C, is positive (+). The result of this sample is confirmed by an individual test. **(B)** linear array. Here the positive pool (in this case, pool 2) must be opened to identify the positive sample (+).

of Health of the Province of Buenos Aires following national and provincial guidance (https://www.argentina.gob.ar/salud/coronavirus-COVID-19/laboratorio, https://www.gba.gob.ar/saludprovincia/noticias/la_provincia_de_buenos_aires_impuls%C3%B3_los_testeos_por_pool_para_evitar_brotes). Informed consent for these diagnostic activities (Public Health activities) is not requested. The VacSal laboratory was chosen to validate and carry out the pool strategy based on the clinical samples obtained from the residents or healthcare workers from closed or semi-closed institutions. At the beginning of the pandemic and when the prevalence of COVID-19 cases was low, sample groupings were performed using a square matrix array (columns and rows). To this end, sample groupings were done in two ways: on the one hand, a group of samples represented a row of the matrix and on the other hand, the samples were grouped again to represent a column of the matrix (**Figure 1A**). Thus, if only one row and one column tested positive, the positive sample could be identified within the pools (**Figure 1A**). When the prevalence of COVID-19 cases increased (after June) or when it was unknown, we used the linear grouping array. In this case, if all pools gave negative results in the RT-qPCR, the experimentation was concluded. In contrast, if a positive result was obtained for a pool of samples, then each sample that is included in the pool was tested individually (**Figure 1B**).

In a 3-month period, 4,936 clinical samples from 153 institutions distributed in 29 municipalities of the Province of Buenos Aires were evaluated.

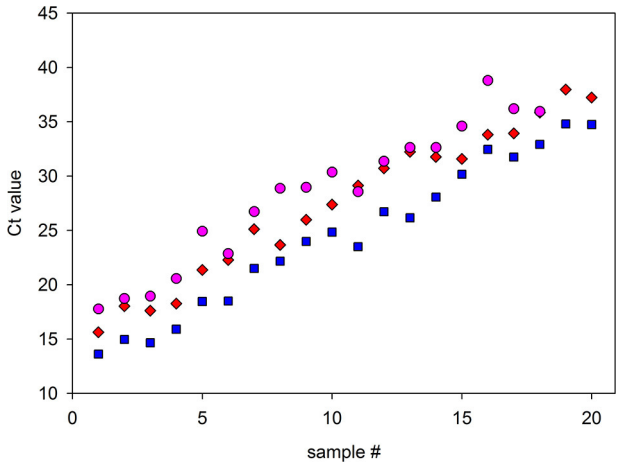

FIGURE 2 | Comparison of RT-qPCR detection of selected samples alone or in pools, and assessment of stability after freezing and thawing. Twenty representative samples with a range of Ct values were subjected to RT-qPCR alone or diluted in 1:20 with negative samples and measured. After dilution, 18 of the 20 samples were detected, while two samples, which possessed the highest Ct values, were not. Red diamonds: Ct values of the samples measured alone (N gene, GeneFinder kit). Pink circles: Ct values of the 1:20 pool. In parallel, Ct values for the same samples measured alone were obtained after freezing and thawing to observe their stability (blue squares). These last two measures were from N gene performed with DisCoVery kit. The average ΔCt value due to the dilution for the measurements at the same run was 4.95, close to the theoretical value (4.3).

RESULTS AND DISCUSSION

Evaluation of Pooling Performance

From 526 independent anonymous SARS-CoV-2 positive RNAs stored at −70°C for setup studies, 20 representative samples were systematically chosen. Samples were ordered in an equispaced manner by initially measured Ct as depicted in **Figure 2**, alongside a second measurement of them after being defrozen, and the respective 1:20 pools prepared from original samples.

To determine the probability of detection near the positive/negative detection boundary, 10 replicates of samples #17 to #20, which possessed the lowest Cts, were analyzed in 1:20 pools. **Figure 3A** indicates that pools belonging to the samples with original Ct 32.4 and 33.3 were detected at 100% rate, while samples with Ct 34.0 and 35.1 were detected at 70 and 50% rate, respectively. To determine the suitability of the pooling method for a range of dilutions, a study was conducted by comparing 20 replicates of different dilution pools, previously known to be near the edge of the detected/undetected result for ORF1ab and N genes. The result indicated a high rate of detection in pools <1:20 (**Figure 3B**).

From the comparison of detection probability and the complete clinical samples Ct histogram, the pool method robustness may be estimated. **Figure 4A** shows the histogram of all 526 samples ordered by N gene-Ct. Despite samples corresponded to initial diagnostic tests, a bimodal distribution is apparent. This two-peak histogram was as previously reported for SARS-Cov-2 infection (23), although, to our knowledge, no explanation was provided yet. Preliminary results indicate that the bimodality is unrelated with symptoms severity, since asymptomatic individuals also present similar histograms. Cts corresponding to high and low viral loads are rather evenly distributed along samples. The line indicates the probability of positive detection of a single sample in a 20-samples pool. **Figure 4B** shows detectable and undetectable samples in the histogram. The coincidence value is 95.3%. It is important to note that the 5% that is lost is not evenly distributed among the samples but corresponds to the lowest specimens' viral loads.

Assessment of Pooling Strategy Sensitivity

The impact of pooling clinical samples aliquots (nasopharyngeal-swabs) prior RNA extraction was tested. To this end, clinical samples with Cts in the ranges of either 20–23 or 30–33 were employed. By combining these samples with negative clinical samples, 1:5 and 1:10 pools were formed. From them, RNA was extracted and RT-qPCR was performed to obtain the Cts. Regardless of individual Ct of the positive RNA extract included in the pool, Cts increased 1.9 ± 1.1 units in 1:5 pools with respect to individual RNA extract, and 2.2 ± 0.3 units in 1:10 pools. Regarding clinical samples whose individual RNA extracts possessed Ct ≥ 36, they turned out negative when pooled together with four or more negative samples.

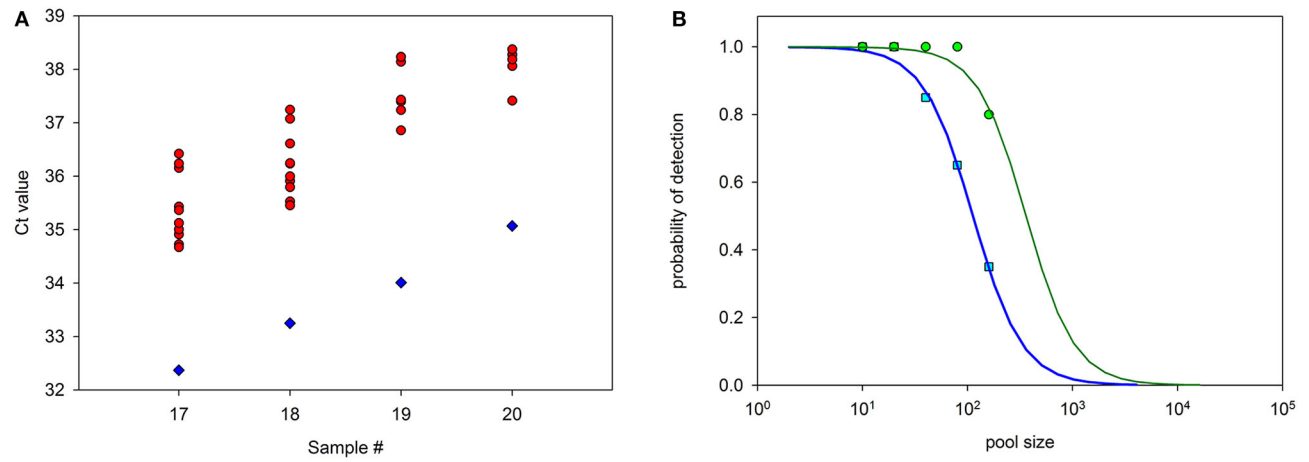

FIGURE 3 | Probability of detection of samples included in pools of different sizes. Samples #17 to #20, which possessed the highest Ct values, were diluted 1:20 with negative samples and measured. **(A)** Diamonds: Original Ct (ORF1) from samples #17 to #20. Circles: Ct values from 10 replicates of the same samples diluted 1:20 with negative samples and measured. Detection with Ct < 40 was positive in all dilutions of samples #17 and #18, 7 out of 10 dilutions of sample #19, and 5 out of 10 dilutions of sample #20 (circles with the same Ct value appear superimposed). **(B)** Probability of detection, as determined in 20 replicas as before, for pools containing one positive sample of Ct = 31.7 (ORF1) diluted at 1:10 to 1:160 with negative samples. Blue squares: ORF1 gene. Green circles: N gene. Lines are best fits of sensitivity curves.

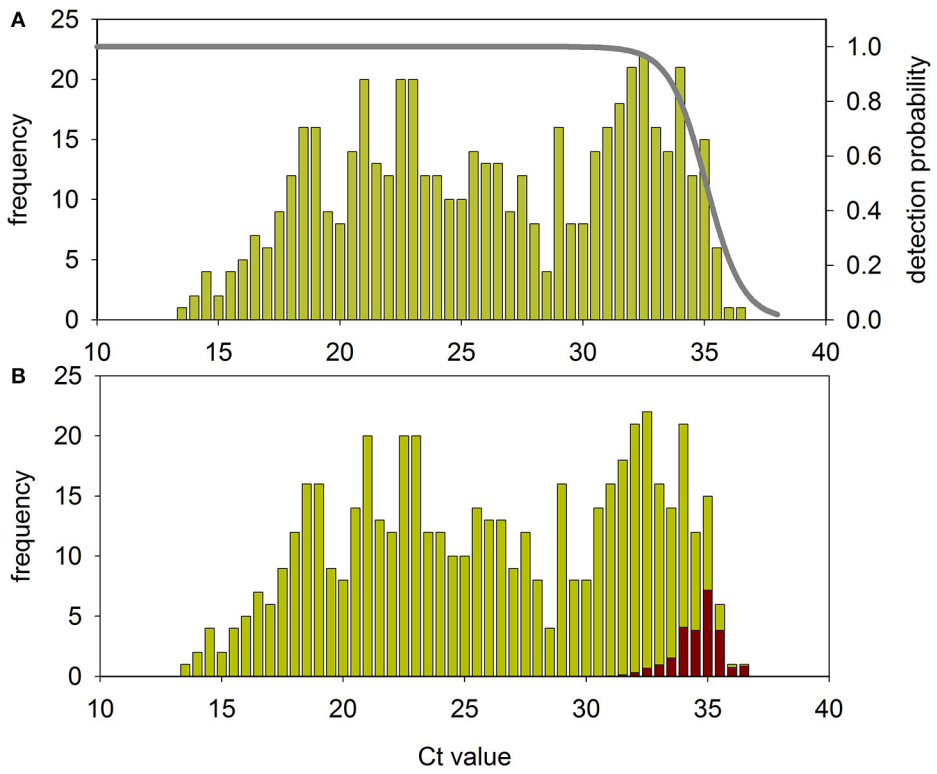

FIGURE 4 | Robustness of the pool method. **(A)** Histogram of the 526 initial diagnostic samples and probability of their individual detection in a 20 samples pool in experiments performed as depicted in **Figures 2**, **3**. **(B)** The same histogram indicating the fraction of samples that will be not detected in a 20 samples pool (red bars).

Pooling Strategy Applied to Active Surveillance of COVID-19 in Closed or Semi-closed Facilities in the Province of Buenos Aires, Argentina

On the basis of these results and considering that contagiousness of individuals with Ct $\geq$ 36 would not impact on COVID-19 epidemiology (24–26), the Ministry of Health of the Province of Buenos Aires decided to apply this methodology to analyze health situation in closed or semi-closed facilities.

The first confirmed case in the Province of Buenos Aires was detected on March 8, 5 days later than the first case detected in Argentina. From this date on, the rate of increase in the week average of total number of cases (N) as well as in the daily reported cases (n) was fast until the end of May, although few cases were still reported. The pooling-strategy was started on May 28, when a significant increase was evident, and data presented here are until August 31. From the slope of plots of $\log_2 N$ against time, N duplication time was deduced for this whole period as 16.1 days; however, N duplication time was increasing from 12.4 days between May 28 and July 7 to 29.9 days between August 18 and 31.

From May 28 to August 31, 4,936 samples were received from 153 institutions distributed in 29 municipalities in the Province of Buenos Aires. Between May 28 and July 7 (duplication time 12.4 days) the prevalence of positives in the analyzed samples did not exceed 4% (40 positives out of 1,052 clinical samples analyzed). In these cases, the clinical samples were pooled mostly applying a matrix clustering where samples are arranged in a square matrix with each row and each column tested in a different pool (**Figure 1A**). Therefore, samples whose row and column are both positive are retested individually. Notice that if there is only one positive sample there will be only one positive row and one positive column. In this scenario the positive sample can be identified at this stage without additional individual testing. So, although this strategy involves more reactions than linear clustering when the number of samples to be tested is small, it allows frequent positives identification without opening pools when prevalence is low. This strategy allowed saving time and kits (66% in average). During a second period comprised between July 8 and August 2 (duplication time 16.9 days) the growth rate in the number of cases was still accelerating for both n and N values. Hence, given that higher percentage of positivity was expected in this epidemiologic context, the advantage of avoiding opening pools through matrix clustering was lost, so linear clustering (**Figure 1B**) was implemented. Although at the beginning of this period pools of five samples for RNA extraction and 10 samples (two pools of five) for RT-qPCR were used, during most of the period analyses were done with pools of five clinical samples for both RNA extraction and RT-qPCR. During this second period 1,730 samples were processed, from which 481 were positive (27.8% prevalence). Finally, during August, when duplication time raised to 22.1 days in the first fortnight, and to 29.9 days in the second 1,262 samples were processed, with 358 positives (28.4%) in the first fortnight and 892 samples with 135 positives (15.1%) in the second Thus, the number of people tested per kit (kit saving) ranged from 2.0 to 7.4 depending on the place of origin, with an average of 3.0. Given the high prevalence, this level of savings is quite better than the values predicted by usual mathematical models. This is because pooled samples are not independent of each other since they were obtained from the same closed or semi-closed facility. In other words, since positive samples are unevenly distributed among facilities, pooling-strategy is more effective than predicted.

Because the strategy was used mostly with samples from care homes, the majority were from elderly population. In the group >75-year-old, 30.2% positive cases were detected (1,415 positives in 4,682 clinical samples). Furthermore, there were 44.7% positives in the group 10–14-year-old, and 42.8% in the group 5–9-year-old, albeit from smaller samples (17 positives out of 38 and nine out of 21, respectively). Almost all positive cases were APO, irrespective of age. Interestingly, in asymptomatic cases, the elder group tended to possess lower Ct than younger groups (**Figure 5A**), being the lowest Cts the most frequent among asymptomatic elders (**Figure 5B**).

Early Detection of Infection Foci

Tracing of infection foci and outbreaks was possible in institutions from which samples were received repeatedly. A first example is a women psychiatric hospital at Temperley (Municipality of Lomas de Zamora) from which 216 samples from 210 people distributed in different rooms—including health workers and resident patients—were received and processed on June 19, 23, and 30, July 3, 6, 14, 23, and 28, and August 14 and 20. Analyses were carried out in 106 reactions with a kit saving of 2.6 ± 2.0.

Until June 23 all samples were negative. On June 30 there were five positives, one of which corresponded to an ambulance driver. Therefore, samples from the other drivers and his close family contacts were asked, all of which, as well as a nurse, resulted positive on July 3 (seven positives). Meanwhile, the other samples, which included 17 healthcare workers and 29 residents, were all negative. In view of this situation, isolation procedures were launched and as result, there were no positives 20 days later. However, three new positive cases were detected on July 28, including a nurse. Epidemiologic investigation showed that this nurse has recently been concluded her preventive quarantine because of being close contact of a symptomatic case. Therefore, all patients that were in contact with this nurse were isolated, and analyzed on August 14. It turned out that all 26 patients were positive, and 21 of them developed symptoms. Strict isolation was undertaken, but one of the patients that had comorbidity conditions deceased. Stringent isolation measures were undertaken because focus dissemination to other rooms of the hospital was detected. On the next survey, carried out August 20 among 18 asymptomatic health workers with close contact among them, only 2 were positive.

In another example, a total of 123 samples from 105 different people were received from a disabled center. This center has two sieges, one at Bernal and the other at Quilmes (both in the Municipality of Quilmes). Samples were from young patients, with mean age 24 ± 4 years in Bernal and 23 ± 9 years in Quilmes.

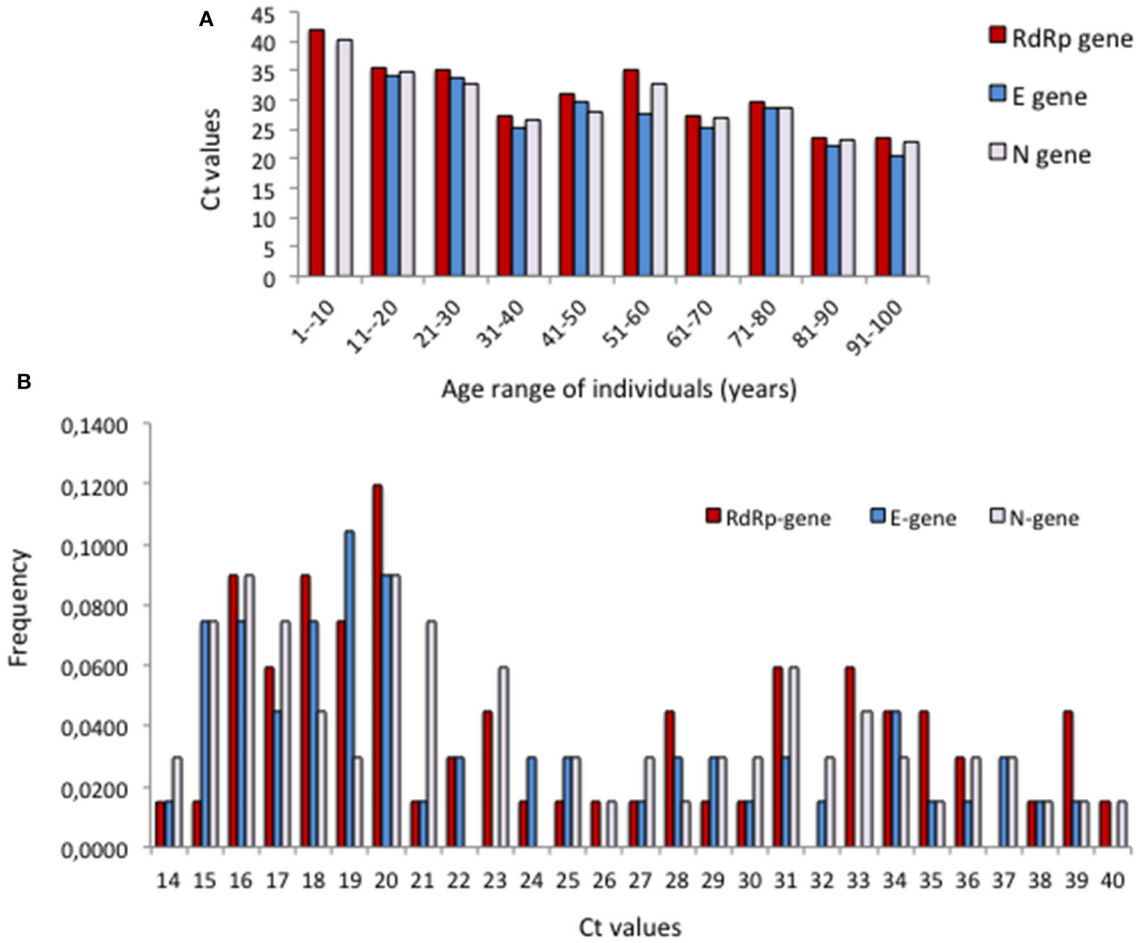

FIGURE 5 | Distribution of Ct values in the clinical samples from asymptomatic individuals. **(A)** Cts according to age for the 10 cohorts between ages 1 and <100 years. **(B)** Frequencies of Cts in the age group 70–100 years.

From Bernal, 35 samples from 28 different patients were processed on July 16 and 21, and August 11 (kit saving: 2.0 ± 1.0). On July 16 there were 7 positives out of 15 total samples (47%), while on July 21 all 13 samples analyzed were positive. Therefore, isolation protocols were applied and all negatives from July 16 were re-analyzed. In this second analysis all patients were negative again.

In turn, 88 samples belonging to 77 different patients were received from Quilmes, which were processed in 28 reactions (kit saving: 3.4 ± 1.4). Eleven samples were received twice each; consecutive analyses of these samples resulted either negative in both instances (three cases) or negative in the first analysis and positive in the second (eight cases). RT-qPCRs were carried out at June 30, July 23 and 30, and August 11. On June 30, all results were negative. The first positive was detected on July 23 in a girl that had cardiac antecedents and presented odynophagia, who was negative in the first analysis on June 30. Therefore, samples were obtained from other patients and analyzed on July 30. All these samples, which amounted 37, were positive. At this moment, several of these patients were hospitalized and prevention measures were further stressed. In the new sampling carried out on August 11, positivity was reduced to four positives detected among 11 samples (36%). Three of the negatives observed at August 11 were previously negative on June 30, showing the control of the focus.

CONCLUSIONS

Pooling effectiveness depends on the prevalence of positive samples (27). Therefore, batch sizes for pool testing or even the decision of pool testing should be taken at the laboratory or regional levels, considering positivity rates, specific groups, and categories being tested. Groups with high pre-test probability or serious manifestations are inadequate for pool testing.

Pooling up to 5–10 samples increased test capacity with existing equipment and test kits and detected positives with Cts ≤ 36 with sufficient diagnostic accuracy (2 Cts increase on average). Remarkably, the use of this strategy in the Province of Buenos Aires allowed early outbreaks detection, and evidenced

that APOs may present Cts as low as those of symptomatic individuals. The role of APOs in virus transmission must be further studied.

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

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 from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

NA, PM, KB, DB, MG, VG, AG, ER, and EZ: investigation, writing-review, and editing. RC and FL: resources, investigation, writing-review, and editing. MD, OF, ML, FM, LR-V, and GS: formal analysis, writing-review, and editing. AL: writing-original draft, formal analysis, validation, and visualization. NP: formal analysis, investigation, writing-review, and editing. AP, RE, and DH: conceptualization, methodology, validation, visualization, investigation, resources, writing-original draft, supervision, funding acquisition, and project administration. All authors contributed to the article and approved the submitted version.

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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.

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Clinical Characteristics and CT Findings in 148 Non-COVID-19 Influenza-Like Illness Cases: A Retrospective Control Study

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Background: This study was to collect clinical features and computed tomography (CT) findings of Influenza-Like Illness (ILI) cases, and to evaluate the correlation between clinical data and the abnormal chest CT in patients with the Influenza-Like Illness symptoms.

Methods: Patients with the Influenza-Like Illness symptoms who attended the emergency department of The Six Medical Center of The PLA General Hospital from February 10 to April 1, 2020 were enrolled. Clinical and imaging data of the enrolled patients were collected and analyzed. The association between clinical characteristics and abnormal chest CT was also analyzed.

Results: A total of 148 cases were enrolled in this study. Abnormalities on chest CT were detected in 61/148 (41.2%) patients. The most common abnormal CT features were as follows: patchy consolidation 22/61(36.1%), ground-glass opacities 21/61(34.4%), multifocal consolidations 17/61(27.9%). The advanced age and underlying diseases were significantly associated with abnormal chest CT.

Conclusions: Abnormal chest CT is a common condition in Influenza-Like Illness cases. The presence of advanced age and concurrent underlying diseases is significantly associated with abnormal chest CT findings in patients with ILI symptoms. The chest CT characteristic of ILI is different from the manifestation of COVID-19 infection, which is helpful for differential diagnosis.

Keywords: computerized tomography, influenza-like illness, influenza, respiratory tract infection, ground glass opacities, consolidation

INTRODUCTION

Seasonal influenza is a global respiratory infectious disease (1). According to the statistics, the incidence of symptomatic seasonal influenza in the general population of the United States was approximately 8% between 2010 and 2016 (2). The disease is usually self-limiting, but it can also cause serious complications and even death in particular populations (3–9).

Based on the statistics, the number of hospitalizations associated with seasonal influenza in the United States is between 140,000 and 960,000 each year (7, 10). The global annual number of deaths caused by respiratory complications related to seasonal influenza is between 290,000 and 640,000 (3). Flu-like symptoms are usually present in such patients when they visit doctor. If the identification of patients in whom respiratory complications have occurred can be achieved as early as possible, the adverse outcomes of delayed diagnosis may be avoided.

Chest CT is an important examination in the diagnosis of lower respiratory tract infections. However, chest imaging, especially chest CT examination, is not usually conducted for the diagnosis and treatment of patients with influenza-like illness (1). Furthermore, existing studies tend to target patients with more serious illness, and there is a paucity of data related to overall lung imaging in influenza-like cases (11–15). Therefore, a general survey of lung imaging is helpful for recognizing the overall condition in the lung imaging of influenza-like cases and for providing a reference for clinical practice.

During the COVID-19 epidemic, in order to screen for patients with respiratory infections, chest CT was used extensively to examine patients with fever at our hospital, which provided us with an opportunity to know about the general characteristics of lung imaging in influenza-like cases. In the COVID-19 epidemic season at the beginning of 2020, we collected and analyzed chest imaging data on influenza-like cases (non-COVID-19) in a non-epidemic area (Ganjiakou area, Haidian District, Beijing, China), to provide a relevant reference for future imaging studies of influenza-like cases. Since lung imaging is an important element in the diagnosis of pneumonia, it plays a significant role in the selection of treatment plans. In this study, influenza-like cases were divided into a normal CT group and an abnormal CT group based on the results of chest CT examination, and a comparison between the groups as well as logistic regression analysis was performed to determine the relevant features with regards to lung imaging abnormalities.

MATERIALS AND METHODS

Patients

Inclusion criteria: Patients who attended the emergency department of The Sixth Medical Center of Chinese PLA General Hospital because of respiratory infection symptom (RTI) from the 10th of February to the 1st April, 2020 were enrolled in this retrospective control study. RTI was defined as one with respiratory tract symptoms (i.e., new onset cough, sore throat, running nose or sputum production) (16).

Exclusion criteria: patients younger than 14 years old; patients without infection; patients with COVID-19 infection; cases without CT examination; patients who did not meet the criteria for ILI (body temperature $\geq 37.5^{\circ}$C, accompanied by cough and/or sore throat) (17, 18). The axillary temperature of all patients were measured using the mercury meter, and the recorded body temperature was the highest value of the body temperature self-reported or measured in the hospital. **Figure 1** showed flowchart of patient selection.

A diagnosis of COVID-19 was excluded in all enrolled patients during this period. Once the patients were suspected of COVID-19, they were first checked by the expert team at our hospital, and respiratory tract specimens were obtained and sent for RT-PCR testing. If the test was positive, a diagnosis of COVID-19 infection was established. If two tests were negative, the diagnosis was excluded.

Standardized data collection forms were used to extract related data such as epidemiological data, demographic data, clinical characteristics, laboratory test results etc. from electronic medical records. The specific data that were collected included the time of consultation, demographic data (gender, age), clinical symptoms, blood test results (routine blood test, C-reactive protein), and comorbidity records. All data were reviewed by two doctors (SWZ and CXX). If there was a difference in interpretation between the two, a third researcher (LDW) acted as an arbiter.

This retrospective study was approved by the Ethics Commission of the Sixth Medical Center of the PLA General Hospital (AF/SC-09/02.1) and the requirement for informed consent was waived by the Ethics Commission.

Chest Imaging Studies

Scanning position: supine position, CT scan range was from the supraclavicular region to the adrenal glands. Philips Brilliance iCT 256-slice CT machine was used, with the following scanning parameters: collimation 128 $\times$ 0.625 mm, reconstruction slice thickness 0.9 mm, slice interval 0.9 mm, matrix 512 $\times$ 512; tube voltage 100 kVp [for scanning patients with body mass index (BMI) $\leq$24 kg/m^2] or 120 kVp (BMI $>$ 24 kg/m^2); X-ray tube rotation speed 0.5 s/turn, pitch 0.992, automatic mAs technology (Dose Right; Philips Healthcare). Iterative model reconstruction (IMR) was adopted for reconstruction of all data. Display window: lung window, window level L-650 HU, window width W1700 HU; mediastinal window, window level L40 HU, window width W400 HU.

For patients undergoing chest CT examination, the radiological images were independently read by two certified radiologists and a report was made. The radiologists were aware that the patients had flu-like symptoms, but they did not know the clinical symptoms, signs, laboratory findings and past history of the patients.

The lung imaging abnormalities were analyzed based on their features, and the results were classified and summarized according to the characteristics below. The criteria for CT morphology were as follows: patchy consolidation, multifocal consolidation, ground-glass opacity, central lobular nodules, bronchial wall thickening, thickening of the interlobular septum, pleural fluid and other CT abnormalities, diagnosed in accordance with the definitions within the glossary of terms by Fleischner (19).

The difference between patchy consolidation and multifocal consolidation lies in the extent and number of lesions. Patchy consolidation lesions have a diameter $>$1 cm or a diameter which

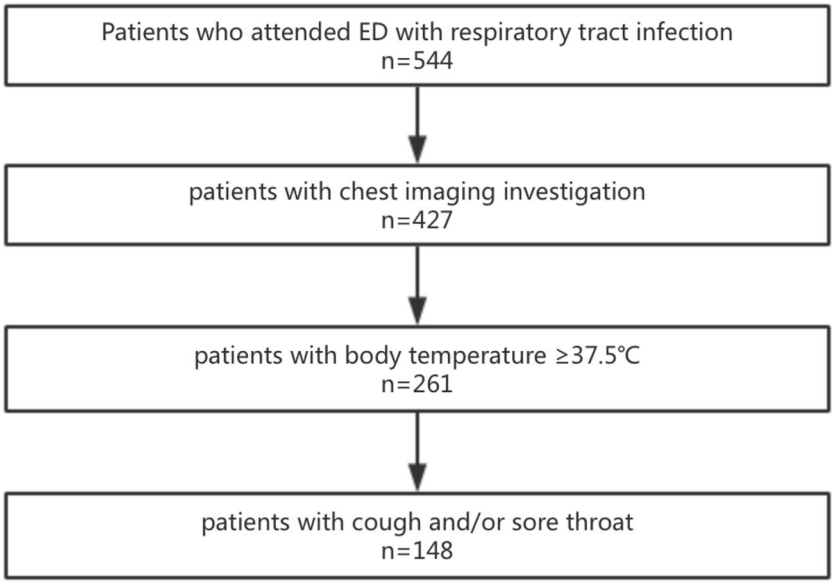

FIGURE 1 | Flowchart of patient selection. 544 patients visited the emergency department with respiratory infection symptoms. 427 patients underwent the chest CT imaging examination. 261 patients with the body temperature ≥37.5°C. 148 patients with the symptoms of cough and/or sore throat.

exceeds a single slice, while multifocal consolidation lesions have a diameter of <1 cm, with three or more lesions present.

A normal appearance or pre-existing lung disease (such as emphysema, bronchiectasis, interstitial lung disease, tumor, old tuberculous foci etc.) without new lesions were defined as non-pathological findings. In addition, the distribution of anatomical lesions was classified into whole lung, upper, middle, or lower lung depending on the main area of the lesion. The location of the lesion was classified as segmental, lobular or diffuse distribution, or random distribution.

Statistics

Continuous and categorical variables were presented as median (IQR) and *n* (%), respectively. SPSS for Windows software package, version 20.0 (SPSS Inc, Chicago, IL) was used. Mann-Whitney *U* test or χ^2 test was used to compare differences between the normal CT group and the abnormal CT group. To explore the risk factors associated with abnormal CT findings, univariate and multivariate logistic regression models were used. By referring to the relevant literature, we selected age, gender, body temperature, underlying diseases, symptoms, white blood cell count, neutrophil count, lymphocyte count, and C-reactive protein as variables for stepwise logistic regression analysis (20). At the same time, parameters of odds ratios that were difficult to estimate were excluded due to the small number of cases as well as the parameters that had collinearity with the underlying disease. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Patients Characteristics

Through HIS system retrieval, a total of 544 adult patients with respiratory tract infection symptoms attended the emergency department of our hospital between February 10 and April 1, 2020, and among them, 117 patients did not undergo imaging investigation. Screening was performed based on the criteria of body temperature ≥37.5°C, and 166 patients with body temperature lower than 37.5°C were excluded. Based on at least one of the symptoms of cough or sore throat, 113 cases were further excluded, and 148 cases were finally included. At this stage, our hospital did not accept patients with COVID-19 infection (diagnosis was based on the diagnosis and treatment guidelines issued by the Ministry of Health of China) (21).

The demographic data and basic medical history of the patients are summarized in **Table 1**. Among the 148 patients who were finally included, the median age was 36 years (26, 58.75), of which 72 were males (48.6%). A total of 23 patients had underlying diseases, including diabetes, tuberculosis, COPD, cerebral stroke sequelae, fractures, bronchiectasis, chronic kidney disease, thyroid disease, hypertension, coronary heart disease, hyperlipidemia, solid tumors, Alzheimer's disease, hematological disease, autoimmune disease etc. In the abnormal imaging group, the median age was 66 years (38.5, 87), and 22 cases (36.1%) had underlying diseases. These two indicators were significantly higher than those of the normal group ($P < 0.01$).

Imaging Analysis

Among the included patients, a total of 61 patients had imaging abnormalities (41.2%). The chest CT findings are summarized in **Table 2**. Among them, there were 22 cases (36.1%) with multifocal consolidations, 21 (34.4%) cases with ground glass lesions, and 17 cases (27.9%) with patchy consolidations. There were 12 (19.7%) cases with bronchial wall thickening, and 8 (13.1%) with centrilobular nodules. There were three cases

TABLE 1 | Patients demographic and basic medical history data.

	Total (n = 148)	Normal (n = 87)	Abnormal (n = 61)	P-value
Sex				0.576
Male	72 (48.6%)	44 (50.6%)	28 (45.9%)	
Female	76 (51.4%)	43 (49.4%)	33 (54.1%)	
Age	36 (26,58.75)	28 (23,38)	66 (38.5,87)	0.000
Underling disease	23 (15.5%)	1 (1.1%)	22 (36.1%)	0.000
Diabetes	1 (0.7%)	0 (0%)	1 (1.6%)	0.231
Tuberculosis	1 (0.7%)	0 (0%)	1 (1.6%)	0.231
COPD	4 (2.7%)	0 (0%)	4 (6.6%)	0.015
Cerebral stroke sequelae	5 (3.4%)	0 (0%)	5 (8.2%)	0.07
Fracture	1 (0.7%)	0 (0%)	1 (1.6%)	0.231
Bronchiectasis	2 (1.4%)	1 (1.1%)	1 (1.6%)	0.799
Chronic kidney disease	3 (2.0%)	0 (0%)	3 (4.9%)	0.037
Thyroid disease	1 (0.7%)	0 (0%)	1 (1.6%)	0.231
Hypertension	2 (1.4%)	0 (0%)	2 (3.3%)	0.089
Coronary heart disease	0 (0%)	0 (0%)	0 (0%)	
Hyperlipidemia	1 (0.7%)	0 (0%)	1 (1.6%)	0.231
Solid tumors	1 (0.7%)	0 (0%)	1 (1.6%)	0.231
Alzheimer's disease	2 (1.4%)	0 (0%)	2 (3.3%)	0.089
Hematological disease	1 (0.7%)	0 (0%)	1 (1.6%)	0.231
Autoimmune disease	1 (0.7%)	0 (0%)	1 (1.6%)	0.231

Data are median (IQR), n (%), or n/N (%). p-values were calculated by Mann-Whitney U test or χ^2 test as appropriate. COPD, chronic obstructive pulmonary disease.

TABLE 2 | CT characteristics in 61 abnormal CT findings.

Abnromal CT findings	Cases (n = 61)	Proportion (%)
Patchy consolidations	17	27.9
Multifocal consolidations	22	36.1
Ground-glass opacities	21	34.4
Centrilobular nodules	8	13.1
Bronchial wall thickening	12	19.7
Interlobular septal thickening	3	4.9
Lymphadenopathy	2	3.3
Pleural effusion	7	11.5
Unilateral	6	9.8
Bilateral	1	1.6
Anatomical distribution zonal predominance		
Upper and mid	18	29.5
Lower	27	44.3
Whole lung	16	26.23
Localization		
Segmental and lobar	49	80.33
Diffuse	12	19.67
Symmetry		
Bilateral	30	49.2
Unilateral	31	50.8

CT, computed tomography.

(4.9%) with interlobular thickening and two cases (3.3%) with lymphadenopathy. Among 61 patients, seven cases (11.48%) had pleural effusion, of which one (1.64%) had bilateral pleural effusion, and six (9.84%) had unilateral pleural effusion. **Figure 2** showed patients with abnormal CT findings.

Based on the statistics related to the distribution characteristics of lesions, it can be seen that there were 31 cases (50.8%) with unilateral lesions, and 30 patients (49.2%) with bilateral lesions. In terms of classification based on the distribution area of the main lesions, there were 18 (29.5%) in the upper lung, 27 (44.3%) in the lower lung, and concurrent involvement of the upper, middle, and lower lung was present in 16 (26.2%). Based on lesion location classification, 49 (80.3%) of the main lesions were distributed along the lobes/segments of the lung, with fewer cases of diffuse distribution (12, 19.7%).

Symptom Analysis

The clinical characteristics of the 148 patients in this study are summarized in **Table 3**. The patient's body temperature was 37.9°C (37.5, 40.2°C) at the time of consultation. The most common clinical symptoms were sore throat 79/148 (53.7%), cough 86/148 (58.1%), and sputum 44/148 (29.7%), followed by fatigue and shortness of breath. Vomiting and diarrhea were relatively rare.

Laboratory Examination Results

In this study, 147 patients underwent routine blood test and 144 patients accepted C-reactive protein test. The results showed that lymphopenia accounted for 27.2%, leukocytosis accounted for

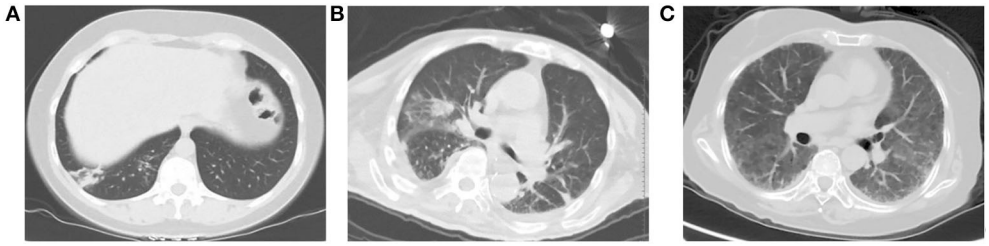

FIGURE 2 | Patients with abnormal CT findings. **(A)** A 43-year-old woman presenting with fever, cough and expectoration. An unenhanced chest CT image shows patchy consolidation in right lower lobe. **(B)** A 85-year-old man presenting with high fever, cough and short of breath. An unenhanced chest CT image shows multifocal areas of consolidation in the bilateral lungs. **(C)** A 73-year-old woman presenting with fever and short of breath. She was diagnosed with Sjogren's syndrome 6 months ago, and she was receiving steroid drugs. An unenhanced chest CT examination shows ground-glass opacity widely scattered across the lungs bilaterally.

39.5%, and leukopenia accounted for only 1.4%. More than half of the patients had elevated C-reactive protein levels.

In univariable analysis, patients with underlying diseases, cough and sputum, had a higher odds ratio of abnormal CT findings. In addition, fatigue, sore throat, advanced age, and elevated C reactive protein were also associated with abnormal chest CT findings (**Table 4**).

In the multivariable logistic regression model, all the variables of 144 patients were included. The results showed that advanced age and underlying disease were related to abnormal chest CT findings (**Table 4**).

DISCUSSION

As we known, ILI is a symptom complex which is a predictive tool for influenza infection. Compared with other clinical definitions, the commonly used definition of ILI is relatively broad to capture influenza-associated illness (22). It is often used as a key indicator for influenza surveillance. However, there is a paucity of data related to chest CT in influenza-like illness patients. We undertook this study to better understand the overall condition in the chest CT examination of ILI cases which may bring new knowledge to the area.

The current study analyzed the influenza-like cases that attended our hospital in the spring of 2020. The demographic characteristics, clinical symptoms, laboratory results, comorbidities, and chest CT imaging data of the enrolled patients were collected. The comparison of relevant indicators as well as logistic regression analysis were conducted on patients with normal chest CT and abnormal chest CT. In this study, the incidence of chest CT abnormalities, the main imaging features as well as the distribution pattern in influenza-like cases were analyzed. In addition, logistic regression analysis was used to identify risk factors associated with abnormal chest CT findings.

After conducting an epidemiological history as well as a survey of symptoms, signs, imaging, and differential diagnoses which included the use of nucleic acid test in accordance with the clinical diagnosis and treatment guidelines in China, COVID-19 infection was excluded in the enrolled cases. Therefore, the data reflected the relevant characteristics of non-COVID-19 influenza-like cases who attended hospital during this period.

In this study, 78.5% (427/544) of patients who presented with the main manifestations of respiratory symptoms underwent chest CT examination. According to our literature search, there have been no specific chest CT imaging studies on ILI cases, and to our knowledge, this study also enrolled the largest coverage of chest CT data in RTI patients (11, 13, 14). There were a few clinical reports about a varies of respiratory infection patients. However, in these studies, the proportion of patients with respiratory symptoms undergoing chest CT examinations did not exceed 20% and, furthermore, the patients were not randomized into groups (12, 16, 23–25). Whereas the limitations of previously research, this study conducted a comprehensive collection of chest CT data for patients with influenza-like symptoms and compared the difference of normal and abnormal chest CT cases. It may be noted that our research results can better reflect the overall characteristics of chest CT in ILI cases.

Among the 148 patients who met the diagnostic criteria for influenza-like cases, the proportion of chest CT abnormalities was 41.2%. Compared with the above-mentioned studies on lower respiratory tract infections involving specific pathogens, the proportion of chest CT positive cases was not high. However, after careful examination of these papers, it can be seen that the proportion of patients who underwent chest CT examination in the existing studies was too small, and thus they cannot reflect the general condition with regards to the group of influenza like illness patients (generally, clinicians only provided chest CT examinations when the condition was severe). Moreover, it can be seen from imaging analysis that abnormal chest CT phenomena in influenza-like cases is not uncommon. This can act as a reminder for clinicians that when treating patients with influenza-like symptoms, they should pay more attention to the changes in the pulmonary condition of patients, and chest CT examination should be performed more readily in high-risk groups.

In order to identify high-risk groups in influenza-like cases with abnormal lung imaging, logistic regression analysis was performed on the case data that were collected. From the results of univariable analysis, it can be seen that advanced age, underlying diseases, cough, sputum and increased CRP levels were risk factors for abnormal chest CT. Sore throat and

TABLE 3 | Symptom and laboratory results.

Symptom	Total ($n = 148$)	Normal ($n = 87$)	Abnormal ($n = 61$)	*P*-value
Body temperature	37.9 (37.6, 38.5)	37.9 (37.6, 38.4)	38.0 (37.6, 38.55)	0.26
Cough	86 (58.1%)	39 (44.8%)	47 (77.0%)	0.000
Sore throat	79 (53.7%)	58 (66.7%)	21 (34.4%)	0.000
Sputum	44 (29.7%)	18 (20.7%)	26 (42.6%)	0.004
Rhinocleisis	9 (6.1%)	6 (6.9%)	3 (4.9%)	0.620
Running nose	20 (13.5%)	15 (17.2%)	5 (8.2%)	0.113
Sneeze	5 (3.4%)	3 (3.4%)	2 (3.2%)	0.955
Headache	14 (9.5%)	11 (12.6%)	3 (4.9%)	0.114
Myalgia	16 (10.8%)	12 (13.8%)	4 (6.6%)	0.163
Fatigue	28 (18.9%)	22 (25.3%)	6 (9.8%)	0.018
Chill	17 (11.5%)	11 (12.6%)	6 (9.8%)	0.598
Diarrhea	3 (2.0%)	2 (2.3%)	1 (1.6%)	0.779
Vomiting	3 (2.0%)	2 (2.3%)	1 (1.6%)	0.779
Chest pain	4 (2.7%)	3 (3.4%)	1 (1.6%)	0.504
Shortness of breath	22 (14.9%)	9 (10.3%)	13 (21.3%)	0.065
Laboratory results				
Leucocyte[a]	8.23 (6.45, 11.52)	8.05 (6.65, 10.98)	8.32 (6.32, 11.92)	0.992
Neutrophil[a]	5.83 (4.12, 9.1)	5.79 (3.93, 8.91)	5.84 (4.27, 9.68)	0.67
Leukomonocyte[a]	1.37 (1.07, 1.96)	1.4 (1.10, 1.98)	1.29 (1.0, 1.93)	0.369
C reactive protein[b]	9.95 (1.33, 45.58)	5.4 (0.73, 27.15)	19.75 (4.23, 66.20)	0.005

Data are median (IQR), n (%), or n/N (%). p-values were calculated by Mann-Whitney U test or χ^2 test as appropriate. [a]One hundred forty seven patients underwent complete blood cell count tests. [b]One hundred forty four patients received C reactive protein examination.

TABLE 4 | Risk factors associated with chest CT findings.

	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Age	1.070	(1.046–1.094)	0.000	1.059	(1.034-1.085)	0.000
Body temperature	1.350	(0.837–2.178)	0.219			
Underling disease	48.513	(6.312–372.877)	0.000	9.379	(1.069–82.249)	0.043
Cough	4.132	(1.989–8.584)	0.000			
Sputum	2.848	(1.378–5.883)	0.005			
Sore throat	0.263	(0.132–0.524)	0.000			
Shortness of breath	2.347	(0.933–5.907)	0.070			
Rhinocleisis	0.698	(0.168–2.907)	0.622			
Running nose	0.429	(0.147–1.250)	0.121			
Sneeze	0.949	(0.154–5.857)	0.955			
Headache	0.357	(0.095–1.340)	0.127			
Myalgia	0.439	(0.134–1.431)	0.172			
Fatigue	0.322	(0.122–0.852)	0.022			
Chill	0.754	(0.263–2.161)	0.599			
Diarrhea	0.708	(0.063–7.991)	0.780			
Vomiting	0.708	(0.063–7.991)	0.780			
Chest pain	0.467	(0.047–4.596)	0.514			
Leukocytosis[a]	0.882	(0.45–1.729)	0.715			
Neutrophilia[b]	0.957	(0.495–1.851)	0.896			
Lymphopenia[c]	0.714	(0.344–1.485)	0.367			
Elevated CRP[d]	2.622	(1.283–5.357)	0.008			

[a]Leukocyte > 9.5 × 10^9/L; [b]neutrophil > 6.3 × 10^9/L; [c]Lymphocyte < 1.1 × 10^9/L; [d] C reactive protein > 5 mg/L.

symptoms of fatigue were associated with normal chest CT. With regards to an analysis of the cause, it may be that these two symptoms tend to appear in the early stages of disease onset, when the condition has not yet progressed to the lower respiratory tract.

After multivariable logistic regression analysis, it was found that advanced age and concurrent underlying diseases were independent risk factors for abnormal chest CT. These results are consistent with those of previous researchers.

A number of studies have suggested that as age increases, the body's immune function changes and the ability to resist pathogen invasion decreases. Saskia et al. found in animal experiments that the elderly macaques exhibited a higher level of systemic inflammatory response following coronavirus infection, but the synthesis and secretion of type I interferon β, which inhibits virus replication, was insufficient, which made it difficult to control the infection (26). It has also been found that the decline of immune function in the elderly is mainly featured by the gradual decrease of the naive CD8 T cell repertoire, and the decline in the ability of CD4 T cells to recognize foreign antigens and generate an immune response (27). As a result of the reduced capacity to resist pathogenic microorganisms such as viruses and bacteria, local infections easily spread to the whole body, leading to a rapid deterioration in the person's condition. This can also explain why the elderly are prone to developing lower respiratory tract infections following the occurrence of upper respiratory tract infections.

In addition, concurrent underlying disease is also a common risk factor of lung disease. Irene RC's research showed that the incidence of community-acquired pneumonia increased sharply with age, and was related to underlying conditions in the patient, particularly chronic obstructive pulmonary disease, asthma, chronic cardiovascular disease, impaired immune function, and neurological diseases (28). A number of recent meta-analyses of retrospective studies on COVID-19 confirmed that advanced age, COPD, hypertension, diabetes, cardiovascular disease and cerebrovascular disease are associated with a significantly increased risk of exacerbation in COVID-19 patients, among which the risk of disease progression in COPD patients is 5.9 times that of non-COPD patients (29, 30).

The results of the current study are also indicative of this feature. Among patients with pulmonary lesions, the incidence of underlying conditions was significantly higher than that of patients with normal lung imaging. However, due to the small number of single disease types within the comorbidities in this study (overall, <4%), single disease subgroup analysis was not performed.

Results of logistic regression analysis showed that the elderly patients with underlying diseases were more likely to develop lower respiratory tract inflammation following upper respiratory tract infection. This also provides a basis for clinicians to distinguish high-risk groups when receiving ILI cases. Therefore, particular attention needs to be paid to this group in clinical practice, and the timing of treatment also needs to be moved forward. However, these conjectures need to be demonstrated by prospective studies with larger sample sizes.

This study also conducted a corresponding analysis of abnormal chest CT findings between ILI cases and COVID-19 patients. According to a literature review, the imaging manifestations of lower respiratory tract infections caused by different pathogens are different. Taking influenza virus pneumonia as an example, the main abnormal CT characteristics are ground glass opacity and consolidation, as well as diffuse distribution in both lungs (16, 31, 32). Kim et al. found that patients with parainfluenza virus infection are more prone to tree-in-bud opacities and bronchial wall thickening (14). At the same time, such changes tend to be in the lower lung and distributed diffusely. Chest CT changes in patients with pneumonia caused by syncytial virus typically involve thickening of the bronchial wall, and lesions are mostly located in the upper and middle lung (16). In lower respiratory tract infections caused by bacteria, the most common chest CT abnormalities are consolidation and ground glass shadows, followed by bronchial wall thickening, and the proportion with a diffuse distribution of lesions is even higher than that of viral infections (16).

The imaging characteristics in this study were different from those in the above reports. Among the 61 cases with imaging changes, over half had chest CT consolidation, while the incidence of ground glass shadows was relatively low (22, 34.4%), and the lesions were more often located in the lower lungs and predominantly distributed in the pulmonary segments and lobes.

However, the performance of COVID-19 is different. In terms of CT features, COVID-19 is associated with ground glass lesions more often than consolidation, reaching as high as 98% (33). And, according to reports, the proportion of lung consolidation in the later stages of the disease gradually increases. With regards to distribution characteristics, the lesions are mostly located under the pleura or along the bronchovascular bundle (34–37).

In addition, according to the literature, the incidence of chest thickening and pleural effusion in non-COVID-19 patients is significantly higher than that of COVID-19 patients (36). However, there are inconsistent views on this issue in different literatures. Some scholars have found that pleural thickening but not accompanied by pleural effusion was often seen in chest CT of COVID-19 patients (38). In our study, the incidence of pleural effusion was low (7, 11.5%), and no pleural thickening was observed. The results suggest that, based only on the evidence of pleural effusion or pleural thickening can not effectively distinguish the two COVID-19 and non-COVID-19 cases, so further research is needed.

Once again, multiple studies have found that the halo and reverse halo signs often appear in COVID-19 patients, but these two signs are rarely seen in non-COVID-19 patients (36, 39–41). However, in the cases enrolled in this study, no halo sign and reverse halo sign was seen in the abnormal signs of chest CT. This result is consistent with previous reports. This means that the appearance of these two signs may indicate that patients are more likely to have COVID-19 infection, which can be used for the differential diagnosis of COVID-19.

Due to limited conditions, it was difficult to obtain respiratory tract samples from patients who attended the emergency

department at our hospital, particularly samples of lower respiratory tract secretions, for nucleic acid testing and pathogen isolation and culture. Therefore, in this study, we did not have a pathogenic basis for performing an analysis of chest CT features based on etiology. However, it can still be seen from the current data that there are still significant differences in chest CT features between influenza-like patients without COVID-19 and patients with COVID-19 infection. CT can thus be used as a rapid screening tool for patients with suspected COVID-19.

There are limitations to this study. First of all, this was a single-center observational study which may not be representative of the general population. Secondly, it needs to be emphasized that influenza-like symptoms are not always caused by viral infections, and other pathogens such as bacteria and mycoplasma as well as non-infectious factors must necessarily be included. However, current data suggest that respiratory viruses are still the most important pathogenic microorganisms in this patient group (42). Moreover, when clinicians are conducting their day-to-day work, pathogen identification is not necessarily the main factor that influences the direction of diagnosis and treatment. Therefore, our results are still helpful for clinicians in their daily work. They can act as a reminder for doctors to focus more on the chest imaging of patients who are elderly and have concurrent underlying conditions. Thirdly, this study was conducted during the novel coronavirus epidemic and, due to precautionary considerations, patients with mild illness were more likely to choose to isolate at home and attend the hospital only when symptoms were more significant. This may have led to an overestimation of the occurrence of abnormal imaging in influenza-like cases. In addition, there were no patients with COVID-19 infection in this study, so direct comparisons could not be made. To overcome these limitations, we plan to conduct multi-center clinical study to address in the next phase.

Despite these limitations, we were able to comprehensively examine the chest CT features of ILI in this study. At the same time, we also believe that this work can prompt physicians to increase the attention they pay to non-COVID-19 ILI cases in clinical practice.

CONCLUSIONS

1. A relatively high proportion of patients with influenza-like illness have chest CT abnormalities.
2. In influenza-like cases, advanced age and concurrent underlying diseases are related to abnormal chest CT findings, indicating that the risk of lower respiratory tract infection is higher in this patient group.
3. At present, the abnormal chest CT manifestations of influenza-like cases in Beijing urban areas are mainly lung consolidation, followed by ground glass lesions. Distinctive differences exist with regards to the distribution characteristics of imaging abnormalities compared to those associated with COVID-19 infection, which is helpful for differential diagnosis.

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 Ethics Commission of the Sixth Medical Center of the PLA General Hospital. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

WS, XC, YS, and DL conceived the study and participated in its design and coordination of the research. WS, XC, and YS contributed to the interpretation of the results. WS and WM developed, modeled, and performed evaluations and statistical analysis. WL and QL provided the radiological analyses and drew the CT conclusion. All authors have contributed to the drafting of the manuscript and have read and approved the final version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.616963/full#supplementary-material

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CASE REPORT
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Case Report: Intrapulmonary Arteriovenous Anastomoses in COVID-19-Related Pulmonary Vascular Changes: A New Player in the Arena?

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Up to now, COVID-19-related vascular changes were mainly described as thrombo-embolic events. A handful of researchers reported another type of vascular abnormality referred to as "vascular thickening" or "vascular enlargement," without specifying whether the dilated vessels are arteries or veins nor providing a physiopathological hypothesis. Our observations indicate that the vascular dilatation occurs in the venous compartment, and underlying mechanisms might include increased blood flow due to inflammation and the activation of arteriovenous anastomoses.

Keywords: COVID-19, computed tomography, perfusion, pulmonary embolism, arteriovenous anastomoses, respiratory failure

INTRODUCTION

Early in the coronavirus disease 2019 (COVID-19) pandemic, a high prevalence of vascular disorders has been reported (1). Such abnormalities were mainly described in the lung and covered a broad spectrum of patterns revealed at histology–including microangiopathy, intussusceptive angiogenesis, and microthrombosis–and at imaging with vessel dilatation, tortuosity, thrombosis, and perfusion abnormalities. Up to now, no convincing theory has helped understand the relationship between virus-induced inflammatory disorders and biological and morphological changes, especially those observed on computed tomography (CT). Furthermore, the refractory hypoxemia observed in COVID-19 patients appears to be driven by more complex processes than alveolar damage with low gas exchange alone because COVID-19 leads to severe respiratory failure despite relatively well-preserved lung gas volume (2). This suggests the contribution of vascular phenomena beyond a simple ventilation–perfusion mismatch.

VASCULAR CHANGES IN COVID-19 PNEUMONIA

Imaging-based morphological vascular abnormalities in the lung described at CT may be categorized into three groups: thromboembolic events (3), vascular dilatation, also known as vascular "thickening" or "engorgement" (4, 5), and perfusional changes (6). Mechanisms leading to vascular remodeling remain unclear, and their prevalence and distribution are a matter of debate. We analyzed CT data from a patient who presented all the three groups of abnormalities

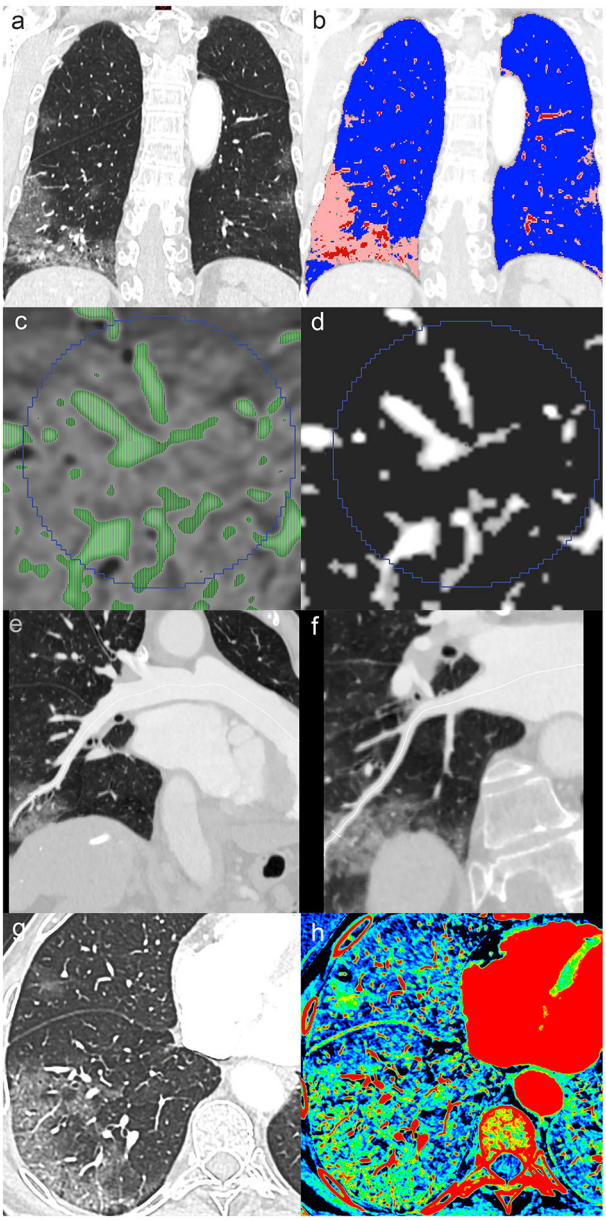

FIGURE 1 | Contrast-enhanced chest CT in a patient admitted for COVID-19. Coronal reformatted image **(a)** shows peripheral ground-glass opacity (GGO) predominantly involving the right lower lobe. Tissue classification **(b)** distinguishing alveolar opacity (peach color code) from normal parenchyma (blue) and vascular components (red) visually indicates vascular enlargement in COVID-19 pneumonia. Specific thresholds to isolate voxels, mostly containing vascular elements **(c,d)**, enable vascular volume extraction in regions-of-interest. Center-line reconstructions of the right posterior basal artery and vein **(e,f)** allow diameter measurement in GGO. Axial conventional image in lung window **(g)** with peripheral COVID-19-related GGO and corresponding dual-energy CT iodine density map **(h)** show increased iodine distribution in GGO consistent with hyperperfusion.

simultaneously and thoroughly assessed vascular findings, trying to understand the different groups' relationships and elucidate the underlying mechanisms.

A man in his 70's with fever, tachypnea, bilateral basal crackling sounds, and reverse transcription-PCR (RT-PCR)-proven severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection underwent dual-energy CT pulmonary angiography to rule out pulmonary embolism. Arterial PaO_2 was 64 mmHg, and SpO_2 was 92% on room air. The examination was carried out on a fast kV-switching dual-energy CT platform (Revolution CT, GE Healthcare), with the following parameters: rotation speed, 0.5 s; tube load, 180 mAs; reconstructed slice thickness, 1.25 mm; and section interval, 1 mm. Using a power injector, 50 ml of iodinated contrast material (Accupaque 300®) was injected through an 18G venous catheter in the right antecubital fossa at a rate of 4 ml/s and followed by a saline chaser. Findings included zones of COVID-19 ground-glass opacity (GGO) surrounded by healthy parenchyma, enlarged blood vessels within GGO, and acute pulmonary embolism in a lung segment without GGO. We applied automatic tissue classification to distinguish alveolar opacity, normal parenchyma, and vascular components (**Figures 1a,b**). In a second step, we used a threshold-based automatic segmentation to extract the (macroscopic) intravascular blood volume in a region-of-interest (ROI) in both normal parenchyma and GGO (**Figures 1c,d**). Calculated intravascular blood volumes showed that in the areas presenting with typical parenchymal changes, the vascular volume was increased by 40% (5.27/300 and 9.0/300 cm^3 vessel-to-tissue ratio in healthy and GGO zones, respectively). Of note, no venous thrombosis was seen. Furthermore, we demonstrate that the increased volume primarily depended on venous dilatation in the involved lung areas (**Figures 1e,f**). Arterial and venous diameters at a sub-segmental level in GGO were 3.6 and 4.9 mm, respectively, whereas in the healthy contralateral posterior basal segment, diameters were 3.0 and 3.1 mm, respectively. The corresponding artery-to-vein ratios are 0.97 (3.0/3.1) in healthy parenchyma and 0.73 (3.6/4.9) in GGO, and the vein-to-vein ratio (GGO vs. healthy segment) was 1.58 (4.9/3.1), indicating marked venous enlargement in COVID-19-related GGO. Note that the artery-to-artery ratio (GGO vs. healthy segment) was 1.2 (3.6/3), indicating a moderate arterial dilatation in GGO consistent with hyperemia.

DISCUSSION

Inflammation-mediated hyperemia is unlikely to be the only factor causing such a marked venous dilatation. We hypothesized that the upregulation of nitric oxide synthase, causing the activation of physiological arteriovenous anastomoses (7, 8) in the involved parenchyma, might explain venous engorgement; these anastomoses create a right-to-left shunt. The existence of pulmonary arteriovenous anastomoses has been suggested and studied by Tobin et al. since the 1950's, and their anatomical location was described as "at the apex of and within the lobular divisions of the lung" (9, 10). Available data suggest that such anastomoses can be activated passively by exercise or supine position, but also actively in the setting of vascular redistribution under both hyperoxia and hypoxia (11). In COVID-19 pneumonia, the consequence of combined

mechanisms is exacerbated hypoxia, giving a better explanation for the discrepancies between the relatively preserved ventilation mechanics, the severity of respiratory failure, and the limited response to invasive ventilation (2). Other injuries, such as endotheliitis (12) and/or distal microthrombosis (13), might potentialize the dysregulation of intrapulmonary arteriovenous anastomoses and the resulting shunting effect. In the case we discuss here, transthoracic saline echocardiography would have been a simple and effective means of evaluating the presence of intrapulmonary anastomoses and should have been performed if possible (14). Furthermore, recent evidence suggests that the recruitment of intrapulmonary arteriovenous anastomoses may be driven by the combination of increased cardiac output and increased pulmonary vascular pressure (8). Unfortunately, we could not provide a meaningful estimation of cardiac output based on the available data.

The observed phenomenon is consistent with previously described increased parenchymal perfusion in COVID-19 GGO with dual-energy CT (6). Likewise, our patient exhibited hyperperfusion in GGO zones on iodine density maps (**Figures 1g,h**). This distal hyperperfusion is attributed to hyperemia induced by the inflammation cascade in COVID-19 pneumonia.

It is also interesting to note that macro-thromboembolic changes (pulmonary embolism) were observed in a different territory than those with parenchymal involvement. This might be another consequence of the vascular shunting effect. This finding is also in agreement with a previous report (15).

In conclusion, our observations indicate that COVID-19-related macroscopic vascular changes depicted *in vivo* are not exclusively due to thromboembolic events. The observed vascular remodeling is mainly related to venous dilatation and might result from combined inflammation-induced hyperemia and dysregulation of arteriovenous anastomoses. Although difficult to establish *in vivo*, vascular shunts could explain the worse than expected clinical course given a relatively modest parenchymal involvement and no visible local thromboembolism. Further studies aiming to characterize those abnormalities in a large series, particularly their distribution and correlation to clinical findings, are needed. The core message of this letter is to frame the hypothesis of intrapulmonary arteriovenous anastomoses as an influencing factor in patients with COVID-19 pneumonia, and the images presented in this case study serve for illustration purposes. An ongoing investigation, the Swiss National Registry COVID-CAVA, is expected to provide relevant insights to address those crucial questions better.

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

SQ performed the literature research and built the concept. DR prepared the figure. All authors were involved in drafting the manuscript and revising it critically.

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Adverse Outcomes Associated With Corticosteroid Use in Critical COVID-19: A Retrospective Multicenter Cohort Study

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Corticosteroid is commonly used to reduce damage from inflammatory reactions in coronavirus disease 2019 (COVID-19). We aim to determine the outcomes of corticosteroid use in critically ill COVID-19 patients. Ninety six critically ill patients, hospitalized in 14 hospitals outside Wuhan from January 16 to March 30, 2020 were enrolled in this study. Among 96 critical patients, 68 were treated with corticosteroid (CS group), while 28 were not treated with corticosteroids (non-CS group). Multivariable logistic regression were performed to determine the possible correlation between corticosteroid use and the treatment outcomes. Forty-six (68%) patients in the CS group died compared to six (21%) of the non-CS group. Corticosteroid use was also associated with the development of ARDS, exacerbation of pulmonary fibrosis, longer hospital stay and virus clearance time. On admission, no difference in laboratory findings between the CS and the non-CS group was observed. After corticosteroid treatment, patients treated with corticosteroids were associated with higher counts of white blood cells, neutrophils, neutrophil-to-lymphocyte ratio, alanine aminotransferase level and Sequential Organ Failure Assessment score. In conclusion, corticosteroid use in critically ill COVID-19 patients was associated with a much higher case fatality rate. Frequent incidence of liver injury and multi-organ failure in corticosteroid treated patients may have contributed to the adverse outcomes. The multi-organ failure is likely caused by more persistent SARS-CoV-2 infection and higher viral load, due to the inhibition of immune surveillance by corticosteroid.

Keywords: SARS-CoV-2, mortality, critical, gluocorticoid, steroid, COVID-19, ARDS, cytokine storm

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INTRODUCTION

The pandemic of Coronavirus Disease 2019 (COVID-19) has caused many deaths, and no therapy with proven efficacy is available. The viral pathogen of COVID-19 is severe acute respiratory syndrome-related coronavirus-2 (SARS-CoV-2), which, together with SARS-CoV, belongs to the species of SARSr-CoV (Severe acute respiratory syndrome-related coronavirus) (1). Both viruses are closely related to MERS-CoV, which causes Middle East respiratory syndrome (MERS).

Study with SARS patients indicated that immune-mediated mechanism rather than virus induced damage drives the clinical progression (2). Consistently, study with macaques indicated that severe symptoms of SARS infection were caused by elevated immune reactions rather than higher viral load, and the anti-inflammatory therapy with type I interferon reduced the respiratory symptoms (3). Similar to SARS and MERS (4), high levels of proinflammatory cytokines and chemokines, the cytokine storm, were observed in the peripheral blood of COVID-19 patients (5). Based on the pathological findings including significantly increased serum level of cytokines, and over-activation of T cells in severe COVID-19 patients, Xu et al. recommended to treat COVID-19 with corticosteroid to control immune reactions (6). Corticosteroid has been commonly administered to COVID-19 patients in (7, 8) and outside China (9).

However, the application of corticosteroids in coronavirus infection has consequences. The use of corticosteroids in SARS patients was associated with serious complications including avascular necrosis, diabetes and psychosis (10). Corticosteroid use also led to delayed viral RNA clearance in SARS (11) and MERS (12). In addition, suppression of the patients' immune system with corticosteroids leads to secondary infection, as observed in clinical trials of septic shock (13) and respiratory failure (14). To understand the beneficial and adverse effects of corticosteroid use in COVID-19, we reviewed the medical records of critical COVID-19 patients from 14 hospitals and found that the use of corticosteroid is associated with more severe respiratory symptoms and a much higher case fatality rate.

METHODS

Study Design and Participants

We reviewed 410 patient charts of suspected COVID-19. Excluding 56 records for negative SARS-CoV-2 nucleic acid test, 24 for duplicated records, and 128 for the lack of relevant data, we identified 202 SARS-CoV-2 RNA positive patients hospitalized at 14 hospitals (**Figure 1**) in Jingzhou, China, from January 16 to March 30, 2020. Our COVID-19 patients were admitted to the hospitals because of fever, cough, dyspnea and chest computed tomography (CT) findings indicating SARS-CoV-2 pneumonia. Diagnosis of COVID-19 was based on positive SARS-CoV-2 nucleic acid test. Ninety-six critically ill patients were enrolled in this study. Critically ill patients were defined as those admitted to the ICU, requiring mechanical ventilation, or had a fraction of inspired oxygen (FiO2) of at least 60% (15, 16).

Treatment of the infection followed the fifth edition of the Diagnosis and Treatment Guideline for COVID-19, National Health Commission of the People's Republic of China. Besides oxygen supplementation and respiratory support, patients were routinely given antibiotics, usually Moxifloxacin, and antivirus drugs, usually Lopinavir and Ritonavir. Mechanical ventilation was conducted when hypoxemia and dyspnea persisted despite non-invasive oxygen supplementation.

The use of corticosteroid in COVID-19 patients is controversial, and this is reflected in the fifth edition of the Diagnosis and Treatment Guideline for COVID-19, National Health Commission of the People's Republic of China, which recommended optional use of corticosteroid when evidence presented for deteriorating (or persistent) dyspnea or chest CT results. Corticosteroid [at a dose not to exceed the equivalent of 1∼2 mg methylprednisolone /kg/day, and should be used for a short period of time (3 to 5 days)] was prescribed according to physicians' preference. Sixty-eight of the critically ill patients were treated with corticosteroids. We compared the demographics, symptoms, treatments and outcomes between critical COVID-19 patients treated with corticosteroids (CS group) and those not treated with corticosteroids (non-CS group).

This study was approved by the Institutional Review Boards of Sun Yat-sen University, and participating hospitals. Informed consent was waived for this retrospective chart review.

Data Collection

Charts were reviewed for demographic, clinical, laboratory, treatment and outcome data. Demographic data included age, gender, and comorbidities including hypertension, diabetes, cardiovascular disease, chronic obstructive pulmonary disease, chronic liver disease and malignancy. Clinical data recorded included vital signs such as temperature, respiratory rate, blood pressure, and oxygen saturation. In addition to fever, cough and dyspnea, other clinical characteristics recorded included sputum production, diarrhea (three or more loose or liquid stools per day), bloody stool (stool positive for occult blood test or white blood cell test), myalgia/muscle fatigue and haemoptysis. Laboratory data included blood cell counts (white blood cell, lymphocyte, neutrophil, monocyte, and platelet), markers for coagulation function (activated partial thromboplastin time (APTT), d-dimer and fibrinogen), infection-related biomarkers (C-reactive protein (CRP), Erythrocyte sedimentation rate (ESR), neutrophil-to-lymphocyte ratio (NLR) and procalcitonin), and other blood biochemistry measurements (aspartate transaminase (AST), alanine aminotransferase (ALT), creatine kinase (CK), creatinine, blood urea nitrogen (BUN), lactate dehydrogenase (LDH), total bilirubin and Sequential Organ Failure Assessment (SOFA) score). Cytokines were not measured for most patients.

The hospital course was reviewed for treatments and severity of disease. The need for supplemental oxygen, respiratory support and admission to the intensive care unit (ICU) were recorded.

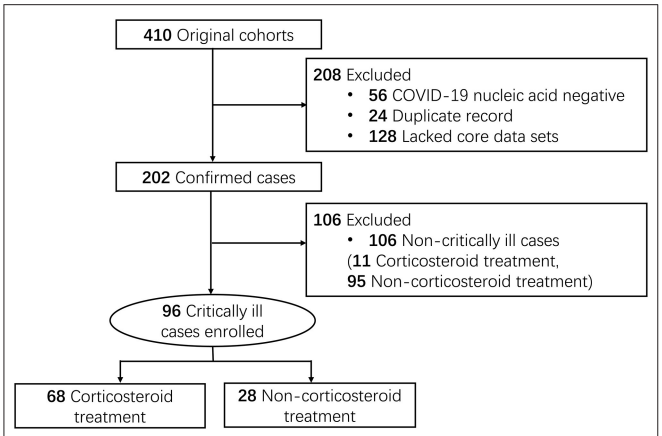

FIGURE 1 | Study flow diagram. Medical records of COVID-19 patients (106 non-critically ill and 96 critically ill) included for the study of the corticosteroid effects were accessed from Jingzhou Hospital of Traditional Chinese Medicine (61 cases), Jianli Hospital of Traditional Chinese Medicine (44), Jingzhou First Hospital (9 cases), Jingzhou Central Hospital (29 cases), Jingzhou First People's Hospital (11 cases), Jingzhou Chest Hospital (9 cases), Gongan People's Hospital (8 cases), Honghu People's Hospital (10 cases), Honghu Hospital for the Control of Schistosomiasis (3 cases), Honghu Central Hospital (2 cases), Jianli People's Hospital (3 cases), Jiangling People's Hospital (2 cases), Shishou Hospital of Traditional Chinese Medicine (5 cases), and Songzi People's Hospital (6 cases).

Statistics

SPSS (Statistical Package for the Social Sciences) version 22.0 software (SPSS Inc.) was used for Student *t* test, Mann-Whitney *U* test, Chi-Square test, Fisher's exact test, multivariable Cox regression analysis, univariable and multivariable logistic regressions. Significance of the differences between two study groups were tested by Student *t* test or Mann-Whitney *U* test for numerical data, when appropriate. For multiple comparisons, *p*-values were adjusted by FDR method in R. Comparisons of categorical data were performed by Chi-Square test or Fisher's exact test, as appropriate. Age was transformed to categorical variable at the threshold of 60. Laboratory findings before discharge were transformed to categorical variables based on reference values. Multivariable logistic regressions were performed to identify clinical features and treatments associated with case fatality and corticosteroid treatment, adjusted for multiple potential confounders identified from univariable regressions. All statistical tests were two sided, with *p*-values of <0.05 considered to be statistically significant.

RESULTS

Demographics, Clinical Characteristics and the Treatments of the Critical COVID-19 Patients

A large proportion (68 of 96, 71%) of the critically ill COVID-19 patients were treated with corticosteroids, compared to 10% (11 of 106) of the non-critical patients (**Figure 1**). Therefore, this study only concerned the critical patients, among which 68 patients were treated with corticosteroids (CS group) while 28 patients were not treated with corticosteroid (non-CS group). Fifty-three patients (78%) in the CS group were 60 or older compared to 14 (50%) in the non-CS group (**Table 1**). No significant difference in gender ratio was found between the two groups.

On admission, the most common symptoms for both groups were fever (87% in the CS group), cough (74%, CS), and dyspnea (46%, CS), while less common symptoms included sputum production (32%, CS), myalgia (26%, CS), diarrhea (18%, CS), cephalalgia (15%, CS), and hemoptysis (1%, CS). No difference in the incidences of these symptoms was observed between the CS group and the non-CS group (**Table 1**).

The most frequently recorded comorbidities for both groups of patients included hypertension (47%, CS), diabetes (16%, CS), and cardiovascular disease (10%, CS). No difference in the incidence of any comorbidity between the CS group and the non-CS group was observed (**Table 1**).

Following the diagnosis and treatment guideline of the National Health Commission (China), the most common treatments included supplemental oxygen (100%, CS), antiviral treatments (90%, CS), and antibiotics (87%, CS). No difference was observed for these treatments between the CS and the non-CS groups (**Table 1**). The two groups were also similarly treated with thymosin α1, mechanical ventilation and ECMO. It is noteworthy that some patients in the CS group were treated with interferon (25%), immunoglobulin (29%) and albumin (7%), which were not used for patients in the non-CS group (**Table 1**).

Outcomes of the Patients Treated With or Without Corticosteroids

Forty-six of the 68 (68%) patients in the CS group died compared to six of the 28 (21%) in the non-CS group (**Table 2**). After adjusting for potential confounding factors including age, gender, hypertension, interferon, immunoglobulin, and thymosin α1, corticosteroids treatment was identified as a risk factor for case fatality in critically ill COVID-19 with an adjusted OR of 4.05 (95%CI: 1.37–11.98, $p = 0.012$, $R^2 = 0.413$) (**Table 3**). We used the threshold of age$>=$60 in the multivariable logistic regression model because of the dichotomized distribution of the age of deceased patients (**Supplementary Figure 1**): out of the 52 deceased patients, 40 were 60 years old or older. This pattern is consistent with the reports that age >60 was a risk factor for critical illness in COVID-19 (17, 18). Similar result was obtained with survival analysis: after adjusting for the effects of age and other confounding factors, mutivariable analysis with the Cox proportional-hazards model indicated a lower survival rate in the CS group (HR 4.52 [95%CI: 1.79–11.41], $p = 0.001$, **Supplementary Table 1**). In line with higher case fatality rate, more of the patients treated with corticosteroids presented ARDS (CS vs. non-CS: 63 vs. 36%) and exacerbation of pulmonary fibrosis (CS vs. non-CS: 56 vs. 21%) than patients not treated with corticosteroids (**Table 2**). A higher proportion of the CS group required mechanical ventilation than the non-CS group, but statistical significance was not achieved. Among the surviving patients, the length of hospital stay was higher in the CS group [24

TABLE 1 | Demographics and clinical characteristics of critical COVID-19 patients on admission.

	Corticosteroid treatment ($n = 68$)	Non-corticosteroid treatment ($n = 28$)	*P*-value
Age, years			
≥60	53 (78%)	14 (50%)	0.063
Sex			
Female	30 (44%)	10 (36%)	1.000
Male	38 (56%)	18 (64%)	
Signs and symptoms			
Fever (≥37.3°C)	59 (87%)	21 (75%)	0.594
Cough	50 (74%)	21 (75%)	1.000
Dyspnea	31 (46%)	12 (43%)	1.000
Sputum	22 (32%)	6 (21%)	0.852
Myalgia	18 (26%)	2 (7%)	0.439
Diarrhea	12 (18%)	3 (11%)	1.000
Cephalalgia	10 (15%)	2 (7%)	1.000
Hemoptysis	1 (1%)	1 (4%)	1.000
Comorbidities			
Hypertension	32 (47%)	12 (43%)	1.000
Diabetes	11 (16%)	5 (18%)	1.000
Digestive tract disease	6 (9%)	3 (11%)	1.000
Cardiovascular disease	7 (10%)	2 (7%)	1.000
Cerebrovascular disease	4 (6%)	1 (4%)	1.000
Malignancy	2 (4%)	1 (4%)	1.000
Liver disease	3 (4%)	1 (4%)	1.000
Chronic lung disease	6 (9%)	0	0.594
Treatments			
Antiviral treatment	61 (90%)	25 (89%)	1.000
Interferon	17 (25%)	0	**0.027**
Antibiotics	59 (87%)	26 (93%)	1.000
Immunoglobulin	20 (29%)	0	**0.027**
Albumin	5 (7%)	0	0.856
Thymosin α1	7 (10%)	2 (7%)	1.000
Supplemental oxygen	68 (100%)	26 (93%)	0.448
Mechanical ventilation	36 (53%)	10 (36%)	0.563
ECMO	1 (1%)	2 (7%)	1.000

Data are n (%). P-values comparing corticosteroid treatment and non-corticosteroid treatment are from Chi-Square test or Fisher's exact test, as appropriate, and adjusted for multiple comparisons by FDR method. COVID-19, coronavirus Disease 2019; ECMO, extracorporeal membrane oxygenation. The bold P-values indicate $P < 0.05$.

(IQR: 18.3–38.8) days] than in the non-CS group [17 (13.8–25.3) days]. The length of virus clearance time was also longer in the CS group [21 (16.8–26.3) days] than in the non-CS group [13.5 (12–16) days].

Laboratory Findings

For better understanding of the elevated case fatality in the CS group, we reviewed the laboratory findings for blood cell counts, markers for coagulation function, inflammatory biomarkers and the markers for cellular, tissue and organ damage.

On admission, no difference in laboratory findings was observed between the CS and the non-CS groups (**Table 4**). Decreased lymphocyte counts in both groups of critical patients reflected the inflammatory characteristics of these patients. Other outstanding observations included the elevated CRP, ESR, and LDH in both groups (**Table 4**), reflecting viral infections, inflammatory reactions and tissue damage.

After corticosteroid treatments, patients treated with corticosteroids were associated with higher counts of white blood cells, neutrophils, and neutrophil-to-lymphocyte ratio (**Table 5**). The differences in the counts of white blood cells remained significant after adjustment for confounding factors (**Table 6**).

No difference was observed for coagulation function markers APTT, fibrinogen or d-dimer (**Table 5**). Serum markers for inflammation CRP and ESR were elevated in both the CS and the non-CS groups, but not different between the study groups.

Regarding markers for tissue and organ damage, multivariate logistic regression revealed higher incidence of elevated ALT and SOFA score > 10 in the CS group (**Table 6**). Levels of LDH, BUN, AST, and total bilirubin were elevated in both the CS and the non-CS group, but not different between the groups (**Table 5**).

DISCUSSION

Our data showed that critical COVID-19 patients treated with corticosteroid had a much higher case fatality rate than those not treated with corticosteroid. This is in line with our observations that increased incidences of ARDS and exacerbation of pulmonary fibrosis, longer hospital stay and virus clearance time were associated with the use of corticosteroid in critical COVID-19 patients. Further, laboratory results provided evidence for increased incidence of tissue damage in corticosteroid users.

One immediate question is which came first, more severe symptoms or the use of corticosteroids? Use of corticosteroids was likely the lead because patients in the two study groups were both critically ill, with similar symptoms and laboratory results on admission. One possible argument is that patients in the CS group were perceived sicker by the physicians, because these patients were significantly older, and many patients in the CS group were treated with interferon, immunoglobulin and albumin, which were not prescribed for the patients in the non-CS group. To address this concern, the influences of age, possible more severe symptoms represented by the use of interferon and immunoglobulin, as well as other confounding factors, were adjusted in the multivariate logistic regression, resulted in an OR of 4.05 (1.37–11.98) for case fatality, indicating that corticosteroid use caused a much higher case fatality in critical patients.

In our hospitals, corticosteroids were usually used in the second week of disease onset when severe symptoms presented. In theory, this is a good timing for the suppression of inflammatory reactions that cause damage in ARDS and viral pneumonia (19). However, the beneficial outcomes seen with SARS patients and other types of pneumonia did not occur with our COVID-19 patients. Instead, we observed higher

TABLE 2 | Outcomes of critical COVID-19 patients on admission.

Outcomes	Corticosteroid treatment ($n = 68$)	Non-corticosteroid treatment ($n = 28$)	*P*-value
ARDS	43 (63%)	10 (36%)	**0.022**
Mechanical ventilation	36 (53%)	10 (36%)	0.143
ICU admission	45 (66%)	21 (75%)	0.397
Case fatality	46 (68%)	6 (21%)	**0.001**
Exacerbation of pulmonary fibrosis	38 (56%)	6 (21%)	**0.005**
SOFA	10.50 (5.25–13.00)	5.00 (4.00–7.00)	**0.006**
Length of hospital stay (survival), days	24.0 (18.3–38.8)	17.0 (13.8–25.3)	**0.041**
Length of virus clearance time (survival), days	21.0 (16.8–26.3)	13.5 (12.0–16.0)	**0.004**

Data are median (IQR) or n (%). P-values comparing corticosteroid treatment and non-corticosteroid treatment are from Mann-Whitney U test, Chi-Square test, or Fisher's exact test, as appropriate, and adjusted for multiple comparisons by FDR method. ARDS, acute respiratory distress syndrome; ICU, intensive care unit; SOFA, sequential organ failure assessment. The bold P-values indicate P < 0.05.

TABLE 3 | Logistic univariate and multivariate regression analyses on the association between the case fatality rate and the clinical features.

	Univariable OR (95% CI)	*P*-value	Multivariable OR (95%CI)	*P*-value
Age ≥ 60years	2.10 (0.87–5.09)	0.101	0.73 (0.21–2.58)	0.630
Sex	0.79 (0.35–1.80)	0.580	0.82 (0.29–2.36)	0.711
Comorbidities				
Hypertension	2.44 (1.06–5.59)	**0.035**	3.29 (1.08–10.00)	**0.036**
Diabetes	1.51 (0.50–4.54)	0.466		
Treatments				
Antiviral	1.21 (0.33–4.47)	0.780		
Interferon	8.51 (1.83–39.72)	**0.006**	2.67 (0.37–19.15)	0.328
Antibiotics	0.98 (0.28–3.47)	0.979		
Corticosteroids	7.67 (2.72–21.60)	**<0.001**	4.05 (1.37–11.98)	**0.012**
Intravenous immunoglobulin	6.64 (1.80–24.54)	**0.005**	3.54 (0.54–23.13)	0.187
Albumin	1.29 (0.21–8.06)	0.788		
Thymosin α1	0.09 (0.01–0.74)	**0.025**	0.025 (0.00–0.36)	**0.007**

OR, odds ratio; CI, confidence interval; ARDS, acute respiratory distress syndrome; SOFA, sequential organ failure assessment. Multivariable logistic regression were adjusted for age, sex, hypertension, interferon, corticosteroids, intravenous immunoglobulin, and thymosin α1. The bold P-values indicate P < 0.05.

case fatality rate and other adverse events in critical patients treated with corticosteroids. Although pulmonary dysfunction was common in our patients treated with corticosteroids, elevated SOFA scores of these patients indicated that multi-organ failure often contributed to corticosteroid related death. In support of this, blood biochemistry revealed frequent incidence of liver injury in corticosteroid treated patients. The multi-organ failure was likely caused by more persistent viral infection and higher viral load, which could be a consequence of the inhibition of the body's immune surveillance in corticosteroid treated patients. Our retrospective study is very limited on relevant data for the evaluation of the corticosteroid effects on patient immunity and subsequent infection risk (viral load, cytokine levels, etc). Available data related to immunity and subsequent infection risk include blood cell counts, inflammation markers and viral clearance time. Before discharge, white blood cell count, lymphocyte count, and NLR were higher in the corticosteroid group, a reflection of being recovered from viral infection. As it is well-established that corticosteroid reduces inflammatory activities in viral pneumonia (19), our data support that, patients' immunity was suppressed by short-term corticosteroid treatment, which may lead to increased viral replication, extended viral clearance, and secondary infections, and therefore subsequent inflammatory reactions in multiple organs. In addition, corticosteroid treatment may directly affect SARS-CoV-2 replication, similarly as it increases viral replication of rhinovirus and influenza A virus through reduced expression of innate anti-viral genes (20). Although data is not available in our patients, known adverse effects of corticosteroids include pancreatitis (21), heart failure, ischemic heart disease (22), and myopathy (23), which may contribute to the elevated SOFA score in corticosteroid treated patients.

Our observations are different from some of the published studies. Zha et al. examined the outcome of 11 mild COVID-19 patients treated with corticosteroid (24). Compared with patients not treated with corticosteroid, the treated patients exhibited no significant difference in every outcome measures, likely due to the small sample size. However, it is noteworthy that the

TABLE 4 | Laboratory findings of critical COVID-19 patients on admission.

	Reference values	Corticosteroid treatment ($n = 68$)	Non-corticosteroid treatment ($n = 28$)	*P*-value
White blood cell count ($\times 10^9$/L)	4.00–10.00	7.79 (5.12–10.95)	7.94 (5.29–11.93)	0.763
Lymphocyte count ($\times 10^9$/L)	1.50–4.00	0.81 (0.61–1.13)	1.01 (0.75–1.59)	0.551
Neutrophil count ($\times 10^9$/L)	2.00–7.00	6.25 (3.55–8.88)	5.72 (3.31–8.99)	0.856
NLR	0.78–3.53	7.05 (4.26–11.71)	3.93 (2.22–9.87)	0.856
Monocyte count ($\times 10^9$/L)	0.12–1.00	0.35 (0.21–0.53)	0.45 (0.26–0.73)	0.551
Platelet count ($\times 10^9$/L)	99.00–303.00	187.00 (157.75–257.00)	244.00 (167.75–285.75)	0.763
APTT(s)	21.00–37.00	29.13 (24.33–35.63)	34.64 (27.50–37.45)	0.551
fibrinogen (g/L)	2.00–4.00	3.46 (2.76–4.47)	3.38 (2.32–4.84)	0.856
D-dimer (μg/ml)	0.00–0.55	0.57 (0.36–1.15)	0.55 (0.34–1.26)	0.856
Procalcitonin (ng/ml)	0.00–0.50	0.26 (0.15–0.44)	0.35 (0.23–0.42)	0.881
CRP(mg/L)	0.00–10.00	24.45 (9.78–100.19)	27.70 (8.97–70.47)	0.763
ESR (mm/1 h)	0.00–30.00	36.00 (26.15–66.50)	32.00 (26.00–49.00)	0.763
Creatine kinase (U/L)	25.00–200.00	150.00 (64.00–175.50)	166.50 (85.90–408.75)	0.551
Lactate dehydrogenase (U/L)	91.00–230.00	230.00 (171.15–350.40)	234.40 (191.00–323.50)	0.856
Creatinine (μmol/L)	44.00–112.00	82.50 (67.50–107.00)	78.70 (61.90–110.40)	0.763
BUN (mmol/L)	2.50–7.10	6.57 (4.80–11.03)	6.52 (5.41–7.50)	0.856
AST (U/L)	0.00–40.00	37.35 (27.00–55.75)	50.45 (32.75–66.50)	0.856
ALT (U/L)	0.00–50.00	42.50 (28.00–63.60)	37.00 (29.25–55.53)	0.763
Total bilirubin (μmol/L)	3.00–21.00	20.55 (14.10–28.65)	17.85 (14.97–24.98)	0.763

Data are median (IQR). P-values comparing corticosteroid treatment and non-corticosteroid treatment are from Mann-Whitney U test or Student's t test, as appropriate, and adjusted for multiple comparisons by FDR method. NLR, neutrophil-to-lymphocyte ratio; APTT, activated partial thromboplastin time; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; BUN, blood urea nitrogen; AST, aspartate transaminase; ALT, alanine aminotransferase.

TABLE 5 | Laboratory findings of critical COVID-19 patients before discharge.

	Reference values	Corticosteroid treatment ($n = 68$)	Non-corticosteroid treatment ($n = 28$)	*P*-value
White blood cell count ($\times 10^9$/L)	4.00–10.00	8.48 (5.88–12.78)	6.90 (4.32–8.46)	**0.015**
Lymphocyte count ($\times 10^9$/L)	1.50–4.00	0.75 (0.54–1.12)	0.86 (0.52–1.33)	0.475
Neutrophil count ($\times 10^9$/L)	2.00–7.00	6.44 (3.90–10.83)	5.19 (2.75–7.29)	**0.015**
NLR	0.78–3.53	9.57 (4.70–16.21)	6.12 (2.43–9.67)	**0.015**
Monocyte count ($\times 10^9$/L)	0.12–1.00	0.45 (0.28–0.65)	0.48 (0.25–0.66)	0.692
Platelet count ($\times 10^9$/L)	99.00–303.00	185.00 (125.75–249.75)	233.00 (128.50–300.50)	0.100
APTT(s)	21.00–37.00	33.00 (25.75–41.00)	35.50 (32.10–49.25)	0.440
fibrinogen (g/L)	2.00–4.00	3.26 (2.03–4.23)	3.50 (2.13–4.48)	0.692
D-dimer (μg/ml)	0.00–0.55	1.05 (0.48–6.66)	0.54 (0.34–2.52)	0.642
Procalcitonin (ng/ml)	0.00–0.50	0.32 (0.12–0.58)	0.34 (0.21–0.44)	0.692
CRP(mg/L)	0.00–10.00	61.70 (24.88–156.85)	42.41 (15.25–122.63)	0.475
ESR (mm/1 h)	0.00–30.00	46.00 (32.00–78.50)	46.00 (31.25–81.75)	0.719
Creatine kinase (U/L)	25.00–200.00	178.00 (50.93–357.25)	187.50 (98.80–384.00)	0.903
Lactate dehydrogenase (U/L)	91.00–230.00	314.00 (214.00–579.00)	309.00 (210.00–393.00)	0.692
Creatinine (μmol/L)	44.00–112.00	83.40 (63.90–145.25)	84.50 (66.13–129.75)	0.692
BUN (mmol/L)	2.50–7.10	7.80 (5.33–18.85)	7.60 (4.99–14.47)	0.692
AST (U/L)	0.00–40.00	64.00 (41.00–112.00)	62.00 (46.75–78.00)	0.692
ALT (U/L)	0.00–50.00	67.00 (46.00–133.00)	61.00 (41.25–114.00)	0.692
Total bilirubin (μmol/L)	3.00–21.00	28.70 (19.65–34.13)	22.15 (17.98–30.80)	0.692
SOFA		10.50 (5.25–13.00)	5.00 (4.00–7.00)	**0.015**

Data are median (IQR). P-values comparing corticosteroid treatment and non-corticosteroid treatment are from Mann-Whitney U test or Student's t test, as appropriate, and adjusted for multiple comparisons by FDR method. NLR, neutrophil-to-lymphocyte ratio; APTT, activated partial thromboplastin time; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; BUN, blood urea nitrogen; AST, aspartate transaminase; ALT, alanine aminotransferase; SOFA, sequential organ failure assessment. The bold P-values indicate $P < 0.05$.

TABLE 6 | Logistic univariate and multivariate regression analyses on the association between the corticosteroid treatment and the clinical features.

	Univariable OR (95% CI)	*P*-value	Multivariable OR (95% CI)	*P*-value
Age≥60 years	3.53 (1.39–9.02)	**0.008**	4.94 (1.49–16.41)	**0.009**
Sex	0.70 (0.28–1.75)	0.449		
Laboratory findings				
White blood cell count ($X10^9$/L)	1.15 (1.02-1.30)	**0.022**	1.19 (1.03-1.37)	**0.019**
4–10	ref			
<4	0.38 (0.10–1.39)	0.142		
>10	2.59 (0.78–8.58)	0.120		
Lymphocyte count ($\times10^9$/L)	0.58 (0.31–1.08)	0.085		
1.5–4.0	ref		ref	
<1.5	2.61 (1.61–4.22)	**<0.001**	1.79 (0.63–5.06)	0.271
>4.0#	/	/		
Neutrophil count ($\times10^9$/L)	1.16 (1.02–1.31)	**0.023**	1.21 (1.04–1.40)	**0.016**
2–7	ref		ref	
<2	1.00 (0.25–4.00)	1.000		
>7	4.43 (1.95–10.06)	**<0.001**	2.32 (0.82–6.61)	0.115
NLR	1.13 (1.03–1.23)	**0.008**	1.16 (1.03–1.29)	**0.011**
0.78–3.53	ref		ref	
<0.78#	/	/		
>3.53	3.22 (1.90–5.47)	**<0.001**	3.51 (1.30–9.49)	**0.013**
Monocyte count ($\times10^9$/L)	1.58 (0.34–7.46)	0.561		
0.12–1.00	ref			
<0.12#	/	/		
>1.00#	/	/		
Platelet count ($\times10^9$/L)	0.99 (0.99–1.00)	**0.029**	0.99 (0.99–1.00)	0.059
99–303	ref			
<99	2.75 (0.88–8.64)	0.083		
>303	0.80 (0.22–2.98)	0.739		
APTT(s)	0.97 (0.94–1.01)	0.136		
21–37	ref			
<21	1.00 (0.63–15.99)	1.000		
>37	1.77 (0.90–3.49)	0.100		
Fibrinogen (g/L)	0.93 (0.70–1.23)	0.596		
2–4	ref			
<2	3.00 (1.09–8.25)	**0.033**	1.40 (0.37–5.34)	0.626
>4	1.55 (0.72–3.30)	0.261		
D-dimer (μg/mL)	1.05 (0.96–1.15)	0.293		
0-0.55	ref		ref	
>0.55	3.14 (1.72–5.74)	**<0.001**	2.67 (1.00–7.15)	0.050
Procalcitonin (ng/mL)	1.22 (0.74–2.03)	0.440		
0-0.50	ref		ref	
>0.50	4.25 (1.43–12.63)	**0.009**	2.27 (0.62–8.40)	0.218
CRP(mg/L)	1.00 (1.00–1.01)	0.193		
0–10	ref		ref	
>10	2.61 (1.61–4.22)	**<0.001**	1.63 (0.59–4.44)	0.344
ESR (mm/1h)	1.00 (0.99–1.01)	0.679		
0-30	ref		ref	
>30	2.05 (1.23–3.41)	**0.006**	1.29 (0.48–3.53)	0.614
Creatine kinase (U/L)	1.00 (1.00–1.00)	0.902		
25–200	ref		ref	
<25	2.00 (0.18–22.06)	0.571		
>200	2.75 (1.22–6.18)	**0.014**	1.30 (0.40–4.28)	0.663

(Continued)

TABLE 1 | Continued

	Univariable OR (95% CI)	*P*-value	Multivariable OR (95% CI)	*P*-value
Lactate dehydrogenase (U/L)	1.00 (1.00–1.00)	0.546		
44–112	ref		ref	
<44#	/	/		
>112	2.06 (1.15–3.68)	**0.015**	1.01 (0.38–2.72)	0.981
Creatinine (μmol/L)	1.00 (1.00–1.01)	0.588		
91–230	ref		ref	
<91#	/	/		
>230	3.12 (1.41–6.93)	**0.005**	2.10 (0.71–6.20)	0.180
BUN (mmol/L)	1.00 (0.99–1.01)	0.627		
2.5–7.1	ref		ref	
<2.5	2.00 (0.18–22.06)	0.571		
>7.1	2.40 (1.31–4.38)	**0.004**	1.38 (0.56–3.42)	0.485
AST (U/L)	1.00 (1.00–1.00)	0.620		
0–40	ref		ref	
>40	2.22 (1.36–3.63)	**0.002**	0.89 (0.32–2.46)	0.817
ALT (U/L)	1.00 (1.00–1.00)	0.620		
0–50	ref		ref	
>50	3.13 (1.75–5.60)	**<0.001**	2.90 (1.06–7.97)	**0.039**
Total bilirubin (μmol/L)	1.01 (0.97–1.05)	0.544		
0–21	ref		ref	
>21	3.29 (1.81–5.98)	**<0.001**	2.36 (0.85–6.55)	0.098
SOFA	1.17 (1.05–1.31)	**0.005**	1.21 (1.07–1.38)	**0.004**
1	ref		ref	
2–5	1.21 (0.60–2.46)	0.591	1.11 (0.31–3.95)	0.872
6–10	2.83 (1.12–7.19)	**0.028**	1.82 (0.38–8.64)	0.453
>10	5.67 (2.34–13.50)	**<0.001**	6.31 (1.34–29.73)	**0.020**

OR, odds ratio; CI, confidence interval. Multivariable logistic regression for age were adjusted for sex and comorbidities. Multivariable logistic regression for laboratory findings were adjusted for age, sex and comorbidities. NLR, neutrophil-to-lymphocyte ratio; APTT, activated partial thromboplastin time; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; BUN, blood urea nitrogen; AST, aspartate transaminase; ALT, alanine aminotransferase; SOFA, sequential organ failure assessment. The bold P-values indicate P < 0.05.
 # Too few cases.

corticosteroid treated patients consistently exhibited a larger median for the virus clearance time, for the length of hospital stay, and for the duration of symptoms.

Recently, in contrast to our findings, two randomized open-label trials reported beneficial effects of corticosteroid therapy on severe COVID-19. The RECOVERY study reported reduced case fatality in patients treated with corticosteroid (25), while the similarly designed REMAP-CAP study observed an increased odds of improvement in organ support-free days within 21 days in the corticosteroid treatment group (26). Inconsistently, data from the REMAP-CAP study also indicated a trend of increased length of ICU stay and hospital stay for the corticosteroid treated patients. One of the limitations in both REMAP-CAP and RECOVERY studies, as reported by the authors, was that 15% of the no corticosteroid group received systemic corticosteroids. Another important limitation in both studies was that they enrolled suspected COVID-19 patients. Without positive viral RNA diagnosis, these studies provide little support for beneficial effects of corticosteroid therapy in COVID-19.

It is worth noting that one earlier review summarizing all data on corticosteroid use with COVID-19 before July 2020 concluded that corticosteroid therapy is associated with the improvement in symptoms and oxygenation for individuals with severe COVID-19, but case fatality rate in the corticosteroid group was significantly higher than that in the non-corticosteroid group (27). This conflict between improved symptoms and increased case fatality rate could be a consequence of the heterogeneous nature of the different studies being reviewed. In contrast, we observed, consistently, higher case fatality rate and higher odds for other adverse outcomes after corticosteroid treatment, which may due to the strict enforcement of our inclusion criteria.

Conflicting results have also been reported on the use of corticosteroids in ARDS and infectious pneumonia. Studies from Meduri' group (28–31), including a randomized, double-blind, placebo-controlled trial (28), and other groups (32–35) reported beneficial effects of methylprednisolone on ARDS. However, in a prospective, randomized, double-blind, placebo-controlled trial in 99 patients with ARDS, high dose of methylprednisolone did not affect case fatality, the reversal of ARDS, or the

incidence of secondary bacterial infection (36). Similarly, in a prospective, randomized, double-blind, placebo-controlled trial of treating severe sepsis and septic shock with high-dose methylprednisolone, no significant difference were found in the prevention or reversal of shock, or overall case fatality of the patients (37). Adverse outcomes from corticosteroid treatment in sepsis were also frequently reported and summarized in a meta-analysis, which concluded that corticosteroid treatment increased case fatality rate and caused a trend of increased rate of secondary infection (38).

In a retrospective cohort study similarly designed as our study, Auyeung et al. examined the effect of corticosteroid in the treatment of SARS patients (39). They reported a 20.7-fold increase in risk of either ICU admission or case fatality, after adjusting for the effects of age and disease severity. Extrapulmonary injury may have contributed to the adverse outcomes, as their corticosteroid treated patients exhibited a trend of elevated lactate dehydrogenase. These results suggest that, although damage caused by inflammation in SARS and COVID-19 pneumonia can be lethal, turning off the immune surveillance with corticosteroids may cause additional adverse outcomes. Both SARS-CoV-2 and SARS-CoV are known to infect lung, intestines and other organs (40, 41), and the use of corticosteroid may exacerbate the viral infections leading to multi-organ failure.

Other limitations of our study include small sample size and imbalanced age between groups. The sample size did not allow sufficient power for many of the comparisons between the study groups. We are lucky that, an R^2 value of 0.413 was achieved in the multivariable logistic regression of the case fatality rate, indicating a decent power for the analysis of the primary outcome. The age of the CS group was apparently older and the difference was statistically significant before adjustment for multiple comparisons. Considering the potential impact of the age on the clinical outcomes, age was included in the multivariate regressions. Our results indicated that adverse outcomes remained significantly associated with corticosteroid use in critical COVID-19 after adjusting for the influence of age and other confounding factors.

In conclusion, corticosteroid use in critically ill COVID-19 patients was associated with a much higher case fatality rate. Frequent incidence of liver injury and multi-organ failure in corticosteroid treated patients may have contributed to the adverse outcomes. The multi-organ failure is likely caused by more persistent SARS-CoV-2 infection and higher viral load, due to the inhibition of immune surveillance by corticosteroid.

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 Institutional Review Board of the Six Affiliated Hospital of Sun Yat-sen University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

GY, LZ, and RZ conceived and designed this study. YL, JL, YZ, LZ, SC, and YH collected data. YL, JK, NJ, JL, LZ, GY, and RZ analyzed data. YL, JK, NJ, and LZ prepared the first draft. All authors critically revised the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.604263/full#supplementary-material

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Design of the Arizona CoVHORT: A Population-Based COVID-19 Cohort

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This study is a prospective, population-based cohort of individuals with a history of SARS-CoV-2 infection and those without past infection through multiple recruitment sources. The main study goal is to track health status over time, within the diverse populations of Arizona and to identify the long-term consequences of COVID-19 on health and well-being. A total of 2,881 study participants (16.2% with a confirmed SARS-CoV-2 infection) have been enrolled as of December 22, 2020, with a target enrollment of 10,000 participants and a planned follow-up of at least 2 years. This manuscript describes a scalable study design that utilizes a wide range of recruitment sources, leveraging electronic data collection to capture and link longitudinal participant data on the current and emerging issues associated with the COVID-19 pandemic. The cohort is built within a collaborative infrastructure that includes new and established partnerships with multiple stakeholders, including the state's public universities, local health departments, tribes, and tribal organizations. Challenges remain for ensuring recruitment of diverse participants and participant retention, although the electronic data management system and timing of participant contact can help to mitigate these problems.

Keywords: COVID-19, SARS-CoV-2, cohort study [or longitudinal study], epidemiology, long-term follow up

INTRODUCTION

SARS-CoV-2, and its associated disease state COVID-19, have been increasing in incidence globally since the first detailed report emerged in February 2020 (1). We are currently in an unprecedented public health response aimed at controlling disease spread (2), and there is an urgent need to capture and evaluate key information about pathogen transmissibility and risk factors associated with severe disease (3). It is equally important to understand symptom duration and the short and long-term health sequelae of SARS-CoV-2 infection. Management and prevention strategies can be developed and

applied for patients to mitigate the adverse impact of COVID-19 on their overall health and well-being.

Early reports of COVID-19 epidemiology identified increasing age and several comorbidities as major risk factors for the development of a severe course of disease (4). Later, more refined data clarified that specific cardiometabolic conditions, including hypertension, diabetes, and cerebrovascular disease, increase the risk of adverse outcomes for COVID-19 patients (5). Reports of the infection in patients from Wuhan, China initially indicated that COVID-19 was largely a respiratory illness, with the majority of patients presenting with shortness of breath, dry cough, and, among the severely ill, Acute Respiratory Distress Syndrome (ARDS) (6). Subsequent studies revealed the involvement of multiple organ systems, including cardiovascular, neurological, and gastrointestinal. Cardiovascular manifestations include unspecified arrhythmia (1, 7), acute cardiac injury (1, 7), cardiomyopathy (6), heart failure (7), and thrombotic complications (7, 8). Additionally, the risk of acute ischemic stroke was found to be higher among COVID-19 patients as compared to patients with influenza (9). Neurological complications and cognitive impairment have been observed in up to 69% of COVID-19 patients including delirium, anosmia, headache, and dizziness, as well as the more severe sequelae of Guillain-Barre Syndrome and encephalitis (10–12). Gastrointestinal (GI) symptoms, initially comparatively rare among patients in China (13), have been more prevalent in patients in other countries with up to 61.3% of patients infected with SARS-CoV-2 presenting with at least one GI symptom in a multi hospital study conducted in the United States (14). However, due to the novelty of the virus and the lack of available treatment options, there is a paucity of knowledge about which chronic health conditions are triggered or aggravated by COVID-19 disease.

In addition to the growing need for data on the conditions that arise from infection with SARS-CoV-2, the duration of symptomatology and heterogeneity in symptom profiles are currently unknown. Among a group of 143 hospitalized COVID-19 patients in Italy, 87.4% continue to experience at least one symptom for up to 60 days post-recovery, of which lasting fatigue and dyspnea were the most prevalent (15). The majority of these patients report significant reductions in their quality of life (QoL) compared to the time prior to their illness (15). A growing number of social media users report their illness as lasting >60 days post-recovery from the acute phase; however, these reports are anecdotal, unconfirmed, and require systematic analysis within the context of a large, prospective epidemiological investigation.

We know from past outbreaks of related coronaviruses that there is a potential for chronic health outcomes following the acute infection. Cohorts established following the 2002–2003 SARS epidemic have identified a range of health conditions among survivors of the infection. Survivors have reported a reduced QoL and persistent fatigue/malaise for up to 1 year after recovery from the acute phase (16). These cohort studies following the SARS epidemic have also reported SARS survivors experiencing an increased susceptibility to lung infection, altered metabolisms, and cardiovascular abnormalities for more than a decade after recovery (17), and lung and bone injury persisting 15 years post-recovery (18). The combination of prolonged sequelae associated with previous coronavirus outbreaks and early reports of sequelae among COVID-19 patients suggests that COVID-19 illness could precipitate a new wave of prolonged chronic diseases accompanying an acute infection symptomology. These past studies underscore the urgency for the rapid development of a rigorous and robust longitudinal study to compare outcomes in patients infected with SARS-CoV-2 to uninfected individuals.

In addition to research gaps related to comorbidities and sequelae associated with COVID-19, the identification of factors that may precipitate a more severe course of disease is urgently needed. A growing number of reports detailing severe COVID-19 cases in young adults with no known underlying conditions underscores the need for carefully designed prospective epidemiology studies to determine why some individuals progress to severe forms of disease and others do not. Further, the short- and long-term health impacts of SARS-CoV-2 infection in asymptomatic individuals is unknown. In one report, abnormal radiological findings of the lung were identified in 66.7% (14/21) of asymptomatic individuals (19), but the comparatively small sample size and short duration of this study evinces the need for a larger epidemiological study with longer follow-up.

In this manuscript, we describe the development of a prospective, population-based cohort of COVID-19 positive participants and uninfected population-based participants designed to (1) determine the contribution of putative risk factors and extant comorbidities to COVID-19 disease severity, (2) identify chronic health conditions that arise following COVID-19 disease, (3) and examine the relationship between disease severity and chronic health outcomes. This Arizona COVID-19 Cohort, dubbed "CoVHORT," will enable us to determine the prevalence of patient-reported health outcomes throughout the acute phase of the illness in baseline assessments, and incident outcomes during the recovery phase. Our prospective approach enables us to identify risk factors or exposures that are implicated in the development of post-COVID-19 health outcomes, including new onset of chronic conditions, exacerbation of existing conditions, or persistence of COVID-19 symptoms. Our inclusive recruitment strategies allow us to reach a diverse population of Arizona residents, increasing the generalizability of our findings to rapidly inform prevention and treatment strategies. A population-based prospective cohort of patients with and without COVID-19 diagnosis is an ideal study design to collectively investigate the COVID-19 disease course including risk factors, disease progression, resolution, and chronic outcomes of infection.

METHODS AND ANALYSIS

Survey Instruments

The baseline survey of the CoVHORT is a 95-item questionnaire using multiple query formats, including multiple choice, multi-select, and free-response text fields. To prepare the survey instruments, we first conducted a review of previously published literature in order to develop survey items that encompass

TABLE 1 | Topic areas included in administered surveys developed for the Arizona CoVHORT.

Topic areas	Example questions
Acute illness	Since the start or your illness, are you feeling back to normal?
Duration	
Exposures	In the 14 days before you started feeling ill, did you have close contact with someone who was positive for COVID-19?
Household transmission	How many people living in your home were confirmed or suspected to have had COVID-19 since your last survey?
Results of diagnostic testing	What were your COVID-19 test results?
Symptoms	Which symptoms did you experience during your illness?
Severity	Were you hospitalized for this illness?
Chronic illness	Have you or a family member missed routine or preventative health care since your last survey? This could include dentist appointments, physicals, eye exams, screening, or vaccination appointments for you or your children.
Changes to routine medical care	
Development of chronic sequelae	Have you been newly diagnosed with any of the following conditions since your last completed survey? Were you diagnosed with this condition(s) before or after your COVID-19 diagnosis?
Exacerbation of pre-existing chronic health conditions	Since your last survey, have you experienced new complications, increases in severity, or changes in medication for any of the following conditions?
Persisting symptoms	Since your last completed survey have you noticed any of the following ongoing or new symptoms?
Financial hardship	How would you describe the money situation in your household right now?
Perceived risks and attitudes	In your opinion, how effective are the following actions for keeping you safe from COVID-19?
Physical activity	If you wear a wearable fitness tracker (e.g., Apple Watch, Fitbit, Garmin), have you noticed that your physical activity has changed compared to before the COVID-19 outbreak?
Pregnancy and reproduction	Have you tried to become pregnant or are you actively trying to become pregnant?
Perception of policy interventions	Should the government quarantine those who might have been exposed to COVID-19 to limit their contact with others?
Sleep quality	Since the start of the pandemic, how often have you had trouble sleeping? (This can be due to reasons such as trouble falling asleep, waking up early/in the middle of the night, trouble breathing/coughing/snoring, feeling too hot/cold, bad dreams, or pain).
Stress and emotional wellness	In the last month, how often have you been upset because of something that happened unexpectedly?

all reported symptoms of the infection. Additional questions, standard to case investigations and studies of infectious diseases, include duration of symptoms and potential exposure routes. Due to the dearth of information regarding household transmission, and particularly transmission from children to adults, the survey includes items to characterize participant household structure and to track spread of infection among household members. In addition to the peer-reviewed literature, public reports of chronic, persistent symptoms were included based on review of symptoms reported by COVID-19 patients using the hashtag "#longcovid" on social media websites. The inclusion of these symptoms allows for a rigorous approach to confirm whether these symptoms are more common in those who have been infected with SARS-CoV-2 as compared to uninfected individuals. We continue to review the COVID-19 literature weekly so that we can update our questionnaires to reflect new information as needed.

A multitude of social, individual, environmental and economic factors influence the acute phase of COVID-19, and these factors also play a significant role in the pathology of other chronic diseases. For this reason, the surveys were designed to capture domains that were impacted by the COVID-19 epidemic in Arizona, as well as information on the impact of COVID-19 on general well-being including financial hardship, risk perception, physical activity, sleep, pregnancy and maternal/fetal outcomes, and stress and emotional wellness (**Tables 1**, **2**). The majority of the survey items were developed from the broad expertise of members of the Arizona CoVHORT research team, expanding on, and modifying previously validated scales.

Risk Factors for Infection and Severe Disease

At baseline, we ask information on behavioral factors currently hypothesized to influence risk of infection including: travel history (international and domestic), close contact with someone who tested positive for COVID-19, quarantine practices (among individuals with SARS-CoV-2 infection), living arrangements, household structure, household member illness history, participation in state-mandated closures (i.e., sheltering in place, working from home), employment-related exposures (i.e., essential worker; number of interactions with other people), employment location (e.g., working from home), volunteer-related exposure (e.g., fire department, school), personal COVID-19 mitigation efforts (e.g., mask wearing), and cigarette smoking/vaping/marijuana use. The questionnaire will further collect information on health and medical history that may contribute to risk of severe COVID-19 disease. Health and medical questions include information on height and weight to calculate body mass index (BMI), and pre-existing conditions. Individuals are asked general information about their ability to obtain regular treatment for controllable conditions including

TABLE 2 | Topic areas included in the Arizona CoVHORT surveys and their assessment frequencies over the course of the 1st year of follow-up.

Topic areas	Baseline	3 months	6 months	9 months	1 year	"Off cycle"
Acute illness						
Duration	•	•	•	•	•	•
Exposures	•	•	•	•	•	
Household transmission	•	•	•	•	•	
Results of diagnostic testing	•	•	•	•	•	
Symptoms	•	•	•	•	•	•
Severity	•	•	•	•	•	•
Chronic illness						
Changes to routine medical care			•		•	
Development of chronic sequelae		•	•	•	•	•
Exacerbation of pre-existing chronic health conditions		•	•	•	•	•
Persisting symptoms	•	•	•	•	•	•
Financial hardship		•		•		
Perceived risks and attitudes		•		•		
Physical activity	•		•		•	
Pregnancy and reproduction	•	•	•	•	•	
Perception of policy interventions			•		•	
Sleep quality	•		•		•	
Stress and emotional wellness		•	•	•	•	

diabetes and high blood pressure to determine if challenges in chronic disease management may influence severity of COVID-19 illness. Information on medications known to lower immune function and whether the participant is actively on dialysis are also collected.

Chronic Disease History and Development

A baseline health history is taken. Among individuals with COVID-19 disease, participants are asked whether they had a history of clinician-diagnosed chronic disease prior to COVID-19 diagnosis. Among participants without a history of COVID-19, we ask about their history of clinician-diagnosed chronic disease prior to enrollment. On each follow-up questionnaire, all our participants are queried regarding changes in their health history and newly clinician-diagnosed conditions. The questionnaire asks specifically about cardiometabolic conditions (i.e., diabetes, pre-diabetes, gestational diabetes, hypertension, hypercholesterolemia, myocardial infarction, angina, congestive heart failure stroke, other cardiac/heart condition), respiratory conditions (i.e., asthma, valley fever, emphysema/chronic bronchitis, chronic obstructive pulmonary disease [COPD]), cancer history, influenza, thyroid disorders, liver disease, chronic kidney disease, gastrointestinal conditions (inflammatory bowel disease, ulcerative colitis, irritable bowel, acid reflux), mental health history (i.e., depression, anxiety), neurologic conditions (Parkinson's disease, lupus, multiple sclerosis), arthritis, and other (open response). Participants are also asked about changes in their condition including any new complications, increases in severity, or changes in the medication used.

Financial Hardship

Measures of financial hardship or deprivation assess whether individuals are excluded from minimally accepted ways of life in society due to a lack of resources (20). In contrast to income or poverty measures which infer exclusion from a lack of resources, financial hardship directly assesses the extent to which individuals or households lack goods, facilities, or services or are unable to engage in activities (21). There is consistent evidence that hardship is associated with psychological distress and common physiological disorders that are expected to be significant comorbidities with COVID-19 (22). We are utilizing a 6-item measure of financial hardship that includes assessments of personal, household, and medical needs that is currently being utilized in comparable COVID-19 projects (23). Both direct and indirect impacts of COVID-19 on financial hardship can be identified in this cohort.

Risk Perception

Contemporary assessments of risk perception focus on multiple dimensions such as affective (degree of concern and emotional attachment), deliberative (the probability of incident), and experience (consequence or impact) to determine the degree to which perception is likely to shape risk-oriented behavior (24). Recent studies have fielded survey instruments that assess that a tripartite factor structure, including all three dimensions, that best capture the degree of worry expressed by individuals about most health outcomes and the likelihood of taking protective measures (25). Our assessment of risk perceptions is pulled from the COVID-19 OBSSR Research Tool and is being utilized in comparable assessments (26, 27) to determine how perceptions

of the risks and consequences of COVID-19 shape behaviors and outcomes.

Physical Activity and Sleep

Our participants are asked about their physical activity and sleep patterns prior to March 2020 and at time of the questionnaire. The questions on physical activity are modeled after the International Physical Activity Questionnaire (IPAQ) (28) and questionnaires used by the Women's Health Initiative (29) and ask about frequency and intensity over the past month. We also ask about information on average time to bed, time to rise, and sleep quality from participants to measure sleep duration and quality, in addition to whether their physical activity and sleep patterns have been affected by the COVID-19 pandemic.

Pregnancy and Maternal/Fetal Outcomes

Female, non-binary and trans male participants between 18 and 49 years of age are asked on each questionnaire whether they are currently pregnant, have become pregnant since their previous questionnaire, are actively trying to become pregnant, or are not trying to become pregnant. If a participant reports that they were pregnant but are no longer pregnant since their previous questionnaire, we collect information regarding the outcomes of their previous pregnancy including: result of their pregnancy (live birth, miscarriage, or termination), due dates, date of miscarriage or termination, date of delivery, and reasons for termination of pregnancy.

Stress

We are utilizing the perceived stress scale-10 (PSS-10) to ask our participants about their perceived stress in regard to COVID-19 over the course of follow-up. The 10-item validated PSS-10 assesses how unpredictable, uncontrollable, and overwhelming an individual may find their circumstances and has been shown to effectively capture stress over the previous 4–8 weeks in community-based samples with at least an 8th grade education (30). The impact of stress on the body is driven by the cognitively mediated responses to a stressful event, rather than the event itself. Thus, the PSS-10 is considered a better measure of relevant stress than objective measures of stressful events (30).

Study Design

The Arizona CoVHORT is a population-based prospective cohort study in which 2,881 participants have been enrolled as of December 22, 2020. The enrollment goal is 10,000 Arizona residents that represent Arizona's geographic and demographic diversity across rural and urban areas. All Arizona residents are eligible to participate. Once enrolled, we determine their previous infection status by asking them to self-report clinical and/or serological results, history of a diagnosis, or symptoms consistent with COVID-19; SARS-CoV-2 infection status is reevaluated at each follow-up survey time point (see SARS-CoV-2 Infection Status and Participant Follow-up and Retention). All study procedures, including advertising, recruitment, consent, enrollment, and follow-up, are available in English and Spanish and conducted in accordance with approval by the University of Arizona Institutional Review Board (#2003521636A002) under the aegis of the University of Arizona Human Subjects Protection Program.

Eligibility Criteria

The eligibility criteria for participation are: (1) current resident of Arizona; (2) ability to complete a survey written in either English or Spanish; and (3) provide informed consent to participate. Participant who wish to participate, but are not current residents of Arizona, are excluded from the cohort. Other than current residence, we have no other exclusion criteria for participation. Individuals under 18 years old may participate in the study, provided that the parent or legal guardian gave informed consent and the minor provides assent to participate. Parent or legal guardians may provide survey data for household members that are younger than 14 years of age. Although all races and ethnicities are eligible for this study, we are not actively recruiting American Indian/Alaska Natives on or off tribal lands in Arizona. We are in the process of consulting with local tribes and tribal health organizations in Arizona to develop potential collaborations.

Recruitment

Participants of the Arizona CoVHORT are recruited via three primary mechanisms, as illustrated in **Figure 1**. The first is composed of academic-public health department partnerships that provides diagnostic testing and conducts case investigations for SARS-CoV-2 positive individuals across Arizona. Prior to the COVID-19 outbreak, and in collaboration with local health departments, the Student Aid for Field Epidemiology Response (SAFER) program at the University of Arizona College of Public Health (31, 32) trained students to conduct case investigations for routine surveillance and outbreak response for various infectious diseases. In response to the COVID-19 pandemic, SAFER leveraged its existing infrastructure to train a large pool of undergraduate and graduate public health student volunteers to conduct case investigations for COVID-19 through established partnerships with the Arizona Department of Health Services and local health departments. Upon completion of each case investigation, cases are given the opportunity to provide their email address and automatically receive the electronic consent form for the Arizona CoVHORT study. Arizona State University's Biodesign Institute has also partnered with the Arizona Department of Health Services to provide free saliva diagnostic testing to underserved Arizona communities (https://biodesign.asu.edu/research/clinical-testing/testing). Individuals who participate in these testing opportunities who indicate interest in taking part in future research are also sent invitations to participate in the study. As of December 22, 2020, we recruit from six of 15 counties that encompass 90% of the state's population. These counties are a mix of rural and urban communities. To date, we have recruited 244 laboratory confirmed COVID-19 positive participants and 592 laboratory confirmed COVID-19 negative participants from this method, or 29.0% of our current total recruitment.

COVID-19 positive and negative participants will also be identified and enrolled through our established collaborations with several COVID-19-related research studies at the University

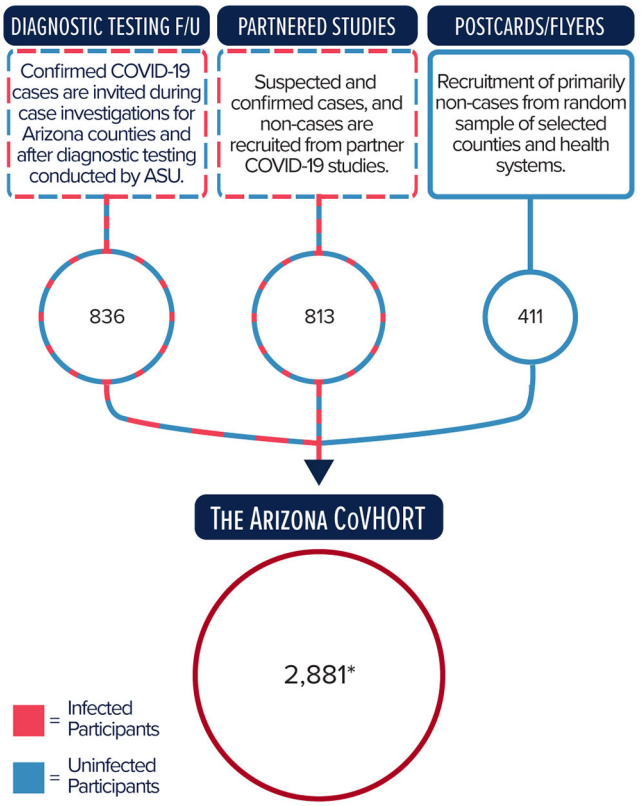

FIGURE 1 | Primary recruitment methods and number of participants enrolled into the Arizona CoVHORT as of December 22, 2020. *Total includes participants also recruited through other methods and by word of mouth.

of Arizona and Arizona State University (**Figure 1**). For example, following IRB approval and participant consent, participants in the State of Arizona COVID-19 Antibody Testing Initiative (https://covid19antibodytesting.arizona.edu/) who indicate interest in taking part in future research are sent invitations to CoVHORT in batches following the receipt of their results. This process is also applied to individuals who are current participants, or are ineligible or opted out of participating in the AZ HEROES study (https://azheroes.arizona.edu/). As of December 22, 2020, 813 of these participants (28.2% of our current total recruitment) have enrolled in the CoVHORT.

The final major recruitment channel is through a phased postcard mailing campaign to establish a population base of individuals who have not yet been infected with SARS-CoV-2 (**Figure 1**). The procedure consists of three rounds of mailed recruitment postcards to a simple random sample of 17,500 residences from each county selected. Phased mailings of recruitment postcards occur every 2 weeks to allow potential participants time to receive the material, review the consent materials, and choose whether or not to enroll in the study. This method enables us to maximize participation and minimize bias (33). We employ participant-provided information from baseline surveys to exclude addresses of those who have enrolled from the mailing list of each subsequent phase of the mailing campaign. Furthermore, each list is screened prior to each mailing to reduce the number of undeliverable postcards. We have completed the phased mailing campaign in Pima County, AZ and completed additional mailing campaigns in Pinal and Yuma County, AZ having contacted more than 51,000 households. We will next expand the postcard mailings to the other counties for which we are conducting case investigations, followed by the remaining Arizona counties. This method has resulted in the enrollment of 403 participants (14.0% of our current total enrollment).

In addition to direct invitations to individuals with laboratory confirmed infections identified through health department investigations, partnering COVID-19 study participants and testing sites, and the postcard mailing campaign, we have also created electronic and print recruitment materials. Recruitment flyers are in circulation within the Veteran's Affairs facilities and other healthcare systems in the greater Phoenix area. Future recruitment efforts will be focused on onboarding additional counties to the CoVHORT through case investigations with other jurisdictions and partnering with additional COVID-19 research studies in Arizona. Postcard mailings will also be directed to each of these counties to continue to expand the cohort. These efforts will be informed by our initial campaigns and tailored as necessary for maximum return and community reach.

SARS-CoV-2 Infection Status

Our partnerships with local health departments and collaborations with the State of Arizona COVID-19 Antibody Testing Initiative allows us to recruit residents who have laboratory confirmed current or past infections with SARS-CoV-2. Participants who are recruited from either case investigations or the antibody testing initiative are imported to our cohort with their linked laboratory test results, so we are treating these individuals as confirmed infections (**Figure 2**). Although other participants may report positive test results, we are not actively verifying self-reported test results with local health departments or the Arizona Department of Health Services. Thus, individuals who report a positive test result or symptoms consistent with COVID-19 illness at any point in time during follow-up are treated as suspected infections. All participants who report no symptoms of COVID-19 and/or receive a negative test result are treated as uninfected residents. While this is a limitation, we will also be able to refer participants who have not received an antibody test to the COVID-19 Antibody Testing Initiative to minimize misclassification. Additionally, at each follow-up survey we ask all uninfected participants to self-report their current SARS-CoV-2 infection status, including any COVID-like symptoms and results of any diagnostic or antibody test received since their last completed survey (see Participant Follow-up and Retention). By doing so, we are able to identify incident SARS-CoV-2 infections among our previously uninfected participants and appropriately assign surveys and survey items developed to track their illness and recovery.

Inclusion of Traditionally Underserved Participants

Stark differences in COVID-19 disease incidence and severity by race/ethnicity have emerged as the pandemic has progressed.

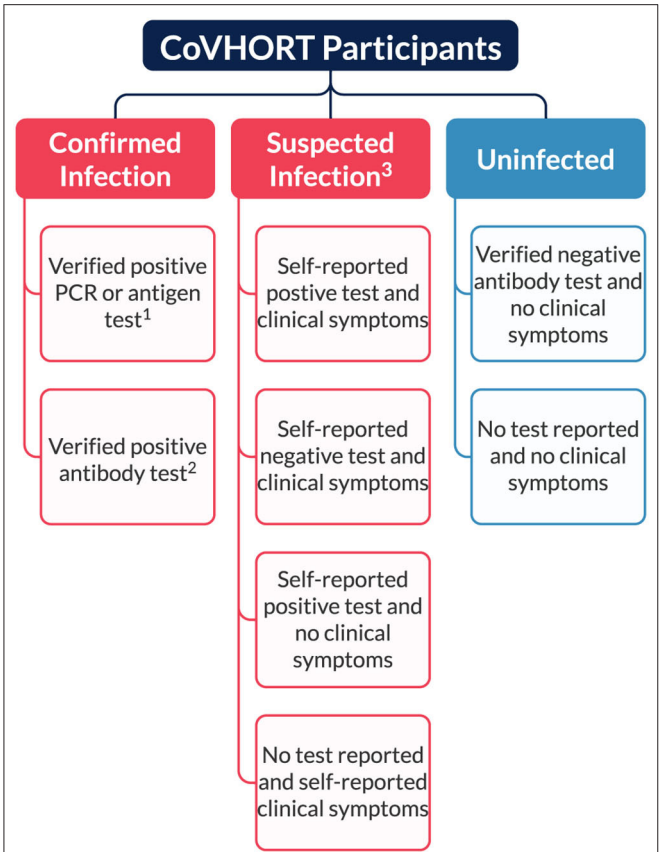

FIGURE 2 | Current classification of participants based on verification of SARS-CoV-2 infection status. [1]Verified through case investigations conducted by SAFER for state and local health departments. [2]Verified through partnerships with the State of Arizona Antibody Testing Initiative. [3]All self-reported test results regardless of tests (PCR, antigen, or antibody) are determined to be suspected due to inability to verify each participant's results.

Black, Indigenous and Latinx persons have higher COVID-19 infection, hospitalization, and mortality rates compared to Whites (34). These disparities arise from a constellation of social inequities that drive underlying disparities in health care access and health outcomes. This is particularly, true in tribal nations where high levels of transmission highlight the structural, social, and health inequities experienced over generations due to colonialism and institutional racism that influence susceptibility to infection and severe disease.

Arizona's diversity is a strength of this cohort, with ∼30% of our residents identifying as Latinx, and 5.3% of the population is a member of one or more of the 21 independent tribal nations that fall within Arizona state boundaries. The Director of Tribal Engagement for CoVHORT (author FCM) has initiated the process to establish and develop partnerships between CoVHORT and tribal lands. While members of Tribal Nations are welcome to join the current cohort, we are not actively recruiting individuals residing on tribal lands until these relationships can be fully developed.

To develop a cohort that reflects the state's demographics, and is inclusive of Arizona's Spanish speaking population, CoVHORT investigators developed materials in both Spanish and English from the outset in collaboration with community partners. All consent forms, surveys, and recruitment materials are available in English and Spanish. Additionally, three of the five counties that the SAFER program currently conducts case investigations for are along the Arizona and Sonora, Mexico border. Latinx populations comprise 40–80% of the total population in these counties.

Participant data are regularly audited for demographic characteristics. These strategies serve as mechanisms to promote inclusion of a wide range of ages, ethnicities, countries of origin, and socioeconomic status.

Enrollment

Regardless of recruitment method, eligible participants are directed to the CoVHORT study website (https://covhort.arizona.edu/) whereupon they are provided an overview of the study, information about what is involved with participation, and a link to begin the informed consent process if they choose to enroll. The consent procedure lists potential risks and benefits of participation and allows participants to withdraw from the study at any time. Following completion of the consent process, participants may begin the baseline survey.

Current Participants

As of December 22, 2020 (day 208 of study recruitment), CoVHORT has enrolled 2,881 participants, 28.8% of our 10,000-participant goal. Of these, 16.2% have a history of COVID-19 determined by self-report of a positive diagnostic test result (**Table 3**). The characteristics of the participants thus far are described in **Table 3**. Briefly, the majority are women (65.9%), and white (87.9%), without a reported history of infection (83.8%). One in five to six, 18.0%, identify as Hispanic, Black, or Indigenous. Another 4.6% identify as multi-racial.

Participant Follow-Up and Retention

Planned follow up for all participants occurs at baseline, 3, 6, 9 months, and 1-year post-enrollment (**Figure 3**). At month 13 following enrollment, we will reduce contact to biannual for the remainder of follow-up. These "on-cycle" surveys, administered to participants with and without a prior COVID-19 diagnosis, allow us to ascertain if there is a higher prevalence of chronic conditions following infection. As one of the primary goals of the CoVHORT is to provide data regarding any long-term COVID-19 disease sequelae, we have planned additional "off-cycle" questionnaires for all participants with suspected or confirmed COVID-19 that are sent at the midpoint between each planned contact to all participants. These "off-cycle" surveys, scheduled at 1.5 months after each quarterly on-cycle survey, are used to assess acute conditions related to SARS-CoV-2 infection. All participants who are COVID-19 positive at enrollment will immediately be scheduled to complete both the on-cycle and off-cycle surveys. We will also schedule the off-cycle surveys for all participants that self-report an incident COVID-19 illness or a positive test result over the course of the follow-up

TABLE 3 | Baseline characteristics of current participants of the Arizona CoVHORT as of December 22, 2020.

	No. (%)
Age (mean ± SD), y	47 ± 16
Gender	
Male	959 (33.3)
Female	1,898 (65.9)
Non-binary	15 (0.5)
Transgender male	3 (0.1)
Transgender female	1 (0.0)
Race	
AI/AN	28 (1.0)
Asian	83 (2.9)
Black	33 (1.1)
NH/PI	3 (0.1)
White	2,532 (87.9)
Mixed race	133 (4.6)
Ethnicity	
Hispanic	458 (15.9)
Non-hispanic	2,331 (80.9)
History of COVID-19 disease[a]	467 (16.2)

%, percent; AI/AN, American Indian/Alaskan Native; NH/PI, Native Hawaiian or Pacific Islander; No., number; SD, standard deviation; y, years.
[a]Determined by self-report of positive PCR test for SARS-CoV-2.

period (**Figure 3**). These off-cycle surveys are administered as long as participants are reporting symptoms. If a participant with a confirmed or suspected SARS-CoV-2 infection reports experiencing no symptoms on two consecutive off-cycle surveys, they are redirected to only receive the on-cycle surveys for the remainder of their follow-up, or until they begin to experience symptoms again (**Figure 3**).

These follow-up methods also allow for the development of sub-studies of specific participants which may ask additional survey questions and/or to provide additional clinical samples. This framework allows flexibility to answer research questions pertaining to special populations, such as children, elderly, or pregnant individuals. As such, the CoVHORT will also function as a pool of Arizona residents who may be recruited for future public health outreach and research efforts conducted by the University of Arizona.

Participant retention poses a major challenge to collect follow-up data longitudinally. In an effort to address this challenge, all of our surveys are designed to take no longer than 30 min to complete and can be completed fully online. However, in the event a participant does not have access to a computer or other device with internet access or they would prefer not to complete the surveys online, we also provide the option for participants to opt in for mailed print versions of the surveys that they would mail back once completed. Understanding the importance of transparency, as well as participant interest in study progress, we are developing a page on the CoVHORT website dedicated to sharing current study descriptive data and findings with participants. We are also discussing the periodic dissemination of an electronic CoVHORT newsletter for active participants that would contain study updates and also reminders to stay active with follow-up surveys. Further, we are exploring various strategies, such as gifts or small monetary incentives, to motivate participants to remain engaged in the study.

Data Management and Analyses

All data will be collected using Research Electronic Data Capture (REDCap), a HIPAA-compliant electronic data collection platform (35). REDCap is a secure, web-based software platform to support data capture and management in research. At the time of each follow-up questionnaire, a unique link is sent by email through REDCap to study participants. The surveys are available in English or Spanish. Additionally, participants who do not wish to complete the survey online, or are unable due to limited or restricted access to a reliable internet connection, are offered the ability to complete print versions of the surveys that can be mailed back to us in a prepaid envelope. Any differences in participants due to method of survey completion (self-administered vs. mailed print) will be evaluated and considered in the analyses. Data is stored in a password-protected database and is accessible only to the PI and paid research staff. Data management and quality control procedures are implemented by the same individuals. We understand that researchers outside of CoVHORT study team may also wish to collaborate with us or wish to have access to our participants' collected data to conduct their own analyses. We review each request on a case by case basis before granting access to our dataset. Any interested researchers should reach out directly to either of the corresponding authors to begin our internal review process.

Statistical Analysis

The CoVHORT is already yielding a rich dataset, and we will provide a large data repository for exploring both known and yet unknown research questions. All analyses will follow best practices in our analytical approaches and reporting, including pre-specified analysis plans, statistically defensible methods for missing data, thoughtful sensitivity analyses, and the careful use of reporting guidelines.

The primary outcome for the first aim is COVID-19 severity, categorized as severe (requiring inpatient care) or not (asymptomatic, mild, moderate). We will perform logistic regression to investigate the association of chronic health conditions (e.g., cardiometabolic conditions such as hypertension, type 2 diabetes, CVD, obesity), behavioral risk factors (mask wearing, quarantine practices, etc.) with severe COVID-19. All models will be adjusted for other known risk factors, including age, sex, race/ethnicity, and stratify analyses by sex if the interaction of comorbid condition and sex is statistically significant; we will categorize age to account for potential non-linearity. We will estimate prevalence of each comorbid condition and behavioral factor amongst persons with COVID-19, stratified by severity, age, sex and race/ethnicity. For each major symptom, symptom trajectory over time will be plotted, stratified by severity, age, sex, and race/ethnicity. With

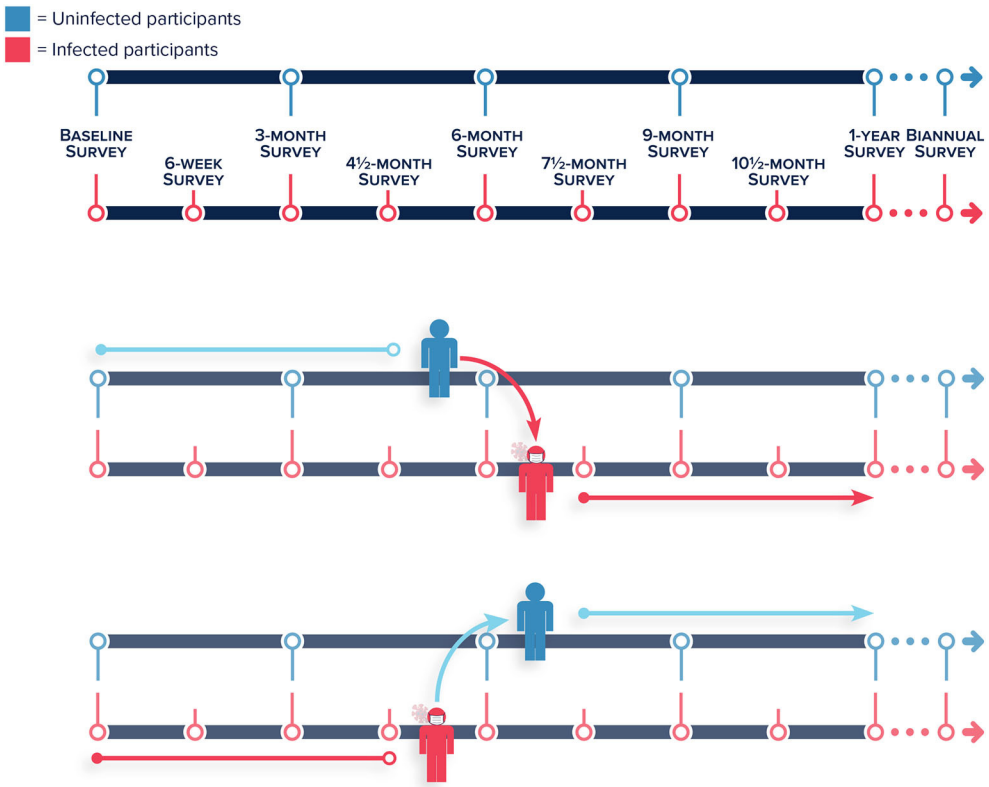

FIGURE 3 | Frequency of on-cycle surveys administered to uninfected and infected participants, and the frequency of off-cycle surveys administered to only infected participants over the course of the follow-up period of the Arizona CoVHORT. (Top and Middle panel) Participants who were uninfected that self-report a COVID-19 illness or a positive test during follow up are shifted to the infected participants survey timeline including the additional "off-cycle" surveys. (Bottom panel) Infected participants that report they are no longer experiencing symptoms over the course of two "off-cycle" surveys are shifted to the uninfected participant survey timeline without the additional "off-cycle" surveys.

appropriate interval-censored survival analysis methods, we will estimate time to a symptom free state.

The primary outcome for the second aim is chronic health sequelae, including impaired lung function, cardiovascular disease, or neurological symptoms. We will model chronic health sequelae using generalized estimating equations (GEE) and compare the odds of chronic health sequelae between individuals with COVID-19 and those without. We will use a binomial distribution, logit link, independent working correlation structure and robust errors. GEE accounts for the longitudinal design and allows for time-varying covariates, including the crossover from COVID-19 negative to positive, while accounting for correlation of repeated measures within subjects. To reduce the effects of confounding we will use propensity score methods. Specifically, the probability of having COVID-19 will be estimated in our sample, and then we will use this propensity score in inverse probability weighting models (36). We will estimate prevalence of each of the comorbid condition amongst persons with COVID-19, stratified by severity, age, sex and race/ethnicity. We estimate that our CoVHORT will consist of 1,200 COVID-19 cases and 8,800 non-cases based on current estimates of positivity inthe state.

We will undertake sensitivity analyses for each of our primary outcomes. To account for missing data, we will use multiple imputation with chained equations; in other words, imputation models that include variables associated with missingness, the outcome, and from the analytic model. Other sensitivity analyses may center around changing definitions of cases and/or symptoms.

Power and Sample Size

For aim 1, we assumed that 12% of our sample of 10,000 would be positive for COVID-19, so that the number of COVID-19 positive cases will be 1,200. We further assumed that 15% of our positive sample will develop severe COVID-19 and used a type I error rate of 0.05. This sample size gives us between 80 and 90% power to detect an odds ratio between 1.55 and 1.60 for severe COVID-19, assuming a comorbid condition rate of 30 to 50% in the reference group (non-severe COVID-19 individuals) at the baseline assessment. For comorbid conditions with lower rates, such as 10%, we will have 80% power to detect an odds ratio of 1.9.

Assuming a 30% attrition and a 1% rate of non-COVID-19 participants developing a comorbid condition during follow-up, there is over 80% power to detect an odds ratio of 2.5 (aim 2). A 5% rate in the non-cases will give more than 90% power to detect odds ratios >2. We believe that the effect sizes on which we have powered the study are both feasible, based on previous research (37), and important from a public health standpoint.

DISCUSSION

The ongoing Arizona CoVHORT study, with 2,881 participants already enrolled, will further the understanding of the acute and chronic effects of SARS-CoV-2 infection in a diverse Southwestern U.S. population. Our prospective approach is ideal for investigating the outcomes of infection and disease course. The data we collect will allow us to identify risk factors and exposures that are associated with both the acute symptomology of COVID-19 and the development of post-COVID-19 health conditions. This study will provide an understanding of the yet uncharacterized chronic sequelae following recovery from COVID-19.

This study has many strengths, one of which is its collaborative population-based approach, which has yet to be implemented for an infectious disease other than coccidioidomycosis (Valley Fever) in the state of Arizona. Our approach includes various recruitment methods in place involving collaborations with local health departments, multiple Arizona universities, and investigators from other COVID-19-related research studies. In addition to the multiple modalities for participation, we have the means to develop a cohort that is representative of the demographic profile of the state of Arizona. This will ultimately lead to more generalizable data and conclusions regarding the causes and consequences of COVID-19. These partnerships and collaborations also allow us to recruit from a population of AZ residents with confirmed COVID-19 by conducting case investigations for state and local health departments. Our research team is comprised of transdisciplinary researchers with a range of expertise. That ensures that the tools we use are appropriate and that we have connections across populations. Furthermore, the participant study schedule and data collected from this cohort form a rich source of recruitment for sub-studies to address more focused research questions pertaining to specific topics or special populations, such as children, the elderly, or pregnant women.

Due to the survey-based nature of our study design, we acknowledge the limitations to data collection in some of the topic areas included in our surveys, including any biases introduced by self-reported data. We utilize validated questionnaires and scales throughout the survey to reduce these biases. Participant retention for the full 2 years of follow-up remains a major potential limitation. However, we are implanting strategies to retain participants for the duration of the study that include texting and email notifications to participants informing them of their next soon arriving questionnaire, reminders if questionnaires are not returned or entered within specified time periods, identification of alternate e-mails, small gift incentives to thank them for their participation, as well as letters conveying our appreciation and the importance of the research. Finally, to address any potential limitations on diversity of the cohort population, we are implementing specific recruitment efforts with tribal nations and Latinx populations of Arizona.

ETHICS STATEMENT

This study involving human participants was reviewed and approved by the Institutional Review Board of the University of Arizona Human Subjects Protection Program. Written informed consent to participate in this study was provided by the participants or the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

KP-B, LF, MJ, MB, RH, ZC, YK, KE, EJ, and EA conceptualized the study and developed the initial study protocol. KH, CC, EA, AS, SK, JRH, and KP-B participated in the design of the protocol. All authors critically reviewed the draft of the manuscript and approved the final version.

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Can ACE2 Receptor Polymorphism Predict Species Susceptibility to SARS-CoV-2?

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A novel severe acute respiratory syndrome coronavirus, SARS-CoV-2, emerged in China in December 2019 and spread worldwide, causing more than 1.3 million deaths in 11 months. Similar to the human SARS-CoV, SARS-CoV-2 shares strong sequence homologies with a sarbecovirus circulating in *Rhinolophus affinis* bats. Because bats are expected to be able to transmit their coronaviruses to intermediate animal hosts that in turn are a source of viruses able to cross species barriers and infect humans (so-called spillover model), the identification of an intermediate animal reservoir was the subject of intense researches. It was claimed that a reptile (*Ophiophagus hannah*) was the intermediate host. This hypothesis was quickly ruled out and replaced by the pangolin (*Manis javanica*) hypothesis. Yet, pangolin was also recently exonerated from SARS-CoV-2 transmission to humans, leaving other animal species as presumed guilty. Guided by the spillover model, several laboratories investigated *in silico* the species polymorphism of the angiotensin I converting enzyme 2 (ACE2) to find the best fits with the SARS-CoV-2 spike receptor-binding site. Following the same strategy, we used multi-sequence alignment, 3-D structure analysis, and electrostatic potential surface generation of ACE2 variants to predict their binding capacity to SARS-CoV-2. We report evidence that such simple *in silico* investigation is a powerful tool to quickly screen which species are potentially susceptible to SARS-CoV-2. However, possible receptor binding does not necessarily lead to successful replication in host. Therefore, we also discuss here the limitations of these *in silico* approaches in our quest on the origins of COVID-19 pandemic.

Keywords: COVID-19, SARS-CoV-2, coronavirus, ACE2, *in silico* analyses

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INTRODUCTION

The recent emergence of SARS-CoV-2, responsible for a respiratory disease named COVID-19 (Coronavirus Disease-2019), threatens public health (1–4). SARS-CoV-2 is responsible for respiratory infections, frequently asymptomatic but sometimes progresses to pneumonia, which, in its most severe forms can lead to death. SARS-CoV-2 is spreading very rapidly worldwide, and since WHO has declared COVID-19 as pandemic, about 54.5 million people have been infected worldwide (https://coronavirus.jhu.edu/map.html; 16 November, 2020). The case fatality rate of COVID-19 (estimated about 3.29%) increases with age and the existence of underlying diseases (2, 3, 5). Recently, Fang et al. (6) reported that the most distinctive comorbidities

TABLE 1 | Viral tropism.

Receptor	Virus	Primary site of disease	Other organs
ACE2	SARS-CoV-1	Lower respiratory tract	Multi-organ failure
	SARS-CoV-2	Lower respiratory tract	Multi-organ failure
	HCoV-NL63	Upper respiratory tract	–
DPP4/CD26	MERS-CoV	Lower respiratory tract	Myocarditis, renal failure
CD13	HCoV-229E	Upper respiratory tract	Gastrointestinal
HLA Class I	HCoV-OC43	Upper respiratory tract	Gastrointestinal
	HCoV-HKU1	Upper respiratory tract	Gastrointestinal

in patients who died from COVID-19 are hypertension, coronary heart diseases, cerebrovascular diseases, and diabetes. Soon after the characterization of SARS-CoV-2, it was demonstrated that this virus uses the angiotensin I converting enzyme 2 (ACE2) expressed on pneumocytes to enter human cells (7, 8). Several recently published papers reported the SARS-CoV-2 entry into target cells through interactions with ACE2 and serine protease TMPRSS2 priming, as well as the three-dimensional (3-D) structures involved in the interactions between the viral spike (S) protein and ACE2 (9–13). The polymorphism of ACE2 in human populations was recently documented, suggesting that these allelic differences could translate into differences in susceptibility to SARS-CoV-2 infection (14, 15). Insofar as the physiological function of ACE2 is to cleave angiotensin II into angiotensin (1–7), SARS-CoV-2 infection could cause a dysregulation of this peptidase leading to risk of malfunction of the Renin–Angiotensin–Aldosterone pathway (16).

SARS-CoV-2 is the 7th human coronavirus (HCoV) reported to date. Previously, the first HCoVs described back in the 1960s were the HCoV-229E (*Alphacoronavirus*) and HCoV-OC43 (*Betacoronavirus* lineage 2a), two agents of common winter cold. In 2003, the coronaviruses gained in notoriety with the emergence in Asia of SARS-CoV (*Betacoronavirus* lineage 2b), proven responsible for a severe acute respiratory syndrome (SARS) in humans with a case fatality rate of 9.6% (17). Within the next couple of years, the HCoV-NL63 (*Alphacoronavirus* lineage 1b) and HCoV-HKU1 (*Betacoronavirus* lineage 2a) were discovered. The HCoV-HKU1 was discovered in Hong Kong. The case fatality rate of the four HCoVs responsible for common winter cold was estimated to be 0.5–1.5% (18–20). In contrast to SARS-CoV and HCoV-HKU1 that emerged in Southeast Asia suggesting that this region is probably a hotspot for coronavirus emergence, the Middle East Respiratory Syndrome (MERS), caused by the MERS-CoV (*Betacoronavirus* lineage 2c), was reported in Saudi Arabia in 2012. This epidemic was characterized by an extremely high case fatality rate of 34.7% (21). The last coronavirus known to infect humans, SARS-CoV-2 (*Betacoronavirus* lineage 2b/Sarbecovirus), emerged in China in 2019 and shows 79.5% nucleotide identity with SARS-CoV (22). It is interesting to highlight that HCoV-NL63, SARS-CoV, and SARS-CoV-2 spike proteins bind ACE2, indicating that several members of the coronavirus family have developed a preferential tropism for this receptor to enter target cells [(23, 24); **Table 1**].

As soon as this new SARS-CoV-2 was discovered, many studies were initiated to understand the viral infection mechanisms and to clarify its origin. Consequently, the search for animal hosts was considered of high urgency for the control of COVID-19. The very first investigations focused on bats (order *Chiroptera*), which are considered a reservoir for coronaviruses (CoV) and can be a source of epizootic and zoonosis (25–27). With 1,230 species, bats have the second highest number of species (after rodents) in the mammal world. They inhabit a multitude of ecological niches and carry a huge number of zoonotic viruses worldwide (28, 29). The probability for CoV to cross species barrier is higher in Southeast Asia where bats are sold in wildlife wet markets. Different species of *Rhinolophus* bats in China carry genetically diverse SARS-like coronaviruses, some of which are direct ancestors of SARS-CoV and SARS-CoV-2 (30, 31). Based on our knowledge of coronaviruses circulating in Chinese bats, it is not a surprise that SARS-CoV-2 was also considered to have originated from *Rhinolophus* bats. This turned out to be confirmed by elegant results showing that SARS-CoV-2 shares 96.2% identity with the BatCoV RaTG13 strain from *Rhinolophus affinis* (22). Then, many laboratories started looking after an intermediate animal host. The snake (*Ophiophagus hannah*) and the pangolin (*Manis javanica*) were claimed to be intermediate hosts. The snake hypothesis was quickly ruled out (32). Although the pangolin hypothesis was the mainstream, it was also recently excluded (33, 34). At the same time, other species were singled out. To quickly study a large number of potential targets without having to grow virus on cells or infect animals with SARS-CoV-2, *in silico* approaches seemed to be a quite appropriate strategy since the three-dimensional structure of the S1 protein of SARS-CoV-2 was resolved, allowing the specification of the amino acids important for binding to ACE2 (9–13, 35). The knowledge previously accumulated on the interaction between ACE2 and the SAR-CoV spike was also of great value (23, 36, 37). Interestingly, K353 and N90 in ACE2 are essential for infection likely due to their effect on the conformation of the α-helix 1 of the receptor.

We revisited here the predicted binding properties between the viral S protein of SARS-CoV-2 and its ACE2 receptor, using *in silico* analysis based on alignment of receptor protein sequences from different species and structural modeling of ACE2 receptors. We found a good match between the *in silico* predictions of virus tropism and the species already considered to be possible intermediates between bats and humans for transmission of SARS-CoV-2. We report that positions K31 and Y41 in the α1 ridge, N82 and N90 in the loop, and α3 and K353 in loop and β5 are those that must be examined in order to predict the possibility of a species to become infected by SARS-CoV-2. In agreement with previous reports suggesting that exchange N90T destroys a major N-glycosylation site in ACE2 (9, 10, 38), we confirm that N90 is likely a critical position in ACE2 for SARS-CoV-2 binding. The analysis of electrostatic potential surface generation of ACE2 variants highlight minor differences in surface charges for mouse and frog sequence insertions compatible with lower susceptibility of these species to SARS-CoV-2. Finally, the broad spectrum of potentially susceptible species argues in favor of

the circulation model (33) rather than in favor of the spillover model (39).

MATERIALS AND METHODS

ACE2 Protein Sequence

ACE2 protein sequences from the NCBI reference sequence database: *Rousettus leschenaultii* (GenBank: ADJ19219.1), *Rousettus leschenaultii* (GenBank: BAF50705.1), *Rousettus aegyptiacus* (NCBI Reference Sequence: XP_015974412.1), *Pteropus alecto* (NCBI Reference Sequence: XP_006911709.1), *Pteropus vampyrus* (NCBI Reference Sequence: XP_011361275.1), *Phyllostomus discolor* (NCBI Reference Sequence: XP_028378317.1), *Desmodus rotundus* (NCBI Reference Sequence: XP_024425698.1), *Miniopterus natalensis* (NCBI Reference Sequence: XP_016058453.1), *Pipistrellus abramus* (GenBank: ACT66266.1), *Eptesicus fuscus* (NCBI Reference Sequence: XP_008153150.1), *Myotis davidii* (NCBI Reference Sequence: XP_015426918.1), *Myotis lucifugus* (NCBI Reference Sequence: XP_023609438.1), *Myotis brandtii* (NCBI Reference Sequence: XP_014399780.1), *Hipposideros armiger* (NCBI Reference Sequence: XP_019522936.1), *Rhinolophus ferrumequinum* (GenBank: ADN93470.1), *Rhinolophus pearsonii* (GenBank: ABU54053.1), *Rhinolophus sinicus* (GenBank: AGZ48803.1), *Rhinolophus pusillus* (GenBank: ADN93477.1), *Rhinolophus macrotis* (GenBank: ADN93471.1), *Homo sapiens* (GenBank: BAB40370.1), *Macaca mulatta* (NCBI Reference Sequence: NP_001129168.1), *Paguma larvata* (GenBank: AAX63775.1), *Felis catus* (GenBank: AAX59005.1), *Mustela putorius furo* (NCBI Reference Sequence: NP_001297119.1), *Sus scrofa domestic* (GenBank: ASK12083.1), *Sus scrofa* (NCBI Reference Sequence: NP_001116542.1), *Rhinolophus sinicus* (GenBank: AGZ48803.1), *Manis javanica* (NCBI Reference Sequence: XP_017505752.1), *Mus musculus* (NCBI Reference Sequence: NP_081562.2), *Rattus rattus* (NCBI Reference Sequence: XP_032746145.1), *Gallus gallus* (GenBank: QEQ50331.1), *Pelodiscus sinensis* (NCBI Reference Sequence: XP_006122891.1), *Xenopus tropicalis* (NCBI Reference Sequence: XP_002938293.2), and *Ophiophagus hannah* (GenBank: ETE61880.1).

Comparison of Sequences

All ACE2 sequences were compared using the Clustal Omega multiple sequence alignment (EMBL-EBI bioinformatic tool; Copyright © EMBL 2020) (https://www.ebi.ac.uk/Tools/msa/clustalo/). The simple Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm of hierarchical clustering available under the Clustal Omega tool was used to produce rooted dendrogram (pairwise similarity matrix to construct a phylogenetic tree).

3-D Analysis and Electrostatic Potential Surface Generation

The 3-D structure of ACE2 was retrieved according to the published data [PDB: 6M1D; (11)]. Three amino acid segments (30–41, 82–93, and 353–358) from *R. sinicus*, *M. musculus*, and *X. tropicalis* ACE2 proteins were inserted into a human ACE2 backbone sequence to determine whether or not these substitutions may change the 3-D structure of ACE2. Protein modeling for these chimeric sequences was performed using the Phyre2 server (40). The PyMOL 1.8.0 software (https://sourceforge.net/projects/pymol/files/pymol/1.8/) and the Adaptative Poisson-Boltzmann Solver (APBS) tools plugin (https://pymolwiki.org/index.php/APBS) were used to generate electrostatic potential surfaces of the human ACE2 and its modified chimeric versions. The red color indicates an excess of negative charges near the surface and the blue color arises from a positively charged surface.

RESULTS

ACE2 Receptor Polymorphism Among Species

Using multiple sequence alignment, we first compared the ACE2 sequences of 18 bat species. We found that the variant ACE2 proteins perfectly grouped in the dendrogram according to the subspecies of bats (**Figure 1A**). When we studied the multiple sequence alignments of ACE2 from bats and examined the regions predicted by crystallography to be the regions of contact with the S1 spike of the SARS-CoV-2 (**Figure 1B**), we observed significant differences between species. *Rhinolophus* bats appeared to be appropriate candidates for binding to SARS-CoV-2-related viruses, yet a species polymorphism was observed among the *Rhinolophus* (i.e., *R. sinicus* with K31, Y41H, N82, N90, and K353 and *R. ferrumequinum* with K31D, Y41H, N82, N90, K353). The K31D variant may possibly alter the binding of the SARS-CoV-2 spike to the ACE2 from *R. ferrumequinum*. Unfortunately, the ACE2 sequence of *R. affinis* hosting the closest relative to SAR-CoV-2, BatCoV-RaTG13, was not available from the database. The ACE2 sequences from other bat species living in various ecosystem and with various geographic distribution, show increased amino acids substitutions at positions considered to be required for viral S1 spike binding (e.g., *D. rotundus* with K31N, Y41, N82T, N90D, and K353N). It is worth noting that the three *Rousettus* and two *Pteropus* ACE2 proteins analyzed in this study were characterized by K31, Y41, and N82T (*Rousettus*) or N82A (*Pteropus*), N90D, and K353. We also found that the three ACE2 proteins from *Myotis* bats were characterized by K31N, Y41H, N82T, N90, and K353, suggesting that these species are unlikely to replicate SARS-CoV-2-like ancestor-related viruses.

The Central Role Played by ACE2 for Interspecies Virus Spread

Unraveling which cellular receptors are used by SARS-CoV-2 for entry should provide insights into viral transmission among species. Before SARS-CoV-2, SARS-like CoV was previously found to circulate in Chinese horseshoe bats and to spread through wild Himalayan palm-civet sold as food in Chinese wildlife markets from Guangdong (41–43). SARS-CoV was also identified in weasels and raccoons in Chinese wet markets (37, 41, 43). Regarding SARS-CoV-2, the question remains how it got to humans. The hypothesis of pangolin (*M. javanica*) as an intermediate host for SARS-CoV-2 quickly became mainstream

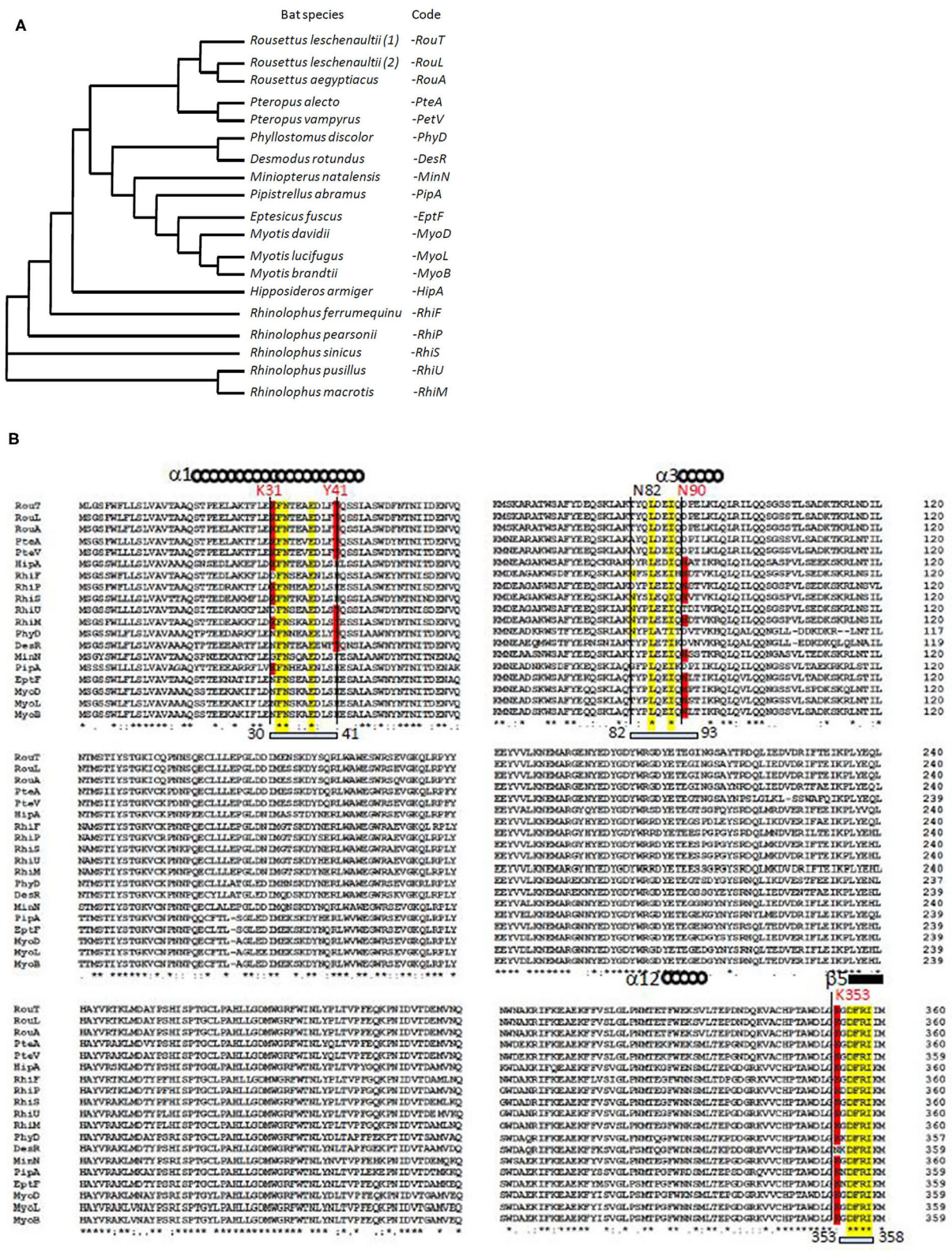

FIGURE 1 | Bat ACE2 sequence alignment. The ACE2 protein sequences from 18 species of bats were obtained from the NCBI reference sequence database: *Rousettus leschenaultii*, *Rousettus aegyptiacus*, *Pteropus alecto*, *Pteropus vampyrus*, *Phyllostomus discolor*, *Desmodus rotundus*, *Miniopterus natalensis*, *Pipistrellus*

(Continued)

FIGURE 1 | *abramus*, *Eptesicus fuscus*, *Myotis davidii*, *Myotis lucifugus*, *Myotis brandtii*, *Hipposideros armiger*, *Rhinolophus ferrumequinum*, *Rhinolophus pearsonii*, *Rhinolophus sinicus*, *Rhinolophus pusillus*, and *Rhinolophus macrotis*. Clustal Omega multiple sequence alignment (EMBL-EBI bioinformatic tool; Copyright © EMBL 2020) was used to compare the ACE2 protein sequences of these mammals considered at the origin of human coronaviruses. **(A)** Phylogenetic tree of bat ACE2 sequences built using the Clustal Omega multiple sequence alignment program and the UPGMA algorithm. The short code is used in **(B)**. **(B)** Sequences alignment of bat ACE2 N-terminal (amino acids 1–360 of 805) protein sequences. Some of the amino acids important for viral tropism are in red (previous studies showed that residues 31 and 41 and regions 82–84 and 353–357 are important for viral spike binding). Within the regions considered important for the interaction with the spike of SARS-CoV-2, the conserved amino acids are in yellow.

(32, 44). We recently demonstrated that pangolin is unlikely to be the intermediate host and that transmission to humans could just as easily have taken place via another animal (33).

We investigated the amino acid substitutions in 14 species of mammals, birds, reptiles, and amphibians, expected to be possible intermediate hosts for SARS-CoV-2 (**Figure 2**). Beside positions K31, Y41, and K353 reported in several studies to have been playing a major role for SARS-CoV-2 spike binding to ACE2, our multisequence alignment suggested that species carrying an N90 are more likely to be susceptible to SARS-CoV-2 infection (it includes *H. sapiens*, *M. mulatta*, *F. catus*, *R. sinicus*, *M. javanica*, and *P. sinensis*) while others should be less susceptible to infection, except if the virus adapts to a second receptor for cellular binding and entry.

Amino Acids K31, Y41, N90, and K353 in ACE2 Are Likely to Confer Susceptibility to SARS-CoV-2

The analysis of 3-D structures of different ACE2 with respect to the amino acids found in regions 30–41, 82–93, and 353–358 was studied after designing a backbone from the *H. sapiens* ACE2 in which the corresponding regions from *R. sinicus*, *M. musculus*, and *X. tropicalis* species were substituted to that from human. We found (**Figure 3A**) that these substitutions did not change the global 3-D structure of the molecule. However, when we analyzed the electrostatic potential surface of ACE2, more particularly in the regions 30–41, 82–93, and 353–358, we found that the substitution of those human ACE2 segments by the corresponding regions from *R. sinicus*, *M. musculus*, and *X. tropicalis* species slightly altered the electrostatic pattern of the molecule (**Figure 3B**). Indeed, in the region where amino acids Y41 and K353 are located in the human ACE2, when this region was substituted by sequences from mouse or frog origins, we observed a shift from neutral to basic electrostatic surface whereas the substitution for bat sequence did not change the electrostatic charge. The electrostatic surface was also different when the region containing K31 was substituted by that from bat or frog. These modifications are likely to be sufficient to reduce the interaction between SARS-CoV-2 spike and the variant ACE2.

DISCUSSION

Soon after the discovery of SARS-CoV-2, the cell surface exopeptidase ACE2 was found to serve as a viral receptor in human, and the first investigation of species susceptibility to this new virus demonstrated that SARS-CoV-2 is able to use Chinese horseshoe bat and swine but not mouse ACE2 to bind host cells (22). Since this pioneering work, several laboratories have intended to predict the utilizing capability by SARS-CoV-2 of ACE2 from different species using amino acid sequence comparisons aimed at identifying the possible intermediate hosts of SARS-CoV-2. This was made possible after published crystallographic analyses had determined which amino acids of ACE2 are essential for the attachment of the viral spike protein (9–11).

Our investigation suggests that SARS-CoV-like ancestral coronaviruses have adapted to the ACE2 receptor to replicate in bats. However, our analysis also suggests that probably not all bat species support SARS-CoV-like coronavirus ACE2 tropism. According to multisequence alignment, *Rhinolophus* bats appear to be appropriate candidates for ACE2 interaction with SARS-CoV-2-related viruses, yet a species polymorphism in ACE2 sequences is observed among the *Rhinolophus*. *R. sinicus* with K31, Y41H, N82, N90, and K353 is a good candidate for SARS-CoV-2-like virus capture whereas *R. ferrumequinum* with K31D, Y41H, N82, N90, and K353 can be predicted less susceptible to the virus binding. ACE2 sequences from other bat species show increased amino acid substitutions at positions considered required for viral spike binding (e.g., *D. rotundus* with K31N, Y41, N82T, N90D, and K353N). In species expressing variant ACE2 not suitable for virus binding, another surface receptor could serve as viral entry into cells, but such viruses will be less likely to cross species barriers using an ACE2 protein as receptor in an intermediate host species. This can support the hypothesis of a long bat and virus co-evolution with bat species that replicate ACE2-tropic viruses like SARS-CoV and other species that replicate CD26-tropic viruses like MERS-CoV.

In order that a SARS-CoV-2-like virus can leave bats to infect another susceptible host, the infected bat must come into contact with an animal expressing an ACE2 receptor adapted to SARS-CoV-2-like virus binding. In agreement with other studies (44–46), our *in silico* search for host species able to pass the SARS-CoV-2 to humans supports the hypothesis that species bearing K31 and K353 amino acids are more likely to bind SARS-CoV-2. For example, ACE2 from *M. javanica*, *M. putorius furo*, and *F. catus*, considered SARS-CoV-2-susceptible species, show K31 and K353 amino acids whereas *M. musculus*, which is considered a SARS-CoV-2-resistant species, shows a K31N and K353H variant. A Y41 also seems to be important, yet *R. sinicus* ACE2 expresses a Y41H variant. It may account for the requirement of an intermediate host before being able to infect humans. A position not particularly stressed out in several SARS-CoV-2 studies that appear important is N90. Indeed, the

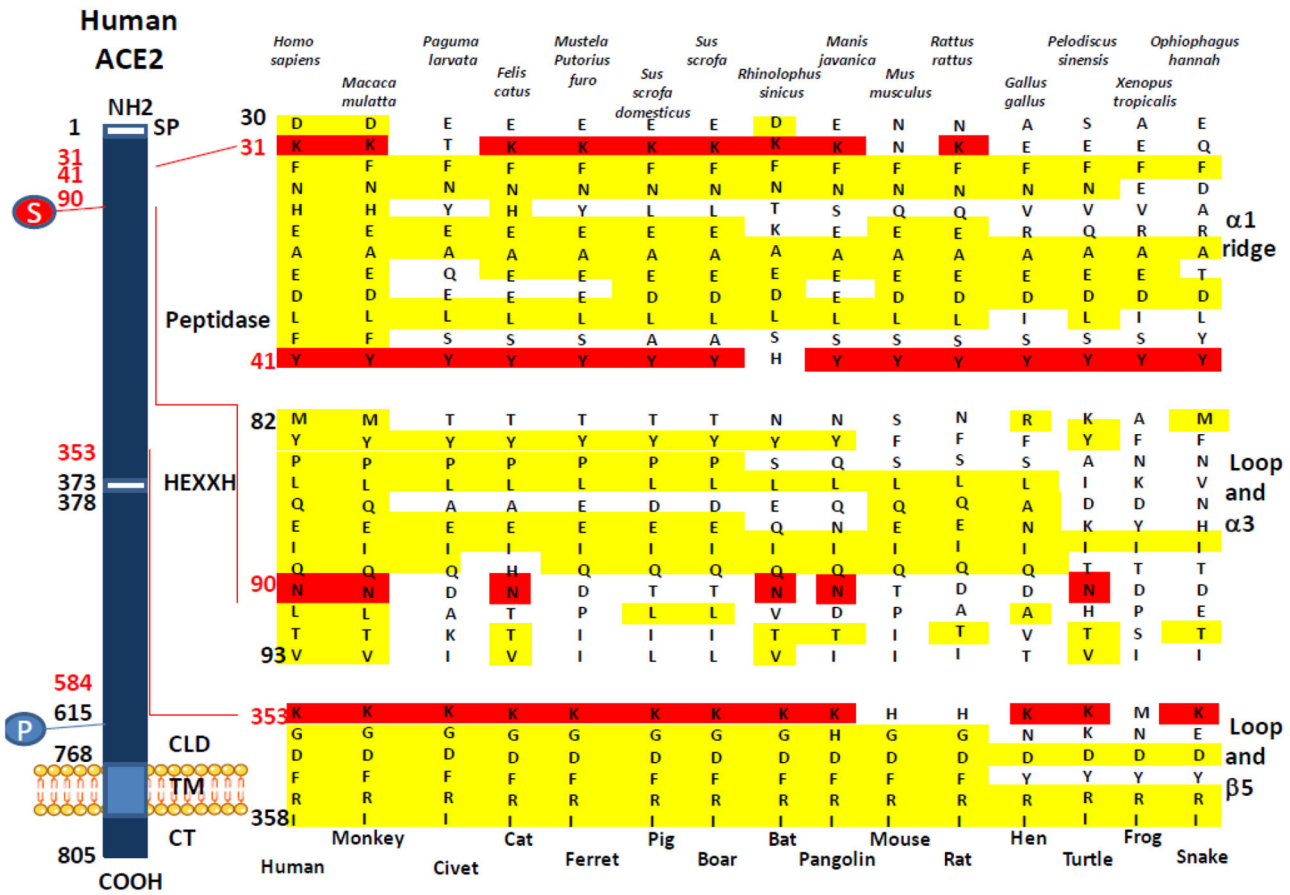

FIGURE 2 | Comparison of the *Homo sapiens* ACE2 protein sequence and sequences from different mammals, birds, amphibians, and reptiles. A schematic representation of the cell surface human ACE2 molecule and its major domains is drawn on left side of the figure. The amino acid positions are in black. Some of the amino acids considered important for viral tropism are in red. S, sugar; P, phosphorylation. A comparison of ACE2 sequences from 15 different species using Clustal Omega multiple sequence alignment is shown in the right side of the figure. All sequences were numbered according to amino acid position on the *H. sapiens* ACE2 protein. All sequences were obtained from the NCBI reference sequence database. They include *Macaca mulatta* (monkey), *Paguma larvata* (palm civet), *Felis catus* (cat), *Mustela putorius furo* (ferret), *Sus scrofa domestic* (pig), *Sus scrofa* (boar), *Rhinolophus sinicus* (bat), *Manis javanica* (pangolin), *Mus musculus* (mouse), *Rattus rattus* (rat), *Gallus gallus* (hen), *Pelodiscus sinensis* (turtle), *Xenopus tropicalis* (frog), and *Ophiophagus hannah* (snake).

N90 that is found in *H. sapiens* final host and *R. sinicus* early host is also found in *M. mulatta*, *M. javanica*, *F. catus*, and *P. sinensis*, previously described susceptible to SARS-CoV-2 and possible intermediate hosts whereas N90D or N90T variants are found in the other species studied. This is also consistent with the earlier observation indicating that N90 was important for SARS-CoV and SARS-CoV-2 binding to ACE2 (9, 10, 23, 38). This may explain why N90 may be very important for infection of host cells. Among the various *in vitro* antiviral activities of chloroquine described to date, it has been suggested that this molecule could prevent the glycosylation of ACE2 (47, 48). We could hypothesize that chloroquine blocks the N-glycosylation at position 90 of the ACE2 sequence, thereby preventing the attachment of SARS-CoV-2 spike to the receptor. However, what is surprising is the sequence of the *P. larvata* with K31T, Y41, N82T, N90D, and K353, since palm civet has been considered as the intermediate host for SARS-CoV and suggested to also serve as a possible intermediate host for SARS-CoV-2 (45), whereas with the absence of K31, in addition to N82 and N90 (which are expected to be glycosylated, thereby favoring interaction with the viral spike), palm civet appears to be an animal unlikely to be infected through ACE2. This discrepancy, also noticed by Wan et al. (35), should be further explored. Another surprising result is the absence of an N90 glycosylation site in the ACE2 from *M. putorius furo* since ferrets are now known to be susceptible to infection with SARS-CoV-2 (49–52). It would be interesting to study the affinity of the SARS-CoV-2 spike for the ferret ACE2. So far, the binding of SARS-CoV-2 to another viral entry receptor than ACE2 cannot be excluded. Very recently, it was reported that the enhanced human spreading of SARS-CoV-2 compared to SARS-CoV could possibly be explained by the presence of a polybasic furin type cleavage site at the S1/S2 junction in the SARS-CoV-2 spike, which is not found in SARS-CoV and that neuropilin-1 (NRP1) known to bind furin-cleaved substrates could be an entry cofactor that potentiates SARS-CoV-2 infectivity (53).

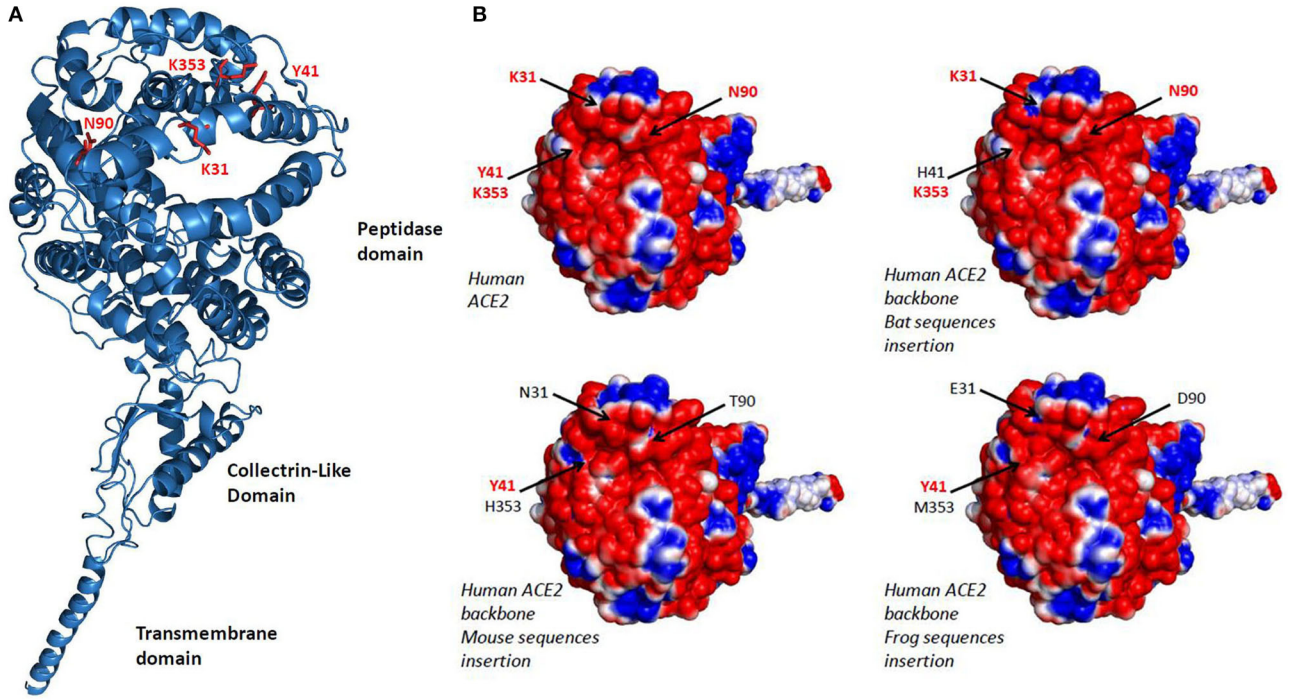

FIGURE 3 | 3-D model of ACE2. **(A)** Variant amino acids segments (30–41, 82–93, and 353–358) from *Rhinolophus sinicus*, *Mus musculus*, and *Xenopus tropicalis* species were superimposed on the *Homo sapiens* ACE2 3-D structure using the Phyre2 server. This model lacks the cytoplasmic tail of ACE2. **(B)** An electrostatic potential surface was generated using the PyMOL 1.8.0 software along with the APBS tool plugin. The upper left panel is a model of *H. sapiens* ACE2 extracellular region with its electrostatic potential distribution (red, acidic; white, neutral; blue, basic). The upper right and the two lower images represent simulation in which the α1 ridge, loop and α3, and loop and β5 (see **Figure 2**, right panel) sequences from *R. sinicus* (bat), *M. musculus* (mouse), and *X. tropicalis* (frog) were substituted to the corresponding human sequences in a *H. sapiens* ACE2 backbone. The locations of amino acids 31, 41, 90, and 353 are indicated by arrows.

Obviously, not all the species expressing an ACE2 predicted to bind SARS-CoV-2 are expected to be susceptible to infection by SARS-CoV-2. *In silico* studies focused on ACE2 protein polymorphism among species together with focused attention on amino acids expected to play a crucial role in the viral spike binding are suitable to predict ACE2 proteins susceptible to bind SARS-CoV-2 and can provide important clues regarding possible intermediate hosts or simply susceptible hosts. The ACE2 protein should contain amino acids essential for the viral spike binding and variants of ACE2 that lack such amino acids are not likely to allow virus binding. An impressive study combining phylogenetic analysis and critical site marking to predict the utilizing capability of ACE2 recently reported by Qiu et al. (46) compared the ACE2 sequences from 250 species with a specific focus on T20, K31, Y41, K68, Y83, S218, A246, K353, D355, R357, M383, P426, T593, N636, A714, R716, and A774 and concluded that SARS-CoV-2 might bind *M. javanica* (pangolin), *F. catus* (cat), *Bos taurus* (cow), *Bubalus* (buffalo), *Capra hircus* (goat), *Ovis aries* (sheep), and *Columba livia* (pigeon) ACE2 but not (*M. musculus*) murine ACE2. They also suggested to pay attention to *Protobothrops mucrosquamatus* (pallas pit viper), a common snake living in the Hubei Province of China. In their study, Luan et al. (45), investigated 42 mammalian ACE2 proteins from the wild animal protection list of Hubei Province. The authors focused on key amino acids K31, E35, D38, M82, and K353. According to their predictions, they considered that beside humans, the mammals whose ACE2 could bind to the S1 protein of SARSCoV-2 are bats (*Rhinolophus macrotis*, *Rhinolophus sinicus*, *Rhinolophus pearsonii*, *Pteropus vampyrus*, and *Rousettus leschenaultii*), pangolin (*Manis javanica*), palm civet (*Paguma larvata*), monkeys (*Macaca mulatta*, *Pan troglodytes*, *Pongo abelii, Papio Anubis,* and *Callithrix jacchus*), cat (*Felis catus*), dog (*Canis lupus familiaris*), ferret (*Mustela putorius furo*), and pig (*Sus scrofa domesticus*), among others (*Rhinopithecus roxellana*, *Mustela erminea*, *Sus scrofa*, *Equus caballus*, *Bos taurus*, *Ovis aries*, *Oryctolagus cuniculus*, *Vulpes*, *Phodopus campbelli*, *Mesocricetus auratus*, *Heterocephalus glaber*, *Ictidomys tridecemlineatus*, and *Cricetulus griseus*). The mammals whose ACE2 appeared unable to bind the S1 protein of SARS-CoV-2 included *Rhinolophus ferrumequinum* bats, rat (*Rattus norvegicus*), mouse (*Mus musculus*), camel (*Camelus dromedarius*), and others (*Procyon lotor*, *Ornithorhynchus anatinus*, *Loxodonta africana*, *Erinaceus europaeus*, *Nyctereutes procyonoides*, *Suricata suricatta*, *Dipodomys ordii*, and *Cavia porcellus*). They draw particular attention to the N82 amino acid in the ACE2 protein. Another study by Liu et al. (44), based on prediction of interactions between the S1 protein of SARS-CoV-2 and ACE2, that investigated monkey (*Gorilla*, *Macaca*), bat (*Rhinolophus sinicus*; *Rhinolophus pearsonii*), pangolin (*Manis javanica*), snake

(*Ophiophagus hannah*), turtles (*Chrysemys picta bellii*, *Chelonia mydas*, and *Pelodiscus. sinensis*), and others (dog, cat, mouse), stressed a possible role as intermediate host animal reservoir for turtles. This study, which focused on positions T27, F28, D30, K31, H34, D38, Y41, Q42, M82, E329, K353, G354, D355, and R357, indicated that mouse and dog ACE2 showed multiple substitutions (>5) among the 14 amino acids that retained their attention, an observation in agreement with the relative resistance of these species to infection by SARS-CoV-2. They suggested K31, Y41, and K353 to be key amino acids for viral spike binding. In recent weeks, several *in silico* studies aimed at finding an intermediate host have been published. Luan et al. (45) ruled out turtle and snake from the potential host list of SARS-CoV-2 and suggested that pangolin ACE2 was predicted to recognize SARS-CoV-2 less efficiently because it only preserved 14 of 20 critical amino acids they investigated, but found that primates, Bovidae, Cricetidae, and Cetacea (*Neophocaena asiaeorientalis asiaeorientalis*, found in the Yangtze River near Wuhan), are capable to recognize the RDB in S1 of SARS-CoV-2. A very elegant work by Damas et al. (54) scored 25 amino acids considered by this team as important for interaction between SARS-CoV-2 spike and ACE2 and they identified possible interaction for 252 mammal species, 72 birds, 65 fishes, 17 reptiles, and 4 amphibian ACE2 orthologs. It is worth noting that species scoring very low in Damas' study included the Chinese pangolin, Sunda pangolin, and white-bellied pangolin. Among Carnivora, 9/43 had the highest score including the domestic cat. Similar approaches that indicate a broad range of possible animal targets for SARS-CoV-2 are currently under evaluation for publication (44, 55). We can therefore also retain from these studies that, according to *in silico* analyses, numerous species are potentially susceptible to infection by SARS-CoV-2. This is a strong argument in favor of the virus circulation model in which there is not a single intermediate host but many susceptible species (33).

Although the *in silico* studies have the advantage of being easy to perform and to allow a quick investigation of the probability of SARS-CoV-2 infection for a large number of species, this strategy has its limits, and possible receptor binding does not necessarily mean successful replication in host. Once in the host, the virus should counteract the cell restriction factors and antiviral immune defense. Nothing can replace *in vitro* and *in vivo* experimentation. *In vitro*, SARS-CoV-2 was found to be able to infect and replicate on human Calu3 and Caco2 cell lines, VeroE6 and FRhK4 from non-human primate cell lines, LLCMK2 (monkey), RK-13 (Rabbit), PK-15 (pig), and CRFK (cat) cell lines (56). Interesting observations reported online (not peer reviewed) indicate that multiple ACE2 orthologs, human (*H. sapiens*), rhesus monkey (*M. mulatta*), dog (*C. lupus familiaris*), cat (*F. catus*), rabbit (*O. cuniculus*), and pangolin, can serve as receptors for SARS-CoV-2 when transiently expressed in 293T cells, whereas rat (*R. norvegicus*) ACE2 does not (8).

Even when cells from a species are susceptible to SARS-CoV-2, this does not always translate into disease. Although it is more fastidious work than *in silico* and *in vitro* approach, evidence supporting that a species is susceptible to SARS-CoV-2 and can develop COVID-19-like symptoms can only be defined after *in vivo* infection. Interestingly golden Syrian hamster (*M. auratus*) and Chinese hamster (*C. griseus*) are known as animal models for SARS-CoV (57, 58). More recently, the golden Syrian hamster has been established as a model to study the transmission of SARS-CoV-2 and the pathogenesis of COVID-19 (59, 60). Monkeys (*M. mulatta*, *Macaca fascicularis*, and *Chlorocebus aethiops*) were also found to be animal models for SARS-CoV with reports of pneumonitis in infected monkeys (61, 62). With SARS-CoV-2, monkeys (*M. mulatta* and *M. fascicularis*) were found to be susceptible to the virus and develop mild disease COVID-19-like signs after infection (63, 64). Ferrets (*M. putorius furo*) were also used as an animal model for SARS-CoV and showed productive infection (65, 66). This species also was found to be susceptible to SARS-CoV-2 and develop mild disease COVID-19-like signs after infection (49**?** –51). It was previously reported that young inbred mice supported SARS-CoV viral replication but failed to show clinical signs of disease (67, 68). Although mouse (*M. musculus*) ACE2 was considered unable to bind SARS-CoV-2 spike (35) and unable to support SARS-CoV-2 replication and disease development (69), it was reported that *M. musculus* transgenic for the human ACE2 gene are susceptible to infection by SARS-CoV-2 and develop mild disease COVID-19-like signs after viral exposure (38, 69). The paper recently published by Shi et al. (52) describes the investigation of the *in vivo* susceptibility of animals to replicate SARS-CoV-2. The authors reported that the virus replicated poorly in dogs, pigs, chickens, and ducks but efficiently infected ferrets and cats. In addition, these authors found that the virus can be transmitted from cat to cat through respiratory droplets. This result agrees with the report of experimental cat-to-cat transmission of SARS-CoV-2 (70) and human-to-cat transmission of SARS-CoV-2 (71). The accidental transmission of SARS-CoV-2 to tigers and lions at the Bronx Zoo (72) and minks (73) was also reported. Finally, Schlottau et al. (50) reported that pig and chickens were not susceptible to SARS-CoV-2 infection, whereas efficient virus replication was found in ferrets and fruit bats. The results obtained in our *in silico* study were compared with those of *in vivo* infection reported by different research teams (**Table 2**), and we observed a good match between the two experimental approaches.

Finally, if the absence of productive infection in animal models makes it possible to exclude certain species from the dynamics of transmission of SARS-CoV-2 to humans, the finding of productive infection provides little information on the origin of the human COVID-19 epidemic and pandemic. Human epidemic can only occur when there is a contact between human and an infected species, when this pathogen is compatible with human, and when human-to-human urban cycle is possible. The spillover model of virus transmission theorizes that the virus is developing into an epizootic stage in an animal population, reaching the threshold requirement for interspecies transmission (39). Thus, based on this model, identifying an animal reservoir appears to be essential to eradicate the disease by eliminating the infected animal host species. However, what we observe from the increasing number of reports aimed at identifying an animal reservoir is that numerous animal species are susceptible to SARS-CoV-2 and that no epizootics was reported with a SARS-CoV-2-like ancestral virus. This is the reason why it was recently

TABLE 2 | Correlation between *in silico* ACE2 binding prediction and *in vivo* SARS-CoV-2 infection.

Species	Probability of SARS-CoV-2 binding to ACE2 (*in silico* prediction and score)	*In vivo* SARS-CoV-2 replication (G clade virus)	Bibliographical references for the *in vivo* experimental infections	Agreement between *in silico* prediction and *in vivo* data
Human	Yes (Score 5)*	COVID-19 outbreak	(1–3)	Reference model
Monkey	Yes (Score 5)	Susceptible (COVID-19-like signs)	(63, 64)	Yes
Civet	No (Score 2)	Not tested		Not applicable
Cat	Yes (Score 4)	Susceptible to infection	(52, 70)	Yes
Ferret	Yes (Score 3)	Susceptible (COVID-19-like signs)	(49, 50, 52)	Yes
Pig	Yes (Score 3)	Susceptible, yet the virus replicates poorly	(50, 52)	Yes
Boar	Yes (Score 3)	Not tested		Not applicable
Bat	Yes (Score 3)	Susceptible to infection	(50)	Yes
Pangolin	Yes (Score 4)	Not tested		Not applicable
Mouse	No (Score 1)	Resistant to infection (hACE2 humanized mice are susceptible to infection and show interstitial pneumonia)	(38, 69, 74)	Yes
Rat	No (Score 2)	Not tested		Not applicable
Hen	No (Score 2)	Not tested		Not applicable
Turtle	Yes (Score 3)	Not tested		Not applicable
Frog	No (Score 1)	Not tested		Not applicable
Snake	No (Score 2)	Not tested		Not applicable

**Score 5: K31, Y41, N90, K353 (+4/4) and no mutation in regions 31–41, 82–93, and 353–358 with respect to the human ACE2 (hACE2) sequence (+1); Score 4: A change for one of the positions K31, Y41,N90, or K353 (+3/4), and no mutation in regions 31–41, 82–93, and/or 353–358 (+1) or K31, Y41, N90, and K353 (+4/4), and mutations in regions 31–41, 82–93, and/or 353–358 (0/1); Score 3: two variants at positions K31, Y41,N90, or K353 (+2/4) and no mutation in regions 31–41, 82–93, and 353–358 (+1), or a change for one of the positions K31, Y41, N90, or K353 (+3/4) and mutations in regions 31–41, 82–93, and/or 353–358 (0/1); Score 2: three variants at positions K31, Y41,N90, or K353 (+1/4) and no mutation in regions 31–41, 82–93, and/or 353–358 (+1) or two variants at positions K31, Y41, N90, or K353 (+2/4) and mutations in regions 31–41, 82–93, and/or 353–358 (0/1); Score 1: three variants at positions K31, Y41, N90, or K353 (+1/4) and mutations in regions 31–41, 82–93, and/or 353–358 (0/1). Arbitrary cut off: it was considered that a score ≥3 is predictive of attachment of the viral spike to ACE2 that can lead to infection.*

suggested to consider a new model, the circulation model, which assumes that there is a broad circulation of virus in different species, and no requirement for zoonotic pressure or epizootic episode prior to the COVID-19 emergence in human (33). According to this new model, if the SARS-CoV-2-like ancestral virus can meet a host, if the virus spike RBD can bind ACE2 molecule even at low affinity, and if the target cells can be productively infected, then the adaptation to the host simply undergoes a quasispecies evolution process. So the scenario that can be suggested here is that the virus was circulating in many species, that following contact between one of these species and humans, a SARS-CoV-2-like virus came into contact with the ACE2 protein at the surface of human lung epithelial cells allowing infection to occur. ACE2 (100-kDa type I cell-surface glycoprotein of 805 amino acids) is expressed on both type I and type II alveolar epithelial lung cells as well as epithelial cells of oral mucosa, enterocytes of the small intestine, and arterial and venous endothelial cells contributing to the COVID-19 disease (38, 75–78). Currently, SARS-CoV-2 is expected to undergo a quasispecies evolution process generating post-infection mutations under host-driven positive selection pressure (32, 79–83).

In conclusion, our results suggest that species carrying a sequence with K31, Y41, N90, and K353 are likely to be susceptible to infection by SARS-CoV-2 (including *H. sapiens*, *M. mulatta*, *F. catus*, *R. sinicus*, *M. javanica*, and *P. sinensis*) while others should be less susceptible or resistant to infection, except if the virus adapts a second receptor for cellular binding and entry. The combination of 3-D structure analysis and electrostatic potential surface indicated that the substitution of human ACE2 regions 30–41, 82–93, and 353–358 by the corresponding regions from *R. sinicus*, *M. musculus*, and *X. tropicalis* species did not significantly change the 3-D structure of ACE2 but slightly modified the electrostatic potential surface of the molecule. These modifications are likely to be sufficient to alter the interaction of SARS-CoV-2 spike with the variants ACE2. The K31 and K353 in the α-helical bundle of the ACE2 interface need to be accommodated in a largely hydrophobic environment to allow interaction with the viral spike RBD (9). The crystal structure analysis of ACE2 also suggested the presence of several hinge regions and N-glycosylations (9, 84), including the glycosylation of N90 considered essential for SARS-CoV-2 binding. The ACE2 NxT/S consensus N-glycosylation motif (54, 85) is altered in 9 out of 19 bat species tested in this study (**Figure 1**). It is also absent on ACE2 from a number of species such as mouse or rat, which are considered resistant to infection, while it is present in species that have been shown to be susceptible to SARS-CoV-2 such as human, monkey, or cat (**Figure 2**). This highlights the *in silico* approach as a simple screening tool to identify species susceptible to SARS-CoV-2 in a given ecosystem (74, 86–88). SARS-CoV-2 infection was recently reported in mink farms and there is evidence that

employees were infected with SARS-CoV-2 after minks became infected, suggesting that mink farms might become a reservoir for future spillover of SARS-CoV-2 to humans (73). We have aligned the mink ACE2 partial sequence available from GenBank (GenBank CCP86723.1) with the human ACE2 and observed that the mink ACE2 carries the K353 amino acid, but it was not possible to compare the other amino acids (K31, Y41, and N90) important for SARS-CoV-2 binding because the N-terminal part (1–318) of the protein is missing (data not shown). Facing the SARS-CoV-2 epidemic in minks, in the last few days, the Danish Government announced the culling of 17 million minks in rearing after researchers in Denmark have identified some 170 mutations (including a Y453F mutation in the viral spike) in samples from 40 mink farms and the report of mink-specific mutations of SARS-CoV-2 found in humans (89). The rationale behind this decision is the risk that these mutations might allow the virus to spread more easily among people, make it more deadly, and negatively impact the deployment of anti-COVID-19 vaccines. However, there is little evidence that these mutations are of particular concern; the real drivers of epidemics and pandemics are human activities, and trying to eradicate all supposed animal sources of infection is probably more fearful than rational (90).

DATA AVAILABILITY STATEMENT

The sequence data presented in this study can be found in the online ncbi database repositories. All sequences can by found at https://www.ncbi.nlm.nih.gov/genbank and the accession numbers are indicated in the Materials and Methods section.

AUTHOR CONTRIBUTIONS

CD performed the Clustal Omega analysis, designed the figures, and wrote the paper. LP performed the 3-D analysis. IO worked on the art design of figures. DR obtained the funding and supervised the study. All authors reviewed and approved the final version of the manuscript. All authors contributed to conceive the manuscript.

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Christmas Festivities and COVID-19: A Foreseeable Risk to Anticipate

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Keywords: COVID-19, holidays, SARS-CoV-2, pandemic, family gathering

With almost 75 million cases of coronavirus disease 2019 (COVID-19) and over 1,650 million related deaths worldwide, we are approaching the end of an unprecedented year (1). The certainties with which the population lived until a few months ago have suddenly been disrupted, and just as some European Countries were beginning to adopt more relaxing approaches in containing the spread of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a second wave of COVID-19 forced most of them to return to containment measures. As Christmas festivities' celebrations are approaching, recommendations from experts and policy makers are abounding on the media, stressing the importance of avoiding any mass family gatherings during this period. A communication from the expert Dr. Anthony Fauci has warned about another surge in COVID cases in the US that could follow the Christmas period, alongside the post-Thanksgiving rise, which is still being currently tackled. This discussion is indeed relevant also for the majority of European Countries, including Italy, France, Spain, and Germany, where the debate about possibly lifting containment interventions adopted from October 2020 has been a burning issue for weeks.

What have we learned from SARS-CoV-2 in terms of household transmission and mass gatherings? Most SARS-CoV-2 infections are spreading due to airborne exposure to infected individuals (including pre-symptomatics, who account for 45–50% of positive subjects), situated within 2 m of distance. The transmission is particularly effective when speaking, shouting, singing, and breathing heavily during exercises within closed poorly ventilated spaces. The Center for Disease Control has recently updated its guidance by acknowledging the potential for airborne spread of SARS-CoV-2 beyond the droplets[1]. Instead, although the transmission through fomites (contaminated surfaces) has not been documented yet, it is still considered possible. Properly worn respiratory masks can reduce the respiratory virus shedding in exhaled breaths, thus they should be continued to be adopted in combination with physical distancing, hand hygiene, and adequate ventilation of indoor spaces. A recent report of the European Center for Disease Control recommends that heating, ventilation, and air-conditioning systems, if well-maintained and adapted for use during the COVID-19 pandemic, may have a complementary role in decreasing potential airborne transmission of SARS-CoV-2[2]. We also know that eating and drinking on-site at locations that offer such options might be important risk factors associated with SARS-CoV-2 transmission, mainly due to people removing their masks once they are seated[3].

[1] Available online at: https://www.cdc.gov/coronavirus/2019-ncov/more/scientific-brief-sars-cov-2.html

[2] Available online at: https://www.ecdc.europa.eu/en/publications-data/heating-ventilation-air-conditioning-systems-covid-19

[3] Available online at: https://www.cdc.gov/mmwr/volumes/69/wr/mm6936a5.htm

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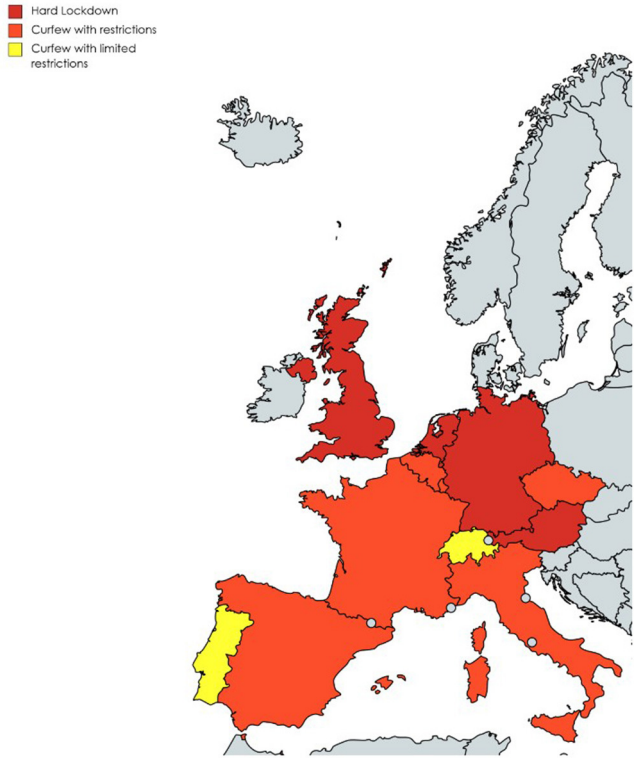

FIGURE 1 | Containment measures in a selected number of the European countries and the UK during Christmas festivities (details according to the colored countries in the Appendix). **Austria, Germany, The Netherlands, United Kingdom:** hard lockdown, closure of non-essential shops, only close family members in the same home; **Italy:** curfew, closure of non-essential shops, no travel between regions, limited number of subjects in the same home, hard lockdown limited to selected days; **Spain:** curfew, closure of non-essential shops, limited travel between regions, limited number of subjects in the same home; **Belgium and Luxembourg: c**urfew, limited number of subjects in the same home; **France:** curfew, closure of non-essential shops, allowed travel between region, limited number of subjects in the same home; **Czech Republic:** schools and all non-essential shops closed, limits on assembly or movement; **Switzerland: e**arlier closure of non-essential shops and public places, bars and restaurants closed on Sundays, limited number of subjects in the same home; **Portugal:** curfew, no limit to subjects (mask required) in the same home for Christmas and travels between regions allowed. Outdoor gatherings limited to six subjects on New Year's eve and no travel between regions.

How do we continue to apply the preventive gold rules for COVID-19 during the coming season's celebrations that, in Europe, are expected to take place in closed spaces because of the winter season? Should we avoid any form of household and mass gatherings indoors and outdoors? These questions are currently at the center of the public debates in the context of the containment measures to undertake in the different EU Countries (https://www.nature.com/articles/d41586-020-03545-1). Familial transmission is responsible for around 70% of SARS-CoV-2 transmission when widespread community control measures are in place (2). We know that household secondary attack rate (SAR) is roughly 27%, which corresponds to a 10 times higher odds of SAR compared to others (2). In Wuhan, the reproduction number (R) dropped from 3.54 to 1.18 after lockdown and cordon sanitaire but reached 0.51 in 2 weeks when complete isolation of cases outside the home was implemented (3). Limiting the size of gatherings is a measure to reduce the likelihood of SARS-CoV-2 spreading to large number of people. A recent analysis reported that the highest reduction in the effective R is achieved when gatherings are limited to 10 people or less (36%; 16–53%) as opposed to 100 or less (21%; 1–39%) (4).

The duration for which people stay indoors is also associated with the attack rate, especially when it comes to community gatherings. For example, in March, during a 2.5 h indoor choir practice in Washington where no preventive measures were adopted, the attack rate was 85.2%[4]. Although in most of the EU Countries wearing masks is mandatory in indoor spaces, this aspect might be especially relevant in the context of holiday season celebrations in churches where singing is a common practice. Outdoor gathering events also represent a risky situation. A recent retrospective analysis of the change in COVID-19 incidence rate during the 2-weeks following outdoor mass gatherings in the US reported an average of 1.5-fold increase (5). In Italy, a large outdoor mass gathering during the UEFA Champions league football match of February 19, followed by extensive celebrations at a time where the first COVID-19 case was not yet detected, is supposed to have contributed to the 567% excess mortality documented in the Bergamo province (6).

With these considerations in mind, some general recommendations might be considered during the upcoming season celebrations in order to avoid the risk of COVID-19: household gatherings with non-cohabitants should be avoided, especially if elderly people are involved, or the number of participants should be limited according to the available space, to maintain proper physical distance; persons should wear masks and avoid eating at tables if this implies removing masks for prolonged periods of time; elderly people might be seated apart; spaces should be properly ventilated if deemed possible according to the outside temperatures; large outdoor mass gathering should be avoided. **Figure 1** reports a map of the different containment measures adopted in the context of Christmas celebrations from selected European Countries and the UK on December 27, 2020.

Although extensive mass vaccination against COVID-19 will start not earlier than mid-2021, we can reasonably assume that the coming season's celebrations will be the last one presenting the COVID-19 pandemic's challenges. In June, the European Commission presented a European strategy to accelerate the development, manufacturing, and deployment of effective and safe vaccines against COVID-19, and it is also committed to ensuring that everyone who needs a vaccine gets it, anywhere in the world and not only at home. As public health professionals, it is time to continue reinforcing the relevance of individual responsibility in containing COVID-19 pandemic also in the coming season' celebrations.

AUTHOR CONTRIBUTIONS

SB designed and wrote this opinion piece.

[4]Available online at: https://www.cdc.gov/mmwr/volumes/69/wr/mm6919e6.htm.

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Sex Differences on Clinical Characteristics, Severity, and Mortality in Adult Patients With COVID-19: A Multicentre Retrospective Study

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Background: Coronavirus disease-2019 (COVID-19) epidemic is spreading globally. Sex differences in the severity and mortality of COVID-19 emerged. This study aims to describe the impact of sex on outcomes in COVOD-19 with a special focus on the effect of estrogen.

Methods: We performed a retrospective cohort study which included 413 patients (230 males and 183 females) with COVID-19 from three designated hospitals in China with a follow up time from January 31, 2020, to April 17, 2020. Women over 55 were considered as postmenopausal patients according to the previous epidemiological data from China. The interaction between age and sex on in-hospital mortality was determined through Cox regression analysis. In addition, multivariate Cox regression models were performed to explore risk factors associated with in-hospital mortality of COVID-19.

Results: Age and sex had significant interaction for the in-hospital mortality ($P < 0.001$). Multivariate Cox regression showed that age (HR 1.041, 95% CI 1.009–1.073, $P = 0.012$), male sex (HR 2.033, 95% CI 1.007–2.098, $P = 0.010$), the interaction between age and sex (HR 1.118, 95% CI 1.003–1.232, $P = 0.018$), and comorbidities (HR 9.845, 95% CI 2.280–42.520, $P = 0.002$) were independently associated with in-hospital mortality of COVID-19 patients. In this multicentre study, female experienced a lower fatality for COVID-19 than male (4.4 vs. 10.0%, $P = 0.031$). Interestingly, stratification by age group revealed no difference in-hospital mortality was noted in women under 55 compared with women over 55 (3.8 vs. 5.2%, $P = 0.144$), as well as in women under 55 compared with the same age men (3.8 vs. 4.0%, $P = 0.918$). However, there was significantly difference in women over 55 with men of the same age group (5.2 vs. 21.0%, $P = 0.007$). Compared with male patients, female patients

had higher lymphocyte ($P < 0.001$) and high-density lipoprotein ($P < 0.001$), lower high sensitive c reaction protein level ($P < 0.001$), and lower incidence rate of acute cardiac injury (6.6 vs. 13.5%, $P = 0.022$).

Conclusion: Male sex is an independent risk factor for COVID-19 in-hospital mortality. Although female mortality in COVID-19 is lower than male, it might not be directly related to the effect of estrogen. Further study is warranted to identify the sex difference in COVID-19 and mechanisms involved.

Keywords: COVID-19, sex, estrogen, menopause, mortality, China

INTRODUCTION

The whole world is currently under the effect of the ongoing epidemic of coronavirus disease-2019 (COVID-19), caused by a novel coronavirus termed severe acute respiratory syndrome coronavirus (SARS-CoV-2). As of 17 June 2020, the World Health Organization (WHO) has reported 8,061,520 confirmed cases and 440,290 deaths in 216 countries and regions, and COVID-19 has become a public health emergency of international concern (PHEIC) (1). Although most of the COVID-19 patients are non-severe and self-limited, there are still 16% severe cases and 3.1% died in China (2–5).

The current studies showed that advanced age and comorbidities were closely related to worse prognosis (6–9). Recently a retrospective multicentre cohort study demonstrated older COVID-19 patients tended to have relatively more severe clinical infections and poorer clinical outcomes associated with COVID-19 compared with younger patients in Jiangsu of China (10). In addition, in a multicentre Italian CORIST study including 3,894 patients with SARS-CoV-2 infection found advanced age at hospital admission was one of powerful predictors of higher in-hospital death (11). Meanwhile, evidence of sex differences in COVID-19 severity emerged, where the morbidity and mortality were all higher among males than females (12–14). A male bias in COVID-19 mortality was reported in 37 of the 38 countries that have provided sex-disaggregated data. Scully et al. showed that the average male case fatality rate (CFR) across 38 countries was 1.7 times higher than the average female CFR (15). Previous epidemiological studies showed the proportion of individuals infected and CFRs in severe acute respiratory syndrome (SARS)-CoV and Middle East respiratory syndrome (MERS)-CoV were higher in males than that of females (16, 17). The causes of Sex differences following virus infections are multifactorial, including differences in steroid hormones, immune response X-linked genes, disease-susceptibility genes in sexes, and gender-related social factors (18–20).

Studies have linked increased susceptibility to infection with circulating steroid hormone concentrations (18, 19, 21). Traditionally, sex steroid hormones, especially the estrogen in females, have been considered for their immunomodulatory properties. Animal study indicated that ovariectomy or treating female mice with estrogen receptor antagonist increased mortality, indicating a protective effect for estrogen receptor signaling in mice infected with SARS-CoV (22). Thus, the lower incidence of severe COVID-19 in female patients might be related to the protective effect of estrogen. However, as yet, few studies have focused on sex differences in clinical characteristics and laboratory tests of COVID-19, especially whether estrogen affects the occurrence and development of COVID-19. Therefore, this study aims to analysis the sex differences on clinical characteristics, severity and mortality in adult patients with COVID-19, and explore possible mechanisms, with a special focus on premenopausal and postmenopausal women.

MATERIALS AND METHODS

Study Design and Participants

The multicentre retrospective cohort study was conducted at three hospitals designated for the treatment of COVID-19, including Jinan Infectious diseases Hospital in Shandong, Shandong Provincial Chest Hospital in Shandong, and Huanggang Central Hospital in Hubei. The recruitment period was from January 31, 2020, to April 17, 2020. The diagnosis of COVID-19 was made based on the National Health Commission of China guidance (23). The presence of SARS-COV-2 in respiratory specimens was confirmed using real-time reverse-transcriptase polymerase chain reaction (RT-PCR) assay were performed in accordance with the protocol described previously (2). The patients that are pregnant or <18 years old were excluded. As of April 17, 2020, all included patients were discharged or died. In addition, the vast majority of city women were postmenopausal by age 55 (24, 25), according to the previous epidemiological data from China, which was consistent with our study. Thus, patients were divided into two groups according to the age of 55 to explore the role of estrogen in the progression of COVID-19. The study was approved by the institutional review board of Jinan Infectious diseases Hospital, Shandong Provincial Chest Hospital, and Huanggang Central Hospital.

Data Collection

Two physicians reviewed clinical electronic medical records and laboratory findings for all patients with SARS-CoV-2 infection, and then a third researcher determined any differences between interpretations of the two primary reviewers. The demographic data, menstrual history of women, clinical characteristics and laboratory results were collected at admission. We also evaluated and gathered complications, treatment and clinical outcomes

(discharged alive or dead) at the end of study, by using a standardized case-report form. For patients with a readmission during the study period, data from the first admission were presented. Sequential Organ Failure Assessment (SOFA) scores were calculated using the worst value of physiological variables within 24 h of presentation.

Diagnostic and Grading Criteria for COVID-19

The disease severity of COVID-19 patients was divided into severe and non-severe conditions, defined according to the American Thoracic Society guidelines for community-acquired pneumonia (26). Severe COVID-19 should reach the following either one major criterion or three or more minor criteria. In detail, Minor criteria included respiratory rate more than 30 breaths per minute, PaO2/FIO2 ratio lower than 250, multilobar infiltrates confusion or disorientation, blood urea nitrogen level more than 7.1 mmol/L, white blood cell count $< 4.0 \times 10^9$ per L, platelet count $< 100 \times 10^{12}$ per L, core temperature lower than 36°C, and hypotension requiring aggressive fluid resuscitation. Major criteria included septic shock with need for vasopressors, or mechanical ventilation. Fever was defined as axillary temperature of at least 37.3°C. Septic shock was defined according to the 2016 Third International Consensus Definition for Sepsis and Septic Shock (27). Acute kidney injury was diagnosed according to the KDIGO clinical practice guidelines (28) and acute respiratory distress syndrome (ARDS) was diagnosed according to the Berlin Definition (29). Acute cardiac injury was diagnosed if serum levels of cardiac biomarkers (e.g., high-sensitive cardiac troponin I) were above the 99th percentile upper reference limit, or if new abnormalities were shown in electrocardiography and echocardiography (2).

Outcomes

The primary outcome was in-hospital mortality. The secondary outcomes were including disease severity, development of acute respiratory distress (ARDS), acute cardiac injury, acute liver injury, sepsis shock and acute kidney injury (AKI).

Statistical Analysis

Patients were divided into two groups according to sex, and the subgroup analysis was performed at the cut-off point of 55 years old according to the age of menopause in women. Female patients and male patients were grouped by age into the younger group(less than or 55 years old) and the older group (above 55 years old) for comparison. Continuous variables were expressed as mean ± standard deviation (SD) or medians (interquartile range, IQR) values. Categorical data were summarized as frequency rates and percentages. The comparison between the two groups was conducted using *t*-tests or Mann-Whitney U tests for continuous variables, and chi-squared tests or Fisher's exact tests for categorical variables. The interaction between age and sex on in-hospital mortality was determined through Cox regression analysis. In addition, multivariate Cox regression models were performed to explore risk factors associated with in-hospital mortality of COVID-19. Considering the total number of death cases ($n = 31$) in this study and to avoid overfitting in the model, four factors with significant association with mortality in univariate regression analyses (sex, age, the interaction between age and sex, and comorbidities) were chosen for multivariate analysis on the basis of previous findings and clinical constraints (8, 10, 12, 13, 30). Hazard ratios (HRs) with 95% confidence intervals (CIs) and the corresponding *P* values were calculated for each risk factor. Kaplan-Meier estimator was generated to estimate the survival curves and log-rank test was used to compare the survival probability between male and female groups. $P < 0.05$ was considered statistically significant. The variables that had >5% of values missing were excluded. Simple data imputation was done for missing data <5%, using the median for skewed distribution data, or the mode for dichotomous data. All analyses were conducted with SPSS software, version 22.0 (SPSS Inc. Chicago, Illinois, United States).

RESULTS

Basic Characteristics

A total of 441 COVID-19 patients (range, 2–89 years) were hospitalized in the three designated hospital from Jan 31, 2020

TABLE 1 | Sex-specific demographic and clinical characteristics of COVID-19 patients.

Variables	Male ($n = 230$)	Female ($n = 183$)	*P*-value
Age, Median (IQR),y	56 (46, 67)	59 (49, 67)	0.094
current or ever smoking	18 (7.8)	0 (0)	**<0.001**
Drinking	17 (7.4)	0 (0)	**<0.001**
Comorbidities			
COPD	6 (2.6)	1 (0.5)	0.139
DM	30 (13.0)	24 (13.1)	0.983
Hypertension	70 (30.4)	47 (25.7)	0.287
Heart disease	15 (6.5)	11 (6.0)	0.832
Kidney disease	6 (2.6)	4 (2.2)	0.781
Liver disease	7 (3.0)	5 (2.7)	0.851
Shock	8 (3.5)	6 (3.3)	0.911
Tumor	6 (2.6)	7 (3.8)	0.482
Immune disease	4 (1.7)	4 (2.2)	0.744
Symptoms			
Fever	208 (90.4)	150 (82.0)	**0.012**
Cough	178 (77.4)	142 (76.1)	0.961
Expectoration	81 (35.2)	57 (31.1)	0.384
Chest distress	110 (47.8)	92 (50.2)	0.621
Chest pain	5 (2.2)	8 (4.4)	0.204
Hemoptysis	4 (1.7)	2 (1.1)	0.697
Headache	12 (5.2)	11 (6.0)	0.727
Myalgia	24 (10.4)	24 (13.1)	0.399
Fatigue	83 (36.1)	71 (38.8)	0.571
Gastrointestinal	33 (14.3)	35 (19.1)	0.193
SOFA Score, median (IQR)	1 (0, 2)	1 (0, 2)	**0.022**

Data are n (%) unless specified otherwise. P < 0.05 was considered statistically significant. COVID-19, coronavirus disease 2019; IQR, interquartile range; COPD, chronic obstructive pulmonary disease; NS, no significance; DM, diabetes mellitus; SOFA, Sequential Organ Failure Assessment. Bold value means P < 0.05.

to Apr 17, 2020. After excluding one pregnant patient, 11 patients < 18 years old, and 16 patients without available key information in their medical records, we included 413 patients in the final analysis, among whom 230 were males and 183 were females. **Table 1** presented the sex-specific demographic and clinical characteristics of the all patients with COVID-19. The median age of all cases was 58 years old (IQR 47–67 years), and it had not difference between males and females. Comorbidities were common for both sexes, but no significant difference. Regarding the symptoms, fever, cough and chest distress were the most common on admission among both men and women. However, a higher percentage of men had fever (90.4% vs. 82.0%, $P = 0.012$). Additionally, SOFA score differed significantly between males and females.

Laboratory Results

Some laboratory results at admission showed significant differences between male and female patients ($P < 0.05$). Male cases had substantially increased hemoglobin, alanine amino transferase (ALT), creatinine, creatine kinase, creatine kinase isoenzyme-MB (CK-MB), and procalcitonin, while significantly decreased lymphocyte counts, platelet counts, total cholesterol (TC) and high-density lipoprotein (HDL). Compared to men, women have lower scrum high sensitive c reaction protein (HS-CRP) levels ($P < 0.001$). The sex-specific laboratory results of the 413 patients with COVID-19 were shown in **Table 2**.

TABLE 2 | Sex-specific laboratory results of COVID-19 patients.

Variables	Normal Range	Male ($n = 230$)	Female ($n = 183$)	*P*-value
White blood cell ($\times 10^9$/L)	3.5–9.5	7.90 (6.68, 10.2)	7.61 (6.54, 9.81)	0.172
Neutrophil ($\times 10^9$/L)	1.8–6.3	6.13 (4.90, 8.37)	5.98 (4.67, 8.55)	0.434
Lymphocyte ($\times 10^9$/L)	1.1–3.2	1.23 (1.0, 1.62)	1.36 (1.11, 1.72)	**<0.001**
Hemoglobin (g/L)	316–354	130 (123, 139)	117 (108, 124)	**<0.001**
Platelet ($\times 10^9$/L)	125–350	251 (220, 313)	265 (233, 313)	**0.030**
D-dimer (μg/ml)	0–1.5	0.80 (0.43, 2.06)	0.92 (0.43, 2.25)	0.483
ALT (U/L)	9–50	36 (22, 53)	22 (15, 35)	**<0.001**
LDH (U/L)	120–250	344 (286, 446)	334 (278, 419)	0.165
IL-6 (pg/ml)	0–7	8.34 (6.08, 11.6)	8.47 (6.59, 10.6)	0.316
Prealbumin (mg/L)	200–430	125 (77, 169)	122 (83, 176)	0.842
Albumin (g/L)	40–55	30.2 (27.0, 33.8)	30.6 (27.3, 34.9)	0.184
Bilirubin (μmol/L)	0–26	12.0 (9.28, 17.2)	11.8 (9.15, 15.3)	0.316
Creatinine (μmol/L)	57–97	77.6 (68.6, 91.7)	56.9 (50.9, 67.0)	**<0.001**
Creatine kinase (U/L)	0–190	96.0 (62.3, 174)	58.0 (38.5, 95.0)	**<0.001**
CK-MB (U/L)	0–24	15.0 (11.0, 19.0)	13.0 (10.0, 16.0)	**<0.001**
HS-CRP (mg/L)	0–3	55.8 (19.3, 115)	25.3 (5.28, 60.9)	**<0.001**
Procalcitonin (ng/mL)	0–0.05	0.05 (0.05, 0.14)	0.05 (0.05, 0.07)	**<0.001**
Troponin (pg/ml)	0–28	4.95 (2.00, 15.2)	4.90 (2.0, 14.8)	0.756
TC (mmol/L)	3.3–5.2	3.52 (3.03, 4.13)	3.79 (3.26, 4.35)	**0.003**
HDL (mmol/L)	1.29–1.55	0.86 (0.70, 1.02)	1.02 (0.84, 1.20)	**<0.001**
LDL (mmol/L)	2.1–3.37	2.06 (1.62, 2.55)	2.05 (1.61, 2.55)	0.849
TG (mmol/L)	0.51–1.70	1.69 (1.49, 2.06)	1.77 (1.55, 2.17)	0.051
ESR (mm/H)	0–15	47.4 (32.8, 63.0)	51.5 (38.5, 67.4)	0.110

Data are median (IQR). $P < 0.05$ was considered statistically significant. COVID-19, coronavirus disease 2019; ALT, alanine amino transferase; LDH, lactate dehydrogenase; IL-6, interleukin-6; CK-MB, creatine kinase isoenzyme-MB; HS-CRP, high sensitive c reaction protein; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglyceride; ESR, erythrocyte sedimentation rate. Bold value means $P < 0.05$.

Clinical Outcomes

The total of 31 patients (7.5%) died during hospitalization. **Figure 1** showed the sex-specific mortality in different age patients. The in-hospital mortality rate was 10.0% for men and 4.4% for women ($P = 0.031$). The cumulative survival rate was significantly different between males and females ($P = 0.018$, log-rank test; **Figure 2A**). In the overall population, 91 cases (22.0%) were diagnosed as severe condition. Although there was no significant difference in the severity of COVID-19 between males and females, severe cases were more likely to be seen in men than women (24.3 vs. 19.1%, $P = 0.203$). There were no sex differences in development of ARDS, sepsis shock, acute kidney injury or acute liver injury (**Table 3**). However, compared with males, females were less likely to develop acute cardiac injury (6.6 vs. 13.5%, $P = 0.022$, **Table 3**).

Multivariate Cox Regression of In-hospital Mortality

Age and sex had significant interaction for the in-hospital mortality ($P < 0.001$) (**Table 4**). Multivariate Cox regression analysis suggested increased in-hospital mortality was associated with age (HR 1.041, 95% CI 1.009–1.073, $P = 0.012$), male sex (HR 2.033, 95% CI 1.007–2.098, $P = 0.010$), the interaction between age and sex (HR 1.118, 95% CI 1.003–1.232, $P = 0.018$), and comorbidities (HR 9.845, 95% CI 2.280–42.520, $P = 0.002$) (**Table 4**).

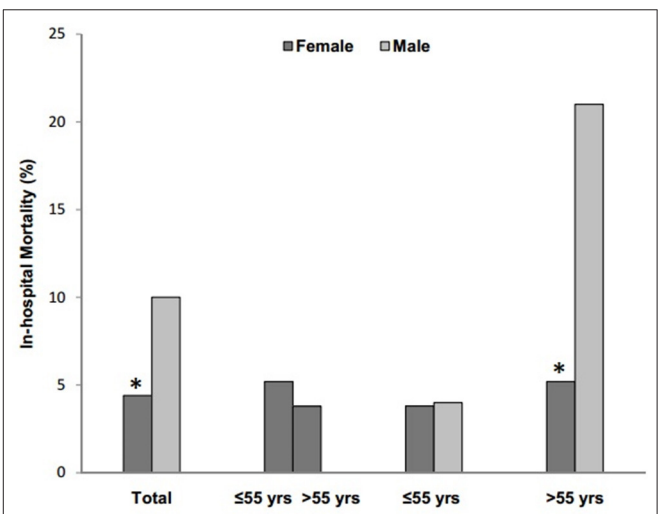

FIGURE 1 | Sex-specific in-hospital mortality of COVID-19 patients and subgroup stratified by age of 55 years. * $P < 0.05$ vs. male by chi-squared tests or Fisher's exact tests.

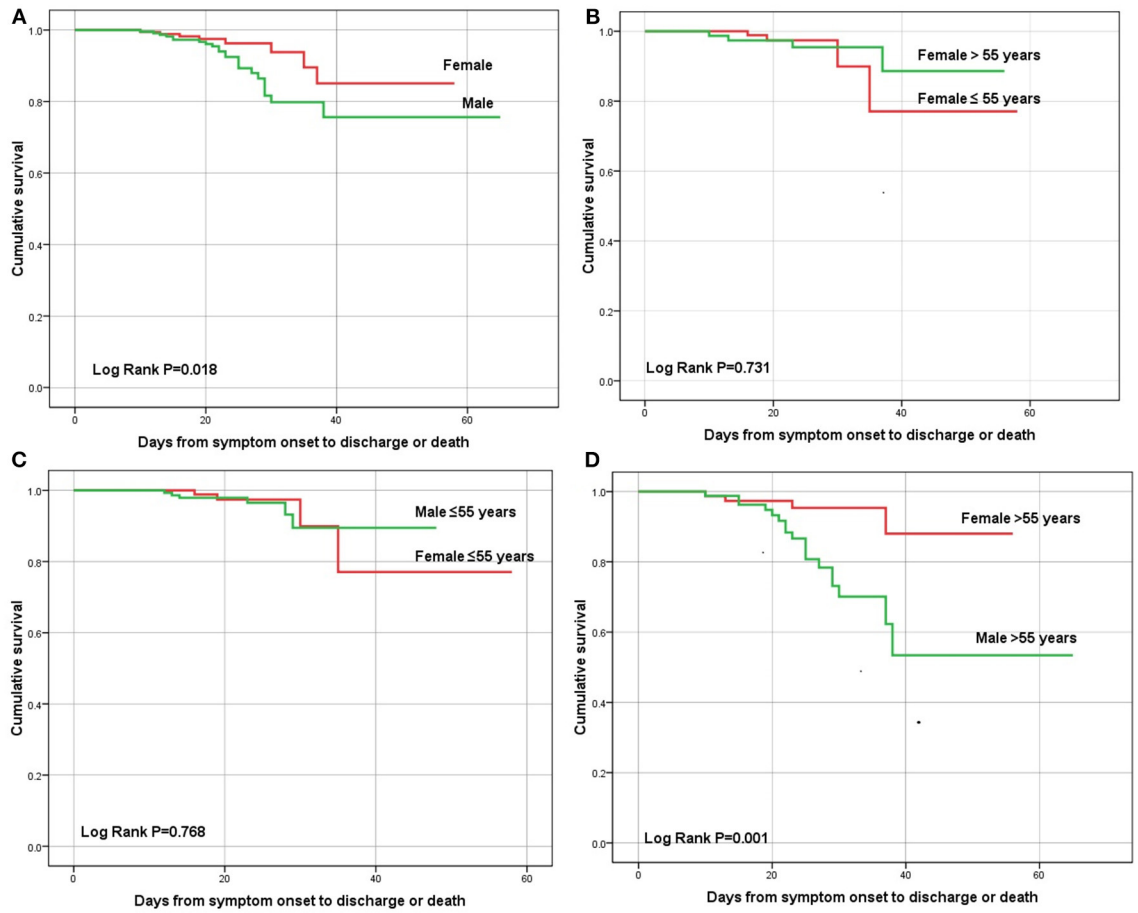

FIGURE 2 | Kalpan-Meier survival curve of male and female patients with COVID-19 in **(A)** male vs. female, **(B)** age ≤ 55 years vs. age > 55 years in female patients, **(C)** male vs. female in age ≤ 55 years old group, and **(D)** male vs. female in age > 55 years old group by log-rank test for all.

TABLE 3 | Sex-specific clinical outcomes of COVID-19 patients.

Clinical outcomes	Male (n = 230)	Female (n = 183)	P-value
Diease severity			
Non-severe	174 (75.7)	148 (80.9)	–
Severe	56 (24.3)	35 (19.1)	0.203
ARDS	62 (27.0)	40 (21.9)	0.233
Time from symptom onset to ARDS, Median(IQR),d	11 (8, 16)	12 (8, 15)	0.748
Sepsis shock	22 (9.6)	12 (6.6)	0.269
Time from symptom onset to shock, Median(IQR),d	18 (13, 24)	17 (13, 23)	0.810
AKI	18 (7.8)	10 (5.5)	0.343
Time from symptom onset to AKI, Median(IQR),d	20 (14, 25)	17 (13, 22)	0.440
Acute liver injury	33 (14.3)	18 (9.8)	0.166
Time from symptom onset to acute liver injury, Median(IQR),d	17 (11, 23)	15 (11, 19)	0.840
Acute cardiac injury	31 (13.5)	12 (6.6)	**0.022**
Time from symptom onset to acute cardiac injury, Median(IQR),d	17 (11, 23)	16 (11, 21)	0.906
In-hospital mortality	23 (10.0)	8 (4.4)	**0.031**

Data are n (%) unless specified otherwise. $P < 0.05$ was considered statistically significant. COVID-19, coronavirus disease 2019; ARDS, acute respiratory distress syndrome; IQR, inter quartile range; AKI, acute kidney injury. Bold value means $P < 0.05$.

TABLE 4 | Cox regression models evaluating risk factors associated with in-hospital mortality in COVID-19 patients.

Variables	Univariate Cox regression		Multivariate Cox regression Model 1		Multivariate Cox regression Model 2	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age	1.054 (1.023–1.087)	**0.001**	1.056 (1.026–1.085)	**0.001**	1.041 (1.009–1.073)	**0.012**
Age >55 yrs (vs. Age ≤ 55 yrs)	3.755 (1.719–8.205)	**0.001**				
Sex						
Male (vs. female)	2.431 (1.061–5.570)	**0.031**	2.027 (1.010–2.043)	**0.028**	2.033 (1.007–2.098)	**0.010**
Age × Sex	1.610 (1.276–2.030)	**<0.001**	1.206 (1.002–1.358)	**0.026**	1.118 (1.003–1.232)	**0.018**
Comorbidities	30.727 (9.125–103.461)	**<0.001**			9.845 (2.280–42.520)	**0.002**
Median time from symptom onset to admission, d	0.672 (0.543–1.384)	0.396				
Lymphocyte	0.164 (0.052–0.518)	**0.002**				
HS-CRP	1.007 (0.992–1.023)	0.353				
Procalcitonin	1.151 (1.007–1.314)	**0.039**				
D-dimer	1.016 (0.991–1.040)	0.210				
Troponin	1.002(0.996–1.009)	0.487				
TC	0.889 (0.524–1.508)	0.663				
HDL	1.356 (0.365–5.041)	0.649				
SOFA	1.881 (1.546–2.289)	**<0.001**				

P < 0.05 was considered statistically significant. COVID-19, coronavirus disease 2019; HS-CRP, high sensitive c reaction protein; TC, total cholesterol; HDL, high-density lipoprotein; SOFA, Sequential Organ Failure Assessment. Comorbidities were defined as having at least one of the followings before diagnosis of COVID-19: chronic obstructive; pulmonary disease, diabetes mellitus, hypertension, coronary heart disease, chronic kidney disease, chronic liver disease, stroke, tumor, autoimmune disease, and HIV infection. Bold value means P < 0.05.

Subgroup Analysis

As mentioned earlier, female patients were divided into premenopausal and postmenopausal groups at the age of 55, and subgroup analysis was performed at the cut-off point of 55 years old. No differences on in-hospital mortality were noted in premenopausal women compared with postmenopausal women (3.8 vs. 5.2%, $P = 0.144$, **Figure 1**). In addition, subgroup analysis revealed no differences in-hospital mortality were noted in premenopausal women compared with men (3.8 vs. 4.0%, $P = 0.918$), but differed significantly in postmenopausal women with men older than 55 (5.2 vs. 21.0%, $P = 0.007$). Additionally, no difference in cumulative survival rate was noted in premenopausal women compared with postmenopausal women ($P = 0.731$, log-rank test; **Figure 2B**), as well as premenopausal women compared with men younger than 55($P = 0.768$, log-rank test; **Figure 2C**), but differed significantly in postmenopausal women with men older than 55($P = 0.001$, log-rank test; **Figure 2D**).

In the subgroup analysis, acute liver injury and acute cardiac injury were less observed complications in women than men older than 55 (10.4 vs. 24.7%, $P = 0.019$; 9.1 vs. 25.9%, $P = 0.006$, respectively). However, the median duration from onset of symptoms to complications had no difference between sexes. Compared with male patients, female patients had higher lymphocyte ($P < 0.001$) and high-density lipoprotein ($P < 0.001$), lower high sensitive c reaction protein level ($P < 0.001$), and lower incidence rate of acute cardiac injury (6.6 vs. 13.5%, $P = 0.022$). The sex differences of clinical characteristics and outcomes were shown in **Tables 5**–**7**.

DISCUSSION

To our best knowledge, this is the first multicentre retrospective cohort study to analyze the sex and estrogen effect on the clinical characteristics and outcomes in adult patients with SARS-COV-2 infection, and the role of estrogen in COVID-19 development. Estrogen modulates immune function in females and may contribute to resistance against infection, while estrogen level is significant difference before and after menopause. To explore the beneficial effect of estrogen in female COVID-19 patients, we made the subgroup comparison according to the age of menopause in women. We documented that fever was more common in men cases, while digestive symptoms were less common. Men with COVID-19 were more prone to develop into the severe condition and die. The same trend was also found in Europe (31). Although no difference in-hospital mortality was noted in women under 55 compared with the same age men, there was significantly difference in women over 55 with men of the same age group, which may be associated with a lower inflammatory response in premenopausal women. However, differences in mortality between premenopausal and postmenopausal women was not significant, suggesting that female mortality in COVID-19 was lower than male might not be directly related to the effect of estrogen. Experimental data showed that male sex was an independent risk factor associated with refractory disease and death (12, 32, 33). We also found that gender-related lifestyle, more chronic diseases, lower lymphocytes on admission, more complications and dyslipidemia may result in higher mortality rate in older men than in older women. These findings contribute to the discussion

TABLE 5 | Sex-specific demographic and clinical characteristics for subgroup stratified by 55 years in COVID-19 patients.

Variables	Age ≤ 55 Y (*n* = 255)			Age > 55 Y (*n* = 158)		
	Male (*n* = 149)	Female (*n* = 106)	*P*-value	Male (*n* = 81)	Female (*n* = 77)	*P*-value
Age, Median(IQR), y	47 (37, 51)	46 (38, 50)	0.494	66 (61, 71)	66 (62, 73)	0.280
current or ever smoking	5 (3.4)	0 (0)	0.078	13 (16.0)	0 (0)	**<0.001**
Drinking	6 (4.0)	0 (0)	**0.043**	9 (11.1)	0 (0)	**0.003**
Comorbidities						
COPD	1 (0.7)	0 (0)	NS	5 (6.2)	1 (1.3)	0.236
DM	7 (4.7)	4 (3.8)	0.720	23 (28.4)	20 (26.0)	0.733
Hypertension	13 (8.7)	6 (5.7)	0.358	57 (70.4)	41 (53.2)	**0.027**
Heart disease	3 (2.0)	2 (1.9)	0.943	12 (14.8)	9 (11.7)	0.563
Kidney disease	1 (0.7)	1 (0.9)	NS	5 (6.2)	3 (3.9)	0.772
Liver disease	2 (1.3)	2 (1.9)	NS	5 (6.2)	3 (3.9)	0.772
Shock	1 (0.7)	0 (0)	NS	7 (8.6)	6 (7.8)	0.846
Tumor	1 (0.7)	1 (0.9)	NS	5 (6.2)	6 (7.8)	0.689
Immune disease	2(1.3)	2(1.9)	NS	2(2.5)	2(2.6)	NS
Symptoms						
Fever	141 (94.6)	86 (81.1)	**0.001**	67 (82.7)	64 (83.1)	0.947
Cough	114 (76.5)	82 (77.4)	0.874	64 (79.0)	60 (77.9)	0.868
Expectoration	56 (37.6)	32 (30.2)	0.221	25 (30.9)	25 (32.5)	0.829
Chest distress	62 (41.6)	43 (40.6)	0.867	48 (59.3)	49 (63.6)	0.572
Chest pain	3 (2.0)	4 (3.8)	0.646	2 (2.5)	4 (5.2)	0.632
Hemoptysis	2 (1.3)	1 (0.9)	NS	2 (2.5)	1 (1.3)	NS
Headache	9 (6.0)	7 (6.6)	0.855	3 (3.7)	4 (5.2)	0.945
Myalgia	15 (10.1)	13 (12.3)	0.580	9 (11.1)	11 (14.3)	0.549
Fatigue	53 (35.6)	32 (30.2)	0.369	30 (37.0)	39 (50.6)	0.085
Gastrointestinal	19 (12.8)	17 (16.0)	0.458	14 (17.3)	18 (23.4)	**<0.001**
SOFA Score, median (IQR)	1 (0, 2)	1 (0, 2)	0.189	2 (1, 3)	1 (0, 2)	**0.009**

Data are n (%) unless specified otherwise. P < 0.05 was considered statistically significant. COVID-19, coronavirus disease 2019; IQR, interquartile range; COPD, chronic obstructive pulmonary disease; NS, no significance; DM, diabetes mellitus; SOFA, Sequential Organ Failure Assessment. Bold value means P < 0.05.

of whether older male patients with SARS-COV-2 infection should be paid more attention.

It has been known that males and females differ in their susceptibility and response to viral infections, resulting in sex differences in incidence and disease severity (18, 34). The reduced susceptibility of females to viral infections could be attributed to the protection from X chromosome and sex hormones, which play an essential role in innate and adaptive immunity (35). SARS-CoV-2 uses ACE2 on pulmonary endothelium as an entry receptor, while the gene for the ACE2 is on the X chromosome (36), which may be the reason for the higher prevalence of COVID-19 in men than in women. In addition, in premenopausal women, estradiol produced by the ovary, is the estrogen in largest quantity (40–400 pg/mL) and most potent. Nevertheless, ovary almost stops producing estradiol after menopause, leading to estradiol level is significant reduced in postmenopausal women (<20 pg/mL), and no difference with men. In our study, the inflammatory marker HS-CRP was lowest in premenopausal women, while HS-CRP may result in cytokine storms and relate to disease severity and mortality (2, 6). Previous study showed hypopituitary women had decreased level of estrogen and increased level of CRP (37). Therefore, the lower morbidity and mortality of premenopausal women may be related to estrogen-mediated low inflammatory response. We also found that no difference in mortality among premenopausal and postmenopausal women. This suggested estrogen influenced the infection with SARS-COV-2 and pathogenesis of COVID-19, but might not be directly related to the lower mortality in women. However, our study had the small sample size, and the majority of younger patients are being non-severe COVID-19 condition and fewer died. Further investigation with larger –scale data is needed to assess the influence of estrogen on COVID-19 patients.

Although advancing age is associated with greater risk of death in both sexes, the male bias remains evident (15). Our study suggested that age, male sex and comorbidities were independently associated with in-hospital mortality, as well as sex and age had significant interaction for in-hospital mortality of COVID-19. The age-related sex differences in patients with COVID-19 were consistent with reported cases of seasonal and pandemic influenza A virus infections in Australia and Japan (38, 39). In the present study, we found the increasing mortality might be associated with higher rates of smoking, hypertension and complications in older men. Smoking rate is higher among men than women worldwide (40), consisting with the result

TABLE 6 | Sex-specific laboratory results for subgroup stratified by 55 years in COVID-19 patients.

Variables	Normal range	Age ≤ 55 Y (*n* = 255)			Age > 55 Y (*n* = 158)		
		Male (*n* = 149)	Female (*n* = 106)	*P*-value	Male (*n* = 81)	Female (*n* = 77)	*P*-value
White blood cell ($\times 10^9$/L)	3.5–9.5	7.77 (6.77, 10.2)	7.45 (6.55, 9.10)	0.341	7.98 (6.76, 11.1)	7.76 (6.71, 9.51)	0.346
Neutrophil ($\times 10^9$/L)	1.8–6.3	5.93 (4.95, 7.90)	5.89 (4.65, 8.03)	0.713	6.27 (4.87, 8.45)	6.00 (4.68, 8.60)	0.464
Lymphocyte ($\times 10^9$/L)	1.1–3.2	1.35 (1.07, 1.77)	1.45 (1.13, 1.74)	0.532	1.13 (0.84, 1.40)	1.31 (1.10, 1.67)	**<0.001**
Hemoglobin (g/L)	316–354	135 (128, 141)	117 (108, 124)	**<0.001**	126 (115, 135)	116 (108, 124)	**<0.001**
Platelet ($\times 10^9$/L)	125–350	274 (225, 349)	262 (234, 311)	0.536	240 (216, 286)	268 (232, 314)	**<0.001**
D-dimer (μg/ml)	0–1.5	0.56 (0.31, 1.15)	0.62 (0.33, 1.17)	0.720	1.11 (0.68, 4.15)	1.25 (0.58, 3.85)	0.803
ALT (U/L)	9–50	37.0 (25.0, 55.0)	22.0 (14.3, 37.5)	**<0.001**	33.0 (19.5, 51.5)	22.0 (15.0, 35.0)	**<0.001**
LDH (U/L)	120–250	331 (284, 397)	327 (281, 404)	0.941	356 (290, 479)	338 (278, 429)	**0.041**
IL-6 (pg/ml)	0–7	7.92 (5.81, 10.5)	7.77 (6.20, 10.7)	0.466	8.93 (6.31, 12.9)	8.91 (6.73, 13.2)	0.616
Prealbumin (mg/L)	200–430	151 (117, 215)	137 (92.5, 194)	0.079	89.0 (56.8, 129)	109 (79.0, 155)	**0.003**
Albumin (g/L)	40–55	30.0 (26.8, 33.7)	30.9 (27.0, 35.4)	0.107	30.3 (27.0, 33.8)	30.4 (27.4, 33.9)	0.795
Bilirubin (μmol/L)	0–26	11.8 (8.83, 18.4)	11.3 (8.90, 14.9)	0.553	12.1 (9.63, 15.4)	12.2 (9.38, 16.1)	0.735
Creatinine (μmol/L)	57–97	75.9 (68.8, 84.9)	54.2 (50.2, 61.1)	**<0.001**	81.2 (68.3, 99.2)	60.0 (52.2, 69.2)	**<0.001**
Creatine kinase (U/L)	0–190	92.5 (58.5, 168)	51.0 (38.3, 84.0)	**<0.001**	103 (67.0, 174)	60.0 (39.0, 117)	**<0.001**
CK-MB (U/L)	0–24	15.0 (11.0, 18.0)	12.0 (9.50, 16.0)	**0.005**	15.0 (12.0, 19.0)	13.0 (10.0, 16.0)	**0.002**
HS-CRP (mg/L)	0–3	26.7 (11.88, 82.4)	12.9 (3.90, 39.3)	**<0.001**	93.6 (44.3, 131)	26.0 (10.1, 63.9)	**<0.001**
Procalcitonin (ng/mL)	0–0.05	0.05 (0.05, 0.09)	0.05 (0.05, 0.05)	**<0.001**	0.07 (0.05, 0.23)	0.05 (0.05, 0.10)	**0.001**
Troponin (pg/ml)	0–28	4.70 (1.78, 14.3)	4.10 (1.80, 13.1)	0.968	6.95 (2.25, 17.5)	5.80 (2.25, 17.4)	0.979
TC (mmol/L)	3.3–5.2	3.62 (3.15, 4.17)	3.64 (3.11, 4.21)	0.883	3.42 (2.93, 4.00)	3.88 (3.38, 4.46)	**<0.001**
HDL (mmol/L)	1.29–1.55	0.85 (0.71, 1.00)	1.00 (0.81, 1.19)	**<0.001**	0.87 (0.70, 1.04)	1.05 (0.85, 1.21)	**<0.001**
LDL (mmol/L)	2.1–3.37	2.32 (1.82, 2.66)	2.01 (1.58, 2.46)	**0.028**	1.85 (1.52, 2.35)	2.13 (1.67, 2.64)	**0.016**
TG (mmol/L)	0.51–1.70	1.73 (1.54, 2.15)	1.78 (1.59, 2.19)	0.444	1.65 (1.45, 1.99)	1.77 (1.52, 2.15)	**0.024**
ESR (mm/H)	0–15	46.7 (35.3, 61.5)	49.5 (29.2, 61.9)	0.725	53.5 (28.1, 63.0)	58.6 (43.5, 72.2)	0.065

Data are median (IQR). P < 0.05 was considered statistically significant. COVID-19, coronavirus disease 2019; ALT, alanine amino transferase; LDH, lactate dehydrogenase; IL-6, interleukin-6; CK-MB, creatine kinase isoenzyme-MB; HS-CRP, high sensitive c reaction protein; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglyceride; ESR, erythrocyte sedimentation rate. Bold value means P < 0.05.

TABLE 7 | Sex-specific clinical outcomes for subgroupstratified by 55 years in COVID-19 patients.

Clinical outcomes	Age ≤ 55 Y (*n* = 255)			Age > 55 Y (*n* = 158)		
	Male (*n* = 149)	Female (*n* = 106)	*P*-value	Male (*n* = 81)	Female (*n* = 77)	*P*-value
Disease severity						
Non-severe	128 (85.9)	93 (87.7)	–	46 (56.8)	55 (71.4)	–
Severe	21 (14.1)	13 (12.3)	0.672	35 (43.2)	22 (28.6)	0.055
ARDS	25 (16.8)	14 (13.2)	0.435	37 (45.7)	26 (33.8)	0.126
Time from symptom onset to ARDS, Median (IQR), d	9 (7, 12)	11 (7, 16)	0.181	13 (8, 17)	12 (8, 16)	0.484
Sepsis shock	1 0 (6.7)	7 (6.6)	0.973	12 (14.8)	5 (6.5)	0.092
Time from symptom onset to shock, Median (IQR), d	17 (11, 20)	23 (14, 33)	0.244	18 (14, 25)	18 (13, 23)	0.828
AKI	5 (3.4)	4 (3.8)	0.859	13 (16.0)	6 (7.8)	0.111
Time from symptom onset to AKI, Median (IQR), d	20 (13, 22)	22 (21, 23)	0.438	16 (13, 22)	18 (14, 26)	0.374
Acute liver injury	13 (8.7)	10 (9.4)	0.846	20 (24.7)	8 (10.4)	**0.019**
Time from symptom onset to acute liver injury, Median (IQR), d	13 (10, 16)	13 (8, 22)	0.657	16 (13, 22)	17 (12, 23)	0.972
Acute cardiac injury	10 (6.7)	5 (4.7)	0.505	21 (25.9)	7 (9.1)	**0.006**
Time from symptom onset to acute cardiac injury, Median (IQR), d	15 (10, 18)	18 (17, 21)	0.145	16 (11, 23)	14 (9, 22)	0.202
In-hospital mortality	6 (4.0)	4 (3.8)	0.918	17 (21.0)	4 (5.2)	**0.007**

Data are n (%) unless specified otherwise. P < 0.05 was considered statistically significant. COVID-19, coronavirus disease 2019; ARDS, acute respiratory distress syndrome; IQR, inter quartile range; AKI, acute kidney injury. Bold value means P < 0.05.

in this study. Such behavior is associated with the risk of developing comorbidities. Simultaneously, smoking is related to higher expression of ACE2 and may be a risk facto rfor disease prevalence and severity (4, 40, 41), but no firm conclusions can be drawn. Hypertension was the most common chronic diseases, particularly in older male patients. Previous epidemiological data indicated an association between hypertension and severe disease or death from COVID-19 (7–9). Besides, the incidence of ARDS and other complications were higher in older men with COVID-19, especially acute liver injury and acute cardiac injury, which might also affect disease severity and prognosis.

In the older group, total lymphocyte count was significantly lower in men than in women. Lymphocytes play a decisive role in maintaining immune homeostasis and inflammatory response throughout the body. Previous studies had suggested that severe COVID-19 patients might have lymphocyte responses. A meta-analysis reported lymphopenia had a 3-fold higher risk of developing severe COVID-19, and lower lymphocyte counts was an effective biomarker in predicting the severity and prognosis in COVID-19 patients (42). Therefore, lymphopenia may be one potential mechanism of age-related sex differences.

In this study, dyslipidemia was found in older patients infected with SARS-COV-2. The TC, HDL, LDL, and TG levels in older male patients showed markedly decreases as compared to older female patients. This goes along well with previously data, that the LDL, HDL and TC levels in COVID-19 patients showed significant decreases at the time on admission (43). Lipids play a central role in viral infection, as they represent the structural foundations of cellular and viral membranes, while LDL levels inversely correlated to disease severities, which could be a predictor for disease progress and poor prognosis (43). The exact mechanisms of dyslipidemia in COVID-19 patients have remained unclear; however, there were several potential causes. Firstly, circulating lipid became oxidized in the inflammatory conditions, which resulted in the loss their protective functions and contributed to ongoing inflammation (44). Meanwhile, inflammation regulated lipoprotein metabolism indirectly via the cytokines, such as resulting in reduced expression and secretion of apoprotein of HDL, remodeling HDL-associated proteome, and promote HDL clearance from plasma (45, 46). Therefore, the measurement of the oxidized LDL and apoprotein of HDL can confirm these notions. Secondly, hepatocytes are the dominant cell type determining systemic LDL levels, and liver injury which is a common complication in older men, can affect the serum LDL levels. Thirdly, older male patients may have exacerbated inflammatory response, while systemic inflammation may accelerate clearance of LDL, which may partly explain the reduction in circulating LDL. Finally, estrogen levels in postmenopausal women affect the normal levels of blood lipids, which lead to increase TC, TG and LDL levels.

LIMITATION

There are several limitations in this study. Firstly, it was a retrospective multicentre study, and there were probably a significant referral bias, recall bias and measurement bias. Besides, this is an observational study and its results are subject to unobserved confounding factors. Secondly, we did not measure estrogen level and collect the history of hormone replacement therapy. However, we refer to previous studies and perform the subgroup analysis based on the age of menopause, to investigate the effect of estrogen in morbidity and mortality in patients with COVID-19 for the first time. Thirdly, only the indexes on admission were selected for analysis, without dynamic monitoring. Further, large-scale clinical studies and basic research are needed to explore risk factors for individualized assessment. Finally, no power calculation was made for the study, so the study is an exploratory study and its results are subject to false positive error and should be interpreted with caution.

In conclusion, older age, male sex and comorbidities were independently associated with in-hospital mortality of COVID-19 patients. Moreover, sex and age are interactively associated with outcome of COVID-19. Although female mortality in COVID-19 is lower than male in this study, it might not be directly related to the effect of estrogen. Further study is warranted to identify the sex difference in COVID-19 and mechanisms involved.

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 Jinan Infectious diseases Hospital, Shandong Provincial Chest Hospital, and Huanggang Central Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YC and MM were involved in study concept and design and revised the final manuscript. JS, GQ, WS, CW, ZZ, XW, and XB collected the epidemiological and clinical data. PW, QY, and JJ processed statistical data. JS and GQ drafted the manuscript. 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.

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Clinical Determinants Differentiating the Severity of SARS-CoV-2 Infection in Cancer Patients: Hospital Care or Home Recovery

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Background: Cancer patients may carry a worse prognosis with SARS-CoV-2 infection. Most of the previous studies described the outcomes of hospitalized cancer patients. We aimed to study the clinical factors differentiating patients requiring hospital care vs. home recovery, and the trajectory of their anti-cancer treatment.

Methods: This study was conducted in a community cancer center in New York City. Eligible patients were those who had cancer history and were diagnosed of SARS-CoV-2 infection between March 1 and May 30, 2020, with confirmatory SARs-CoV-2 virus test or antibody test. Four groups were constructed: (A) hospitalized and survived, (B) hospitalized requiring intubation and/or deceased, (C) non-hospitalized, asymptomatic, with suspicious CT image findings, close exposure, or positive antibody test, and (D) non-hospitalized and symptomatic.

Results: One hundred and six patients were included in the analysis. Thirty-five patients (33.0%) required hospitalization and 13 (12.3%) died. Thirty (28.3%) patients were asymptomatic and 41 (38.7%) were symptomatic and recovered at home. Comparing to patients who recovered at home, hospitalized patients were composed of older patients (median age 71 vs. 63 years old, $p = 0.000299$), more who received negative impact treatment (62.9 vs. 32.4%, $p = 0.0036$) that mostly represented myelosuppressive chemotherapy (45.7 vs. 23.9%, $p = 0.0275$), and more patients with poorer baseline performance status (PS $\geq$ 2 25.7 vs. 2.8%, $p = 0.0007$). Hypoxemia (35% in group A vs. 73.3% in group B, $p = 0.0271$) at presentation was significant to predict mortality in hospitalized patients. The median cumulative hospital stay for discharged patients was 16 days (range 5–60). The median duration of persistent positivity of SARS-CoV-2 RNA was 28 days (range 10–86). About 52.9% of patients who survived hospitalization and required anti-cancer treatment reinitiated therapy. Ninety-two percent

of the asymptomatic patients and 51.7% of the symptomatic patients who recovered at home continued treatment on schedule and almost all reinitiated treatment after recovery.

Conclusions: Cancer patients may have a more severe status of SARS-CoV-2 infection after receiving myelosuppressive chemotherapy. Avoidance should be considered in older patients with poor performance status. More than two thirds of patients exhibit minimal to moderate symptoms, and many of them can continue or restart their anti-cancer treatment upon recovery.

Keywords: COVID-19, cancer, chemotherapy, immunosuppression, asymptomatic, treatment of negative impact

INTRODUCTION

The unprecedented COVID-19 pandemic has presented a public health challenge globally. As of May 31, 2020, 1,778,515 confirmed cases and 104,051 deaths were reported in the US (1). Patients with advanced age and comorbidities appeared to have poorer outcomes with the SARS-CoV-2 infection (2, 3). Cancer patients, as a group, also showed higher fatality rates (2–5). Presumably, factors such as the presence or absence of disease, recent therapy with possible myelosuppressive or immunosuppressive potentials, and types of cancer may play important roles in influencing their outcomes. Most of the previous studies concentrated on the examination of patients who developed severe symptoms and required hospitalization.

In this registry study, we analyzed clinical factors including presence of disease, cancer-related treatment, interval between cancer treatments to clinical diagnosis of SARS-CoV-2 infection or admission, baseline performance status, and immune status of all patients, and compared these frequencies between hospitalized patients and non-hospitalized patients. We hypothesized that anti-cancer treatments with potential negative impact to the immune system and the interval between its administration and the onset of COVID-19 symptoms may be critical. We defined this category of therapy to include myelosuppressive, immunosuppressive, and immune modulating agents. We also evaluated time kinetics of virus clearance and time kinetics of re-initiation of anti-cancer treatment. We further followed the trajectory of recovery of the patients and studied the time course of persistent positivity of SARS-CoV-2 RNA.

METHODS

This was a prospective observational registry study established in March 2020 and approved by Institutional Review Board (IRB). Patients were eligible if they had a confirmed or suspicious diagnosis of SARS-CoV-2 infection between March 1, 2020 and June 15, 2020, as well as active cancer history. Two cohorts of patients were enrolled. In cohort 1, patients were identified by health care providers between March and May of 2020 when they presented with suspicious COVID-19 symptoms, or had a known close contact to a known COVID-19 case, or suspicious radiological findings on CT or X-rays performed for the purpose of their cancer staging. A suspicious radiographic reading was defined as "peripheral ground-grass opacities," possibly compatible with SARS-CoV-2 infection. As COVID-19 reverse transcriptase-polymerase chain reaction (RT-PCR) tests were only offered to patients who met the hospitalization criteria (usually hypoxemia with oxygen saturation <92% at room air), some patients did not get RT-PCR test at the time of suspicion, and were then followed up and offered COVID-19 antibody tests when it became available starting May 2020. Only those who had confirmed SARS-CoV-2 diagnosis, defined by positive COVID-19 RT-PCR or positive COVID-19 antibody test were included in the main analysis. One exception was a patient who died quickly in the hospital and did not have an opportunity for testing but carried highest clinical suspicion. In cohort 2, patients were retrospectively identified through electronic medical records by their positive COVID-19 RT-PCR test, positive point of care virus test, or positive COVID-19 antibody test, which were performed between May 20 and June 30, 2020. Medical records were reviewed for documentation of symptoms, treatment history, laboratory and radiological findings, and admission records. For the patients in cohort 2, if the COVID-19 related symptoms were not documented in the medical records, health care providers conducted interviews by phone-calls to help patients to recall their symptoms potentially associated with their past SARS-CoV-2 infection, and to get contact history.

Nasopharyngeal swabs were collected and tested for SARS-CoV-2 RNA with RT-PCR assay using the XPERT XPRESS SARS-COV-2 test kit in our hospital laboratory. Nasopharyngeal swabs were also collected for the molecular point-of-care test for SARS-CoV-2 virus detection using Abbott ID NOW^{TM} kit. Antibody tests (IgG and IgM) were sent out and tested at Lenco Diagnostics Laboratory, Brooklyn, NY. The data entry cut off was 7/1/2020.

Study Group Definition

Patients in cohort 1 and 2 were combined and divided into 4 groups. Group A patients were hospitalized with no intubation events, discharged, and survived. Group B patients were hospitalized and required intubation or hospitalized and were deceased. Group C patients were asymptomatic who were tested for a suspicious CT scan result, had a history of close exposure to a known case, or did not recall any symptoms after showing presence

of positive COVID-19 Ig G or Ig M antibodies. Group D patients exhibited symptoms consistent with SARS-CoV-2 infection, though these symptoms were not severe enough for hospital admission.

Definition of Performance Status and Baseline Immune Status

The performance status scale (PS) was based on Eastern Cooperative Oncology Group (ECOG) scale. PS 0: fully active, no performance restrictions; PS1: restricted in strenuous physical activity, fully ambulatory and able to carry out light work; PS2: Capable of all self-care but unable to carry out any work activities, up and about >50% of waking hours; PS3: Capable of only limited self-care, confined to bed or chair >50% of the waking hours. PS4: Completely disabled, cannot carry out any self-care; totally confined to bed or chair (6).

The baseline immune status was estimated by taking lab results performed in February 2020 or prior, including absolute neutrophil count, absolute lymphocyte count, and albumin level. Any abnormal value among the three tests, defined as lower than the lower limit of the normal range, was considered abnormal.

Treatment Category Definition

Myelosuppressive regimens included all routine chemotherapy drugs. Exceptions include therapeutic antibodies (trastuzumab, bevacizumab), oral targeted therapies (erlotinib, osimertinib), and hormonal treatments (luteinizing hormone releasing hormone (LHRH) agonists, fulvestrant, tamoxifen, and aromatase inhibitors). Immunosuppressive drugs included rituximab, lenalidomide, high dose steroids, and daratumumab. Immune modulating agents included the immune checkpoint inhibitors that target PD-1 (programmed cell death protein 1) or PD-L1, such as pembrolizumab, nivolumab, and durvalumab. Negative impact treatment denotes treatments with potential negative impact on the immune system (any regimens in the myelosuppressive, immunosuppressive, or immune modulating categories).

Definition of Treatment Duration

The start of any anti-cancer treatment until the day of diagnosis of SARS-CoV-2 infection. If there was a treatment break of more than 3 months, then the treatment before the break was not counted. If the continuation of treatment included hormonal or non-myelosuppressive treatment followed by treatment with negative impact, then the start day of treatment with negative impact treatment was chosen for the start day.

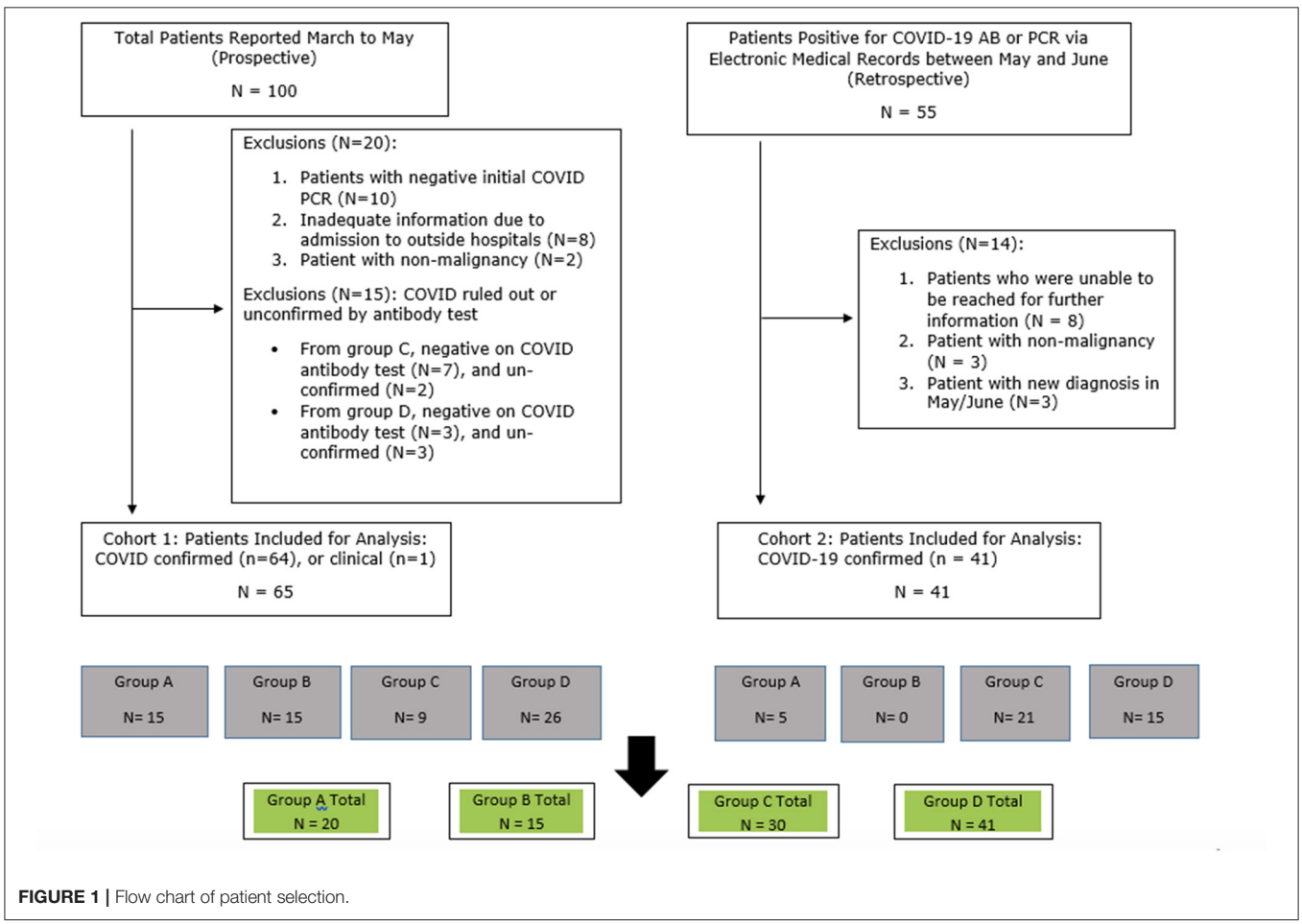

FIGURE 1 | Flow chart of patient selection.

TABLE 1 | Patient demographics and disease characteristics.

Characteristics	Total number (%)
Total number	106
Age	
Median	65
Range	31–94
Gender	
Male	32 (30.2)
Female	74 (69.8)
Ethnicity	
Caucasian	47 (44.3)
African American	32 (30.2)
Asian	10 (9.4)
Hispanic	15 (14.2)
Mid-Eastern	2 (1.9)
Cancer types	
Solid tumors	**95 (89.6)**
Breast	39 (36.8)
GYN	13 (12.3)
Gastrointestinal	12 (11.3)
Lung	12 (11.3)
Head/Neck	5 (4.7)
GU	12 (11.3)
Brain	1 (1.5)
Osteosarcoma	1 (1.5)
Hematological malignancy	**11 (10.4)**
Presence of disease	
No evidence of disease (NED)	**47 (44.3)**
Presence of disease	**59 (55.7)**
Localized disease	19 (17.9)
Metastatic disease	40 (37.7)
Treatment	
No treatment	28 (26.4)
Active treatment*	78 (73.6)
Negative impact treatment	45 (42.5, 45/106)
Treatment within 30 days	36 (34.0, 36/106)
Diagnosis month	
March	22 (20.8)
April	54 (50.9)
May	11 (10.4)
June	1 (0.9)
Unknown	18 (17.0)
Hospitalized	35 (33.0)
Staying home	71 (67.0)

**Defined as last treatment (any anti-cancer) treatment within 3 months of SARS-CoV-2 diagnosis.*

Sampling Frequency Study of the Time Kinetics of Persistent SARS-CoV-2 Virus Status

Patients were screened for whether or not a repeat COVID-19 RT-PCR test were performed. Most of those tests were done at unplanned intervals mainly to get a negative result to qualify patients to resume anti-cancer treatment. The days between the initial positive test and the last positive test was defined as "positive duration."

Statistical Analysis

We first compared the clinical features of hospitalized patients (Group A vs. B). Next, we compared the clinical features of the hospitalized patients to at-home patients (Group A + B vs. Group C + D). Wilcoxon rank sum test or Student's *t*-test were used to compare continuous data. Fisher's exact test was performed for the categorical variables. Next, we assessed risk factors for hospitalization—a more severe status of SARS-CoV-2 infection. We conducted multivariate analyses by utilizing logistic regression with the inclusion of variables significant in univariate analysis. The multivariate logistic model was built from a two-sided stepwise regression based on the Akaike Information Criterion (AIC). AIC is an estimator of out-of-sample prediction error and thereby relative quality of statistical models for a given set of data. Given a collection of models for the data, AIC estimates the quality of each model, relative to each of the other models. Thus, AIC provides a means for model selection.

Odds ratios for a more severe status of SARS-CoV-2 infection were calculated via logistic regression. Two-sided significance level 0.05 was used. All statistical analyses were done using R (version 3.5.3; The R Foundation).

RESULTS

Patient Demographics

Out of the 100 cases with clinical suspicious diagnosis of SARS-CoV-2 infection which were reported to the study team between March and May 2020, 65 patients were included in cohort 1 of this analysis (**Figure 1**). Out of the 55 patients identified retrospectively by electronic medical records, 41 were included in the analysis (**Figure 1**). For all of the 106 eligible patients, the median age was 65 years old (range 31–94). There was female predominance (69.8%), and 44.3% were Caucasian (**Table 1**). The female predominance was present in all 4 groups and was numerically highest in group D (**Table 2**). Ninety-five (89.6%) patients had solid tumors, with the most common being breast cancer (36.8% of total). Forty-seven (44.3%) patients had no evidence of disease (NED). Fifty-nine patients (55.7%) had presence of tumor, either localized (17.9%) or metastatic (37.7%). Seventy-eight (73.6%) patients were receiving active treatment within 3 months of diagnosis, and 45 (42.5%) were receiving negative impact treatment. Thirty-six (34.0%) who were receiving negative impact treatment received last dose of therapy within 30 days of SARS-CoV-2 diagnosis. Thirty-five (33.0%) patients were hospitalized and the remainder recovered at home (**Table 1**). The detailed breakdowns of the demographic and clinical characteristics in the four groups are listed in **Table 2**.

TABLE 2 | Clinical characteristics of patients divided by groups of severity of SARS-CoV-2 infection.

Characteristics	Group A Hospitalized	Group B Intubated and/or Deceased	*p*-Value *A* vs. *B*	Group C Asymptomatic Suspicious CT	Group D Symptomatic, Home Isolation	Group A + B	Group C + D	*p*-Value A + B vs. C + D
Total number	20 (18.9)	15 (14.2)		30 (28.3)	41 (38.7)	35 (33.0)	71 (67.0)	
Confirmed*	20 (4 AB*)	14		30 (27 AB)	41 (35 AB)	34 (4 AB)	71 (62 AB)	
Unconfirmed	0	1		0	0	1	0	
Age								
Median	74	68	0.4593	68	60	71	63	**0.000299**
Range	44–88	48–83		31–87	43–94	44–88	31–94	
Gender								
Male	8 (40)	6 (40)	1	11 (36.7)	7 (17.1)	14 (40)	18 (25.4)	0.1764
Female	12 (60)	9 (60)		19 (63.3)	34 (82.9)	21 (60)	53 (74.6)	
Cancer types								
Solid tumors	16 (80)	13 (86.7)	0.6804	29 (96.7)	37 (90.2)	29 (82.9)	66 (93)	0.172
Hematological	4 (20)	2 (13.3)		1 (3.3)	4 (9.8)	6 (17.1)	5 (7)	
Disease status								
NED	9 (45.0)	3 (20)	0.1629	16 (53.3)	19 (46.3)	12 (34.3)	35 (49.3)	0.1534
Presence of disease	11 (55.0)	12 (80)		14 (46.7)	22 (53.7)	23 (65.7)	36 (50.7)	
Localized	4 (20)	3 (20)		7 (23.3)	5 (12.2)	7 (20)	12 (16.9)	
Metastatic	7 (35.0)	9 (60)		7 (23.3)	17 (41.5)	16 (45.7)	24 (33.8)	
Treatment and disease								
NED no Tx	4(20)	1 (6.7)	0.511	4 (13.3)	8 (19.5)	5 (14.3)	12 (16.9)	1
NED + Tx	5 (25.0)	2 (13.3)		12 (40)	11 (26.8)	7 (20)	23 (32.4)	0.2522
+ disease no Tx	2 (10.0)	2 (13.3)		3 (10)	4 (9.8)	4 (11.4)	7 (9.9)	1
+ disease + Tx	9 (45.0)	10 (66.7)		11 (36.7)	18 (43.9)	19 (54.3)	29 (40.8)	0.2175
Treatment								
Yes	14 (70.0)	12 (80)	0.7	23 (76.7)	29 (70.7)	26 (74.3)	52 (73.2)	1
No	6 (30)	3 (20)		7 (23.3)	12 (29.3)	9 (25.7)	19 (26.8)	
Treatment								
Myelosuppressive	8 (40.0)	8 (53.3)	0.506	6 (20)	11 (26.8)	16 (45.7)	17 (23.9)	**0.0275**
Immunosuppressive	1 (5.0)	0	1	0	1 (2.4)	1 (2.9)	1 (1.4)	1
Immunomodulating	3 (15)	2 (13.3)	1	3 (10)	2 (4.9)	5 (14.3)	5 (7.0)	0.2926
Any above negative impact	12 (60)	10 (66.7)	0.7372	9 (30)	14 (34.1)	22 (62.9)	23 (32.4)	**0.0036**
Negative Impact <30 days	10 (50)	7 (46.7)	1	8 (26.7)	11 (26.8)	17 (48.6)	19 (26.8)	**0.0171**
Time duration of active treatment (m)								
Median (range)	3.1 (0.6–81.3)	3.0 (0–46.1)	0.2364	9.8 (0.7–115.4)	13.4 (1.6–63.9)	3.0 (0–81.3)	11.2 (0.7–115.4)	**0.0058**
Mean (SD)	14.9 (0.47)	6.5 (0.41)		17.0 (0.43)	19.8 (0.46)	11.0 (0.44)	18.6 (0.45)	
Time duration of tx with negative impact (m)								
Median (range)	2.7 (0.6–33.1)	3.0 (0–46.1)	0.4281	8.5 (0.7–115.4)	6.5 (1.8–48.5)	2.7 (0–46.1)	8.0 (0.7–115.4)	0.0823
Mean (SD)	10.3 (0.50)	7.0 (0.49)		20.0 (0.47)	12.4 (0.48)	8.8 (0.49)	15.4 (0.47)	
Chemotherapy before admission								
Median (days)	7	13.5	0.1112					
Range (days)	1–64	1–79						
Less than 14 Days	9 (45)	6 (40)	1					
Greater than 14 Days	3 (15)	4 (26.7)	0.4301					
No treatment	8 (40)	5 (33.3)	0.7372					

(Continued)

TABLE 2 | Continued

Characteristics	Group A Hospitalized	Group B Intubated and/or Deceased	*p*-Value *A* vs. *B*	Group C Asymptomatic Suspicious CT	Group D Symptomatic, Home Isolation	Group A + B	Group C + D	*p*-Value A + B vs. C + D
Hypoxemia on presentation to the hospital								
Yes	7 (35)	11 (73.3)	**0.0271**					
No	6 (30)	0						
Unknown	7 (35)	4 (26.7)						
Comorbidities								
0 or 1 factor	7 (35)	2 (13.3)	0.2444	16 (53.3)	21 (51.2)	9 (25.7)	37 (52.1)	**0.0124**
2 or more factors	13 (65)	13 (86.7)		14 (46.7)	20 (48.8)	26 (74.3)	34 (47.9)	
HTN	13 (65)	14 (93.3)	0.1009	18 (60)	24 (58.5)	27 (77.1)	42 (59.2)	0.0844
DM	6 (30)	7 (46.7)	0.481	8 (26.7)	13 (31.7)	13 (37.1)	21 (29.6)	0.5085
HLD	9 (45)	8 (53.3)	0.738	11 (36.7)	8 (19.5)	17 (48.6)	19 (26.8)	**0.0309**
History of PE/DVT	4 (20)	1 (6.7)	0.365	1 (3.3)	3 (7.3)	5 (14.3)	4 (5.6)	0.1526
COPD/ILD/asthma	4 (20)	2 (13.3)	0.6804	6 (20)	5 (12.2)	6 (17.1)	11 (15.5)	1
Baseline lab for immune status								
Abnormal	8 (40)	3 (20)	0.2814	7 (23.3)	7 (17.1)	11 (31.4)	14 (19.7)	0.2254
Normal	12 (60)	11 (73.3)	0.4885	23 (76.7)	29 (70.7)	23 (65.7)	52 (73.2)	0.4975
Unknown	0	1 (6.7)	0.4286	0	5 (12.2)	1 (2.9)	5 (7)	0.6611
Performance status (PS)								
PS 0–1	13 (65)	11 (73.3)	0.721	29 (96.7)	36 (87.8)	24 (68.6)	65 (91.5)	**0.0042**
PS ≥2	6 (30)	3 (20)	0.7003	1 (3.3)	1 (2.4)	9 (25.7)	2 (2.8)	**0.0007**
Unknown	1 (5)	1 (6.7)	1	0	4 (9.8)	2 (5.7)	4 (5.6)	1

**Confirmed by PCR test. (AB): confirmed by positive SARS-CoV-2 antibody test.*
NED, no evidence of disease; Tx, treatment; SD, standard deviation; m, months; COPD, chronic obstructive pulmonary disease; ILD, interstitial lung disease. Bolded values represented significant results in the p-values.

Clinical Features of the Hospitalized Patients

Thirty-five patients required hospitalization. Twenty patients did not require intubation and survived (group A). Among 15 patients in group B, 7 required intubation and 13 were deceased. The intubation rate was 46.7% and only 1 intubated patient survived (14.3%). Two patients died at a second admission, and 1 died in a rehabilitation center after discharge. The case fatality rate based on the hospitalized patients was 37.1 % (13 out of 35) and 12.3 % (13 out of 106) based on all cases.

The tumor and treatment characteristics of those patients in group B are shown in **Table 3**. The tumor types were breast ($n = 5$), lung ($n = 3$), lymphoma ($n = 2$), GYN ($n = 2$), GI ($n = 2$), and 1 case of unknown primary. Twelve patients received any type of anti-cancer treatment within 3 months, while 6 (40%) received negative impact chemotherapy ≤14 days. Most patients had advanced cancer ($n = 12$), while three patients had no evidence of disease (NED). Of note, 13 of the 15 patients in group B had 2 or more comorbidities.

At the time of admission, excluding those who were admitted to outside hospitals with incomplete information, 7 (35%) and 11 (73.3%) patients in group A and B had hypoxemia (room air oxygen saturation <92%) (**Table 4**). Fourteen patients in group A also met other criteria for admission: anemia with or without bleeding from the gastrointestinal tract requiring transfusion, neutropenia with or without fever, mental status changes, or syncope with a burn (**Table 4**). Three patients in groups A were admitted for other reasons and had incidental findings of positive SARS-CoV-2 infection (**Table 4** legend). All admitted patients in group B with available medical records showed respiratory compromise. More than 50% of patients in group A ($n = 11$) required oxygen supplement during the hospital course, and most patients in group B progressed with worsening respiratory status, with 7 requiring mechanical ventilation, all for respiratory failure (**Table 4**). A number of patients also developed other complications including renal insufficiency, liver function abnormalities, venous or arterial thrombosis and sepsis (**Table 4**). Lymphopenia, elevation of LDH, and elevation of D-Dimer were very common (**Table 4**).

In comparing the clinical characteristics to differentiate patients in group A from group B, there were no statistical differences in age, active cancer status, treatment status, number of comorbidities, whether chemotherapy with negative impact had been given or not, or given within 30 days (**Table 2**). There was no statistical difference between group A and B in terms of the duration of any treatment or the duration of treatment with negative impact agents prior to the diagnosis of SARS-CoV-2 infection (**Table 2**). The difference in the percentage

TABLE 3 | Clinical characteristics of patients in group B, hospitalized and diseased or requiring intubation.

	Age	Gender	2 or more comorbidities	Primary tumor	Localized tumor or metastatic site	Cancer treatment	Last treatment to admission day	Hospital Days	Was intubation required?	Outcome
1	68	Female	✓	Endometrial	Peritoneum	None	N/A	No info		Deceased
2	83	Female	✓	NSCLC	Liver	Dabrafenib*, Trametinib*	N/A	3		Deceased
3	68	Male	✓	Esophageal	Bilateral supraclavicular and lower cervical lymphadenopathy	Cisplatin, Irinotecan	11 days	8	✓	Deceased
4	62	Female	✓	Breast	Chest wall, bone, mediastinal, retroperitoneal LNs	Paclitaxel, Atezolizumab	6 days	12 2nd admission 3 days		Deceased
5	64	Female	✓	Breast	Bone, lung, and pleura	Ibrance, Fulvestrant	28 days	14	✓	Deceased
6	71	Male	✓	Gastric	Stomach	Docetaxel, Oxaliplatin, Leucovorin, 5-FU	14 days	30	✓	Deceased
7	80	Female	✓	Breast	Localized	Paclitaxel	1 day	22 2nd admission 16 days		Deceased
8	65	Male		NSCLC	Mediastinal soft tissue, visceral pleura	Tagrisso, Carboplatin, Alimta	51 days	12		Deceased
9	69	Male	✓	SCLC	LNs, liver, and bone	Carboplatin, Etoposide, Durvalumab	13 days	6		Deceased
10	66	Male	✓	Unknown primary	Lung	Gemcitabine, Carboplatin	51 days	3		Deceased
11	83	Female	✓	Endometrial	NED	Carboplatin, Paclitaxel	79 days	2		Deceased
12	48	Male		Lymphoplasmacytic Lymphoma, WM	Lymph nodes	None	N/A	60	✓	Alive
13	67	Female	✓	DLBCL	NED	None	N/A	23	✓	Deceased
14	77	Female	✓	Breast	Lung/Brain	Adriamycin	10 days	24	✓	Deceased
15	65	Female	✓	Breast	NED	Herceptin*, Letrozole*	N/A	Discharged April 2020	✓	Alive

NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; DLBCL, diffuse large B cell lymphoma; WM, Waldenstroms Macroglobulinemia; NED, no evidence of disease.

of 2 or more comorbidities between group A and B was not statistically significant (**Table 2**). However, hypoxemia at presentation was significantly more common in group B than in group A ($p = 0.0271$), suggesting that a respiratory compromise at presentation was an important adverse predictive factor for poor survival.

We examined the hospital admission days for all the patients who were admitted and discharged (group A + 2 patients from group B) (**Figure 2**). The median duration of days for their first admission was 10, ranging between 5 and 60 days. Five patients (27.8%) had a second admission, while 2 of the 5 patients then had a third admission. The next admission dates were 1–27 days from the last discharge, with a median of 5 days. The median duration of cumulative hospitalized days (including all admissions) was 16 with a range of 5–60 days.

Characteristics of Patients Under Home Isolation

The right side of **Table 2** shows the clinical characteristics of patients in groups C and D. There were 30 patients in group C (6 due to suspicious surveillance CT scan, 3 due to history of close exposure and 21 due to retrospective identification with positive COVID-19 antibody test). Those patients did not show detectable symptoms during subsequent follow up

TABLE 4 | Presentation and admission criteria for admitted patients, as well as their hospital course and complications.

	Group A *N* = 20 Number/Available (%)	Group B *N* = 15 Number/Available (%)
Hypoxia at presentation[a]		
Yes	7 (35)	11 (73.3)
No	6 (30)	0 (0)
Unknown	7 (35)	4 (26.6)
Other criteria for admission at presentation		
Anemia requiring transfusion	3 (15)	0 (0)
GI bleeding and anemia	2 (10)	0 (0)
Neutropenia ± fever	3 (15)	1 (6.7)
Mental status changes	3 (15)	5 (33.3)
Syncope	1 (5)	0 (0)
Incidental finding[b]	3 (15)	0 (0)
Unavailable information	3 (15)	3 (20)
Hospital course with progression of hypoxemia		
Unknown	6 (30)	2 (13.2)
Room air only	3 (15)	0 (0)
Oxygen supplement by:		
NC or Ventruri mask	8 (40)	2 (13.2)
HFNC	2 (10)	0 (0)
100% NRB	1 (5)	4 (26.6)
Ventilator	0 (0)	7 (46.6)
Prophylactic intubation	0 (0)	0 (0)
Intubation due to respiratory failure	0 (0)	7 (100)
Hospital course with other complications[c]		
Renal insufficiency[d]	10/14 (71.4)	7/10 (70)
LFT elevations	10/14 (71.4)	10/10 (100)
DVT or PE	1 (5)	0 (0)
A-Fib or MI	1 (5)	2 (13.2)
Bacterial pneumonia, bacteremia	1 (5)	3 (20)
Other lab abnormalities[c]		
Lymphopenia	13/14 (92.9)	10/10 (100)
Elevation of LDH	11/12 (91.7)	10/10 (100)
Elevation of D-Dimer	11/12 (91.7)	9/9 (100)

[a] *Hypoxia is defined as oxygen saturation at room air to be < 92%.*
[b] *The reasons of admission for the 3 patients who had incidental findings of SARS-CoV-2 infection status were: social admission (n = 1), displaced nephrostomy tube (n = 1), and large neck mass requiring emergency tracheostomy (n = 1).*
[c] *The percentages in those categories are calculated based on the number of patients with available information, not the total number of patients in the respective groups.*
[d] *Abnormal lab values: any value outside the upper or lower limit of normal reference value per hospital lab.*
GI, gastrointestinal; NC, nasal cannula; HFNC, High flow nasal cannula; NRB, non-rebreather; LFT, liver function tests; DVT, deep vein thrombosis; PE, pulmonary embolism; A-Fib, atrial fibrillation; MI, myocardial infarction; LDH, Lactate dehydrogenase.

or retrospective recall. The months of diagnosis was not able to be ascertained in 18 patients, as they were captured by positive COVID-19 antibody tests, but could not recall any previous symptoms or close contact. Group C was noted to have the numerically lowest percentage of patients having presence of disease (46.7%), and the numerically highest percentage of patients in the category of NED + treatment (40%). Group D had 41 patients who had various degrees and constellations of COVID-19 symptoms with subsequent recovery. Although 76.7% and 70.7% patients in group C and D were receiving active treatments, only 20% and 26.8% were receiving myelosuppressive treatments, respectively, and an additional 10 and 4.9% were receiving immunomodulating treatments, respectively; the rest of the patients were receiving endocrine therapy. Although there was a numerical increase in patients taking negative impact treatment in group C vs. group D, it was not statistically significant ($p = 0.8002$).

Comparison of Clinical Factors Between the Hospitalized Patients and At-Home Recovery Patients

We then compared the clinical characteristics of patients who were hospitalized (groups A + B) to patients who recovered at home (groups C + D) (**Table 2**). Patients in Groups A + B were older patients (mean age 71 vs. 63 years old, $p = 0.000299$). There was no difference between groups A + B vs. groups C + D regarding the factors of having active disease, receiving anti-cancer treatment, or both. Receiving negative impact therapies was a distinguishing factor (62.9 vs. 32.4%, $p = 0.0036$), which was driven mainly by receiving myelosuppressive treatment (45.7 vs. 23.9%, $p = 0.0275$), while receiving immunosuppressive or immunomodulating treatments did not show difference between groups A + B vs. groups C + D (**Table 2**). Patients taking negative impact treatment within 30 days of admission was 48.6% in groups A + B and 36.6% in groups C + D, which was also statistically significant ($p = 0.017$). Groups A + B have significantly more patients harboring 2 or more other comorbidities (74.3 vs. 47.9%, $p = 0.0124$).

Patients in group C + D had a longer median duration on treatment (11.2 months) than that in group A + B (3.0 months), which was statistically significant, although opposite to the intuitive prediction. Another way of examination by mean + standard deviation (SD) showed similar trend and same *p*-value. The duration of negative impact treatments between Group A + B vs. group C + D did not show statistical significance.

At a baseline assessment of the performance status (PS) of the patients, patients in groups A + B had more patients with PS of 2 or above than groups C + D, indicating patients who needed hospital care had poorer performance status at baseline. The baseline immune status, taking into account of absolute neutrophil counts, or absolute lymphocyte counts or albumin levels, was similar in all groups.

A multivariate analysis was carried out to explore the most important risk factors that were associated with a more severe form of SARS-CoV-2 infection with hospitalization (group A + B) vs. home recovery (group C + D) (**Table 5**), we observed that older age (Odds Ratio, OR = 1.01, 95% CI: 1.00–1.02) ($p = 0.004$) was significant. According to this statistical model, OR>1 indicates more likelihood for hospitalization, and the odds increases by 1% for each additional year of age. Being on active myelosuppressive treatment (OR = 0.78, 95% CI: 0.66–0.93)

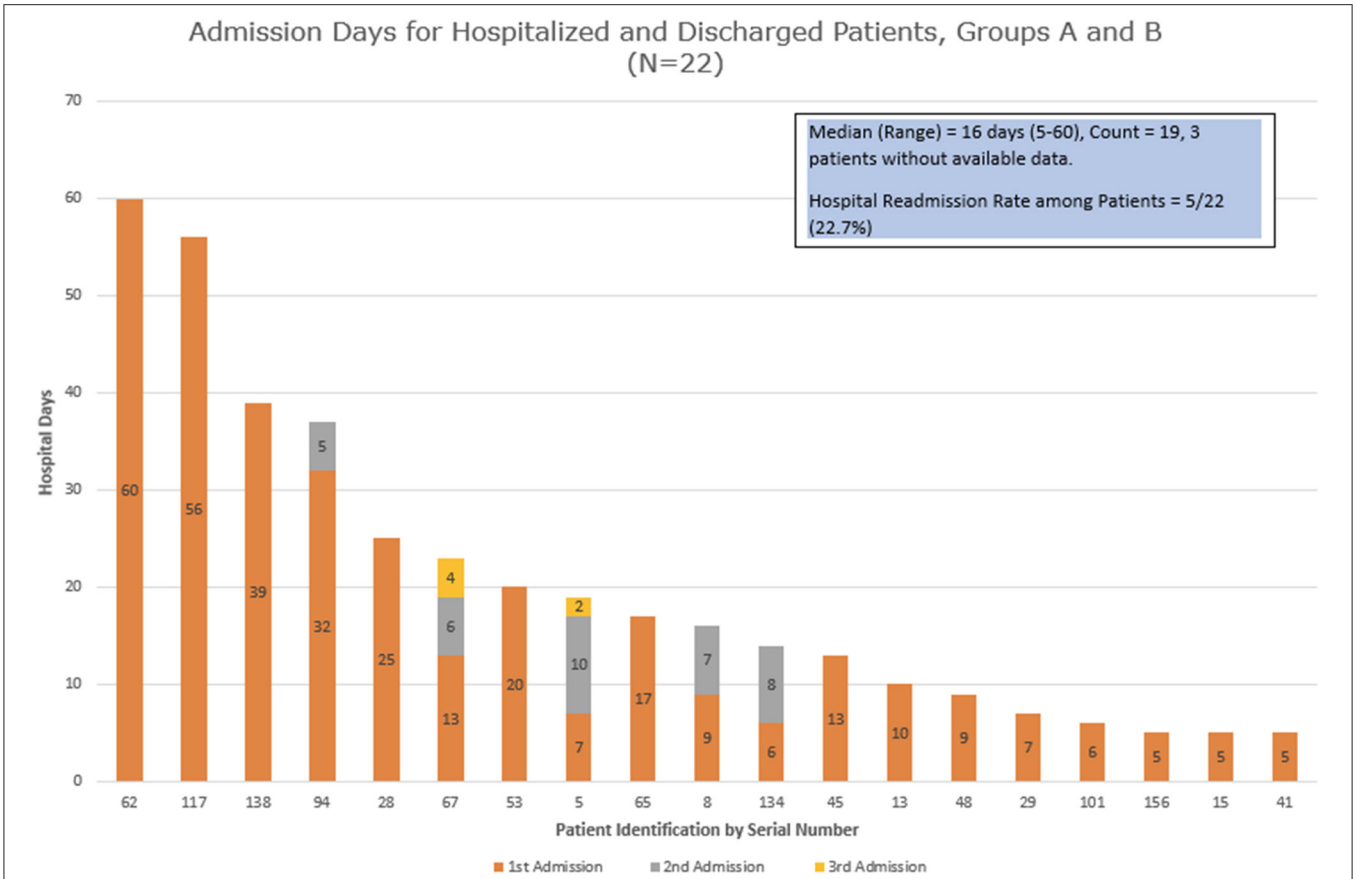

FIGURE 2 | Admission days for hospitalized and discharged patients.

($p = 0.006$) and having a poorer performance status ≥ 2 vs. PS 0–1 (OR = 0.64, 95% CI: 0.49–0.84) ($p = 0.002$) were significantly associated with a more severe status of infection. Negative impact treatment as a whole, and 2 or more comorbidities, although significant predictors in univariate analysis, were not significant in multivariate analysis.

Time Kinetics of Persistent SARS-CoV-2 Virus Status

Thirteen patients who were discharged home from groups A + B, and 2 patients from group D had a subsequent repeat positive COVID-19 RT-PCR test. The days between the initial positive test and the last positive test was defined as "positive duration." The median duration for the positive duration was 28 days, with a range of 10–86 days (**Figure 3**).

Re-initiation of Cancer Treatment

The details of patients receiving treatment prior, during and after their SARS-CoV-2 diagnosis are shown in **Table 6**. Among the 15 patients in group A + B who survived hospital admission and were receiving treatment prior to SARS-CoV-2 infection and the 2 patients waiting to start new treatment, 9 (52.9%) patients started treatment after recovery, with a median delay of 40 days (range 14–75 days). In group C, 22 patients were receiving treatment and 2 were waiting to start treatment, and all of them continued or started treatment as planned during their presumable SARS-CoV-2 infection duration. Two patients were supposed to start adjuvant chemotherapy after surgery, and they had a delayed start. The on-schedule rate was 92.3% (24/26). In group D, 29 patients were on treatment prior to SARS-CoV-2 diagnosis, and 15 continued on schedule, while 13 restarted after a delay, with an on-schedule rate of 51.7%, and continuation rate of 96.6%. The median duration of delay was 17 days (range 6–31) based on cohort 1 group D data only, as the recall data for the duration of symptoms from cohort 2 group D patients was considered inaccurate.

DISCUSSION

We report results from a registry study at a community cancer center located in New York City, the epicenter for COVID-19 pandemic in the United States. Among 106 patients who had a confirmed SARS-CoV-2 diagnosis, 33.0% of the patients required hospitalization and the case fatality rate was 37.1% among those hospitalized and 12.3% for the entire cohort. Among the patients who required hospitalization, not having hypoxemia at presentation appeared to be a significant factor for survival. Other than that, we could

TABLE 5 | Multivariate logistic regression analysis on risk factors associated with a more severe status of SARS-CoV-2 infection: group A + B (hospitalized patients) vs. group C + D (home recovery patients).

Characteristics	Univariate		Multivariate	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Age	1.06 (1.03–1.11)	0.001	**1.01 (1.00–1.02)**	**0.004**
Gender:				
Male (vs. Female)	1.96 (0.82–4.67)	0.125		
Cancer types:				
Hematological (vs. Solid tumors)	2.73 (0.76–10.17)	0.119		
Disease status:				
Presence of disease (vs. NED)	1.86 (0.82–4.41)	0.146		
Treatment and disease:				
NED + Tx (vs. NED no Tx)	0.73 (0.19–2.93)	0.701		
Disease no Tx (vs. NED no Tx)	1.37 (0.26–7.02)	0.647		
Disease + Tx (vs. NED no Tx)	1.57 (0.50–5.60)	0.457		
Treatment:				
No treatment (vs. treatment)	0.95 (0.36–2.34)	0.909		
Treatment: (No vs. Yes)				
Myelosuppressive	0.37 (0.16–0.88)	0.025	**0.78 (0.66–0.93)**	**0.006**
Immunosuppressive	0.92 (0.41–2.09)	0.842		
Immunomodulating	0.45 (0.12–1.75)	0.239		
Any above negative impact	0.28 (0.12–0.65)	0.004		
Negative Impact <30 days	0.35 (0.15–0.80)	0.014		
Time duration of active treatment (m)	0.98 (0.94–1.00)	0.138		
Time duration of tx with negative impact (m)	0.98 (0.93–1.01)	0.298		
Comorbidities:				
2 or more factors (vs. 0 or 1 factor)	3.14 (1.33–7.98)	0.012		
HTN (No vs. Yes)	0.43 (0.16–1.04)	0.072		
DM (No vs. Yes)	0.71 (0.30–1.69)	0.433		
HLD (No vs. Yes)	0.39 (0.16–0.90)	0.028		
h/o PE/DVT (No vs. Yes)	0.36 (0.08–1.44)	0.146		
COPD/ILD/asthma (No vs. Yes)	0.89 (0.31–2.79)	0.828		
Baseline lab for immune status:				
Normal (vs. abnormal)	0.56 (0.22–1.44)	0.226		
Unknown (vs. abnormal)	0.25 (0.01–1.89)	0.241		
Performance				
PS ≥2 (vs. PS 0–1)	12.19 (2.89–83.82)	0.002	**1.57 (1.19–2.06)**	**0.002**

Bolded values represented significant results in the p-values.

not identify distinguishing factors such as age, comorbidity, tumor characteristics, or treatment characteristics to predict mortality. However, we did identify multiple factors that were associated with a more severe status of SARS-CoV-2 infection for those who required hospitalization vs. those who recovered at home. These factors were: (1) older age, (2) use of treatments with potential negative impact to immune system, which was represented mainly by patients receiving myelosuppressive therapies, (3) having more than 2 comorbidities, and (4) a baseline poor performance status. Among them, older age, having myelosuppressive chemotherapy, and a baseline poor performance status emerged in multivariate analysis as strong, significant risk factors for a severe form of SARS-CoV-2 infection.

The relationship of cancer and outcome from SARS-CoV-2 infection has been the topic of multiple studies (2, 5, 7–11). The vulnerability of cancer patients to severe SARS-CoV-2 complications and increased mortality is presumably owing to immunosuppression from either the presence of disease and/or the detrimental effects from anti-cancer treatment. The tumor burden may presumably induce secondary decline in metabolism, nutritional status, and even more immunosuppression, which is not well-defined. In that regard, the study from Dai et al. suggested that patients with metastatic disease had the highest frequency of severe events, where the outcome was similar in patients without metastatic disease to patients without cancer history (12). However, multiple published studies generated conflicting results of whether

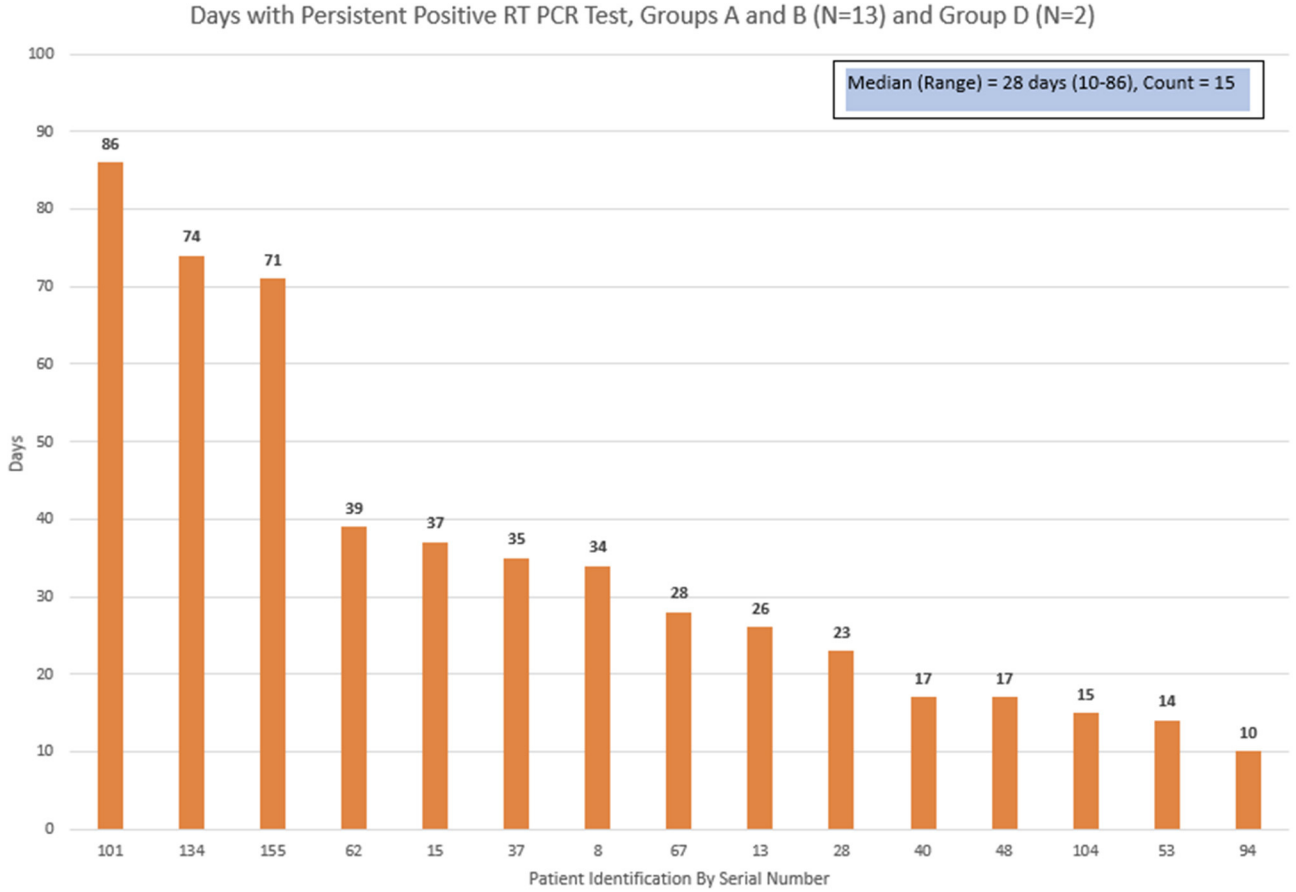

FIGURE 3 | Time kinetics in persistent positivity of COVID PCR test.

chemotherapy alone stands as a sole negative factor for the severity of the infection. While multicenter studies from China suggested detrimental effects from anti-cancer treatment (2, 12), studies from the COVID-19 and Cancer Consortium (CCC) and MSKCC did not support it (9, 10). Even in multiple myeloma patients, the study from Mount Sinai revealed no bearing of treatment drug exposure (13).

Our observations provide evidence to support the above presumptions and show that treatment with negative impacts therapies resulted in worse outcomes. Importantly, myelosuppressive chemotherapy was clearly a differentiating factor, while immunomudulating therapies (namely immune check-point inhibitors) were not. Our study was unique in multiple ways. We focused on chemotherapies with negative impact instead of on all anti-cancer treatment, as a large portion of these treatments are of endocrine or non-myelosuppressive nature which may not negatively affect a patient's immune defense. While most of the previous studies analyzed data on symptomatic hospitalized patients, we selected our control group to include patients who recovered at home and asymptomatic (group C), or mildly to moderately symptomatic (group D). We demonstrated that treatments with negative impact resulted in worse symptoms leading to hospital care but did not differentiate outcomes leading to death or survival. Age and comorbidity in cancer patients fared as unfavorable factors as well, as depicted in all other published studies for both cancer patients and for the general population. Our study did not show a relationship of metastatic disease with worse outcomes. Performance status is a way to quantify the general well-being and activities of daily living in cancer patients. This measure has been widely used in determination of a patient's eligibility for aggressive chemotherapy. Interestingly, patients in groups A + B had a higher percentage of decreased performance status, suggesting "compromised functional status" as a predictive marker for severe infection as well. Patients in group C + D had a statistically significant longer median duration on treatment (11.2 months) than that in group A + B (3.0 months). Although this observation may be opposite to the intuitive prediction, one could attempt a presumptive hypothesis that the patients in group C + D had undergone a natural selection to be those who had preserved PS to continue treatment for a prolonged time.

Based on the findings in our study, we would recommend consideration of decreasing the exposure to treatments with negative impact on patients who are older, or with compromised functional status and have comorbidities during the height of the COVID-19 pandemic. To address the concerns mentioned

TABLE 6 | Treatment before, during, and re-initiation after SARS-CoV-2 diagnosis.

	Group A + B	Group C	Group D
Total patients	35	30	41
Total on treatdment before COVID-19 pandemic	15 (42.9)	22 (73.3)	29 (70.7)
Myelosuppressive	8 (22.9)	5 (16.7)	11 (26.8)
Immunosuppressive	2 (5.7)	0	1 (2.4)
Immunomodulating	2 (5.7)	3 (10)	2 (4.9)
Non-myelosuppressive or endocrine or radiation	3 (8.6)	14 (46.7)	15 (36.6)
Waiting to start treatment before or during COVID-19 pandemic	2 (5.7)	2 (6.7)	3 (7.3)
Treatment during pandemic and SARS-CoV-2	0	24 (80)	15 (36.6)
Diagnosis			
On schedule	0	22 (73.3)	15 (36.6)
New start	0	2 (6.7)	0
Myelosuppressive	0	7 (23.3)	4 (9.8)
Immunosuppressive	0	0	0
Immunomodulating	0	3 (10)	0
Non-myelosuppressive or endocrine or radiation	0	14 (46.7)	11 (26.8)
Delayed: Re-initiation after SARS-CoV-2 diagnosis	9 (25.7)	0	13 (31.7)
Or start new treatment	0	2 (6.7)	3 (7.3)
Myelosuppressive	7 (20)	2 (6.7)	6 (14.6)
Immunosuppressive	0	0	1 (2.4)
Immunomodulating	1 (2.9)	0	2 (4.9)
Non-myelosuppressive or endocrine or radiation	1 (2.9)	0	7 (17.1)
Duration of delay (from discharge to restart): Median (range)	40 (14–75)	57.5 (37–78)	17 (6–31)*
Restart rate (%)	52.9 (9/17)	N/A	N/A
On-schedule rate (%)	0	92.3 (24/26)	51.7 (15/29)
Continuation rate (%)	n/a	100 (26/26)	96.6 (28/29)

Based on cohort 1 data only.

above, the medical oncology community has already developed guidelines for treatment modifications and adopted practice-changing strategies. Most of them considered individualized assessment incorporating both disease and treatment factors (14).

The case fatality rate (CFR) in our study was 37.1% among hospitalized patients and 12.3% among all the study population. This is comparable to other studies conducted at New York City, most notably the study from Montefiore Hospital system, which reported a CFR of 28% among mainly hospitalized cancer patients (5). Memorial Sloan Kettering Cancer Center reported CFR of 12% in overall symptomatic cancer patients; considering a reported 40% admission rate, the CFR would be 30.1% among hospitalized/admitted patients (9). Socioeconomic status, racial disparity, timely pursuit of medical care, and access to critical care resources in an overwhelmed community hospital may contribute to the poor outcome of our hospitalized patients, in addition to their medical conditions. On the other hand, the CFR in the general population in the New York City during the same period was 5.2% and 6% from 2 reports (5, 9), much lower than that of the cancer patients. Furthermore, our cancer patients who contracted SARS-CoV-2 infection and required hospital admission had a median cumulative admission of 16 days, while the New York City average was 3.9 days (15). In addition, they required a longer time for virus clearance (median of 28 days), while it was 4–17 days in general population (16, 17). As we defined positive virus interval to be between 2 positive RT-PCR results, this becomes an underestimation of the true virus clearance time. Other studies also reported similar observation of prolonged virus clearance (13, 18). The above results collectively confirmed that cancer patients had a higher risk for more severe events when contracted with SARS-CoV-2 infection.

In this study, we reported for the first time that cancer patients with SARS-CoV-2 infection might exhibit no symptoms. They not only appeared "healthy," but also actually remained healthy, as many of them were able to receive planned anti-cancer treatment on schedule. This group (group C) is comprised of 28.3% (30/106) of the all patients, with 46.6% of them having presence of tumor and 76.7% of them taking treatment. Likewise, patients with similar characteristics might have mild symptoms who were able to recover at home and almost all resumed planned treatment. It is interesting to note that the asymptomatic carrier rate of 28.3% in cancer patients was moderately lower than the 40% in the public that was reported by the Center of Disease Control (CDC) in July 2020 (19).

We also attempted to give assessment of the patients' immune status at baseline. The function of immune system may be measured by both humoral and cellular immunity. Judging from the routine clinical labs, the immune status may be partially measured by the absolute neutrophil count (ANC) and the absolute lymphocyte count (ALC). The quantitative immunoglobin level is a more direct assessment of the humoral immunity, but it is not routinely tested. With a composite assessment of ANC, ALC, and albumin values taken at a visit prior to COVID-19 pandemic, we noted that about 60–70% patients had all normal labs, and no difference was found among different groups, or between groups A + B vs. C + D. These results suggest that not all patients with cancer and/or on treatment are rendered a status of severe immunosuppression to the point of being unable to fight the coronavirus. In fact, in our previous study on the generation of protective neutralizing antibodies after H1N1 vaccination in 2015, cancer patients' response to vaccination was as good as the healthy controls (20). Similarly, in the patient cohort of multiple myeloma at Mount Sinai hospital, a majority of the patients also mounted anti-COVID-19 antibodies (13). Furthermore, 52.9% of patients (groups A + B) who required hospital admission and 96.6% of patients who had mild symptoms (group D) were able to start or restart anti-cancer treatment after a hiatus. This observation demonstrated the proportion and degree of complete recovery of cancer patients from SARS-CoV-2 infection, albeit with a longer time course.

The strength of this study is the inclusion and analysis of the characteristics of asymptomatic patients. Some of those rose to attention due to suspicious CT findings or a history of close

exposure; and a large portion was discovered with antibody screening in late May and June. This part of the study cohort is not covered in most of the published studies (2, 5, 8, 9, 13). This research methodology enabled us to have a glimpse of the base of the pyramid, to those who had cancer and mild symptoms and to examine their cancer burden and treatment status. The inclusion of cohort 2, with consecutive patients identified by electronic medical records for positive COVID-19 antibody tests, also significantly reduced a selection bias of not encompassing all patients who recovered at home with no or mild symptoms. Another strength is the analysis of patients' baseline immune status and performance status prior to the pandemic and the identification of a baseline performance status as a predictive marker for the severity of disease.

There are multiple limitations in our study. First, our data collection reflected a relatively small sample size. Second, about 69.8% of patients in this study were females, and breast cancer was the most prevalent diagnosis. Very small numbers of hematological malignancies were represented here, therefore raising cautions in generalizing the conclusion to patients with other malignancies. Third, we also excluded patients who were admitted to outside hospitals who lacked confirmatory information. Lastly, we excluded patients with suspicious clinical findings but negative or unconfirmed COVID-19 antibody test results. It is possible that some patients with true infection did not develop antibodies to COVID-19, or the presence of antibodies in some asymptomatic and mildly symptomatic patients was transient and diminished at the time of the test. In a recent publication, 40% of asymptomatic patients vs. 12.9% of symptomatic patients became seronegative in the early convalescent phase (21).

Overall, our observations should add valuable information to the rapidly accumulating world evidence of cancer and SARS-CoV-2 infection. Systemic anti-cancer treatment with a potential of negative impact to the immune system, particularly myelosuppressive chemotherapy, advanced age, with compromised functional status, and having more than 2 comorbidities were unfavorable factors associated with more severe infection status and hospital admissions but not for in-hospital mortality. Cancer patients not only have a higher mortality rate than the general population, but they also have longer hospital admission stay and protracted virus clearance time. On the other hand, patients with cancer on active treatment still may have mild disease, improve without hospitalization, and re-initiate anti-cancer treatment after recovery from SARS-CoV-2 infection. Proactive mitigation of modifiable factors and a careful balance of benefits and risks associated with anti-cancer treatment is warranted to safely navigate our cancer patients' course during the COVID-19 pandemic.

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 Maimonides Medical Center Institutional Review Board (IRB # 2020-04-05). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

DL, NS, BD, and YX: conception of research. KB, SB-R, JD'S, YH, JL, TM, LM, PM, PR, BD, and YX: provision of patient information. DL, YW, ST, VN, BD, and YX: data collection and analysis. YW: statistical analysis. DL, YW, BD, and YX: principal manuscript writing. All authors contributed to the manuscript writing and approval.

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frontiers in Medicine

SYSTEMATIC REVIEW
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Skin Manifestations in COVID-19 Patients: Are They Indicators for Disease Severity? A Systematic Review

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Introduction: Until now, there are several reports on cutaneous manifestations in COVID-19 patients. However, the link between skin manifestations and the severity of the disease remains debatable. We conducted a systematic review to evaluate the temporal relationship between different types of skin lesions and the severity of COVID-19.

Methods: A systematic search was conducted for relevant studies published between January and July 2020 using Pubmed/Medline, Embase, and Web of knowledge. The following keywords were used: "SARS-CoV-2" or "COVID-19" or "new coronavirus" or "Wuhan Coronavirus" or "coronavirus disease 2019" and "skin disease" or "skin manifestation" or "cutaneous manifestation."

Results: Out of 381 articles, 47 meet the inclusion criteria and a total of 1,847 patients with confirmed COVID-19 were examined. The overall frequency of cutaneous manifestations in COVID-19 patients was 5.95%. The maculopapular rash was the main reported skin involvement (37.3%) commonly occurred in middle-aged females with intermediate severity of the disease. Forty-eight percentage of the patients had a mild, 32% a moderate, and 20% a severe COVID-19 disease. The mild disease was mainly correlated with chilblain-like and urticaria-like lesions and patients with vascular lesions experienced a more severe disease. Seventy-two percentage of patients with chilblain-like lesions improved without any medication. The overall mortality rate was 4.5%. Patients with vascular lesions had the highest mortality rate (18.2%) and patients with urticaria-like lesions had the lowest mortality rate (2.2%).

Conclusion: The mere occurrence of skin manifestations in COVID-19 patients is not an indicator for the disease severity, and it highly depends on the type of skin lesions. Chilblain-like and vascular lesions are the ends of a spectrum in which from chilblain-like to vascular lesions, the severity of the disease increases, and the patient's prognosis worsens. Those with vascular lesions should also be considered as high-priority patients for further medical care.

Keywords: COVID-19, coronavirus – COVID-19, skin manifestations, skin - pathology, systematic literature search, disease severity, mortality, prognosis

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INTRODUCTION

A viral outbreak caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged from Wuhan, China in late December 2019 (1). The disease was named coronavirus disease 2019 (COVID-19) by World Health Organization (WHO) and was declared as a pandemic on 11 March 2020 (2). After 1 year from the beginning of the pandemic, the full spectrum of COVID-19 presentations and its relationship with disease severity is still unknown. Fever, cough, chills, dyspnea, myalgia, and sore throat are the most common clinical presentations of COVID-19 and as time goes on, different other manifestations have been reported (3). Recently, skin lesions have been described as potential manifestations of COVID-19 (4–6). The cutaneous changes reported to date include maculopapular rash, vesicular lesions, urticaria-like lesions, and chilblain-like lesions (4–8). Some of these skin manifestations arise before the signs and symptoms more commonly associated with COVID-19, suggesting that they could be presenting signs of COVID-19 (9). However, the link between skin manifestations and the severity of the disease remains debatable. Due to the great variety of reported dermatologic presentations as well as the inconsistency of data on the association between skin presentations of COVID-19 with poor outcome, we aimed to conduct a comprehensive systematic review on the clinical and histopathological characteristics of skin manifestations in relation to other features of confirmed COVID-19 patients and to evaluate the temporal relationship between different types of skin lesions and the severity of COVID-19.

METHODS

This review conforms to the "Preferred Reporting Items for Systematic Reviews and Meta-Analyses" (PRISMA) statement (10). Registration: PROSPERO (pending registration ID: 215422).

Search Strategy and Selection Criteria

To investigate the prevalence and characteristics of cutaneous manifestations in COVID-19 patients, a systematic search was conducted for relevant studies published between January and July 2020 using Pubmed, Embase, and Web of knowledge.

The following search terms were used (designed using MeSH keywords and Emtree terms): "SARS-CoV-2" or "COVID-19" or "new coronavirus" or "Wuhan Coronavirus" or "coronavirus disease 2019" and "skin disease" or "skin manifestation" or "cutaneous manifestation." Only studies included if they contained data about the skin manifestation in patients with confirmed COVID-19. There were no language restrictions. We got help from the Google Translate system for non-English papers. Review articles, duplicate publications, and articles with no relevant data were excluded from the analysis. Two authors independently screened the remaining articles. Finally, selected data were extracted from the full-texts of eligible publication by other investigators of the team.

Data Extraction

Data about the first author's name, date of publication, country, number of COVID-19 patients, number of cases with skin manifestations, age, gender, location and type of skin manifestations, associated cutaneous symptoms, the onset of skin lesions with systemic symptoms, the median duration of the lesions, treatment strategies and main histological findings of the lesions as well as comorbidities, associated symptoms, drug history, laboratory findings, severity and outcome of the patients were selected for further analysis. All cutaneous presentations related to COVID-19 were categorized into six groups: chilblain-like, vesicular, urticaria-like, maculopapular, vascular, and miscellaneous (lesions that we couldn't subscribe to any of the groups). Petechiae, purpura, livedo, and necrosis were classified into vascular lesions. Two authors (PJ, BH) independently extracted the data from the selected studies. The data was jointly reconciled, and disagreements were discussed and resolved between review authors (PJ, BH, MJN).

Quality Assessment

The critical appraisal checklist for case reports provided by the Joanna Briggs Institute (JBI) was used to perform a quality assessment of the studies (11).

RESULTS

At the first round of review, 381 articles were selected. After removing the duplicates and studies that did not meet the entry criteria, 88 full texts were finally selected for further assessment. Of these, only 47 articles had the characteristics appropriated for systematic review and were entered into the data extraction (**Figure 1**). Most of the studies were case reports (47%, N: 22) followed by case series (42.4%, N: 20), retrospective hospital/private section-based study (6.4%, N: 3), and cross-sectional (4.2%, N: 2). Thirteen articles were originated from Italy, 11 from Spain, 10 from France, 5 from the USA, and others from Belgium, China, Thailand, Kuwait, Indonesia, Russia, Turkey, and Singapore. Information of the 47 analyzed articles can be found in **Table 1**.

A total of 1,847 patients with confirmed COVID-19 (based on positive RT-PCR or positive antibody tests) were examined in 47 articles, of which 597 patients had different skin manifestations. The overall frequency of cutaneous manifestations in COVID-19 patients was 5.95%.

Characteristics of the Cutaneous Lesions in Confirmed COVID-19 Patients

The maculopapular rash was the main reported skin involvement (37.3%) followed by chilblain-like lesions (18.4%). The prevalence rate of vesicular and urticaria-like lesions was 15% (**Table 4**).

The mean age of patients with cutaneous manifestations was 53.3 (ranging from 16 to 92) years. Chilblain-like lesions were more common in younger patients (mean age: 40.7 years) and vascular lesions were more common in the elderly (mean age: 72.3 years).

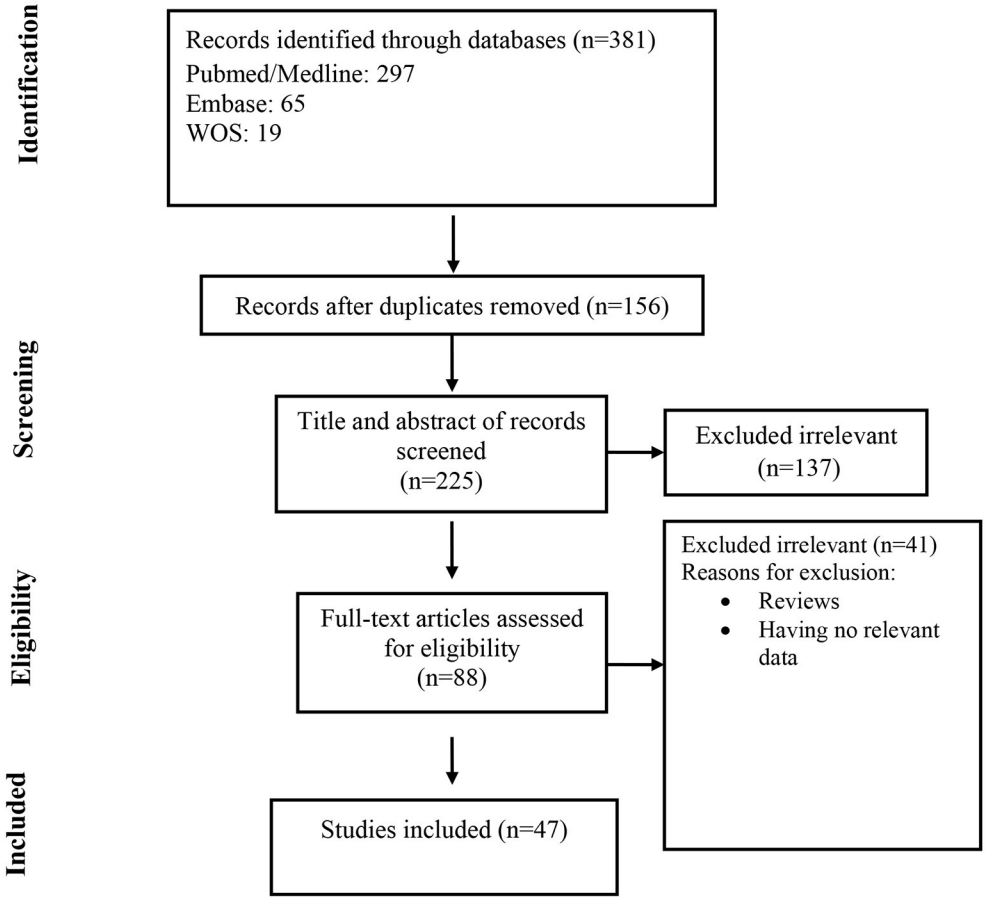

FIGURE 1 | Flow chart of study selection for inclusion in the systematic review and meta-analysis.

The prevalence of skin lesions was slightly higher in females than males (54 vs. 46%). Urticaria-like, chilblain- like and miscellaneous lesions were more frequent among females (**Table 4**). Vascular lesions were more frequent in males (61%). The prevalence of vesicular and maculopapular lesions was almost the same in men and women (51 and 49%).

Trunk, lower limb, and upper limb were the main involved regions. Chilblain-like and vascular lesions were more common in acral areas and except for maculopapular lesions, others were commonly located in the trunk. The maculopapular lesions were more common in extremities. The involvement of palms and soles were rare. Mucous membrane involvement was reported in all types of skin lesions particularly maculopapular and vascular lesions, but it was not reported in chilblain-like lesions (**Table 4**). Vesicular rashes could have diffused polymorphic or localized monomorphic patterns (27, 37).

Out of 597, 397 (66%) of the patients had associated cutaneous symptoms. Pruritus was the most prominent (238, 60%) particularly in vesicular lesions (89%). Pain was the most frequent symptom in chilblain-like lesions (63.5%) (see **Table 4**).

In the majority of patients (89.5%), dermatologic manifestations presented after (55%) or at the same time (34.5%) with the onset of systemic symptoms of COVID-19. Urticaria-like lesions appeared usually as a concomitant symptom (47%). In 3.5% of patients particularly with chilblain-like lesions, skin manifestations were the only presentation of COVID-19. In 7% of patients, skin manifestations occurred before the systemic symptoms, particularly in chilblain-like lesions (**Table 4**).

The median duration of skin lesions was about 9 days ranging from 1 to 18 days (**Table 4**). Urticaria-like lesions had the least duration (5 days) and chilblain-like lesions had the most duration (14 days).

No skin biopsy or histological examination of urticaria-like lesions was performed. Therefore, the following results are related to other types of skin lesions.

Perivascular lymphocytic infiltration, spongiotic and interface dermatitis, and vacuolization or keratinocyte necrosis were the common histologic findings in skin biopsies, except for vesicular lesions. In vesicular lesions, the absence of inflammatory infiltrates, atrophic epidermis, and hyperkeratosis was reported. In almost all types of lesions (except maculopapular and vesicular lesions) thrombotic vasculopathy and red blood cell extravasation were present. Langerhans cell aggregations

TABLE 1 | Characteristics of the included studies.

First author	Country	Published time	Type of study	Mean age	Male/ Female	No. of confirmed COVID-19 patient(s)	No. of the patient(s) with skin manifestations
Hunt, M. (12)	USA	Mar 28-2020	Case report	20	1 M	1	1
*Recalcati, S. (6)	Italy	Apr 7-2020	Retrospective hospital-based study	–	–	88	18
*Joob, B. (13)	Thailand	Apr 7-2020	Retrospective hospital-based study	40.5	4 M, 37 F	41	1
Zhang, Y. (14)	China	Apr 7-2020	Case series	59	4 M, 3 F	7	7
Fiehn, C. (15)	Spain	Apr 15-2020	Case report	28	1 F	1	1
Mahé, A. (16)	France	Apr 16-2020	Case report	46	1 F	1	1
Zulfiqar, A. A. (17)	France	Apr 16-2020	Case report	65	1 F	1	1
*Magro, C. (18)	USA	Apr 18-2020	Case series	54.6	3 M, 2 F	5	3
Morey-Olive, M. (19)	Spain	Apr 22-2020	Case series	Boy: 6 years Girl: 2 months	1 M, 1 F	2	2
Najarian, D. J. (20)	USA	Apr 22-2020	Case report	58	1 M	1	1
Gianotti, R. (21)	Italy	Apr 29-2020	Case series	68	1 M, 2 F	3	3
Gianotti, R. (22)	Italy	Apr 30-2020	Case series	–	–	5**	5**
Sanchez, A. (23)	France	Apr 30-2020	Case report	Elderly	–	1	1
Galván Casas, C. (24)	Spain	Apr 30-2020	Cross-sectional	56.3	113 M, 121 F	234	234
Ahouach, B. (25)	France	May 1-2020	Case report	57	1 F	1	1
Quintana-Castanedo, L. (26)	Spain	May 1-2020	Case report	61	1 M	1	1
Marzano, A. V. (27)	Italy	May 1-2020	Case series	56.4	16 M, 6 F	22	22
Zengarini, C. (28)	Italy	May 3-2020	Case report	67	1 F	1	1
Alramthan, A. (29)	Kuwait	May 5-2020	Case series	31	2 F	2	2
Henry, D. (30)	France	May 5-2020	Case report	27	1 F	1	1
*Recalcati, S. (31)	Italy	May 6-2020	Case series	72.2	58 M, 49 F	107	3
*Tammaro, A. (32)	Italy	May 6-2020	Case series	–	–	130 + X***	2 + 1***
van Damme, C. (33)	Belgium	May 6-2020	Case report	71	1 M	1	1
Avellana Moreno, R. (34)	Spain	May 6-2020	Case report	32	1 F	1	1
Amatore, F. (35)	France	May 6-2020	Case report	39	1 M	1	1
Suarez-Valle, A. (36)	Spain	May 8-2020	Case series	–	–	3	3
Fernandez-Nieto, D. (37)	Spain	May 8-2020	Case series	45	6 M, 18 F	24	24
Paolino, G. (38)	Italy	May 8-2020	Case report	37	1 F	1	1
Diaz-Guimaraens, B. (7)	Spain	May 8-2020	Case report	48	1 M	1	1
Bouaziz, J. D. (39)	France	May 8-2020	Case series	–	–	14	14
Locatelli, A. G. (40)	Italy	May 9-2020	Case report	16	1 M	1	1
Jimenez-Cauhe, J. (41)	Spain	May 9-2020	Case series	66.7	4 F	4	4
Robustelli Test, E. (42)	Italy	May 10-2020	Case report	70	1 F	1	1
Gunawan, C. (43)	Indonesia	May 10-2020	Case report	51	1 M	1	1
*de Masson, A. (44)	France	May 28-2020	Retrospective private practices-based study	–	–	25	7
Freeman, E. E. (45)	USA	May 30-2020	Case series	41	12 M, 11 F	23	23
Bosch-Amate, X. (46)	Spain	June 03-2020	Case report	79	1 F	1	1
Reymundo, A. (47)	Spain	June 04-2020	Case series	66.6	2 M, 5 F	7	7
Gargiulo, L. (48)	Italy	June 07-2020	Case report	72	1 F	1	1
Freeman, E. E. (45)	USA	June 23-2020	Case series	44	78 M, 93 F	165****	165****
*Askin, O. (49)	Turkey	June 24-2020	Cross-sectional	NM	NM	122	34
Ciccarese, G. (50)	Italy	June 24-2020	Case report	19	1 F	1	1
*Matar, S. (51)	France	June 26-2020	Case series	55.6	6 M, 2 F	759	8
Ho, W. Y. B. (52)	Singapore	June 26-2020	Case series	59	1 M, 1 F	2	2
Potekaev, N. N. (53)	Russia	July 03-2020	Case series	62.2	7 M, 5 F	12	12

(Continued)

TABLE 1 | Continued

First author	Country	Published time	Type of study	Mean age	Male/ Female	No. of confirmed COVID-19 patient(s)	No. of the patient(s) with skin manifestations
Le Cleach, L. (54)	France	July 06-2020	Case series	34	3 M, 7 F	10	10
Proietti, I. (55)	Italy	July 22-2020	Case report	6 months	1 M	1	1

**Articles that are included for calculating the prevalence of cutaneous manifestations in confirmed COVID-19 patients.*
***Total population of cases was 8 but data of 5 patients were available only.*
****Tammaro et al. visited 130 patients in a hospital in Rome in which 2 patients had cutaneous manifestations. Also, they visited undetermined (X) patients in Barcelona in which there was a patient with cutaneous manifestation. Note that for calculating the prevalence number we excluded the latter patient (because of the undetermined number of total case population).*
*****Total population of confirmed COVID-19 patients was 171 but data of 165 patients were available only.*

were seen within the epidermis in maculopapular lesions. Telangiectatic blood vessels were seen within the dermis of vascular and miscellaneous lesions. Virally-induced cytopathic alterations were absent according to reports on the miscellaneous category. Striking vascular and dermal deposits of complement factors (C5b-9, C3d, C4d) and IgM were present in four vascular rashes. Some studies performed an RT-PCR test on skin samples of maculopapular and vesicular lesions and the results were all negative for SARS-CoV-2. More details can be found in **Table 2**.

Most lesions required systemic corticosteroids (47%) or had spontaneous remission (23.5%). Antihistamines were the most widely used medication especially for urticaria-like lesions (57%). Systemic corticosteroids were commonly used in vascular lesions (71%) (**Tables 2**, **4**).

Characteristics of the Confirmed COVID-19 Patients With Skin Manifestations

The overall prevalence of comorbidities among patients with skin manifestations was 17.9% (**Table 4**). Hypertension (39%), diabetes (23%), and dermatologic diseases (20%) were the most frequent comorbidities, respectively. Utmost cases with comorbidity were across the patients with maculopapular lesions (40%). Previous dermatologic illnesses were most common in patients with vesicular lesions (**Table 4**). Cardiovascular disease, hypertension, and obstructive lung diseases were common comorbidities amongst patients with vascular lesions (**Table 4**). Rheumatologic diseases were more frequent in patients with chilblain-like lesions (30%). Diabetes was seen commonly in patients with urticaria-like lesions (46%).

Fever (72%), cough (61%), fatigue/myalgia (51%), and dyspnea (46%) were the most common associated symptoms amongst the patients. Fever was more frequent in patients with vascular lesions (84%) and less frequent in patients with chilblain-like lesions (39.5%). Headache (41%), dysosmia/hyposmia (27.5%), nasal congestion/coryza (19%), and irritability/confusion (10%) were mainly seen in patients with vesicular lesions. Seventeen percentage of patients with chilblain-like lesions, 5% of patients with urticaria-like lesions, and 1% of patients with maculopapular lesions were asymptomatic. Bleeding presentations like epistaxis were seen just in patients with vascular lesions (**Tables 3**, **4**).

Elevated D-dimer was the main laboratory finding in most of the cases, especially in patients with chilblain-like (100%) and vascular (46%) lesions. Disruption of coagulation condition (increase in PT, INR, and fibrinogen) was reported in patients with chilblain-like and vascular lesions (**Tables 3**, **4**).

Regarding the drug history and medication regimen used for COVID-19, data of 389 out of 597 cases were available, most of which related to maculopapular and urticaria-like lesions. Fifty-two percentage of all cases and 72% of cases with chilblain-like lesions underwent symptomatic treatment with paracetamol, etc., or recovered without any medication. Chloroquine/hydroxychloroquine was the most common medication used in patients (45%). Details in **Tables 3**, **4**.

Most patients had mild disease (48%). The majority of patients with chilblain-like lesions had mild disease (82%) and the majority of patients with vascular lesions had severe disease (68%). Also, most of the patients with maculopapular lesions were moderate (43%) regarding severity (**Tables 3**, **4**).

The overall mortality rate among COVID-19 patients with cutaneous manifestations was 4.5%. Patients with vascular lesions had the highest mortality rate (18.2%) and patients with urticaria-like lesions had the lowest mortality rate (2.2%).

Details indicating characteristics of the lesions and the patients are shown in **Tables 2**–**4**.

DISCUSSION

After 1 year from the beginning of COVID-19 pandemic, the world is still facing a crisis. According to the current literature, more than half of the patients are asymptomatic leading to uncontrolled transmission of the virus (57–60). Recognizing COVID-19 related cutaneous manifestations may assist clinicians in early diagnosis of disease, before the development of respiratory symptoms, and may also be used to identify complications requiring treatment. The current study found that 10.5% of the COVID-19 patients reported skin lesions before the initiation of other symptoms or as their chief complaint. On the other hand, considering cutaneous manifestations is important to make the right diagnosis; as Joob et al. reported a COVID-19 patient with petechiae misdiagnosed with dengue fever (13). Our data demonstrated that 34.5% of cutaneous manifestations occurred at the same time with other symptoms particularly urticaria-like lesions (47%). It may suggest that urticaria-like lesions may be a diagnostic sign for COVID-19. The rest of the skin manifestations appeared later in the course of the disease

TABLE 2 | Characteristics of the cutaneous lesions in confirmed COVID-19 patients.

First author	Category	Location	Description	Associated cutaneous symptoms	Rash onset with other symptoms	Median duration	Main histologic findings	Rash treatment
Locatelli, A. G. (40)	Chilblain-like	Dorsal aspects of the fingers	Erythematous-oedematous macules and plaques (Chilblain-like)	Asymptomatic	After	–	Oedema of the papillary dermis, superficial and deep lymphocytic infiltrate in a peri-vascular and strong peri-eccrine pattern; no signs of endothelial damage. consistent with a diagnosis of chilblains	–
Alramthan, A. (29)	Chilblain-like	Dorsal aspect of fingers bilaterally, subungual area of the thumb	Red-purple papules, diffused erythema in the subungual area	–	Chief complaint	–	–	–
Suarez-Valle, A. (36)	Chilblain-like	Toes (3), soles (1) (sparing palms and mucous membranes)	Rounded reddish-purple plaques, measuring between 0.5 and 1 cm, sharply defined, with no retiform borders	–	23 days after	14 days	Ischemic necrosis affecting the epidermis and dermis with signs of re-epithelialization. vasculitis or microthrombi were not found.	–
de Masson, A. (44)	Chilblain-like	Hands and feet	Acral lesions (chilblains)	–	–	–	Lichenoid dermatitis with a perivascular and eccrine mononuclear infiltrate, vascular microthrombi	–
Freeman, E. E. (45)	Chilblain-like	Hand (7), foot (20)	Pernio-like acral skin lesions	Pruritus (8), Pain/Burning (16)	Before (4), After (11), At the same time (3), No other COVID-19 symptoms (5)	14 days	Mild vacuolar interface dermatitis with dense superficial and deep lymphocytic inflammation, consistent with pernio vs. connective tissue disease. No thrombi were noted.	–
Le Cleach, L. (54)	Chilblain-like	Acral area of hand and foot, dorsum of toes and soles, lateral part of the foot	Typical chilblains, severe form with bullae, erythema multiform-like lesions, punctiform purpuric lesions, diffuse vascular erythema, and oedema	–	After/Before	–	Vacuolization or apoptosis of keratinocytes, superficial and deep infiltrates mainly of lymphocytes, perieccrine, and perivascular reinforcement, superficial capillary thrombosis, dermal oedema	Without treatment, topical corticosteroids
Najarian, D. J. (20)	Maculopapular	Legs, thighs, forearms, arms, shoulders, back, chest, and abdomen (sparing the face, hands, feet, and mucosa)	Morbilliform erythematous macules and patches	Pruritus	After	–	–	Triamcinolone 0.1%

(Continued)

TABLE 2 | Continued

First author	Category	Location	Description	Associated cutaneous symptoms	Rash onset with other symptoms	Median duration	Main histologic findings	Rash treatment
Sanchez, A. (23)	Maculopapular	Trunk, back, thighs, arms	Digitatepapulosquamous eruption and erythematous periumbilical patch (digitate scaly thin plaques)	–	7 days after	7 days	Spongiosis, parakeratosis, a few rounded spongiotic vesicles containing aggregates of lymphocytes and Langerhans cells. moderate lymphohistiocytic infiltration, negative COVID-19 RT-PCR on a fresh skin biopsy specimen	No
Hunt, M. (12)	Maculopapular	Trunk and extremities (sparing the face, mucosal or ocular involvement)	Diffuse, morbilliform, maculopapular (consistent with a viral exanthem)	Non-pruritic	At the same time	–	–	–
Ahouach, B. (25)	Maculopapular	Trunk, limbs	Diffuse fixed erythematous blanching maculopapular lesions	Burning	–	–	Spongiosis, basal cell vacuolation and mild perivascular lymphocytic infiltrate, negative PCR on whole-skin biopsy specimen for SARS-CoV-2.	–
AvellanaMoreno, R. (34)	Maculopapular	Face, neck, thorax, abdomen, buttocks, extremities including folds and scalp, respecting the palmoplantar region and mucosa	Generalized, pruritic morbilliform rash cephalocaudal progress (petechial and maculopapular on an erythematous base), a scaly reaction occurred on the fourth day after the rash started	Pruritus	6 day after	4 days	–	–
Reymundo, A. (47)	Maculopapular	Trunk (7), proximal upper limbs (6), proximal lower limbs (1)	–	–	–	–	Mild superficial perivascular lymphocytic infiltrate	Without treatment (1), systemic corticosteroid (6)
Morey-Olive, M. (19)	Maculopapular (1), Urticaria-like (1)	*Maculopapular:* trunk, neck spreading to the cheeks, upper and lower extremities (involving the palms) *Urticaria-like:* face, upper extremities spreading to the trunk and lower extremities (sparing palms and soles)	*Maculopapular:* erythematous, confluent, non-pruritic maculopapular exanthem *Urticaria-like:* pruritic Urticaria-like exanthem	Pruritus (1)	After (1), At the same time (1)	5 days	–	No

(Continued)

TABLE 2 | Continued

First author	Category	Location	Description	Associated cutaneous symptoms	Rash onset with other symptoms	Median duration	Main histologic findings	Rash treatment
Gianotti, R. (22)	Maculopapular (3), Vascular (2)	Trunk, limb	*Maculopapular:*diffusemaculo-papulovesicular rash and hemorrhagic dot-like area, sligthlypapular erythematous exanthema, erythematous papular eruption with crusted and erosive lesions mimicking Grover disease *Vascular:* diffuse macular livedoid hemorrhagic lesions		After (5)	–	*Maculopapular:* classic dyskeratotic cells, ballooning multinucleated cells and sparse necrotic keratinocytes with lymphocytic satellitosis, edematous dermis with many eosinophils, lymphocytic vasculitis *Vascular:* diffuse telangiectatic small blood vessels in the dermis, spongiotic dermatitis with exocytosis along with a large nest of Langerhans cells and a dense perivascular lymphocytic and eosinophilic infiltration, lymphocytic vasculitis, Intravascular microthrombi in the small dermal vessels.	–
Mahé, A. (16)	Miscellaneous	Both antecubital fossae extended to the trunk and axillary folds	Erythematous rash	–	4 days after	5 days	–	–
Robustelli Test, E. (42)	Miscellaneous	Face, trunk, upper and lower limbs (sparing the mucous membranes, palms, and soles)	Diffuse pustular eruption: widespread eruption on an erythematous-oedematous base, with scattered pinhead-sized pustules and scales, Targetoid lesions studded with small pustules in a symmetric pattern	Pruritus	After	–	Subcorneal pustule with mild focal acanthosis and spongiosis, neutrophilic exocytosis, sparse keratinocyte necrosis, perivascular lymphocytic infiltrate with rare neutrophils and eosinophils (consistent with AGEP)	–
Zengarini, C. (28)	Miscellaneous	Neck, trunk, back, proximal portions of limbs (sparing the palmoplantar skin, face, and mucous membranes)	Erythematous confluent rash, with undefined margins, bleaching	Moderate pruritus	After	7 days	Slight superficial perivascular lymphocytic infiltrate extremely dilated vessel in the papillary and mid dermis.	–

(Continued)

TABLE 2 | Continued

First author	Category	Location	Description	Associated cutaneous symptoms	Rash onset with other symptoms	Median duration	Main histologic findings	Rash treatment
Amatore, F. (35)	Miscellaneous	Upper limbs, chest, neck, abdomen, and palms (sparing the face and mucous membranes)	Erythematous and edematous annular fixed plaques	Non-pruritic	At the same time	7 days	Predominantly superficial perivascular infiltrate of lymphocytes without eosinophils, papillary dermal edema, subtle epidermal spongiosis, lichenoid, and vacuolar interface dermatitis with occasional dyskeratotic keratinocytes in the basal layer. No virally-induced cytopathic alterations or intranuclear inclusions were present. Direct immunofluorescence was negative.	HCQ
Gargiulo, L. (48)	Miscellaneous	Trunk, upper and lower limbs	Erythema multiforme-like (erythematous and slightly edematous patches, along with some isolated typical target lesions)	Pruritus	10 days Before	–	Mixed perivascular and interstitial infiltrate including lymphocytes, granulocytes, histiocytes, plasma cells, and mast cells.	Systemic corticosteroid
Ciccarese, G. (50)	Miscellaneous	Lower limbs, Inner surface of the lips, platelet, gingiva	*Cutaneous lesions:* erythematous macules, papules, and petechiae *Oropharyngeal lesions:* erosions, ulcerations, blood crusts, petechiae	Asymptomatic	5 days after	12 days	–	–
Recalcati, S. (6)	Miscellaneous (14), Urticaria-like (3), Vesiclular (1)	Trunk	Erythematous rash (14), widespread urticaria (3), chickenpox-like vesicles (1)	Low or absent pruritus	At the same time (8), After (10)	Few days	–	–
Gianotti, R. (21)	Maculopapular (3)	Arms, trunk, lower limbs	Widespread erythematous macules, erythematous crusted macules, and papules	Pruritus (1)	After (2), Before (1)	5, 8, 10 days	Perivascular dermatitis with slight lymphocytic exocytosis in a vasculitic pattern. vascular thrombosis, Swollen thrombosed vessels with neutrophils, eosinophils and nuclear debris, extravasated red blood cells, focal acantholyticsuprabasal clefts, dyskeratotic and ballooning herpes-like keratinocytes, swollen vessels in the dermis with dense lymphocyte infiltration, mixed with rare eosinophils. a nest of Langerhans within the epidermis.	No (3)

(Continued)

TABLE 2 | Continued

First author	Category	Location	Description	Associated cutaneous symptoms	Rash onset with other symptoms	Median duration	Main histologic findings	Rash treatment
Jimenez-Cauhe, J. (41)	Miscellaneous (4)	Upper trunk, coalescing in the back, and then spread to the face and limbs within 1 week (without the involvement of palms and soles), platal macules and petechiae (3)	Erythematous papules that progressively turned to erythemato-violaceous patches with a dusky center, and a pseudo-vesicle in the middle, typical target lesions (2).	–	19.5 days after	17.5 days	Normal basket-weave stratum corneum, mild to moderate spongiosis in the epidermis, dilated vessels filled with neutrophils, extravasation of red blood cells, lymphocytic perivascular and interstitial infiltrate, Basal vacuolar changes with interface dermatitis, lymphocytic exocytosis	Systemic corticosteroids
Galván Casas, C. (24)	Chilblain-like (29), Vesicular (17), Urticaria-like (49), Maculopapular (122), Vascular (17)	*Chilblain-like:* acral areas of hands and feet. usually asymmetrical *Vesicular*: trunk, limbs *Urticaria-like:* mostly trunk, a few cases were palmar *Maculopapular*: extremities, mostly dorsum of the hands *Livedo/necrosis:* trunk, acral area	Pseudo-chilblain (29), Vesicular (17), Urticarial (49), Maculopapules (122), Livedo/necrosis (17)	–	Before (9), At the same time (147), After (77)	*Chilblain-like:* 12.7 days *Vesicular:* 10.4 days *Urticaria-like:* 6.8 days *Maculopapular*: 8.6 days	–	–
Fiehn, C. (15)	Urticaria-like	Both heels	Confluent erythematous-yellowish papules and plaques	Pruritus	13 days After	–	–	–
Gunawan, C. (43)	Urticaria-like	Face	Urticaria	Pruritus	5 days After	1		Loratadine
Quintana-Castanedo, L. (26)	Urticaria-like	Thighs, arms, and forearms (sparing the palms and soles)	Urticarial rash consisting of confluent, edematous, and erythematous papules	Mild pruritus	Chief complaint	7 days	–	Antihistamine
Henry, D. (30)	Urticaria-like	Face, acral area, palm	Disseminated erythematous plaques (Urticaria), papules in palm	Pruritus	Before	–	–	Antihistamine
van Damme, C. (33)	Urticaria-like	–	Extensive acute urticarial rash	–	At the same time	–	–	Bilastine
Paolino, G. (38)	Urticaria-like	Trunk, neck, face, lower limbs	An urticaria-like lesion with craniocaudal development	Non-pruritic	3 days after	8 days	–	–
Proietti, I. (55)	Urticaria-like	Trunk, limbs	Giant urticaria with multiple lesions	–	14 days after RT-PCR test (no associated symptoms)	–	–	Oral betamethasone
Zulfiqar, A. A. (17)	Vascular	Lower extremity	Purpura	–	5 days after	13 days	–	IVIG, Prednisolone, Platelet transfusion
Joob, B. (13)	Vascular	–	Petechiae	–	–	–	–	No

(Continued)

TABLE 2 | Continued

First author	Category	Location	Description	Associated cutaneous symptoms	Rash onset with other symptoms	Median duration	Main histologic findings	Rash treatment
Diaz-Guimaraens, B. (7)	Vascular	Symmetric periflexural distribution: buttocks, popliteal fossae, proximal anterior thighs, and lower abdomen (sparing the crural folds and mucosa)	Confluent erythematous macules, papules, and petechiae	Slightly pruritic	3 days after	5 days	Perivascular lymphocytic infiltrate, red cell extravasation, and focal papillary edema, along with focal parakeratosis and isolated dyskeratotic cells. No features of thrombotic vasculopathy were present	0.05% Betamethasone dipropionate cream, Loratadine
Magro, C. (18)	Vascular	Buttocks, palms and soles, chest, legs, and arms	Purpuric reticulated eruptions with surrounding inflammation (Livedoracemosa)	–	4 days after	–	Thrombogenic vasculopathy, extensive necrosis of the epidermis and adnexal structures, interstitial and perivascular neutrophilia with prominent leukocytoclasia, superficial vascular ectasia, perivascular lymphocytic infiltration, absence of clear vasculitis, Significant vascular deposits of C5b-9, C3d, and C4d (in all cases)	–
Recalcati, S. (6)	Vascular	Acral area, foot	Acrocyanosis (2), foot thrombosis (1)	–	–	–	–	–
Zhang, Y (14)	Vascular	Finger/toe	Acro-ischemia including cyanosis, bulla, and dry gangrene	–	–	12 days to death	–	–
Bosch-Amate, X. (46)	Vascular	Both legs	Retiform purpuric-violaceous patches of 15 cm with some hemorrhagic blisters and crusts suggestive of retiform purpura	Pain	–	–	Multiple thrombi occluding most small-sized vessels of the superficial and mid-dermis, deposition of IgM, C3, C9, and fibrinogen within superficial-to-deep dermal blood vessel walls.	–
Bouaziz, J. D. (39)	Vesicular (2), Urticaria-like (1), Chilblain-like (2), Vascular (3), Miscellaneous (6)		Inflammatory lesions were reported in 7 patients: exanthema (4), chickenpox like vesicles (2), cold urticaria (1), Vascular lesions were reported in 7 patients: violaceous macules with "porcelain-like" appearance (1), livedo (1), nonnecroticpurpura (1), necrotic purpura (1), chilblain appearance with Raynaud's phenomenon (1), chilblain (1), eruptive cherry angioma (1).	–	A few days after	–	–	–

(Continued)

TABLE 2 | Continued

First author	Category	Location	Description	Associated cutaneous symptoms	Rash onset with other symptoms	Median duration	Main histologic findings	Rash treatment
Marzano, A. V. (27)	Vesicular (22)	Trunk (22), limbs (4)	Scattered (16), diffuse (6), Predominance of vesicles (12), varicella-like exanthem	Mild pruritus (9)	3 days after	8 days	Basket-wave hyperkeratosis, absence of inflammatory infiltrate, atrophic epidermis, vacuolar alteration with disorganized keratinocytes lacking orderly maturation, enlarged and multinucleate keratinocytes with dyskeratotic (apoptotic) cells.	–
Fernandez-Nieto, D. (37)	Vesicular (24)	Head (4), anterior trunk (21), posterior trunk (14), arms (8), legs (10), palms-soles (2)	18 disseminated pattern (small papules, vesicles, and pustules with varying sizes of up to 7–8 mm diameter, different stages of the lesions appeared simultaneously), 6 localized pattern (monomorphic lesions, of up to 3–4 mm diameter, at the same stage of evolution, mostly trunk involvement)	Pruritus (20), Asymptomatic (4)	Before (2), At the same time (3), After (19)	10 days	Intraepidermal vesicles with mild acantolisis and ballooned keratinocytes consistent with a viral infection, negative SARS-CoV-2 RT-PCR on fluid content of the vesicles	–
Tammaro, A. (32)	Vesicular	Trunk, back	Isolated herpetiform lesions: lesions were characterized by vesicles surrounded by erythematous halos. In one of the patients, the vesicles had started to form crusts, numerous vesicular isolated lesions on her back.	Mild pruritus	After	–	–	–

(Continued)

TABLE 2 | Continued

First author	Category	Location	Description	Associated cutaneous symptoms	Rash onset with other symptoms	Median duration	Main histologic findings	Rash treatment
Potekaev, N. N. (53)	Chilblain-like (1), Vesicular (2), Urticaria-like (1), Maculopapular (4), Vascular (4)	Lower limb (6), upper limb (5), trunk (4), first MTP joints (1), ankles and dorsal surfaces of the feet and toes (1)	*Vascular:* papulonecrotic rash with hemorrhagic crusts, polymorphic cutaneous vasculitis, dense petechial and ecchymotic rash *Chilblain-like:* hyperemic pernio-like lesions *Maculopapular:* spotted elements of bright pink color, papulosquamous rash (pytriasis rosea-like, absence of the herald patch), disseminated pink-red maculopapular rash resembling that of measles, large bright red foci *Vesicular:* papulovesicular eruptions with surface erosions	Pain (1), Pruritus (1)	Before (1), At the same time (1), After (5)	–	–	Without treatment (1), Systemic corticosteroid (3)
Freeman, E. E. (56)	Chilblain-like (31), Vesicular (18), Urticaria-like (27), Maculopapular (78), Vascular (11)	Hand (38), foot (51), face (32), head (11), neck (26), chest (49), abdomen (63), back (62), arm (66), genitals (7), leg/buttocks (72), entire body (9)	*Maculopapular:* morbilliform rash, macular erythema, papulosquamous *Vascular:* retiform purpura	Pruritus (97), Burning/pain (55)	Before (17), After (107), At the same time (29), Chief complaint (10)	–	*Vascular:* thrombotic vasculopathy, leukocytoclastic vasculitis *Maculopapular:* spongiosis and dermal inflammation *Chilblain-like:* vacuolar interface dermatitis, subepidermal edema, and superficial and deep lymphocytic inflammation *Miscellaneous (actually distributed petechial, macular, and urticarial eruption):* numerous dyskeratotic keratinocytes, sparse perivascular lymphohistiocytic inflammation, and rare dermal eosinophils.	–
Matar, S. (51)	Maculopapular, Vesicular, Miscellaneous	-	Disseminated maculopapular exanthema, digitate papulosquamous rash, herpes recurrence, papulovesicular rash, Grover's disease	–	13 days after	–	–	–

(Continued)

TABLE 2 | Continued

First author	Category	Location	Description	Associated cutaneous symptoms	Rash onset with other symptoms	Median duration	Main histologic findings	Rash treatment
Ho, W. Y. B. (52)	Miscellaneous (1), Vascular (1)	*Miscellaneous:* Trunk, proximal thighs, intertriginous areas including bilateral axillae and groin *Vascular:* abdomen, back	*Miscellaneous:* erythematous, blanchable, non-follicular papules, non-follicular pinpoint pustules within the intertriginous areas *Vascular:* purpuric plaques	–	*Miscellaneous:* 12 days after *Vascular:* 15 days after	7 days, 10 days	*Miscellaneous:* spongiotic and interface dermatitis, superficial perivascular infiltrate of predominantly lymphocytes, focal erythrocyte extravasation without vasculitis. There were no viral cytopathic or herpetic changes.	Topical corticosteroids (2)

and mainly after the initiation of systemic symptoms (55%) in our review. Galván Casas et al. suggested the chilblain-like and vesicular lesions as epidemiological markers for the disease (24). However, in our study, vesicular lesions (74%) were the most important cutaneous manifestations usually appearing after systemic symptoms of the disease.

Most of the patients with skin manifestations were middle-aged females, while, patients with chilblain-like lesions were younger (mean age: 40.7 years) and patients with vascular lesions were older individuals (mean age: 72.3 years). These findings are along with other studies about the chilblain-like lesions (6, 19, 24, 40). Maculopapular lesions were the most common dermatologic presentation of COVID-19 patients that commonly appeared at extremities. It occurred most often in middle-aged patients and was associated with moderate COVID-19 severity.

The overall mortality rate between the COVID-19 patients with skin presentations was 4.5%, with the point that there was the lowest mortality rate among the patients with urticaria-like lesions (2.2%) and contradictory, there was the highest mortality rate among the patients with vascular lesions (18.2%). Previous studies showed a pooled mortality rate of 3.2–6% in patients with COVID-19 (61, 62). Thus, the mortality rate of COVID-19 patients with skin manifestations is proportionate to the overall mortality rate of the disease.

Regardless of the type of skin lesions, 80% of COVID-19 patients with cutaneous manifestations experienced a mild and moderate, and 20% a severe COVID-19 disease. A previous study from the Chinese Center for Disease Control and Prevention reported that 81% of COVID-19 patients had a mild, 14% a severe, and 5% a critical disease (63). We don't have any specific data on patients without skin manifestations but comparing the COVID-19 severity in patients with skin manifestations and COVID-19 patients, regardless of their symptoms, demonstrates no obvious difference. Future cohort studies are required to compare the disease severity and outcome of COVID-19 patients with and without skin manifestations.

There is a wide range of cutaneous manifestations related to COVID-19 that in terms of age, associated symptoms, comorbidity, medication, severity, and mortality, chilblain-like lesions, and vascular lesions are the ends of this spectrum. Chilblain-like, urticaria-like, vesicular, maculopapular, miscellaneous, and vascular lesions are associated with an increase in COVID-19 severity and worsening the prognosis, respectively. Vascular lesions were more prevalent in males (61%) compared to females (39%). Considering the more severe disease and higher mortality rate in patients with vascular lesions, we can conclude that COVID-19 is more severe in males compared to females. This finding is compatible with our recent article, in which we assessed the sex-specific risk of mortality in COVID-19 patients (62).

Up to date, there is conflicting information about the potential possibility of transmitting the virus through the skin (37, 40, 64). Further investigations are required to identify the pathophysiology of SARS-COV-2 and to determine whether patients with long-lasting skin lesions (e.g., chilblain-like lesions) are capable of infecting other individuals through skin contact or not.

TABLE 3 | Characteristics of the confirmed COVID-19 patients with skin manifestations.

First author	Comorbidity	Associated symptoms	Drug history	Laboratory findings	Severity/outcome
Locatelli, A. G. (40)	–	Dysgeusia, mild diarrhea	–	–	Non-severe
Alramthan, A. (29)	–	Asymptomatic	–	–	–
Suarez-Valle A. (36)	–	–	–	D-dimer↑, fibrinogen↑	Non-severe
de Masson, A. (44)	–	–	–	–	–
Freeman EE. (45)	HTN (2), obstructive lung disease (2), reumatologic disease (2)	Fever (9), cough (9), dyspnea (6), sore throat (5), headache (7), malaise (4), asymptomatic (5)	–	–	Outpatient care only (18), Hospitalized (5), Death (2)
Le Cleach L. (54)	Raynaud syndrome (2)	Fever (2), cough (2), dyspnea (3), asthenia (5), myalgia (3), headache (7), odynophagia (3), anosmia/ageusia (5), asymptomatic (3)	–	–	Outpatient (10)
Najarian, D. J. (20)	–	Cough, pain in leg and hands	Azithromycin, Benzonatate	–	Non-severe
Sanchez, A. (23)	T2D, HTN, peripheral artery disease, chronic renal failure	Fatigue, fever, dyspnea, acute respiratory distress	Cefpodoxime	EBV PCR positive (reactivation of EBV)	Severe/ Death
Hunt, M. (12)	–	Fever	–	Lymphopenia, CRP↑	Severe
Ahouach, B. (25)	–	Fever, dry cough	Paracetamol	–	–
Avellana Moreno, R. (34)	–	Fever, myalgia, asthenia, cough, diarrhea	Paracetamol	–	–
Reymundo A. (47)	–	–	–	–	–
Morey-Olive, M. (19)	Cholestatic liver disease (1)	Low-grade fever	Oral symptomatic treatment	Worsening of the markers for cholestasis	–
Gianotti, R. (22)	–	Fever, sore throat, cough	Levofloxacin (3), HCQ (3)	–	Mild (2), Moderate (2), Severe (1)
Mahé, A. (16)	T2D	Fever, asthenia, cough	Paracetamol	–	Non-severe/Survived
Robustelli Test, E. (42)	–	–	Lopinavir/ritonavir, HCQ (3 weeks before)	–	–
Zengarini, C. (28)	Moderate obesity, a history of alcoholism, and various chronic morbidities	Progressive dyspnoea, fever	HCQ, Omeprazole, Piperacillin/Tazobactam, Remdesevir, Potassium canreonate, and Enoxaparine.	–	Severe
Amatore, F. (35)	–	Fever	HCQ	Blood count: NL, electrolytes: NL, CRP: NL, anti-DNA antibodies: NL	
Gargiulo L. (48)	–	Fever	Paracetamol, Darunavir/cobicistat, HCQ	–	Severe, Death
Ciccarese G. (50)	–	Fever, sore throat, fatigue, hyposmia	Cefixime (3 days earlier discontinued), IVIG, Methylprednisolone	Leukocytosis, Lymphocytosis, severe Thrombocytopenia, LFT↑, LDH↑	–
Recalcati S. (6)	–	–	–	–	–

(Continued)

TABLE 3 | Continued

First author	Comorbidity	Associated symptoms	Drug history	Laboratory findings	Severity/outcome
Gianotti, R. (21)	–	Fever (2), cough (2), headache (1), arthralgias (1)	Lopinavir/Ritonavir (1), Heparin (1), Levofloxacin (2), Ceftriaxone (1), Azithromycin (1), HCQ (1)	CRP↑, fibrinogen↑, ALT↑, AST↑	Mild (1), Severe (2)
Jimenez-Cauhe J. (41)	–	–	Lopinavir/Ritonavir, HCQ, Azithromycin, Corticosteroids, Ceftriaxone	Laboratory tests at the time of skin lesions showed worsening of one or more parameters compared to those at the time of discharge (CRP↑, D-dimer↑, lymphocyte count↓)	–
Galván Casas C. (24)	–	Cough, dyspnea, fever, asthenia, headache, nausea/vomiting/diarrhea, anosmia, ageusia, pneumonia	–	–	*Pseudo-chilblain:* less severe *Vesicular lesions:* intermedium severity *Urticarial and maculopapular lesions:* more severe COVID-19 disease *Livedoid/necrotic lesions:* the most severe disease
Estébanez, A. (5)	–	Dry cough, nasal congestion, fatigue, myalgias, arthralgias, diarrhea, ageusia, anosmia	Paracetamol	–	–
Gunawan, C. (43)	HTN, diabetes, dyslipidemia, hyperuricemia	Fever, cough, dyspnea, diarrhea	Azithromycin, HCQ, Cefoperazone-sulbactam, Omeprazole, medicines for his comorbidities	–	Non-severe
Quintana-Castanedo, L. (26)	–	4-day history of progressive cutaneous rash	–	–	–
Henry, D. (30)	–	Odynophagia, diffuse arthralgia, chills, chest pain, fever	Paracetamol	Moderate lymphopenia, CRP↑	Non-severe
van Damme, C. (33)	Obesity, T1D, hypercholesterolemia, HTN, obstructive sleep apnea-hypopnea syndrome, stroke 18 months ago without further sequelae, kidney failure on dialysis	General weakness, fever	–	Mild lymphopenia, CRP↑, LFT (GOT, GPT, LDH, GGT)↑	Severe/Death
Paolino, G. (38)	–	7th postpartum day, fever, dry cough, myalgia, arthralgia	Paracetamol	–	–
Proietti I. (55)	–	Asymptomatic	–	Normal	–
Zulfiqar, A. A. (17)	HTN, autoimmune hypothyroidism	Fatigue, fever, dry cough, abdominal discomfort, epistaxis	IV Amoxicillin–Clavulanic acid, LMWH	CRP↑, LFT showed cholestasis, progressive thrombocytopenia, fibrinogen↑, TPO↑	–
Joob, B. (13)	–	Fever, pneumonia, bleeding presentation (firstly missed diagnosed to be dengue)	–	–	–

(Continued)

TABLE 3 | Continued

First author	Comorbidity	Associated symptoms	Drug history	Laboratory findings	Severity/outcome
Diaz-Guimaraens, B. (7)	HTN	Fever, pleuritic chest pain, shortness of breath	Telmizartan, HCQ, LR, Azithromycin	Lymphopenia, CRP↑, D-dimer↑	Non-severe
Magro, C. (18)	Obesity-associated sleep apnea, anabolic steroid use	Fever, cough, dyspnea, diarrhea, chest pain, myalgia	HCQ, Azithromycin, Remdesivir, prophylactic Enoxaparin	D-dimer↑, INR↑, CH50↑, C3↑, C4↑, Thrombocytopenia	Severe
Recalcati S. (6)	–	–	–	–	–
Zhang, Y (14)	HTN, DM, CAD	Fever, cough, dyspnea, diarrhea	LMWH	D-dimer↑, fibrinogen↑, FDP↑, PT↑	Severe/Death (5)
Bosch-Amate X. (46)	–	Fever, asthenia, cough, shortness of breath	HCQ, Azithromycin, LR, LMWH, Fondaparinux	Leukopenia, CRP↑, D-dimer↑	Hospitalized, Survival
Bouaziz, J. D. (39)	–	–	–	–	–
Marzano, A. V. (27)	–	Fever (21), cough (16), headache (11), weakness (11), coryza (10), dyspnea (9), hyposmia (4), hypogeusia (4), pharyngodynia (1), diarrhea (1), myalgia (1)	–	–	Mild (10), Moderate (2), Severe (10)/ Death (3)
Fernandez-Nieto, D. (37)	Atopic dermatitis (5), chronic urticaria (2)	–	7 patients: Lopinavir/Ritonavir (5), HCQ (6), Azithromycin (2)	–	Mild (14), Moderate (9), Severe (1)
Tammaro A. (32)	–	–	–	–	–
Potekaev NN. (53)	–	Fever (2), cough (1), weakness (1), shortness of breath (1)	HCQ (1)	–	Severe (2)
Freeman EE. (56)	HTN (32), diabetes (19), obstructive lung disease (14), Non-obstructive lung disease (9), cardiovascular disease (5), kidney disease (5), reumatologic disease (5), hidradenitis suppurativa (2), contact dermatitis (5), alopecia areata (4), melanoma (3)	Fever (103), cough (92), dyspnea (64), sore throat (62), headache (54), diarrhea, vomiting or nausea (51), malaise (45), myalgia (35), irratibility/confusion (27), chest pain (27), abdominal pain (23), anosmia (18), dysgeusia (12), arthralgia (16), rhinorrhea (14), asymptomatic (11)	Bevacizumab (12), Remdesivir (9), Lopinavir/Ritonavir (2), supportive care only (96), anti-malarials (41), antibiotics (40), serpin inhibitors (6), IL-6 inhibitors (4), JAK inhibitors (2)	–	Out-patient (95), Hospitalized (17), Non-invasive ventilation or high flow oxygen, ventilator and/or ECMO required (24), Death (8)
Matar S. (51)	–	–	–	–	–
Ho WYB. (52)	–	–	Lopinavir/Ritonavir	–	–

TABLE 4 | Summary of characteristics of the patients based on the type of lesions.

Characteristics	Chilblain-like lesions	Vesicular lesions	Urticaria-like lesions	Maculopapular lesions	Vascular lesions	Miscellaneous	Total
N (%)	110 (18.4)	89 (15)	89 (15)	223 (37.3)	55 (9.2)	31 (5.2)	597
Sex, *N**	97	83	85	219	46	11	541
Male, *n* (%)	43 (44)	42 (51)	28 (33)	107 (49)	28 (61)	2 (18)	250 (46)
Female, *n* (%)	54 (56)	41 (49)	57 (67)	112 (51)	18 (39)	9 (82)	291 (54)
Age, mean	40.7	56.1	46.3	56.4	72.3	48	53.3
Rash location Trunk, *n*	0	85	91	143	21	23	363
Upper Limb, *n*	24	44	29	194	8	9	308
Lower Limb, *n*	53	42	30	188	18	10	341
Head/Neck, *n*	0	12	20	42	0	7	81
Palms/Soles, *n*	1	2	2	1	1	1	8
Acral area (Finger, Toe), *n*	69	0	1	0	18	0	88
The mucous membrane, *n*	0	2	1	4	4	2	13
Associated cutaneous symptoms, *n* (%)	74 (67)	62 (70)	80 (90)	159 (71)	18 (33)	4 (13)	397 (66)
Pruritus, *n* (%)	28 (38)	55 (89)	22 (27.5)	126 (79)	4 (22)	3 (75)	238 (60)
Burning, *n* (%)	40 (54)	11 (18)	7 (9)	23 (14)	2 (11)	0 (0)	83 (21)
Pain, *n* (%)	47 (63.5)	9 (14.5)	6 (7.5)	19 (27)	3 (17)	0 (0)	84 (21)
The onset of the lesions in relation toother symptoms, *N*	91	86	85	222	41	17	542
Before, *n* (%)	10 (11)	5 (6)	5 (6)	14 (6)	1 (2)	1 (6)	36 (7)
Chief complaint, *n* (%)	13 (14)	0 (0)	3 (3.5)	3 (1)	0 (0)	0 (0)	19 (3.5)
At the same time, *n* (%)	21 (23)	17 (20)	40 (47)	92 (41)	16 (39)	1 (6)	187 (34.5)
After, *n* (%)	45 (49)	64 (74)	37 (43.5)	113 (51)	24 (58.5)	15 (88)	298 (55)
Median duration of skin lesions, day	14	9	5.25	7.4	9.5	9.3	9
Rash treatment, *N***	0	0	7	15	5	7	34
Without treatment, *n* (%)	0 (0)	0 (0)	0 (0)	7 (47)	1 (20)	0 (0)	8 (23.5)
Antihistamines, *n* (%)	0 (0)	0 (0)	4 (57)	0 (0)	1 (20)	0 (0)	5 (15)
Topical corticosteroids, *n* (%)	0 (0)	0 (0)	1 (14)	1 (7)	2 (40)	1 (14)	5 (15)
Systemic corticosteroids, *n* (%)	0 (0)	0 (0)	2 (28.5)	7 (47)	2 (40)	5 (71)	16 (47)
Hydroxychloroquine, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (14)	1 (3)
Comrbidity, *N** (%)**	20 (19)	14 (13)	13 (12)	43 (40)	15 (14)	2 (2)	107 (100)
Hypertension	6 (30)	3 (21)	5 (38)	16 (37)	12 (80)	0 (0)	42 (39)
Diabetes	0 (0)	2 (14)	6 (46)	10(23)	6 (40)	1 (50)	25 (23)
Previous dermatologic illness****	2 (10)	8 (57)	1 (8)	10 (23)	0 (0)	0 (0)	21(20)
Obstructive lung disease	2 (10)	1 (7)	1 (8)	6 (14)	6 (40)	0 (0)	16 (15)
Non-obstructive lung disease	1 (5)	1 (7)	3 (23)	4 (9)	0 (0)	0 (0)	9 (8)
Rheumatologic disease	6 (30)	1 (7)	0 (0)	1 (2)	1 (7)	0 (0)	9 (8)
Chronic kidney disease	0 (0)	0 (0)	1 (8)	6 (14)	0 (0)	0 (0)	7 (6.5)
Cardiovascular disease	1 (5)	0 (0)	0 (0)	3 (7)	2 (13)	0 (0)	6 (6)
Obesity	0 (0)	0 (0)	1 (8)	0 (0)	1 (7)	1 (50)	3 (3)
Obstructive sleep apnea	0 (0)	0 (0)	1 (8)	0 (0)	1 (7)	0 (0)	2 (2)
Dyslipidemia	0 (0)	0 (0)	2 (15)	0 (0)	0 (0)	0 (0)	2 (2)
Liver disease	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)	0 (0)	1 (1)
Peripheral artery disease	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)	0 (0)	1 (1)
Autoimmune hypothyroidism	0 (0)	0 (0)	0 (0)	0 (0)	1 (7)	0 (0)	1 (1)
Hyperuricemia	0 (0)	0 (0)	1 (8)	0 (0)	0 (0)	0 (0)	1 (1)
Stroke	0 (0)	0 (0)	1 (8)	0 (0)	0 (0)	0 (0)	1 (1)
Alcoholism	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)	1 (1)

(Continued)

TABLE 4 | Continued

Characteristics	Chilblain-like lesions	Vesicular lesions	Urticaria-like lesions	Maculopapular lesions	Vascular lesions	Miscellaneous	Total
Associated symptoms, *N******	96	58	83	210	43	6	496
Fever, *n* (%)	38 (39.5)	48 (83)	63 (76)	169 (80)	36 (84)	5 (83)	359 (72)
Cough, *n* (%)	35 (36)	40 (69)	51 (61)	146 (69.5)	31 (72)	1 (17)	304 (61)
Fatigue/Myalgia, *n* (%)	42 (44)	33 (57)	46 (55)	113 (54)	15 (35)	2 (33)	251 (51)
Dyspnea, *n* (%)	27 (28)	22 (38)	33 (40)	120 (57)	27 (63)	1 (17)	230 (46)
Headache, *n* (%)	32 (33)	24 (41)	26 (31)	64 (30)	7 (16)	0 (0)	153 (31)
Nausea/Vomiting/Diarrhea/Abdominal discomfort, *n* (%)	17 (18)	14 (24)	31 (37)	77 (37)	14 (32.5)	0 (0)	153 (31)
Dysosmia/Dysgeusia, *n* (%)	21 (22)	16 (27.5)	22 (26.5)	39 (18.5)	3 (7)	1 (17)	102 (20.5)
Sore throat, *n* (%)	13 (13.5)	10 (17)	11 (13)	33 (16)	3 (7)	1 (17)	71 (14)
Chest pain, *n* (%)	2 (2)	4 (7)	8 (10)	12 (6)	2 (5)	0 (0)	28 (6)
Nasal congestion/Coryza, *n* (%)	5 (5)	11 (19)	5 (6)	4 (2)	0 (0)	0 (0)	25 (5)
Irritability/Confusion	3 (3)	6 (10)	5 (6)	13 (6)	0 (0)	0 (0)	27 (5)
Arthralgia, *n* (%)	1 (1)	3 (5)	6 (7)	10 (5)	1 (2)	0 (0)	21 (4)
Bleeding presentation, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	2 (5)	0 (0)	2 (0.4)
Chills, *n* (%)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	1 (0.2)
Odynophagia, *n* (%)	3 (3)	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	4 (0.8)
Asymptomatic, *n* (%)	16 (17)	0 (0)	4 (5)	3 (1)	0 (0)	0 (0)	23 (5)
Laboratory Findings, *N*******	3	0	2	10	26	12	53
D-dimer increase, *n* (%)	3 (100)	0 (0)	0 (0)	0 (0)	12 (46)	3 (25)	18 (34)
Fibrinogen increase, *n* (%)	2 (67)	0 (0)	0 (0)	0 (0)	8 (31)	0 (0)	10 (19)
FDP increase, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	7 (27)	0 (0)	7 (13)
PT/INR increase, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	6 (23)	0 (0)	6 (11)
CRP increase, *n* (%)	0 (0)	0 (0)	1 (50)	3 (30)	3 (11.5)	2 (17)	9 (17)
Leukopenia, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)	1 (2)
Leukocytosis, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (8)	1 (2)
Lymphopenia, *n* (%)	0 (0)	0 (0)	1 (50)	2 (20)	1 (4)	2 (17)	6 (11)
Thrombocytopenia, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	2 (8)	1 (8)	3 (6)
LFT increase, *n* (%)	0 (0)	0 (0)	0 (0)	3 (30)	1 (4)	1 (8)	5 (9)
CH50, C3, C4increase, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)	1 (2)
COVID-19 treatment, *n* (%)	46	40	69	185	39	10	389
Paracetamol, symptomatic or without treatment, *n* (%)	33 (72)	21 (52.5)	39 (56.5)	103 (56)	5 (13)	3 (30)	204 (52)
Chloroquine/Hydroxychloroquine, *n* (%)	9 (19.5)	16 (40)	27 (39)	90 (49)	26 (67)	8 (80)	176 (45)
Lopinavir/Ritonavir, *n* (%)	3 (6.5)	7 (17.5)	14 (20)	52 (28)	9 (23)	5 (50)	90 (23)
Azithromycin, *n* (%)	2 (4)	7 (17.5)	13 (20)	38 (20.5)	4 (10)	4 (40)	68 (17)
Other antibiotics*******, *n* (%)	4 (9)	4 (10)	7 (10)	22 (12)	12 (31)	4 (40)	53 (14)
Systemic corticosteroids, *n* (%)	1 (2)	3 (7.5)	5 (7)	19 (10)	5 (13)	4 (40)	37 (9.5)
NSAIDs, *n* (%)	4 (9)	1 (2.5)	3 (4)	10 (5)	17 (43.5)	0 (0)	35 (9)
Tocilizumab (IL-6 inhibitors), *n* (%)	2 (4)	2 (5)	4 (6)	9 (5)	5 (13)	0 (0)	22 (6)
Bevacizumab, *n* (%)	0 (0)	2 (5)	4 (6)	6 (3)	0 (0)	0 (0)	12 (3)
LMWH, *n* (%)	0 (0)	0 (0)	0 (0)	1 (0.5)	9 (23)	1 (10)	11 (3)
Remdesivir, *n* (%)	1 (2)	1 (2.5)	2 (3)	4 (2)	2 (5)	1 (10)	11 (3)
Serpin inhibitors, *n* (%)	1 (2)	1 (2.5)	1 (1)	3 (2)	0 (0)	0 (0)	6 (1.5)
Omeprazole, *n* (%)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	1 (10)	2 (0.5)
JAK inhibitors, *n* (%)	2 (4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (0.5)
Telmizartan, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2.5)	0 (0)	1 (0.2)
Fondaparinux, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2.5)	0 (0)	1 (0.2)
Darunavir/cobicistat, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	1 (0.2)
IVIG, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	1 (0.2)

(Continued)

TABLE 4 | Continued

Characteristics	Chilblain-like lesions	Vesicular lesions	Urticaria-like lesions	Maculopapular lesions	Vascular lesions	Miscellaneous	Total
COVID-19 severity******, *N***	96	63	79	201	44	4	487
Mild, *n* (%)	79 (82)	32 (51)	40 (51)	79 (39)	2 (5)	2 (50)	234 (48)
Moderate, *n* (%)	13 (14)	18 (29)	28 (35)	86 (43)	12 (27)	0 (0)	157 (32)
Severe, *n* (%)	4 (4)	13 (21)	11 (14)	36 (18)	30 (68)	2 (50)	96 (20)
Death, *n* (%)*********	4 (3.6)	3 (3.4)	2 (2.2)	7 (3.1)	10 (18.2)	1 (3.2)	27 (4.5)

FDP, fibrinogen degradation product; PT, prothrombin time; LFT, liver function test.
**Number of patients that their gender is reported in the articles.*
***Number of patients that articles mentioned their specific treatment for the lesions.*
****Number of patients reported having comorbidities in the articles.*
*****E.g., atopic dermatitis, chronic urticaria, melanoma, alopecia areata, hidradenitis suppurativa.*
******Number of patients that articles mentioned their associated symptoms.*
*******Number of patients that their laboratory findings are reported in the articles.*
********Including mainly Levofloxacin, Amoxicillin–clavulanic acid, Cefpodoxime, Ceftriaxone, Piperacillin/tazobactam, Cefoperazone-sulbactam.*
*********Mild: outpatients, Moderate: hospitalized patients (with or without supplemental oxygen), Severe: ICU added patients, non-invasive/invasive ventilation or ECMO required, patients with acute respiratory distress syndrome (ARDS).*
**********N is the total number of cases in each category; Chilblain-like lesions (110), Vesicular lesions (89), Urticaria-like lesions (89), Maculopapular lesions (223), Vascular lesions (55), Miscellaneous (31), Total (597).*

The overall frequency of cutaneous manifestations in COVID-19 patients was 5.95%, with a range from 0.2% up to 20.4% in different studies (6, 65).

Although skin presentations of COVID-19 are well described, the pathogenesis of skin lesions remains unknown. The direct viral invasion of the skin cells may be one possibility. Angiotensin-converting enzyme 2 (ACE2) is known as a ligand for the Spike protein of SARS-CoV-2 for entering human cells (66). There is a high expression of ACE2 on keratinocytes and sweat gland cells, respectively (67, 68). Thus, SARS-CoV-2 can directly infect keratinocytes resulting in necrosis. This hypothesis is consistent with our histologic findings which demonstrated the epidermal and adnexal necrosis in all skin lesions except vesicular rashes. According to Amatore et al., neither viral-induced cytopathic alterations nor intranuclear inclusions were seen in skin biopsies (35). However, SARS-CoV-2 spike and envelope proteins were detected in the endothelial cells of damaged skin in two cases with purpuric rashes (22). RT-PCR for SARS-CoV-2 was performed on skin samples of some patients and was negative in all of them. Since the nasopharyngeal swabs of these patients were positive simultaneously, we assume that it can be a false negative result due to a small viral load or technical problems. Further research is urgently needed.

Skin lesions during SARS-CoV2 infection might be immune-related phenomena. It has been shown that the presence of virus RNA in blood is related to greater severity of infection (69). Viremia is also associated with the levels of cytokines and growth factors in a dose-dependent manner with markedly higher levels in patients suffering from more severe COVID-19 (69). Recognition of the viral RNA by Toll-free receptors like TLR7 stimulates the intracellular signaling pathways which in turn enhance the cytokine secretion (69).

In a group of patients, with the end of the first week of the infection, a sharp increase in inflammatory cytokines such as interleukin (IL)1, IL2, IL7, IL10, granulocyte colony-stimulating factor (G-CSF), tumor necrosis factor (TNF) α and interferon (IFN)-g occurs. Overactivation of immune responses followed by pro-inflammatory cytokines increase may result in a "cytokine storm" which is an immune pathological condition (69–71). Increased cytokines allow them to access the skin, where they stimulate various cells, including lymphocytes, dendritic cells, macrophages, neutrophils, monocytes, and Langerhans cells to cause various skin manifestations (22, 69). Maybe a hyperviremia state is responsible for vascular lesions in severe COVID-19 patients. We suggest further investigations on the viral load levels among patients with vascular lesions compared with other skin manifestations.

The antigen-antibody complex can lead to complement activation and subsequent mast cell degranulation. This mechanism is suggested particularly for the urticaria-like lesions (43).

A low or delayed interferon response may result in uncontrolled viral replication followed by a subsequent cytokine storm which can lead to severe disease (72). Activation of the host immune system in response to viral antigen deposition may result in vascular damage in COVID-19 infection (73). It seems that high levels of type 1 interferon response, a critical factor in immunity against viral agents, is associated with chilblain-like lesions and mild disease (15, 72, 74). Activation and aggregation of cytotoxic CD8+ T cells and B cells also lead to lymphocytic thrombophilic arteritis and destruction of keratinocytes (21, 22). Nests of Langerhans cells are seen in most of the COVID-19 skin lesion biopsies and have been also reported in another viral-induced skin dermatitis-like pytriasis rosea (75).

Coinfection with other viruses is another potential possibility for COVID-19 related cutaneous manifestations. Some skin lesions in COVID-19 patients are very similar to rashes induced by other viruses like parvovirus18, herpes simplex virus type 1 and 2 (HSV-1, HSV-2), varicella-zoster virus (VZV), and poxviruses, both clinically and histologically. It is probable that because of the attenuation of the immune system, COVID-19 patients are susceptible to coinfection with or relapse of the other viral exanthems. This hypothesis is strongly suggested for vesicular and some miscellaneous lesions (e.g., erythema

multiform) due to their unique histologic findings compared to other skin lesions of COVID-19 (24, 32, 37). A study reported four COVID-19 patients presenting diffuse vesicular lesions which microbiological and serological investigations demonstrated varicella infection (24). Thus, in COVID-19 patients with vesicular lesions, physicians need to investigate other possible etiological factors other than SARS-CoV-2.

Coagulopathy and vasculitis are other possible reasons for skin lesions during COVID-19. Evidence shows that COVID-19 patients are predisposed to coagulopathy and subsequent thrombotic events (76). It seems to be a result of inflammatory cytokine release, hypoxia, and other illness or therapeutic risk factors (76). Microvascular thrombosis of dermal vessels leads to ischemia or vasculitis mainly seen in chilblain-like or vascular lesions. Magro et al. focused on the role of the complement factors activation, especially alternative and lectin pathways, and subsequent thrombotic microvascular injuries (22). Evidence for this hypothesis is the elevated levels of CH50, C3, and C4 in blood samples as well as significant vascular depositions of C5b-9, C3d, and C4d in the dermis of skin specimens (22). According to our histologic findings mentioned in RESULT, vascular thrombosis was reported in almost all skin biopsies (except vesicular lesions). This finding across with the increased level of D-dimer, fibrinogen, and prolonged PT and INR in most patients is in favor of this hypothesis. Another presentation of coagulopathy in COVID-19 patients is hemorrhagic events and subsequent dermatologic manifestations (petechiae, purpura, and livedo). These manifestations are not specific to SARS-CoV-2. Schneider et al. reported a petechial rash associated with coronavirus NL63 (77, 78).

Extremely dilated blood vessels were introduced as a diagnostic histological finding for SARS-CoV-2 by Zengarini et al. (28). There are other reports of vasodilation and telangiectatic vessels in the dermis. With this finding, Magro et al. explained a possible pathway in which dysfunction of ACE2 (due to SARS-CoV-2 binding) and subsequent elevated level of angiotensin2 can result in high activation of endothelial nitric oxide synthase (eNOS) and ensuing vasodilation (22).

Drug-induced eruptions may occur during COVID-19. COVID-19 patients usually use a set of medications that potentially can cause cutaneous rashes. The current study found that paracetamol, azithromycin, hydroxychloroquine, lopinavir/ritonavir, and remdesivir were the most common medications used for COVID-19 patients. Paracetamol has been reported to cause asymmetrical drug-related intertriginous and flexural exanthema (STRIFE) (16). However, in Mahé et al. study, despite keeping the drug, skin lesions disappeared; that is very uncommon in drug reactions (16). Najarian et al. mentioned that maculopapular lesions of their patient could be according to azithromycin use or hypersensitivity reaction to azithromycin due to concurrent viral infection (20).

Hydroxychloroquine that has been used in 45% of all the cases (mentioned in Result) is one of the most likely medications to cause different skin rashes. Acute generalized exanthematous pustulosis (AGEP), erythroderma, urticaria, and erythema multiform are some of the skin lesions that have been reported in connection with hydroxychloroquine (79–81). However, Robustelli et al. mentioned that the skin lesion developed 3 weeks after discontinuation of the drug (42). As a conclusion, most of our reviewed articles considered the potential possibility of drug-induced exanthems but in almost all cases, dermatologic manifestations preceded the drug intake or the rashes disappeared despite the continuation of drugs (5, 7, 16, 20, 23, 37, 42, 43). So it is very unlikely that current COVID-19 medications are responsible for the reported skin lesions.

In our study, the prevalence of comorbidities in COVID-19 patients with skin manifestations is about 17.9% mainly reported in patients with maculopapular lesions. History of serious comorbidities like cardiovascular disease, hypertension, and obstructive lung disease was mostly reported in patients with vascular lesions; suggesting that patients with these skin manifestations are more complicated cases and need more attention. Interestingly, immune disorders were more common in patients with chilblain-like lesions. This finding is not reported yet and we suggest it to be focused on due to the possible relationship with the etiology and pathophysiology of these lesions.

Fever, cough, and dyspnea were more frequent in patients with vascular lesions and less frequent in patients with chilblain-like lesions. Also, 17% of patients with chilblain-like lesions were asymptomatic regarding systemic symptoms. Astonishingly, headache, dysosmia/dysgeusia, nasal congestion/coryza, and irritability/confusion were more common in patients with vesicular lesions. This finding can demonstrate the probable link between vesicular lesions and neurological manifestations. Future investigations are required to clarify the issue.

LIMITATIONS

There were limited articles that mentioned complete data about all the items including the disease severity and outcome of the COVID-19 patients with dermatologic presentations. Another limitation was the absence of data about the COVID-19 patients without skin manifestations. Future cohort studies are required to compare the severity and prognosis of the disease in patients with and without skin manifestations, considering other related characteristics. Such studies help to better understand the prognostic value of the cutaneous manifestations in COVID-19 patients.

CONCLUSIONS

Cutaneous lesions occur most often in middle age individuals at the same time or after the systemic symptoms of COVID-19. Urticaria-like lesions commonly (47%) occurred at the same time with other symptoms. It may suggest that urticaria-like lesions may be a diagnostic sign for COVID-19. A maculopapular rash is the main reported skin involvement in COVID-19 patients and is associated with intermediate severity of the disease. The mere occurrence of skin manifestations in COVID-19 patients is not an indicator for the disease severity, and it highly

depends on the type of skin lesions. Chilblain-like and vascular lesions are the ends of a spectrum in which from chilblain-like to vascular lesions, the severity of the disease increases, and the patient's prognosis worsens. We highly suggest emergency and general practitioners to evaluate the suspected COVID-19 patients for any cutaneous manifestations. Those with vascular lesions should also be considered as high-priority patients for further medical care.

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

PJ, MN, and MM designed the study. PJ and BH performed the review literatures, collected the data, and wrote the first draft of the manuscript. PJ, HV, and MD helped in manuscript preparation. MM critically reviewed the manuscript.

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Cesarean Section or Vaginal Delivery to Prevent Possible Vertical Transmission From a Pregnant Mother Confirmed With COVID-19 to a Neonate: A Systematic Review

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Background: The impact of delivery mode on the infection rates of Coronavirus disease 2019 (COVID-19) in the newborn remains unknown. We aimed to summarize the existing literature on COVID-19 infection during pregnancy to evaluate which mode of delivery is better for preventing possible vertical transmission from a pregnant mother confirmed with COVID-19 to a neonate.

Methods: We performed a comprehensive literature search of PubMed, Embase, Cochrane Library, Web of Science, Google Scholar, and the Chinese Biomedical Literature database (CBM) from 31 December 2019 to 18 June 2020. We applied no language restrictions. We screened abstracts for relevance, extracted data, and assessed the risk of bias in duplicate. We rated the certainty of evidence using the GRADE approach. The primary outcome was severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) test positivity in neonates born to mothers with confirmed COVID-19 following different delivery modes. Secondary outcomes were neonatal deaths and maternal deaths. This study is registered with PROSPERO, CRD42020194049.

Results: Sixty-eight observational studies meeting inclusion criteria were included in the current study, with no randomized controlled trials. In total, information on the mode of delivery, detailed neonatal outcomes, and SARS-CoV-2 status were available for 1,019 pregnant women and 1,035 neonates. Six hundred and eighteen (59.71%) neonates were born through cesarean section and 417(40.29%) through vaginal delivery. Probable congenital SARS-CoV-2 infections were reported in 34/1,035 (3.29%) neonates. Of babies born vaginally, 9/417 (2.16%) were tested positive compared with 25/618 (4.05%) born by cesarean. Of babies born vaginally, 0/417 (0.00%) neonatal deaths were reported compared with 6/618 (0.97%) born by cesarean. Of women who delivered vaginally, 1/416 (0.24%) maternal deaths were reported compared with 11/603 (1.82%) delivered by cesarean. Two women died before delivery. Sensitivity analyses and subgroup analyses showed similar findings.

Conclusions: The rate of neonatal COVID-19 infection, neonatal deaths, and maternal deaths are no greater when the mother gave birth through vaginal delivery. Based on the evidence available, there is no sufficient evidence supporting that the cesarean section is better than vaginal delivery in preventing possible vertical transmission from a pregnant mother confirmed with COVID-19 to a neonate. The mode of birth should be individualized and based on disease severity and obstetric indications. Additional good-quality studies with comprehensive serial tests from multiple specimens are urgently needed.

Study registration: PROSPERO CRD42020194049.

Keywords: coronavirus disease 2019, COVID-19, SARS-CoV-2, pregnancy, mode of delivery, vertical transmission

INTRODUCTION

Since the outbreak of a cluster of patients with pneumonia of unknown cause in Wuhan, Hubei Province, China in December 2019 (1). The disease was later named Coronavirus disease 2019 (COVID-19), caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) quickly spreading in China and other countries (2).

COVID-19 is the third coronavirus outbreak in the twenty-first century, and the other two are severe acute respiratory syndrome coronavirus (SARS-CoV) outbreaks in 2002 and Middle East respiratory syndrome coronavirus (MERS-CoV) outbreaks in 2012 (3–5), both can cause severe complications during pregnancy (6–9). Pregnant women might be at increased risk of severe infections considering that the COVID-19 seems to have a similar pathogenic potential as SARS-CoV and MERS-CoV. Pregnant women are generally susceptible to COVID-19 considering they are in a particular state of immune suppression and more susceptible to respiratory pathogens (10, 11). Because of decreased lung volumes caused by increases in uterus size during pregnancy, patients might be more prone to have a more rapid clinical deterioration with COVID-19 during pregnancy, which may increase the risk of adverse pregnancy outcomes.

There is a concern about the vertical transmission of SARS-CoV-2 due to the limited data on COVID-19 (12). Until now, vertical transmission from a pregnant mother confirmed with COVID-19 to a neonate, and the delivery mode which can best prevent this from happening is still unknown. Expert consensus has stated that there is no clear evidence that cesarean delivery prevents vertical transmission at the time of delivery (13). Whether vaginal delivery increases the risk of mother-to-child intrapartum transmission and whether uterine contraction could increase the possibility of the virus ascending needs to be further investigated.

Therefore, this review aims to determine which mode of delivery is better for preventing possible vertical transmission from COVID-19 positive pregnant women to the neonate.

METHODS

We wrote the review based on Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (14). The protocol was registered in the International Prospective Register of Systematic Reviews (known as PROSPERO; registration number: CRD42020194049).

Data Sources, Search Strategy, and Eligibility Criteria

We conducted a comprehensive literature search of PubMed, Embase, Cochrane Library, Web of Science, Google Scholar, and the Chinese Biomedical Literature database (CBM) from 31 December 2019 (when COVID-19 was first reported from Wuhan, China) to 18 June 2020. We also searched the references of selected studies. We placed no limits or filters on the searches. Combinations of the following keywords and MeSH terms were used: 2019-nCov, COVID-19, coronavirus disease 2019, severe acute respiratory syndrome coronavirus 2, SARS-CoV-2, pregnancy, pregnant, gravidity, gestation, maternal, mothers, fetal, fetus, neonate, newborn, vertical transmission, maternal-fetal transmission, intrauterine transmission, delivery. A detailed search strategy can be seen in **Appendix 1**. Eligibility criteria were randomized controlled studies, observational studies (including cohort, case-control studies, case series, and case reports), studies involving laboratory-confirmed and/or clinically diagnosed COVID-19 during pregnancy, studies involving neonates born to mothers with confirmed COVID-19 infection, studies with available clinical characteristics, including neonatal outcomes, clinical studies, studies reporting original data, studies reporting SARS-CoV-2 infected women who have delivered. Exclusion criteria were as follows: Studies involving mothers with suspected COVID-19 infection, studies with unreported neonatal outcomes, unpublished reports, studies suspected of including duplicate reporting, review, guidelines, opinions, and comments.

Suspected case defined as a person who meets the clinical **AND** epidemiological criteria (15):

Clinical Criteria

- Acute onset of fever AND cough; OR
- Acute onset of ANY THREE OR MORE of the following signs or symptoms: fever, cough, general weakness/fatigue, headache, myalgia, sore throat, coryza, dyspnoea, anorexia/nausea/vomiting, diarrhea, altered mental status (signs separated with a slash are to be counted as one sign).

Epidemiological Criteria

- Residing or working in an area with a high risk of transmission of virus: closed residential settings, humanitarian settings such as camp and camp-like settings for displaced persons; anytime within the 14 days prior to symptom onset; OR
- Residing or travel to an area with community transmission anytime within the 14 days prior to symptom onset; OR
- Working in any health care setting, including within health facilities or within the community; any time within the 14 days prior to symptom onset.

A clinically diagnosed case was defined as a suspected case with manifestations with pneumonia image features on computerized tomography (CT) scan (Another potential cause of pneumonia was rule out before diagnosis) (16).

A laboratory-confirmed case was defined as a positive result to reverse transcriptase-polymerase chain reaction (RT-PCR) nasopharyngeal swab and/or antibody testing SARS-CoV-2 (15).

A positive result of antibody testing of SARS-CoV-2 was defined as elevated concentrations of immunoglobulin M (the normal IgM level: <10 AU/mL).

Study Selection

Two independent reviewers (B.W and H.W) evaluated articles for potential inclusion by screening titles and abstracts. The full texts of those identified as being relevant were assessed to determine eligibility for final inclusion. Between each assessment, we discussed the results to reach a consensus on interpreting the inclusion criteria. We resolved any disagreements regarding study eligibility by consensus, and a third reviewer (D.Z) was consulted, if necessary. If the information required to assess eligibility is unavailable or unclear, the relevant study authors were contacted for clarification. Duplicate publications were identified and removed using EndNote software version X7 (Clarivate Analytics). The identified paper(s) were analyzed using criteria based on the largest sample size, the maximum correspondence with the inclusion criteria, and minimal risk of bias. When a hospital had published their cases more than once, if the periods of recruitment overlapped, we included the paper with the biggest data to minimize the possibility of double counting.

Data Extraction and Synthesis

We extracted data from the studies selected for inclusion, as follows: general characteristics of included studies (author names, title, publication date, source of funding, and reported conflicts of interests), type of the study, sample size, study subject characteristics (demographic characteristics, gestational age, mode of delivery), outcome measures and analyses (neonatal outcomes, number of positive samples, maternal deaths). Two authors (H.X.Z.and H.L.) extracted the data independently and in duplicate. We resolved discrepancies through discussion to achieve a consensus. Study authors were contacted to obtain missing information or to clarify the information available. However, at the time of submission, we received no responses. The SARS-CoV-2 test positivity in neonates born to mothers with confirmed COVID-19 following different delivery modes was the primary outcome. Secondary outcomes were neonatal deaths and maternal deaths. Our search did not identify any randomized trials. We did a narrative synthesis of the findings when the meta-analysis was not possible or appropriate from the included studies.

Risk of Bias and Grade Certainty Assessment

The risk of bias was assessed using the Newcastle-Ottawa scale (NOS) for observational cohort and case-control studies, and Joanna Briggs Institute (JBI) critical appraisal tools for case reports and case series studies (17, 18). For cohort and case-control studies, there were three grouping items as follows: selection, comparability, exposure/outcomes. A study can be awarded a maximum of one star for each numbered item within the selection and outcome categories. A maximum of two stars can be given for comparability (17). More stars are equalling lower risk. Case reports and case series studies were categorized according to the percentage of positive answers to each of the questions. Low risk of bias indicated more than 70% of positive answers; moderate risk of bias ranged between 50 and 69%, and high risk of bias represented <49% of positive answers (19). We graded the certainty of evidence using the GRADE approach. We used the GRADEpro guideline development tool (GDT) app to rate evidence and present it in a summary of findings table (20).

Data Analysis

Characteristics of each study, and results were described and tabulated. We also performed additional sensitivity analyses to assess the robustness of our findings. Sensitivity analyses for the primary outcome and secondary outcomes included: (1) we excluded cases reported neonate with only elevated IgM levels for SARS-CoV-2 but negative for RT-PCR considering the possibility of false-positive results for the serological test (21); (2) we conducted subgroup analyses by pregnant women who were symptomatic or asymptomatic before delivery. Clinically, since an asymptomatic patient identified at screening might not have any delivery complications as opposed to a symptomatic one, where the mode of delivery might be because of maternal indication. Thus, perinatal transmission study might be biased; (3) the infection moment when pregnant women confirmed COVID-19 (i.e., infection on first, second, third trimester). All sensitivity analyses were considered exploratory. No other statistical analyses were anticipated.

RESULTS

We identified 880 studies, 94 full-text articles assessed for eligibility, including 21 duplicates (cases reported by one or more of the same hospitals and study dates overlap), three unreported neonatal outcomes, one withdrawn at the request of the author, one study reported pregnant women with suspected COVID-19 infection (**Figure 1**). The detailed information on these excluded studies can be seen in **Appendix S7**.

In total, sixty-eight studies from 21 countries that meet the eligibility were included in our systematic review. Studies were all observational in nature. We identified no randomized

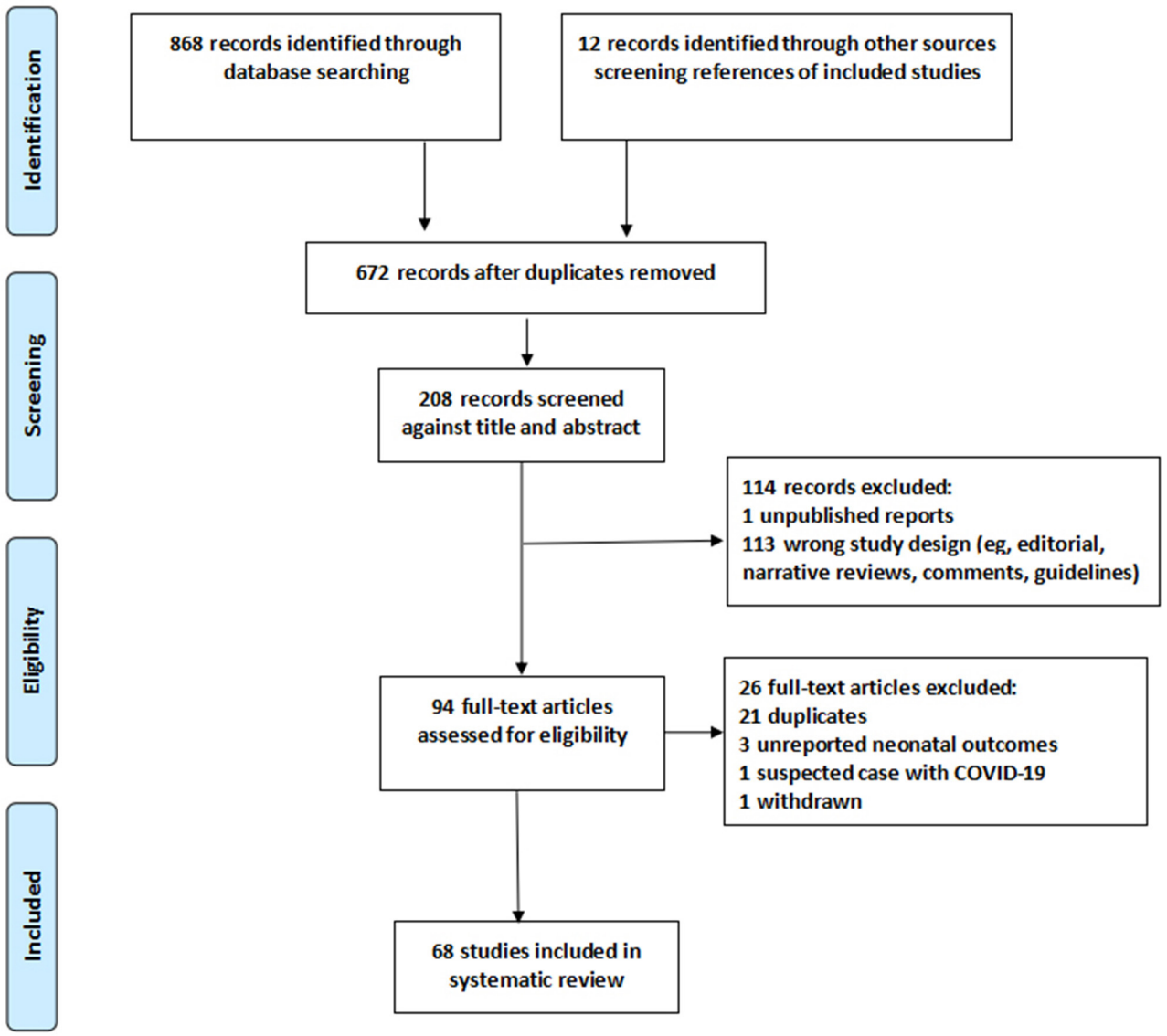

FIGURE 1 | PRISMA flow diagram.

controlled trials in the search strategy. These were published from 6 February 2020 to 12 June 2020. Forty-one were case report studies, 22 were case series studies, four cohort studies, and one case-control study (**Table 1**).

Among the five comparative studies (cohort and case-control), one study compared maternal and neonatal outcomes of pregnant women with and without COVID-19 infections. Three studies compared the clinical course of pregnant women with mild, severe, or critical COVID-19 pneumonia. Only one cohort study estimates associations between delivery mode (vaginal vs. cesarean delivery) and maternal and neonatal outcomes among SARS-CoV-2–infected women giving birth. It was impossible to perform a meta-analysis in this systematic review. Thus, we did a narrative synthesis of the findings from the included studies.

The maternal age of the reported cases ranged from 16 to 48 years, gestational age at diagnosis ranged from 16 to 41 weeks. A total of 1,019 women and 1,035 neonates had detailed information on the delivery mode and infant infection status, including 14 sets of twins and one set of triplets. Among fifteen multiple pregnancies, one woman had a vaginal birth for twins, and the others had a cesarean section.

Of the 1,035 neonates, 618 (59.71%) were born through cesarean section and 417 (40.29%) through vaginal delivery (**Table 2**). SARS-CoV-2 infections were reported in 34/1,035 (3.29%) neonates, included thirty-one RT-PCR positive neonates. The other three neonates had elevated levels of IgM for SARS-CoV-2 but negative for RT-PCR. Of the 416 women who delivered vaginally, 9/417 (2.16%) neonates tested positive for

TABLE 1 | Characteristics of the included studies.

Study	Date of publication	Country	Study design	Language of publication	Study period	No. of pregnant women confirmed with COVID-19 (n)	Maternal age, y (range)	Gestational age at delivery, week (range)	Funding
Zhu et al. (22)	Feb. 6, 2020	China	Case series	English	1.20∼2.05	9	25∼35	31∼39	No
Li et al. (23)	Feb. 19, 2020	China	Case report	Chinese	1.29∼2.02	1	27	38	No
Wang et al. (24)	Feb. 28, 2020	China	Case report	English	2.05∼2.18	1	28	30	Yes
Chen et al. (25)	Mar. 2, 2020	China	Case reports	Chinese	1.21∼2.25	3	23∼34	35∼38+6/7	No
Lei et al. (26)	Mar. 2, 2020	China	Case series	Chinese	1.22∼2.01	9	29∼35	34+2/7∼37 +5/7	No
Yao et al. (27)	Mar. 2, 2020	China	Case report	Chinese	2.11∼2.25	1	22	38+2/7	No
Zhao et al. (28)	Mar. 6, 2020	China	Case report	Chinese	1.31∼2.13	1	Not reported	35+5/7	No
Bai et al. (29)	Mar. 6, 2020	China	Case report	Chinese	2.01∼2.15	1	Not reported	37+1/7	No
Kang et al. (30)	Mar. 11, 2020	China	Case report	Chinese	2.06∼2.24	1	30	35+4/7	No
Chen et al. (31)	Mar. 16, 2020	China	Case reports	English	Not mention	4	23∼34	37+2/7∼39	Yes
Zhou et al. (32)	Mar. 16, 2020	China	Case report	Chinese	2.12∼2.21	1	30	37+3/7	No
Khan et al. (33)	Mar. 19, 2020	China	Case reports	English	1.28∼3.01	3	27∼33	34+6/7∼39 +1/7	Yes
Li et al. (34)	March 20, 2020	China	Case-control study	English	1.24∼2.29	16	26∼37	33+6/7∼40 +4/7	Yes
Yu et al. (35)	March 24, 2020	China	Case series	English	1.01∼2.08	7	29∼34	37∼41+2/7	Yes
Zeng et al. (36)	Mar. 26, 2020	China	Case series	English	1.30∼2.15	33	Not reported	31+2/7∼ 40+4/7	No
Zeng et al. (37)	Mar. 26, 2020	China	Case series	English	2.16∼3.06	6	Not reported	Not reported	Yes
Dong et al. (38)	Mar. 27, 2020	China	Case report	English	1.28∼2.22	1	29	34+2/7	Yes
Chen et al. (39)	Mar. 28, 2020	China	Case series	English	1.20∼2.10	5	25∼31	38+6/7∼40 +4/7	Yes
Baud et al. (40)	Mar. 30, 2020	Switzerland	Case report	English	3.18∼3.22	1	28	19	No
Lee et al. (41)	Mar. 31, 2020	Korea	Case report	English	3.06∼3.11	1	28	37+6/7	No
Kalafat et al. (42)	Apr. 6, 2020	Turkey	Case report	English	3.20-3.28	1	32	35+3/7	No
Gidlöf et al. (43)	Apr. 6, 2020	Sweden	Case report	English	Not mention	1	34	36+2/7	No
Peng et al. (44)	Apr. 6, 2020	China	Case report	English	2.05∼2.19	1	25	35+3/7	No
Breslin et al. (45)	Apr. 9, 2020	USA	Case series	English	3.13∼3.27	43	20∼39	32∼39	No
Xiong et al. (46)	Apr. 10, 2020	China	Case report	English	3.7∼3.10	1	25	38+4/7	No
Khassawneh et al. (47)	Apr. 14, 2020	Jordan	Case report	English	3.23∼3.26	1	30	36+3/7	No

(Continued)

TABLE 1 | Continued

Study	Date of publication	Country	Study design	Language of publication	Study period	No. of pregnant women confirmed with COVID-19 (n)	Maternal age, y (range)	Gestational age at delivery, week (range)	Funding
Schnettler et al. (48)	Apr. 14, 2020	USA	Case report	English	3.24-4.10	1	39	34+1/7	No
Liu et al. (49)	Apr. 14, 2020	China	Case series	English	1.31∼2.29	19	26∼38	35+2/7∼41+2/7	No
Carosso et al. (50)	Apr. 14, 2020	Italy	Case report	English	Not mention	1	28	37	No
González Romero (51)	Apr. 17, 2020	Spain	Case report	Spanish	Not mention	1	44	29+2/7	No
Koumoutsea et al. (52)	Apr. 17, 2020	Canada	Case report	English	Not mention	2	23∼40	35+3/7∼35+5/7	No
Zamaniyan et al. (53)	Apr. 17, 2020	Iran	Case report	English	3.7∼3.26	1	22	32	No
Alzamora et al. (54)	Apr. 18, 2020	Peru	Case report	English	3.29∼4.03	1	41	33	No
Lyra et al. (55)	Apr. 20, 2020	Portugal	Case report	English	Not mention	1	35	39+6/7	No
Al-kuraishy et al. (56)	Apr. 21, 2020	Iraq	Case report	English	3.13∼3.30	1	25	30	No
Lu et al. (57)	Apr. 24, 2020	China	Case report	English	2.11∼2.17	1	22	38	Yes
Ferrazzi et al. (58)	Apr. 27, 2020	Italy	Case series	English	3.01∼3.20	42	21∼44	Not reported	No
Hantoushzadeh et al. (59)	Apr. 28, 2020	Iran	Case series	English	2.15∼3.15	9	Not reported	28∼38+3/7	Yes
Penfield et al. (60)	May 3, 2020	USA	Case series	English	3.01∼4.20	32	22∼40	26+5/7∼41+3/7	No
Wu et al. (61)	May 5, 2020	China	Case series	English	1.31∼3.09	13	26∼40	16∼38+4/7	Yes
Piersigilli et al. (62)	May 7, 2020	Belgium	Case report	English	3.01∼3.15	1	Not reported	26+4/7	No
Blauvelt et al. (63)	May 8, 2020	USA	Case report	English	Not mention	1	34	28+6/7	Yes
Pierce-Williams et al. (64)	May 8, 2020	USA	Cohort study	English	4.20∼5.05	64	Not reported	Not reported	No
Valente et al. (65)	May 10, 2020	Portugal	Case report	English	3.17∼3.19	1	31	38	No
Liu et al. (66)	May 11, 2020	China	Case series	English	1.20∼3.03	51	Not reported	35+1/7∼41+2/7	Yes
Perrone et al. (67)	May 11, 2020	Italy	Case reports	English	3.01∼4.30	4	26∼36	38+2/7∼40+4/7	No
Baergen et al. (68)	May 12, 2020	USA	Case series	English	Not mention	20	16∼41	32+2/7∼40+4/7	No
Taghizadieh et al. (69)	May 13, 2020	Iran	Case report	English	Not mention	1	33	34	No
Patanè et al. (70)	May 14, 2020	Italy	Case series	English	3.05∼4.21	22	Not reported	Not reported	No
Kirtsman et al. (71)	May 14, 2020	Canada	Case report	English	Not mention	1	40	35+5/7	No
Dória et al. (72)	May 15, 2020	Portugal	Case series	English	3.25∼4.15	10	27∼40	37∼41	No

(Continued)

TABLE 1 | Continued

Study	Date of publication	Country	Study design	Language of publication	Study period	No. of pregnant women confirmed with COVID-19 (n)	Maternal age, y (range)	Gestational age at delivery, week (range)	Funding
Mehta et al. (73)	May 16, 2020	USA	Case report	English	Not mention	1	39	27	No
Chen et al. (74)	May 16, 2020	China	Case series	English	Not mention	17	Not reported	Not reported	No
Sharma et al. (75)	May 17, 2020	India	Case report	English	Not mention	1	Not reported	38+6/7	No
Xia et al. (76)	May 17, 2020	China	Case report	English	1.23∼2.20	1	27	37+2/7	Yes
Panichaya et al. (77)	May 18, 2020	Thailand	Case report	English	Not mention	1	43	18	No
Lokken et al. (78)	May 18, 2020	USA	Case series	English	1.21∼4.17	46	26∼34	37+3/7∼39 +6/7	Yes
London et al. (79)	May 19, 2020	USA	Cohort study	English	3.15∼4.15	68	24∼34	Not reported	No
Li et al. (80)	May 19, 2020	China	Case report	English	2.06∼2.19	1	30	35	No
Qadri et al. (81)	May 20, 2020	USA	Case series	English	Not mention	16	20∼40	22∼40+3/7	No
Tang et al. (82)	May 23, 2020	Netherlands	Case report	English	4.01∼4.30	1	Not reported	41	No
Lowe et al. (83)	May 28, 2020	Australia	Case report	English	Not mention	1	31	40+3/7	No
Kayem et al. (84)	Jun. 4, 2020	France	Case series	English	3.01∼4.14	617	Not reported	Not reported	No
Martínez-Perez et al. (85)	Jun. 8, 2020	Spain	Cohort study	English	3.12∼4.06	82	19∼48	25∼41+4/7	Yes
Wang et al. (86)	Jun. 8, 2020	China	Case series	English	12.08∼4.01	30	26∼33	30∼40+6/7	No
Knight et al. (87)	Jun. 8, 2020	UK	Cohort study	English	3.01∼4.14	427	Not reported	Not reported	Yes
Pereira et al. (88)	Jun. 10, 2020	Spain	Case series	English	3.14∼4.14	60	22∼43	27∼41	No
Bani Hani et al. (89)	Jun. 12, 2020	Jordan	Case report	English	3.28∼4.12	1	29	37+4/7	No

COVID-19. Of the 603 women who had a cesarean section, 25/618 (4.05%) neonates were found to be positive for COVID-19. A total of six neonatal deaths (including one set of twins) and nine stillbirths (including one set of twins) have been reported. Of babies born vaginally, 0/417 (0.00%) neonatal deaths were reported compared with 6/618 (0.97%) born by cesarean. A total of fourteen maternal deaths have been reported. Of women who delivered vaginally, 1/416 (0.24%) maternal deaths were reported compared with 11/603 (1.82%) delivered by cesarean. Two women died in the second trimester before delivery.

The risk of bias was mostly low-to-moderate after considering the observational designs. The results for each quality assessment by the study are presented in **Appendix S2–S4**.

Sensitivity analyses and subgroup analyses showed similar findings (**Appendix S6**). After excluded 3 (All three neonates were born through cesarean section) elevated levels of IgM for SARS-CoV-2 but negative for RT-PCR, 9/417 (2.16%) neonates born by vaginally tested positive compared with 22/615 (3.58%) neonates born by cesarean. Of the 394 women who were asymptomatic before delivery, 0/220 (0%) maternal deaths were reported with vaginal delivery compared with 2/174 (1.15%) with cesarean delivery. Of the 625 women who were symptomatic before delivery, 1/196 (0.51%) maternal deaths with vaginal delivery were reported compared with 9/429 (2.10%) with cesarean delivery. Nearly all pregnant women delivered in the third trimester except three who delivered vaginally in the second trimester. Of women who delivered vaginally in the third trimester, 1/413 (0.24%) maternal deaths were reported compared with 11/603 (1.82%) delivered by cesarean in the third trimester.

TABLE 2 | Maternal and neonatal outcomes by mode of delivery.

	Vaginal delivery No. (%) (n = 416)[a]	Cesarean delivery No. (%) (n = 603)[b]
Comorbidities[c]	32 (29.36%)	60 (27.91%)
INDICATIONS FOR CESAREAN DELIVERY[d]		
Due to obstetrical indications	NA	407 (68.52%)
Due to concern about Covid-19	NA	187 (31.48%)
MATERNAL OUTCOMES Maternal deaths[e] (secondary outcome)	1 (0.24%)	11 (1.82%)
NEONATAL OUTCOMES SARS-CoV-2 test positivity[f] (primary outcome)	9 (2.16%)	25 (4.05%)
Neonatal deaths[g] (secondary outcome)	0 (0.00%)	6 (0.97%)

SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2; NA, Not applicable.
[a]416 pregnant women gave birth vaginally, including one set of twins.
[b]603 women gave birth by cesarean section, including one set of triplets and thirteen sets of twins.
[c]Detailed information on comorbidities was available for 324 pregnant women. Of the 109 women who delivered vaginally, 32 had one or more comorbidities. Of the 215 women who had a cesarean delivery, 60 had one or more comorbidities.
[d]Detailed information on the indication for cesarean section was available for 594 pregnant women.
[e]Excluding two women who died in the second trimester before delivery.
[f]Including 31 reverse transcriptase-polymerase chain reaction positive neonates and three elevated Immunoglobulin M levels for SARS-CoV-2 neonates.
[g]Excluding nine stillbirths.

DISCUSSION

Our results have shown that SARS-CoV-2 infections were rare in neonates. The rate of neonatal COVID-19 infection, neonatal deaths, and maternal deaths is no greater when the mother gave birth through vaginal delivery. Second, the vertical transmission of SARS-CoV-2 infection is possible in the third trimester but relatively low. Third, there has been duplicate reporting of pregnant women confirmed with COVID-19 from China and other countries.

Vertical transmission refers to how pathogens are transmitted from mother to offspring before and after birth. It includes transmission via placental blood during pregnancy, via the birth canal during delivery, and via breastmilk during postpartum breastfeeding (22). Placenta, cord blood, amniotic fluid, and vaginal secretion are intrauterine tissue samples that are essential for assessing vertical transmission (90). It is necessary to collect more kinds of specimens of SARS-CoV-2 infected pregnant women and their newborns to better evaluating the possibility of vertical transmission of SARS-CoV-2. It is noteworthy that these samples should be collected immediately after birth to avoid contamination condition (91). But very few of the included studies have met these criteria. Thus, additional good-quality studies with comprehensive serial tests from multiple specimens are urgently needed.

A total of 34 neonates were born in the third trimester with possible congenital SARS-CoV-2 infections were reported, suggesting that vertical transmission of COVID-19 is possible in the third trimester. Only three pregnant women gave birth vaginally in the second trimester. All neonates' samples tested negative by RT-PCR, suggesting that no intrauterine fetal infection occurred during the second trimester of pregnancy. A recent study suggested that the SARS-CoV-2-infected mother-to-fetus transmission ratio will be significantly lower than that of the Zika virus. Because the expression of angiotensin-converting enzyme 2 (ACE2), which is the receptor that SARS-CoV-2 enters the cell, is deficient in all kinds of early maternal-fetal interface cells (92). And this may explain why SARS-CoV-2 can be found in human saliva rather than in vaginal secretions (93), which could also partially explain why the risk of intrauterine mother-to-child transmission for SARS-CoV-2 is low.

Thirty-one neonates tested positive for SARS-CoV-2 by RT-PCR. The remaining three neonates had elevated IgM levels for SARS-CoV-2 but negative by RT-PCR. These three cases deserve additional details. Two studies reported three neonates with elevated IgM antibody values to SARS-CoV-2 born to mothers with COVID-19 from separate research teams in China (37, 94). All mothers wore masks during the cesarean delivery in negative pressure isolation rooms, and all medical staff wore protective suits and double masks. After birth, all infants were isolated from their mothers immediately. Neonatal blood was collected to test IgG and IgM antibodies to SARS-CoV-2 at 0 and 2 h after birth, respectively. However, all of the three neonatal respiratory samples tested negative for SARS-CoV-2 RNA, and there was no information provided by testing cord blood or placenta.

It is worth noting that IgM antibodies are not usually transferred from mother to fetus via the placenta because of the larger macromolecular structure (95). IgM is generally the first responded antibody that eliminating pathogens before sufficient IgG is produced (96). IgM positive results tend to indicate recent exposure to SARS-CoV-2. In contrast, the detection of COVID-19 IgG antibodies means virus exposure some time ago. IgM antibodies usually take days to appear after infection. IgM antibodies can be detected after a median of 5 days following the onset of symptoms (97). Most guidelines using nucleic acid tests as the gold standard for the diagnosis of COVID-19 due to the time lag between the onset of symptoms and IgM's appearance in serum and a lower sensitivity and specificity of serological tests (98, 99). Caution in interpreting these findings has been suggested, including the possibility of false-positive results (100). Thus, sensitivity analyses were performed by excluded reported elevated levels of IgM for SARS-CoV-2 but negative for RT-PCR. Sensitivity analyses showed the rate of neonatal COVID-19 infection still lower when the baby is born vaginally. Additional two sensitivity analyses showed similar findings regarding the moment of the infection and if pregnant women were symptomatic or asymptomatic before delivery.

Most of the guidelines for managing pregnant women with COVID-19 are based on previous SARS and MERS experience (13, 101, 102). Suggestions on the selection of delivery methods in pregnancies with COVID-19 are contradictory (13). There were no confirmed cases of vertical transmission for SARS-CoV and

MERS (103). Despite causing approximately one billion annual infections globally, the influenza virus has only a few cases of confirmed or suspected intrauterine fetal infections reported (104). Evidence for intrauterine influenza transmission exists from antigen and antibody testing in the infant brain, amniotic fluid, fetal heart, and cord blood (105). Nevertheless, which delivery mode is better for preventing vertical transmission from a pregnant woman with influenza to a neonate remains unknown.

While we have presented the data from a robust search of the literature for 1,019 women and 1,035 neonates, the given number did not control some confounding factors. For example, the mothers' COVID-19 infection severity was not presented due to the missing information of the included studies. What's more, the baseline conditions of pregnant women undergoing cesarean section and vaginal delivery are different, so we remind the reader to interpret the data in light of these biases would weaken the conclusions of current studies. The overall rate of cesarean section in the included studies was 59.18% (603/1,019), much higher than cesarean birth rates in the United States (31.9%) and China (36.7%) (106, 107). About 31.48% (187/594) of the cesarean deliveries were performed among women with COVID-19 due to concern about Covid-19 without obstetrical indications. According to a WHO report, (108) the rates of complications during pregnancy were similar between women who delivered vaginally (18.36%) or by cesarean section (19.57%). The neonatal death rates were similar between babies born vaginally (0.59%) or by cesarean section (0.79%). However, for maternal mortality, cesarean sections were associated with a significantly increased risk of maternal mortality than vaginal delivery (adjusted odds ratio 2.1, 95% CI 1.7–2.6). Furthermore, cesarean delivery is associated with increased morbidity in the immediate postpartum period because of the increased risks of thromboembolic disease, blood loss, and infections (109). Currently, there is no sufficient evidence supporting that cesarean section improves outcomes among patients with COVID-19 and prevent possible vertical transmission from a pregnant mother confirmed with COVID-19 to a neonate. Our findings suggest that COVID-19 infection should not be an indication for a cesarean birth. We advise that cesarean delivery be performed in women with COVID-19 only after a careful evaluation of the disease severity and obstetrical indications. We believe our findings are reassuring and relevant to pregnant women confirmed with COVID-19 and obstetricians. Especially pregnant women with COVID-19 who want to give birth by vaginal delivery.

We identified 21 duplicate studies, some articles have been published in different languages, and some authors have reported features of pregnant women with COVID-19 from different perspectives. The detailed information on these duplicates studies can be seen in **Appendix S7**. There have several concerns about duplicate reporting of cases of COVID-19 been described (110, 111). Reporting the duplicates in different articles creates an inaccurate scientific record, precludes valid meta-analyses considering double-counting, and may affect understanding the disease and its epidemiology (110). To minimize the possibility of double counting, we reviewed the hospital and periods of recruitment. If they overlapped, only the study with the biggest data was included.

LIMITATIONS OF STUDY

Our article has some limitations. Firstly, we didn't search for the LILACS or SciELO database, which means the data on pregnant women from Latin America, the Caribbean region, and Brazil are scarce. Brazil has the third-largest number of COVID-19 cases after the United States and India. Secondly, we didn't perform analysis according to the severity of the COVID-19 infection of the mothers due to the missing information of the included studies. Pregnant women with more severe COVID-19 infection appear to prefer delivery by cesarean delivery rather than vaginal birth (112, 113). What's more, all patients in the study who give birth were recruited in their second and third trimester, so we were unable to ascertain the possibility of intrauterine vertical transmission during the first trimester. For example, rubella infection in the first trimester can affect more than 50% of fetuses via intrauterine infection. In contrast, by the end of the second trimester, the incidence rate is reduced by half (114).

CONCLUSIONS

The rate of neonatal COVID-19 infection, neonatal deaths, and maternal deaths is no greater when the mother gave birth through vaginal delivery. Based on the evidence available, there is no sufficient evidence supporting that the cesarean section is better than vaginal delivery in preventing possible vertical transmission from a pregnant mother confirmed with COVID-19 to a neonate. The mode of birth should be individualized and based on disease severity and obstetric indications. Additional good-quality studies with comprehensive serial tests from multiple specimens are urgently needed.

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 authors.

AUTHOR CONTRIBUTIONS

JC had the idea for the article. DZ and HL contributed to the design of the search strategy. HW and BW did data selection. YY and RZ had roles in the assessment of the risk of bias in the included studies. All authors reviewed and approved the final version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.634949/full#supplementary-material

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BRIEF RESEARCH REPORT
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Mortality of the COVID-19 Outbreak in Sweden in Relation to Previous Severe Disease Outbreaks

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Influenza viruses have caused disease outbreaks in human societies for a long time. Influenza often has rapid onset and relatively short duration, both in the individual and in the population. The case fatality rate varies for different strains of the virus, as do the effects on total mortality. Outbreaks related to coronavirus infections have recently become a global concern but much less is known about the dynamics of these outbreaks and their effects on mortality. In this work, disease outbreaks in Sweden, in the time period of 1860–2020, are characterized and compared to the currently ongoing COVID-19 outbreak. The focus is on outbreaks with a sharp increase in all-cause mortality. Outbreak onset is defined as the time point when death counts start to increase consistently for a period of at least 10 days. The duration of the outbreak is defined as the time period in which mortality rates are elevated. Excess mortality is estimated by standard methods. In total there were 15 outbreaks detected in the time period, the first 14 were likely caused by influenza virus infections, the last by SARS-CoV-2. The mortality dynamics of the SARS-CoV-2 outbreak is shown to be similar to outbreaks due to influenza virus, and in terms of the number of excess deaths, it is the worst outbreak in Sweden since the "Spanish flu" of 1918–1919.

Keywords: COVID-19, mortality, all-cause (overall) mortality, disease outbreak, influenza, SARS-CoV-2

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1. INTRODUCTION

Influenza viruses of type A are known to have caused disease outbreaks at least since 19th century, and probably for much longer (1). The severity of the disease caused by an influenza virus infection depends both on the properties of the virus (which strain) and on the acquired immunity and general health status of the infected individual [e.g., (2)]. The great majority recover completely from an infection, however, each year a number of persons die from consequences of influenza infections. The death toll at the level of the population depends on vaccination programs and non-pharmaceutical interventions aiming to reduce the spread of the virus (3).

Outbreaks caused by coronaviruses are thought to be a more recent phenomenon; the first reported outbreak was caused by SARS-CoV-1 in 2003 (4), and the most recent is the still ongoing pandemic caused by SARS-CoV-2 (5). Vaccines against coronaviruses are still under development (6) and measures available to control the outbreaks have so far been limited to non-pharmaceutical interventions.

It has long been recognized that influenza outbreaks often are associated with an increase in all-cause mortality that exceeds the increase directly attributed to influenza and pneumonia [e.g., (7, 8)]. Indeed, influenza seasons, and outbreaks, can be reliably detected from all-cause mortality

data [e.g., (7, 9)]. In fact, many countries and regions of the world monitor influenza by, among other things, detecting when, and by how much, the number of deaths per week exceed a preset, model based, threshold. Outbreaks, such as the 1918–1920 influenza pandemic are characterized by a high attack rate and often lead to a rapidly increasing number of deaths during a short time period. Consequently, with access to daily death counts, it should be possible to detect outbreaks by looking at the local rate of change of the number of recorded deaths. Here such an approach is developed and applied to daily death counts from Sweden in the time period of 1860–2020. The excess mortality caused by the ongoing outbreak of COVID-19 is related to the 14 most severe outbreaks during the previous 160 years.

2. METHODS

2.1. Data

Two sources of daily counts of deaths from all causes were used. For the years 1860–2014, data were obtained from "Swedish Book of Death" issued by the The Federation of Swedish Genealogical Societies (10). This is a database compiled from a range of official sources and contains information on times and places of births and deaths for persons that have died in Sweden since 1860. The coverage is almost complete. Mortality data from 2015 until 31th of August 2020 was obtained from the website of Statistics Sweden (`www.scb.se`) on November 9th 2020. Data on total population size for the years 1859–2019 were obtained from Statistics Sweden (`www.scb.se`). The complete time series used in this work is available on GitHub: https://github.com/aledberg/outbreaks.

2.2. Outbreak Detection

Outbreaks were detected by analyzing the rate of change (time derivative) of daily death counts. A period of a rapid increase in death counts corresponds to a period where the derivative is consistently positive. Since the mortality data were relatively noisy, the daily deaths counts were first smoothed with a 21-point truncated Gaussian kernel and the time derivative was approximated by the first-order difference applied to the smoothed time series.

Time intervals, 10 days or longer, where the rate of change exceeded a threshold value of 2.9 were selected as candidate outbreaks. The onset of the outbreak was defined as the first time point where the derivative exceeded the threshold. The offset of the outbreak was defined as the time point at which the derivative returned to zero from below. This procedure would, in principle, accurately detect the onset and offset of an outbreak described by a smooth function with a single local maximum[1]. The values of the three parameters involved in this procedure: i.e., width of the Gaussian smoothing kernel (standard deviation 1.5), the threshold value of the derivative (2.9), and the minimum number of consecutive days (10), were determined by applying the procedure to data from 1860 to 2017, i.e., not using data from the COVID-19 outbreak. Two of the outbreaks detected using this method, both occurring before 1886, were after visual inspection determined not to qualify as outbreaks (the mortality did not exceed the background level). In some cases, the offsets of the outbreaks needed to be adjusted manually since the automatic detection based on the derivative sometimes led to an overestimate the duration, and sometimes (for the 1918–1919 outbreak) underestimated the duration due to the presence of multiple local maxima.

[1]Such a shape would result, for example, from simple compartmental models, such as the SIR-model if the death counts are assumed proportional to the number of infectious people.

2.3. Excess Mortality Estimation

Excess mortality caused by a disease outbreak is usually defined as the observed number of deaths minus the expected number of deaths. To estimate the expected number of deaths a variant of the regression method first described by Robert Serfling (11) is often used, and this approach was also adopted here. This consists in fitting a regression model to data where time periods corresponding to the outbreaks have been removed. The model is then used to predict (forecast) values for the time period corresponding to the outbreak, and excess mortality is taken as the difference between observed death counts and counts predicted from the model.

In this work, the number of deaths per day, N_t, was assumed to follow a Poisson distribution with a time-dependent expected value obeying the following model

$$\log\left\{E(N_t)\right\} = \mu + \beta t + \alpha \text{month}(t), \tag{1}$$

where month(t) is a categorical variable denoting the month corresponding to time t, and μ, β, and α are the parameters used to fit the model to data. Note that time, t, is expressed in units of days. Separate models for each outbreak were fitted to data from 5 years prior to the onset of the outbreak.

Excess mortality during the outbreaks was then estimated as the sum of the differences between observed and expected mortality for the days of the outbreak. Since the Swedish population has increased substantially over the time period, excess mortality was also expressed in terms of per 100,000 population. The number for the total population size was taken as the population the last of December the year before the onset of the outbreak.

Supplementary Figure 1 illustrates the method used to detect outbreaks and estimate excess mortality.

2.4. Classification of Outbreaks

To investigate if influenza virus infections might have caused the detected outbreaks, official Swedish records reporting on causes of death for the corresponding years were used. For the years prior to 1911 this information was published in the annual publication "Bidrag till Sveriges officiella statistik. A. Befolkningsstatistik (BiSOS A)," for the years 1911–1996, causes of death were published annually in "Dödsorsaker" both issued by Statistics Sweden and available online on their website www.scb.se.

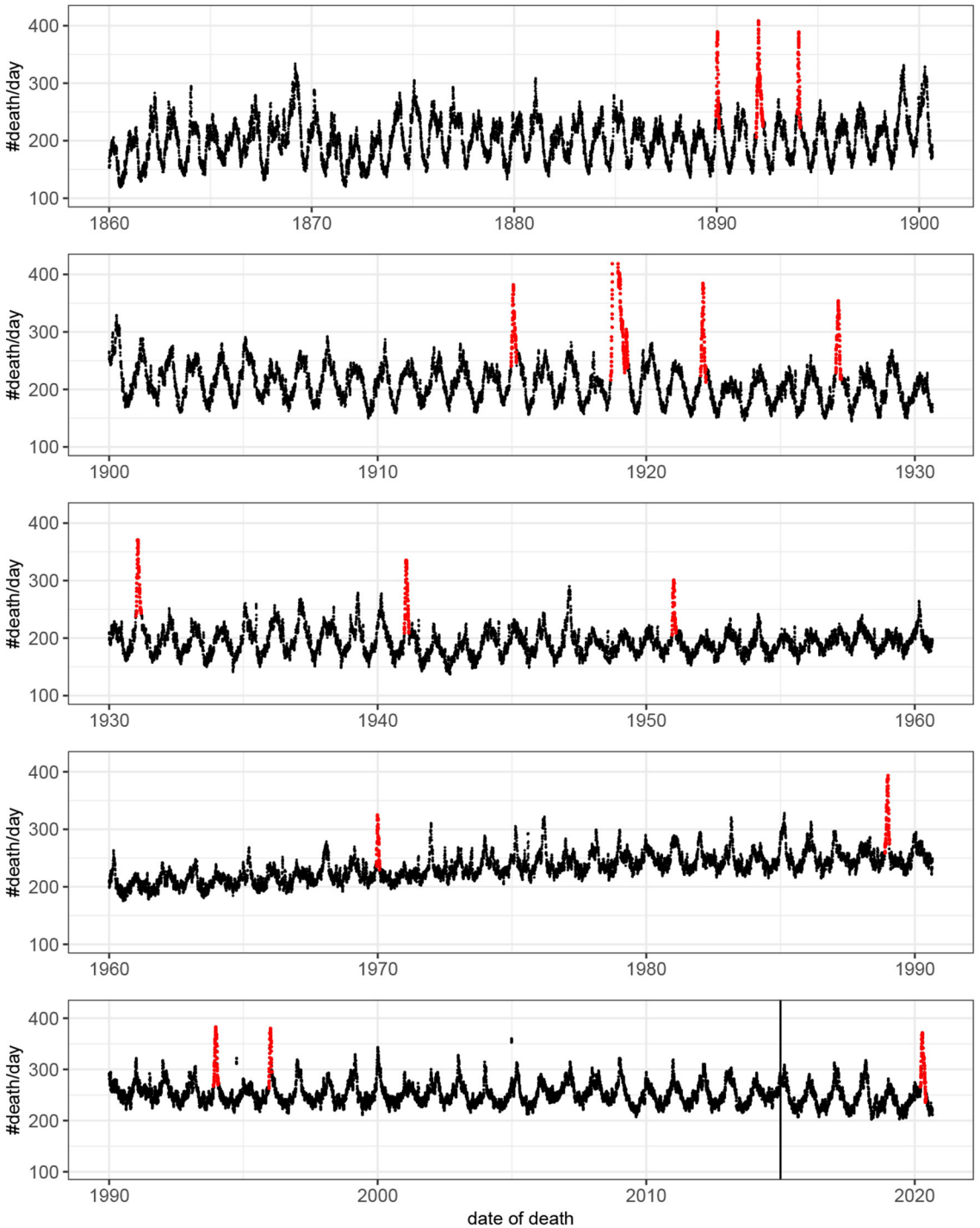

FIGURE 1 | Number of deaths per day as a function of time. For visualization the original count data have been smoothed by rectangular window of length seven. That is, each value represent the mean value of the neighboring seven values. Detected outbreaks are shown in red. Note that the peak of the outbreak of 1918–1919 has been truncated. The vertical line indicates the time point at which there was a change in data source (see Methods).

3. RESULTS

Fifteen outbreaks were detected in the 160 years of mortality data analyzed (**Figure 1**). These outbreaks correspond to the 15 highest peaks in the data. The method used to estimate excess mortality is illustrated in **Figure 2**, which also shows the COVID-19 outbreak in more detail. Data characterizing the 15 outbreaks are tabulated in **Table 1**. In terms of excess deaths,

TABLE 1 | Outbreaks detected in the data.

Onset	Offset	Duration (days)	Excess Mortality	Excess mortality (per 10^5 pop.)	Common name
Dec 21 1889	Feb 13 1890	54	5,011	105.5	"Russian flu"
Dec 16 1891	Apr 14 1892	120	7,186	150.2	"Russian flu"
Dec 29 1893	Feb 13 1894	46	2,664	55.4	"Russian flu"
Dec 22 1914	Mar 04 1915	72	5,058	89.7	
Sep 08 1918	Apr 25 1919	229	39,391	679.1	"Spanish flu"
Jan 11 1922	Mar 23 1922	71	3,527	59.2	
Jan 21 1927	Apr 03 1927	72	4,587	75.5	
Jan 04 1931	Mar 15 1931	70	4,571	74.4	
Dec 29 1940	Mar 01 1941	62	3,145	49.6	
Dec 20 1950	Feb 10 1951	52	2,363	33.8	
Dec 15 1969	Jan 25 1970	41	1,345	17.0	
Nov 22 1988	Jan 19 1989	58	2,997	35.6	
Nov 22 1993	Jan 24 1994	63	2,877	33.1	
Dec 13 1995	Jan 20 1996	38	2,543	28.8	
Mar 19 2020	May 31 2020	73	5,216	50.5	COVID-19

Excess mortality is expressed as the difference between observed and expected deaths (see Methods), and is show both in absolute numbers as well as standardized to the size of the total population.

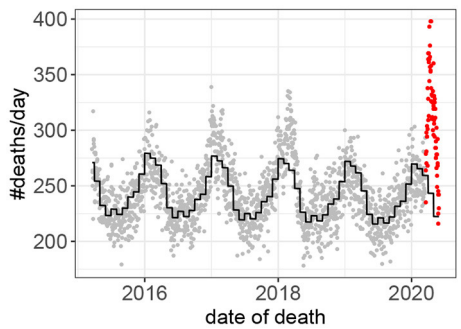

FIGURE 2 | Death counts prior to, and during, the COVID-19 outbreak. Observed values prior to the outbreak in gray. Values during the outbreak in red. The predicted values based on the Poisson model (Equation 1) are shown in black.

the COVID-19 outbreak (last outbreak) is the worst since the outbreak in 1918–1919, and when standardized by the total population size, it is the worst outbreak since 1931.

4. DISCUSSION

Using daily death counts from 1860 until present, 15 disease outbreaks, characterized by rapidly increasing all-cause mortality, were detected. Official Swedish records on causes of death (see Methods) clearly indicate that the outbreaks occurring before 1960 all coincided with influenza epidemics or pandemics. Four of these outbreaks coincided with previously characterized pandemics (see **Table 1**), but in most cases the influenza virus strains causing the outbreaks are not known with certainty[2]. Likely, the outbreaks between 1960 and 2000 were also caused by influenza virus, but here the official records give less clear support. The last outbreak detected was caused by SARS-CoV-2. In terms of all-cause mortality the time-course is similar for all the 15 outbreaks; a rapid onset and a slower return to baseline. Note that the algorithm used to detect outbreaks was fine-tuned using data not including the COVID-19 outbreak, and that this outbreak was readily detected demonstrate that the dynamics is similar to outbreaks caused by influenza viruses. Most outbreaks were relatively short in duration; all except two were <3 months long. The 1918–1919 outbreak (part of the "Spanish flu" pandemic) was exceptional both in excess mortality and duration, and lasted for more than half a year.

A disease outbreak might be reasonably defined as a sudden increase in the number of cases of the disease. Consequently, the outbreaks detected in this work are, of course, just a subset of all outbreaks in Sweden during the time period. Many disease outbreaks are not associated with a substantial increase in mortality rates and such outbreaks cannot be detected using mortality data. Furthermore, by looking at all-cause mortality from the entire Swedish population, local disease outbreaks, even with a marked increase in mortality, might be hard to detect. The focus, in this work, on outbreaks with rapid increases in death counts was partly a consequence of the available data. Indeed, the most conspicuous outbreaks present in the data were of this kind (**Figure 1**). However, outbreaks having less rapid onsets, smaller peaks, and longer durations might not be detected with the approach used here, even if their contributions to the total death count would be of comparable magnitudes. Note that the singular

[2]There is still a debate on what virus actually caused the 1890 pandemic. The Swedish records at the time classified this as "influenza" but this of course was based on the symptoms of the disease, and not on an identification of the causal agent.

mortality increases caused by the sinking of MS Estonia the 28th of September 1994 and by the Indian Ocean tsunami of 26th of December 2004 were not classified as outbreaks by the method, even if the casualties, more than 500 at each occasion, led to a very sharp increase in the number of deaths around these dates. Taken together, this shows that using information local in time it is possible to reliably detect onsets of disease outbreaks from all-cause mortality data. This approach might have advantages compared to model-based approaches that defines an epidemic threshold based on data from, in some cases, several years in the past [e.g., (11)]. Furthermore, when outbreaks occur outside of the classical influenza season (as was the case with COVID-19), a method not requiring a predefined time interval is beneficial.

There is no clear threshold at which an increase in deaths become an "outbreak"; changing the three parameters of the method would lead to more (or fewer) peaks being so classified. Data and code are publicly available https://github.com/aledberg/outbreaks, and the curious reader can easily try out other parameter combinations. It should be emphasized that the results obtained with respect to excess mortality are not very sensitive to the exact delimitation of the outbreak: when the observed death counts return to the expected counts, the contribution to the excess mortality is minor.

Of the eleven outbreaks that were detected in the 20th century the five first were the most severe in terms of cases per 100,000 population, and they all occurred before 1932. This decrease in severity likely has several causes, including better treatment of those infected as well as the development of influenza vaccines. It is interesting to note that the amplitude of the overall seasonality of deaths decreased under the same time period (12), supporting the notion that infectious diseases are one main driver of the seasonal fluctuations in mortality (8). The SARS-CoV-2-related outbreak in 2020 seems to be an exception from this trend of decreasing severity. In terms of absolute number of excess deaths this outbreak is the most severe since the Spanish flu in 1918–1919. Furthermore, the COVID-19 pandemic is far from over, and the final number of excess deaths will likely be much higher than the 5,200 reported here.

DATA AVAILABILITY STATEMENT

Data and R-code are available at https://github.com/aledberg/outbreaks.

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

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.579948/full#supplementary-material

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CANPT Score: A Tool to Predict Severe COVID-19 on Admission

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Background and Aims: Patients with critical coronavirus disease 2019 (COVID-19) have a mortality rate higher than 50%. The purpose of this study was to establish a model for the prediction of the risk of severe disease and/or death in patients with COVID-19 on admission.

Materials and Methods: Patients diagnosed with COVID-19 in four hospitals in China from January 22, 2020 to April 15, 2020 were retrospectively enrolled. The demographic, laboratory, and clinical data of the patients with COVID-19 were collected. The independent risk factors related to the severity of and death due to COVID-19 were identified with a multivariate logistic regression; a nomogram and prediction model were established. The area under the receiver operating characteristic curve (AUROC) and predictive accuracy were used to evaluate the model's effectiveness.

Results: In total, 582 patients with COVID-19, including 116 patients with severe disease, were enrolled. Their comorbidities, body temperature, neutrophil-to-lymphocyte ratio (NLR), platelet (PLT) count, and levels of total bilirubin (Tbil), creatinine (Cr), creatine kinase (CK), and albumin (Alb) were independent risk factors for severe disease. A nomogram was generated based on these eight variables with a predictive accuracy of 85.9% and an AUROC of 0.858 (95% CI, 0.823–0.893). Based on the nomogram, the CANPT score was established with cut-off values of 12 and 16. The percentages of patients with severe disease in the groups with CANPT scores <12, ≥ 12, and <16, and ≥ 16 were 4.15, 27.43, and 69.64%, respectively. Seventeen patients died. NLR, Cr, CK, and Alb were independent risk factors for mortality, and the CAN score was established to predict mortality. With a cut-off value of 15, the predictive accuracy was 97.4%, and the AUROC was 0.903 (95% CI 0.832, 0.974).

Conclusions: The CANPT and CAN scores can predict the risk of severe disease and mortality in COVID-19 patients on admission.

Keywords: SARS-CoV-2, COVID-19, severe illness, prediction, nomogram

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by severe respiratory syndrome coronavirus 2 (SARS-CoV-2), and pneumonia is the main clinical manifestation (1–3). SARS-CoV-2 is highly transmissible (4, 5), and the COVID-19 pandemic has spread to every country. With the rapid increase in the number of confirmed cases, medical resources have been inadequate (6).

As of December 30, 2020, the cumulative number of confirmed cases worldwide exceeded 80 million, and more than 1.78 million patients had died; the daily number of newly diagnosed patients is still rising. Although many clinical trials have been performed in the treatment of COVID-19 patients, so far, only dexamethasone has been validated in reducing the mortality rate of critically ill patients, and no specific medicine is available for COVID-19 (7). The demand for intensive care unit (ICU) beds, ventilators, protective equipment, other medical resources and medical staff exceed the existing supply by 10-fold (6). The early identification of patients at risk for severe disease and death, the timely initiation of interventions and admission to the ICU can prevent disease progression and reduce the mortality rate. Patients with mild COVID-19 require access to only limited medical resources for isolation and general symptomatic treatment. Therefore, it is very important to establish models predicting the prognoses of patients with COVID-19. More than 700 prognosis-related articles have been published in journals and on preprint platforms; most articles have only provided the risk factors for a poor outcome in COVID-19 patients; and ∼50 prognostic models have been reported (8). Yuan et al. reported a model with good predictive efficacy [area under the receiver operating characteristic curve (AUROC) = 0.901] in predicting the risk of mortality in patients with COVID-19 using chest computed tomography (CT) scores. However, this model is not sufficiently representative and generalizable because only 27 patients from Wuhan were included (9). Other models were constructed using data only from patients in Wuhan (9–13), and many models have been presented as nomograms, which are inconvenient for clinical application and have not been verified in populations of other COVID-19 patients (12, 14–16).

Liang et al. established a model based on 1,590 COVID-19 patients from 31 provinces in China and validated this model in another 710 patients with COVID-19; the model is available on a web page (http://118.126.104.170/). The model showed good predictive ability for a poor prognosis of COVID-19 in both the development cohort and the external validation cohort (AUROC = 0.880). The following 10 variables were included the model: X-ray abnormalities, age, hemoptysis, dyspnea, unconsciousness, number of comorbidities, cancer history, neutrophil-to-lymphocyte ratio (NLR), lactate dehydrogenase (LDH) level, and direct bilirubin level; however, only the NLR, LDH level, and direct bilirubin level are biochemical indicators, which are inadequate for enabling a comprehensive evaluation of renal, heart, and coagulation function in patients with COVID-19. This model and several other models used ICU admission, invasive ventilation, and death as the composite outcome (14, 16–18), although not all patients with critical disease require ICU admission and/or invasive ventilation, and more than 50% of patients with critical disease will die (6); thus, individual models are needed for the precise prediction of severe disease or death. Wu et al. reported a model with good predictive efficacy (16) that was established based on data from 299 patients from Wuhan, China, and verified in 426 patients with COVID-19 from China, Italy, and Belgium. However, Collins et al. believed that the sample size of patients in their study was relatively small and that it was unreasonable to use 239 patients for model development and 60 patients for internal validation. The patients with a predicted risk of a poor prognosis from 21.00 to 80.00% were classified into the medium-risk group, further casting doubt regarding the basis of risk stratification in the study (19). The CALL score developed by Ji et al. includes comorbidities, lymphocytes, age, and LDH, has good predictive efficacy and is convenient for clinical use. However, the sample size in their study was relatively small, and there was no external validation (20). The NLR has been reported to be a prognostic factor in COVID-19 (21, 22); however, the NLR can only reflect the status of the immune system and is insufficient for assessing the comprehensive situation in COVID-19 patients because COVID-19 is a systemic disease (23, 24). In summary, these models have a risk of bias, and their reliability in clinical application has not been verified (8, 25). As the COVID-19 epidemic continues to spread, it is necessary to develop a reliable and clinically applicable prognostic model for COVID-19.

In this study, by comparing the demographic, clinical, and blood biochemical characteristics of COVID-19 patients with and without severe disease on admission, the risk factors for severe disease and mortality were identified, and risk prediction models for severe disease and death in COVID-19 patients were established.

MATERIALS AND METHODS

Patient Selection

This study retrospectively included patients with COVID-19 diagnosed at Shiyan Taihe Hospital, Ankang Central Hospital, Ningbo Hwamei Hospital, and Yichang Central People's Hospital from January 22, 2020 to April 15, 2020. The criteria used for the diagnosis and classification of confirmed cases of COVID-19 were provided in the "Guidance for 2019 coronavirus disease prevention, control, diagnosis and management" (26). The clinical classifications were as follow. (1) Mild: the clinical symptoms were mild, and no pneumonia manifestations were observed on imaging. (2) Moderate: patients had symptoms, such as fever and respiratory tract symptoms, and pneumonia manifestations were observed on imaging. (3) Severe: any of the following criteria were met: (1) respiratory distress, respiration rate (RR) ≥30 breaths/min; (2) pulse oxygen saturation (SpO2) ≤93% on room air at rest; or (3) arterial partial pressure of oxygen (PaO2)/oxygen concentration (FiO2) ≤300 mmHg. In regions with a high altitude (more than 1 kilometer above sea level), the PaO2/FiO2 values were adjusted based on the following: equation of PaO2/FiO2 × [atmospheric pressure (mm Hg)/760]. Patients with >50% lesion progression within 24 to 48 h on pulmonary imaging were treated as having severe cases. And (4) Critical: any of the following criteria were met: (1) respiratory failure

needing mechanical ventilation; (2) shock; or (3) other organ failure requiring monitoring and treatment in the ICU. In this study, the severe and critical cases were classified as having severe disease, while the mild and moderate cases were classified as having non-severe disease. Patients diagnosed with severe disease on admission were only included in the mortality risk analysis. This study was approved by the Medical Ethics Committee of Shiyan Taihe Hospital. The approval number is 2020KS018.

Data Collection

Clinical data pertaining to COVID-19 patients on admission were retrieved from the medical record databases of Shiyan Taihe Hospital, Ankang Central Hospital, Ningbo Hwamei Hospital, and Yichang Central People's Hospital. The data included the patients' epidemiological histories, comorbidities, vital signs (heart rate, RR, blood pressure, and body temperature), signs and symptoms (fever), laboratory tests (liver and kidney function, routine blood tests, C-reactive protein (CRP) levels, and chest CT findings), and outcome at discharge. The patients with COVID-19 who progressed to severe or critical disease during hospitalization were included in the analysis of severe disease. Survival at discharge was the final outcome of this study. The included comorbidities were hypertension, diabetes, cardiocerebrovascular disease, malignant tumor, chronic liver disease, chronic kidney disease, and chronic lung disease.

Statistical Analysis

The normally and non-normally distributed continuous variables are presented as the means ± standard deviations (SDs) and medians (interquartile ranges, IQRs), respectively. The categorical variables are presented as *n* (%). *t*-tests, chi-square tests and Mann-Whitney *U*-tests were used to compare the differences in various indicators between the two groups. To ensure that the variables conformed to a normal distribution to the greatest extent possible, natural logarithmic transformation was applied to the white blood cell (WBC) count, procalcitonin (PCT) level, CRP level and other variables. Then, we obtained the natural logarithm (ln) of WBC [ln (WBC)], ln (NLR), ln (PLT), ln (Alb), ln (Tbil), ln (Cr), etc. During the modeling process, variables with more than 10% missing values were excluded from the analysis, and variables with <10% missing values were addressed with multiple imputation. The independent prognostic risk factors were selected by a logistic regression analysis and included in the nomogram, which was used to establish the prediction model. For convenience in clinical application, the independent risk factors identified by the logistic regression analysis were converted into dichotomous variables with a cut-off value determined by receiver operating characteristic (ROC) curve analysis. A logistic regression was performed to determine the weight of the influence of the variables on disease progression and establish a new scoring model. The best cut-off value was determined according to the Youden index, sensitivity, specificity, predictive value, and likelihood ratio. The leave-one-out cross validation method was used for internal validation, and 1,000 bootstrap resamplings were performed. The AUROC and Hosmer-Lemeshow test were used to evaluate the predictive efficacy of the model. SPSS software, version 22.0 (SPSS, Inc., Chicago, IL, USA), was used for the data analysis. The nomogram was established using R software, version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). A two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

Baseline Characteristics of COVID-19 Patients

In total, 582 patients with COVID-19, including 202 from Shiyan Taihe Hospital, 40 from Ankang Central Hospital, 108 from Ningbo Hwamei Hospital, and 232 from Yichang Central People's Hospital, were enrolled in this study. During hospitalization, 116 patients developed severe disease, and 17 patients died. There were 466 patients with non-severe COVID-19, including 25 with mild cases and 441 with moderate cases (**Figure 1**). The median age of the patients with severe disease was significantly higher than that of the patients with non-severe disease (63.00 *vs.* 47.00, $P < 0.001$). The proportion of patients with comorbidities in the severe disease group was almost two times greater than that in the non-severe disease group (59.48 *vs.* 24.68%, $P < 0.001$). Hypertension was the most common comorbidity in the patients with COVID-19 (20.10%), followed by diabetes (9.28%) and malignant tumors (2.23%), which were more common in the patients with severe disease than those with non-severe disease (**Table 1**). Among the 582 patients with COVID-19, fever was the most common symptom (74.05%), and the incidence of fever in the patients with severe disease was higher than that in the patients with non-severe disease, although the difference was not statistically significant (80.17 *vs.* 72.53%, $P = 0.093$). However, the proportion of patients with a body temperature ≥38.5°C in the group with severe disease was significantly higher than that in the group with non-severe disease (41.38 *vs.* 22.32%, $P < 0.001$); intrapulmonary ground-glass opacities (GGOs) were observed on CT in 559 /582 (96.05%) patients with COVID-19, including all patients with severe disease, and 443/466 (95.06%) patients with non-severe disease. There was no significant difference between the two groups in terms of sex, respiration, heart rate, or blood pressure on admission (**Table 1**).

The lymphocyte (LY) count was reduced in 300/582 (51.55%) patients with COVID-19, and a reduced LY count was more common in the group with severe disease than in the group with non-severe disease (76.72 *vs.* 45.92%, $P < 0.001$). However, the group with severe disease had a significantly larger proportion of patients with elevated WBC counts, especially neutrophils (NE), than the group with non-severe disease (11.21 *vs.* 3.22%, $P < 0.001$; 23.28 *vs.* 7.73%, $P < 0.001$). Therefore, the patients with severe disease had a significantly higher NLR than those with non-severe disease (4.20 *vs.* 2.64, $P < 0.001$). The proportions of patients with reduced hemoglobin (HGB) and PLT levels were two times higher in the group with severe disease than in the group with non-severe disease (14.66 *vs.* 7.30%, $P = 0.012$; 29.31 *vs.* 13.52%, $P < 0.001$). The rates of abnormal aspartate aminotransferase (AST) and γ-glutamyl transpeptidase (GGT) levels were significantly higher in the patients with severe disease than in the patients with non-severe disease. A reduced albumin

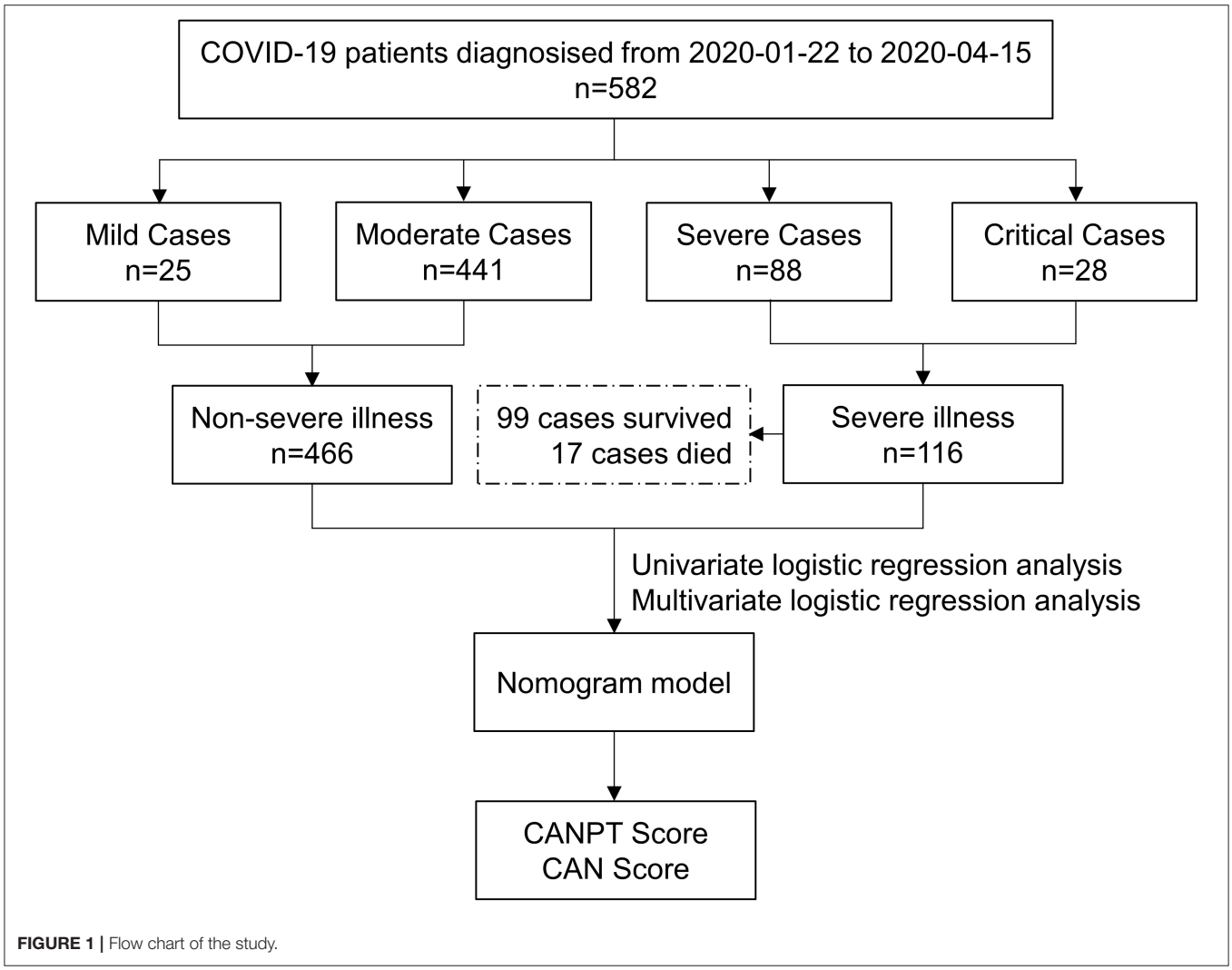

FIGURE 1 | Flow chart of the study.

TABLE 1 | Baseline demographics and characteristics of the COVID-19 patients.

Characteristics	All patients $n = 582$	Severe disease $n = 116$	Non-severe disease $n = 466$	$t/z/\chi^2$ value	*P*-value
Sex (male)	286 (49.1%)	62 (53.5%)	224 (48.1%)	1.08	0.300
Age, years	50.0 (36.0, 63.0)	63.0 (50.0, 71.0)	47.0 (34.0, 57.0)	−7.72	<0.001
Heart rate, beats per minute	86.0 (78.0, 97.0)	88.0 (80.0, 101.0)	85.0 (78.0, 96.0)	−1.55	0.121
Respiratory rate, breaths per minute	20.0 (18.0, 20.0)	20.0 (18.0, 21.0)	20.0 (18.0, 20.0)	−2.16	0.033
MAP, mm Hg	93.0 (87.0, 102.0)	93.0 (87.0, 102.0)	93.0 (87.0, 102.0)	0.84	0.402
Comorbidities	184 (31.6%)	69 (59.5%)	115 (24.7%)	52.04	<0.001
Hypertension	117 (20.1%)	43 (37.1%)	74 (15.9%)	25.96	<0.001
Diabetes	54 (9.3%)	24 (20.7%)	30 (6.4%)	22.41	<0.001
Malignant tumor	13 (2.2%)	7 (6.0%)	6 (1.3%)	9.58	0.002
Clinical symptoms					
Fever	431 (74.1%)	93 (80.2%)	338 (72.5%)	2.82	0.093
Highest temperature ≥38.5°C	152 (26.1%)	48 (41.4%)	104 (22.3%)	17.49	<0.001

MAP, mean arterial pressure.

TABLE 2 | Baseline blood and biochemical indices of the COVID-19 patients at baseline.

Biochemical indexes	Abnormal standard	All patients $n = 582$	Severe disease $n = 116$	Non-severe disease $n = 466$	$t/z/\chi^2$ value	*P*-value
Routine blood tests						
White blood cell count, $\times 10^9$/L	≥9.5	28 (4.81%)	13 (11.21%)	15 (3.22%)	12.941	<0.001
Neutrophil count, $\times 10^9$/L	≥6.3	63 (10.82%)	27 (23.28%)	36 (7.73%)	23.268	<0.001
Lymphocyte count, $\times 10^9$/L	≤1.1	300 (51.55%)	89 (76.72%)	214 (45.92%)	35.307	<0.001
NLR		2.86 (2.00, 4.58)	4.20 (2.50, 8.32)	2.64 (1.86, 4.18)	−6.148	<0.001
Hemoglobin, g/L	≤110	51 (8.76%)	17 (14.66%)	34 (7.30%)	6.291	0.012
Platelet count, $\times 10^9$/L	≤125	97 (16.67%)	34 (29.31%)	63 (13.52%)	16.675	<0.001
Liver function						
Alanine aminotransferase, U/L	≥40	101 (17.35%)	24 (20.69%)	77 (16.52%)	1.124	0.289
Aspartate aminotransferase, U/L	≥40	93 (15.98%)	33 (28.45%)	60 (12.88%)	16.777	<0.001
γ-glutamyl transpeptidase, U/L	≥50	89 (16.79%)	24 (26.97%)	65 (14.74%)	7.924	0.005
Alkaline phosphatase, U/L	≥100	101 (20.74%)	23 (28.05%)	78 (19.26%)	3.205	0.073
Albumin, g/L	≤40	258 (44.33%)	83 (71.55%)	175 (37.55%)	43.502	<0.001
Total bilirubin, μmol/L	≥21	62 (10.67%)	16 (13.91%)	46 (9.87%)	1.581	0.209
Cut-off	≥11	346 (59.45%)	79 (68.10%)	267 (57.30%)	4.500	0.034
Renal function						
Blood urea nitrogen, mmol/L	≥7.6	36 (6.02%)	23 (20.00%)	16 (3.43%)	40.426	<0.001
Creatinine, μmol/L	≥104	53 (9.11%)	26 (22.41%)	27 (5.79%)	30.995	<0.001
Cut-off	≥85	153 (26.29%)	51 (43.97%)	102 (21.89%)	23.362	<0.001
Myocardium						
Creatine kinase, U/L	≥171	82 (14.14%)	30 (25.86%)	52 (11.21%)	16.419	<0.001
Cut-off	≥104	194 (33.33%)	56 (48.28%)	138 (29.61%)	14.556	<0.001
Creatine kinase-MB, U/L	≥25	13 (2.74%)	4 (3.96%)	9 (2.41%)	0.714	0.398
Lactate dehydrogenase, U/L	≥243	165 (34.96%)	56 (55.45%)	109 (29.38%)	23.722	<0.001
Coagulation function						
Prothrombin time, s	≥13	65 (16.09%)	19 (20.88%)	46 (14.70%)	1.996	0.158
Activated partial thromboplastin time, s	≥36.5	68 (16.83%)	24 (26.37%)	44 (14.06%)	7.64	0.006
International normalized ratio	≥1.5	1 (0.20%)	1 (0.94%)	0 (0.00%)	3.157	0.076
D-dimer, mg/L	≥0.25	295 (66.74%)	77 (89.53%)	218 (61.24%)	24.99	<0.001
Inflammatory indexes						
Procalcitonin, ug/L	≥0.5	5 (1.08%)	4 (4.30%)	1 (0.27%)	8.425	<0.001
C-reactive protein, mg/L	≥5	419 (72.62%)	105 (92.11%)	314 (67.82%)	27.135	<0.001
Erythrocyte sedimentation rate, mm/1 h	≥15	244 (56.74%)	54 (66.67%)	190 (54.44%)	4.003	0.045

Missing data of baseline blood and biochemical indices: variables with missing values exceeding 10% include: procalcitonin (118 cases), erythrocyte sedimentation rate (152 cases), alkaline phosphatase (95 cases), creatine kinase-MB (108 cases), lactate dehydrogenase (110 cases), prothrombin time and activated partial thromboplastin time (178 cases), and D-dimer (140 cases).
NLR, neutrophil-to-lymphocyte ratio.

(Alb) level was more common in the patients with severe disease than those with non-severe disease (71.55 *vs.* 37.55%, $P < 0.001$). There were larger proportions of patients with abnormal levels of blood urea nitrogen (BUN), creatinine (Cr), creatine kinase (CK), LDH, D-dimer, PCT, and CRP; activated partial thromboplastin time (APTT); and erythrocyte sedimentation rate (ESR) in the group with severe disease than in the group with non-severe disease (**Table 2**).

Independent Risk Factors for Severe COVID-19

According to univariate logistic regression analysis, 24 variables, including age, comorbidities, fever, and ln (CRP), were associated with the severity of COVID-19 and included in the multivariate logistic regression analysis (forward likelihood method). A body temperature ≥38.5°C, ln (NLR), ln (PLT), ln (Alb), ln (Tbil), ln (Cr), and ln (CK) were independent risk factors for severe COVID-19. The risk of severe illness in the patients with a body temperature ≥38.5°C was 2.37 (95% CI, 1.39, 4.03) times that in the patients with a body temperature <38.5°C. Comorbidities and high values of ln (NLR), ln (Tbil), ln (Cr), and ln (CK), and low values of ln (PLT) and ln (Alb) were associated with an increased risk of severe COVID-19 (**Table 3**).

Predictive Nomogram for Severe COVID-19

Based on the aforementioned eight variables, a nomogram model was established to predict the risk of severe COVID-19 (**Figure 2**) with a prediction accuracy of 85.9%, a leave-one-out cross

TABLE 3 | Risk factors for severe COVID-19 and mortality.

Variable	Crude OR (95% CI)	Adjusted OR (95% CI)	*P*-value (Wald's test)	*P*-value (LR-test)
Risk factors for severe COVID-19				
Comorbidity	4.48 (2.93, 6.86)	2.69 (1.61, 4.49)	<0.001	<0.001
Temperature ≥38.5°C	2.46 (1.6, 3.77)	2.37 (1.39, 4.03)	0.002	0.002
ln (NLR)	2.75 (2.04, 3.71)	1.64 (1.13, 2.37)	0.009	0.008
ln (PLT)	0.27 (0.15, 0.48)	0.45 (0.23, 0.9)	0.024	0.023
ln (Alb)	0.00 (0.00, 0.00)	0.00 (0.00, 0.02)	<0.001	<0.001
ln (Tbil)	2.04 (1.29, 3.21)	1.92 (1.04, 3.52)	0.036	0.032
ln (Cr)	3.54 (1.96, 6.4)	2.68 (1.39, 5.17)	0.003	0.001
ln (CK)	1.86 (1.4, 2.48)	1.44 (1.03, 2.02)	0.034	0.034
Risk factors for mortality				
ln (NLR)	3.78 (2.17, 6.60)	2.67 (1.27, 5.62)	<0.001	0.01
ln (Alb)	0.01 (0.00, 0.01)	0.01 (0.00, 0.05)	<0.001	<0.001
ln (Cr)	7.28 (3.06, 17.31)	7.23 (2.89, 18.10)	<0.001	<0.001
ln (CK)	3.29 (1.93, 5.61)	3.00 (1.60, 5.61)	<0.001	<0.001

NLR, neutrophil-to-lymphocyte ratio; PLT, platelet; Alb, albumin; Tbil, total bilirubin; Cr, creatinine; CK, creatine kinase; ln, natural logarithm.

validation accuracy of 81.6%, an AUROC of 0.858 (95% CI, 0.823–0.893), and a Hosmer-Lemeshow test *P*-value of 0.237.

CANPT Score: A Novel Scoring Model for the Prediction of the Risk of Severe COVID-19

For convenience in clinical use, a novel scoring model was constructed based on the nomogram model and named the CANPT score, with scores ranging from 8 to 20 (**Table 4**). The AUROC of the CANPT score was 0.841 (95% CI, 0.804, 0.879), with a positive predictive value of 35.49% (95% CI, 30.23%, 41.13%), and a negative predictive value of 95.85% (95% CI, 92.81%, 97.68%) when 12 was used as the first cut-off value, and a positive predictive value of 69.64% (95% CI, 56.6%, 80.16%) and a negative predictive value of 85.36% (95% CI, 82.07%, 88.14%) when 16 was used as the second cut-off value (**Table 5**). In this study, 12/289 patients with a CANPT score <12 developed severe disease; 65/237 patients with a CANPT score ≥12 and <16 developed severe disease; and 39/56 patients with a CANPT score ≥16 developed severe disease. The actual incidence of severe disease in the COVID-19 patients with CANPT scores <12, ≥12 and <16, and ≥16 were 4.15, 27.43, and 69.64%, respectively. Thus, with cut-off values of 12 and 16, COVID-19 patients could be classified into low-risk, medium-risk, and high-risk groups with corresponding risks of developing severe COVID-19 of <5, 30, and 70%, respectively.

In this study, the CALL scores were calculated for 472 COVID-19 patients who had measurements of serum LDH levels on admission, and the predictive efficacies of the CALL score and the NLR were verified among these patients and compared with the efficacy of the CANPT score. The results showed that the AUROCs of the CANPT score, CALL score, and NLR were 0.835 (95% CI, 0.794, 0.876), 0.795 (95% CI, 0.747, 0.844), and 0.669 (95% CI, 0.607, 0.730), respectively. The predictive performance of the CANPT score was better than that of the CALL score and NLR (**Figure 3**).

Model for the Prediction of the Risk of Mortality in COVID-19 Patients

Multivariate logistic regression analysis showed that the NLR and the levels of Alb, Cr, and CK were independent risk factors for mortality in patients with COVID-19 (**Table 3**). The CAN score was established to predict the risk of mortality in patients with COVID-19. The CAN score ranged from 4 to 19 (**Table 4**), with a prediction accuracy of 97.3%, a leave-one-out cross validation accuracy of 96.2%, an AUROC of 0.903 (95% CI, 0.832, 0.974), and a Hosmer-Lemeshow test *P*-value of 0.173. The cut-off value was determined by maximizing the Youden index at 15 points, with a sensitivity of 76.47% (95% CI, 52.23%, 90.95%), a specificity of 93.63% (95% CI, 91.28%, 95.38%), a positive predictive value of 26.53% (95% CI, 16.10%, 40.37%), a negative predictive value of 99.25% (95% CI, 98.01%, 99.78%), a positive likelihood ratio of 12.00 (95% CI, 11.12, 12.95), and a negative likelihood ratio of 0.25 (95% CI, 0.21, 0.29). In this study, 49/582 patients with COVID-19 had a CAN score ≥15; of these patients, 13 died. The actual mortality rates were 26.53% in the patients with a CAN score ≥15 and only 0.75% in those with a CAN score <15. Among the patients with severe disease, the actual mortality rate was 43.33% in the patients with a CAN score ≥15.

DISCUSSION

We enrolled 582 COVID-19 pneumonia patients in this study from four hospitals in three provinces, 74.57% of whom were from Hubei Province, and 25.43% of whom were from outside Hubei Province; thus, we reduced patient selection bias. Previous studies have shown that COVID-19 is a systemic disease with damage occurring not only in the lungs but also in many other systems, including the circulatory, cardiovascular, renal, gastrointestinal, endocrine, nervous, and integumentary systems (23, 24). In this study, some patients with COVID-19 had increased WBC and NE counts; increased ESRs; increased APTTs; increased levels of D-dimer, PCT, CRP, AST, GGT, BUN, Cr, CK, and LDH; decreased levels of HGB and Alb; and decreased PLT counts, further confirming the presence of multisystem damage in COVID-19 patients. Moreover, the incidence and degree of abnormalities in the above indicators in the patients with severe disease were significantly higher than those in the patients with non-severe disease, and the number of damaged systems and degree of damage were related to the severity of COVID-19. In this study, comorbidities, a body temperature ≥38.5°C, ln (NLR), ln (PLT), ln (Alb), ln (Tbil), ln (Cr), and ln (CK) were found to be independent risk factors for severe COVID-19, and the CANPT score comprehensively reflected the presence and degree of damage to the immune system, circulatory system, liver, kidneys, and heart in the patients with COVID-19, thereby accurately predicting the risk of severe disease.

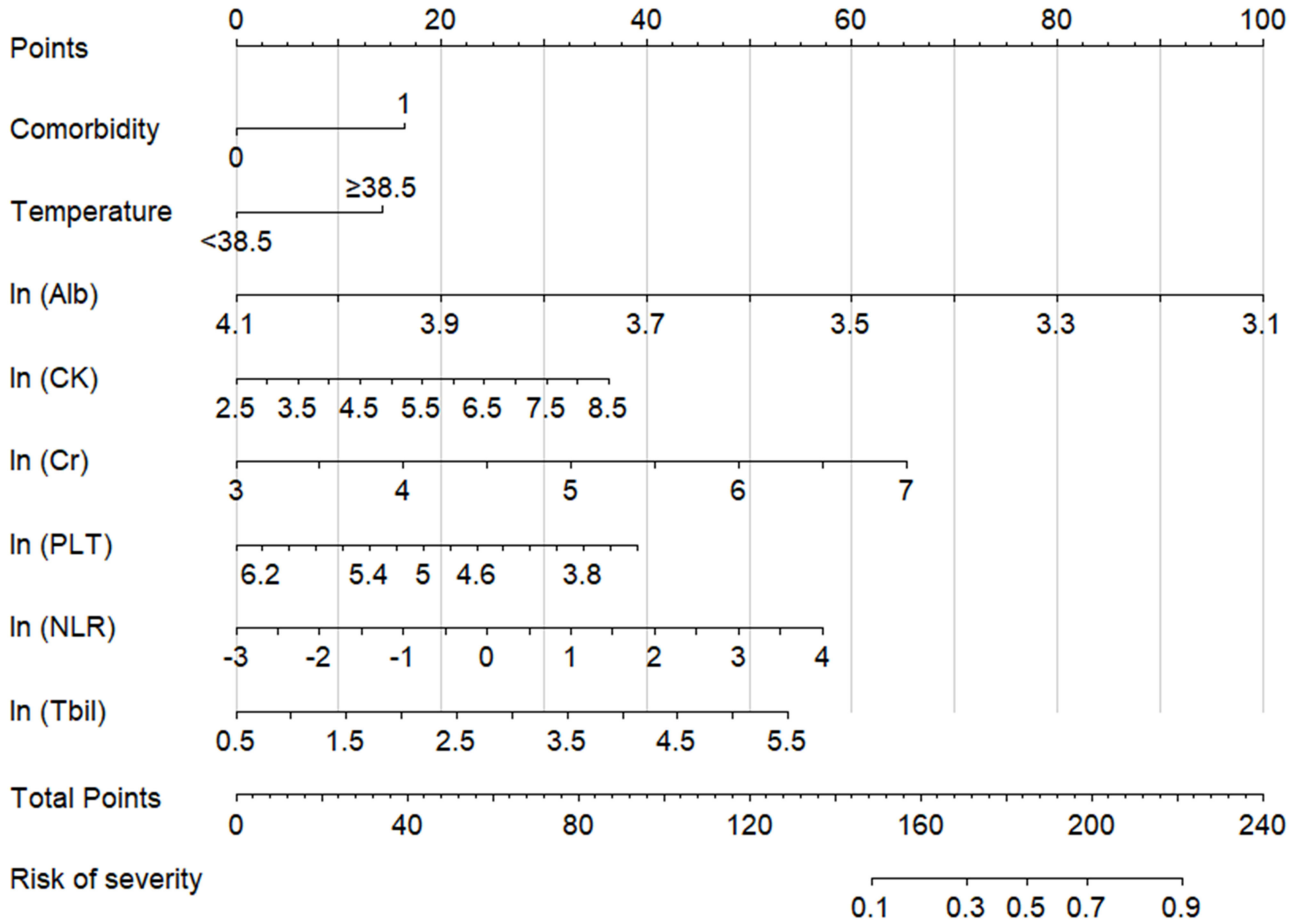

FIGURE 2 | Nomogram model for assessing the risk of severe COVID-19. Total points are calculated by adding each point of the factors, such as comorbidities, temperature, ln (Alb), ln (CK), ln (Cr), ln (PLT), ln (NLR), and ln (Tbil), and the severity risk stratum of a patient can be obtained by projecting the total points downward. The higher that the score is, the higher that the probability of severe disease is. Regarding comorbidities, "0" indicates absent, and "1" indicates present. NLR, neutrophil-to-lymphocyte ratio; PLT, platelet; Alb, albumin; Tbil, total bilirubin; Cr, creatinine; CK, creatine kinase.

Current studies have confirmed that patients with severe COVID-19 develop SARS-CoV-2-related cytokine storms and systemic inflammatory response syndrome (SIRS) (27–29). SIRS often leads to dysfunction in the lungs, kidneys, liver, heart, etc., and even multiple organ failure syndrome (MOFS). Angiotensin converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2) are the receptors by which SARS-CoV-2 invades cells. In addition to the respiratory system, organs and tissues, such as the kidneys, heart, and bile duct epithelium, express ACE2/TMPRSS2 and therefore are potential target organs of SARS-CoV-2 that can be directly damaged (30, 31). Acute kidney injury (AKI) and myocardial injury have been observed in patients with critical COVID-19 (32–34). Therefore, organs, such as the kidneys, liver, and heart, can be affected by both direct damage from SARS-CoV-2 and indirect damage mediated by SIRS. Indicators of the function of these organs, such as Alb, Tbil, and Cr, were given relatively greater weight in the CANPT score to reflect that the degree of organ dysfunction plays an important role in the progression and severity of COVID-19.

Previous studies have shown that AKI is a common complication in patients with COVID-19 and that patients with kidney disease have a significantly higher risk of in-hospital mortality (35). Kidney biopsies from 17 patients with COVID-19 complicated with kidney injury did not show SARS-CoV-2 in the kidney tissue, suggesting that kidney injury in COVID-19 patients is mainly caused by SARS-CoV-2-associated SIRS, rather than direct renal damage caused by SARS-CoV-2 (36). A previous study found that the level of Cr is an independent risk factor for severe COVID-19; thus, Cr was given relatively greater weight in the CANPT score. Therefore, CANPT score could accurately reflect the extent of renal damage in COVID-19 patients early on admission.

Recent studies have shown that almost all hospitalized patients with COVID-19 have elevated levels of serum CK and LDH (28, 37, 38). Autopsies of patients who died of COVID-19 showed cardiomyocyte necrosis and monocyte infiltration (26). Persistently elevated CK indicates the occurrence and progression of myocardial injury in patients with COVID-19.

TABLE 4 | Calculation of the CANPT and CAN scores.

Variable	Adjusted OR (95% CI)	*P*-value (Wald's test)	Points
CANPT score			
Comorbidities			
Present	3.24 (1.98, 5.29)	<0.001	3
Absent			1
Highest temperature			
≥38.5°C	2.18 (1.30, 3.65)	0.003	2
<38.5°C			1
NLR			
≥3.7	1.90 (1.17, 3.11)	0.01	2
<3.7			1
PLT			
≥155			1
<155	1.95 (1.19, 3.18)	0.008	2
Alb			
≥38			1
<38	4.14 (2.49, 6.88)	<0.001	4
Tbil			
≥11	1.37 (0.82, 2.28)	0.229	2
<11			1
Cr			
≥85	3.13 (1.86, 5.29)	<0.001	3
<85			1
CK			
≥104	1.69 (1.03, 2.76)	0.036	2
<104			1
CAN score			
NLR			
≥7	4.92 (1.51, 16.10)	0.008	5
<7			1
Alb			
≥38			1
<38	2.50 (1.47, 4.24)	0.001	2
Cr			
≥80	8.62 (2.24, 33.24)	0.002	8
<80			1
CK			
≥106	4.34 (1.32, 14.28)	0.016	4
<106			1

NLR, neutrophil-to-lymphocyte ratio; PLT, platelet; Alb, albumin; Tbil, total bilirubin; Cr, creatinine; CK, creatine kinase.

TABLE 5 | Accuracy of the CANPT score in estimating the risk of disease progression.

Variable	Enrolled patients ($n = 582$)
AUROC	0.841 (0.804, 0.879)
Cut-off value (95% CI)	12
Sensitivity, %	89.66 (82.65, 94.12)
Specificity, %	59.44 (54.92, 63.81)
Positive predictive value, %	35.49 (30.23, 41.13)
Negative predictive value, %	95.85 (92.81, 97.68)
Positive likelihood ratio	2.21 (2.12, 2.31)
Negative likelihood ratio	0.17 (0.16, 0.19)
Cut-off value (95% CI)	16
Sensitivity, %	33.62 (25.66, 42.64)
Specificity, %	96.35 (94.2, 97.75)
Positive predictive value, %	69.64 (56.6, 80.16)
Negative predictive value, %	85.36 (82.07, 88.14)
Positive likelihood ratio	9.22 (8.69, 9.78)
Negative likelihood ratio	0.689 (0.671, 0.706)

AUROC, area under the receiver operating characteristic curve; CI, confidence interval.

CK is a prognostic marker for severe COVID-19, and the cut-off value is less than the upper limit of normal (ULN) (39). In this study, elevated CK levels were found in 14.14% of the patients with COVID-19 and was more common in patients with severe disease than those with non-severe disease (25.86 *vs.* 11.21%, $P < 0.001$). CK was given a weight of 2 in the CANPT score, reflecting myocardial damage in patients with COVID-19.

The activation of the coagulation system is very common in inflammatory and anti-inflammatory reactions and readily leads to diffuse intravascular coagulation (DIC), which plays an important role in the occurrence and development of organ damage (40). Abnormal coagulation function can be observed in patients with COVID-19, and almost all patients with severe COVID-19 have coagulation disorders. Several studies have shown that abnormal coagulation parameters are closely related to a poor prognosis of COVID-19 (41–46), especially D-dimer, which was not included in the modeling analysis due to the missing value of D-dimer being >10%. Previous studies have reported that the PLT count can be used as a marker of the progression of COVID-19 (47–51). In this study, a reduced PLT count was a risk factor for severe COVID-19, consistent with the results of previous studies. On the one hand, the reduced PLT count in patients with COVID-19 is due to the massive consumption of PLTs in the DIC process; autopsies of patients who died from COVID-19 showed that microthrombi formed in the pulmonary capillaries (26). On the other hand, SARS-CoV-2 can also directly infect bone marrow components, causing hematopoietic abnormalities or triggering an autoimmune response to blood cells (52, 53), further leading to a reduction in the PLT count. Therefore, the decrease in the PLT count reflects abnormal coagulation function, even DIC, in patients with COVID-19.

The presence of comorbidities is also an independent risk factor for severe COVID-19. Patients with hypertension, diabetes, structural lung disease, chronic kidney disease, etc., are more likely to develop ARDS and multiple organ dysfunction syndrome (MODS) in response to SIRS because of preexisting organ structural abnormalities and/or dysfunction (54–57). Due to the high levels of ACE2 expression on the surface, vascular endothelial cells may suffer from direct damage by SARS-CoV-2 and indirect damage due to SARS-CoV-2-associated SIRS. Patients with preexisting vascular endothelial cell damage due to diabetes, hypertension, or chronic kidney disease are predisposed to experiencing more damage to vascular endothelial cells by SARS-CoV-2, which could play a key role in the development of MOFS and severe disease (58).

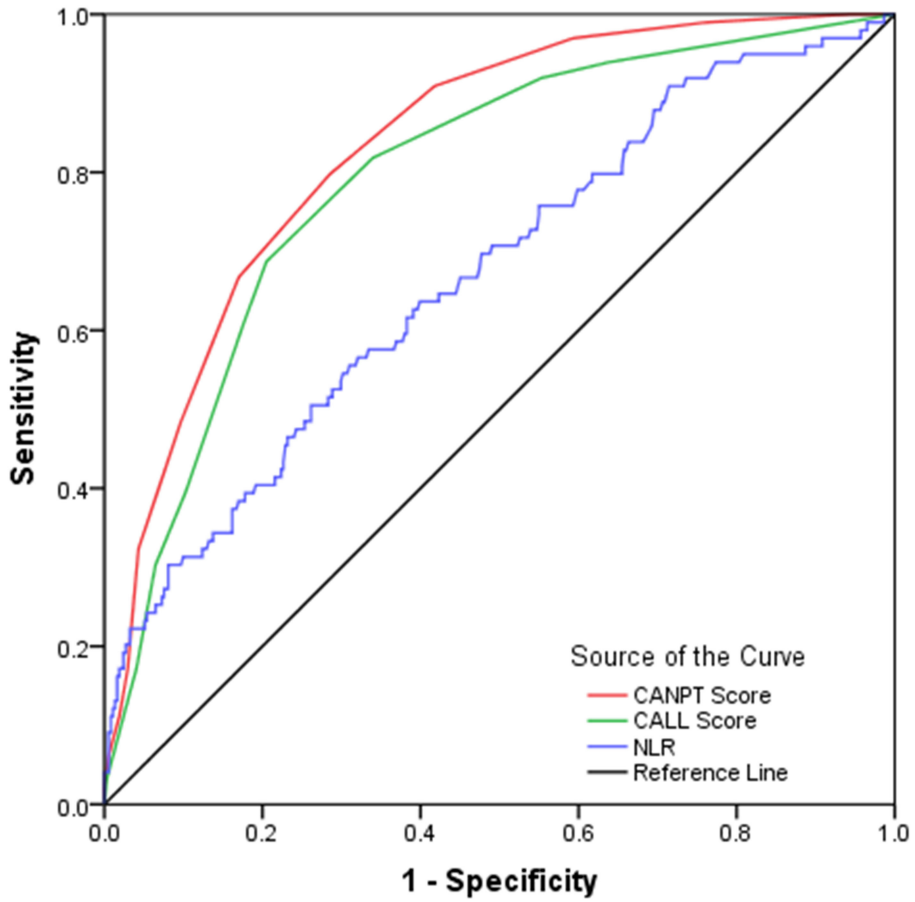

FIGURE 3 | ROC curves of the CANPT score, CALL score, and the NLR. The area under the receiver operating characteristic curves of the CANPT score, CALL score, and the NLR were 0.835 (95% CI, 0.794, 0.876), 0.795 (95% CI, 0.747, 0.844), and 0.669 (95% CI, 0.607, 0.730), respectively. ROC, receiver operating characteristic curve; CI, confidence interval; NLR, neutrophil-to-lymphocyte ratio.

In this study, a high NLR was a risk factor for severe disease in patients with COVID-19, consistent with the results of previous studies (10, 21, 59). The elevation in the NLR is related to immune disorders in patients with COVID-19. The invasion of SARS-CoV-2 triggers cytokine storms and SIRS in the body, resulting in an increased NE count. The LY count was found to be significantly reduced in peripheral blood from patients with COVID-19 due to recruitment and translocation in the local inflammatory system, which is especially pronounced in patients with severe disease (60). Another study found that SARS-CoV-2 could promote T lymphocyte apoptosis by activating the STAT1/IRF3 pathway (61). Thus, an elevated NE count and a reduced LY count in patients with COVID-19 lead to an increase in the NLR, reflecting the degree of the immune response and SIRS in patients with COVID-19.

Fever is a common symptom in patients with COVID-19. The immune response to SARS-CoV2 leads to systemic inflammation and even SIRS, with the consequent release of endogenous pyrogens, and the severity of fever represents the severity of SIRS (62). In this study, a body temperature $\geq$38.5°C was found to be more common in the group with severe disease than in the group with non-severe disease and was an independent risk factor for severe COVID-19.

ARDS is a clinical characteristic of severe COVID-19 and occurs in more than 71.2% of patients with severe COVID-19 in the ICU (63). The Murray score is used to evaluate the severity of acute lung injury and the risk of ARDS. The higher the Murray score, the more severe the acute lung injury, and the higher the risk of ARDS (64). Previous studies have shown that the Alb level in patients with COVID-19 is negatively correlated with the SARS-CoV-2 load and Murray score, and the higher the SARS-CoV-2 load in patients, the more critical the patient's condition (65). Recent studies have found that a low Alb level is an independent risk factor for disease progression in patients with COVID-19 (51, 66–68). In this study, a low Alb level was an important predictor of severe disease in patients with COVID-19 and was given a weight of 4 in the CANPT score, further validating previous research.

There have been reports of severe liver damage in patients with severe COVID-19 (69–71). Chai et al. suggested that liver injury in patients with COVID-19 could be caused by SARS-CoV-2-mediated injury in bile duct cells (72). However,

SARS-CoV-2 has not been found in bile duct cells from patients who died of COVID-19, suggesting that liver damage in patients with COVID-19 is mediated by the SARS-CoV-2-associated cytokine storm and SIRS (73). Elevated ALT and AST levels suggest liver cell damage, while an elevated Tbil level indicates hepatocyte necrosis after the exclusion of bile duct obstruction or hemolysis. Several studies have found that an elevated Tbil level is significantly correlated with adverse outcomes of COVID-19 (69, 70, 74, 75); Tbil, which was given a weight of 2 in the CANPT score, reflects the severity of liver injury in patients with COVID-19.

Interestingly, the cut-off values of CK, Cr, and Tbil determined in this study were markedly lower than their ULNs. The rationale for CK, Cr, and Tbil levels being elevated is high risk for severe COVID-19, but the cut-off chosen for the CANPT score is lower than the ULN can be explained for the following reasons: (1) patients with higher values of CK, Cr, and Tbil than the cut-off levels could at high risk for according organ damage; and (2) higher values of CK, Cr, and Tbil may be resulted from an increase from much lower baseline levels due to organ injury; i.e., a significant increase in levels of CK, Cr, and Tbil could be important indicators of organ injury. The report from Qin et al. showed similar results in COVID-19 patients. In this study, cut-off values for high-sensitivity cardiac troponin I (hs-cTnI), creatine phosphokinase-MB (CK-MB), CK, and myoglobin (MYO) equivalent to ∼49% ULN and a cut-off value of N-terminal pro-brain natriuretic peptide (NT-proBNP) equivalent of ∼18.9% ULN were established for the prediction of adverse outcomes in COVID-19 patients, and patients with higher hs-cTnI, CK-MB, CK, MYO, and (NT-pro) BNP levels than the cut-off values were correlated with increased risk of death (39). Therefore, COVID-19 patients with CK, Cr, and Tbil levels higher than the cut-off values determined in this study, although within normal ranges, would still be at high risk for adverse outcomes and require more attention. Further, CANPT/CAN scoring could be helpful in identifying COVID-19 patients who are at high risk for severe disease.

In this study, <5% of the COVID-19 patients with a CANPT score <12 developed severe disease. Thus, the patients with a CANPT score <12 were considered low-risk patients, and the recommendation is to place these low-risk patients in a mobile cabin hospital or have them isolate at home with general symptomatic treatment with oral medication. In total, 27.43% of the patients with a CANPT score ≥12 and <16 developed severe disease and were considered at intermediate risk; therefore, people with a score within this range should be admitted to an isolation ward for respiratory monitoring and receive antiviral, anti-inflammatory, and symptomatic treatment. Nearly 70% of the patients with a CANPT score ≥16 developed severe disease. These patients should be considered at a high risk and should be transferred to an isolation ICU to receive comprehensive antiviral and symptomatic supportive treatment and respiratory support.

When the CAN score was used to predict the risk of mortality, only 0.75% of the patients with a CAN score <15 died, while among those with a CAN score ≥15, 26.53% of all patients, and 43.33% of the patients with severe disease eventually died. Thus, the CAN score could be used to identify patients who are at a high risk for mortality. Patients with a CAN score <15 are relatively safe, while those with a CAN score ≥15 are at a high risk for mortality regardless of whether they have severe disease and should be treated in the ICU.

The CALL score, which considers comorbidities, the LY count, age, and the LDH level, has been reported to have good predictive efficacy and is convenient for use in clinical practice (20). Studies have also reported that the NLR is an independent predictor of poor prognosis in patients with COVID-19 (21, 22). In this study, the CANPT score was compared with the CALL score and the NLR. The predictive performance of the CANPT score was significantly superior to that of the CALL score and the NLR with a larger AUROC.

The use of comorbidities and routine indicators, including body temperature, the NLR, the PLT count, and the levels of Alb, Tbil, Cr, and CK, renders the CANPT and CAN scores easy to calculate, and these scores are efficient in predicting the risk of severe illness and death in patients with COVID-19. These scores could be used to help clinicians to identify patients at high risk for poor outcomes or mortality soon after admission. Providing intensive care to the small proportions of patients who at high risk could improve their outcomes and reduce the mortality rate, and the rational allocation of limited medical staff and equipment could alleviate the serious shortages of medical resources. There were some limitations of this study. First, the practice for identification of severe and critically ill COVID-19 might differ in different hospitals, which could bias the results of the analysis. Second, this study only included Chinese patients; thus, the performance of the CANPT score and CAN score in patients of other ethnicities must be validated. Third, only 17 of the 582 COVID-19 patients included in this study died; therefore, the CAN model might not be sufficiently accurate to predict the risk of death in COVID-19 patients. Finally, this study was a retrospective study with a sample size of 582 without external validation, and the CANPT and CAN scores must be further validated in a large sample of patients from different regions.

The CANPT and CAN scores can be used to identify patients with COVID-19 who are at a high risk for severe disease or death soon after admission, guiding patient management and the rational allocation of limited medical resources based on patient risk stratification.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: Clinical data were retrieved from the medical records databases of Shiyan Taihe Hospital, Ankang Central Hospital, Ningbo Hwamei Hospital, and Yichang Central People's Hospital. Requests to access these datasets should be directed to Yuanyuan Chen, cyy15871089714@163.com.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Shiyan Taihe Hospital. The approval number is 2020KS018. Written

informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

YC and ZM designed and coordinated the research, contributed to the statistical analysis, and interpretation and the writing of the manuscript. ZM reviewed and edited the manuscript. JZ guided the data analysis. YC, HY, XZ, HH, ZJ, and SL collected the data. All authors contributed to and approved the submitted version of this manuscript.

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Recent Advances in the Evaluation of Serological Assays for the Diagnosis of SARS-CoV-2 Infection and COVID-19

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Introduction: Few data on the diagnostic performance of serological tests for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection are currently available. We evaluated sensitivity and specificity of five different widely used commercial serological assays for the detection of SARS-CoV-2–specific IgG, IgM, and IgA antibodies using reverse transcriptase-PCR assay in nasopharyngeal swab as reference standard test.

Methods: A total of 337 plasma samples collected in the period April–June 2020 from SARS-CoV-2 RT-PCR positive (n = 207) and negative (n = 130) subjects were investigated by one point-of-care lateral flow immunochromatographic assay (LFIA IgG and IgM, Technogenetics) and four fully automated assays: two chemiluminescence immunoassays (CLIA-iFlash IgG and IgM, Shenzhen YHLO Biotech and CLIA-LIAISON® XL IgG, DiaSorin), one electrochemiluminescence immunoassay (ECLIA-Elecsys® total predominant IgG, Roche), and one enzyme-linked immunosorbent assay (ELISA IgA, Euroimmune).

Results: The overall sensitivity of all IgG serological assays was >80% and the specificity was >97%. The sensitivity of IgG assays was lower within 2 weeks from the onset of symptoms ranging from 70.8 to 80%. The LFIA and CLIA-iFlash IgM showed an overall low sensitivity of 47.6 and 54.6%, while the specificity was 98.5 and 96.2%, respectively. The ELISA IgA yielded a sensitivity of 84.3% and specificity of 81.7%. However, the ELISA IgA result was indeterminate in 11.7% of cases.

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Conclusions: IgG serological assays seem to be a reliable tool for the retrospective diagnosis of SARS-CoV-2 infection. IgM assays seem to have a low sensitivity and IgA assay is limited by a substantial rate of indeterminate results.

Keywords: SARS-CoV-2 infection, COVID-19, SARS-CoV-2 RT-PCR, SARS-CoV-2-specific antibodies, LFIA, CLIA, ECLIA and ELISA, sensitivity and specificity

INTRODUCTION

Since emerging in late December 2019 in Wuhan, China, the novel coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread rapidly worldwide resulting in a pandemic (1). According to the World Health Organization, as of October 20, 2020, more than 40,000,000 laboratory-confirmed cases and over 1,000,000 deaths have been globally reported (2). Currently, the laboratory confirmation of possible and probable cases of coronavirus disease 2019 (COVID-19) is based on the detection of the viral genome in respiratory tract specimens by nucleic acid amplification tests such as the real-time reverse transcriptase (RT)-PCR assay (3). However, the diagnostic accuracy of the molecular testing may be affected by some factors such as the time window of viral replication, the magnitude of viral load at the site of sample collection, and the quality of sample collection (4). Concerning the serological testing, though clinical utility is currently unclear (5), it is known that validated serological assays have important application areas, for instance for patient contact tracing and for epidemiological studies (6). In this regard, although many serological assays have been rapidly developed and made commercially available during this pandemic, only limited clinical validations considering different groups of subjects such as those who developed an asymptomatic infection or with probable COVID-19 as well as the timing of sample collection in relation to symptoms onset has been currently performed (7). The aim of this study was to evaluate the diagnostic performance of five different widely used commercial serological assays for the detection of SARS-CoV-2–specific IgG, IgM, and IgA antibodies using the US Center for Disease Prevention and Control SARS-CoV-2 real-time RT-PCR in nasopharyngeal swab as reference standard test. A secondary aim was to assess the agreement between different serological assays by class of immunoglobulin detected (IgG or IgM).

MATERIALS AND METHODS

Study Design

This is a retrospective case-control study evaluating the sensitivity and specificity of a point-of-care (POC) lateral flow immunochromatographic assay (LFIA) and four fully automated assays, two chemiluminescence immunoassays (CLIAs), an electrochemiluminescence immunoassay (ECLIA), and an enzyme-linked immunosorbent assay (ELISA), for the detection of SARS-CoV-2–specific IgG, IgM, and IgA antibodies in blood samples.

Study Sample

Residual frozen plasma samples from asymptomatic and symptomatic individuals with positive or negative SARS-CoV-2 RNA nasopharyngeal swab were collected from April to June 2020 during routine serological investigations performed at the Operative Unit of Clinical Microbiology of the IRCCS St. Orsola Polyclinic, University of Bologna, Italy. Asymptomatic subjects underwent molecular testing for SARS-CoV-2 infection given that they met at least one of the two epidemiological criteria for coronavirus disease 2019 of the European Center for Disease Prevention and Control (8). We excluded subjects with a negative SARS-CoV-2 RT-PCR who met clinical and epidemiological or imaging criteria of COVID-19 (probable COVID-19–positive patients) (8). The study was approved by the Ethical Committee of the University of Bologna.

Serological Assays

Blood samples were collected in ethylenediamine tetraacetic acid–anticoagulated tubes and plasma sample leftovers were prospectively stored at −80°C until testing. The tests' procedures and the interpretation of results adopted were reported in the manufacturer instructions for all the assays. The evaluation of serological assays was simultaneous.

Qualitative Detection of SARS-CoV-2 IgG and IgM

The nCOVID-19 IgG and IgM POCT (Technogenetics S.r.l., Milan, Italy) LFIA and the SARS-CoV-2 IgM and IgG CLIA kits (Shenzhen YHLO Biotech Co., Ltd., China) were used; these assays are CE marked. Briefly, the POC test for the rapid detection of both IgG and IgM antibodies in human serum, plasma, and whole blood samples was performed in laboratory by testing plasma samples. The assay had a turnaround time (TAT) of 15 min; results were evaluated independently by two different investigators and faint banding for IgG and/or IgM was considered positive. The assay detects antibodies to the nucleocapsid protein of SARS-CoV-2. The CLIA assays (hereinafter named CLIA-iFlash) were performed on the iFlash3000 CLIA analyzer (Shenzhen YHLO Biotech Co., Ltd., China); these are high-throughput assays with an estimated TAT of 40 min per sample. The amount of SARS-CoV-2 IgM or IgG in the serum/plasma sample is in proportion to the relative light unit (RLU) measured by the CLIA analyzer that automatically calculates the antibody concentration (in arbitrary

Abbreviations: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; COVID-19, coronavirus disease 2019; RT-PCR, reverse transcriptase-PCR; POC, point-of-care; LFIA, lateral flow immunochromatographic assay; CLIA, chemiluminescence immunoassays; ECLIA, electrochemiluminescence immunoassay; ELISA, enzyme-linked immunosorbent assay; POCT, point-of-care test; TAT, turnaround time; RLU, relative light unit; AU, arbitrary units; EUA, emergency use authorization; COI, cut-off index; CI, confidence interval.

units (AU)/ml) on the basis of the RLU and the calibration curve. The cut-off value for reactivity (positivity) is equal to 10.0 AU/mL for both IgG and IgM. The magnetic beads of these assays are coated with recombinant antigens representing SARS-CoV-2 nucleocapsid protein and spike protein.

Quantitative Detection of SARS-CoV-2 IgG

The LIAISON® SARS-CoV-2 S1/S2 IgG CLIA assay (hereinafter named CLIA-LIAISON® XL; DiaSorin S.p.A., Saluggia, Italy) performed on the LIAISON® XL Analyzer (DiaSorin) was used. It is a high-throughput assay with an estimated TAT of 40 min per sample. The assay is CE marked and in late April 2020, received the Food and Drug Administration's Emergency Use Authorization (EUA). Antibody concentration in serum/plasma sample, expressed as AU/ml, was automatically calculated by the analyzer on the basis of the RLU and the calibration curve. The cut-off value for a positive result is equal to 15 AU/ml. The magnetic beads of the assay are coated with recombinant antigens representing the S1 and S2 subunits of the spike protein of SARS-CoV-2. Given the assay's target, potential neutralizing antibodies could be detected. In this regard, some authors showed that this assay provided the detection of neutralizing antibodies with 94.4% positive agreement and 97.8% negative agreement to plaque reduction neutralization test (9).

Qualitative Detection of SARS-CoV-2 Total (Predominantly IgG) Antibodies

The Elecsys® Anti-SARS-CoV-2 ECLIA assay (Roche Diagnostics AG, Rotkreuz, Switzerland) performed on the cobas e 801 analyzer (Roche Diagnostics) was used. This is a high-throughput assay with an estimated TAT of 20 min per sample. The assay is CE marked and at the beginning of May 2020, received the Food and Drug Administration's EUA. Results [in cut-off index (COI)] are determined automatically by the analyzer's software that compares the electrochemiluminescence signal obtained from the reaction product of the serum/plasma sample with the signal of the cut-off value previously obtained by calibration. The cut-off value for reactivity (positivity) is equal to 1.0 COI. The assay uses a recombinant protein representing the nucleocapsid antigen, and its format favors the preferential detection of late, mature, and high affinity antibodies. Therefore, despite that this assay detects all classes of immunoglobulin (IgA, IgM, and IgG), it detects predominantly IgG (10).

Semiquantitative Detection of SARS-CoV-2 IgA

The Anti-SARS-CoV-2 IgA ELISA assay (Euroimmun Medizinische Labordiagnostika, Lübeck, Germany) performed on EUROIMMUN Analyzer I was used. This is a midvolume assay with an estimated TAT of 4 h per 96-well plate; the assay is CE marked. The results are expressed as a ratio between the extinction of the serum/plasma sample, and the calibrator that is automatically calculated by the analyzer. A ratio $\geq$0.8 to $<$1.1 identify an equivocal (indeterminate) result; a ratio $>$1.1 identifies a positive result. The assay uses a recombinant protein representing the S1 subunit of the spike protein of SARS-CoV-2.

Statistical Analysis

Sensitivity and specificity with 95% confidence interval (CI) of each serological assay were calculated using 2 × 2 tables. Sensitivity and specificity are, respectively, the percentage of subjects with positive and negative SARS-CoV-2 RT-PCR correctly identified by serological assay. The accuracy of each test, that is the percentage of individuals for whom both the serological test and reference standard give the same result, was quantified by receiver operating characteristic (ROC) analysis. Plasma samples with indeterminate results by ELISA IgA were excluded from sensitivity and specificity analyses of this test. Sensitivity and specificity of serological assays was also assessed separately in asymptomatic and symptomatic subjects and by the time elapsed from the onset of symptoms and blood collection ($<$14 vs. $>$14 days). We assessed the agreement between serological assays by class of immunoglobulin detected (IgG or IgM) using Kappa statistic. Continuous variables were described using mean and standard deviation (SD). A $p < 0.05$ was considered statistically significant. All statistical analyses were performed using STATA version 15 (StataCorp, College Station, TX, USA).

RESULTS

During the study period, plasma samples from 361 subjects were collected. Of these, 24 symptomatic subjects with a negative SARS-CoV-2 RT-PCR were excluded as they were probable COVID-19–positive patients. A total of 337 subjects [mean age 59.3, SD 23.8; males: 158 (46.9%)], 284 with symptoms and 53 without symptoms, were included in the study. Of these, 207 were RT-PCR positive (188 with symptoms) and 130 RT-PCR negative (96 with symptoms). Of the RT-PCR–positive subjects, one was not tested by the LFIA IgG and IgM and four by ELISA IgA due to insufficient sample volume.

Diagnostic Performance

Of the 202 SARS-CoV-2 RT-PCR–positive subjects who underwent all the serological assays, only 17 (8.4%) resulted negative for IgG, IgM, or IgA.

Table 1 shows the sensitivity and specificity of each serological assay. The overall sensitivity of all IgG serological assays was $>$80% and the specificity was $>$95%. In particular, the overall sensitivity of IgG serological assays ranged from 81.6% (95% CI, 75.7–86.7) with CLIA-LIAISON® XL to 89.9% (95% CI, 84.9–93.6) with CLIA-iFlash, and the specificity from 97.7% (95% CI, 93.4–99.5) with CLIA-LIAISON® XL to 100% (95% CI, 97.2–100) with ECLIA-Elecsys®. The overall sensitivity of IgM serological tests was very low being 47.6% (95% CI, 40.6–54.6) and 54.6% (95% CI, 47.5–61.5) with LFIA and CLIA-iFlash, respectively, while the specificity was 98.5% (95% CI, 94.6–99.8) and 96.2% (95% CI, 91.3–98.7).

As expected, the overall accuracy of IgG serological assays was significantly higher than IgM with both CLIA-iFlash (94.2 vs. 75.4%, $p < 0.0001$) and LFIA (91.6 vs. 73%, $p < 0.0001$) (**Figure 1**). The ELISA IgA had a sensitivity of 84.3% (95% CI, 78.3–89.2) and specificity of 81.7% (95% CI, 73.1–88.4). However, the result of ELISA IgA was indeterminate in 39 out

TABLE 1 | Overall sensitivity and specificity of the serological assays for the diagnosis of SARS-CoV-2 infection using RT-PCR as reference standard.

Serological assays			No. of samples	RT-PCR positive	RT-PCR negative	True positive	False positive	True negative	False negative	Sensitivity % (95% CI)	Specificity % (95% CI)
IgG	LFIA	POCT	336	206	130	173	1	129	33	84.0 (78.2–88.7)	99.2 (95.8–100)
	CLIA	iFlash	337	207	130	186	2	128	21	89.9 (84.9–93.6)	98.5 (94.6–99.8)
		LIAISON® XL	337	207	130	169	3	127	38	81.6 (75.7–86.7)	97.7 (93.4–99.5)
	ECLIA	Elecsys®	337	207	130	179	0	130	28	86.5 (81.0–90.8)	100 (97.2–100)
IgM	LFIA	POCT	336	206	130	98	2	128	108	47.6 (40.6–54.6)	98.5 (94.6–99.8)
	CLIA	iFlash	337	207	130	113	5	125	94	54.6 (47.5–61.5)	96.2 (91.3–98.7)
IgA	ELISA	Euroimmune I	294	185	109	156	20	89	29	84.3 (78.3–89.2)	81.7 (73.1–88.4)

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RT-PCR, reverse transcriptase-PCR; CI, confidence interval; LFIA, lateral flow immunochromatographic assay; POCT, point-of-care test; CLIA, chemiluminescence immunoassays; ECLIA, electrochemiluminescence immunoassay; ELISA, enzyme-linked immunosorbent assay.

of 333 (11.7%) individuals, whose 18 out of 203 (8.9%) had RT-PCR positive and 21 out of 130 (16.1%) had RT-PCR negative. If we consider all indeterminate tests as being false negative (in those with RT-PCR positive) or false positive (in those with RT-PCR negative) (worst-case scenario), the sensitivity of ELISA IgA would drop to 76.8% (156/203) and the specificity to 68.4% (89/130).

Table 2 shows the diagnostic performance of serological assays by presence of symptoms. The sensitivity of all tests was lower in asymptomatic than symptomatic individuals, while the specificity was similar. However, in asymptomatic subjects, all IgG serological assays showed a sensitivity around 80%, a part LFIA that yielded a sensitivity of 68.4%.

Table 3 shows the sensitivity of serological assays stratified by time from the onset of symptoms. The sensitivity of all serological assays was lower in subjects with onset of symptoms within the 14 days from the blood collection than in those where the onset of symptoms was >14 days. In particular, the sensitivity of LFIA, CLIAs, and ECLIA IgG was 73.8 vs. 91.8%, 80.0 vs. 95.9% (CLIA-iFlash), 70.8 vs. 87.8% (CLIA-LIAISON® XL), and 72.3 vs. 95.1%, in subjects with onset of symptoms within and after 14 days from blood collection, respectively.

We found a good agreement between the results of the IgG serological assays with k values ranging from 0.78 (LFIA vs. CLIA-LIAISON® XL) to 0.94 (LFIA vs. ECLIA-Elecsys®), while the agreement was moderate between the IgM assays ($k = 0.57$) (**Table 4**).

Finally, of the 24 patients with RT-PCR negative but considered COVID-19 probable cases, 11 (45.8%) were IgG positive with LFIA, 12 (50%) with CLIA-iFlash, 10 (41.6%) with CLIA-LIAISON® XL, and 11 (45.8%) with ECLIA-Elecsys®, while 6 (25%) and 7 (29.2%) were IgM positive with LFIA and CLIA-iFlash, respectively, and 10 (41.6%) with ELISA IgA.

DISCUSSION

A key aspect for controlling the COVID-19 outbreak is the availability of diagnostic methods that ensure an early and accurate diagnosis of the viral infection (4). To date, few data on serological diagnosis of SARS-CoV-2 infection are currently available (11). In the present study, the diagnostic performances of one point-of-care lateral flow immunochromatographic test and four widely used fully automatic tests for the detection of IgG, IgM, and IgA against SARS-CoV-2 were evaluated by testing plasma samples from subjects with positive and negative SARS-CoV-2 RNA nasopharyngeal swab. The use of a unique and large clinical sample panel to perform the head-to-head comparison of the different serological assays is the strength of our study.

High sensitivities were observed for all four IgG assays, with the CLIA-iFlash resulting to have the highest, with a value equal to 89.9%; the other three assays showed sensitivities not < 80%. Sensitivity stratified by the timing of sample collection in relation to symptoms onset demonstrated that all the IgG assays performed better after 2 weeks from onset of symptoms, with values of sensitivity from 87.8% with CLIA-LIAISON® XL up to 95.9% with CLIA-iFlash. An increase in the IgG-positive rate with time was expected, as IgG are antibodies characteristic of the late stages of infection. Very low values of sensitivity were observed for the two IgM assays that in plasma samples collected after 14 days from the onset of symptoms identified as seropositive approximately half of the RT-PCR SARS-CoV-2 positives. Finally, the sensitivity of ELISA IgA was equal to 84.3% and in symptomatic patients improved overtime ($p < 0.001$), resulting in a sensitivity of 94.6%.

Among the plasma samples from subjects with positive SARS-CoV-2 RNA nasopharyngeal swab about 8% ($n = 17$) was negative by the IgG, IgM, and IgA assays. In particular, three samples were from patients with asymptomatic infection, seven samples were from patients with blood collected during the very early stage of infection (i.e., <7 days after onset symptomatology), and seven plasma samples were from patients with a mean age equal to 83 years. The type of infection, the timing of blood collection, and old age suggest that these patients might have produced low virus-specific antibody levels, not detectable by the serological assays (12–15).

A very good performance in terms of specificity was observed for IgG and IgM assays, with specificities not <97% and equal to 100% by ECLIA-Elecsys® IgG. These findings are in line with those of other studies evaluating SARS-CoV-2 commercial serological methods that reported specificities of IgG and IgM assays ranging from more than 90% up to 100% (14, 16, 17). A lower specificity (81.7%) was observed for ELISA IgA. In addition, according to other authors (18, 19), a significant overall percentage (i.e., 11.7%) of indeterminate results was obtained.

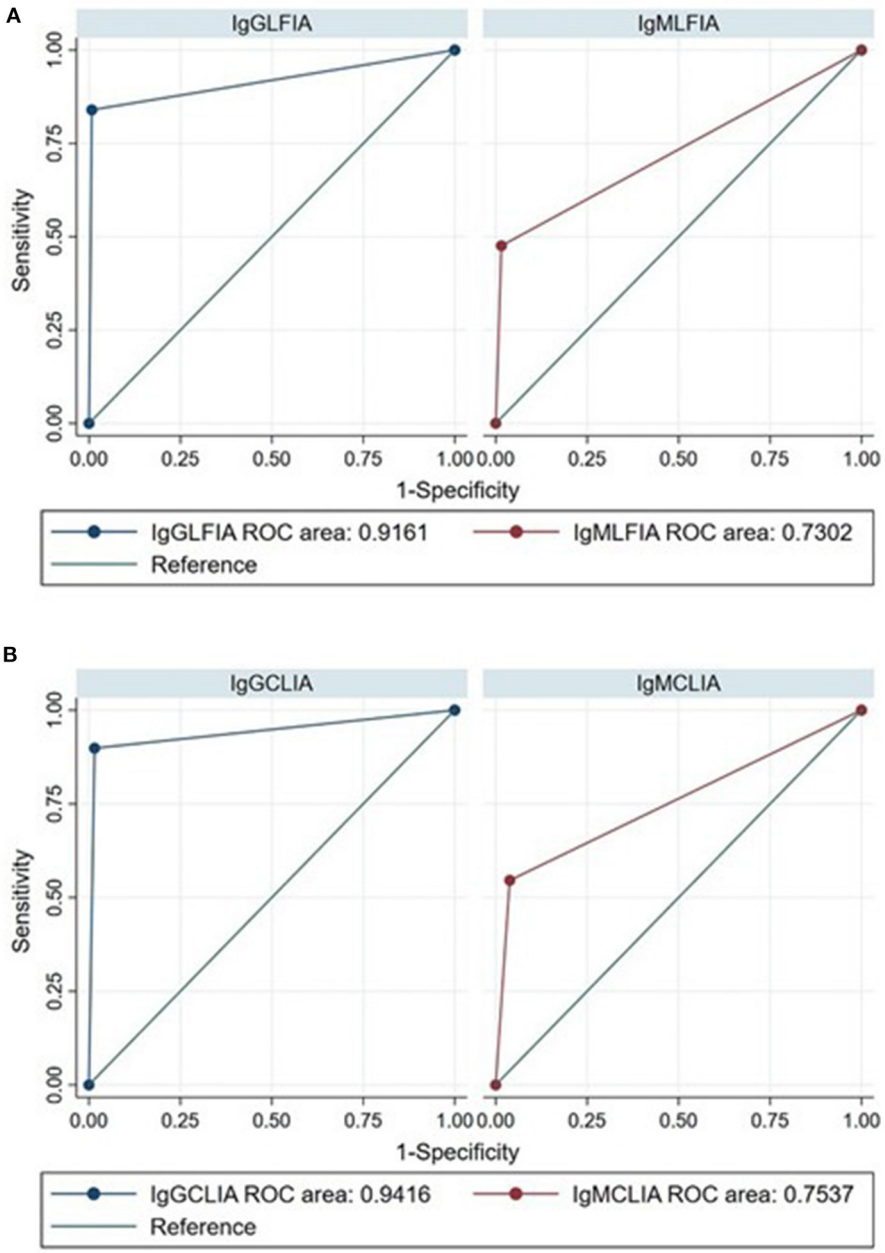

FIGURE 1 | Receiver operating characteristic (ROC) curves for the diagnosis of SARS-CoV-2 infection by IgG and IgM LFIA **(A)** and CLIA-iFlash **(B)** using RT-PCR as reference standard ROC area IgG vs. IgM LFIA, $p < 0.0001$; ROC area IgG vs. IgM CLIA-iFlash, $p < 0.0001$.

The highest indeterminate rate of IgA (i.e., 16.1%) was obtained by testing plasma samples from SARS-CoV-2 RT-PCR–negative cases. Cross-reactivity of ELISA IgA with other respiratory viruses such as influenza A and B and the four common human coronaviruses was reported by some studies (6, 7, 14). We obtained overlapping assays' specificity values by preliminarily investigating a group of 300 archived serum samples collected from healthy blood donors and pregnant women during the pre-pandemic period (i.e., September–October 2019) (data not shown). In this group of samples, the issue of the diagnostic accuracy of the molecular testing in terms of false negatives as well as the possible presence of subjects with a past SARS-CoV-2 infection were overcome.

More variability in sensitivity data was found among studies, particularly for the LFIAs; i.e., sensitivity from 14.4 to 93.1% and from 3 to 69% (95% CI, 60.6–76.3) were reported for the IgG and IgM LFIAs, respectively (14, 20, 21). Sensitivities ranging from 75.4 to 88.9% and equal to 71% were reported for the CLIA IgG and the ECLIA, respectively (14, 16, 17). Sensitivities of 48.1 and 72.1% were reported for the IgM CLIAs (16, 17);

TABLE 2 | Sensitivity and specificity of the serological assays for the diagnosis of SARS-CoV-2 infection in symptomatic and asymptomatic individuals.

Serological assays			No. of samples	RT-PCR positive	RT-PCR negative	True positive	False positive	True negative	False negative	Sensitivity % (95% CI)	Specificity % (95% CI)
Symptomatic individuals											
IgG	LFIA	POCT	283	187	96	160	1	95	27	85.6 (79.7–90.3)	99.0 (94.3–100)
	CLIA	iFlash	284	188	96	170	2	94	18	90.4 (85.3–94.2)	97.9 (92.7–99.7)
		LIAISON® XL	284	188	96	154	2	94	34	81.9 (75.7–87.1)	97.9 (92.7–99.7)
	ECLIA	Elecsys®	284	188	96	164	0	96	24	87.2 (81.6–91.6)	100 (96.2–100)
IgM	LFIA	POCT	283	187	96	94	2	94	93	50.3 (42.9–57.6)	97.9 (92.7–99.7)
	CLIA	iFlash	284	188	96	107	5	91	81	56.9 (49.5–64.1)	94.8 (88.3–98.3)
IgA	ELISA	Euroimmune I	244	168	76	144	19	57	24	85.7 (79.5–90.6)	75.0 (63.7–84.2)
Asymptomatic individuals											
IgG	LFIA	POCT	53	19	34	13	0	34	6	68.4 (43.4–87.4)	100 (89.7–100)
	CLIA	iFlash	53	19	34	16	0	34	3	84.2 (60.4–96.6)	100 (89.7–100)
		LIAISON® XL	53	19	34	15	1	33	4	78.9 (54.4–93.9)	97.1 (84.7–99.9)
	ECLIA	Elecsys®	53	19	34	15	0	34	4	78.9 (54.4–93.9)	100 (89.7–100)
IgM	LFIA	POCT	53	19	34	4	0	34	15	21.1 (6.0–45.6)	100 (89.7–100)
	CLIA	iFlash	53	19	34	6	0	34	13	31.6 (12.6–56.6)	100 (89.7–100)
IgA	ELISA	Euroimmune I	50	17	33	12	1	32	5	70.6 (44.0–89.7)	97.0 (84.2–99.9)

RT-PCR, reverse transcriptase-PCR; CI, confidence interval.

TABLE 3 | Sensitivity of the serological assays for the diagnosis of SARS-CoV-2 infection by onset of symptoms.

Serological assays			Time elapsed from symptoms onset and blood sample collection					
			≤14 days			>14 days		
			No. of RT-PCR positive	True positive	Sensitivity (95% CI)	No. RT-PCR positive	True positive	Sensitivity (95% CI)
IgG	LFIA	POCT	65	48	73.8 (61.5–84.0)	122	112	91.8 (85.4–96.0)
	$CLIA_S$	iFlash	65	52	80 (68.2–88.9)	123	118	95.9 (90.8–98.7)
		LIAISON® XL	65	46	70.8 (58.2–81.4)	123	108	87.8 (80.7–93.0)
	ECLIA	Elecsys®	65	47	72.3 (59.8–82.7)	123	117	95.1 (89.7–98.2)
IgM	LFIA	POCT	65	23	35.4 (23.9–48.2)	122	71	58.2 (48.9–67.1)
	CLIA	iFlash	65	35	53.8 (41–66.3)	123	72	58.5 (49.3–67.3)
IgA	ELISA	EUROIMMUNE I	57	39	68.4 (54.8–80.1)	111	105	94.6 (88.6–98.0)

RT-PCR, reverse transcriptase-PCR; CI, confidence interval.

TABLE 4 | Agreement between serological assays.

Serological assays			% of agreement	Cohen's kappa coefficient
IgG	LFIA-CLIAs	POCT-iFlash	94.9	0.90
		POCT-LIAISON® XL	88.9	0.78
	LFIA-ECLIA	POCT-Elecsys®	97	0.94
	$CLIA_S$	iFlash-LIAISON® XL	92.8	0.85
	$CLIA_S$-ECLIA	iFlash-Elecsys®	96.7	0.93
		LIAISON® XL-Elecsys®	91.4	0.82
IgM	LFIA-CLIA	POCT-iFlash	81.2	0.57

finally, sensitivities ranging from 93.3 to 75% were reported for the IgA ELISA (7, 14, 18). It can be hypothesized that this heterogeneity in sensitivity, in addition to the different assays' targets and the problem of the subjective reading of the band in LFIAs (mainly if faint), could also be due to the characteristics of the study populations selected for estimating the assay's

TABLE 5 | Main characteristics of the studies included in the manuscript.

Study	Study population (number of investigated samples)		Clinical setting	Sample collection (days after symptoms onset)	Serological methods* (antigens)
	Sensitivity assessment	Specificity assessment			
Okba et al. (6)	Confirmed COVID-19 cases ($n = 41$)	Healthy blood donors ($n = 45$); Pts with laboratory-confirmed other virus infection ($n = 150$)	Severe and mild cases	3–27	Commercial and in-house IgG and IgA ELISAs (S, N protein)
Lassaunière et al. (7)	Confirmed COVID-19 cases ($n = 30$)	Healthy individuals before the pandemic ($n = 10$); Pts with laboratory-confirmed other virus infection ($n = 72$)	Inpatients, 100% ICU	7 to >21	Total Ig (S protein), IgG and IgA ELISAs (S protein), IgG-IgM POCTs (not reported)
Charlton et al. (14)	Confirmed COVID-19 cases ($n = 46$)	Healthy individuals before the pandemic ($n = 50$); Pts with laboratory-confirmed other respiratory virus infection ($n = 62$)	Inpatients, 93% (35% ICU); ambulatory, 7%	Mean time: 16; range: 2–48	IgG CMIA (N protein), IgG ECLIA (N protein), IgG CLIA (S1 and S2 domains of S protein), IgG, IgM, and IgA ELISAs (S protein; N protein), IgG-IgM POCTs (not reported; N, S protein)
Infantino et al. (16)	Confirmed COVID-19 cases ($n = 61$)	Healthy individuals before the pandemic ($n = 20$); Pts before the pandemic with rheumatic ($n = 31$) and infectious diseases ($n = 13$)	Inpatients, 100%; 50.8% ICU; 49.2% mild to moderate symptoms	Mean time: 12; range: 8–17	IgG and IgM CLIAs (N, S protein)
Jin et al. (17)	Pts with laboratory-confirmed SARS-CoV-2 ($n = 43$)	Pts with suspected SARS-CoV-2 infection ($n = 33$)	Inpatients, not specified	Median time: 18.0; IQR 11–23	IgG and IgM CLIAs (N, S protein)
Nicol et al. (18)	Pts with laboratory-confirmed SARS-CoV-2 infection ($n = 141$) Pts with probable COVID-19 ($n = 57$)	Pts before the pandemic ($n = 50$); pts with laboratory-confirmed other virus infection ($n = 25$); pregnant women ($n = 10$) and pts with positive rheumatoid factor ($n = 10$)	Majority of pts with symptoms	0 to >15	IgG CLIA (N protein), IgG and IgA ELISAs (S protein), IgG-IgM POCT (N protein)
Van Elslande et al. (19)	Confirmed COVID-19 cases ($n = 167$)	Pts before the pandemic with laboratory-confirmed other respiratory virus infection ($n = 63$) and laboratory-confirmed other virus infection ($n = 40$)	Inpatients, 35% in critical conditions	0–25	IgG and IgA ELISAs (S protein); IgG-IgM POCTs (not reported; N protein)
Imai et al. (20)	Confirmed COVID-19 cases ($n = 139$)	Pts before the pandemic ($n = 48$)	Inpatients, not specified	Median time: 5; IQR, 2–7	IgG-IgM POCT (N, S protein)
Hoffman et al. (21)	Confirmed COVID-19 cases ($n = 29$)	Healthy individuals before the pandemic ($n = 124$)	Pts with symptoms	9–29	IgG-IgM POCT (N, S protein)

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Pts, patients; ICU, intensive care unit; IQR, interquartile range; NA, not available; S, spike; N, nucleocapsid.

sensitivity (**Table 5**). In fact, variations in the dynamics of the antibody response depending on presence/absence of disease and severity of disease were reported (12, 13). In particular, it was suggested that asymptomatic or paucisymptomatic patients might have low antibody concentrations that could give false-negative results (12). This is in line with our findings given that lower values of sensitivities were observed by investigating plasma from asymptomatic than symptomatic subjects. However, due to the small sample size, no conclusions about the sensitivity performance of the serological assays in asymptomatic population can be drawn from this study. This study has two limitations, i.e., the assays' diagnostic performance was mainly evaluated on symptomatic cases and the case-control study design may have introduced a selection bias. Furthermore, given that a good number of serial serum samples from patients was not available, the kinetics of IgG, IgM, and IgA antibody detection were not analyzed. The results of serological investigations for the diagnosis of SARS-CoV-2 infection have only been examined at two different time-points, during the first 2 weeks and after 14 days after symptoms onset.

In addition to the very good analytical performances observed for the IgG assays, a good agreement between the different assay formats was found, with k values up to 0.94. Conversely,

moderate agreement was observed between the two IgM assays ($k = 0.57$).

Plasma samples from probable COVID-19 cases were also investigated by the five serological assays, and it is noteworthy to mention the seropositivity analysis in this group of patients showed that up to 50% of the cases resulted laboratory confirmed by means of positive serological testing. Larger analyses are advocated given the small sample size here investigated.

In conclusion, the high number of false negatives obtained by the IgM assays seems to limit the use of IgM detection as a marker of acute infection and the high number of indeterminate results obtained by ELISA IgA makes it difficult to clearly define the application area of the search of this class of immunoglobulin. On the other hand, the very good analytical performances in terms of sensitivity, particularly in sera from convalescent phase, and specificity observed for the fully automated high throughput assays for IgG detection, indicate that the search of IgG may represent a reliable tool for epidemiological serosurveys and for retrospective diagnosis of SARS-CoV-2 infection in targeted populations. Moreover, given the ability of the serological assays to detect antibodies in probable COVID-19 group, serological testing could be an important complement to molecular assay for the diagnosis of SARS-CoV-2 infection in this type of patients.

Our findings highlight the potential of serological testing in improving epidemiological control and clinical management of COVID-19. Future studies are certainly required since many questions remain currently unanswered such as the role, pathogenic or protective, of antibody responses during infection, how long antibodies persist after infection, and if the infection results in an immune response that protects individuals from future infections or illness.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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Implications of Laboratory Tests in Disease Grading and Death Risk Stratification of COVID-19: A Retrospective Study in Wuhan, China

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Background: Although laboratory tests have become an indispensable part in clinical practice, its application in severity classification and death risk stratification of COVID-19 remains unvalidated. This study aims to explore the significance of laboratory tests in the management of COVID-19.

Methods: In 3,342 hospitalized patients with COVID-19, those of mild or moderate subtype were categorized into the non-severe group, while those of severe or critical subtype were categorized into the severe group. Initial laboratory data were analyzed and compared according to disease severity and outcome. Diagnostic models for the severe group were generated on risk factors identified by logistic regression and receiver operating characteristic (ROC) analyses. Cox regression and ROC analyses on risk factors were utilized to construct prognostic models.

Results: In identification of patients in the severe group, while age, neutrophil-to-lymphocyte ratio, and α-hydroxybutyrate dehydrogenase were identified as independent predictors, the value of combination of them appears modest [area under the curve (AUC) = 0.694]. Further ROC analyses indicated that among patients in the severe group, laboratory indices had a favorable value in identifying patients of critical subtype rather than severe subtype. For death outcome, IL-6, co-existing cerebrovascular disease, prothrombin time activity, and urea nitrogen were independent risk factors. An IL-6 single-parameter model was finalized for distinguishing between fatal and recovered individuals (AUC = 0.953). Finally, a modified death risk stratification strategy based on clinical severity and IL-6 levels enables more identification of non-survivors in patients with non-critical disease.

Conclusions: Laboratory screening provides a useful tool for COVID-19 management in identifying patients with critical condition and stratifying risk levels of death.

Keywords: SARS-CoV-2, clinical classification, risk-stratification, prediction model, IL-6

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a pandemic infectious disease caused by severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) (1). Since its outbreak from Wuhan in December 2019, COVID-19 has affected over 35 million patients and caused more than 1 million deaths according to the latest report from the World Health Organization (2). The clinical spectrum of COVID-19 varies from asymptomatic or paucisymptomatic forms to severe clinical conditions characterized by dyspnea and lethal complications such as acute respiratory distress syndrome (ARDS), multi-organ failure, and septic shock (3, 4). While mild or moderate disease was exhibited by ∼80% patients, severe and critical conditions were diagnosed in the remaining 20% (5). Although the accurate case-fatality rate (CFR) across various disease severity remains unclear, the CFR in patients with critical disease was reported up to 49% (3, 5). Therefore, precise identification of disease severity and underlying risk factors for mortality is of paramount importance to initiate individualized therapeutics and improve patient outcomes.

Laboratory tests performed on blood samples reflect individual physiological and biochemical states. Accumulating laboratory data have revealed a variety of abnormities such as coagulopathy, myocardial injury, liver damage, kidney injury, and immune dysfunction in patients with severe COVID-19 (6–8), particularly in those fatal cases (9–11). Despite the significance in COVID-19, laboratory items have not been included in the current clinical classification of COVID-19, which is mainly based on clinical manifestations and radiologic features (12). Given that disease severity is directly linked to treatment decision and prognosis, we hypothesized that, in addition to current clinical criteria, abnormal laboratory variables may provide an alternative tool to grade patients and, meanwhile, predict survival. Surprisingly, few studies have reported this before. The first area that experienced COVID-19 outbreak, Wuhan, has a large number of patients on whom broad and basic laboratory screening was exclusively performed. Therefore, we revisited patient datasets in Wuhan to investigate the significance of laboratory tests in disease grading along with the prognosis of COVID-19.

METHODS

Study Participants

We reviewed a total of 3,477 medical records of COVID-19 from Wuhan Huoshenshan Hospital, Hubei Maternal and Child Health Hospital, and General Hospital of Central Theater Command from 5 February to 15 March 2020. These three tertiary hospitals, in Wuhan of Hubei Province, were specifically requisitioned to treat patients with COVID-19 during the outbreak in China.

The diagnosis of COVID-19 was based on World Health Organization interim guidance (13). Disease severity was defined according to the guideline of diagnosis and management for COVID-19 (sixth edition, in Chinese) released by the National Health Commission of China. The mild subtype was diagnosed if patients had slight clinical symptoms without pneumonia on radiography. The moderate subtype was confirmed when patients presented with fever and/or respiratory symptoms plus pneumonia on radiography. While patients were classified into the severe subtype if they exhibited dyspnea (respiratory frequency $\geq$ 30/min), blood oxygen saturation $\leq$ 93%, or $PaO_2/FiO_2 \leq 300$ mmHg, patients with respiratory failure, multi-organ failure or shock and requirement of mechanical ventilation and intensive care unit admission were categorized into the critical subtype (12). In this study, patients with mild or moderate disease were classified into the non-severe group, whereas the severe group included those with severe or critical condition (**Figure 1**). All patients were followed up till recovery or death.

Data Collection

Clinical data including demography, medical history, clinical manifestations, laboratory blood test results, and outcomes were collected and independently reviewed by two attending physicians. We focused on the comprehensive laboratory results including the following seven categories: complete blood cell count, coagulative state, myocardial injury markers, liver function markers, kidney function markers, electrolyte and glucose test, as well as inflammatory factors including C-reactive protein (CRP) and IL-6 from each patient on admission (**Supplementary Table 1**).

Statistical Analysis

No imputation was made for variables with missing data. Quantitative data with non-normal distribution were expressed in median [interquartile range (IQR)] and statistically compared by Mann-Whitney U non-parametric test. Percentage (%) of enumeration data were calculated and compared using the χ^2 test or Fisher's exact test. Survival curves were plotted using the Kaplan–Meier method with log-rank test.

Risk factors associated with disease severity in demography and laboratory variables were analyzed using univariate logistic regression analysis followed by receiver operating characteristic (ROC) curve analyses. To avoid excessive laboratory variables in subsequent multivariate analyses, one or two risk factors in each category of laboratory tests meeting the following requirements were selected: (1) significant variables identified in univariate analyses; (2) variables with scientific and clinical merits or proven to relate to disease severity in prior studies; and (3) variables with high AUC value identified in ROC analyses. Considering that the elderly, especially those with comorbidities, could easily progress from dyspnea to critical condition and even death (5, 14), all significant demographic variables identified in univariate analyses were selected as potential confounding variables in the multivariable models with a forward stepwise approach. Similarly, survival prediction models were developed using univariate and multivariable Cox regression analyses.

Further, a nomogram for predicting survival was built and evaluated by the AUC value and calibration plots. All statistical analyses were performed using SPSS software (version 22.0, IBM Corp) and R software (version 3.3.1, R Foundation). $P < 0.05$ were considered statistically significant.

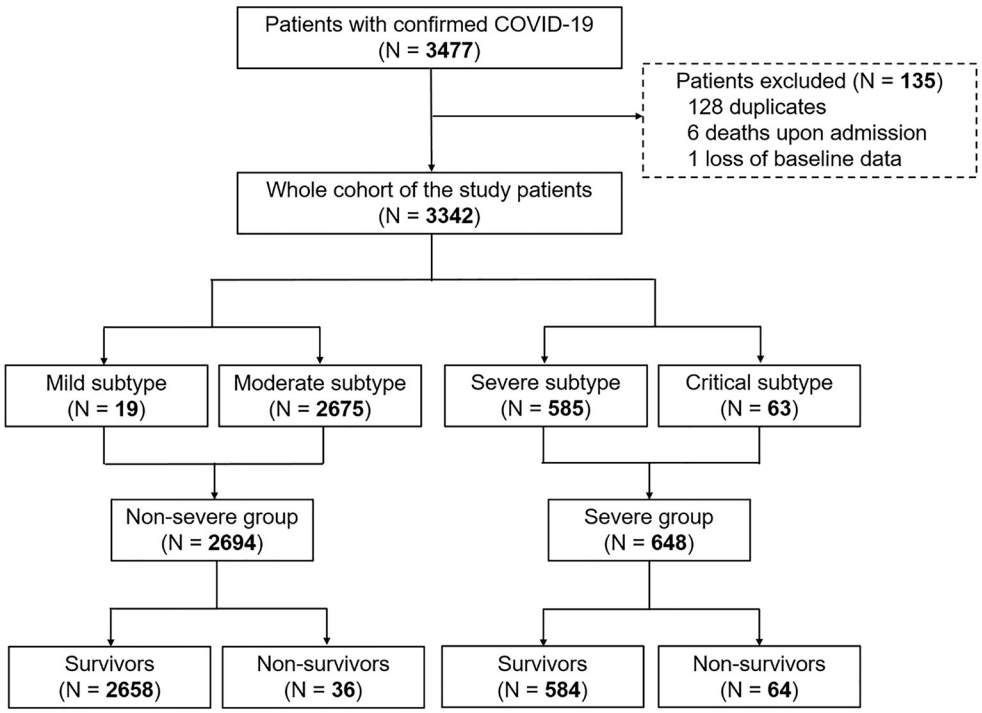

FIGURE 1 | Flowchart of patient recruitment.

RESULTS

Patient Characteristics, Laboratory Findings, and Clinical Outcomes

Data of 3,342 patients with COVID-19 from a total of 3,477 medical records were analyzed (**Figure 1**). Overall, 19 patients, 2,675 patients, 585 patients, and 63 patients were classified into mild subtype, moderate subtype, severe subtype, and critical subtype, respectively. Accordingly, 2,694 patients (80.6%) and 648 patients (19.4%) were categorized into the non-severe group and severe group, respectively (**Figure 1**). The death rate was 2.99% (100/3,342) in the entire cohort. The CFR of 9.88% (64/648) in the severe group was significantly higher than that of 1.34% in the non-severe group ($P < 0.001$; **Figure 1**).

Compared with the non-severe group, the severe group had an older median age and was composed of a higher proportion of male and those with various comorbidities (**Supplementary Table 1**). By analyzing laboratory blood tests, we found that coagulopathy, myocardial injury, kidney injury, and increased CRP and IL-6 levels were exhibited more frequently in the severe group than in the non-severe group (**Supplementary Figure 1A**). After separating patients according to outcome, we observed a similar tendency of the above demographic and laboratory characteristics in non-survivors compared to survivors. Notably, liver injury with a higher frequency in non-survivors was the only feature that was not significantly different between the severe group and the non-severe group (**Supplementary Figure 1B**; **Supplementary Table 1**).

As expected, patients in the severe group had a longer hospitalization stay [median (IQR), 15 (8–23) vs. 13 (8–18) days; $P < 0.001$] than those in the non-severe group. A significantly escalated risk of mortality was also revealed by Kaplan–Meier curve for patients in the severe group [hazard ratio (HR), 5.641; **Figure 2A**] or of critical subtype (HR, 33.981; **Figure 2B**).

Predictive Laboratory Factors for Identifying Patients in Severe Group

Initial univariate logistic analysis identified 46 significant risk factors for the severe group (**Supplementary Table 2**). Among them, the onset age had the highest predictive accuracy, but with the AUC value of only 0.657 (**Supplementary Table 2**). Thereafter, a multivariate model, including all significant demographic variables and 10 laboratory variables with highest AUCs that represent multi-organ injury, was established, indicating that age [odds ratio (OR), 1.032], neutrophil-to-lymphocyte ratio (NLR; OR, 1.090), and α-hydroxybutyrate dehydrogenase (OR, 1.004) were independent risk factors for severe COVID-19 (**Supplementary Table 3**).

Further, we developed three single-parameter models and one multi-parameter model based on the above independent predictors to differentiate the severe group and the non-severe group (**Supplementary Table 4**). However, these models possessed undesirable discrimination as the highest AUC in Model 4 was only 0.694 (**Figure 3A**), suggesting that laboratory data may not be strongly associated with clinical severity classification.

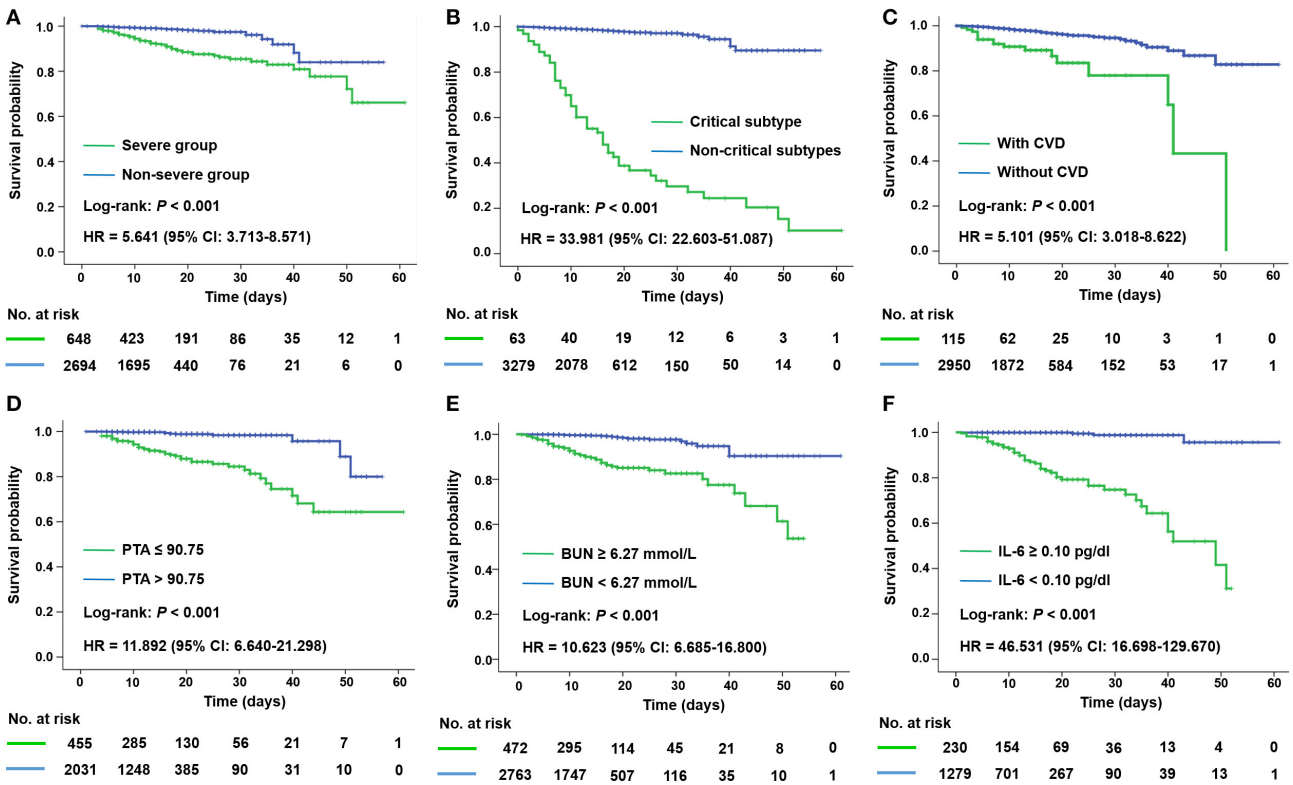

FIGURE 2 | Kaplan–Meier plots for disease severity and different prognostic factors of all patients with COVID-19. Mortality was significantly higher in patients of the severe group **(A)**, of critical subtype **(B)**, and with CVD **(C)**, PTA ≤ 90.75 **(D)**, BUN ≥ 6.27 mmol/L **(E)**, and IL-6 ≥ 0.10 pg/dl **(F)** according to corresponding ROC cutoffs. BUN, blood urea nitrogen; CVD, cerebrovascular disease; HR, hazard ratio; IL-6, interleukin-6; PTA, prothrombin activity.

Prognostic Laboratory Factors for Mortality in the Whole Cohort

Univariate Cox regression analysis identified 48 significant risk factors for mortality in all patients (**Supplementary Table 5**). We incorporated 21 items with relatively high AUC into a multivariable model and found that cerebrovascular disease (CVD; HR, 6.162), prothrombin activity (PTA; HR, 0.912), blood urea nitrogen (BUN; HR, 1.207), and IL-6 (HR, 1.085) were independent predictors for fatality (**Supplementary Table 6**). Kaplan–Meier analysis (**Figures 2C–F**) revealed a poorer prognosis in patients with pre-existing CVD, decreased PTA (≤90.75 vs. >90.75), elevated BUN (≥6.27 vs. <6.27 mmol/L), or elevated IL-6 (≥0.10 vs. <0.10 pg/dl) according to corresponding ROC cutoffs.

Further, four single-parameter models and four multi-parameter models were developed (**Supplementary Table 7**). Unlike the models in differentiation of disease severity, models aiming at predicting survival possessed a favorable performance (**Figure 3B**). Notably, among these candidates, a single-parameter model based on IL-6 levels had the highest discrimination (AUC = 0.953) and a good calibration (**Figure 3B**; **Supplementary Figure 2**). At the optimal cutoff of 0.10 pg/dl, the sensitivity and specificity was 91.8% and 86.3%, respectively. Therefore, a nomogram based on serum IL-6 levels was constructed to predict survival for further clinical practice (**Figure 3C**).

Prognostic Value of IL-6 for Mortality in Severe Group

Given that severe patients have higher risk of poor prognosis (3, 5), we sought to investigate whether IL-6 remains effective in survival prediction specifically among patients in the severe group. Again, non-survivors exhibited significantly higher levels of IL-6 than those survivors ($P < 0.001$; **Supplementary Table 8**). Subsequent Cox regression analyses revealed that IL-6 (HR, 1.114), together with pre-existing chronic kidney disease (CKD), increased NLR, and decreased PTA, was the independent predictor for fatal outcome in the severe group (**Supplementary Tables 9, 10**; **Supplementary Figure 3**). Among all the candidate models (**Supplementary Table 11**), IL-6 still had a relatively high performance (AUC = 0.914), with a sensitivity of 87.5% and a specificity of 84.7% at the cutoff of 0.17 pg/dl.

Predictive Value of IL-6 for Patients of Varying Severity

We next sought to investigate the reason for the discrepancy in predictive value of laboratory indices when distinguishing

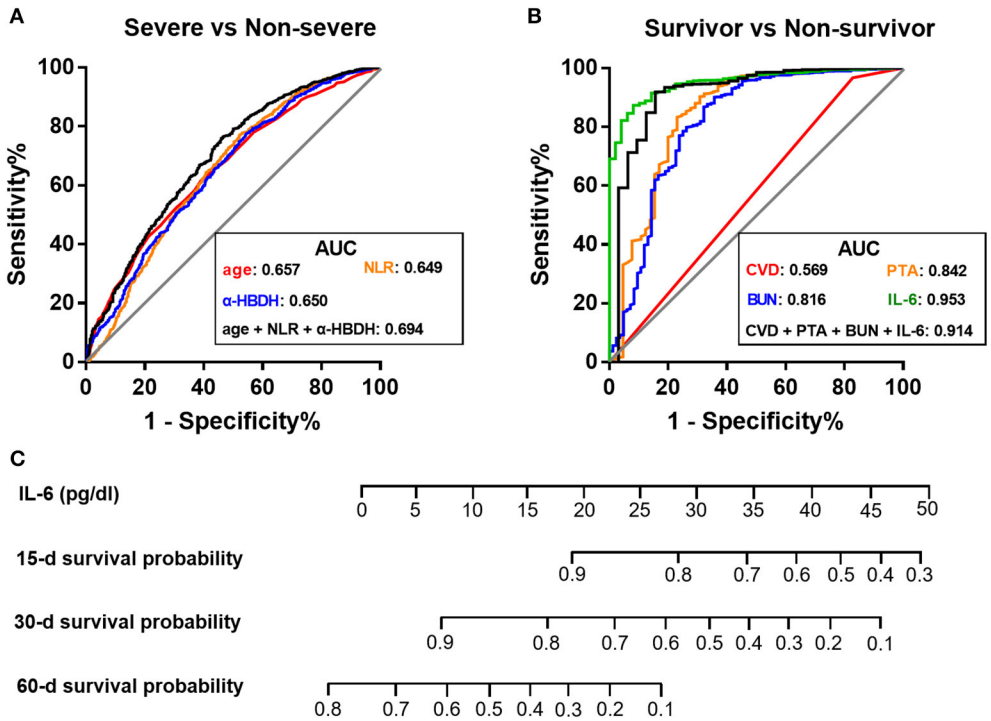

FIGURE 3 | Comparison of receiver operating characteristic curves of laboratory models for disease grading and prognosis of COVID-19. **(A,B)** ROC curves for the classification of severe and non-severe medical conditions **(A)**, and the prediction of 60-day survival probability **(B)**. **(C)** Prognostic nomogram based on IL-6 for predicting survival probability. AUC, area under the curve; BUN, blood urea nitrogen; CVD, cerebrovascular disease; α-HBDH, α-hydroxybutyrate dehydrogenase; IL-6, interleukin-6; NLR, neutrophil-to-lymphocyte ratio; PTA, prothrombin activity; ROC, receiver operating characteristic.

non-survivors and those in the severe group. IL-6, PTA, and BUN, independent laboratory predictors for overall mortality mentioned before (**Supplementary Table 6**), were adopted for the analyses herein. The predictive values of IL-6 for differentiating four subpopulations of COVID-19 patients, including non-survivors, critical subtype, severe group (including both critical and severe subtypes), and severe subtype, from the non-severe group were compared. Strikingly, we observed a substantial decline in AUC from non-survivors (AUC = 0.958) and critical subtype (AUC = 0.951) to the severe group (AUC = 0.649) and severe subtype (AUC = 0.616; **Figure 4A**). Similar phenomenon was also present in PTA and BUN (**Figures 4B,C**). In addition, IL-6 outperformed PTA and BUN when identifying those with fatal outcome or in critical condition (**Figure 4**). Taken together, these results indicate that laboratory results have a favorable value in identifying patients of critical subtype rather than severe subtype. Since patients of severe subtype occupied the vast majority of the severe group (585/648), its role in disease grading was weakened by the inefficacy in identifying severe subtype.

A Modified Risk Stratification Strategy for COVID-19

Based on the current clinical classification system, we found that CFR was not dramatically different between patients of severe subtype (3.08%) and in the non-severe group (1.34%). Thus, we sought to investigate whether death risk stratification could be improved with the introduction of laboratory variables. Given the good performance in identifying patients with fatal outcome and in critical condition, we integrated IL-6 assessment into the current clinical classification system. In a cohort of 1,509 patients with the initial IL-6 test, the non-severe group, severe subtype, and critical subtype had 1,151 (CFR = 1.48%, 17/1,151), 323 patients (CFR = 2.17%, 7/323), and 35 patients (CFR = 71.43%, 25/35; **Figure 5A**), respectively. Given the dramatically high CFR in patients of critical subtype, we ranked high-risk level to them without further modifications. Therefore, the death cases in the high-risk group occupied 51.02% (25/49) of total death cases (**Figures 5B,C**). In addition, 30 patients had IL-6 levels $\geq$ 0.1 pg/dl in this group. Further, using the cutoff value of 0.1 pg/dl, we found 1,280 patients with IL-6 levels $<$ 0.1 pg/dl (CFR = 0.23%, 3/1,280) and 194 patients with IL-6 levels $\geq$ 0.1 pg/dl (CFR = 10.82%, 21/194) in those non-critical patients (**Figure 5B**). By introducing IL-6 levels, we surprisingly found that the death composition ratio was altered, in which patients with IL-6 levels $\geq$ 0.1 pg/dl took 42.86%, while those with IL-6 levels $<$ 0.1 pg/dl occupied just 6.12% (**Figure 5C**). Therefore, low-risk and medium-risk groups were defined using the IL-6 of 0.1 pg/dl (**Figure 5B**). Compared with clinical classification, this strategy could identify more non-critical patients with fatal

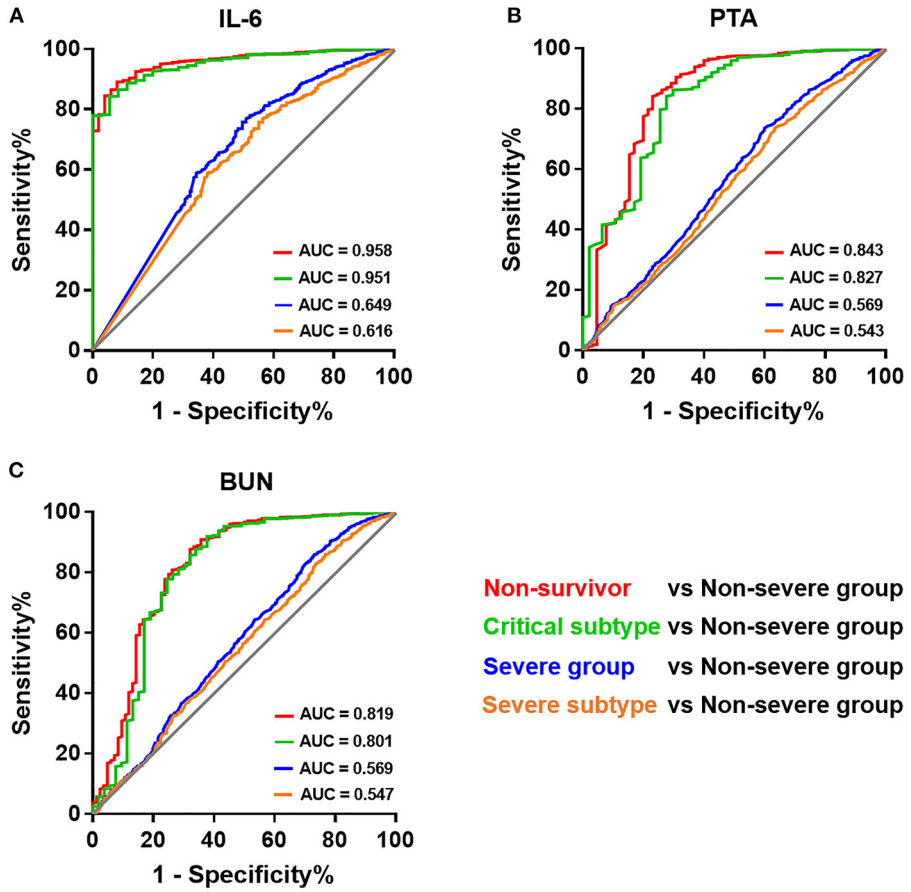

FIGURE 4 | Receiver operating characteristic curves of different laboratory prognostic factors for identifying patients of varying severity. Receiver operating characteristic curves of IL-6 **(A)**, PTA **(B)**, and BUN **(C)** for identifying patients of varying severity. BUN, blood urea nitrogen; IL-6, interleukin-6; PTA, prothrombin activity.

outcome in the medium-risk group, with a higher sensitivity (87.50 vs. 29.17%; $P < 0.001$) and positive predictive value (10.82 vs. 2.17%; $P < 0.001$; **Figure 5D**).

DISCUSSION

Our study determines the associations of basic laboratory screening with disease severity and prognosis of COVID-19. One surprising finding is that laboratory variables, alone or in combination, had a better performance in predicting survival than identifying patients in the severe group. Further analysis indicated that laboratory tests showed excellent performance in identifying patients of critical subtype rather than severe subtype. One possible explanation is that patients of severe subtype displayed only symptoms of hypoxia, instead of ARDS, multi-organ failure, or septic shock that frequently occurred in critical or deceased patients (10, 11), so no dramatic change was induced in these indices reflecting inflammation, multi-organ function, and homeostasis. Therefore, the inclusion of laboratory test should be considered for the diagnosis of critical COVID-19.

In accordance with prior studies concerning prognostic models of COVID-19 (15), we found that laboratory tests showed strong advantages in predicting survival, among which IL-6 stands out as the most appealing one owing to its superior discrimination. The presence of raised circulating levels of IL-6 has been shown closely relating to disease deterioration and fatal outcome of COVID-19 (8, 16–19). Our results support previous findings by finalizing a single-parameter IL-6 prognostic nomogram. Although it did not possess the highest discrimination compared with previous models (summarized in **Supplementary Table 12**), it may outperform them for its simplicity in clinical practice.

The classification of COVID-19 guides management decisions (12), but may not closely relate to risk stratification, owing to no huge difference observed in CFR between the severe subtype and the non-severe group. Therefore, we established a grading system for COVID-19 by combining serum IL-6 levels and current classification criteria. Excitingly, this modification was capable of identifying more non-critical patients with fatal outcome in the medium-risk group. Since our study

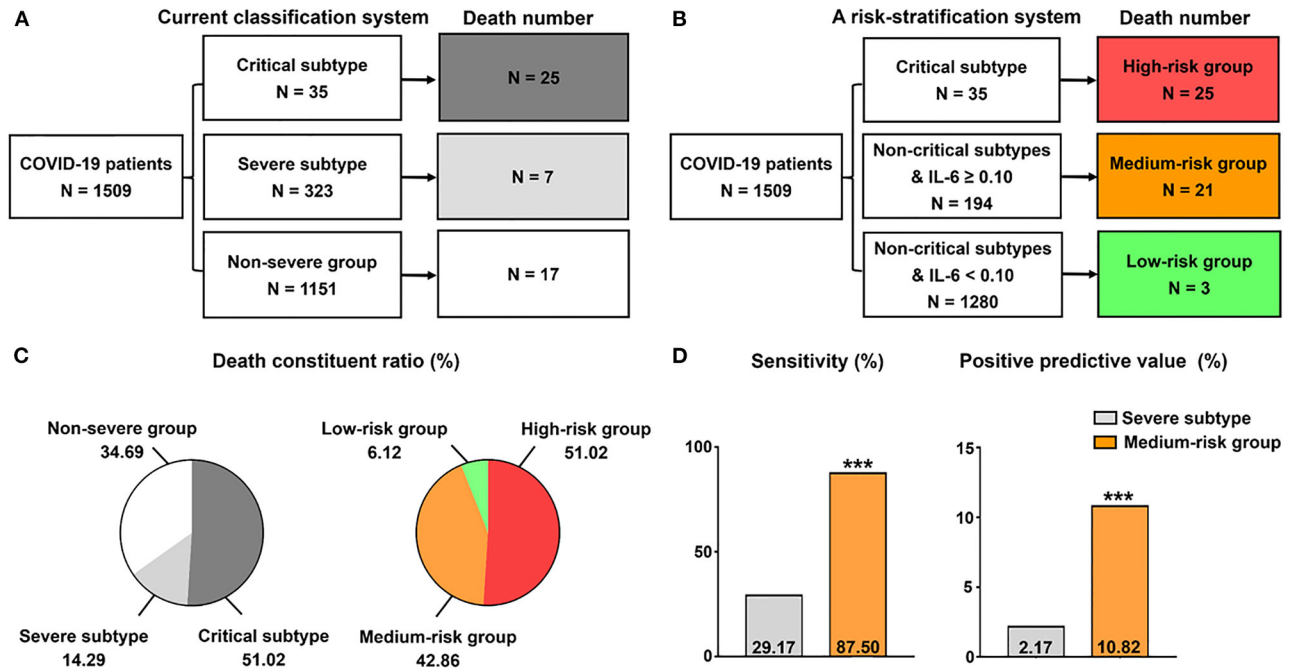

FIGURE 5 | A modified grading strategy for COVID-19. **(A)** Current classification system. **(B)** A risk stratification system on the basis of IL-6 and current classification system. **(C)** Death constituent ratio of the two grading methods. The data in the pie chart indicate death constituent ratio (%) in each group. **(D)** Comparison of the efficacy of the medium-risk group in identifying non-critical patients with fatal outcome between the two methods. ***$P < 0.001$.

focuses on routine bloodwork tests, whether the collaboration of IL-6 and other specific laboratory tests (20), such as virus tilter measurements, anti-SARS-CoV-2 antibody levels, and other immunological biomarkers could optimize the risk stratification of COVID-19 is an interesting question of future inquiries.

Severe COVID-19 is considered as a virally induced hyper-inflammatory condition with multi-organ involvement *via* the cytokine storm (20). IL-6 has been recognized as an important pro-inflammatory cytokine involved in this process, which impairs immune cell cytotoxicity, maintains antigen stimulation, and leads to sustained cytokine production (21, 22). The finalized IL-6 nomogram highlights cytokine storm as a core mechanism for COVID-19-related death, which is further supported by the fact that nomograms incorporating cytokine indices dominated the top of the ranking in predictive value for mortality (**Supplementary Table 12**). It is worth mentioning that IL-6 does not contradict with other laboratory indicators reflecting multi-organ function in prior prognostic models (**Supplementary Table 12**), since cytokine storm is the main culprit for multi-organ injury in COVID-19 (20). Hence, the control of cytokine storm is specifically emphasized in the treatment of critical patients (23).

The etiology of kidney injury is multifactorial, including direct cytopathic effect of SARS-CoV-2, cytokine storm, and systemic effects of lung inflammation (24, 25). Abnormal kidney function upon admission is considered a negative prognostic factor for survival (26, 27). Patients with CKD were also reported to have a poorer prognosis (28), since they were in a pro-inflammatory state with deficits in immune function and thus vulnerable to respiratory infection (29). Herein, we identified CKD and BUN as independent predictors for mortality, supporting the pivotal position of kidney damage in pathophysiology of this pandemic. Thus, prompt identification and intervention of kidney dysfunction is necessary during treatment.

The prevalence of initial hepatic dysfunction is also high in COVID-19, but overt liver failure as the cause of death rarely occurs (30). Liver injury is related to the hyper-inflammatory status (31, 32) instead of direct cytopathic effect (33, 34). Consistent with prior data (35, 36), we identified liver function indices, DBIL and PTA, as independent predictors for mortality, which reflects the immune dysregulation status from the perspective of hepatology. Hence, more attention should be paid toward immune dysfunction control than liver protecting therapy when dealing with liver injury (37).

The coagulopathy of COVID-19 is essentially an endothelial disease induced by cytokine storm, which contributes to the formation of hypercoagulable status and subsequent multi-organ ischemic/hemorrhagic complications in the late stage of COVID-19 (38). During this process, IL-6 facilitates clot formation by promoting the synthesis of coagulation factors and inhibiting the endogenous fibrinolytic system (39). In agreement with prior data (35, 36, 40), a coagulation marker PTA was proven to be associated with fatal outcome herein.

Additionally, pre-existing CVD was also identified to be predictive of fatality. One possible explanation is that CVD usually reflects a condition of endothelial and hemorheological disorder, rendering patients more prone to negative vascular events (41). Thus, personalized medication in consideration of comorbidities should be advocated to minimize the occurrence of complications.

There are several limitations in this study. First, the IL-6 prognostic model was constructed based on all 3,342 patients in the cohort, which was validated in an internal cohort including all patients with severe disease. Additionally, we also randomly split the data into a training cohort (N = 1,678) and a validation cohort (N = 1,664). An IL-6 prognostic model was still finalized according to the methods described herein, with a high discrimination in both the training (AUC = 0.948) and validation (AUC = 0.961) cohorts (data not shown). However, external and prospective validations of this model are urgently needed. Second, since all participants were from Wuhan in the early days of the outbreak, the findings may not be generalized to other regions with diverse epidemiological characteristics worldwide. Third, this study only focused on the implications of laboratory tests in the prognosis of COVID-19, while other factors, such as the heterogeneities of admission time, therapeutic strategy, and medical treatment level in different hospitals, should not be ignored. Fourth, due to the limits of medical resources, not every item in laboratory tests was performed, especially in those with mild or moderate illness. The existence of missing data would inevitably contribute bias to our findings. Notwithstanding this, each laboratory variable still has results from at least 1,500 individuals, which we feel is sufficient for statistical analysis. Last but not least, despite the inclusion of broad laboratory variables, as we delve deeper in understanding COVID-19, more valid laboratory tests will emerge or even replace those we found herein.

In summary, our retrospective study suggests that laboratory findings have the potential for disease grading and survival prediction in COVID-19. A prognostic nomogram based on IL-6 highlights the key role of cytokines in COVID-19 pathophysiology. Our findings shed new light on the understanding and management of this pandemic.

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 authors.

ETHICS STATEMENT

This study was approved by the National Health Commission of China and the Institutional Review Board in these hospitals. Written informed consent was waived by the ethics committee of the designated hospitals for patients with emerging infectious diseases.

AUTHOR CONTRIBUTIONS

LL, GL, and FW had full access to all of the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis. YB, EW, LL, and GL conceptualized the article. YZhu, YZha, SZ, YB, LC, and HL collected the data. YB, EW, SZ, and JL analyzed the data. YB and FW co-wrote the manuscript with all authors providing critical feedback and edits to the subsequent revisions. All authors approved the final draft of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.629296/full#supplementary-material

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CASE REPORT
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Case Report: Identification of SARS-CoV-2 in Cerebrospinal Fluid by Ultrahigh-Depth Sequencing in a Patient With Coronavirus Disease 2019 and Neurological Dysfunction

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We reported that the complete genome sequence of SARS-Coronavirus-2 (SARS-CoV-2) was obtained from a cerebrospinal fluid (CSF) sample by ultrahigh-depth sequencing. Fourteen days after onset, seizures, maxillofacial convulsions, intractable hiccups and a significant increase in intracranial pressure developed in an adult coronavirus disease 2019 patient. The complete genome sequence of SARS-CoV-2 obtained from the cerebrospinal fluid indicates that SARS-CoV-2 can invade the central nervous system. In future, along with nervous system assessment, the pathogen genome detection and other indicators are needed for studying possible nervous system infection of SARS-CoV-2.

Keywords: SARS-CoV-2, COVID-19, central nervous system, infection, metagenomic next-generation sequencing

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a new illness that has become a pandemic. As of October 18th 2020, there have been more than 39.3 million confirmed cases and more than 1.1 million deaths worldwide. Severe acute respiratory syndrome-Coronavirus-2 (SARS-CoV-2), the causative pathogen of COVID-19, pre-dominantly affects the lungs and causes respiratory illness (1). However, this virus also infects the intestinal tract, urinary system, blood, and other organ systems (2). Furthermore, SARS-CoV-2 is also associated with meningitis/encephalitis (3). In a study of hospitalized COVID-19 patients in Wuhan, China, 36.4% of patients experienced neurological symptoms, including headache, anosmia, ageusia, confusion, seizure, and encephalopathy (4). A recent study reported that SARS-CoV-2 can productively infect human neural progenitor cells and brain organoids, highlighting the potential of direct viral involvement in neurological symptoms in COVID-19 patients (5). SARS-CoV-2 has been detected by real-time reverse transcription-polymerase chain reaction (RT-PCR) in COVID-19 cases with central nervous system (CNS) symptoms

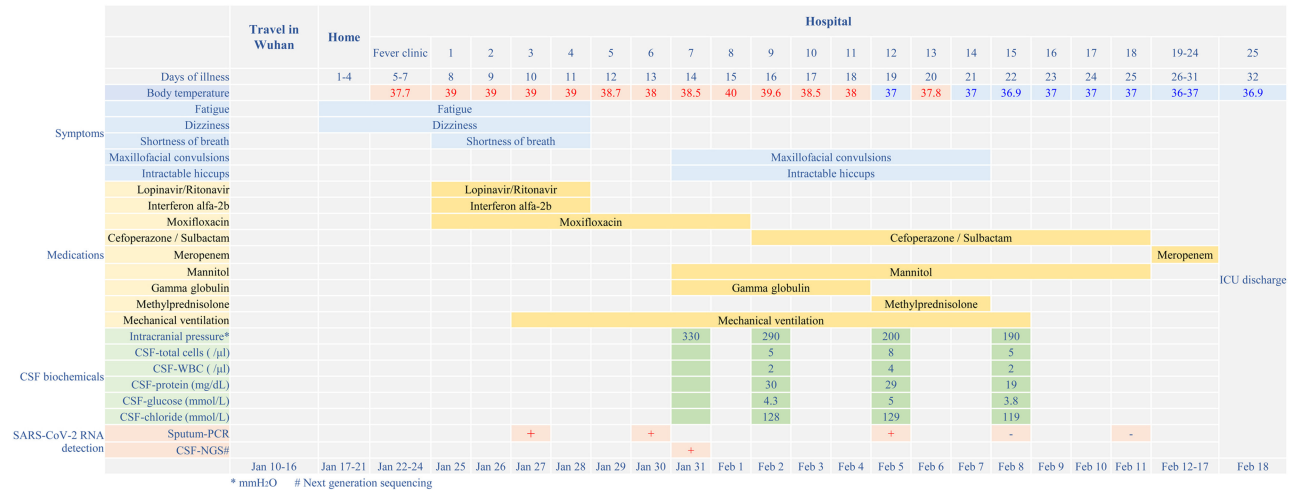

FIGURE 1 | Disease course timeline from the initial presentation of illness to days spent in hospital.

in Japan. However, no complete sequence of SARS-CoV-2 from cerebrospinal fluid (CSF) has been reported (6–8). Here, we used ultrahigh-depth metagenomic next generation sequencing (mNGS) to determine the complete genome sequence of SARS-CoV-2 from the CSF of a COVID-19 case with CNS symptoms. This provides evidence for the existence of SARS-CoV-2 in the CNS.

CASE REPORT

On 24 January 2020, a 56-year-old man was admitted to hospital experiencing fatigue and dizziness for 7 days, and fever for 3 days (**Figure 1**). He had a history of hypertension in the preceding 3 years but his blood pressure was under normal control after treatment with amlodipine besylate. He had no lung disease, epilepsy, or familial psychosocial history. The patient had traveled to Wuhan 14 days prior to hospitalization and one of his relatives, who he had direct contact with, was a COVID-19 case. A computed tomography (CT) scan of the chest revealed a large area of ground-glass opacities (GGOs) dominated by extraneous areas in both lungs (**Figure 2**). A throat swab RT-PCR test for SARS-CoV-2 was positive. COVID-19 with respiratory failure then developed. A nasal catheter was inserted and oxygen was administered at 5 L/min. He was given antiviral therapy with lopinavir/ritonavir (500 mg twice daily) combined with interferon alfa-2b (5 million units twice daily, atomization inhalation), and moxifloxacin (0.4 g once daily, intravenously) to prevent secondary infection (**Figure 1**). After admission, his dyspnea symptoms continued to worsen. On the 10th day of illness, chest CT showed an enlarged GGO area and partial opacities in both lungs. Short-term high-flow nasal oxygen was briefly administered with a gas flow rate of 50 L/min and oxygen concentration of 90%. The patient continued to exhibit respiratory distress, with a respiratory rate of 50 times/min and oxygen saturation (SpO_2) 85%. Therefore, endotracheal intubation was performed in the intensive care unit (ICU) and mechanical ventilation was conducted according to the respiratory ventilation protocol for severe acute respiratory distress syndrome.

After mechanical ventilation for 96 h, frequent maxillofacial and oral spasms accompanied by persistent hiccoughs were observed. Physical examination revealed positive neck-resistance, bilateral pupils of equal size (3 mm diameter) with slow response to light, increased muscle tension in the extremities, hyperreflexia in both knees, and positive bilateral Babinski sign and ankle clonus. The CSF pressure was >330 mmH_2O and had a clear colorless appearance. On the 14th day of illness, the patient was treated with gamma globulin (20 g daily for 5 days), mannitol dehydration (250 mL/6 h) to control intracranial pressure, chlorpromazine to control frequent hiccups, and midazolam (20 mg/h) to control seizures. Three days later, CSF pressure was >290 mmH_2O, the CSF cell count was 5/mL, protein was 30 mg/mL and glucose was 4.3 mmol/L (**Figure 1**). No abnormalities were found on brain CT (**Figure 3**). Therefore, methylprednisolone (500 mg daily for 3 days) was given as shock therapy. Subsequently, the patient's hiccups disappeared and body temperature returned to normal. After discontinuation of midazolam on 6 February, the patient regained clear awareness of his surroundings and clinical seizures did not recur.

Meanwhile, pulmonary lesions were improved by mechanical ventilation for 14 days. The endotracheal intubation was removed on the 24th day of illness and the patient was discharged from ICU on the 32nd day of illness. Head magnetic resonance imaging on May 6 (day 82 of the illness) showed high signal shadows in the hippocampus and bilateral temporal lobe, which may indicate lesions (**Figure 1**). The patient had no cognitive or memory impairment after discharge.

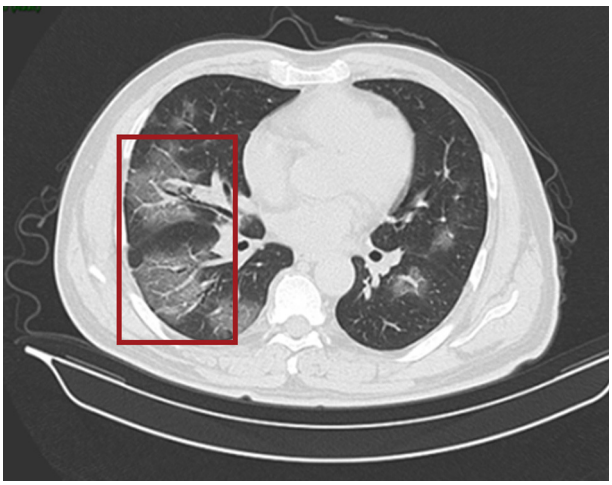

FIGURE 2 | CT scan of the chest. Both lungs show scattered and patchy ground-glass opacities (in red box) on 24th January (day 7 of the illness).

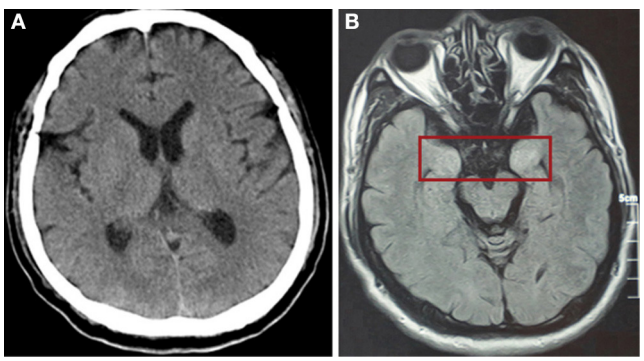

FIGURE 3 | (A) CT scan of the brain showed no abnormally high or low opacity on 31st January (day 14 of the illness). **(B)** Magnetic resonance scan of the brain after discharge showed abnormalities (in red box) in the bilateral temporal lobe and hippocampus on 5th May (day 81 of the illness).

Metagenomic Sequencing

Total genomic DNA and RNA were extracted from samples using the QIAamp DNA Mini Kit and QIAamp Viral RNA Kit (Qiagen, USA), respectively, according to the manufacturer's protocols. Libraries were constructed with the MGIEasy FS DNA Library Prep Set, MGIEasy rRNA Depletion Kit, and MGIEasy RNA Library Prep Set (MGI, China). The prepared libraries were quantified using an Agilent 2100 (Agilent, USA) and sequenced on the MGISEQ-200RS and MGISEQ-2000RS sequencing platforms (MGI). NGS data were processed using Kraken (9), and the Pathogeny Fast Identification (PFI) pipeline for metagenomic identification of pathogens. Bowtie2 and samtools were used to extract the SARS-CoV-2 related reads. Available complete genomes of SARS-CoV-2 in the NCBI database were used as a reference. Based on the extracted SARS-CoV-2-related reads, we assembled the SARS-CoV-2 genome using SPAdes (10). To determine the Nextstrain clade of the sequence obtained in this study, 29 SARS-CoV-2 genome sequences belong to 19A/19B/20A/20B/20C/20D, according to the clade classification scheme of SARS-CoV-2 genomes by Nextstrain, were retrieved from GISAID (https://www.gisaid.org/). The genome sequences were aligned using mafft v7.45, and Mega v6.06 was used to infer the maximum likelihood tree.

Total genomic DNA and RNA were extracted from CSF and used to identify potential pathogens by RT-PCR assays and mNGS. The detection of SARS-CoV-2 by RT-PCR was negative, according to CT values >40. Ultrahigh-depth mNGS produced a full data set of 209,119,576 raw reads from the RNA library, and 10,116 reads corresponded to SARS-CoV-2. The reads were mapped to the SARS-CoV-2 reference genome NC_045512.2 and approximately 100% coverage was obtained with a mean depth of 31.6. No other pathogens were detected. We obtained a 29,857 bp SARS-CoV-2 genome (EPI_ISL_412386) (**Figure 4**) belonging to Nextstrain clade 19A. Furthermore, 29,003 and 15,790 bp SARS-CoV-2 genomes were assembled based on 61,224,674 and 8,800,232 raw reads from sputum and blood samples, respectively. No single nucleotide polymorphisms were found among the three assembled SARS-CoV-2 sequences from CSF, sputum and blood. Compared with the reference genome, MN908947, three nucleotide variations in EPI_ISL_412386 were found, all of which caused amino acid variations, including in the S2 subunit of the spike glycoprotein (D1168N), ORF3a (G251V), and ORF8 (V62L). No mutation was found in the receptor binding domain (**Figure 4**).

DISCUSSION

Human coronaviruses are recognized as respiratory viruses. However, some recognized human respiratory pathogens, including HCoV-OC43, HCoV-229E, and SARS-CoV, are associated with triggering or exacerbating neurological diseases because viral RNA or infectious virus can be detected in human brains (11–13). Preliminary reports showed some COVID-19 patients with CNS manifestations, such as dizziness, headache, nausea, vomiting, impaired consciousness, acute cerebrovascular disease, ataxia, and seizure, which indicated the neuroinvasive potential of SARS-CoV-2 (14, 15). Similar to SARS-CoV, SARS-CoV-2 also binds to the angiotensin-converting enzyme 2 (ACE2) receptor to enter human cells (16). Many types of cell in the brain, such as neurons and glial cells, express ACE2 and are thus vulnerable to SARS-CoV-2 infection. *In vitro* experiments show that SARS-CoV-2 can infect human neural progenitor cells and brain organoids (5). SARS-CoV-2 infection damages the choroid plexus epithelium, leading to leakage across this important barrier, which normally prevents entry of pathogens, immune cells, and cytokines into the CSF and the brain (17). Several case reports and post-mortem examinations of brain tissue have demonstrated SARS-CoV-2 nucleic acid in the CSF and brain tissue of infected and deceased individuals (18). Post-mortem of 43 COVID-19 patients showed fresh territorial ischemic lesions in 14% of patients, while 86% of patients had astrogliosis in all assessed regions. Activation of microglia and infiltration by cytotoxic T lymphocytes was most

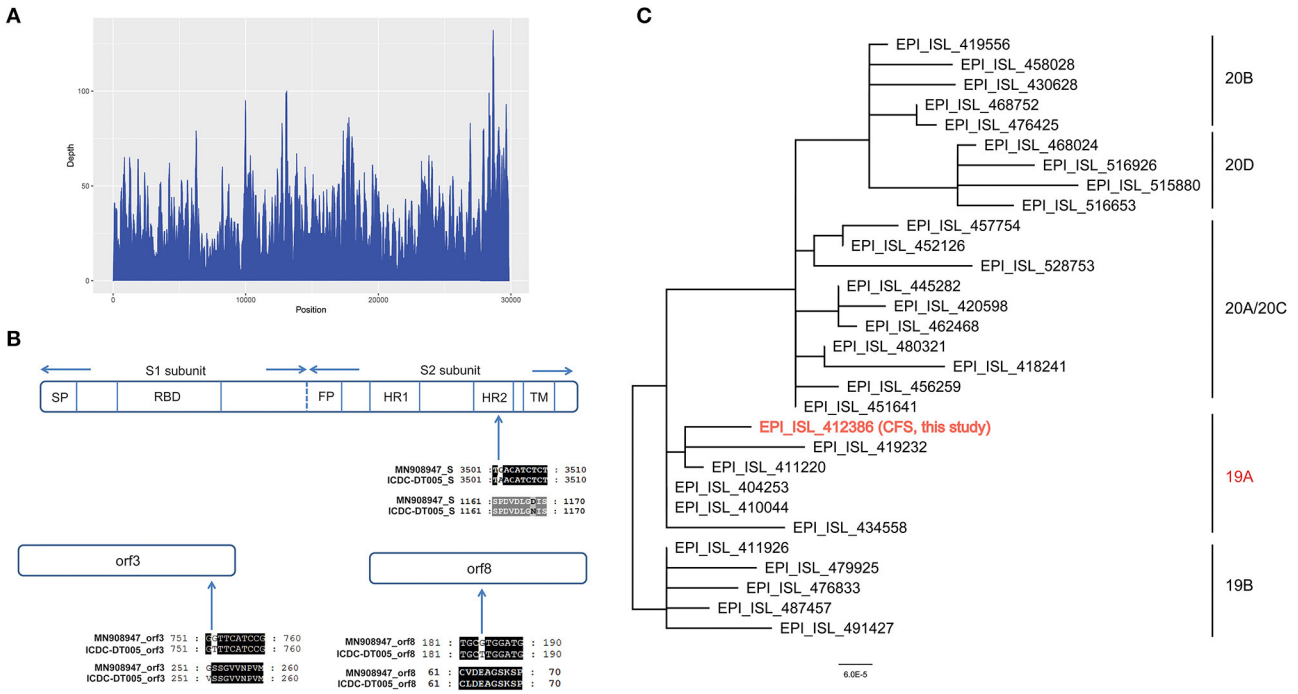

FIGURE 4 | (A) Sequencing depth of the SARS-CoV-2 genome obtained in this study (EPI_ISL_412386). A total of 10,116 sequence reads derived from the patient's cerebrospinal fluid sample were mapped to the SARS-CoV-2 genome. **(B)** Alignment of the S gene, ORF3a and ORF8 of the SARS-CoV-2 genome obtained in this study (EPI_ISL_412386) with the reference genome of Wuhan-Hu-1. Only the alignment block containing a variation is shown for each protein. **(C)** Phylogenetic tree based on whole genome sequences of the SARS-CoV-2 genome obtained in this study (EPI_ISL_412386) and the 29 SARS-CoV-2 genomes which belong to 19A/19B/20A/20B/20C/20D obtained from GISAID.

pronounced in the brainstem and cerebellum, and meningeal cytotoxic T lymphocyte infiltration was seen in 79% of patients (19). One case report patient, whose CSF was positive for SARS-CoV-2 based on RT-PCR, showed neurological symptoms of demyelinating disease (20). RT-PCR is currently the most popular method to detect SARS-CoV-2 because it is specific, rapid, and economical. However, SARS-CoV-2 infection is usually confirmed by monitoring one or two sites. Therefore, RT-PCR shows a high false negative rate in clinical assessments (21). Of 61 suspicious COVID-19 samples, 22 tested negative or inconclusive by RT-PCR but were identified as positive by sequencing (21). Therefore, sequencing has great potential for identifying viruses (22). Other methods can be used in parallel with mNGS. Intrathecal SARS-CoV-2 IgG and specific IgM in CSF have been found in RT-PCR assay-negative COVID-19 cases. These markers may be promising for the diagnosis of COVID-19 in cases with CNS symptoms (23). Calculation of the IgG antibody index (AI) is based on Reiber's method (24). In addition to analyzing the presence of viruses in CFS, its inflammatory profile, including white blood cell count and CSF/blood albumin ratio should also be determined as a supplement to mNGS (25). Among the COVID-19 patients in our care, the main neurological symptoms are maxillofacial convulsion, intractable burping, significantly increased intracranial pressure combined with neck resistance, positive bilateral Babinski sign and ankle clonus, which all indicate neurological dysfunction. There were no additional pathogens identified by examination of CSF. The whole genome sequence of SARS-CoV-2 was obtained from CSF by ultrahigh-depth mNGS, which revealed that SARS-CoV-2 had invaded the CNS. Not all neurological symptoms of COVID-19 occur because of direct viral action. Other causes can also lead to encephalopathy during viral infections, such as auto-immune encephalopathy; therefore, direct associations between encephalopathy symptoms and SARS-CoV-2 require further investigation. During the clinical treatment of this patient, autoimmune antibodies were not tested, and magnetic resonance imaging was not performed at the acute stage. Therefore, a causal relationship between the symptoms and the viral infection was not confirmed. We suggest that future studies include the detection of pleocytosis, high level of protein, blood-CSF barrier dysfunction, and intrathecal synthesis of immunoglobulins, which can be used to assess the inflammatory profile. Albuminocytological dissociation (high CSF protein levels without pleocytosis) can be assessed. Immunological and molecular examinations can be evaluated to exclude autoimmune encephalitis (23).

In summary, we present the first determination of the complete SARS-CoV-2 sequence based on ultrahigh-depth mNGS of a CSF sample, although a direct association between the symptoms of encephalopathy and SARS-CoV-2 requires further investigation. Our case confirmed that SARS-CoV-2 can invade the CNS, highlighting the need for physicians to pay close attention to nervous system symptoms of COVID-19 patients. Moreover, our results

indicate that NGS can be used as a clinical approach for the diagnosis of a specific infectious disease caused by an uncommon pathogen. For COVID-19 patients with neurological dysfunction, detection of SARS-CoV-2 in CSF by NGS will provide a more comprehensive understanding of SARS-CoV-2 infection. This will help reduce the mortality of severely ill patients and lower the risk of transmission resulting from missed diagnosis.

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 at: GISAID, Accession ID: EPI_ISL_412386.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Beijing Ditan Hospital, Capital Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BK, YW, and JL design the study. PX, XX, LG, HW, HX, RL, LP, FJ, CL, MZ, JT, YS, YL, HG, JH, YW, and JL supplied the clinical data. JL, PX, LG, HX, LP, CL, MZ, JT, YS, FZ, YL, HG, and JH evaluated and treated the patients. XL, YX, TQ, HR, JY, JG, XC, HZhe, FZ, XH, and HZho did the metagenomic next generation sequencing. HZho, PX, XX, XL, LG, ZL, and BK analyzed the data. HZho, PX, XL, and LG wrote the paper. BK, YW, and JL reviewed the paper. All authors contributed to the article and approved the submitted version.

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Prospective Comparison of Saliva and Nasopharyngeal Swab Sampling for Mass Screening for COVID-19

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Current testing for COVID-19 relies on reverse-transcriptase polymerase chain reaction from a nasopharyngeal swab specimen. Saliva samples have advantages regarding ease and painlessness of collection, which does not require trained staff and may allow self-sampling. We enrolled 776 persons at various field-testing sites and collected nasopharyngeal and pooled saliva samples. One hundred sixty two had a positive COVID-19 RT-PCR, 61% were mildly symptomatic and 39% asymptomatic. The sensitivity of RT-PCR on saliva samples vs. nasopharygeal swabs varied depending on the patient groups considered or on Ct thresholds. There were 10 (6.2%) patients with a positive saliva sample and a negative nasopharyngeal swab, all of whom had Ct values <25 for three genes. For symptomatic patients for whom the interval between symptoms onset and sampling was <10 days sensitivity was 77% but when excluding persons with isolated N gene positivity (54/162), sensitivity was 90%. In asymptomatic patients, the sensitivity was only 24%. When we looked at patients with Cts <30, sensitivity was 83 or 88.9% when considering two genes. The relatively good performance for patients with low Cts suggests that Saliva testing could be a useful and acceptable tool to identify infectious persons in mass screening contexts, a strategically important task for contact tracing and isolation in the community.

Keywords: COVID-19, saliva, sensitivity, PCR, nasopharyngeal

INTRODUCTION

Current testing for COVID-19 relies on reverse-transcriptase polymerase chain reaction (RT-PCR) from a nasopharyngeal swab specimen (1). Nasopharyngeal sampling requires human resources and training, personal protective equipment and swabs, and time, generating testing bottlenecks and potential exposure to transmission at crowded testing sites. Moreover, the unpleasantness of the procedure and the long waiting delays for swab collection and results may dissuade some persons

to get tested or to repeat tests when they are negative. There is an urgent need for innovative testing strategies to rapidly identify cases, reduce waiting delays, and facilitate mass screening. Saliva samples have advantages regarding ease and painlessness of collection, which does not require trained staff and may allow self-sampling. The comparison of real time PCR results on salivary and nasopharyngeal samples has shown discrepancies between studies, with most finding greater sensitivity and lower RT-PCR Cts in nasopharyngeal swab samples (2–4) whereas others found greater sensitivity in saliva samples (5, 6). The sources of variation may have been the study population (hospitalized patients vs. screening of contacts or mildly symptomatic patients), saliva collection techniques and timing, conditioning and delays in processing raw saliva samples, or differences in the RT-PCR techniques used.

French Guiana is an Overseas French territory between Brazil and Suriname. Although it has a French Health System, it is isolated and its limited hospital capacity is vulnerable to the COVID 19 epidemic surge. As the epidemic peaked in July 2020, intense efforts were undertaken to expand hospital and ICU capacity, to continue contact tracing and offer a place to quarantine for patients that were unable to isolate themselves at home, and to expand COVID-19 testing and reduce testing bottlenecks at the public and private laboratories on the territory and the ensuing renouncement to get tested. We here report the first prospective study of the performance of saliva testing compared to nasopharyngeal swabs in a field context of mass screening in French Guiana.

METHODS

Context

This French territory neighboring Amapa state in Brazil has been highly impacted by COVID-19 with 3.2% of the population having had a confirmed infection, notably among the poorest populations (7). In this context, testing and tracking were implemented throughout the epidemic, testing tents and mobile testing teams including the remote health centers, the Red Cross, Médecins du Monde, and the reinforcements from the Réserve Sanitaire were coordinated by the regional health agency to investigate around clusters of cases. The testing efforts for this small population peaked to nearly 0.5% of the population screened in a day.

Study Conduct

Between July 22th and September 10th, we prospectively enrolled consecutive, persons aged 3 years or more with mild symptoms suggestive of COVID-19 and asymptomatic persons with a testing indication at various testing sites and mobile testing brigades in French Guiana reaching remote sites up to 240 km in the Amazonian Forest. During screening missions, mobile teams, consisting of Healthcare personnel (doctors, nurses) were coordinated by the Health Regional Agency of French Guiana, targeting villages, neighborhoods, where the virus was circulating collected persons often out of doors or in health centers. These mobile teams were made up of staff from the Red Cross, Médecins du Monde, the Cayenne hospital PASS, the Maripa Soula health center, and the health reserve. Team travel was coordinated and decided by the health regional agency of French Guiana each week during a weekly update and was guided the knowledge of current clusters of cases which triggered screening campaigns in the concerned neighborhoods—urban or rural, and usually socially disadvantaged; in addition, patients requiring hospitalization for non-COVID reasons (for example a fractured limb) were screened to rule out infectiousness; during the peak of the epidemic drive through testing services were also deployed to offer testing to any person requesting a test. Inclusion criteria were: males or females with an indication to perform a COVID diagnostic test (symptomatology, contact case, systematic screening, etc.), aged at least 3 years old. Non-inclusion criteria were refusal of the patient or his/her legal representative, person taking treatments that reduce salivary volume (anticholinergic activity), impossibility of carrying out the Nasopharyngeal swab, and persons under guardianship or curatorship, or placed under protective measures. All study participants were enrolled and sampled in accordance with the protocol. An investigator explained the objectives of the study and obtain the oral consent of the patient or his/her legal representative. The form was completed by the investigator or a person delegated by the investigator. The trained nurse present during the testing mission performed the nasopharyngeal swab and collected the salivary sputum sample in a urine container. A trained agent carried out a short questionnaire. At the end of each day, all completed forms and samples were sent to Cayenne hospital and stored at 4°C before analysis. Samples and participant information were non-individually identifiable and collected with a unique identifying number.

Laboratory Analysis

The same technique was used for the two samples throughout the study: the QIAsymphony and GeneFinder kit, a Real-time PCR assay. GeneFinder™ COVID-19 detects SARS-CoV-2 by amplification of RdRp gene, E gene, and N gene according to WHO's recommended protocol. Viral nucleic acid was extracted by using the QIAamp DSP viral kit on the QIAsymphony RGQ, an integrated fully automated nucleic acid extraction (chemical lysis and paramagnetic bead binding) and sample preparation platform (Qiagen GmbH, Germany). The real-time PCR assays for SARS Cov2 were performed with an Applied 7500 cycler (Thermofisher) with the Genefinder kit (Ellitech group) that could detect the N gene, RdRp and E gene, which is not specific to COVID-19. As the Nucleic acid extraction methods could affect the results of viral nucleic acid amplification tests, we treated the couple saliva-nasopharyngeal specimens with the same method and most of the time in the same series, the eluates were obtained from 200 µl of specimens (300 µL – 100 µL dead volume). The remainder of each sample was divided into paired aliquots kept in a biorepository for further studies evaluating new screening tools.

Statistical Analysis

Statistical analysis was performed using STATA® 16 (Stata corporation, College Station, Texas, USA). Cross tabulations considering different subgroups was performed. We considered the RdRp and N genes, specific for SARS-Cov2, to calculate

different Ct categories. The raw data can be accessed at https://doi.org/10.7910/DVN/KPLJ9A.

Ethical

The protocol received ethical approval from the Comité de Protection des Personnes under the number 2020-A02009-30/SI:20.07.07.54744.

RESULTS

We included 776 patients between July 22th and September 10th. The sex ratio (M/F) was 1.6, the mean age was 40 (standard deviation = 16.8). Overall, 61% were mildly symptomatic and 39% were asymptomatic. For symptomatic patients, 84% had a symptoms onset <10 days, and 4% were hospitalized within 2 weeks after inclusion.

Patients With Positive RT-PCR

The crude analysis showed that 152 had a positive RT-PCR on the nasopharyngeal sample and 86 had a positive RT-PCR on the saliva sample; 76 persons had both a positive Nasopharyngeal and Saliva RT-PCR result, while 76 had a positive nasopharyngeal RT-PCR but a negative saliva RT-PCR; Finally, 10 patients (6.2%) had a negative Nasopharyngeal RT-PCR but a positive saliva RT-PCR

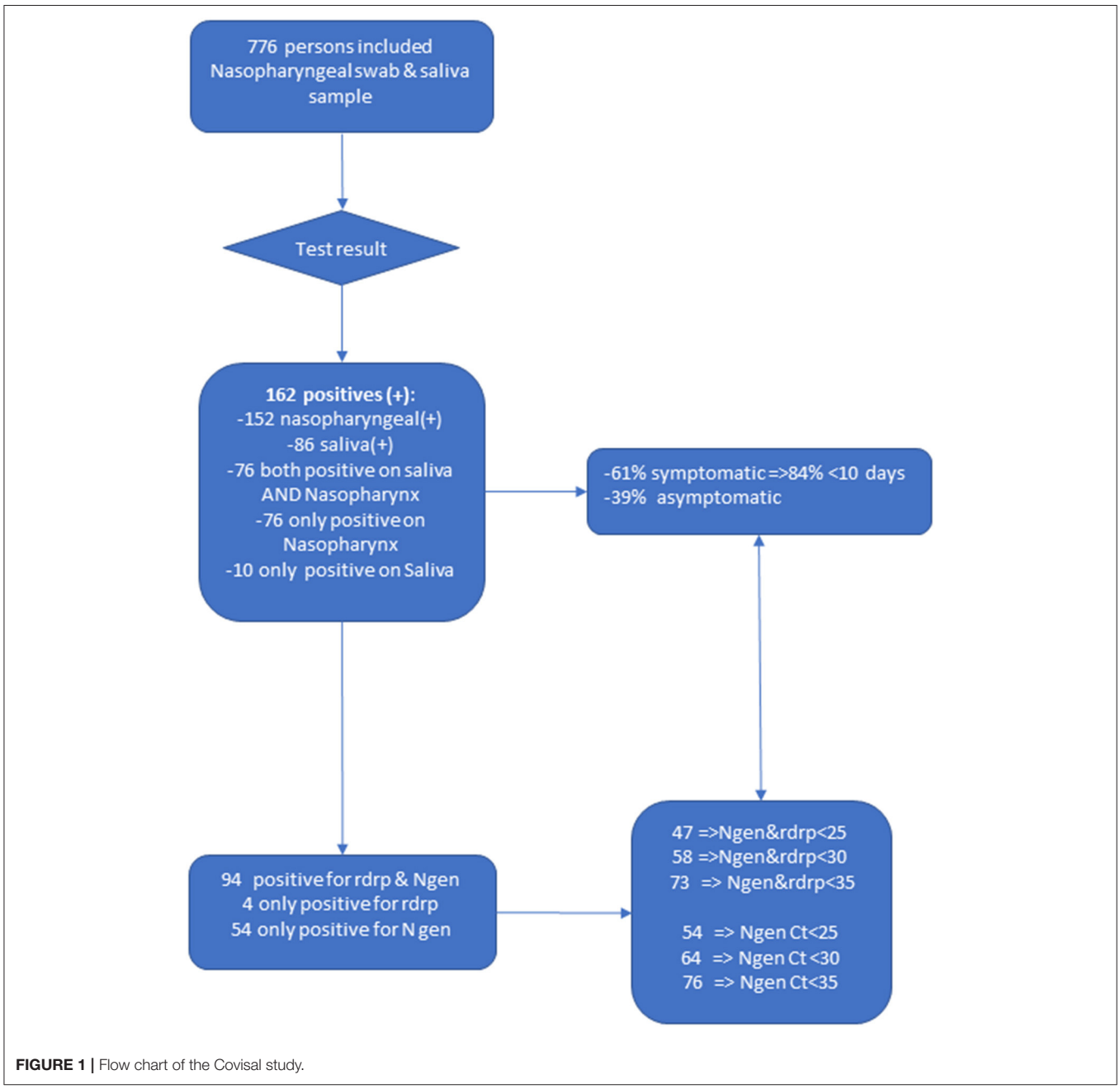

FIGURE 1 | Flow chart of the Covisal study.

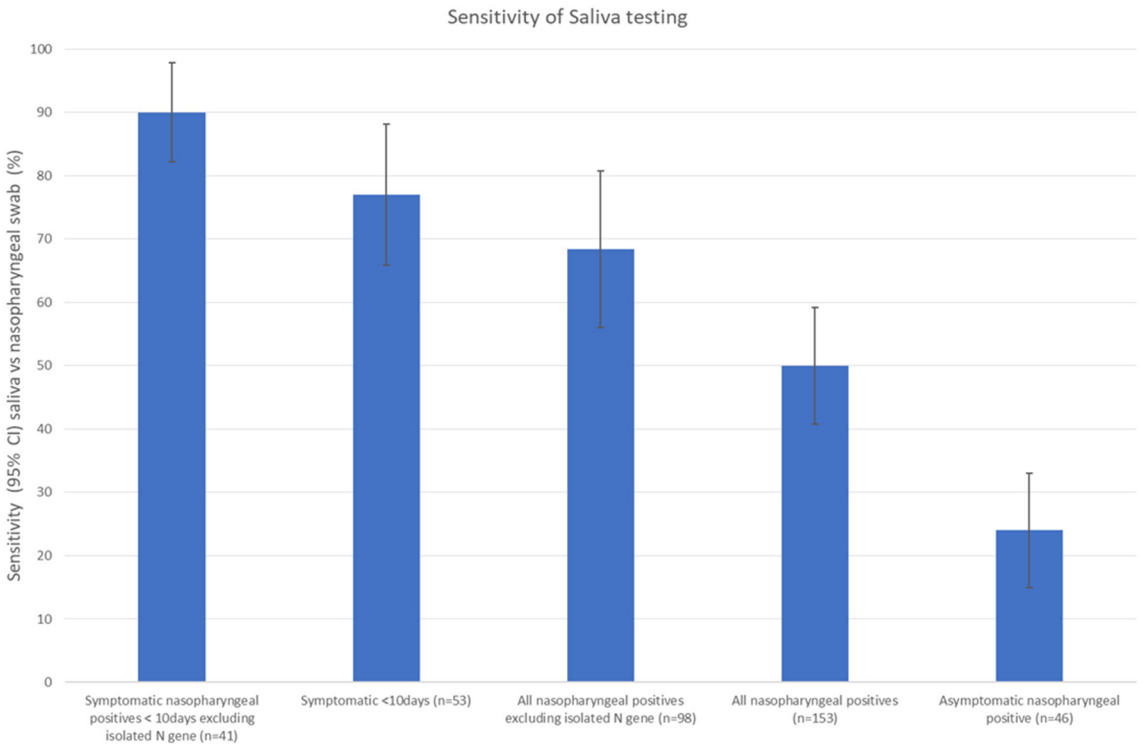

FIGURE 2 | Sensitivity of saliva testing vs. nasopharyngeal swabs for RT-PCR for different groups in a community screening context.

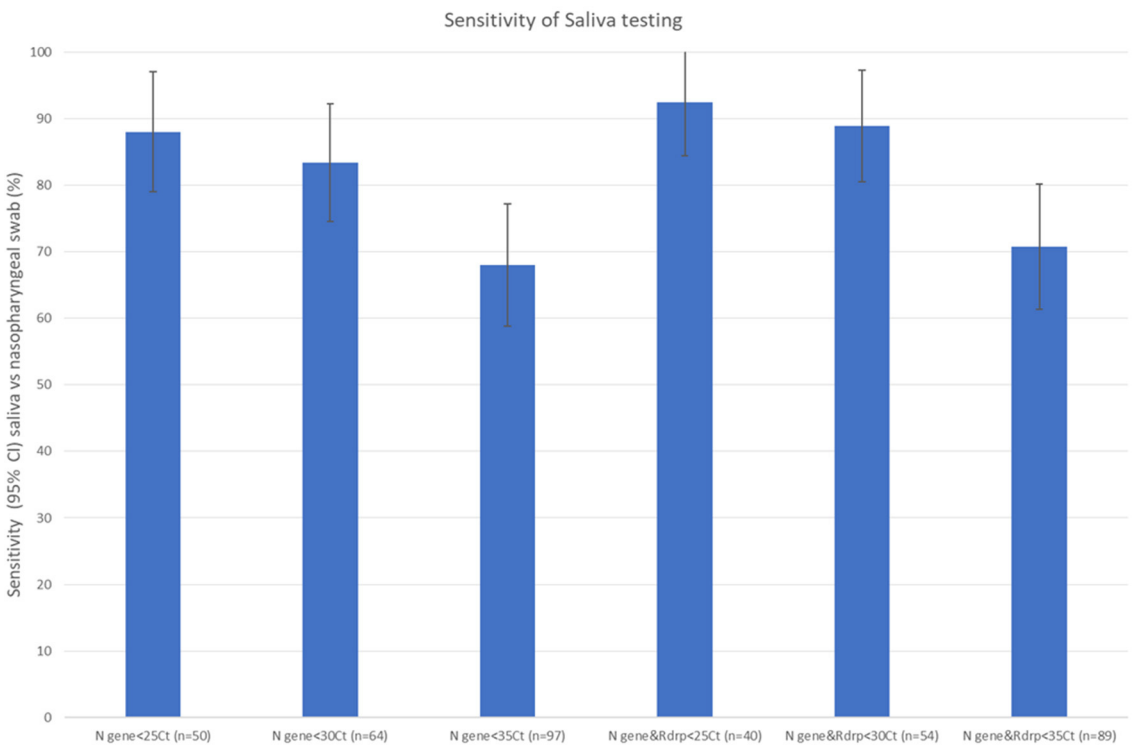

FIGURE 3 | Sensitivity of saliva testing vs. nasopharyngeal swabs for different RT-PCR Cts in a community screening context.

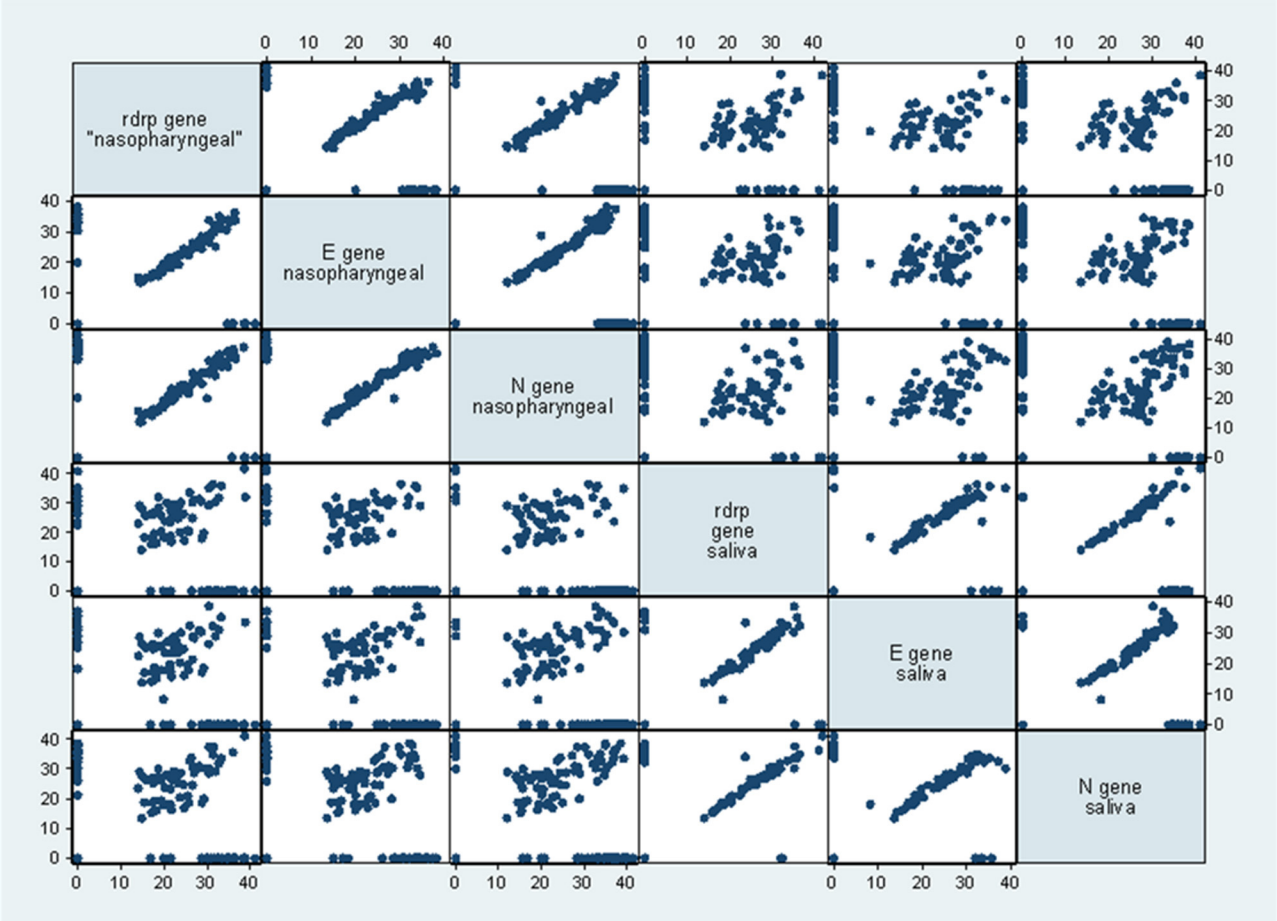

FIGURE 4 | Scatterplot matrix for the Ct of different genes in the nasopharyngeal and saliva samples. There was a "fanning" pattern with greater dispersion at higher Ct values for different genes in the nasopharyngeal and saliva samples.

(**Figure 1**). In total 162 (20.9%) of patients had a positive result on either the Nasopharyngeal or the saliva sample.

Sensitivity, Symptoms, and Ct Values

The sensitivity of RT-PCR on saliva samples vs. nasopharygeal swabs varied depending on the patient groups considered (**Figure 2**) or on Ct thresholds (**Figure 3**). When considering all patients with at least one gene amplification—irrespective of delays, symptoms, or Cts, sensitivity was low (50%); For symptomatic patients with an interval between symptoms onset and sampling under 10 days sensitivity was 77%; however, when excluding persons with isolated N gene positivity (54/162) from this subgroup, sensitivity was 90%.For asymptomatic patients, the sensitivity was only 24%, the lowest of all studied groups (**Figure 2**).

Recent studies have argued that transmission potential - estimated by the capacity to infect cell cultures- was restrained to those with low Cts (8, 9), a proxy for high viral load. When we looked at patients with Cts <30, sensitivity was 83 or 88.9% when considering two genes. Among the 10 patients with a positive saliva sample and a negative nasopharyngeal swab, all had Ct values <25. **Figure 4** shows increasing dispersion for the higher Ct values of the nasopharyngeal vs. saliva sample scatterplots for the different genes amplified by RT PCR emphasizing the greater discordance between samples among patients with lower viral loads.

DISCUSSION

Contrarily to two studies suggesting a greater positivity rate for saliva (5, 6), we observed that saliva testing was less sensitive than nasopharyngeal swabs. Whereas, most studies were hospital-based collecting saliva in the early morning before mouth rinsing and breakfast, our study was a screening study that was performed in difficult field conditions targeting hard to reach populations after breakfast and teeth brushing, moreover out of doors in a tropical context. These realistic conditions were however also a limitation because of the heterogeneity of inclusion sites. Since the main objective was to compute sensitivity, in order to shorten the time allocated to each inclusion, there was limited clinical/epidemiology data from tested individuals and no data on possible repeated testing. The study started after the epidemic peak and hence inclusion of

positive patients became increasingly difficult, and the number of positives was hence insufficient to conduct stratified analyses on subgroups, and particularly for asymptomatic persons with positive RT-PCR who may pool active infections and residual shedding but no clear time frame that could allow to disentangle the two. The poor sensitivity on asymptomatic positive nasopharyngeal swabs was thus presumably also linked to the inclusion of non-infectious patients in the denominator. A third of positives only had a positive N gene, the RdRp and E gene being negative. Based on the empirical experience of the laboratory, such patients were considered to be at later stages of the infection.

The relatively good performance for patients with low Cts suggests that Saliva testing could be a useful and acceptable tool to identify infectious persons in mass screening contexts, a strategically important task for contact tracing and isolation in the community. With the considerable testing bottlenecks, alleviating the workload and shortening the sample collection time would be improvements that could reduce waiting times to get tested and human-resource costs. The sensitivity saliva samples for asymptomatic persons seemed insufficient but without any temporal indication about the onset of infection, it should be further studied by Ct values with a larger sample size. In view of the present results the French Health authorities have officially declared that saliva testing may be used on symptomatic patients only when nasopharyngeal tests cannot be used (10).

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 Protection des Personnes. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MN and MD: conception. MM-F, DB, OB, TP, PM, VS-R, VV, BT, AM, MS, SS, MGu, MGa, BB, WF, LC, AF, DR, NV, AV, and MD: investigation. MN: analysis and first draft writing. MN, MM-F, and MD: review and editing. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Effect of Spironolactone on COVID-19 in Patients With Underlying Liver Cirrhosis: A Nationwide Case-Control Study in South Korea

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Purpose: On the basis that spironolactone is involved in ACE2 expression and TMPRSS2 activity, previous studies have suggested that spironolactone may influence the infectivity of COVID-19. Research has suggested that cell entry of SARS-CoV-2, the virus that induces COVID-19, is associated with the ACE2 receptor and TMPRSS2. The purpose of this study was to investigate whether spironolactone has a protective effect against COVID-19 and the development of associated complications in patients with liver cirrhosis.

Methods: We conducted a nationwide case-control study on liver cirrhosis patients with or without COVID-19 from the population-based data acquired from the National Health Insurance Systems of Republic of Korea. After 1:5 case-control matching, multivariable adjusted conditional logistic regression analysis was performed.

Results: Among the patients with liver cirrhosis, the case group with COVID-19 was found to be significantly less exposed to spironolactone compared with the control group without COVID-19. The adjusted odds ratio (OR) and 95% confidence interval (CI) between the two groups was 0.20 (0.07–0.54). In addition, regardless of cumulative dose of spironolactone, exposure to spironolactone was associated with lower COVID-19 infection. In terms of the development of complications due to COVID-19, spironolactone did not show any significant association between the patients with and without complications ($P = 0.43$). The adjusted OR and 95% CI between the two groups was 1.714 (0.246–11.938).

Conclusion: We conclude that spironolactone may reduce susceptibility to COVID-19 but does not affect the development of its associated complications; however, further studies are needed to confirm the exact association between spironolactone and COVID-19 infection.

Keywords: coronavirus disease 2019, spironolactone, liver cirrhosis, infectivity, susceptibility

INTRODUCTION

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is a novel coronavirus that causes coronavirus disease 2019 (COVID-19). COVID-19 has rapidly spread globally, and the World Health Organization declared COVID-19 a pandemic on March 11, 2020. The mortality rate based on cumulative data is around 3.4% in China and 0.4% outside of China (1). Despite the relatively low mortality rate, COVID-19 can cause severe complications such as acute respiratory distress syndrome (ARDS), with elderly patients being of particularly high risk (2).

Spironolactone is used primarily to treat heart failure, edematous conditions such as ascites in severe liver diseases, secondary hyperaldosteronism due to liver cirrhosis, and essential hypertension (3). The pharmacodynamics of spironolactone are diverse; for example, it is a mineralocorticoid receptor antagonist that tends to disclose favorable patterns of renin-angiotensin-aldosterone system (RAAS) and angiotensin-converting enzyme-2 (ACE2) expression. It also reduces transmembrane serine protease 2 (TMPRSS2) activity through its antiandrogenic activity (4–6). Previous studies have noted that cell penetration of SARS-CoV-2 is associated with the ACE2 receptor and TMPRSS2 (7–9). Research has therefore suggested that spironolactone may influence the infectivity of COVID-19 (4, 10, 11).

In light of this theory, we have conducted a nationwide case-control study investigating whether spironolactone exposure could be associated with SARS-CoV-2's infectivity and complication rate in COVID-19 patients with liver cirrhosis. The null hypothesis was that there are no differences between patients with or without spironolactone exposure in terms of SARS-CoV-2's infectivity and complication rate of COVID-19.

MATERIALS AND METHODS

Data Source and Study Population

This study was approved by the Institutional Review Board of Asan Medical Center (IRB number: 2020-1153) and written informed consent was waived by the board due to the de-identified nature of the data. The anonymized data obtained from the National Health Insurance claims of Republic of Korea were analyzed. The flow of the population in this case-control study is represented in **Figure 1**.

In detail, the population-based dataset comprised all patients tested for COVID-19 from January 20, 2020, when the first case of COVID-19 was observed in South Korea, to May 15, 2020, including suspected and confirmed cases, with demographic information and medical services history for the past 3 years. The analysis was performed on 234,427 patients tested for COVID-19 with the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10) diagnosis codes of B342, B972, Z208, Z290, U18, U181, Z038, Z115, U071, and U072. Screening was conducted by performing polymerase chain reaction amplification of the viral E gene and the RdRp region of the ORF1b gene was amplified to confirm COVID-19. Among the total 234,427 patients with COVID-19 screening test results, 6,462 subjects were confirmed to have liver cirrhosis over 19 years. The presence of liver cirrhosis was established based on ICD-10 codes for liver cirrhosis (K702, K703, K704, K717, K720, K721, K729, K740-K746, K761, K766-K767, R18, I850, I859, I864, I868, I982, I983) (12). Among patients with liver cirrhosis, there were 67 (1.0%) confirmed COVID-19 cases in the case group and 6,395 (99.0%) uninfected cases in the control group. Cases and controls were matched according to a 1:5 ratio based on covariates such as sex, age, region, and tested hospital, considering the explosive outbreak in Daegu and Gyeongbuk regions (13, 14). Patients were classified to either Daegu and Gyeongbuk regions or other regions, and hospitals in which patients had been tested were classified to tertiary hospitals and others. Patients' covariates were matched, but the nearest neighbor matching was performed on age, with a caliper width of 0.1 in propensity scores. The final numbers of cases and controls were 67 and 332, respectively. Then, whether the subjects were exposed to spironolactone within 1 year from when the patients were tested for COVID-19 was evaluated.

Further subgroup analysis for complication rate was done on the case group. Complications due to severe COVID-19 disease were defined as cases requiring intervention, such as oxygen therapy, anti-viral therapy, vasopressors, admission to the intensive care unit, continuous renal replacement therapy, or death (15) (**Supplementary Table 1**). Patients were divided into two groups: those with complications and those without complications (16). There were 35 and 32 patients with and without complications, respectively.

Exposure to Spironolactone

Exposure to spironolactone was defined as the administration of spironolactone at least once within 1 year before the date of COVID-19 testing. Two additional sensitivity analyses were performed to verify the robustness of the study findings. With at least one claim within 6 months and 3 months for prescription of spironolactone, we classified these according to exposure to spironolactone and performed additional analyses. In addition, to quantify the exposure to spironolactone and to determine the dose-response association, the cumulative defined daily dose (cDDD) of spironolactone during the exposure period was calculated ($\leq$30 cDDD or $>$30 cDDD) (17). The DDD was used for measuring a prescribed amount of a given drug and was considered the assumed average daily maintenance dose of a drug according to its main indication in adults (determined from the ATC/DDD system of the WHO Collaborating Center for Drug Statistics and Methodology) (18). For spironolactone, the WHO DDD is 75 mg. cDDD was calculated as the total amount of drug divided by the amount of that drug in DDD. The illustration for the study design and spironolactone exposure is presented in **Supplementary Figure 1**.

Abbreviations: ARDS, Acute respiratory distress syndrome; CCI, Charlson Comorbidity Index; CI, Confidence interval; ESRD, End-stage renal disease; OR, Odds ratio; RAAS, Renin-angiotensin-aldosterone system; DDD, Defined daily dose; SARS-CoV-2, Severe acute respiratory syndrome coronavirus-2.

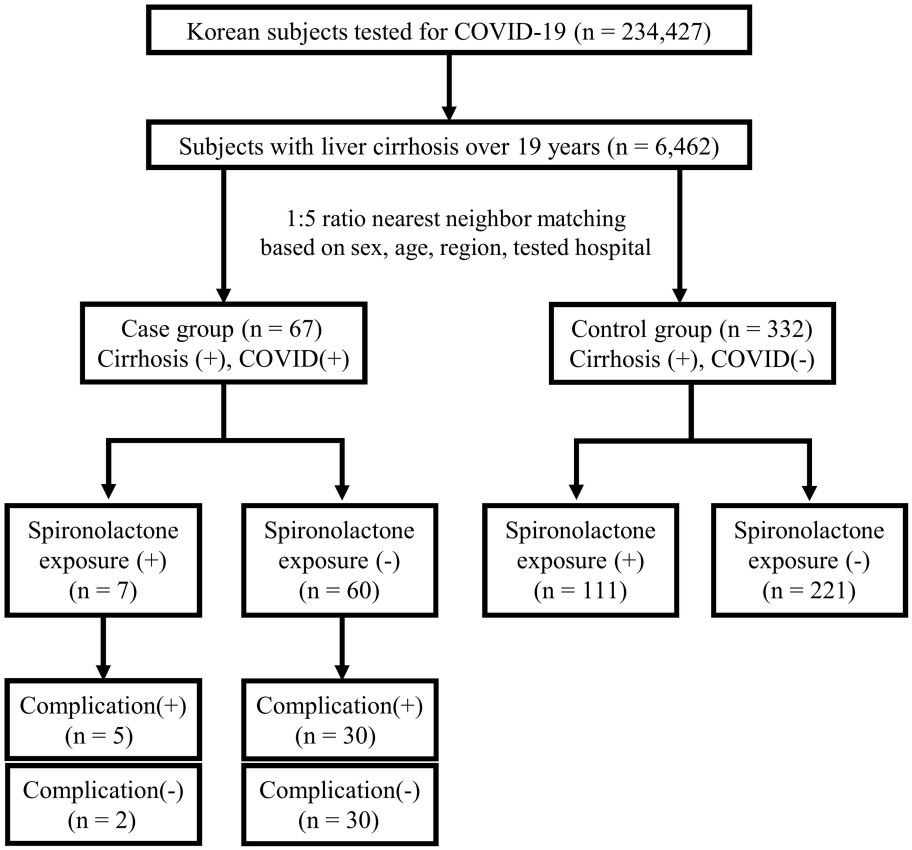

FIGURE 1 | Study flow diagram.

Definitions of Covariates

Underlying diseases were established based on diagnosis codes of the ICD-10. The considered comorbidities were decompensated liver cirrhosis, diabetes, hypertension, dyslipidemia, cardiovascular disease including myocardial infarction and stroke, cancer, lung disease including chronic obstructive pulmonary disease and asthma, end-stage renal disease (ESRD) with dialysis, and immunocompromised status including autoimmune diseases and human immunodeficiency virus infections. These comorbidities in the present study were chosen based on the announcement of Centers for Disease Control and Prevention in the U.S that these comorbidities increased risk of severe illness from COVID-19 infection (19) (**Supplementary Table 1**) The Charlson Comorbidity Index (CCI) was also used as a covariate (20), and a higher CCI score indicated a greater likelihood that the predicted outcome would result in mortality.

Statistical Analysis

Baseline characteristics of case and control groups were presented as mean with standard deviation for continuous variables, and the number with percentage (%) for categorical variables. Comparisons between both groups were performed using Student's *t*-tests for continuous variables and chi-squared or Fisher's exact tests for categorical variables. After 1:5 ratio case-control matching, the odds ratio (OR) and 95% confidence interval (CI) were calculated with conditional logistic regression analyses. For multivariable-adjusted analysis according to COVID-19 status, two models were used because of the limited study population. Model 1 was adjusted for hypertension, dyslipidemia, and CCI because CCI does not include hypertension and dyslipidemia. Model 2 was adjusted for decompensated liver cirrhosis, hypertension, cardiovascular disease, cancer, lung disease, ESRD with dialysis, and CCI, which were significant at the $P < 0.10$ level for the univariable analysis. Subgroup analysis was performed for COVID-19 status by dividing the study group by sex (male and female) and age (age $\geq$60 and $<$60 years). For multivariable-adjusted analysis according to the presence of complications, the model was adjusted for age, diabetes, hypertension, cancer, and CCI, which were significant at the $P < 0.10$ level in univariable analysis. The statistical software SAS for version 9.4 (SAS Inc., Cary, NC, USA) was used to perform all statistical analyses. A $P < 0.05$ was considered to be statistically significant.

RESULTS

Baseline Characteristics

Before matching, the number of patients in the case and control groups were 67 and 6,395, respectively. After matching, a total

TABLE 1 | Baseline characteristics of patients with liver cirrhosis, according to COVID-19.

Total (n = 399)	Patients with liver cirrhosis and COVID-19 (n = 67)	Patients with liver cirrhosis but not COVID-19 (n = 332)	P-value
Demographics			
Sex, male, n (%)	40 (59.7)	197 (59.3)	1.00
Age (years), mean (SD)	59.9 (15.7)	60.3 (15.3)	0.85
Region of diagnosis			
Daegu and Gyeongbuk, n (%)	43 (64.2)	212 (63.9)	1.00
Tested hospital			
Tertiary hospital, n (%)	9 (13.4)	45 (13.6)	0.98
Comorbidities			
Decompensated liver cirrhosis, n (%)	19 (28.4)	154 (46.4)	0.01
Diabetes, n (%)	21 (31.3)	121 (36.5)	0.43
Hypertension, n (%)	27 (40.3)	185 (55.7)	0.02
Dyslipidemia, n (%)	19 (28.4)	127 (38.3)	0.13
Cardiovascular disease, n (%)	9 (13.4)	82 (24.7)	0.04
Cancer, n (%)	12 (17.9)	113 (34.0)	0.01
Lung disease, n (%)	17 (25.4)	120 (36.1)	0.09
ESRD with dialysis, n (%)	0 (0)	21 (6.3)	0.03
Immunocompromised status, n (%)	9 (13.4)	31 (9.3)	0.31
Charlson Comorbidity Index, mean (SD)	4.3 (2.7)	6.3 (3.8)	<0.0001
Complications	35 (52.2)	55 (16.6)	<0.0001
Oxygen therapy, n (%)	12 (17.9)	32 (9.6)	0.04
Antiviral therapy, n (%)	28 (41.8)	1 (0.3)	<0.0001
Vasopressors, n (%)	4 (6.0)	14 (4.2)	0.52
Admission to the intensive care unit, n (%)	2 (3.0)	9 (2.7)	1.00
Continuous renal replacement therapy, n (%)	1 (1.5)	1 (0.3)	0.31
Death, n (%)	6 (9.0)	32 (9.6)	0.86
Exposure to spironolactone	7 (10.5)	111 (33.4)	0.0002
Non-user	60 (89.5)	221 (66.5)	0.0008
cDDD ≤30	3 (4.5)	51 (15.4)	
cDDD >30	4 (6.0)	60 (18.1)	

ESRD, end-stage renal disease; SD, standard deviation; cDDD, cumulative defined daily dose.

of 399 subjects were analyzed. The baseline characteristics of the study population are presented in **Table 1**. The mean age was 60.2 years, and the proportion of male subjects was 59.4%. The proportions of decompensated liver cirrhosis, hypertension, cardiovascular disease, cancer, lung disease, and ESRD with dialysis were significantly higher in the control group compared with the case group. The CCI was higher in the control group than case group (6.3 vs. 4.3). The complication rate was 52.2% in the case group and 16.6% in the control group ($P < 0.0001$). Among complications, the presence of oxygen therapy and anti-viral therapy was significantly higher in the case group. The proportion of spironolactone exposure was 10.5% in the case group and 33.4% in the control group ($P = 0.0002$). Of the patients exposed to spironolactone, four case and 60 control patients had a spironolactone cDDD of >30, whereas, three case and 51 control patients had a spironolactone cDDD of ≤30.

Association Between Exposure to Spironolactone and Risk of Infection With COVID-19

The results of the logistic regression analysis for COVID-19 infection according to exposure to spironolactone are shown in **Table 2**. The adjusted OR (95% CI) in model 2 for COVID-19 between patients who were and were not exposed to spironolactone within 1 year was 0.20 (0.07–0.54). Additional analyses within 6 months and 3 months also show a significant difference between case and control groups ($P < 0.05$). Using non-users as reference, the adjusted ORs for patients with a spironolactone cDDD of ≤30 and >30 were significant regardless of different definitions for the timing of spironolactone exposure. However, a dose-response relationship was not shown for the association between spironolactone exposure and COVID-19 (**Table 2**).

TABLE 2 | Odds ratios and 95% confidence intervals for COVID-19 according to exposure to spironolactone.

	Case (%)	Control (%)	Crude OR (95% CI)	*P*-value	Adjusted OR* (95% CI)	*P*-value	Adjusted OR† (95% CI)	*P*-value
Within 1 year								
Total	67 (100)	332 (100)						
Without exposure to spironolactone	60 (89.5)	221 (66.6)	1.00		1.00		1.00	
Exposure to Spironolactone	7 (10.5)	111 (33.4)	0.19 (0.08–0.47)	0.0003	0.21 (0.08–0.55)	0.001	0.20 (0.07–0.54)	0.002
cDDD for spironolactone								
Non-user	60 (89.5)	221 (66.5)	1.00		1.00		1.00	
cDDD ≤30	3 (4.5)	51 (15.4)	0.22 (0.07–0.72)	0.01	0.25 (0.08–0.86)	0.03	0.23 (0.07–0.78)	0.02
cDDD >30	4 (6.0)	60 (18.1)	0.25 (0.09–0.70)	0.009	0.32 (0.11–0.93)	0.04	0.30 (0.10–0.89)	0.03
Within 6 months								
Total	58 (100)	287 (100)						
Without exposure to Spironolactone	52 (89.7)	187 (65.2)	1.00		1.00		1.00	
Exposure to Spironolactone	6 (10.3)	100 (34.8)	0.17 (0.06–0.45)	0.0004	0.198 (0.071–0.555)	0.002	0.17 (0.06–0.49)	0.001
cDDD for spironolactone								
Non-user	52 (89.6)	187 (65.1)	1.00		1.00		1.00	
cDDD ≤30	3 (5.2)	51 (17.8)	0.21 (0.06–0.71)	0.01	0.26 (0.07–0.88)	0.03	0.25 (0.07–0.87)	0.03
cDDD >30	3 (5.2)	49 (17.1)	0.22 (0.07–0.74)	0.01	0.27 (0.08–0.92)	0.04	0.27 (0.08–0.93)	0.04
Within 3 months								
Total	49 (100)	245 (100)						
Without exposure to Spironolactone	43 (87.8)	156 (63.7)	1.00		1.00		1.00	
Exposure to Spironolactone	6 (12.2)	89 (36.3)	0.22 (0.09–0.56)	0.002	0.26 (0.10–0.68)	0.006	0.23 (0.08–0.64)	0.005
cDDD for spironolactone								
Non-user	43 (87.8)	156 (63.7)	1.00		1.00		1.00	
cDDD ≤30	3 (6.1)	48 (19.6)	0.23 (0.07–0.76)	0.02	0.25 (0.07–0.86)	0.03	0.26 (0.08–0.90)	0.03
cDDD >30	3 (6.1)	41 (16.7)	0.27 (0.08–0.90)	0.03	0.31 (0.09–1.05)	0.06	0.28 (0.08–1.00)	0.05

**Model 1: adjusted for hypertension, dyslipidemia, and Charlson Comorbidity Index.*
† Model 2: adjusted for decompensated liver cirrhosis, hypertension, cardiovascular disease, cancer, lung disease, ESRD with dialysis, and Charlson comorbidity index.
OR, odds ratio; CI, confidence interval; ESRD, end-stage renal disease; cDDD, cumulative defined daily dose.

Subgroup Analysis for COVID-19 Status According to Sex and Age

For risk stratification, subgroup analyses for COVID-19 status were performed by stratifying the study population by sex and age. The results of these analyses are shown in **Supplementary Table 2**. Importantly, most of the adjusted ORs and 95% CIs were found to be significant, especially in men and patients over 60 years of age.

Comparison Between the Complication and No Complication Groups of Patients With Liver Cirrhosis and COVID-19

Baseline characteristics of the complication and no complication groups of patients with liver cirrhosis and COVID-19 infection are shown in **Table 3**. The proportions of diabetes, hypertension, and cancer were significantly higher in the complication group than in the no complication group. There was no significant difference in the proportion of patients exposed to spironolactone between the complication and no complication groups ($P = 0.43$). The crude and adjusted ORs (95% CI) of spironolactone exposure for the development of COVID-19-related complications were 2.50 (0.45–13.91) and 1.714 (0.25–11.94), respectively.

DISCUSSION

To summarize, the results showed that a significantly low proportion of cirrhosis patients with COVID-19 had previous exposure to spironolactone. Spironolactone was not significantly associated with complications. The factors associated with complications in cirrhotic patients with COVID-19 were

TABLE 3 | Baseline characteristics of patients with liver cirrhosis and COVID-19.

Total ($n = 67$)	Patients with complications ($n = 35$)	Patients without complications ($n = 32$)	*P*-value
Demographics			
Sex, male, n (%)	21 (60.0)	19 (59.4)	0.96
Age (years), mean (SD)	63.5 (15.8)	56.0 (14.8)	0.05
Region of diagnosis			
Daegu and Gyeongbuk, n (%)	21 (60.0)	22 (68.8)	0.46
Tested hospital			
Tertiary hospital, n (%)	5 (14.3)	4 (12.5)	1.00
Comorbidities			
Decompensated liver cirrhosis, n (%)	13 (37.1)	6 (18.8)	0.10
Diabetes, n (%)	15 (42.9)	6 (18.8)	0.03
Hypertension, n (%)	19 (54.3)	8 (25.0)	0.01
Dyslipidemia, n (%)	9 (25.7)	10 (31.3)	0.62
Cardiovascular disease, n (%)	4 (11.4)	5 (15.6)	0.73
Cancer, n (%)	10 (28.6)	2 (6.3)	0.02
Lung disease, n (%)	10 (28.6)	7 (21.9)	0.53
ESRD with dialysis, n (%)	0 (0)	0 (0)	-
Immunocompromised status, n (%)	4 (11.4)	5 (15.6)	0.73
Charlson Comorbidity Index, mean (SD)	5.0 (2.9)	3.5 (2.3)	0.02
Complications			
Oxygen therapy, n (%)	12 (17.9)	–	–
Antiviral therapy, n (%)	28 (41.8)	–	–
Vasopressors, n (%)	4 (6.0)	–	–
Admission for intensive care unit, n (%)	2 (3.0)	–	–
Continuous renal replacement therapy, n (%)	1 (1.5)	–	–
Death, n (%)	6 (9.0)	–	–
Exposure to spironolactone	5 (14.3)	2 (6.3)	0.43
Non-user	30 (85.7)	30 (90.9)	0.12
cDDD $\leq$30	1 (2.9)	2 (9.1)	
cDDD >30	4 (11.4)	0 (0)	

ESRD, end-stage renal disease; SD, standard deviation; cDDD, cumulative defined daily dose.

diabetes, hypertension, cancer, and CCI score. This result of high-risk factors coincides with those indicated in previous studies (21, 22). Therefore, the null hypothesis was partially accepted and partially rejected.

The value of our study is that it provides theoretical evidence for the role of spironolactone in terms of COVID-19 susceptibility. A previous study by Cadegiani et al. (4) has proposed that spironolactone may have protective effects against COVID-19. Cadegiani et al. suggested that spironolactone could be a plausible candidate for prophylactic and early treatment of COVID-19. This was based on the theory that spironolactone could avoid SARS-CoV-2 cell entry by modulation of ACE2 expression, decreasing viral priming by reducing TMPRSS2 activity, attenuating the damage caused by the overexpression of angiotensin II-AT-1 axis, and inducing anti-inflammatory effects in the lungs through pleiotropy. Our study has shown that patient cases with COVID-19 had statistically significant lower exposure to spironolactone compared with patients without COVID-19 in liver cirrhosis controls. Considering that decompensated liver cirrhosis, hypertension, cardiovascular disease, cancer, ESRD, and CCI were higher in patients without COVID-19, it can be concluded that spironolactone may have protective effects against SARS-CoV-2's infectivity.

In our study, the result showed that there were no statistically significant correlations between complication rate and spironolactone exposure. This result could be distorted because there were only 35 patients in the complication group, which were too small, and comorbidities were unequally distributed, specifically the significantly higher CCI score of the complication group compared with the no complication group, which could raise the complication rate. When baseline characteristics from previous studies were analyzed (diabetes, hypertension, cancer, and CCI) as risk factors for COVID-19 complications, they were higher in patients in the complication group compared with those in the without complication group (21, 22). For these reasons, the protective effect against COVID-19 complication of spironolactone could be masked.

We acknowledge the limitations of our study. First, we used data from national health insurance claims, which potentially caused some discrepancies between actual therapeutic practices. In addition, due to the nature of the present study, biases from the unequal distribution of comorbidities between the two

groups might have affected the association between the use of spironolactone and COVID-19, despite statistical adjustments. Second, it was challenging to define ARDS, so complications induced by this condition included cases treated with oxygen therapy and other severe complications related to the disease. Third, the susceptibility of contagious diseases can be affected by multiple factors such as sociocultural factors, which can be difficult to anticipate. We were also not able to gather information regarding patients' lifestyle-related factors such as smoking and alcohol drinking, which might affect the outcome of our study. Additionally, there was a small number of COVID-19 cases in patients with liver cirrhosis. Moreover, our study lacked detailed information about severity or stage of liver cirrhosis. Therefore, our results should be interpreted with caution because only complications in patients with COVID-19 and liver cirrhosis, and whether these patients were exposed to spironolactone, were investigated. Our results should therefore be validated in a larger cohort study.

Our study is the first to investigate the impact of spironolactone on patient susceptibility to COVID-19, and the prevalence of its associated complications. Based on relevant statistical analysis, patients who were infected by COVID-19 with underlying liver cirrhosis showed significantly lower spironolactone exposure rate compared to patients who were not infected by COVID-19 with underlying liver cirrhosis. Therefore, our results suggested that exposure of spironolactone may reduce susceptibility to COVID-19 in patients with liver cirrhosis. Further studies are needed to confirm the exact association between spironolactone and COVID-19.

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 at: https://hira-covid19.net/.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Asan Medical Center, Seoul, Republic of Korea (IRB number: 2020-1153). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

DJ, MS, and JC were responsible for the conception and design of the study, acquisition, analysis and interpretation of the data, and drafting of the manuscript. MS performed the statistical analyses. All authors have full access to all data used in the study and take responsibility for the integrity of the data and the accuracy of the data analysis, and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.629176/full#supplementary-material

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ORIGINAL RESEARCH
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Acceptability of a COVID-19 Vaccine Among Healthcare Workers in the Kingdom of Saudi Arabia

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Objective: This study aims to determine the acceptability of a COVID-19 vaccine among healthcare workers in Saudi Arabia and the factors affecting their intention to accept the vaccine.

Methods: The study used data from an online cross-sectional survey that was conducted in Saudi Arabia between 8 December 2020 and 14 December 2020. This study employed bivariate and multivariable regression analyses. The bivariate was used to describe and tabulate the frequency of all the variables, including the sociodemographic characteristics, the risk perception and the acceptance of the COVID-19 vaccination and a chi-squared test of independence was calculated. Multivariable logistic regression models were employed to examine and identify the factors associated with an intention to have the COVID-19 vaccination and the factors associated with its immediate acceptance.

Results: Of the total of 736 healthcare workers who began the online questionnaire, 673 completed it (a 91.44% completion rate). Among the study participants, 50.52% were willing to have the COVID-19 vaccine, of which 49.71% intended to have the vaccine as soon as it becomes available in the country, while 50.29% would delay until the vaccine's safety is confirmed. Being a male healthcare worker, perceiving a high risk of infection, and believing that the COVID-19 vaccine should be compulsory for all citizens and residents in the country increased the probability of intention to vaccinate against COVID-19 and the probability of accepting the COVID-19 vaccination as soon as possible.

Conclusion: This study calls for more health-related education among healthcare workers to alleviate any fears that might be associated with the COVID-19 vaccine.

Keywords: acceptability, COVID-19, healthcare workers, hesitancy, Saudi Arabia, vaccine

INTRODUCTION

The world is witnessing a major global humanitarian disaster due to the spread of the Coronavirus disease 2019 (COVID-19), which has affected all aspects of life across the planet. Countries around the world have implemented strict precautions and controls to contain the outbreak of COVID-19, which, among others, include social distancing and mandatory use of face coverings (1, 2). However, it is recognized that such preventive measures may neither be enough nor sufficient to halt the spread of COVID-19. Therefore, the vaccine's development and deployment is one of the most promising health intervention strategies to mitigate the spread of COVID-19 (3, 4).

COVID-19 vaccines are finally becoming available and many countries, including the Kingdom of Saudi Arabia (KSA), are already reserving supplies of the long-awaited vaccine. Following the Saudi Food and Drug Authority approval of the Pfizer-BioNTech COVID-19 vaccine, the country is set to introduce a phased vaccine rollout. Healthcare workers, the elderly, and patients with chronic and autoimmune diseases are scheduled to be early recipients of the vaccine (5). However, the success of any vaccination programme depends on high vaccine acceptance and uptake, and the main challenge that now lies ahead is building public confidence in an emergency-released vaccine. Without such confidence, vaccine hesitancy is immanent (6).

Vaccine hesitancy is defined as "the delay in acceptance or refusal of vaccination despite the availability of vaccination services," and it is a global concern and a crucial factor in under-vaccination (7). Vaccine hesitancy presents a barrier to immunization program success and, in fact, has been identified by the World Health Organization (WHO) as one of the top 10 global health threats in 2019 (8). Despite the global effort to bring an end to the pandemic, anti-vaccination sentiments that spread misinformation on the dangers and consequences of vaccination cause hesitancy in immunization against preventable infectious diseases (9).

Healthcare workers play an important role in immunization program success and research has shown that their knowledge and attitudes in relation to vaccines determine their intentions for vaccine uptake and their recommendation of the vaccine (10, 11). There is a wealth of literature showing that healthcare workers can themselves be vaccine hesitant and their hesitancy levels can thus impact hesitancy and aversion to receiving the vaccine among the general public (12–14). Additionally, it has been reported that healthcare workers who have negative attitudes, are averted, or are hesitant about vaccinations share these unfavorable attitudes and tend to recommend vaccination to their patients infrequently (15).

Research studies assessing the uptake of seasonal and/or pandemic influenza vaccines among healthcare workers found that vaccine acceptance among this population is low. Various factors were found to underlie this behavior, which include low perceived benefits, low perceived risk of infection, fear of side effects and concerns surrounding safety and efficacy (16–19). Given the significant role of vaccinated healthcare workers on shaping the general population's decisions to vaccinate (20, 21), and as the availability of the vaccine does not necessarily translate into its adoption, this study thus aims to determine the COVID-19 vaccine's acceptability among healthcare workers in the KSA and to identify the factors affecting their intention to accept it. In this paper, healthcare workers are those who work in healthcare settings and deliver care and services to the sick and ailing either directly or indirectly such as physicians, dentists, nurses, pharmacists, and allied health professionals.

This study lands at a critical time for the Saudi health authorities as it is undertaken during the COVID-19 pandemic, specifically following the approval and before the arrival of the vaccine to the KSA. The results of this study are expected to provide insight into projected vaccine uptake and underlying drivers of vaccine-related decision making among healthcare workers. By understanding this, effective strategies can be developed to enhance COVID-19 vaccine uptake in the KSA, as well as in other countries in the Arabian Gulf. This study contributes to the limited literature on the demand (acceptability) of the novel COVID-19 vaccine in several ways. First, it assesses the demand for the vaccine across the healthcare workers who are not only at an increased risk of contracting and transmitting COVID-19 but whose acceptance of the vaccine is significant in preventing the transmission of the virus between medical personnel and patients. Second, this study represents one of the first findings on this matter in the KSA which is among the few countries that was able to successfully maintain a handle on the virus.

MATERIALS AND METHODS

Study Design and Sample

This study used data from a cross-sectional survey that was conducted on the acceptability of a COVID-19 vaccine among the public and healthcare practitioners in the KSA from 8 December 2020 to 14 December 2020. The study recruited all participants from an online survey, via a self-reported questionnaire, using SurveyMonkey. Invitations to participate in the study were distributed to the respondents via Twitter and the WhatsApp communication platform. The participants were recruited using a simplified-snowball sampling technique where the invited participants were requested to pass the invitations to their WhatsApp contacts. The online approach is currently being used in order to avoid further physical contact as it might pose a risk of spreading the COVID-19 infection.

The target population was individuals aged 18 years or older and currently living in the KSA. Online informed consents were obtained from all participants before proceeding with the questions. The informed consent provided two options: "yes" for those who volunteered to participate in the study and "no" for those who did not wish to. Only those who selected the affirmative response were taken to the questionnaire page to complete the survey. The respondents were clearly informed about the study's aim and objectives and were also advised that they were free to withdraw from the study at any time, without giving a reason, and that all information and opinions provided would be anonymous and confidential.

Measures

The self-reported questionnaire was designed and adapted by the authors based on similar studies and frameworks to assess vaccine acceptance for newly emerging infectious diseases (2, 7, 10, 22–24). The questionnaire was originally in English. M.K.A and N.A. translated the questions into Arabic, while A.M.N.Q and O.A. translated it back to English to ensure that the translation preserved the meaning captured by the original English version. The survey then used the Arabic text to administer the study.

The questionnaire consisted of 3 primary sections. The first section gathered information on the respondents' sociodemographic characteristics, including age, gender, marital status, education level, region in which they were currently residing, income level, and whether the healthcare practitioner was working on the front line in facing COVID-19. The second section collected information on the respondents' health status, vaccination history and perceived COVID-19 risk. The third section collected information on the acceptability of a COVID-19 vaccine.

Statistical Analyses

The survey's primary outcome was the acceptance of the COVID-19 vaccination. In order to measure vaccination intention, the participants were asked about their willingness to be vaccinated. The respondents were provided with an informative statement that "scientists around the world are currently working on a vaccine that could prevent people from getting infected with COVID-19. It is hoped that the vaccine will become available in a few months." The participants were then asked the following question "In the case that a COVID-19 vaccine becomes available in the next few months, with an effective rate of the COVID-19 vaccine between 90 and 95%, would you be willing to get the COVID-19 vaccine if it was provided free by the government?". The respondents' options included "yes" or "no." Respondents who stated "no," that they are not willing to be vaccinated, were asked to indicate the main reason for their unwillingness to be vaccinated. Respondents who stated "yes," and thereby showed a willingness to be vaccinated, were asked whether they would be willing to have the COVID-19 vaccine (to be vaccinated) as soon as possible when it became available or to delay vaccination until the vaccine safety was confirmed.

Some explanatory variables were collected. Respondents were asked about their sociodemographic characteristics, including their age, gender, marital status, the region in which they were residing, monthly income and whether they were working on the front line in facing COVID-19. The age variable was divided into five categories: 18–29 (the reference category), 30–39, 40–49, 50–59, and ≥60. Gender was coded as a dummy variable, with one for male and zero for female. Marital status was captured as binary, and a value of one was used for marriage and zero for otherwise (including single, widowed and divorced). Monthly income (Saudi Riyal, SR 1 = USD 0.27) was grouped into eight categories: <SR 3,000 (the reference category), SR 3,000 to <5,000, SR 5,000 to <7,000, SR 7,000 to <10,000, SR 10,000 to <15,000, SR 15,000 to <20,000, SR 20,000 to <30,000, and ≥SR 30,000. The healthcare workers status in relation to COVID-19 was also coded as one for those who are frontline healthcare workers and zero for otherwise. The region status covered all of the 13 administrative regions in the KSA, including Riyadh, Mekkah, Almadina Almonawra, Qaseem, Eastern Region, Aseer, Tabouk, Haiel, Northern Borders, Jazan, Najran, Albaha, and Aljouf, and was grouped into five categories, which are Central, West, East, North, and South.

Information was also collected on the healthcare worker respondents' health status, vaccination history and perceived COVID-19 risk. Respondents were asked whether they had a chronic illness that made them clinically vulnerable to serious illness from COVID-19 (yes, no), if they had been vaccinated for seasonal influenza (yes, no) and if they had ever refused a vaccine recommended by a physician because of doubts about it (yes, no).

The participants were also asked about psychological factors. The respondents were asked to what extent they thought COVID-19 poses a risk to people in Saudi Arabia, on a five-point Likert scale, from "no risk at all" to "major risk." Additionally they were asked to what extent they are concerned about getting infected with COVID-19, on a five-point Likert scale, from "very low" to "very high." They were also asked whether they have been infected with, or currently have, COVID-19 (yes, no), if any of their family members have been, or currently are, infected with COVID-19 (yes, no) and if any of their friends have been, or currently are, infected with COVID-19 (yes, no). The healthcare worker respondents were also asked whether they support that the COVID-19 vaccine should be compulsory for all citizens and residents inside Saudi Arabia or not.

This study employed bivariate and multivariable regression analyses. The bivariate analysis was done as cross-tabulation between all the variables and our dependent variable of interest using chi-squared tests. A multivariable logistic regression analysis was employed to examine and identify the variables associated with an intention to have the COVID-19 vaccination, with the odds ratio (OR), and a 95% confidence interval (CI) being calculated. Additionally, a multivariable logistic regression analysis was also performed to examine and identify factors associated with the vaccine demand group (immediate acceptance and delayed acceptance). All analyses were conducted using STATA 15.1 software (StataCorp LP, Texas, USA).

Ethical Considerations

All procedures performed in this study involving human participants complied with the institutional and/or national research committee ethical standards and the 1964 Helsinki declaration and subsequent amendments or equivalent ethical standards. This research has been reviewed and given a favorable opinion by King Abdulaziz University. The study was designed and conducted in accordance with the ethical principles established by King Abdulaziz University and, therefore, ethical approval was obtained from the Biomedical Ethics Research Committee, Faculty of Medicine, King Abdulaziz University (Ref-628-20).

TABLE 1 | Frequency distribution and chi-square analysis of intentions of COVID-19 vaccination acceptance.

Variable	Willing to accept COVID-19 vaccination 340 (50.52%)		Not willing to accept COVID-19 vaccination 333 (49.48%)		Total		*P*-value
	A		B		C		D
Age							
18–29	80	(23.53%)	67	(20.12%)	147	(21.84%)	0.242
30–39	147	(43.24%)	158	(47.45%)	305	(45.32%)	
40–49	79	(23.24%)	62	(18.62%)	141	(20.95%)	
50–59	23	(6.76%)	33	(9.91%)	56	(8.32%)	
≥60	11	(3.24%)	13	(3.90%)	24	(3.57%)	
Gender							
Female	112	(32.94%)	156	(46.85%)	268	(39.82%)	0.000***
Male	228	(67.06%)	177	(53.15%)	405	(60.18%)	
Marital status							
Unmarried	106	(31.18%)	97	(29.13%)	203	(30.16%)	0.563
Married	234	(68.82%)	236	(70.87%)	470	(69.84%)	
Location							
Central	43	(12.65%)	52	(15.62%)	95	(14.12%)	0.000***
South	86	(25.29%)	37	(11.11%)	123	(18.28%)	
East	32	(9.41%)	42	(12.61%)	74	(11.00%)	
North	13	(3.82%)	9	(2.70%)	22	(3.27%)	
West	166	(48.82%)	193	(57.96%)	359	(53.34%)	
Monthly income							
<SR 3,000	43	(12.65%)	28	(8.41%)	71	(10.55%)	0.169
SR 3,000 to <SR 5,000	17	(5.00%)	9	(2.70%)	26	(3.86%)	
SR 5,000 to <SR 7,000	22	(6.47%)	15	(4.50%)	37	(5.50%)	
SR 7,000 to <SR 10,000	39	(11.47%)	49	(14.71%)	88	(13.08%)	
SR 10,000 to <SR 15,000	99	(29.12%)	97	(29.13%)	196	(29.12%)	
SR 15,000 to < SR 20,000	64	(18.82%)	68	(20.42%)	132	(19.61%)	
SR 20,000 to < SR 30,000	29	(8.53%)	28	(8.41%)	57	(8.47%)	
≥SR 30,000	27	(7.94%)	39	(11.71%)	66	(9.81%)	
Frontline healthcare worker							
No	157	(46.18%)	189	(56.76%)	346	(51.41%)	0.006***
Yes	183	(53.82%)	144	(43.24%)	327	(48.59%)	
Having chronic conditions							
No	270	(79.41%)	272	(81.68%)	542	(80.53%)	0.457
Yes	70	(20.59%)	61	(18.32%)	131	(19.47%)	
Received flu vaccination in the past							
No	79	(23.24%)	114	(34.23%)	193	(28.68%)	0.002***
Yes	261	(76.76%)	219	(65.77%)	480	(71.32%)	
Refused vaccination in the past							
No	312	(91.76%)	225	(67.57%)	537	(79.79%)	0.000***
Yes	28	(8.24%)	108	(32.43%)	136	(20.21%)	
Infected with COVID-19							
No	278	(81.76%)	281	(84.38%)	559	(83.06%)	0.365
Yes	62	(18.24%)	52	(15.62%)	114	(16.94%)	
Family infected with COVID-19							
No	201	(59.12%)	197	(59.16%)	398	(59.14%)	0.991
Yes	139	(40.88%)	136	(40.84%)	275	(40.86%)	

(Continued)

TABLE 1 | Continued

Variable	Willing to accept COVID-19 vaccination 340 (50.52%)		Not willing to accept COVID-19 vaccination 333 (49.48%)		Total		*P*-value
	A		B		C		D
Friends infected with COVID-19							
No	27	(7.94%)	35	(10.51%)	62	(9.21%)	0.249
Yes	313	(92.06%)	298	(89.49%)	611	(90.79%)	
Perceived risk of COVID-19 to people in Saudi Arabia							
Minor risk or no risk	52	(15.29%)	70	(21.02%)	122	(18.13%)	0.010**
Moderate risk	118	(34.71%)	134	(40.24%)	252	(37.44%)	
Significant or major risk	170	(50.00%)	129	(38.74%)	299	(44.43%)	
Concerned about getting infected with COVID-19							
Low or very low	124	(36.47%)	158	(47.45%)	282	(41.90%)	0.000***
Fair	109	(32.06%)	116	(34.83%)	225	(33.43%)	
High or very high	107	(31.47%)	59	(17.72%)	166	(24.67%)	
A COVID-19 vaccine should be compulsory for all citizens and residents inside Saudi Arabia							
No	92	(27.06%)	314	(94.29%)	406	(60.33%)	0.000***
Yes	248	(72.94%)	19	(5.71%)	267	(39.67%)	

$^{***}p < 0.01$, $^{**}p < 0.05$.

RESULTS

Of the total of 736 healthcare workers who began the online questionnaire, 673 completed it (a 91.44% completion rate). Among the 673 participants, 340 (50.52%) respondents were willing to have the COVID-19 vaccine if it was provided free by the government, while 333 (49.48%) were not willing to be vaccinated. **Table 1** shows the frequency distribution of the intentions of COVID-19 vaccination acceptance by different the healthcare worker participants' characteristics and the factors that influence vaccination acceptance.

Most of the healthcare worker participants were aged 30–49 (45.32%) and were male (60.18%). More than half of the respondents (60.18%) were married. About 29% of participants indicated that their income was in the range SR 10,000 to <SR 15,000, while only 10.55% of the participants were within the lower-income category of <SR 3,000.

327 respondents were frontline healthcare workers, thereby representing 48.59% of the sample. Four hundred eighty of the respondents (71.32%) received a flu vaccine in the past. About 17% had a history of being infected with COVID-19 and 20.21% had previously refused a vaccination recommended by a physician. Regarding the perceived risk of COVID-19 to people in Saudi Arabia, a majority (44.43%) perceived that it poses a significant or major risk to the people of Saudi Arabia, although many of the respondents (41.90%) thought that they had a minor or no risk of catching COVID-19. Suffice to say, a majority (60.33%) thought that the vaccine should not be compulsory [see column (C)].

As can be seen in **Table 1**, from column (D), it was found that gender, location, being a frontline healthcare worker, having received the flu vaccination in the past, having refused a vaccination recommended by a physician in the past, the perceived risk of COVID-19 to people in Saudi Arabia, the concern of being infected with COVID-19 and the participants' belief that the COVID-19 vaccine should be compulsory for all citizens and residents inside Saudi Arabia were all statistically significant.

It was also found that no significant association across age groups. Among those who showed a willingness to be vaccinated, more were male (67.06%), whereas, among those who said that they were not willing to be vaccinated, 46.85% were females. No significant association was observed across all income categories. **Table 1** also lists additional results regarding the distribution of the other variables.

Table 2 shows the distribution of the vaccine demand group (the immediate acceptance group and vaccine delayed acceptance group) and the factors that influence vaccination–immediate or delayed–acceptance. Among 340 healthcare workers who were willing to be vaccinated, 169 (49.71%) respondents were willing to be vaccinated as soon as possible once the vaccine becomes available. On the other hand, 171 (50.29%) respondents would delay the vaccination until the vaccine's safety was confirmed.

As can be seen in **Table 2**, it was found that gender, being a frontline healthcare worker, being concerned about getting infected with COVID-19 and the participants' belief that the COVID-19 vaccine should be compulsory for all citizens and residents inside Saudi Arabia were all statistically significant. More males (72.19%) were willing to be vaccinated as soon as the vaccine becomes available than females. **Table 2** also lists additional results regarding the distribution of the other variables.

Havin narrated the bivariate analysis, the next step is to present the multivariable logistic regression regarding the factors that are associated with the willingness to be vaccinated. These findings are reported in **Table 3**. For most age groups,

TABLE 2 | Frequency distribution and chi-square analysis of the vaccine demand group (immediate or delayed acceptance).

Variable	Immediate acceptance 169 (49.71%)		Delayed acceptance 171 (50.29%)		Total		*P*-value
Age							
18–29	38	(22.49%)	42	(24.56%)	80	(23.53%)	0.899
30–39	73	(43.20%)	74	(43.27%)	147	(43.24%)	
40–49	40	(23.67%)	39	(22.81%)	79	(23.24%)	
50–59	11	(6.51%)	12	(7.02%)	23	(6.76%)	
≥60	7	(4.14%)	4	(2.34%)	11	(3.24%)	
Gender							
Female	47	(27.81%)	65	(38.01%)	112	(32.94%)	0.045**
Male	122	(72.19%)	106	(61.99%)	228	(67.06%)	
Marital status							
Unmarried	50	(29.59%)	56	(32.75%)	106	(31.18%)	0.529
Married	119	(70.41%)	115	(67.25%)	234	(68.82%)	
Location							
Central	18	(10.65%)	25	(14.62%)	43	(12.65%)	0.370
South	43	(25.44%)	43	(25.15%)	86	(25.29%)	
East	19	(11.24%)	13	(7.60%)	32	(9.41%)	
North	4	(2.37%)	9	(5.26%)	13	(3.82%)	
West	85	(50.30%)	81	(47.37%)	166	(48.82%)	
Monthly income							
<SR 3,000	17	(10.06%)	26	(15.20%)	43	(12.65%)	0.657
SR 3,000 to <SR 5,000	9	(5.33%)	8	(4.68%)	17	(5%)	
SR 5,000 to <SR 7,000	8	(4.73%)	14	(8.19%)	22	(6.47%)	
SR 7,000 to <SR 10,000	21	(12.43%)	18	(10.5%3)	39	(11.47%)	
SR 10,000 to <SR 15,000	54	(31.95%)	45	(26.32%)	99	(29.12%)	
SR 15,000 to <SR 20,000	31	(18.34%)	33	(19.30%)	64	(18.82%)	
SR 20,000 to <SR 30,000	16	(9.47%)	13	(7.60%)	29	(8.53%)	
≥SR 30,000	13	(7.69%)	14	(8.19%)	27	(7.94%)	
Frontline healthcare worker							
No	70	(41.42%)	87	(50.88%)	157	(46.18%)	0.080*
Yes	99	(58.58%)	84	(49.12%)	183	(53.82%)	
Having chronic conditions							
No	127	(75.15%)	143	(83.63%)	270	(79.41%)	0.0530*
Yes	42	(24.85%)	28	(16.37%)	70	(20.59%)	
Received flu vaccination in the past							
No	35	(20.71%)	44	(25.73%)	79	(23.24%)	0.2730
Yes	134	(79.29%)	127	(74.27%)	261	(76.76%)	
Refused vaccination in the past							
No	158	(93.49%)	154	(90.06%)	312	(91.76%)	0.250
Yes	11	(6.51%)	17	(9.94%)	28	(8.24%)	
Infected with COVID-19							
No	141	(83.43%)	137	(80.12%)	278	(81.76%)	0.429
Yes	28	(16.57%)	34	(19.88%)	62	(18.24%)	
Family infected with COVID-19							
No	106	(62.72%)	95	(55.56%)	201	(59.12%)	0.179
Yes	63	(37.28%)	76	(44.44%)	139	(40.88%)	
Friends infected with COVID-19							
No	14	(8.28%)	13	(7.60%)	27	(7.94%)	0.816
Yes	155	(91.72%)	158	(92.40%)	313	(92.06%)	

(Continued)

TABLE 2 | Continued

Variable	Immediate acceptance 169 (49.71%)		Delayed acceptance 171 (50.29%)		Total		*P*-value
Perceived risk of COVID-19 to people in Saudi Arabia							
Minor risk or no risk	28	(16.57%)	24	(14.04%)	52	(15.29%)	0.217
Moderate risk	51	(30.18%)	67	(39.18%)	118	(34.71%)	
Significant or major risk	90	(53.25%)	80	(46.78%)	170	(50.00%)	
Concerned about getting infected with COVID-19							
Low or very low	52	(30.77%)	72	(42.11%)	124	(36.47%)	0.081*
Fair	57	(33.73%)	52	(30.41%)	109	(32.06%)	
High or very high	60	(35.50%)	47	(27.49%)	107	(31.47%)	
A COVID-19 vaccine should be compulsory for all citizens and residents inside Saudi Arabia							
No	26	(15.38%)	66	(38.60%)	92	(27.06%)	0.000***
Yes	143	(84.62%)	105	(61.40%)	248	(72.94%)	

$^{***}p < 0.01$, $^{**}p < 0.05$, $^{*}p < 0.1$.

there was no significant difference among the age groups, except for the 40–49 years old category, who were more likely to get vaccinated than those in the 18–29 years old category (OR: 2.226; 95% CI: 0.957–5.176). Furthermore, males were more likely to get vaccinated than females (OR: 1.609; 95% CI: 0.971–2.665). Healthcare workers living in the South were more likely to accept the vaccine (OR: 2.458; 95% CI: 1.047–5.775) compared to the people who indicated that they live in the Central region. In addition to the above, it is interesting to observe that no significant differences were observed across income quintiles, or being a frontline healthcare worker, having a chronic disease, and receiving a flu vaccine in the past.

As can be seen in **Table 3**, healthcare workers who had ever refused a vaccine recommended by a physician because of doubts about it were less likely to be willing to be vaccinated (OR: 0.252; 95% CI: 0.129–0.493) compared with those who had never refused a vaccination. Another interesting result is concerning those who indicated that they were infected with COVID-19 in the past, as it showed that they were more likely to be vaccinated compared to those who had never been infected with COVID-19 (OR: 1.841; 95% CI: 0.893–3.795). The perceived risk of COVID-19 to people of Saudi Arabia and the concerns regarding catching COVID-19 were also associated with higher willingness to be vaccinated, as opposed to those who perceived the COVID-19 risk to people in Saudi Arabia as minor or no risk and those having low or very low concern of getting infected with COVID-19. Lastly, those who support that the vaccine for COVID-19 should be mandatory were more likely to express that they were willing to be vaccinated (OR: 43.654; 95% CI: 24.592–77.502).

Table 4 shows the analysis for the group that had shown willingness to be vaccinated only ($n = 340$). Multivariate logistic regression was performed between the immediate acceptance group ($n = 169$) and the delayed acceptance group ($n = 171$) to identify the factors that influence vaccination acceptance (immediate or delayed acceptance).

Among those who would accept vaccination, males (OR: 1.706; 95% CI: 0.986–2.952) were more likely to accept the COVID-19 vaccination as soon as possible once it becomes available when compared to females. Healthcare workers who perceived a high or very high risk of infection with COVID-19 (OR: 1.888; 95% CI: 0.893–3995) were more likely to accept COVID-19 vaccination as soon as possible once it becomes available than those who had a low or very low concern. Moreover, those who had the perception that a COVID-19 vaccine should be compulsory for all citizens and residents inside Saudi Arabia (OR: 3.666; 95% CI: 2.03–6.608) were also more likely to be willing to be vaccinated as soon as possible once it becomes available when compared to those who thought that the vaccine should not be mandatory.

Moving away from the bivariate and logistic regression analysis, it is also imperative to look into the reasons why people were not willing to get vaccinated and the findings pertaining to this aspect are shown in **Table 5**. Of the reasons put forward, many cited fears of adverse side effects from the vaccine (26.73%). The short duration of the clinical trials was also cited as a cause for concern (20.72%), which was followed by fear about the vaccine's safety, and efficacy (16.82%). The least among the reasons was that some thought that COVID-19 does not actually exist.

DISCUSSION

This study represents one of the first estimates of COVID-19 vaccination intention among healthcare workers in the KSA. Our findings can be used to guide future projections of vaccine uptake. Promoting the uptake of an emergency-released vaccine across a targeted population can pose significant challenges to public health authorities and in the context of the COVID-19 pandemic, failure to address such challenges could impede the country's unprecedented efforts in managing the pandemic. Thus, identifying the factors that can either be a facilitator or a barrier in influencing intentions to uptake or decline the COVID-19 vaccine is important.

The results reveal that almost half of the healthcare worker respondents in this study were unwilling to be vaccinated

TABLE 3 | Logistic regression estimates of factors associated with acceptance of a COVID-19 vaccine.

Variable	OR	95% CI	*p*-value
Age			
18–29	1		
30–39	0.969	0.486–1.931	0.929
40–49	2.226	0.957–5.176	0.063*
50–59	1.403	0.484–4.069	0.533
≥60	1.534	0.462–5.096	0.485
Gender			
Female	1		
Male	1.609	0.971–2.665	0.065*
Marital status			
Unmarried	1		
Married	0.802	0.418–1.539	0.506
Location			
Central	1		
South	2.458	1.047–5.775	0.039**
East	1.019	0.430–2.413	0.966
North	2.53	0.789–8.112	0.119
West	0.896	0.447–1.798	0.758
Monthly income			
<SR 3,000	1		
SR 3,000 to <5,000	1.763	0.453–6.864	0.413
SR 5,000 to <7,000	0.566	0.197–1.630	0.292
SR 7,000 to <10,000	0.5	0.173–1.444	0.200
SR 10,000 to <15,000	0.545	0.245–1.215	0.138
SR 15,000 to <20,000	0.669	0.274–1.631	0.377
SR 20,000 to <30,000	0.681	0.220–2.113	0.506
≥SR 30,000	0.799	0.269–2.372	0.686
Frontline healthcare worker			
No	1		
Yes	1.092	0.671–1.778	0.724
Having chronic conditions			
No	1		
Yes	0.658	0.342–1.268	0.212
Received flu vaccination in the past			
No	1		
Yes	1.328	0.804–2.193	0.268
Refused vaccination in the past			
No	1		
Yes	0.252	0.129–0.493	0.000***
Infected with COVID-19			
No	1		
Yes	1.841	0.893–3.795	0.098*
Family infected with COVID-19			
No	1		
Yes	1.19	0.72–1.97	0.497
Friends infected with COVID-19			
No	1		
Yes	1.402	0.633–3.103	0.405
Perceived risk of COVID-19 to people in Saudi Arabia			
Minor risk or no risk	1		
Moderate risk	1.521	0.803–2.883	0.198
Significant or major risk	1.86	0.955–3.624	0.068*
Concerned about getting infected with COVID-19			
Low or very low	1		
Fair	1.246	0.697–2.227	0.458
High or very high	2.091	1.068–4.092	0.031**
A COVID-19 vaccine should be compulsory for all citizens and residents inside Saudi Arabia			
No	1		
Yes	43.657	24.592–77.502	0.000***

(Continued)

TABLE 3 | Continued

****p < 0.01, **p < 0.05, *p < 0.1; OR, odds ratio; CI, confidence interval.*

against COVID-19. 50.52% of the sample were willing to have the COVID-19 vaccine if it was provided free by the Saudi government, of which 49.71% were willing to be vaccinated as soon as the vaccine becomes available in the country, while 50.29% would delay vaccination until the vaccine's safety is confirmed. The vaccination acceptance rate was lower compared to earlier studies conducted in Saudi Arabia prior to the country's approval of the vaccine (25) or even before the vaccine was available (26).

Two reasons could explain this observed low rate. First, this study was conducted at the time when the Saudi government had just approved the COVID-19 vaccine. During that period, the dissemination of anti-vaccination misinformation on different social media platforms had intensified and this might have caused the creation of doubt about the novel vaccine. Second, the daily confirmed new COVID-19 cases in the country had started to decline at that time which could in turn resulted in alleviated worries among healthcare workers and contributed to weaker intentions to vaccinate.

Consistent with other previous findings from the United States of America (USA) (27), Australia (28), and Turkey (29) concerning the acceptance of the COVID-19 and influenza vaccinations, this study found that concerns regarding the vaccine's safety and efficacy and fear of adverse reactions were the most important predictors of vaccine refusal. Healthcare workers have also identified the expedited vaccine trials as a reason for lack of intent to vaccinate. Taken together these findings reaffirm results from previous studies of vaccine uptake during the influenza pandemic (30).

In the KSA, health authorities have highlighted that the Saudi Food and Drug Authority has stringent procedures in place to ensure the safety, effectiveness, and strengths of COVID-19 vaccine before permitting its use. They have also emphasized that approval came only after reviewing all scientific data that confirms the safety and efficacy of the vaccine, however uncertainties still exist (31). While there is a need to tailor effective outreach strategies aimed at addressing concerns related to vaccine safety and efficacy particularly among healthcare workers, the findings indicate that they need to be supplemented

TABLE 4 | Logistic regression estimates of factors associated with immediate or delayed acceptance of a COVID-19 vaccine.

Variable	OR	95% CI	*p*-value
Age			
18–29	1		
30–39	0.677	0.321–1.425	0.304
40–49	0.718	0.297–1.736	0.462
50–59	0.616	0.219–1.736	0.360
≥60	1.425	0.227–8.949	0.705
Gender			
Female	1		
Male	1.706	0.986–2.952	0.056*
Marital status			
Unmarried	1		
Married	0.97	0.505–1.863	0.927
Location			
Central	1		
South	1.465	0.626–3.431	0.379
East	2.082	0.732–5.921	0.169
North	0.573	0.119–2.770	0.489
West	1.599	0.750–3.411	0.224
Monthly income			
<SR 3,000	1		
SR 3,000 to <5,000	1.974	0.565–6.895	0.286
SR 5,000 to <7,000	1.001	0.310–3.230	0.999
SR 7,000 to <10,000	2.454	0.796–7.562	0.118
SR 10,000 to <15,000	2.176	0.847–5.588	0.106
SR 15,000 to <20,000	1.775	0.631–4.997	0.277
SR 20,000 to <30,000	2.724	0.820–9.049	0.102
≥SR 30,000	2.547	0.696–9.312	0.158
Frontline healthcare worker			
No	1		
Yes	1.189	0.715–1.977	0.504
Having chronic conditions			
No	1		
Yes	1.375	0.725–2.607	0.330
Received flu vaccination in the past			
No	1		
Yes	1.09	0.603–1.970	0.776
Refused vaccination in the past			
No	1		
Yes	0.874	0.374–2.041	0.755
Infected with COVID-19			
No	1		
Yes	1.331	0.538–3.292	0.536
Family infected with COVID-19			
No	1		
Yes	0.849	0.512–1.408	0.525
Friends infected with COVID-19			
No	1		
Yes	1.111	0.456–2.710	0.816
Perceived risk of COVID-19 to people in Saudi Arabia			
Minor risk or no risk	1		
Moderate risk	0.59	0.291–1.193	0.142
Significant or major risk	0.763	0.391–1.491	0.429
Concerned about getting infected with COVID-19			
Low or very low	1		
Fair	1.72	0.825–3.586	0.148
High or very high	1.888	0.893–3.995	0.096*
A COVID-19 vaccine should be compulsory for all citizens and residents inside Saudi Arabia			
No	1		
Yes	3.666	2.034–6.608	0.00***

****p < 0.01, *p < 0.1; OR, odds ratio; CI, confidence interval.*

TABLE 5 | Reasons for not accepting the COVID-19 vaccination.

	N	%
Fear of adverse side effects	89	26.73
Safety and efficacy concerns	56	16.82
The speed of making the vaccine	13	3.9
The short duration of clinical trials	69	20.72
Personal desire not to be vaccinated	30	9.01
I think the vaccine is a plot	32	9.61
I do not believe in the existence of COVID-19	2	0.6
I feel that masks and sanitisers are sufficient for protection	23	6.91
Other	19	5.71
Total	333	100

with building trust and ensuring transparency in the process of vaccine approval to achieve confidence and consequently improve vaccine acceptance.

In line with other studies (30, 32), the results of this study suggest an association between vaccine intention and healthcare workers' greater perceived risk of COVID-19 to themselves. It can thus be argued that the perceived risk of COVID-19 might remain even after being infected with the virus. The significant positive association between being previously infected with COVID-19 and vaccine intention found in this study supports this speculation.

Additionally, this study has found that vaccination intention was associated with a high-risk perception of COVID-19 to the country. The impact of the pandemic on the country's economic and social well-being had devastating consequences (33). Thus, it has been suggested that vaccination campaigns highlighting the pandemic's consequences on the overall country's well-being, including the social, economic and public cost of the disease, could be an effective strategy in encouraging vaccination (34). This strategy is especially important in Saudi Arabia, where coronavirus-related treatment has been offered to both residents and expatriates at no cost to curb the spread of the virus.

Given the global attention on COVID-19 vaccine nowadays, healthcare workers who believed that the COVID-19 vaccination should be mandatory were more willing to accept the vaccine. This could be stemming from the perception that the vaccine is the "seatbelt against the disease" and the potential solution in protecting oneself and others and achieving greater good at minimal cost (35).

In terms of vaccination history, vaccine intention was found to be correlated with previous acceptance of a certain type of vaccine. Earlier studies have identified habit (past vaccination behavior) as a strong determinant of future vaccination behavior (36). Previous study on influenza vaccine acceptance among healthcare workers in the KSA showed that the influenza vaccine uptake was low among healthcare workers, ranging from 3% in 2010 to 44.1% in 2015 (19). It has also been shown that the acceptance of a previous vaccination in Australia increased the intention to immunize, with participants who had accepted previous influenza vaccines being 5 times more likely to accept a pandemic vaccine (37).

There is some evidence suggesting that vaccination intention is likely to be higher than the actual vaccine uptake (38). In this study, almost 51% of those who were willing to be vaccinated intend to delay vaccination until the vaccine's safety is confirmed. Concerns regarding the safety of newly developed vaccines are well-documented (39–41). For example, 47% of Chinese people who showed an intention to accept the COVID-19 vaccination plan to delay immunization to see if there are associated side effects (23).

However, as the other half of the healthcare worker respondents who were willing to be vaccinated have the intention to vaccinate as soon as possible, it is important to identify the factors associated with immediate vaccination intention. Support for a mandatory vaccine was a significant predictor for immediate vaccination intention and healthcare workers who believe that vaccination should be mandatory were more likely to accept vaccination as soon as possible once the vaccine becomes available. Our results also confirmed risk perception's importance in accepting immediate vaccination, which concurs with the findings of other studies (23).

Furthermore, given that males are at high risk from COVID-19 (42), it was not unexpected that male healthcare workers were more willing to accept the COVID-19 vaccine compared to females healthcare workers. This finding is in line with several other studies (10, 23, 43). Additionally, we observed regional differences in COVID-19 vaccine acceptability. Healthcare workers residing in the Southern region of Saudi Arabia were more likely to report an intention to immunize against COVID-19 than residents of the Central region. While the reason behind this is unclear, it is important to note that the Southern region was among the worst-affected regions in the country and this could have played a role in promoting COVID-19 vaccination intention.

This study's strengths include the large sample size, participants from the 13 administrative regions in Saudi Arabia and the examination of a wide range of possible correlates. However, it is worthwhile looking at the possible limitations of the study and a key limitation is the study's cross-sectional design and lack of available data on non-respondents. Another limitation is that this study does not imply causality, given that it does not use causal identification methods. Finally, as the use of an online survey might impact the study's generalisability, it is worth noting that the sample of healthcare workers in this study is skewed toward the male gender (60.18% male, 30.82% female). According to the latest yearly statistical book by MOH in 2018 (44), the total male healthcare workers (including physicians, dentists, nurses, pharmacists, and allied health professionals) are 49.5% while the total female healthcare workers are 50.5%.

CONCLUSION

This study provides early insight into the acceptability of the COVID-19 vaccine among healthcare workers in Saudi Arabia. Given that only half of the sample would be willing to be vaccinated, of which only half were willing to be vaccinated as soon as possible, it is worrying that the other half do not intend to be vaccinated, even though healthcare workers are expected to be more knowledgeable and aware of the benefits and risks of vaccination. There is an urgent need, therefore, to design effective and evidence-based strategies to promote the COVID-19 vaccine's uptake among healthcare workers. Healthcare workers are at great risk of contracting and spreading the disease and, unless wide-acceptance of the vaccine is achieved, the transmission of the virus would continue and recovery strategies would be hard to accomplish. Of particular importance is also the need for more health-related education among healthcare workers in order to alleviate any fears associated with the vaccine.

DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the current study are not publicly available due to privacy and confidentiality agreements as well as other restrictions, but are available from the corresponding author (Mohammed Khaled Al-Hanawi) on reasonable request.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data, took part in drafting the article or revising it critically for important intellectual content, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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Angiotensin-Converting Enzyme (ACE) Inhibitors and Angiotensin Receptor Blockers (ARB) Are Protective Against ICU Admission and Mortality for Patients With COVID-19 Disease

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Introduction: Corona Virus disease 2019 (COVID-19) caused by the Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) has become a global pandemic. The aim of this study was to investigate the impact of being on an Angiotensin-Converting Enzyme Inhibitors (ACEI) and/or Angiotensin Receptor Blockers (ARB) on hospital admission, on the following COVID-19 outcomes: disease severity, ICU admission, and mortality.

Methods: The charts of all patients consecutively diagnosed with COVID-19 from the 24th of February to the 16th of June of the year 2020 in Jaber Al-Ahmed Al-Sabah hospital in Kuwait were checked. All related patient information and clinical data was retrieved from the hospitals electronic medical record system. The primary outcome was COVID-19 disease severity defined as the need for Intensive Care Unit (ICU) admission. Secondary outcome was mortality.

Results: A total of 4,019 COVID-19 patients were included, of which 325 patients (8.1%) used ACEI/ARB, users of ACEI/ARB were found to be significantly older (54.4 vs. 40.5 years). ACEI/ARB users were found to have more co-morbidities; diabetes (45.8 vs. 14.8%) and hypertension (92.9 vs. 13.0%). ACEI/ARB use was found to be significantly associated with greater risk of ICU admission in the unadjusted analysis [OR, 1.51 (95% CI: 1.04–2.19), $p = 0.028$]. After adjustment for age, gender, nationality, coronary artery disease, diabetes and hypertension, ICU admission was found to be inversely associated with ACEI use [OR, 0.57 (95% CI: 0.34–0.88), $p = 0.01$] and inversely associated with mortality [OR, 0.56 (95% CI: 0.33–0.95), $p = 0.032$].

Conclusion: The current evidence in the literature supports continuation of ACEI/ARB medications for patients with co-morbidities that acquire COVID-19 infection. Although,

the protective effects of such medications on COVID-19 disease severity and mortality remain unclear, the findings of the present study support the use of ACEI/ARB medication.

Keywords: ACEi (angiotensin conversing enzyme inhibitor), ARB (angiotensin II AT1 receptor blocker), ICU–intensive care unit, COVID−19, mortality

INTRODUCTION

Corona Virus disease 2019 (COVID-19) caused by the Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) has become a global pandemic (1). Although the disease is easily transmissible, clinical presentation ranges from being asymptomatic to multi-organ involvement and death (2–5). Disease severity has been found to be associated with certain risk factors like older age, male gender, and co-morbidities (6, 7). The overall mortality rate has been found to range from 1 to 5% (6). However, in the presence of cardiovascular disease, diabetes, chronic respiratory disease, or hypertension, the mortality rate is found to increase dramatically (8, 9).

SARS-CoV-2 enters human cell through the angiotensin-converting enzyme 2 (ACE2) receptor, a membrane receptor that is broadly expressed in the respiratory system, the gastrointestinal tracts, the heart, and the kidney (10–12). Due to its close association with the ACE2 receptor, concerns were raised about the effect of using antihypertensive medications like angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin receptor blockers (ARBs) in patients with COVID-19. It has been hypothesized that the use of such drugs could upregulate the ACE2 receptor expression in alveolar 2 cells (13) and render patients more susceptible to infection and disease propagation. On the other hand, it was suggested that the use of such drugs may inhibit the ACE2 receptor and prevent virus entry to the cell, thus posing a protective effect (14). The aim of this study was to investigate the impact of being on an Angiotensin-Converting Enzyme Inhibitors (ACEI) and/or Angiotensin Receptor Blockers (ARB) on hospital admission, on the following COVID-19 outcomes: disease severity, ICU admission, and mortality.

METHODS

Study Design and Data Collection

For the present retrospective study, all patients consecutively diagnosed with COVID-19 from the 24th of February to the 16th of June of the year 2020 in Jaber Al-Ahmed Al-Sabah hospital in Kuwait were included in the study. Inclusion criteria included patients of all ages diagnosed with COVID-19 using PCR testing, in accordance with the World Health Organization (WHO) interim guidance (15). All related patient information and clinical data was retrieved from the hospitals electronic medical record system. These included sociodemographic factors (age, gender, nationality), clinical indicators (temperature on admission, blood pressure), and presence of co-morbidities (diabetes, hypertension, asthma, coronary artery disease).

Laboratory Investigations

All diagnostic tests were performed in Jaber Al-Ahmad Al-Sabah hospital in Kuwait. COVID-19 was confirmed via real-time reverse-transcriptase-polymerase chain-reaction (RT-PCR) assay of specimens obtained via nasopharyngeal swabs (16).

Outcome

For the present study the two investigated outcomes were compared across ACEI/ARB and non-ACEI/ARB users. The primary outcome was COVID-19 disease severity measured as the need for Intensive Care Unit (ICU) admission. The secondary outcome was mortality. All patient mortalities were attributed to COVID-19 since only SARS-CoV-2 positive patients were admitted and subsequently included in the present study. Criteria for ICU admission was based upon patients need for mechanical ventilation and/or vasopressors which was determined based upon evaluation by a rapid response COVID-19 team who assess individual patients with certain risk factors: age $>$ 60 years old, heart rate $>$ 100, systolic blood pressure $<$ 90 or mean arterial pressure $<$ 65, temperature $>$38.1, respiratory rate $>$26–30, saturation of oxygen $<$92% on room air, or any pulmonary infiltrate not considered chronic changes. The presence of any 3 of the previous criteria alerts the COVID-19 team to discuss with the ICU consultant on-call for decision regarding ICU admission. Our center's ICU beds were never fully occupied, and any patient with the above stated indications was admitted to ICU.

Patients who were ACE/ARB users on hospital admission, were compared to those who were not on those medications when they first presented to the hospital.

Ethical Considerations and Role of Funding

Ethical approval for conduction of this study was granted by the Ministry of Health Ethical Review Board in Kuwait (No. 2020/1402). A research grant (Grant No.: Cor-prop-35) was awarded by the Kuwait Foundation for the Advancement of Science (KFAS) and was utilized for assistance in data collection, statistical analysis, and publication.

Statistical Analysis

Data was analyzed using R (version 4) (17). Descriptive statistics were used to report mean and standard deviations for continuous data, and frequency statistics were used to calculate numbers and percentages for categorical variables.

Patient characteristics of ACE/ARB and non-ACE/ARB users were analyzed using independent t-test for continuous variables and, chi-squared test for categorical variables.

Logistic regression was used to identify significant predictors of ACE and non-ACE users, in both unadjusted and adjusted models. Unadjusted models were first run separately for each

factor, followed by multiple logistic regression models. Models were adjusted for the following covariates: age, gender, non-Kuwaiti, CAD, diabetes and hypertension. Odds Ratio (OR) with 95% Confidence Intervals (CI) was calculated. Statistical significance was set at p value <5%.

RESULTS

In the present retrospective cohort study 4,019 COVID-19 patients were included, of which 325 patients (8.1%) used ACEI/ARB, whilst 3,694 (91.9%) did not. Baseline patient characteristics are shown in **Table 1**. Users of ACEI/ARB were found to be significantly older than non-ACEI/ARB users (54.4 vs. 40.5 years), were more often male (70.5 vs. 29.5%) and non-Kuwaiti (40.3 vs. 59.7%). Additionally, ACEI/ARB users were found to have more co-morbidities, for instance; diabetes (45.8 vs. 14.8%) and hypertension (92.9 vs. 13.0%). The proportion of patients on ACEI/ARB admitted into the ICU were proportionally more than non-ACEI/ARB users (11.1 vs. 7.6%). Even more, mortality was greater among ACEI/ARB users (6.5%) when compared to non-ACEI/ARB users (4.3%).

Table 2 shows the unadjusted and adjusted odds ratios from the logistic regression analysis. ACEI/ARB use was found to be significantly associated with greater odds of ICU admission compared to non-users in the unadjusted analysis [OR, 1.51 (95% CI: 1.04–2.19), $p = 0.028$]. However, after adjustment for confounding factors (age, gender, non-Kuwaiti, coronary artery disease, diabetes, and hypertension), ICU admission was found to be inversely associated with ACEI use [OR, 0.57 (95% CI: 0.34–0.88), $p = 0.01$]. Following the adjustment for confounding factors, ACEI/ARBs use was found to be inversely associated with mortality [OR, 0.56 (95% CI: 0.33–0.95), $p = 0.032$].

Table 3 shows the adjusted analysis restricted to patients aged over 40 years old with or without coronary artery disease (CAD). For patients over 40 years old, logistic regression analysis showed that ICU admission was inversely associated with ACEI/ARB use [OR, 0.51 (95% CI: 0.33–0.78), $p = 0.002$]. This association was also observed when analysis was restricted to patients >40 years with a history of CAD [OR, 0.54 (95% CI: 0.35–0.84), $p = 0.006$]. Mortality was also inversely associated with ACEI/ARB use, for those over 40 years [OR, 0.47 (95% CI: 0.27–0.80), $p = 0.005$] and >40 years with a history of CAD (OR, 0.44 [95% CI: 0.25–0.78), $p = 0.005$].

DISCUSSION

Among COVID-19 positive patients, the present study found a significant inverse association between ACEI/ARBs use, ICU admission, and mortality following the adjustment for baseline demographics and co-morbidities. Several studies have postulated that the use of ACEIs/ARBs may influence COVID-19 severity (18–20). However, the mechanism by which these drugs affect the pathogenesis of COVID-19 disease remains unclear, and there is need for clinical studies to guide the usage of such drugs in patients with COVID-19 disease (21).

SARS-CoV-2 uses angiotensin-converting enzyme 2 (ACE2) receptor to reach and replicate in the mucosal epithelium of the respiratory tract (8). Because previous research on animal studies (22, 23) have shown that the use of ACEI/ARBs can upregulate ACE2 receptor expression (13), concerns were raised on the subsequent effect of this on COVID-19 disease propagation (24). It was postulated that the use of such drugs may increase patient susceptibility of acquiring COVID-19 disease and/or having a more severe clinical course (24) and thus requiring the discontinuation of these drugs in suspected cases. However, the findings from animal studies remain equivocal, and the consequences of ACE2 receptor upregulation requires further investigation. Conversely, some studies have indicated that ACE 2 receptor upregulation may initiate an anti-inflammatory state by augmenting vasodilatation and providing antioxidant protective effects (21, 25, 26). These effects may be enhanced through a mechanism by which an increase in Angiotensin I (Ang 1–7) production exerts anti-inflammatory and antioxidant properties once bound to its receptor (26). The protective anti-inflammatory effects of ACE2 and Ang 1–7 were evident in studies conducted on animal lung injury models (21, 27), and those involving cardiac myocytes, in part due to their role in regulating cardiac contractility and hypoxia-induced cardiac genes (28).

The continued use of ACEI/ARBs for patients diagnosed with COVID-19 disease was initially concerning because a few studies reported it to be associated with worse outcomes (18–20). This was a result of their crude analysis showing these drugs to be associated with increased COVID-19 disease severity. In fact, the effect of demographics and co-morbidities on disease severity in COVID-19 is well established. For example, the overall mortality of COVID-19 is reported to be 1–5%, but when stratified by age, it can go as high as 14.8% for those who are over 80-years. In addition, in a cohort of 44,672 confirmed cases, the case-fatality rate of patients with COVID-19 disease who have co-morbidities was found to be higher than average, these include cardiovascular disease (10.5%), diabetes (7.3%), chronic respiratory disease (6.3%), and hypertension (6%) (8, 9). For this reason, when reporting associations relating to COVID-19 disease, demographics, and co-morbidity status has to be taken into consideration.

Although a retrospective study on 1,178 hospitalized COVID-19 disease patients in Wuhan City found that the use of ACEIs/ARBs was not associated with COVID-19 disease severity or mortality, the study did not adjust for confounding variables (29). Similarly, Tetlow et al. (30) reported that ACEI/ARB use was not associated with acute kidney injury, macrovascular thrombi, or mortality when studying 558 hospital inpatients admitted with COVID-19 disease. Moreover, an observational study by Braude et al. (31) on 1,371 patients from 11 hospitals in the United Kingdom, found that although ACEI/ARB use was not associated with increased inpatient mortality, their use was found to be associated with shorter length of in-hospital stay, in particular the effect was stronger in hypertensive patients.

After adjusting for confounding variables, the potential beneficial effect of ACEI/ARBs use becomes more evident (24). For instance, Zhang et al. (32) reported a lower mortality risk

TABLE 1 | Baseline characteristics of COVID-19 patients by ACE/ARB and non-ACE/ARB use.

	ACE/ARB users		**Non-ACE/ARB users**		
	***n* = 325 (8.1%)**		***n* = 3694 (91.9%)**		***P*-value**
Age, y	54.4	(10.2)	40.5	(16.8)	<0.001
<18 years	1	(0.3)	293	(7.9)	
18-29	5	(1.5)	660	(17.9)	
30-39	19	(5.8)	974	(26.4)	
40-49 years	85	(26.2)	725	(19.7)	
50-59 years	115	(35.4)	540	(14.6)	
60 years and above	100	(30.8)	495	(13.4)	
Gender					
Female	96	(29.5)	1093	(29.5)	0.99
Male	229	(70.5)	2601	(70.4)	
Nationality					
Kuwaiti	194	(59.7)	2175	(58.9)	0.78
Non-Kuwaiti	131	(40.3)	1519	(41.1)	
Systolic Blood Pressure, mmHg	137.1	(18.3)	125.1	(16.1)	<0.001
Diastolic Blood Pressure, mmHg	81.7	(10.6)	77.1	(9.8)	<0.001
Heart Rate, bpm	88.1	(14.2)	88.2	(15.0)	0.92
Diabetes, *n* (%)	149	(45.8)	585	(14.8)	<0.001
Hypertension, *n* (%)	302	(92.9)	480	(13.0)	<0.001
Coronary artery disease, *n* (%)	36	(11.1)	132	(3.6)	<0.001
Chronic Obstructive Pulmonary Disease, *n* (%)	4	(1.2)	13	(0.4)	0.02
Asthma, *n* (%)	25	(7.7)	212	(5.7)	0.15
Temperature on admission	37.0	(0.6)	36.9	(0.6)	0.05
ICU admission, *n* (%)	36	(11.1)	282	(7.6)	0.03
Mortality, *n* (%)	21	(6.5)	160	(4.3)	0.76

Statistical significance set at $p < 0.05$.
ICU; Intensive Care Unit.

TABLE 2 | Odds ratios for ACE/ARB use vs. Non-ACE/ARB use with ICU admission and mortality.

	Unadjusted model		***P* value**	**Fully adjusted model**		***P* value**
	OR	**(95% CI)**		**OR**	**(95% CI)**	
Mortality	1.52	(0.95–2.44)	0.078	0.56	(0.33–0.95)	0.032
ICU Admission	1.51	(1.04–2.18)	0.028	0.57	(0.34–0.88)	0.010

Fully adjusted model includes the following covariates: age, gender, non-Kuwaiti, CAD, diabetes, hypertension.
Statistical significance set at $p < 0.05$.

in the ACEI/ARB group as compared to non-ACEI/ARB group after adjusting for age, gender, comorbidities, and in-hospital medications [HR, 0.42 (95% CI, 0.19-0.92); $P = 0.03$] but the study population was limited to hypertensive patients, which in turn limits generalizability. Fosbol et al. (24), on the other hand, conducted a study on 4,480 patients with COVID-19 disease, and reported that ACEI/ARB was not found to be associated with increased COVID-19 disease severity after adjusting for age and co-morbidities [HR, 1.15 (95% CI 0.95–1.41)]. The results of our study replicate this finding and also reported ACEI/ARBs to be protective against severe COVID-19 disease, with decreased need for ICU admission [OR, 0.57 (95% CI: 0.34–0.88), $p = 0.01$]. Fosbol et al. (24) also reported their findings on the association of ACEI with COVID-19 disease-related mortality. Although ACEI/ARBs were found to be significantly associated with mortality in unadjusted analysis [HR, 2.65 (95% CI 2.18–3.23)], this association was lost when adjusted for age and medical co-morbidities [HR, 0.83 (95% CI 0.67–1.03)]. Our study was able to replicate this finding with a greater effect size as our adjusted OR for mortality was 0.56 and can be as low as 0.33 when comparing patients with COVID-19 disease using ACEI/ARBs to those who do not use ACEI/ARBs. As presented in our regression model, we have adjusted for nationality (Kuwaiti vs. Non-Kuwaiti) as a confounding variable based on the findings of our previous study, which found Non-Kuwaitis to have a two-fold higher odds of death or ICU admission, which is explained

TABLE 3 | Odds ratios for ACE/ARB use vs. non-ACE/ARB use with ICU admission and Mortality for patients over 40 years.

	Fully adjusted model		*P* value
	OR	(95% CI)	
Mortality[a]	0.47	(0.27–0.80)	0.005
Mortality[b]	0.44	(0.25–0.78)	0.005
ICU Admission[a]	0.51	(0.33–078)	0.002
ICU Admission[b]	0.54	(0.35–0.84)	0.006

Fully adjusted model includes the following covariates: age, gender, non-Kuwaiti, CAD, diabetes, hypertension.

[a] *Restricted to patients over 40 years old (n = 2,067).*

[b] *Restricted to patients over 40 years old with history of cardiovascular disease (n = 775). Statistical significance set at p < 0.05.*

by the differences in socioeconomic status, living and working conditions, and health care access between the two groups (33).

Similarly, a retrospective study by Senkal et al. (34) on 611 COVID-19 patients in Istanbul found that a total of 165 patients had severe disease (hospitalization for >14 days, ICU admission, or death), and the use ACEI was found to be significantly associated with lower disease severity [OR, 0.37 (95% CI 0.15–0.87)], milder infiltrations on CT, lower level of inflammatory markers (C-reactive protein and ferritin), and shorter hospital stay. Moreover, although their study also found ARB exposure to be associated with lower odds of severe disease, this association failed to reach significance [OR, 0.6 (95% CI 0.27–1.36) $p = 0.31$].

Comparable to previous findings, Zhou et al. (35) assessed 15,504 patients from 17 different hospitals in China to investigate the association between in hospital use of ACEI/ARB with 28-day all cause death of COVID-19 in 3,572 patients. The authors reported the results of their propensity score-matched analysis, in which patients were matched for age, gender, disease severity, co-morbidities, and calcium channel blocker usage after adjustment for imbalanced variables and in-hospital medications. They found that in-hospital ACEI/ARB use was associated with decreased risk of 28-day all-cause mortality from COVID-19 in patients with hypertension [OR, 0.11 (95% CI 0.15–0.66), $p = 0.002$], hypertension and coronary artery disease (CAD) [OR, 0.11 (95% CI 0.04–0.31) $p < 0.001$], and CAD [OR, 0.38 (95% CI 0.16–0.89) $p = 0.03$].

More recently, similar to the present study, Bean et al. (36) evaluated the risk of COVID-19 disease severity among 1,200 patients. A total of 33% ($n = 399$) of the patients were found to be using ACEI or ARB and the adjusted risk of ICU admission or death was lower among users [OR, 0.63 (95% CI 0.47–0.84)].

Comparatively, the beneficial effects of ACE use have been reported in heterogenous patient cohorts from different demographic backgrounds. A more recent meta-analysis of 12 studies, including 19,000 COVID-19 positive patients found that ACEI/ARB use did not increase the risk of disease severity (OR = 0.98; 95 % CI, 0.87–1.09; $p = 0.69$) or mortality (OR = 0.73, 95 %CI, 0.5–1.07; $p = 0.111$) in patients with COVID-19, and the use of ACEI/ARB was found to protective against mortality among hypertensive patients when compared with other antihypertension medications [OR = 0.48, (95 % CI, 0.29–0.81); $p = 0.006$] (37). Another meta-analysis by Flacco et al. (38) combined the results of 10 studies with a total of 9,890 patients with hypertension to assess if ACEI/ARB use was associated with severe or lethal COVID-19 disease and found no significant association with either ACEI [OR: 0.9 (95% CI 0.65–1.26)] or ARB [OR: 0.92 (95% CI 0.75–1.12)] use. The available findings in the literature in conjunction with the findings of these studies supports the statements made by professional societies about continuation of ACEI/ARBs for patients with COVID-19 disease (39).

Although the present study did not assess the relationship between ACEI/ARB use and COVID-19 incidence, it was postulated that the use of ACEI/ARBs may potentially increase the risk of acquiring COVID-19 disease. However, evidence is limited and remains equivocal. For example, in one study by Reynolds et al. (40) that included 12,594 COVID-19 positive found no association with any medication class, which included ACEIs/ARBs, calcium channel blockers, beta blockers, and thiazide diuretics. Reynolds et al. (40) also reported that none of the examined drug classes were associated with an increased severity of COVID-19 disease (40).

The association of ACEI and ARBs with COVID-19 disease is complex and further studies are needed to explore the mechanism of interaction of SARS-CoV-2 virus with ACE2 receptor and its implications on COVID-19 disease pathophysiology. Bellone and Calvisi (41) have discussed the association of specific ACE2 polymorphisms on the aggressiveness of COVID-19 disease and suggested that Ins/Del and Del/Del polymorphisms may be associated with severe clinical disease and mortality from Acute Respiratory Distress Syndrome (ARDS). It was also proposed that ACE and Angiotensin II may be a therapeutic target for COVID-19 patients through their effects on the previously discussed polymorphisms (41). Recently, Vaduganathan et al. (21) reviewed the involvement of the RAAS system in COVID-19 disease pathophysiology and indicated the benefits of ACEI/ARB use to outweigh the hypothesized risks of these medication classes on COVID-19 disease incidence or severity among patients, in otherwise stable condition, with indications to take these drugs. Altogether, the current evidence in the literature is in conjunction with the findings of this study and supports the statements made by professional societies about continuation of ACEI/ARBs for patients with COVID-19 disease (39, 42).

LIMITATIONS

The present study has several limitations. Firstly, the observational nature of the study does not confer causality but rather our results are reported as associations. Secondly, this study was limited to a single hospital in Kuwait so generalizability could be affected. Although the medication list for all patients is accurately reported in the system, indication for ACEI/ARB use was not gathered for all patients. Those not on ARB/ACE may be on an alternate treatment

or on no treatment, which could result in some residual confounding. We also did not collect data on medication dose, duration of therapy, and have not investigated differences between the use of ACEI or ARB, which add up to the study's limitations.

CONCLUSION

The current evidence in the literature supports continuation of ACEI/ARB medications for patients with co-morbidities that acquire COVID-19 infection. With time, the protective effects of such medications on COVID-19 disease severity and mortality is becoming clearer, and the findings of the present study support the use of ACEI/ARB medication in such 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

Ethical approval for conduction of this study was granted by the Ministry of Health Ethical Review Board in Kuwait. All participants provided written informed consent.

AUTHOR CONTRIBUTIONS

SAlS: conception of idea, overseeing project, and proofreading. RE and DA: collecting data and writing. SAlY: analyzing data and writing of paper. HB: analyzing data and writing. SAlm: analyzing data, writing, and proofreading. MA-H and MJ: overseeing project and proofreading. All authors contributed to the article and approved the submitted version.

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Clinical, Serological, Whole Genome Sequence Analyses to Confirm SARS-CoV-2 Reinfection in Patients From Mumbai, India

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Background: SARS-CoV-2 infection may not provide long lasting post-infection immunity. While hundreds of reinfections have reported only a few have been confirmed. Whole genome sequencing (WGS) of the viral isolates from the different episodes is mandatory to establish reinfection.

Methods: Nasopharyngeal (NP), oropharyngeal (OP) and whole blood (WB) samples were collected from paired samples of four individuals who were suspected of SARS-CoV-2 reinfection based on distinct clinical episodes and RT-PCR tests. Details from their case record files and investigations were documented. RNA was extracted from the NP and OP samples and subjected to WGS, and the nucleotide and amino acid sequences were subjected to genome and protein-based functional annotation analyses. Serial serology was performed for Anti-N IgG, Anti- S1 RBD IgG, and sVNT (surrogate virus neutralizing test).

Findings: Three patients were more symptomatic with lower Ct values and longer duration of illness. Seroconversion was detected soon after the second episode in three patients. WGS generated a genome coverage ranging from 80.07 to 99.7%. Phylogenetic analysis revealed sequences belonged to G, GR and "Other" clades. A total of 42 mutations were identified in all the samples, consisting of 22 non-synonymous, 17 synonymous, two in upstream, and one in downstream regions of the SARS-CoV-2 genome. Comparative genomic and protein-based annotation analyses revealed differences in the presence and absence of specific mutations in the virus sequences from the two episodes in all four paired samples.

Interpretation: Based on the criteria of genome variations identified by whole genome sequencing and supported by clinical presentation, molecular and serological tests, we were able to confirm reinfections in two patients, provide weak evidence of reinfection in the third patient and unable to rule out a prolonged infection in the fourth. This study emphasizes the importance of detailed analyses of clinical and serological information as well as the virus's genomic variations while assessing cases of SARS-CoV-2 reinfection.

Keywords: SARS-CoV-2, COVID-19, reinfection, whole genome sequencing, seroconversion

INTRODUCTION

In December 2019, a novel coronavirus (n-CoV-19) sparked an outbreak in Wuhan, China. This virus was subsequently named SARS-CoV-2 and the disease COVID-19. On 11th March 2020, there were 1,18,000 cases in 114 countries with 4,291 deaths and the World Health Organization (WHO) declared that COVID-19 was a pandemic (1).

In August, the first report of reinfection by a phylogenetically distinct strain of SARS-CoV-2 was confirmed in Hong Kong (2) and subsequently Nevada reported a confirmed reinfection in USA (3). While there have been many reports of putative reinfections based on RT-PCR positivity, this has been confounded by prolonged shedding of viral RNA in the absence of replication competent virus (4) which can continue to cause RT-PCR positivity for up to at least 83 days (5). Nevertheless, the samples from the two episodes can be sequenced and genomic analysis may demonstrate genetic variation that can't be explained by short term *in vivo* evolution, which when combined with epidemiological and clinical evidence, may confirm reinfection (2, 3).

The present study was undertaken using samples collected from individuals tested for SARS-COV-2 as standard of care either for contact tracing or diagnostic purposes in symptomatic individuals. We report a case series of four individuals who had asymptomatic or mild RT-PCR proven COVID-19 followed by a second symptomatic RT-PCR positive episode with lower Ct values and varying degrees of increased clinical severity in the second episode.

MATERIALS AND METHODS

Study Design and Participants

We identified four individuals who had tested RT-PCR positive for SARS-CoV-2 between April to June 2020 and who tested RT-PCR positive for SARS-CoV-2 once again between July to September after presenting with symptoms suggestive of COVID-19. Based on the RT-PCR results and clinical presentation of the patients, we suspected reinfection with SARS-CoV-2. Upon confirmation of the RT-PCR findings, whole genome sequencing was performed on the stored paired samples. Clinical findings and investigations were retrieved from their case records. Blood samples were collected prior to and after the second episode for anti-SARS-CoV-2 serology including anti-N, anti-S1 RBD, sVNT (surrogate virus neutralization test). The study was approved by the Institutional Review Board of Kasturba Hospital of Infectious Diseases; IRB number 015/2020. The patients provided written informed consent.

Procedures

Sample Collection

Nasopharyngeal (NP) and oropharyngeal (OP) samples for SARS-CoV-2 RT-PCR were collected, aliquoted and stored for future use as detailed in the **Supplementary Table 1**. Phlebotomy was performed and blood was collected in dipotassium EDTA tubes for anti-SARS-CoV-2 serology at time points between the first and second episode, early in the second episode and a longitudinal sample as described in **Table 1**.

RT-PCR

One of the aliquots was used for RNA extraction and tested by multiplex real time RT-PCR TaqPath™ COVID19 RTPCR kit for the qualitative detection of nucleic acid of SARS-CoV-2 from Applied Biosystems. Additional details of RT-PCR testing are described in **Supplementary Table 1**.

Serology

Anti-N protein IgG antibodies were tested by qualitative ARCHITECT chemiluminescence microparticle immunoassay (Abbott Laboratories, USA). Anti-S1 RBD antibodies were tested using SARS-CoV-2 Total antibody test on Atellica IM analyzer (Siemens, Germany). Neutralizing antibodies were tested by SARS-CoV-2 Surrogate Virus Neutralization test (GenScript USA, Inc).

Whole Genome Sequencing

Extracted RNA from all four paired stored samples was transported at −80°C for whole genome sequencing. Sample preparation, sequencing, and data analysis was performed by previously published protocols (6). Briefly, double-stranded cDNA was synthesized from 50 ng of total RNA for all the SARS-CoV-2 positive samples. The first strand of cDNA was synthesized using Superscript IV followed by RNA digestion with RNase H for second strand synthesis using DNA Polymerase I Large fragment (Klenow fragment). One hundred nanograms of purified double-stranded cDNA for both pools of ARTIC tiling PCR primers (V3 Primer pools) were taken forward. Post-amplification, pool 1 and 2 amplicons were pooled and purified using 1x AMpure beads (AMPure XP, Beckman Coulter, Cat. No. A63881). Further, 200 ng of each purified sample of multiplexed PCR amplicons obtained was taken for library preparation using Oxford Nanopore Technology (ONT) as per document no. PTC_9096_V109_REVf_06fEB2020. This included End Repair/dA tailing, Native Barcode Ligation, and Adapter Ligation of the PCR amplicons. One hundred nanograms of the pooled and purified library was sequenced using ONT's MinION Mk1B platform.

Phylogenetic and Comparative genomic analysis

Samples were base called and demultiplexed using Guppy basecaller (https://community.nanoporetech.com). Reads having phead quality score <7 were discarded to filter the low-quality reads. The resulting fastq files were normalized by read length (300–500) and reads were aligned using Minimap2 (v2.17) (7) to the reference (MN908947.3). Variants were called using Nanopolish (8) from the aligned reads and further creating consensus fasta using bcftools (v1.8) (9). Assembled fasta files from the SARS CoV-2 were aligned using CLC workbench and a UPGMA tree was constructed using default parameters. A secondary tree was generated after downloading whole genome sequences from VIPR (10) database from India submitted during the period from March 2020 to June 2020. Phylogenetic Analysis was done on all the compiled datasets using Vipr.

TABLE 1 | Clinical course, RT-PCR, and serology.

Patient nomenclature	Date of RT-PCR	Clinical features and duration of illness	Ct values			NC CLIA IgG	S1 RBD CLIA IgG	sVNT >20% positive <20% negative
			N gene	ORF1ab	S gene			
Patient A	+ve 15/5/20 –ve 19/05/20	Sore throat, nasal congestion and rhinitis. Symptoms resolved in 2 days	32	32	Nil	01/07/2020 negative 0.02	–	–
Patient A f/u	+ve 19/7/20 –ve 29/7/20	Myalgia, fever, non-productive cough, fatigue. Symptoms resolved in 1 week	25	23	23	23/7/2020 negative 0.05 16/9/2020 positive 3.32	23/07/2020 non-reactive 0.02 16/9/2020 reactive >20.00	23/07/2020 positive 25% 16/9/2020 positive 93%
Patient B	+ve 15/5/20 –ve 18/5/20	None	33	Nil	32	01/07/2020 negative 0.05	–	–
Patient B f/u	+ve 18/7/20 –ve 25/7/20	Myalgia, malaise. Symptoms resolved in 2 days	36	38	Nil	23/7/2020 negative 0.02 16/9/2020 negative 0.02	23/07/2020 non-reactive 0.00 16/9/2020 non-reactive 0.01	23/07/2020 negative 12% 16/9/2020 positive 22%
Patient D	+ve 14/5/20 –ve N/A	Sore throat, rhinitis and myalgia. Symptoms resolved in 5 days	32	34	35	4/6/2020 negative 0.04	4/6/2020 non-reactive 0.03	4/6/2020 negative 11%
Patient D f/u	+ve 7/7/20 –ve N/A	Fever, myalgia, rhinitis, sore throat, non-productive cough and fatigue. Prolonged course, unable to return to work for 3 weeks	17	18	21	8/7/2020 positive 1.4 17/9/2020 positive 2.44	8/7/2020 reactive 2.37 17/9/2020 reactive 6.39	8/7/2020 positive 60% 17/9/2020 positive 91%
Patient E	+ve 20/04/20 –ve 23/04/20	Fever, myalgia, dry cough. Symptoms resolved in 1 week	31	31	–31	02/09/2020 negative 0.03	02/09/2020 non-reactive 0.01	02/09/2020 negative 6%
Patient E f/u	+ve 04/09/20 –ve 18/09/20	Fever, myalgia, dry cough, nausea, abdominal pain, breathlessness on exertion. Prolonged source, unable to return to work for 6 weeks. Breathlessness on exertion and fatigue persisted	22	22	22	18/09/2020 positive 3.62	18/09/2020 reactive 1.91	18/09/2020 positive 74%

**Nil = not detected.*

Lineage Analysis

Further, the assembled SARS-CoV-2 genomes were assigned lineages using the package Phylogenetic Assignment of Named Global Outbreak LINeages (PANGOLIN) (11).

Protein-Based Annotation

In order to categorize the specific amino acid variants present, the genomes were annotated by SnpEff version 4.5 (12). NC_045512 was taken as the reference genome of SARS-CoV-2 (13). The synonymous variants were filtered out from the analysis. The global frequency data for these 12 unique missense variations present across the four pairs was taken from cov-GLUE database which lists amino acid changes observed in GISAID SARS-CoV-2 sequences (14, 15). Total number of GISAID sequences retrieved at the time of analysis was 82,927, out of which 75,734 passed the exclusion criteria of CoV-GLUE.

Role of the Funding Source

The funder of the study had no role in 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.

RESULTS

A timeline summary of the clinical presentation during the two episodes, RT-PCR testing and serology are provided in **Figure 1**.

Clinical Analysis Reveals Increased Severity in the Second SARS-CoV-2 Episode

The four patients included in the study were assigned the IDs of Patient A, Patient B, Patient D and Patient E and their follow

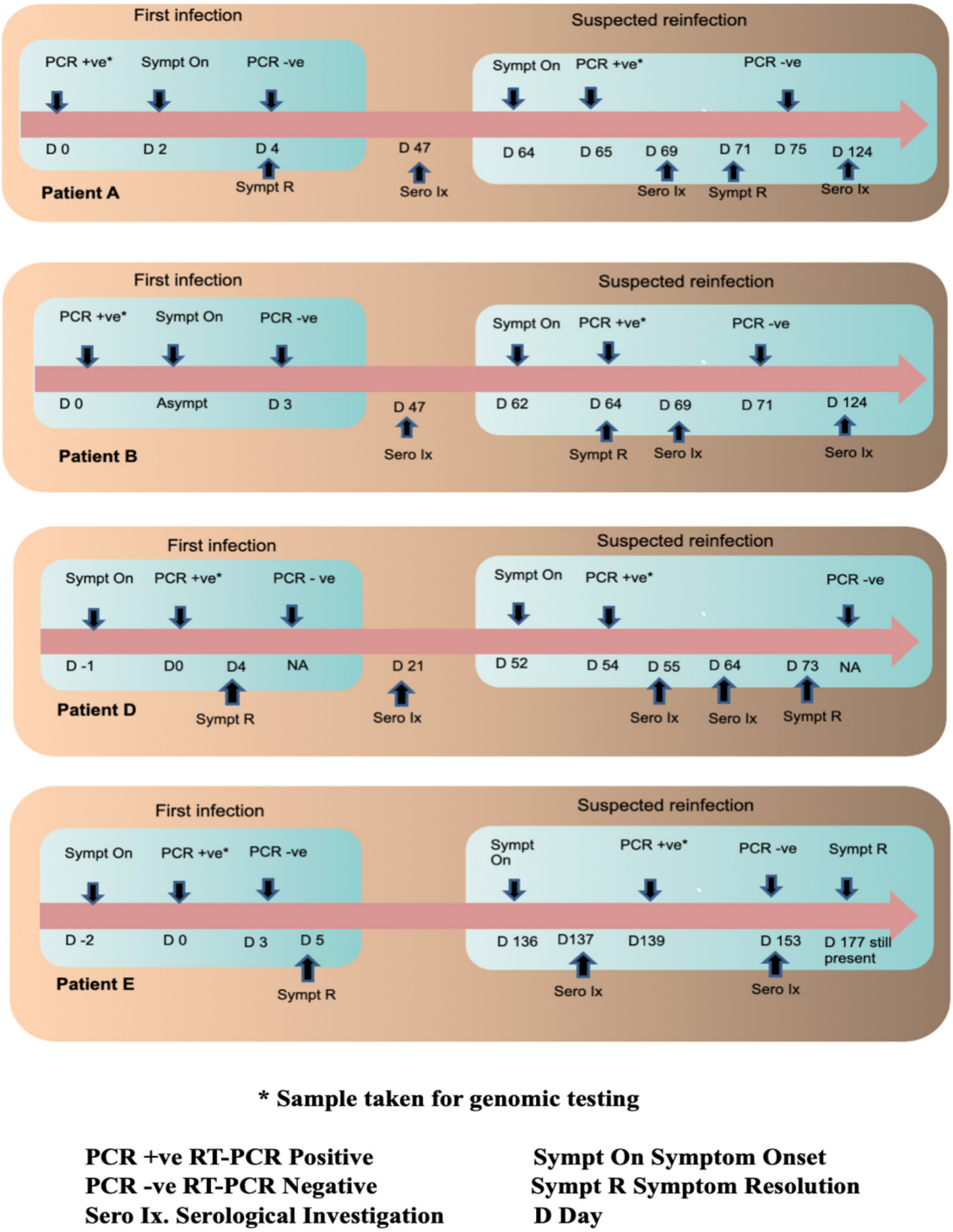

FIGURE 1 | Timeline of infections in the four patients along with their serological and RT PCR investigations.

up samples were suffixed with f/u after each ID. Patient A (aged 27, male), B (aged 31, male) and D (aged 24, female) had no history of pre-existing illnesses or immunodeficiency. They were all directly involved in the clinical care of COVID-19 patients. Patient E (aged 51, female) was a controlled hypertensive, had no history of other pre-existing illnesses or immunodeficiency

and worked as a technician in a COVID-19 diagnostic laboratory. Patients A and B were tested as part of a contact tracing exercise. Patient A developed sore throat and rhinitis 2 days after testing positive and recovered completely in 2 days. Patient B remained asymptomatic. Counting from their first positive RT-PCR tests A and B tested RT-PCR negative 4 and 3 days later, respectively. On day 64 and 62, respectively, they both developed COVID-19 like symptoms. Patient A tested RT-PCR positive on day 65 (1 day after symptom onset) and patient B on day 64 (2 days after symptoms onset). Patient A had fever, cough, myalgia and fatigue that lasted a week while Patient B had myalgia that lasted 2 days. Patient D's first episode was symptomatic and she tested RT-PCR positive a day after symptom onset. Symptoms included sore throat, rhinitis, and myalgia and lasted 5 days. Counting from the first positive RT-PCR, Patient D developed symptoms compatible with COVID-19 on day 52 and 2 days later on Day 54 tested positive by RT-PCR. Symptoms included sore throat, rhinitis, cough, fever, myalgia, and fatigue. Most symptoms resolved in 3 weeks but fatigue persisted for over a month. Patients A, B, and D were hospitalized during both episodes for isolation and monitoring. All three had normal respiratory rates, pulse oximetry and chest X-rays during both episodes. During the first episode, Patient E developed cough, fever, myalgia, and tested RT-PCR positive 2 days after symptoms onset. Fever remitted in 5 days but fatigue persisted. Counting from the first positive test, on day 3, RT-PCR was negative. On day 136, Patient E developed symptoms compatible with COVID-19 and 3 days later on day 139, tested RT-PCR positive. Symptoms included fever, cough, breathlessness, myalgia, nausea, and abdominal pain. Fever lasted 8 days, but breathlessness on exertion and fatigue persisted for more than 6 weeks. She was hospitalized for isolation and monitoring in the first episode but was managed as an outpatient during the second episode. Her respiratory rate and pulse oximetry were normal during both episodes but a HRCT of the chest during the second episode demonstrated pneumonia and pulmonary fibrosis. In all four patients, the second episode was more symptomatic and lasted longer in duration. All four reported that their second episodes were subjectively worse.

RT-PCR samples were collected within 3 days of symptom onset for all patients during both episodes. Patient A's sample was collected 2 days before and 1 day after symptom onset in the first and second episodes, respectively. Patient B was asymptomatic during the first episode and the sample was collected 2 days after symptom onset in the second episode. Patient D's samples were collected 1 day and 2 days after symptom onset in the first and second episodes, respectively. Patient E's samples were collected 2 days and 3 days after symptom onset in the first and second episodes, respectively. Similar time points of sample collection for the first and second episodes for the patients along with harmonized RT-PCR sample collection, processing and testing methodology allowed us to compare Ct values despite the short window for RT-PCR positivity in some COVID-19 patients. Patients A, D, and E had lower Ct values in the second episode compared to the first. Patient B's Ct values were higher during the second episode. Details of Ct values are presented in **Table 1**.

Seroconversion Detected After the Second Episode

Three serological tests performed, anti-N IgG, anti-S1 RBD IgG, and neutralizing antibodies by sVNT. Counting from the first positive RT-PCR test, on day 47 Patients A and B were both negative for anti-N IgG antibodies. Their plasma samples drawn on day 47 were not stored for additional tests (which became available later). On day 69 both patients had already developed symptoms for the second time and serological sampling was repeated. Patient A became symptomatic 5 days prior and RT-PCR positive 4 days prior to serological sampling. Patient A's sample was sVNT was positive but anti-N and anti-S1 RBD IgG were both negative. Patient B became symptomatic 7 days prior and RT-PCR positive 5 days prior to serological sampling. All three serological tests were negative on day 69. A third sample was drawn for both A and B on day 124. All three serological tests were positive for Patient A. Patient B was positive by sVNT but negative for anti-N and anti-S1 RBD IgG. Counting from the first positive RT-PCR, on day 21 Patient D was negative for all three antibodies. On day 55, just 3 days after symptom onset and 1 day after RT-PCR positivity in the second episode, Patient D was positive for all three serological tests. A longitudinal sample collected on day 73 was more strongly positive for all three tests. Counting from the first positive RT-PCR test, Patient E tested negative for all three antibodies on day 137 (1 day after symptom onset in the second episode). On day 153 (17 days after symptom onset in the second episode) Patient E was positive for all three antibodies.

Genome Analysis Reveals Clade Change and/or Distinct Mutations in the Virus Populations Between Episodes

Genome sequencing generated genome coverage of 80.07–99.7% (**Table 2**).The assembled genomes were curated and taken for further analysis. Phylogenetic tree analysis of the eight sequences, along with 160 complete viral genome sequences submitted from India in GISAID between the months of May to September 2020 because both phases of samples used for the study has been collected in this duration, revealed two samples (Patients A and B) sub-clustered together with their f/u samples respectively while samples Patient D and E and their f/u sequences clustered in different clades (**Figure 2**).

Clade based analysis revealed that two of eight sequences belonged to the G clade while one sequence belonged to clade GR while the remaining five sequences categorized under "Other" category. Further, analysis of lineage by PANGOLIN revealed distribution of the eight with variations of B lineages including B, B.1, B.1.80, and B.1.1.32 (**Table 2**).

The samples from the first and second episode of infection of the four patients are predominantly from the SARS-CoV-2 clade 19A and 20A. The clades from the first and second episode, respectively, were 20A and 19A in Patient A, 20B and 20B in patient B, 19A and 20B in Patient D, and 19A and 20B in Patient D.

TABLE 2 | Clade, lineage of patients with reinfections ($n = 4$).

Sequence name	GISAID ID	Genome coverage	Sequencing depth	Lineage (PANGOLIN)	Most common countries (PANGOLIN)	Nextclade	GISAID
Patient_A	EPI_ISL_528419	80.37	220	B	UK, China, USA	20A	Other
Patient_A_f/u	EPI_ISL_528420	83.01	270	B.1.80	India, Australia, Luxembourg	19A	Other
Patient_B	EPI_ISL_528421	97.78	1345	B.1	UK, USA, Australia	20B	G
Patient_B_f/u	EPI_ISL_528422	90.87	351	B.1	UK, USA, Australia	20B	Other
Patient_D	EPI_ISL_528425	85.22	311	B.1	UK, USA, Australia	19A	Other
Patient_D_f/u	EPI_ISL_528426	98.26	2299	B.1.1.32	India, UK, Spain	20B	GR
Patient_E	EPI_ISL_801538	83.99	376	B.1.5	UK, USA, Australia	19A	Other
Patient_E_f/u	EPI_ISL_676509	90.16	1233	B.1	UK, Brazil, Finland	20A	G

Mutation analysis of the samples revealed distinct mutations in all the samples (**Table 2**). Interestingly, we observed a higher number of mutations in the follow-up samples except Pair-B, which had 10 mutations in first infection compared to three in the follow-up. Pair-E had the highest number of 13 mutations in the follow-up sample compared to two in the first sample, followed by Pair-D with 10 mutations in follow-up and one in the first sample and lastly by Pair-A with two in follow-up and one in the first sample. A total of 42 (**Figure 3**) mutations were observed in our sample set of four patients. Twenty-two non-synonymous, 17 synonymous, and 2 upstream UTR and 1 downstream UTR mutation is observed. Interestingly the non-synonymous mutation P323L in the nsp 12 RNA-dependent RNA polymerase gene has been reported to be concurrently present with D614G mutation in the spike protein, is observed in all patient samples, whereas D614G mutation was observed only in four samples (16, 17). In the nsp3 region, part of the replicase complex, two synonymous mutations F924F, N1123N, and one non-synonymous mutation A1812D observed in mild cases of COVID-19 (18) were observed in Patient E, Patient B, and Patient B f/u samples, respectively.

To evaluate amino-acid alterations, we performed protein-based annotation of the 22 non-synonymous mutations found from our genome analysis of the four pair of samples (**Figure 4**). It was observed that Pair 1, i.e., Patient A shows minor variations, with common ones occurring within Nsp12. With respect to the other patients, interestingly, we found heterogeneity within mutations in both episodes. For instance, in Patient B, the mutations within Spike protein (D614G, Q677H) in the first episode were missing in the followup sample. Similarly, in Patients D and E, we found presence of additional mutations in samples of followup. Interestingly, in re-infection cases, a higher number of mutations were found in non-structural proteins, including nsp1, nsp2, nsp3, nsp5, nsp6, and nsp12, and nsp 14. Further, we also performed correlations of these mutations with viral genomes from world-wide populations (∼1,44,426) to understand their relative frequency (**Figure 2**). While P323L mutation within nsp12 was found in all samples without exception, other frequent mutations showed abrupt patterns. In particular, D614G mutation within the Spike protein was consistently present in both infections in Patient E but was present only in one of the episodes in Patients B and D.

Sequence Submission

All SARS-CoV-2 sequences from eight patients were submitted to GISAID under the accession number EPI_ISL_528419 and EPI_ISL_528420 for patient A, A_f/u, EPI_ISL_528421, and EPI_ISL_528422 for patient B, B_f/u, EPI_ISL_528425, and EPI_ISL_528426 for patient D, D_f/u, EPI_ISL_801538, and EPI_ISL_676509 for patient E, E_f/u.

DISCUSSION

Clinically SARS-CoV-2 infection can present with or without symptoms and severity has been categorized into four types ranging from asymptomatic to critical illness based on symptoms, clinical findings, chest imaging and blood gases as presented in **Supplementary Figure 1** (19). New immunological evidence is enriching our knowledge of the immune response to infection (20) and duration of immunity following infection (21). Emerging evidence suggests Ct values and viral loads at the time of diagnosis maybe implicated in pathogenesis and disease severity (22). A handful of confirmed SARS-CoV-2 reinfection have been published on the basis of genome variation observed in the viruses between the two episodes with varying clinical manifestations between the episodes (2, 3, 23, 24). The European Center for Disease Control and Prevention (ECDC) (25) and United States Center for Disease Control and Prevention (US CDC) (26) have considered multiple criteria to investigate a case of suspected reinfection.

On the basis of these criteria, we discuss our patients and confirm or reject a case as SARS-CoV-2 reinfection. As per the US CDC, SARS-CoV-2 reinfection should be considered in individuals with COVID-19 like symptoms and a positive RT-PCR for SARS-CoV-2 with a Ct value <33 at least 45 days after the first positive RT-PCR. There should not be an obvious alternative etiology for the symptomatic second episode. Paired samples from the two episodes should undergo genomic testing that includes evaluation of single nucleotide variations (SNV) and clades to distinguish between viral persistence within host evolution vs. reinfections. In patients meeting the above criteria, genomic testing revealing differing clades as defined in Nextstrain (27) and GISAID of SARS-CoV-2 between the first

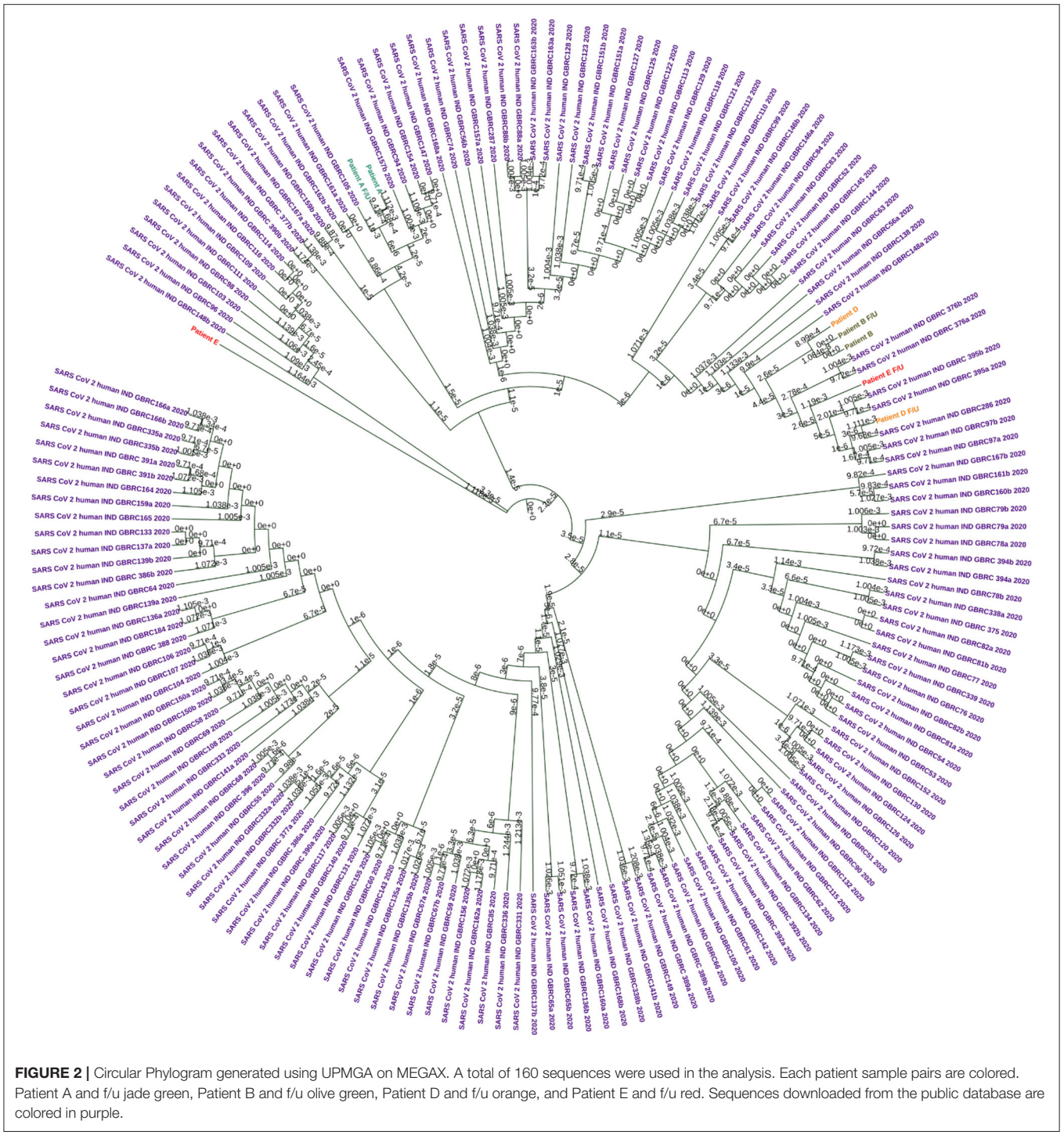

FIGURE 2 | Circular Phylogram generated using UPMGA on MEGAX. A total of 160 sequences were used in the analysis. Each patient sample pairs are colored. Patient A and f/u jade green, Patient B and f/u olive green, Patient D and f/u orange, and Patient E and f/u red. Sequences downloaded from the public database are colored in purple.

and second infection is considered the best evidence of SARS-CoV-2 reinfection. More than two nucleotide differences per month in consensus between sequences that meet quality metrics is considered moderate evidence. The US CDC also recommends serial serological testing.

Accordingly our present study evaluates clinical, RT-PCR, genomic and serological information to evaluate reinfections in four patients who presented with repeat episodes of SARS-CoV-2 infections. Of the four patients in the study, Patients A, D, and E had COVID-19 like symptoms during both first episodes and second episode and did not have an obvious alternate etiology for their COVID-19 like symptoms. Their symptoms were also accompanied by a positive RT-PCR for COVID-19 over 45 days from the first positive RT-PCR. Interestingly, Patients A, D, and E

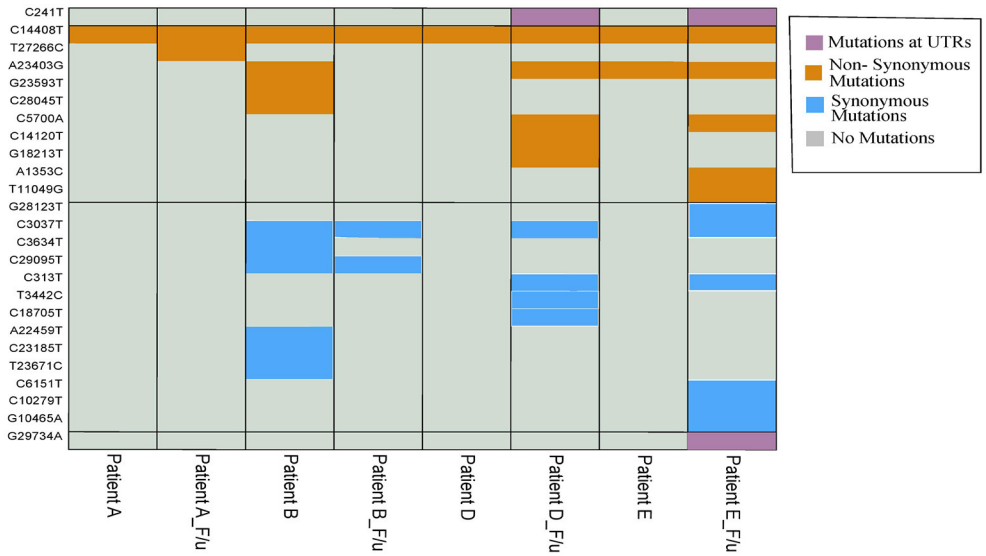

FIGURE 3 | Heatmap with 42 overall mutations (unique set of 25 mutations). Purple, mustard yellow, and sky blue colors show the presence of mutation in samples while gray color shows the absence of mutation in samples.

FIGURE 4 | Mapping of amino-acid substitutions within SARS-CoV-2 genome of four pairs of samples. The upper plot demonstrates the seven proteins in different colors that harbor 12 non-synonymous mutations shown in dots. The Y-axis shows the four pair of patient samples. The blue and red dot indicates the presence of the mutation in the first and second episode of infections respectively. The lower plot shows the frequency of that particular mutation in 82,927 genomes deposited in GISAID.

had increased clinical severity and lower Ct values in the second episode. All three had Ct values not exceeding 23. Such Ct values correlate with active viral replication and positively correlate with virus culture positivity (28). Analysis of whole genome sequence data generated from the samples of both episodes of Patients A, D and E revealed that the two paired samples clustered in different clades and belonged to different lineages.

Patient A's paired samples contained viruses from different clades but were separated by a single mutation. Moreover, the sample from the second episode had low Ct values (23 in confirmatory gene) and the clinical picture strongly suggested active SARS-CoV-2 infection. Crucially, Patient A was positive for neutralizing antibodies just 5 and 4 days after symptom onset and RT-PCR positivity during the second episode. While WGS showed a single distinct mutation in consensus sequences, the clinical picture, low Ct values, difference in clade and presence of neutralizing antibodies within 5 days of symptom onset supports reinfection. It should be noted that Patient A's first sample

genome coverage was 80.37 and in the second episode was 83.01. This could have resulted in detection of fewer mutations. Despite the clade change, clinical picture, lower Ct values, and nAb positivity, with the caveat of genomic coverage and based on the CDC criteria for defining reinfection, we determined the evidence as weak evidence for assigning the second episode of Patient A as a reinfection.

Patient B was asymptomatic in the first episode and but had a symptomatic second episode about 2 months later with myalgia and malaise. The Ct value from samples for RT-PCR was 33 in the first episode but 36 in the second episode. The genome analysis of the paired samples of this patient further showed no clade or lineage difference. However, mutation analysis revealed difference in mutations observed including the presence of the D614G mutation only in the sample from the first episode. There were addition/deletion of both synonymous and non-synonymous mutations between the samples of the two episodes as was observed in the functional protein annotation analysis. Most of the mutations were found in the spike protein, the region most likely to undergo mutations to escape immune pressure during prolonged infections. Three synonymous and two non-synonymous mutations occurred in the spike region. Additionally, in the second episode, 7 and 5 days after symptom onset and RT-PCR positivity all three antibody tests (anti-N, anti-S1 RBD, and sVNT) were negative. All these analyses put together make it difficult to differentiate between a prolonged infection and a reinfection in Patient B.

Both patient D and E had symptoms compatible with COVID-19 during both episodes and the clinical picture was strongly suggestive of COVID-19. Both had lower Ct values in the second episode suggestive of active viral replication. Additionally, during the second episode Patient E had radiological evidence of acute pulmonary infection (pneumonitis) superimposed on COVID-19 pulmonary sequelae (pulmonary fibrosis). Paired samples from both Patient D and E contained viruses from different clades and had distinct mutations exceeding the cut off requiring >2 distinct mutations per month between consensus sequences clearly confirming SARS-CoV-2 reinfection.

In the present study, we found priming of immunity in the first episode leading to a boosting effect following the second episode by production of neutralizing antibodies early in the second episode. Analysis of the serological profiles of all the patients failed to reveal seroconversion after the first episode but during the second episode, neutralizing antibodies were detected 5 and 3 days after symptom onset as seen in Patients A and D, respectively. Further, longitudinal samples of these patients revealed increasing titers of neutralizing antibodies. In the case of Patient E, seroconversion was not detected early in the second episode but was observed two and a half weeks after symptoms onset. While most individuals do seroconvert following SARS-CoV-2 infection, some individuals do not seroconvert (20). It is possible that the patient sin our study had failure of humoral immunity which may explain the absence of detectable antibodies. It is possible that the absence of seroconversion predisposed them to reinfection.

While our study found that the second episode was more symptomatic with a longer duration of illness, our study was not designed to identify reasons for increased severity in the second episode. Nevertheless, we hypothesize a few possible reasons for the observed increased severity in the second episode.

Some evidence from animal studies suggests that increased inoculum size or a higher infecting dose may result in increased clinical severity (29). Owing to their status as health care workers caring for COVID-19 patients or handling their samples all four patients had an occupational risk of exposure. It is possible that the participants in our study were exposed to a larger infecting dose in the second episode as compared the primary infection. Another aspect to consider is the impact of mutations in the viral genome. Recent detection of SARS-CoV-2 variants has raised important questions about the impact of S gene mutations and deletions on increased transmissibility, ACE-2 receptor affinity, viral loads, immune escape, and severity. S variants of SARS-CoV-2 have been associated with significantly lower median Ct values suggesting that changes in the S protein RBD may result in increased viral loads (30). While our sample size and absence of viral culture studies does not allow us to make determinations about the impact of S gene mutations and deletions on clinical severity and viral load, it is possible that mutations at the Spike gene may explain lower Ct values and increased severity in the second episodes.

Some experimental *in vitro* studies suggest the possibility of antibody dependent enhancement of SARS-CoV-2 (31, 32) which has also been observed in other coronaviruses. It is possible immune enhancement may have increased the severity of the second episode.

Taken altogether, our present study provides a level of evidence classified by US CDC as best evidence of reinfection in two patients (Patients D and E), weak evidence with possible reinfection in one patient (Patient A), and we were unable to differentiate between prolonged infection and reinfection in the case of Patient B. Our study adds to the growing body of evidence of SARS-CoV-2 reinfections and demonstrates the value of serial serological data in supporting reinfection claims. Our study highlights that SARS-CoV-2 reinfections do occur, and individuals who have recovered from SARS-CoV-2 infection should continue to take infection prevention precautions.

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 Institutional Review Board of Kasturba Hospital of Infectious Diseases; IRB number 015/2020. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JS conceptualized and designed the study. JS and LP identified the study participants. SP and SA collected and compiled data from different sources. NC and MP performed RNA extraction, aliquoting, and RT-PCR. RP, VA, JSV, AK, RM, and SF performed genome sequencing. RP, VA, JSV, AK, RM, SF, LT, SS, SC, and CS performed genomic and linage analyses. SP, SS, and JS drafted and revised the manuscript. JS, SS, and AA provided resources and participated in overall supervision. All authors contributed to data interpretation, critically reviewed the manuscript, provided contributions to tables, figures and text in the manuscript, and approved the final manuscript for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.631769/full#supplementary-material

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BRIEF RESEARCH REPORT
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COVID-19 Mortality and Case-Fatality Rates in Sergipe State, Northeast Brazil, From April to June 2020

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Information on how coronavirus disease 2019 (COVID-19) mortality is related to population characteristics in low- and middle-income countries is still limited. We described the deaths from COVID-19 in Sergipe state, Northeast Brazil, from April 2 to June 27, 2020. For this purpose, we conducted a study composed of (i) a case series study of all deaths due to COVID-19 and (ii) a population-based study to verify the behavior of the mortality and case-fatality rates (CFR) related to COVID-19. Data from 605 deaths due to COVID-19 were used to describe the characteristics of individuals with the disease, as well as the differences in gender, age, and comorbidities. Additionally, population data were extracted to estimate the mortality and CFR by population stratum. We also performed an adjusted CFR analysis including a time lag of 14 days between the onset of symptoms and reporting deaths. Of the 605 patients included in this study, 321 (53.1%) were males and the median age was 67.0 years. Most patients ($n = 447$, 73.9%) who died from COVID-19 had at least one pre-existing clinical condition. The mortality rate was 29.3 deaths per 100,000 inhabitants and the crude CRF was 2.6% (95% CI 2.4–2.8). CFR was higher in males (3.1%, 95% CI 2.8–3.4; $p < 0.001$) and people aged ≥60 years (14.2%, 95% CI 13.0–15.6; $p = 0.042$). About 25% of patients died during the first 24-h post-hospital admission. The adjusted CFR for a 14-day time lag was ∼2-fold higher than the crude CFR over the study period.

Keywords: COVID-19, mortality, Brazil, SARS-CoV-2, coronavirus

INTRODUCTION

Coronavirus disease 2019 (COVID-19), an infectious disease caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), has emerged in China in December 2019 and is currently a global public health concern. More than 10 million cases and more than 500,000 deaths due to COVID-19 were registered up to the first half of 2020. COVID-19 mortality has been higher in men, older people, and among those with some comorbidities, including hypertension, diabetes, and heart disease (1). These associations have emerged from studies performed in the United States and in high-income countries in Asia and Europe. However, information on how COVID-19 mortality

is related to population characteristics in low- and middle-income countries (LMIC) is still limited. In this sense, as the recognition of target groups most at risk of death is a valuable tool for disease control measures, we described the deaths from COVID-19 in a population of Northeast Brazil.

METHODS

Study Design

This study comprised (i) a case series study of all deaths from COVID-19 and (ii) a population-based study to verify the behavior of the mortality and case-fatality rates (CFR) related to COVID-19 in Sergipe state, Northeast Brazil, from April 2 to June 27, 2020.

Study Area

Sergipe is the smallest Brazilian state with 21,925,424 km^2, a population of ∼2.3 million people, and a Human Development Index (HDI) of 0.665. In Sergipe, the first case of COVID-19 was reported on March 14, 2020, and by June 27, 23,319 COVID-19 cases had been registered.

Case Series Study

Data on COVID-19 cases and deaths were extracted from the microdata catalog of the State Health Secretariat. Sergipe's health surveillance service has registered all deaths due to COVID-19 in the state. In this study, we included all patients with laboratory confirmation for SARS-CoV-2 infection, defined as a positive result on real-time reverse transcription polymerase chain reaction (RT-PCR) assay of respiratory tract samples based on the World Health Organization (WHO)'s interim guidelines (2).

Data retrieved included age, gender, pre-existing medical conditions, date of initial symptoms prior to diagnosis, date of hospitalization, and date of death. Pre-existing health conditions were categorized as systemic arterial hypertension, diabetes, obesity, liver, kidney, heart, neurodegenerative and chronic pulmonary diseases, stroke, cancer, high-impact communicable diseases [e.g., tuberculosis, human immunodeficiency virus (HIV), and neglected tropical diseases], and non-HIV immunocompromised conditions.

Population-Based Study

Data collected were obtained from two information systems: (1) Population data were obtained from the Brazilian Institute of Geography and Statistics (IBGE, acronym in Portuguese) and (2) the number of COVID-19 cases and deaths were obtained from the surveillance system of the State Health Secretariat of Sergipe. From these data, mortality and CFR related to COVID-19 were estimated.

Data Analysis

Categorical variables were described as absolute frequencies and percentages, and continuous variables were described as median and interquartile range (IQR). χ^2 test, Cochran-Armitage test for trend, or Fisher's exact test was used to compare proportions between groups, where appropriate. Mann–Whitney *U* test was used for comparisons of differences in medians. The significance level was set as 0.05.

Mortality and CFR were stratified by gender and age (0–19 years, 20–39 years, 40–59 years, and ≥60 years). Mortality rates per 100,000 inhabitants were calculated according to the general population, while CFR with associated 95% confidence interval (CI) was defined as the number of deaths from COVID-19 divided by the total number of confirmed cases. An adjusted CFR estimate was also calculated from this population-level data including a time lag of 14 days between the onset of symptoms and reporting deaths (3, 4). Data were analyzed by using JASP software version 0.13 (JASP Team, Amsterdam, Netherlands).

Ethical Consideration

Institutional review board approval and informed consent were not required because all data were obtained from secondary data sources and data were deidentified.

RESULTS

A total of 605 deaths (321 males and 284 females) due to COVID-19 were registered between April 2 and June 27, 2020. The characteristics of individuals who died from COVID-19 are shown in **Table 1**. The proportion of deaths by gender was similar (males: 53.1% *vs.* females: 46.9%). The individuals' age ranged from 2 days to 105 years and the median age was 67 years (IQR 54.0–79.0) without differences between genders (males: 67.0 years, IQR 52.3–77.3; females: 67.0 years, IQR 54.0–79.0; $p = 0.697$). Only 20 (3.3%) deaths were observed in individuals aged <20 years and most cases occurred over 60 years of age ($n = 395$, 65.4%). Four hundred and forty-seven patients (73.9%) had at least one pre-existing medical condition. Hypertension ($n = 229$; 37.9%), diabetes ($n = 199$; 32.9%), and heart disease ($n = 85$; 14.1%) were the most common comorbidities. Obesity was more frequent among female patients ($p = 0.038$) (**Table 1**).

Complete time-to-event data were retrieved from 509 patients. The median duration from symptoms onset to death was 10 days (IQR 6.0–17.0). The time interval between first symptoms and hospital admission was 4 days (IQR 2.0–8.0) and that from admission to death was 4 days (IQR 1.0–9.0). About 25% ($n = 150$) of patients died during the first 24 h after hospital admission.

The evolution of the accumulated deaths over the study period was also analyzed, as shown in **Figure 1**. Between May 27 and June 27, 2020, the number of deaths due to COVID-19 increased by 375% in Sergipe. Mortality rate was 29.3 deaths per 100,000 population and the crude CFR was 2.6% (95% CI 2.4–2.8). CRF was higher in males (3.1%, 95% CI 2.8–3.4) and in individuals aged over 60 years (14.2%, 95% CI 13.0–15.6) (**Table 2**). The adjusted CFR using 14-day time lag from symptoms onset to death was 4.3% (95% CI 4.0–4.7).

DISCUSSION

This study describes the deaths from COVID-19 in a poor area of Northeast Brazil. Similar to other localities (5, 6), most patients who died due to COVID-19 were older males with at

TABLE 1 | Characteristics of individuals who died due to COVID-19 in Sergipe state, Northeast Brazil, from April 2 to June 27, 2020.

Variable	All* (*n* = 605)	Male(*n* = 321)	Female (*n* = 284)	*P*-value
Age, median (IQR)	67.0 (54.0–79.0)	67.0 (52.3–77.3)	67.0 (54.0–79.0)	0.478
Age group, *n* (%)				
0–19 years	20 (3.3)	10 (3.1)	10 (3.5)	0.780
20–39 years	43 (7.1)	23 (7.2)	20 (7.0)	0.920
40–59 years	146 (24.2)	80 (25.0)	66 (23.3)	0.624
≥60 years	395 (65.4)	207 (64.7)	188 (66.2)	0.697
Comorbidity, *n* (%)	447 (73.9)	231 (72.0)	216 (76.1)	0.250
Specific-type comorbidity				
Hypertension, *n* (%)	229 (37.9)	118 (36.8)	111 (39.1)	0.562
Diabetes, *n* (%)	199 (32.9)	103 (32.1)	96 (33.8)	0.660
Heart disease, *n* (%)	85 (14.1)	46 (14.3)	39 (13.7)	0.834
Obesity, *n* (%)	38 (6.3)	14 (4.4)	24 (8.5)	**0.038**¥
Kidney disorder, *n* (%)	40 (6.6)	24 (7.5)	16 (5.6)	0.347
Cancer, *n* (%)	30 (5.0)	16 (5.0)	14 (4.9)	0.952
Chronic pulmonary disease, *n* (%)	35 (5.8)	18 (5.6)	17 (6.0)	0.834
Non-HIV immunocompromised condition, *n* (%)	16 (2.6)	8 (2.5)	8 (2.8)	0.818
Stroke, *n* (%)	17 (2.8)	12 (3.7)	5 (1.8)	0.159
Neurodegenerative disease, *n* (%)	17 (2.8)	11 (3.4)	6 (2.1)	0.332
Liver disease, *n* (%)	11 (1.8)	8 (2.5)	3 (1.1)	0.201
High-impact communicable diseases, *n* (%)	5 (0.8)	4 (1.2)	1 (0.4)	0.276
Others, *n* (%)	30 (5.0)	14 (4.4)	16 (5.6)	0.497

**In one case, age was not identified in a male patient.* ¥*p-values <0.05 were considered statistically significant.*

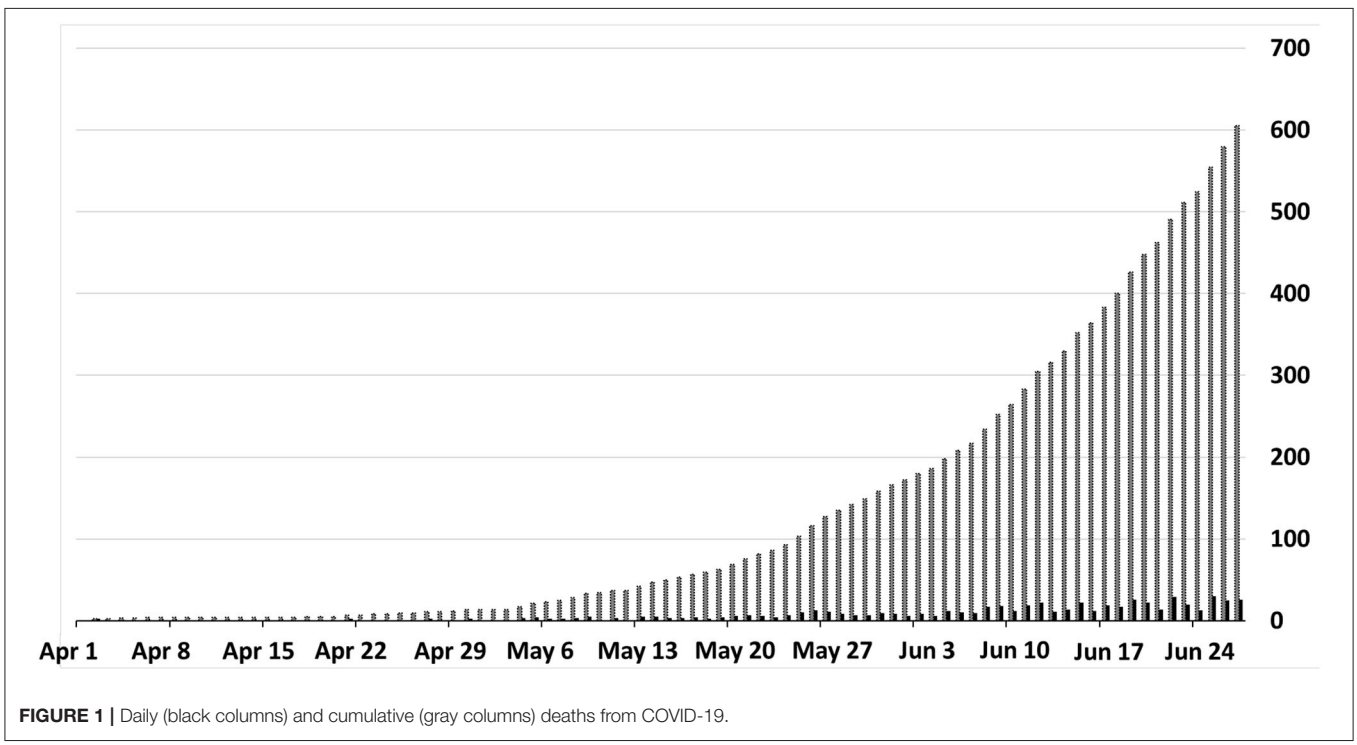

FIGURE 1 | Daily (black columns) and cumulative (gray columns) deaths from COVID-19.

least one pre-existing clinical condition. Moreover, we found that 25% of deaths have occurred in the first 24 h of hospital admission. Finally, a higher fatality rate (4.3%) was found when we adjusted for a 14-day time lag between the symptoms onset and death compared to the crude CFR (2.6%) over the study period.

TABLE 2 | Mortality rate and crude case-fatality rate for COVID-19 in Sergipe state, Brazil.

Variable	Cases of COVID-19	Deaths	Mortality rate (per 100,000 inhabitants)	Crude CFR(%) (95% CI)	*p*-value
Gender					
Male	10,457	321	31.9	3.1 (2.8–3.4)	<0.001
Female	12,862	284	26.7	2.2 (2.0–2.5)	
Age					0.042
0–19 years	1493	20	2.6	1.3 (0.9–2.1)	
20–39 years	10,851	43	6.1	0.4 (0.3–0.5)	
40–59 years	8195	146	35.2	1.8 (1.5–2.1)	
≥ 60 years	2779	395	212.4	14.2 (13.0–15.6)	
Total	23,319*	605*	29.3	2.6 (2.4–2.8)	

*CFR, case-fatality rate. CI, confidence interval. *In one case, age was not identified in a male patient.*

There is a wide variation in CFR for COVID-19 across countries, which can be explained by differences in age structure, prevalence of pre-existing clinical conditions, testing capacity, preparedness and public health response to COVID-19, and methodology used to calculate the CFR (if general or adjusted by period and population groups). For example, in January 2020, the WHO estimated an overall CFR of 2% for COVID-19, but at that time, WHO did not consider some important factors such as the dynamics and fast spreading of the SARS-CoV-2, population groups, and the time lag between symptoms onset and deaths. In this study, the adjusted CFR for a 14-day time lag was ∼2-fold higher than the crude CFR in June 27, 2020. This means that the CFR varies with the moment of the COVID-19 pandemic and its adjustment can provide more accurate information to assist policymakers in controlling the disease.

In this study, the time between the admission and death was short (a median of 4 days) and higher mortality and CFR rates were found among older people. Older adults are highly susceptible to life-threatening respiratory and systemic conditions associated with SARS-CoV-2 infection, which may be related to changes in immune function, a decline in physiological reserve capacity, and the diversity of pre-existing clinical conditions that appear to increase the risk of mortality from COVID-19. Surprisingly, a quarter of deaths in Sergipe occurred within the first 24 h of hospitalization. From this finding, some explanations can be offered, such as lack of a well-established protocol for the management of a new emerging disease, difficulties in access to diagnostic tests and health services, especially for the poorest population, and potential overload of hospital services in the months immediately after the beginning of the COVID-19 pandemic. Furthermore, health inequalities in disadvantaged populations may be related to the high mortality rate in this setting. In a recent neighborhood-level analysis in Aracaju municipality (capital of Sergipe state), we found that poor communities have shown limited testing resources and higher fatality rates from COVID-19 compared with communities with better living conditions (7).

In Sergipe, most patients who died from COVID-19 presented underlying clinical conditions or other recognized risk factors for severe outcomes from respiratory infections. Similar results were reported by the US Centers for Disease Control and Prevention (CDC), which found that among intensive care unit (ICU) admissions and deaths, 78 and 94%, respectively, occurred among patients with one or more underlying health conditions (8). These findings are also consistent with previous Italian (5) and Chinese (6) reports, suggesting that key specific strategies to protect individuals with pre-existing medical conditions should be implemented to decrease the risk of death from COVID-19.

Studies in high-income countries have also shown that hypertension, diabetes, and heart disease are associated with an increased risk of death from COVID-19. However, the interaction between COVID-19 and other infectious diseases, especially HIV and tuberculosis, which are quite common in LMIC, is still unknown. Recently, we described the clinical characteristics and outcomes in patients with COVID-19 and leprosy in Aracaju, which is considered an endemic area for this neglected tropical disease. All co-infected patients died, and they had the lepromatous form of disease (9). In the present study, we found a rate of 0.8% of patients who died co-infected with high-impact communicable diseases. As the disease spreads through settings with a high burden of communicable diseases, and more data are revealed, we will be able to know how co-infections can influence the outcomes in patients with COVID-19.

The findings of the present study should be interpreted with caution. Our data were obtained from surveillance information systems and the underreporting of pre-existing conditions may have occurred. Furthermore, aggregated data do not allow for examination of confounding factors, so that our analysis needs to be supplemented by prospectively collected data.

CONCLUSION

In Sergipe state, Northeast Brazil, the CFR for COVID-19 was higher in males and in older people, with a quarter of deaths occurring within the first 24 h of hospitalization. The adjusted CFR for a 14-day time lag between the symptoms onset and death was ∼2-fold higher than the crude CFR over the study period.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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

PM-F and VS conceptualized and designed the study, performed the data analysis, and drafted the manuscript. MG and MS collected the data. All authors performed the interpretation of data, critically revised the article for important intellectual content, and approved the final version of the manuscript.

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D-Limonene Is a Potential Monoterpene to Inhibit PI3K/Akt/IKK-α/NF-κB p65 Signaling Pathway in Coronavirus Disease 2019 Pulmonary Fibrosis

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At the time of the prevalence of coronavirus disease 2019 (COVID-19), pulmonary fibrosis (PF) related to COVID-19 has become the main sequela. However, the mechanism of PF related to COVID (COVID-PF) is unknown. This study aimed to explore the key targets in the development of COVID-PF and the mechanism of D-limonene in the COVID-PF treatment. The differentially expressed genes of COVID-PF were downloaded from the GeneCards database, and their pathways were analyzed. D-Limonene was molecularly docked with related proteins to screen its pharmacological targets, and a rat lung fibrosis model was established to verify D-limonene's effect on COVID-PF-related targets. The results showed that the imbalance between collagen breakdown and metabolism, inflammatory response, and angiogenesis are the core processes of COVID-PF; and PI3K/AKT signaling pathways are the key targets of the treatment of COVID-PF. The ability of D-limonene to protect against PF induced by bleomycin in rats was reported. The mechanism is related to the binding of PI3K and NF-κB p65, and the inhibition of PI3K/Akt/IKK-α/NF-κB p65 signaling pathway expression and phosphorylation. These results confirmed the relationship between the PI3K–Akt signaling pathway and COVID-PF, showing that D-limonene has a potential therapeutic value for COVID-PF.

Keywords: D-limonene, coronavirus disease 2019, coronavirus disease related pulmonary fibrosis, severe acute respiratory syndrome, PI3K/Akt signaling pathway

INTRODUCTION

Since 2003, coronavirus has caused multiple major public health events that resulted in global epidemics, such as severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), and coronavirus disease 2019 (COVID-19). Since December 2019 to date, SARS coronavirus 2 (SARS-CoV-2) has caused the most severe pandemic of *Coronaviridae* to date, but there is currently no specific drugs for COVID-19.

Pneumonia is the main manifestation of COVID-19. In general, persistent inflammatory damage to lung tissue caused by various reasons develops into pulmonary fibrosis (PF) (1) and further leads to pulmonary dysfunction and reduced quality of life after recovery. Although PF changes are occasionally observed as sequelae of other respiratory viral infections, they appear to be more common after COVID (2). For example, in 1-year follow-up studies, PF was observed in the lungs of 27.5% of SARS survivors ($n = 97$) (3). Long-term follow-up studies have shown that many survivors of SARS-CoV infection show signs of fibrosis in their lungs (4–6). MERS coronavirus (MERS-CoV) infection can also be responsible for PF (7, 8). Pathological analysis revealed that the alveolar septum of MERS patients was destroyed and expanded, and type II alveolar epithelial cells proliferated and shed. The clinical manifestations and severity of COVID-19 are similar to those of SARS (9). A large amount of evidence supports that COVID-19 can contribute to PF (10). The pathological changes in the early lungs of COVID-19 can be manifested as viral interstitial pneumonia, suggesting that it is imperative to start anti-fibrosis treatment in the early clinical stage (11). Pirfenidone (PFD) is a commonly used drug for the clinical treatment of PF, but it cannot effectively prolong the survival of patients (12, 13). PFD has side effects of gastrointestinal reactions, rash, and photosensitivity (14, 15). At present, the mechanism of occurrence and development of COVID-related PF (COVID-PF) is not yet clear. Due to this lack of therapeutic options, there is a critical need to understand the molecular pathways involved in the development of COVID-PF (16, 17), thus helping to identify novel targets for therapy and develop new drugs.

Based on the research of GeneChip bioinformatics, sequence comparison, and cluster analysis are utilized to extract the biological information generated by gene chip technology. These endeavors will enable more comprehensive and systematic study for diseases. With a spurt of progress in high-throughput GeneChips and sequencing technologies in recent years, it has been made possible to reveal the gene expression profile of COVID-PF and the changes in PF tissue and cell key genes. On this basis, there have been successful examples in other fields to screen potential drugs by docking small molecular compounds with proteins according to their core differentially expressed gene proteins. D-Limonene is a terpenoid compound extracted from the essential oils of several citrus plants, and it is widely used in the food industry (17). Systematic reviews and pharmacological studies have found that D-limonene can prevent and control respiratory system damage through its anti-inflammatory and antioxidant activities (18). It has also been reported that D-limonene can improve the pulmonary tissue remodeling that occurs in animal models of pulmonary hypertension and asthma. Respiratory system damage and lung tissue structure remodeling are mutually causal and vicious circles and play a key role in forming COVID-PF. However, the mechanism of action of D-limonene on PF is unknown. This study aimed to analyze the key signaling pathways of the COVID-PF differentially expressed genes and explore the mechanism of D-limonene in a rat model.

MATERIALS AND METHODS

Difference Analysis of Key Biological Processes and Signal Pathways Between Coronavirus Disease-Related Pulmonary Fibrosis and Pulmonary Fibrosis

GeneCards (https://www.genecards.org/) is a comprehensive database that integrates human genomic, transcriptomic, proteomic, clinical, and functional information. We used "corona virus disease related pulmonary fibrosis" and "pulmonary fibrosis" as keywords to search the key targets of COVID-PF and PF in the GeneCards database and import them into the Reactome Pathway Database (https://reactome.org/). The latter is a tool for comprehensive analysis and visualization of biological processes and signal pathways, which can compare the differences between COVID-PF and PF.

Coronavirus Disease-Related Pulmonary Fibrosis Protein–Protein Interaction Construction, Gene Ontology, Pathway Enrichment, and Module Analysis

COVID-PF key target genes were imported into the STRING (https://string-db.org/) and Metascape (http://metascape.org/gp/index.html#/main/step1) platforms. Protein types were defined as *Homo sapiens* and screened to obtain protein interaction network diagrams and module analysis. The R software package was used to draw a bar graph to show the frequency of key target interactions in the network. Target genes were analyzed for Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment. GO functional analysis items with similar functions were clustered and constructed an interactive network, and the target pathway network was drawn based on the results of KEGG enrichment analysis.

Molecular Docking

Based on the differential expression results of the above genes and proteins, and based on the pathway enrichment analysis, the core protein was selected for forwarding molecular docking with D-limonene. D-Limonene molecular structure data were obtained from the PubChem website (https://pubchem.ncbi.nlm.nih.gov/), and related protein crystal structure data from the RCSB website (http://www.rcsb.org/). We used Discovery Studio 2016 to calculate the molecular docking, and we drew 2D and 3D effect pictures. The results of molecular docking suggest the mechanism of the effect of D-limonene on COVID-PF and guided the selection of related detection indicators in subsequent animal experiments.

Antibodies and Reagents

D-Limonene was purchased from Sigma-Aldrich Co., Ltd. (183164). Bleomycin (BLM) was obtained from Cool Chemical Technology (Beijing) Co., Ltd. (S656455V), and PFD was obtained from Beijing Continent Pharmaceutical Co., Ltd. (190806). The Masson Tricolor staining kit (G1006-100), H&E staining kit (GP1031), reactive oxygen species (ROS) test kit

(ROS, 2019-07), and the detection kits for hydroxyproline (HYP; 201900711), malondialdehyde (MDA; 20190830), superoxide dismutase (SOD; 20191125), and total protein quantification [by bicinchoninic acid (BCA) method, 20190711] were all purchased from Nanjing Jiancheng Bioengineering Research Institute. Rabbit anti-phosphatidylinositol 3-kinase (PI3K) antibody (bs-10657R), rabbit anti-p-PI3K p110-Ser1070 antibody (bs-6417R), rabbit anti-AKT1 antibody (bs-0115R), rabbit anti-p-AKT1-S473 antibody (bs-12456R), and rabbit anti-p-NF-κB p65-S536 antibody (bs-0982R) were purchased from Biosynthesis Biotechnology Inc. (Beijing, China). Anti-IκBα kinase (IKK)-α antibody (A2062), rabbit anti-IκBα antibody (A1187), and anti-β-actin antibody (AC026) were obtained from Wuhan ABclonal Biotechnology Co., Ltd. Rabbit anti-p-AKT1-T308 antibody (GB13459), rabbit anti-nuclear factor-κB (NF-κB) p65 antibody (GB11997), rabbit anti-α-SMA antibody (GB11044), rabbit anti-COL-I-A1 antibody (COL1A1, GB11022-3), rabbit anti-COL-III antibody (COL3A1, GB13023-2), and horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG antibodies (GB23303) were purchased from Wuhan Servicebio Technology Co., Ltd. The rat TGF-β1 ELISA kit was purchased from CUSABIO BIOTECH CO., Ltd. Rat interleukin (IL)-6, IL-1β, TNF-α, and VEGF ELISA kits were purchased from Wuhan Huamei Bioengineering Co., Ltd.

Animal Grouping and Modeling

The study protocol was approved by the Research Ethics Committee of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine (Approval No. AWE-2019-046) and met the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). Male Sprague Dawley (SD) rats [180–220 g, grade specific pathogen free (SPF)] were purchased from Jinan Pengyue Experimental Animal Breeding Co., Ltd. (Certificate No. SCXK[Lu]2014-0007, Jinan, China) and were placed in an environment with 12-h lighting and 12-h darkness per day and free feeding and drinking. After 7 days of adaptive breeding, the rats were randomly divided into six groups (six in each group): (1) saline (NS) group, (2) BLM + NS group, (3) BLM + D-limonene (25 mg/kg/day) group, (4) BLM + D-limonene (50 mg/kg/day) group, (5) BLM + D-limonene (100 mg/kg/day) group, and (6) BLM + PFD (150 mg/kg/day) group. The single intratracheal instillation of BLM (5 mg/kg) was used to induce PF in rats. After the modeling, the rats in the D-limonene group were injected intraperitoneally with the corresponding concentration of drugs. The rats in the PFD group were given intragastric administration of PFD and were sacrificed 28 days later. The rat blood, which was taken from the abdominal aorta, was separated at 5,000 rpm for 10 min at 4°C, and the serum was stored at −80°C. Lung tissues were collected and weighed. The formula lung index = (lung weight (g)/[body weight (g)] × 100% was calculated and obtained. The whole lung was lavaged three times using 2 ml of physiological saline, and bronchoalveolar lavage fluid (BALF) was then collected. Part of the lung tissues was placed in 4% paraformaldehyde, with the rest frozen in liquid nitrogen and stored at −80°C for further examination.

Morphological and Histological Analyses

The lung tissues were fixed using 4% paraformaldehyde for 48 h, embedded in paraffin, and sliced with a thickness of 5 μm. The slices were stained with H&E and Masson trichrome to evaluate the pathological changes of lung tissues. At the same time, we obtained images that were magnified 200 times using an optical microscope. According to the Szapiel scoring standard and Ashcroft scoring standard, the degree of alveolitis and PF was scored, respectively (19, 20).

Measurement of Hydroxyproline, Malondialdehyde, Reactive Oxygen Species Content, and Superoxide Dismutase Activity

The lung tissues were ground in cold physiological saline to obtain a 10% lung tissue homogenate. The homogenate was separated at 3,500 rpm 10 min at 4°C, and the supernatant was retained for the detection of HYP, MDA content, ROS level, and SOD activity. Both were determined according to the corresponding kit instructions.

Enzyme-Linked Immunosorbent Assay

BALF and serum were prepared for ELISA. An ELISA kit was used to detect the contents of TNF-α, IL-1β, and IL-6 in rat BALF, and TGF-β1, and VEGF in serum. According to the manufacturer's instructions, samples were added to wells of an assay plate coated with a capture antibody. After incubation, the assay plate was washed three times, and a detection antibody was added. After 1-h incubation at room temperature and washing the plate three times, streptavidin–HRP was added to the wells. The color was developed with the tetramethylbenzidine substrate, and the absorbance was measured by enzyme-labeled instrument.

Western Blot

After the total protein was extracted from the lung tissues using radioimmunoprecipitation assay (RIPA) lysis buffer containing 0.1% phenylmethylsulfonyl fluoride (PMSF), we extracted the protein from the lung tissues according to the manufacturer's instructions. We used the BCA protein detection kit to measure protein concentration. Equal amounts of protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking, 5% non-fat milk in TBST was incubated with primary antibodies against PI3K (diluted 1:1,000), p-PI3K (diluted 1:800), AKT (diluted 1:1,000), p-AKT1-S473 (diluted 1:1,000), p-AKT1-T308 (diluted 1:1,000), NF-κB p65 (diluted 1:800), p-NF-κB p65 (diluted 1:1,000), IκBα (diluted 1:800), and IKK-α (diluted 1:800) at 4°C overnight. After being washed four times with TBST, we incubated with goat anti-rabbit secondary antibody for 1.5 h at room temperature and then washed four times with TBST, forming protein bands on the membrane with enhanced chemiluminescence reagent.

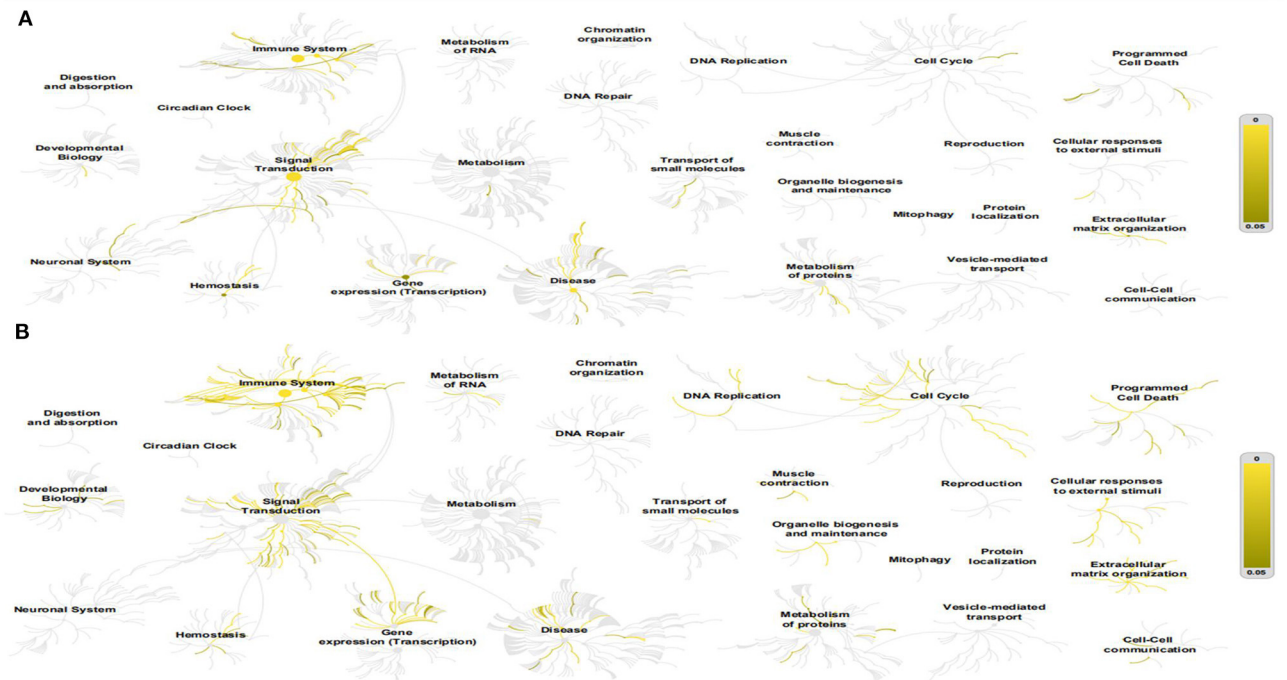

FIGURE 1 | This figure shows a genome-wide overview of the results of coronavirus disease-related pulmonary fibrosis (COVID-PF) **(A)** and PF **(B)**. Reactome pathways are arranged in a hierarchy. The center of each of the circular "bursts" is the root of one top-level pathway. Each step away from the center represents the next level lower in the pathway hierarchy. The color code denotes the overrepresentation of that pathway in your input dataset. Light gray signifies pathways that are not significantly overrepresented.

ImageJ software was used to detect the gray value of the protein bands.

Quantitative Real-Time Polymerase Chain Reaction

mRNA was extracted from the superficial dorsal horn using a universal RT-PCR Kit (Solarbio Science & Technology Co., Ltd., Shanghai, China) following the manufacturer's instructions. Samples were treated with DNase and then purified using an RNeasy kit (Qiagen, Hilden, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal reference. PCR primer sequences included the following: IL-6: forward primer: 5′-ATGAAGTTTCT CTCCGCAAGAGACTTCCAGCCAG-3′; reverse primer: 5′-CTAGGTTTGCCGAGTAGACCTCATAGTGACC-3′, TNF-α: forward primer: 5′-CTCCCAGAAAAGCAAGCAAC-3′; reverse primer: 5′-CGAGCAGGAATGAGAAGAGG-3′, IL-1β: forward primer: 5′-ATGCCTCGTGCTGTCTGAC-3′; reverse primer: 5′-TCCCGACCATTGCTGTTTCC-3′, VEGF: forward primer: 5′-GGCTCTGAAACCATGAACTTTCT-3′; reverse primer: 5′-GCAGTAGCTGCGCTGGTAGAC-3′, NF-κB p65: forward primer: 5′-GACGAGGCTCGGAGAGCCCA-3′; reverse primer: 5′-CTGGGGCGGCTGACCGAATG-3′, PI3K: forward primer: 5′-TGCTATGCCTGCTCTGTAGTGGT-3′; reverse primer: 5′-GTGTGACATTGAGGGAGTCGTTG-3′, AKT: forward primer: 5′-GTGCTGGAGGACAATGACTACGG-3′; reverse primer: 5′-AGCAGCCCTGAAAGCAAGGA-3′, GAPDH: forward primer: 5′-TGATGACATCAAGAAGGTGG TGAAG-3′; reverse primer: 5′-TCCTTGGAGGCCATGTGGGC CAT-3′.

Immunohistochemical Analysis

After being dewaxed, the lung slices were subjected to antigen recovery with citrate buffer under microwave heating. The slices were cooled down to room temperature and then sealed with 3% bovine serum albumin (BSA) for 30 min and were incubated overnight with primary antibody at 4°C. The primary antibodies used were rabbit anti-α-SMA antibody (diluted 1:1,000), rabbit anti-COL1A1 antibody (diluted 1:1,000), rabbit anti-COL3A1 antibody (diluted 1:200), rabbit anti-PI3K antibody (diluted 1:200), rabbit anti-AKT antibody (diluted 1:250), and rabbit anti-NF-κB p65 antibody (diluted 1:100). The slices were then washed with PBS and incubated with goat anti-rabbit secondary antibody (diluted 1:200) at 37°C for 50 min. After being rinsed with PBS, the slices were visualized with diaminobenzidine and counterstained with hematoxylin. The average optical density was measured using ImageJ.

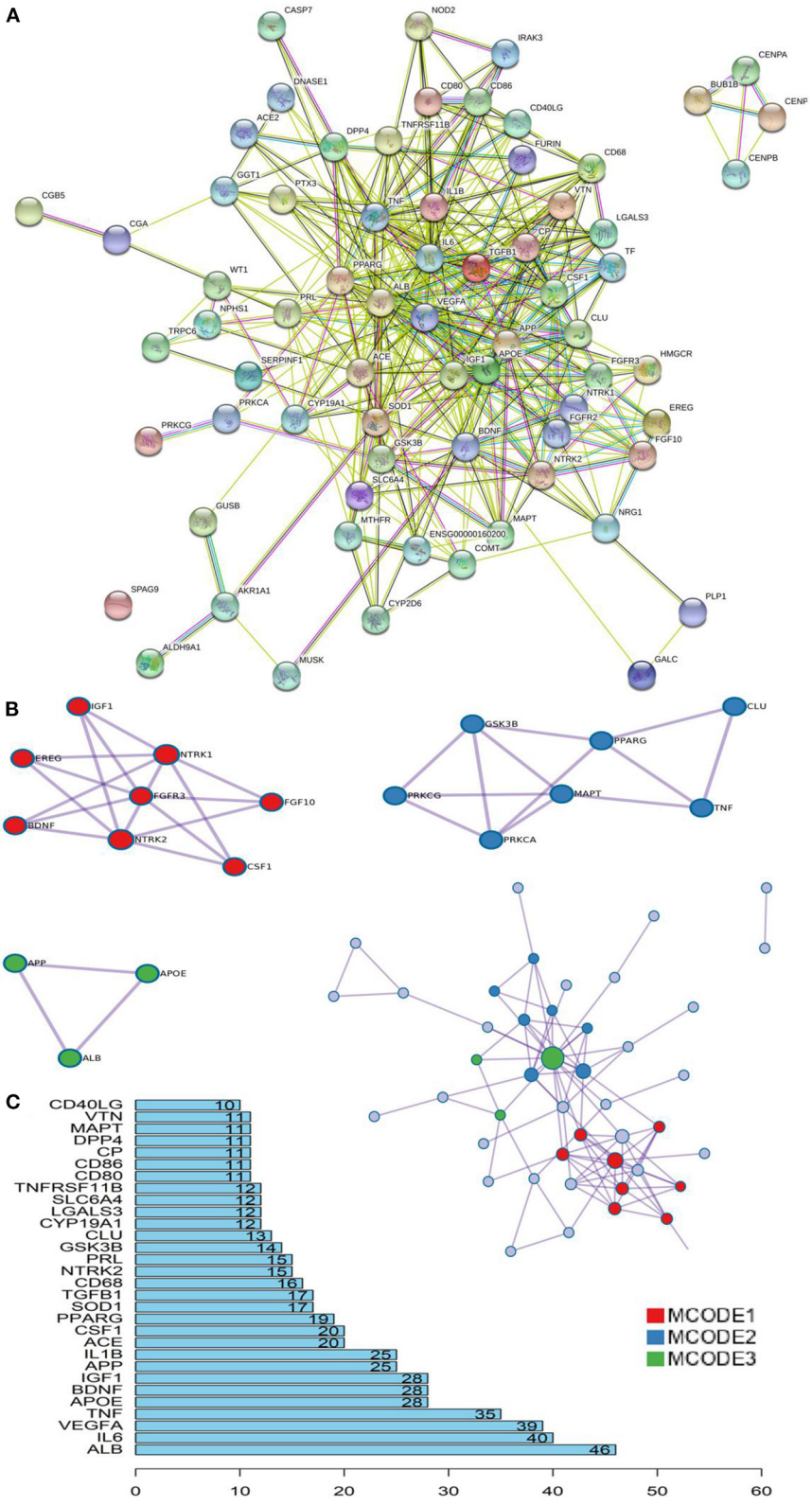

FIGURE 2 | Key proteins and interactions in coronavirus disease-related pulmonary fibrosis (COVID-PF). **(A)** Protein–protein interaction (PPI) network of differential expression protein of COVID-PF. **(B)** Three important core modules and their relationship displayed in MCODE module analysis. **(C)** Ranking of correlation degree of key proteins.

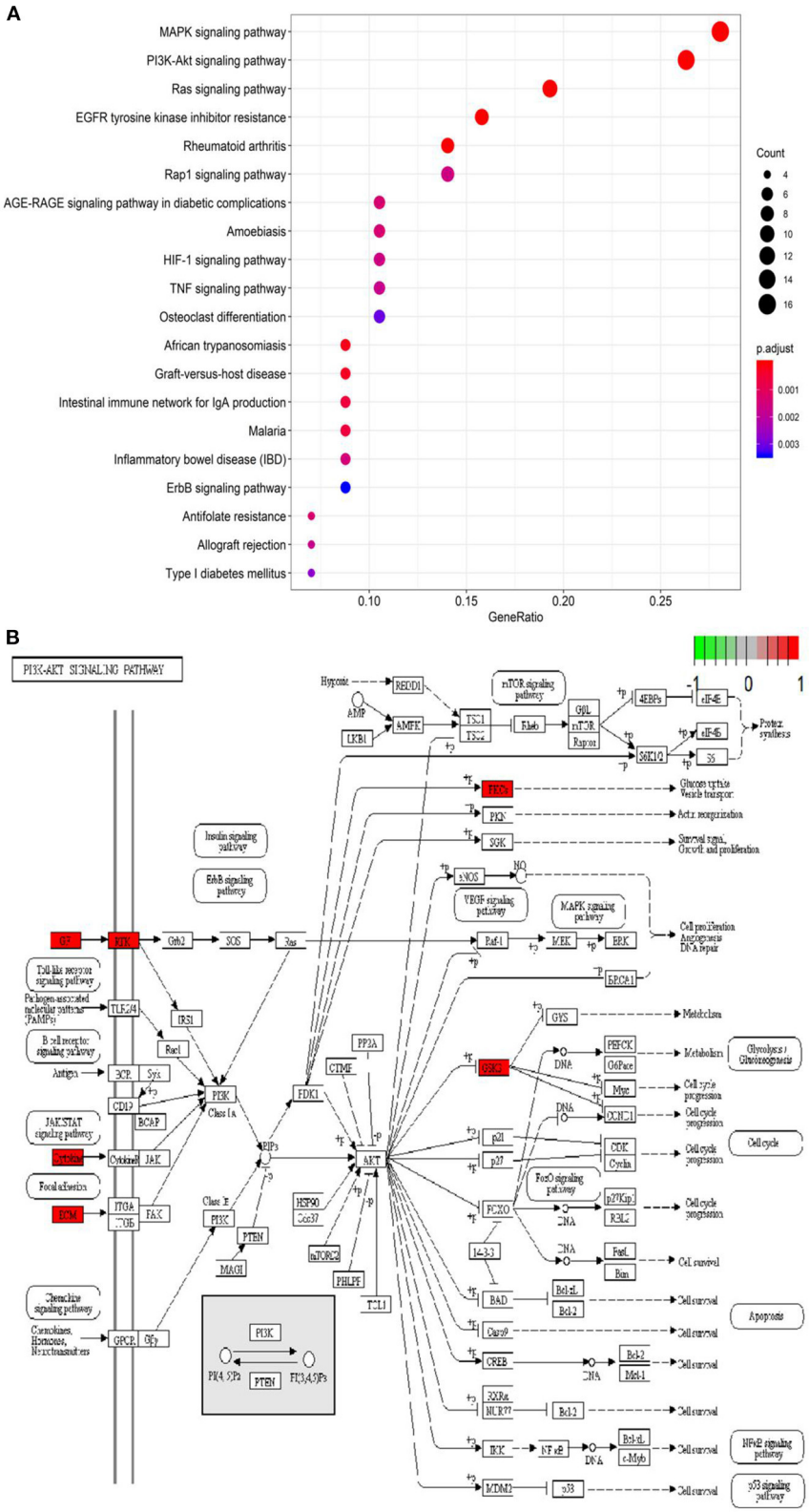

FIGURE 3 | Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. **(A)** Bubble chart of enrichment analysis results of KEGG pathway analysis. **(B)** The key target of the PI3K/AKT signaling pathway in coronavirus disease-related pulmonary fibrosis (COVID-PF).

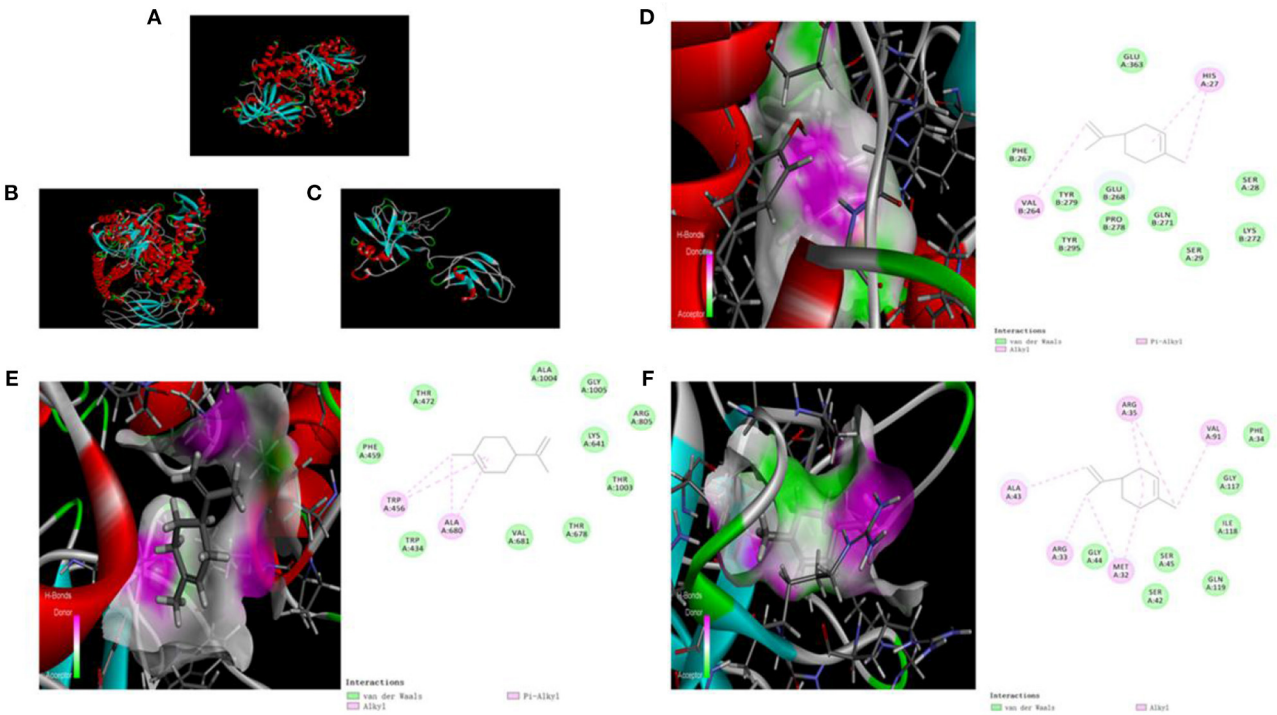

FIGURE 4 | The upper left corner shows the 3D schematic diagram of D-limonene binding to PI3Kδ **(A)**, PI3Kβ **(B)**, and NF-κB p65 **(C)**. **(D)** 3D schematic diagram of active space binding of D-limonene and PI3Kδ. **(E)** 3D schematic diagram of active space binding of D-limonene and PI3Kβ. **(F)** 3D schematic diagram of active space binding of D-limonene and NF-κB p65.

Statistical Analysis

Data are shown as mean ± standard deviation (SD). Differences between the groups were evaluated using one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) *post-hoc* test. A $P < 0.05$ was considered statistically significant. Statistical analyses and figures were obtained using IBM SPSS Statistics 23.0 (IBM SPSS Software, NY, USA) and GraphPad Prism Version 8.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

The Difference Between the Key Biological Processes of Coronavirus Disease-Related Pulmonary Fibrosis and Pulmonary Fibrosis

The Reactome Pathway Database shows a genome-wide overview of the results of COVID-PF and PF (**Figure 1**). The first five core paths of COVID-PF are IL-10 signaling; constitutive signaling by aberrant PI3K in cancer; PI3K/AKT signaling in cancer; PI5P, PP2A, and IER3 regulate the PI3K/AKT signaling; and transcriptional regulation by the AP-2 (TFAP2) family of transcription factors. The first five core paths of PF are IL-4 and IL-13 signaling, signaling by ILs, cytokine signaling in immune system, immune system, and antigen processing–cross-presentation. This suggests that the biological pathways of COVID-PF and PF caused by other reasons are different. The PI3K/AKT signaling pathway plays a key role in the occurrence of COVID-PF.

Protein–Protein Interaction Network Analysis, Gene Ontology Function Enrichment Analysis, and Kyoto Encyclopedia of Genes and Genomes Pathway Analysis

Based on the STRING database and Metascape platform, a PPI network related to COVID-PF was constructed (**Figure 2A**). MCODE module analysis showed that the functions of three important core modules were mainly focused on vascular remodeling, inflammatory response, and PI3K/Akt signaling pathway-related proteins. The interaction between the three core modules is shown in **Figure 2B**. The sequence of the first 30 key proteins is shown in **Figure 2C**. The first five key proteins included ALB, IL-6, VEGFA, TNF, and APOE (**Figure 2C**). Biological process analysis suggested that the humoral immune response of COVID-PF patients was unbalanced, characterized by the high expression of inflammatory mediators (**Supplementary Figure 1**). KEGG pathway analysis revealed that the MAPK signaling pathway and PI3K/Akt signaling

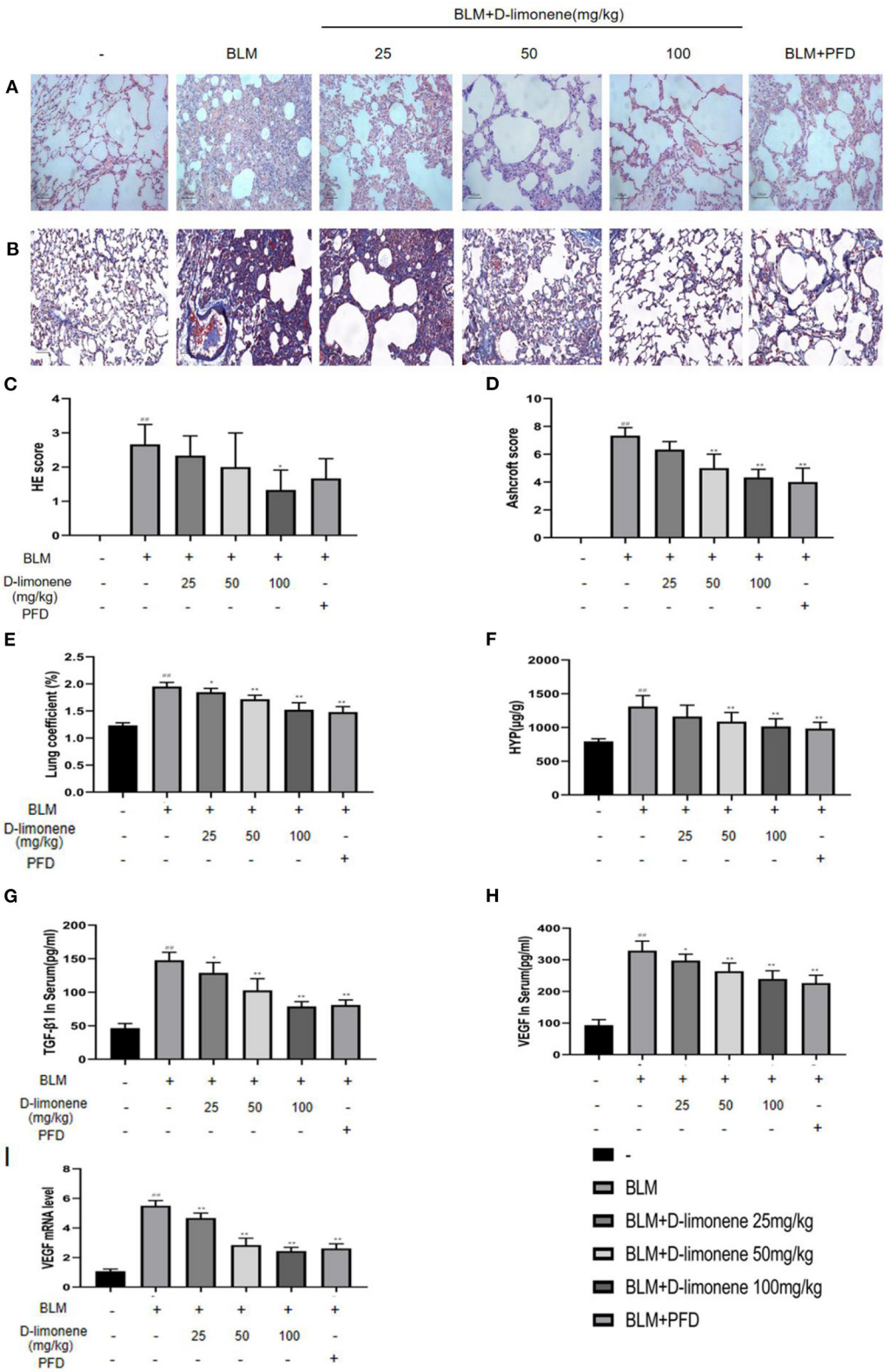

FIGURE 5 | The protective effect of D-limonene on bleomycin (BLM)-induced pulmonary fibrosis (PF) in rats. **(A)** Photomicrographs of lung sections stained with H&E. **(B)** Photomicrographs of lung sections stained with Masson trichrome staining. **(C)** Alveolitis score of each group. **(D)** Statistics of the PF area in each group.

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FIGURE 5 | (E) Measurement of lung coefficient in each group. **(F)** Determination of hydroxyproline content in lung tissues of each group. **(G)** Determination of TGF-β1 content in the serum of each group. **(H)** Determination of VEGF content in the serum of each group. **(I)** The mRNA expression levels of VEGF in each group detected by qRT-PCR. Data are presented as the means ± SD ($n = 3$ or 6), $^{\#}$comparison with the control group, and *comparison with the BLM group. $^{\#\#}P < 0.01$; $^{*}P < 0.05$; and $^{**}P < 0.01$.

pathway were the core pathways of COVID-PF, which was consistent with the conclusion of the Reactome Pathway Database (**Figures 3A,B**).

Molecular Docking

We used molecular docking technology to dock key proteins suggested by PPI network analysis and KEGG pathway analysis with D-limonene. The CDOCKER experiment revealed that D-limonene can be docked effectively with PI3Kδ (PDB code: 4XEO), PI3Kβ (PDB code: 2Y3A), and NF-κB p65 (PDB code: 1VKX). Furthermore, the binding energies were −22.26, −14.54, and −18.77 kcal/mol. Molecular docking also revealed the binding sites of D-limonene and each protein, as shown in **Figures 4A–F**.

D-Limonene Improves Bleomycin-Induced Pulmonary Fibrosis

PF was successfully induced by intratracheal instillation of BLM (5 mg/kg) in rats. H&E staining confirmed that the structure of rat lung tissues in the BLM group was disordered, with thickened alveolar walls; a large number of inflammatory cells had infiltrated the alveolar cavity and the interstitial fluid; and some of the alveoli disappeared. Masson trichrome staining revealed a large amount of collagen deposition (**Figures 5A,B**). However, D-limonene significantly improved lung tissue structural damage caused by BLM (**Figures 5C,D**). The protective effect of the 100 mg/kg dose group was similar to that of PFD (150 mg/kg), and the lung coefficient was significantly reduced in a dose-dependent manner (**Figure 5E**). HYP is the main component of collagen, VEGF is upregulated in lung fibroblasts under hypoxia, and TGF-β1 can induce the fibroblasts to synthesize a large amount of collagen. After treatment with D-limonene at a dose of 25–100 mg/kg, the levels of HYP in the lung tissues of lung fibrosis rats and the TGF-β1 in the serum were reduced compared with those in the BLM group. The expressions of VEGF and VEGF mRNA were downregulated (**Figures 5F–I**). There was no significant difference between the 100 mg/kg group and PFD (150 mg/kg) groups.

D-Limonene Alleviates Pulmonary Fibrosis by Inhibiting the PI3K/Akt/IKK-α/NF-κB p65 Signaling Pathway

After BLM (5 mg/kg) induced the PF rat model successfully, the expressions of PI3K, Akt, IKK-α, and NF-κB p65 in the BLM group were all upregulated ($P < 0.05$), and IκBα expression was downregulated ($P < 0.01$), indicating that the PI3K/Akt/IKK-α/NF-κB p65 signaling pathway was activated (**Figures 6A,B,E**). After being administered PFD and different doses of D-limonene, the expressions of PI3K, Akt, IKK-α, and NF-κB p65 decreased in a dose-dependent manner, and the expression of IκBα was upregulated. In addition, the expressions of PI3K mRNA, Akt mRNA, and NF-κB p65 mRNA were downregulated (**Figure 6C**), indicating that D-limonene can inhibit the activation of the PI3K/Akt/IKK-α/NF-κB p65 signaling pathway in PF rats. The immunohistochemistry results also proved this (**Figures 6D,J–L**), and the effect of D-limonene at a dose of 100 mg/kg was similar to that of PFD. The study of phosphorylated proteins found that BLM induced the phosphorylation of PI3K in the rat model and also induced the rapid and sustained phosphorylation of AKT at Thr308, Ser473, and NF-κB p65 at Ser536. Different doses of D-limonene downregulated the expressions of p-PI3K, p-AKT Thr308, and p-NF-κB p65 Ser536 (**Figures 6E–I**). PFD downregulated the expressions of p-AKT Thr308 and p-AKT Ser473 (**Figures 6G,H**). However, we did not observe a significant change in p-AKT Ser473 expression in the D-limonene group (**Figure 6H**).

D-Limonene Reduces Inflammatory Response, Oxidative Stress, and Collagen Deposition in Lung Tissue of Pulmonary Fibrosis Rats

In order to verify the anti-inflammatory and anti-oxidant capacity of D-limonene, we used ELISA to detect relevant biomarkers. As expected, 28 days after successful modeling, there were increases in the levels of inflammatory mediators and oxidative stress in the lung tissue of the BLM Group ($P < 0.01$). At the same time, D-limonene reduced the levels of IL-1β, TNF-α, IL-6, and MDA in the lung tissues of PF rats and ROS levels; downregulated the expressions of IL-1β mRNA, TNF-α mRNA, and IL-6 mRNA; and increased the activity of SOD. The effects of 100 mg/kg D-limonene and PFD groups were similar or significantly different (**Figures 7A–G**). Collagen deposition is a significant manifestation of PF. Immunohistochemistry showed that the expressions of COL1A1, COL3A1, and α-SMA were upregulated in the BLM group ($P < 0.05$), while PFD and D-limonene decreased their expressions. The effect of D-limonene was more obvious than that of PFD, and it was dose-dependent (**Figures 7H–K**).

DISCUSSION

In this study, the key biological processes and signaling pathways of COVID-PF differentially expressed genes were analyzed. The mechanism of action of D-limonene in a rat PF model was discussed. We found that the occurrence of COVID-PF is closely related to the PI3K/AKT signaling pathway. D-Limonene can significantly improve the collagen deposition and oxidative stress levels of PF rats and inhibit inflammation and angiogenesis. The

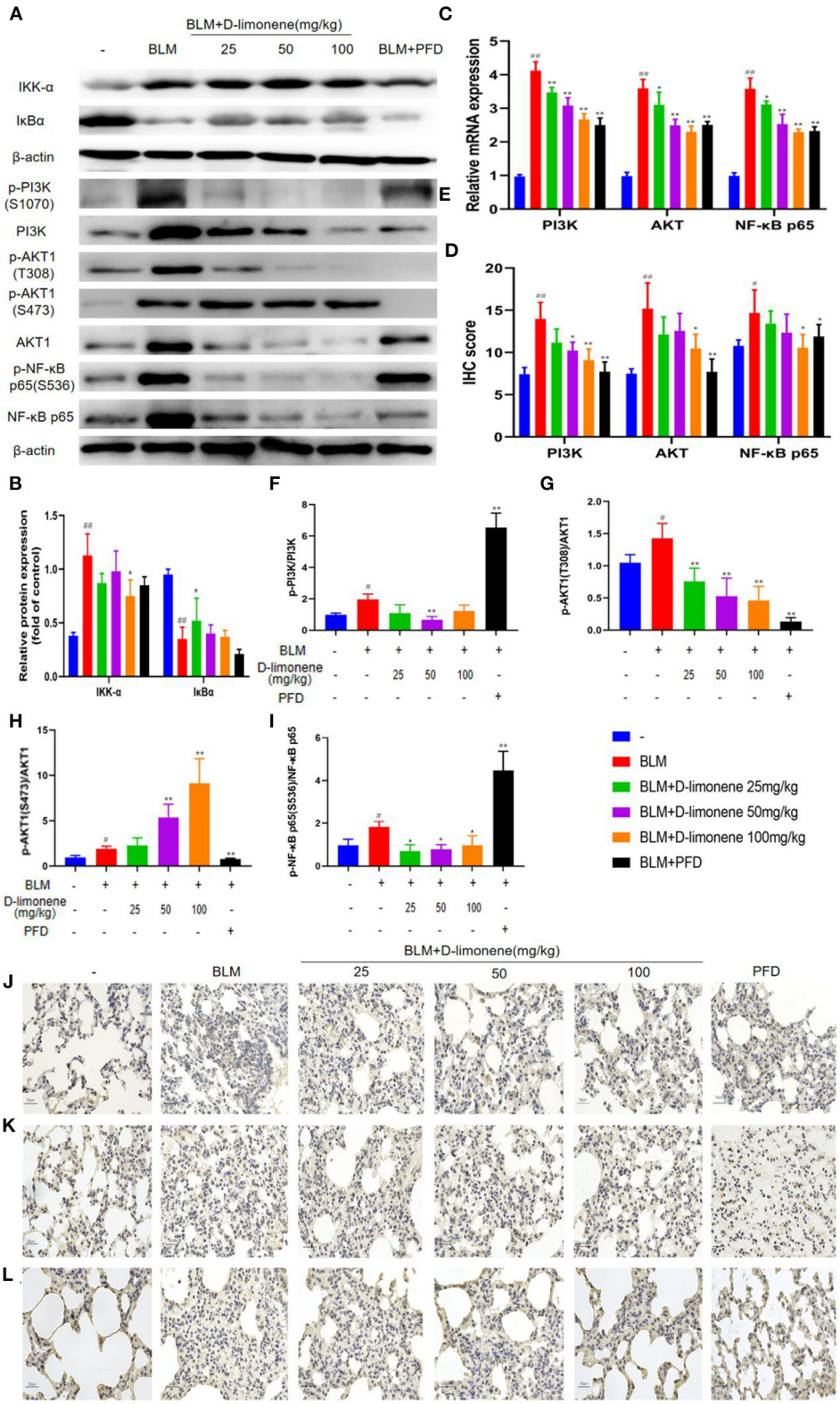

FIGURE 6 | D-Limonene alleviates pulmonary fibrosis (PF) by inhibiting PI3K/Akt/IKK-α/NF-κB p65 signaling pathways. **(A)** Western blot analysis of the protein levels of IKK-α and IκBα in lung tissues. **(B)** Densitometric analysis of IKK-α and IκBα in the immunoblots, using β-actin as the internal reference. **(C)** The mRNA expression

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FIGURE 6 | levels of PI3K, Akt, and NF-κB p65 in each group detected by qRT-PCR. **(D)** The average optical density of PI3K, Akt, and NF-κB p65. **(E–I)** Western blot analysis of PI3K **(F)**, Akt **(G,H)**, NF-κB p65 **(I)** protein expression, and phosphorylation level in lung tissue. **(J–L)** Immunohistochemical staining of PI3K- **(J)**, AKT- **(K)**, and NF-κB p65 **(L)**-positive cells in the lungs. Data are presented as the means ± SD ($n = 3$), #comparison with the control group, and *comparison with the bleomycin (BLM) group. #$P < 0.05$, ##$P < 0.01$; *$P < 0.05$; and **$P < 0.01$.

mechanism may be mediated by inhibiting the PI3K/Akt/IKK-α/NF-κB p65 signaling pathway.

As a "global pandemic" disease announced by WHO, the number of COVID-19-related infections continues to rise. Coronavirus not only has a higher fibrogenic potential than common respiratory viruses but also makes patients more likely to enter a dangerous acute respiratory distress syndrome (ARDS) state. Patients with ARDS require mechanical ventilation to maintain respiratory function during treatment to improve the patient's hypoxic state, and mechanical ventilation-related lung injury is a major adverse reaction caused by the ventilator to patients (21). The harmful effect of mechanical ventilation is not only mediated by the systemic release of local inflammatory cytokines but also induced by mechanical stress, which can lead to the transformation of epithelial stroma and the release of fibrogenic mediators caused by cell stretching and mechanical ventilation, which then develops into PF (22, 23). Therefore, the factors of continuous lung damage in the course of COVID-19 are complicated, and PF may become the main complication after the completion of this outbreak. Bioinformatics research suggests that inflammatory response, oxidative stress, angiogenesis, and other biological processes and the PI3K/AKT signaling pathway are closely related to COVID-PF. The PI3K/AKT signaling pathway is involved in many cellular processes such as cell differentiation, proliferation, apoptosis, and angiogenesis (24–26). Studies have shown that the PI3K/Akt pathway, as a form of "adaptive strategy," is involved in the immune response process of the host cell to counteract viral invasion (27). This partly explains the preference of COVID-PF as a complication and sequela of viral infection for PI3K/Akt signaling pathway.

Although COVID is not unique to humans, animal models of coronavirus infection show different disease characteristics than humans. Although several other animal models of SARS-CoV infection have been described, these models rarely show lethality (28–32). Although studies have predicted that the survival of lethally infected aged mice could be extended to the fibrotic phase of ARDS using sublethal infection, the model has clear fibrosis characteristics, which are very different from the clinical manifestations of human patients (33). Therefore, this study selected BLM to induce PF in rats at the start of the study. Our study found that the PI3K/Akt signaling pathway in the lung tissue of PF rats was significantly activated, consistent with the predictions of bioinformatics. AKT is the main effector kinase of the PI3K signaling pathway. AKT activated by phosphorylation has important biological significance. Many growth factors, hormones, and cytokines activate AKT by binding their homologous receptor tyrosine kinase or by triggering the activation of lipid kinase PI3K, thereby generating PIP3 in the plasma membrane of the cell. AKT binds to PIP3 through its PH domain, causing AKT to translocate to the cell membrane and phosphorylated by the double phosphorylation mechanism. PDK1, which is also translocated to the cell membrane due to its PH domain, can also phosphorylate AKT by activating the Thr308 site. The secondary phosphorylation of Ser473 at the carboxyl terminus of AKT is also necessary for activity and is performed by mTORC2 (34, 35). Studies have confirmed that PI3K/Akt signaling pathway is involved in the pathogenesis of PF, and blocking the PI3K/Akt pathway can reduce BLM-induced inflammation and fibrosis (36). NF-κB is a nuclear transcription factor that is involved in the expression of inflammatory cytokine genes. At rest, the NF-κB dimer binds to IκBα and exists in the cytoplasm in an inactive form. Under the stimulation of lipopolysaccharide (LPS) and TNF-α (37), IKK is activated to phosphorylate serine residues at specific sites of IκBα and then ubiquitinates and degrades. At the same time, Ser536 of NF-κB p65 transcription activation domain can also be phosphorylated by IKK to enhance its transcriptional activity. In contrast, the process of LPS activation of NF-κB p65 is initiated by upstream kinases such as PI3K/AKT (38, 39). This study found that D-limonene can dose-dependently inhibit the expression of PI3K, Akt, and NF-κB p65; can inhibit the upregulation of IKK-α and the degradation of IκBα; and can inhibit the phosphorylation of PI3K, AKT (Thr308 site), and NF-κB p65 (Ser536 site), which is consistent with previous reports on the mechanism of action of D-limonene (40–42).

It has been reported that a hypoxic environment can promote the progression of fibrosis through epithelial–stromal transformation. Furthermore (43), hypoxia not only can directly cause lung tissue damage but also can aggravate the inflammatory response and oxidative stress. In the inflammatory state, the production of oxygen free radicals increases, and the body cannot produce enough SOD and catalase to eliminate them in time, which aggravates the damage, as mentioned above (44). Moreover, activation of the PI3K/AKT cascade is triggered by ROS (45). Our study found that D-limonene can effectively inhibit the secretion of inflammatory mediators and reduce the level of oxidative stress, which is consistent with previous reports (46). Hypoxia is the inevitable state of ARDS. In the middle and late stages of ARDS, with the initiation of lung injury repair mechanism, collagen deposition, and fibrosis promotion level increase (47). TGF-β, a major fibrogenic factor, is also one of the promoters of the PI3K/Akt signaling pathway (48). In this process, the necessary vascular remodeling and generation processes are very significant in COVID-PF. Some studies have shown that epidermal growth factor receptor (EGFR) signaling is a key regulator of SARS-CoV-induced lung

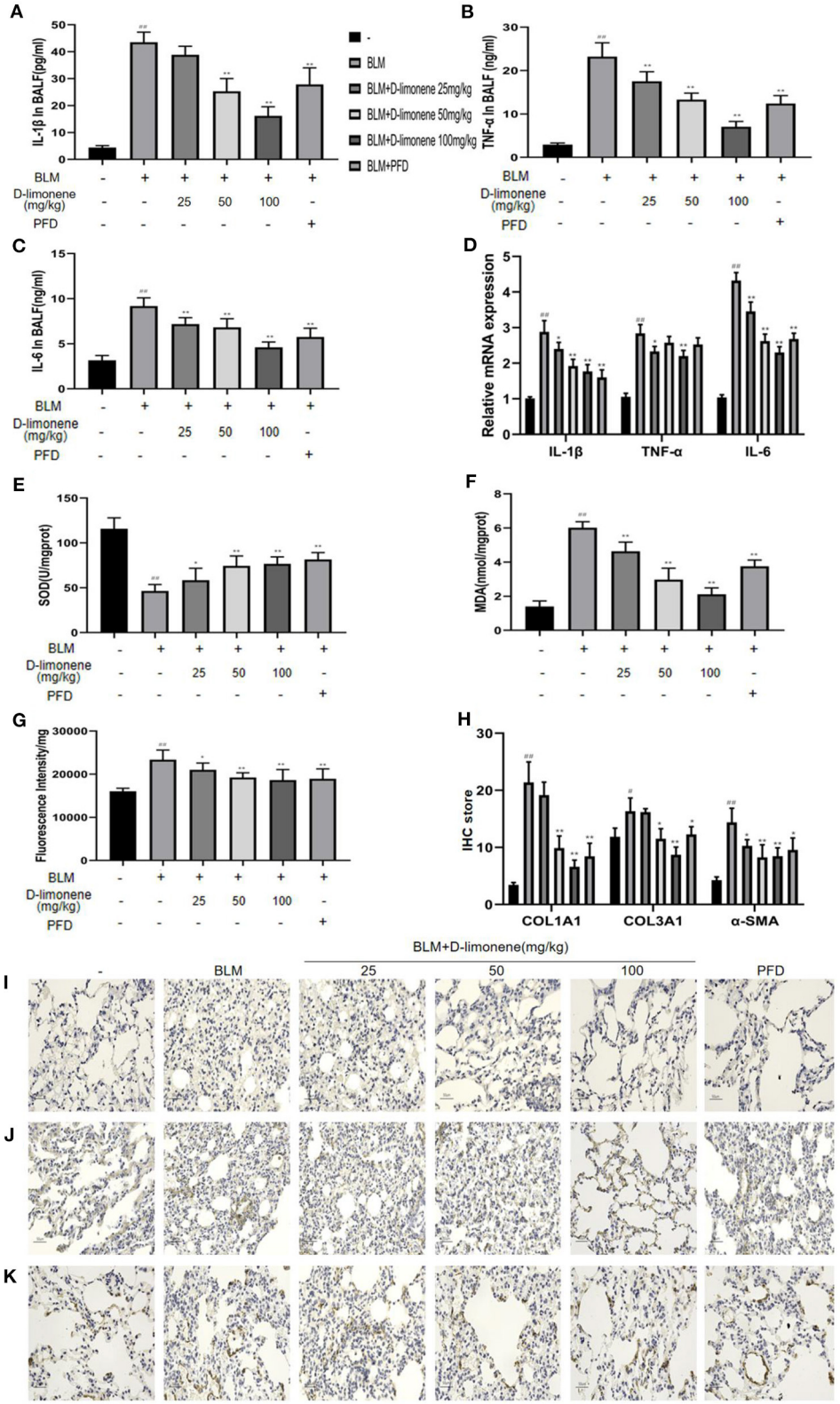

FIGURE 7 | D-Limonene reduces inflammation, oxidative stress, and collagen deposition in the lung tissue of rats with pulmonary fibrosis (PF). **(A)** Determination of IL-1β in bronchoalveolar lavage fluid (BALF) of each group. **(B)** Determination of TNF-α in BALF of each group. **(C)** Determination of IL-6 in BALF of each group.

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FIGURE 7 | (D) The mRNA expression levels of IL-1β, TNF-α, and IL-6 in each group detected by qRT-PCR. **(E)** Determination of superoxide dismutase (SOD) in the lung tissue of each group. **(F)** Determination of malondialdehyde (MDA) in the lung tissue of each group. **(G)** Determination of reactive oxygen species (ROS) in the lung tissue of each group. **(H)** The average optical density of COL1A1, COL3A1, and α-SMA. **(I,K)** Immunohistochemical staining of COL1A1 **(I)**, COL3A1 **(J)**, and α-SMA **(K)**-positive cells in the lungs. Data are presented as the means ± SD ($n = 3$ or 6), #comparison with the control group, and *comparison with the bleomycin (BLM) group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; $^{*}P < 0.05$; and $^{**}P < 0.01$.

damage leading to fibrosis (49) and mainly regulated by the PI3K/Akt signaling pathway. D-Limonene can downregulate the expression of fibrotic markers such as COL1A1, COL3A1, and α-SMA and can reduce the content of TGF-β1 and VEGF mRNA in tissues, which is of positive significance for COVID-PF and even ARDS in the middle and late stages. Although there are inherent difficulties in preparing animal models of COVID-PF as mentioned above, this study did not proceed directly on the relevant animal models, so only D-limonene can be called a "potential" effective natural compound. In addition, there are few studies on COVID-PF at present. The differential gene expression data collected in the public database may not fully reflect all the characteristics of COVID-PF. Moreover, due to factors such as lack of time, we have not yet established a sufficient number of pathologically confirmed COVID-19-related PF patient databases to support our research conclusions. The above items are the main limitations of this research. In the current imaging analysis of these patients, we found that their PF can show two patterns of usual interstitial pneumonia or non-specific interstitial pneumonia—these are two completely different outcomes. Therefore, studying the pathological characteristics and prognosis of COVID-19-related PF in different populations and the therapeutic value of existing drugs for other fibrotic lung diseases on COVID-19-related PF should be the next research direction. Therefore, in future studies, we will continue to pay attention to the research progress of this disease in order to fully understand its pathogenesis.

CONCLUSIONS

It is proved that the imbalance between collagen breakdown and metabolism, inflammatory response, and angiogenesis are the core processes of COVID-PF, and PI3K/AKT signaling pathways and related signal transduction molecules are the key targets of the COVID-PF treatment. The ability of D-limonene was reported for the first time to protect against lung fibrosis induced by BLM in rats. The mechanism is related to the binding of PI3K and NF-κB p65 and the inhibition of PI3K/Akt/IKK-α/NF-κB p65 signaling pathway expression and phosphorylation. Additionally, new insights are provided into the potential value of D-limonene in the treatment of COVID-PF. However, at this time, the research on the differential gene expression of COVID-19 leaves some room for improvement, so our research on COVID-PF cannot fully summarize the characteristics of COVID-19-PF. It remains to be further explored.

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 animal study was reviewed and approved by Research Ethics Committee of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine.

AUTHOR CONTRIBUTIONS

Experimental design was carried out by FY and WL. Validation was performed by ZH and RCa. Data curation was done by XC and HZ. Writing-original draft preparation was done by GZ and YL. Writing-review and editing was performed by RCh and WZ. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.591830/full#supplementary-material

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COVID-19 Is Not the Flu: Four Graphs From Four Countries

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Background: COVID-19 has caused a global public health emergency. Government mitigation strategies included a series of behavior-based prevention policies that had a likely impact on the spread of other contagious respiratory illnesses, such as seasonal influenza. Our aim was to explore how 2019–2020 influenza tracked onto COVID-19 pandemic and its mitigation methods.

Materials and Methods: We linked the WHO FluNet database and COVID-19 confirmed cases (Johns Hopkins University) for four countries across the northern (Canada, the United States) and southern hemispheres (Australia, Brazil) for the period 2016–2020. Graphical presentations of longitudinal data were provided.

Results: There was a notable reduction in influenza cases for the 2019–2020 season. Northern hemisphere countries experienced a quicker ending to the 2019–2020 seasonal influenza cases (shortened by 4–7 weeks) and virtually no 2020 fall influenza season. Countries from the southern hemisphere experienced drastically low levels of seasonal influenza, with consistent trends that were approaching zero cases after the introduction of COVID-19 measures.

Conclusions: It is likely that the COVID-19 mitigation measures played a notable role in the marked decrease in influenza, with little to no influenza activity in both the northern and southern hemispheres. In spite of this reduction in influenza cases, there was still community spread of COVID-19, highlighting the contagiousness of SARS-CoV-2 compared to influenza. These results, together with the higher mortality rate from SARS-CoV-2 compared to influenza, highlight that COVID-19 is a far greater health threat than influenza.

Keywords: COVID-19, influenza, transmission, epidemiology, behavior-based policy, behavior change

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INTRODUCTION

On March 11th, 2020, the World Health Organization (WHO) declared COVID-19 a pandemic, a disease caused by the SARS-CoV-2 virus (1). As of December 31st, 2020, there have been around 83.52 million cases in 188 countries, areas, or territories, with a death toll of ∼1.82 million individuals (2). COVID-19 prevention measures have relied upon widespread adherence to

behavior-based policies, like physical distancing, mask wearing, and hand washing, to reduce virus transmission, even with the current introduction of vaccines in numerous countries (3–5). Theoretically, these mitigation measures should also have positive impacts on other transmissible infectious diseases such as the influenza virus.

Seasonal influenza A and B epidemics generally occur between November and April across the northern hemisphere, and between late May and October across the southern hemisphere (6), time periods which have overlapped with various COVID-19 waves in the countries in both hemispheres. As such, we aimed to explore how the epidemiological pattern of the 2019–2020 influenza tracked onto the evolution of the COVID-19 pandemic and the first introduction of behavior-based mitigation methods to prevent its transmission in four countries (Australia, Brazil, Canada, and the United States). Another aim was to provide evidence to combat the miss-information being spread (7) that the impact of COVID-19 is no different to influenza. These have propagated messages within some communities that adherence to the behavior-based prevention policies are unnecessary resulting in non-adherence and in some areas, public protesting against the policies (8).

MATERIALS AND METHODS

Data Sources

We accessed FluNet data on influenza virological surveillance coordinated and provided by the WHO, between January 1st, 2016, and December 27th, 2020 (9). Influenza number of cases (A [H1]; A [H1N1] pdm09; A[H3]; A[H5]; A[not subtyped]; B [Yamagata lineage]; B [Victoria lineage]; B [lineage not determined]) and total number of influenza positive/negative viruses for Australia, Brazil, Canada, and the United States were downloaded for the period 2016–2020. Most recent COVID-19 epidemiological data, between January 1st and December 25th, 2020, were obtained from the Johns Hopkins Coronavirus Resources Center (2, 10). In order to obtain data on policies related to COVID-19, the Coronavirus Government Response Tracker was used to obtain the dates when the governments had first put in place closures and containment measures (school closing, workplace closing, international travel controls, canceling of public events) (11). For the northern hemisphere, the first containment measures included international travel controls on January 22nd and February 22nd for Canada and the United States, respectively. For the southern hemisphere, the international travel controls in Australia (February 2nd) and the school and workplace closing in Brazil (March 12th) were first introduced. Influenza and COVID-19 datasets were merged based on the weeks of the year (i.e., week 1 = the first week in January), and graphical presentations of the raw longitudinal data are provided in order to obtain instantaneous visual insight and discuss the potential influence of COVID-19 outbreak on influenza rates across the individual countries.

RESULT

Northern Hemisphere—Examples of Canada and the United States

Between 2016–2019, the average influenza season occurred between October (week 40) and May (week 19) for both Canada and the United States, with peaks around weeks 7 and 8 (end of February). As seen in **Figure 1**, there was a significant reduction in influenza cases during the first months of 2020 (solid blue curve) compared to the average number of influenza cases (dashed dark blue curve) after the COVID-19 mitigation measures were introduced (solid black line). Furthermore, this notable decrease in influenza cases (lower than 500 cases/week) meant that the influenza season seemed to end 7 and 4 weeks earlier for the United States and Canada, respectively (week 15; between April 6 and 12, 2020) compared to previous years. In contrast, the number of cases of COVID-19 in both countries (solid red curve) started to increase dramatically during week 11 of 2020 (March 9–15, 2020), while there was a more rapid than usual decline in the number of influenza cases between weeks 12 and 13 (March 16–29, 2020). Finally, we observed persistently low numbers of influenza cases, approaching zero values, in both countries throughout the start of the 2020–2021 influenza season, which contrasts the consistent increases in cases and the second wave of COVID-19 (see **Figures 1A,B**).

Southern Hemisphere- Examples of Australia and Brazil

In the southern hemisphere, the average influenza season runs from May (week 19) to November (week 45) in Australia and from March (week 9) to August (week 31) in Brazil, with peaks occurring around week 34 and week 14, respectively (see **Figure 2**). This pattern is notably different from the northern hemisphere. In Australia, the influenza case rates remained around zero from around week 16 up to when we stopped capturing data (December 27, 2020; week 40). More notable, is that the usual peak in influenza cases (around week 34) did not occur in 2020, this is in spite of there being a 2nd COVID-19 wave which covered this period (weeks 24–40, peak at week 31). In Brazil, the initial phases of the 2020 influenza season followed a normal pattern until week 12 (1 week after the introduction of the COVID-19 mitigation measures of the 4 countries included). By week 16 (mid-April, 2020) there were minimal influenza cases and by week 19 (early May, 2020) the case rate dropped to zero, where it remained until the end of data capture (December 27, 2020; week 40). In contrast, the number of COVID-19 cases started to increase around week 13, and have remained elevated ever since.

DISCUSSION

Our descriptive analysis of four countries across the southern and the northern hemispheres provides compelling evidence of the potential association between the waves of the COVID-19 pandemic, the introduction of behavior-based COVID-19 mitigation measures, and a reduction in influenza transmission. There are two notable implications stemming from our

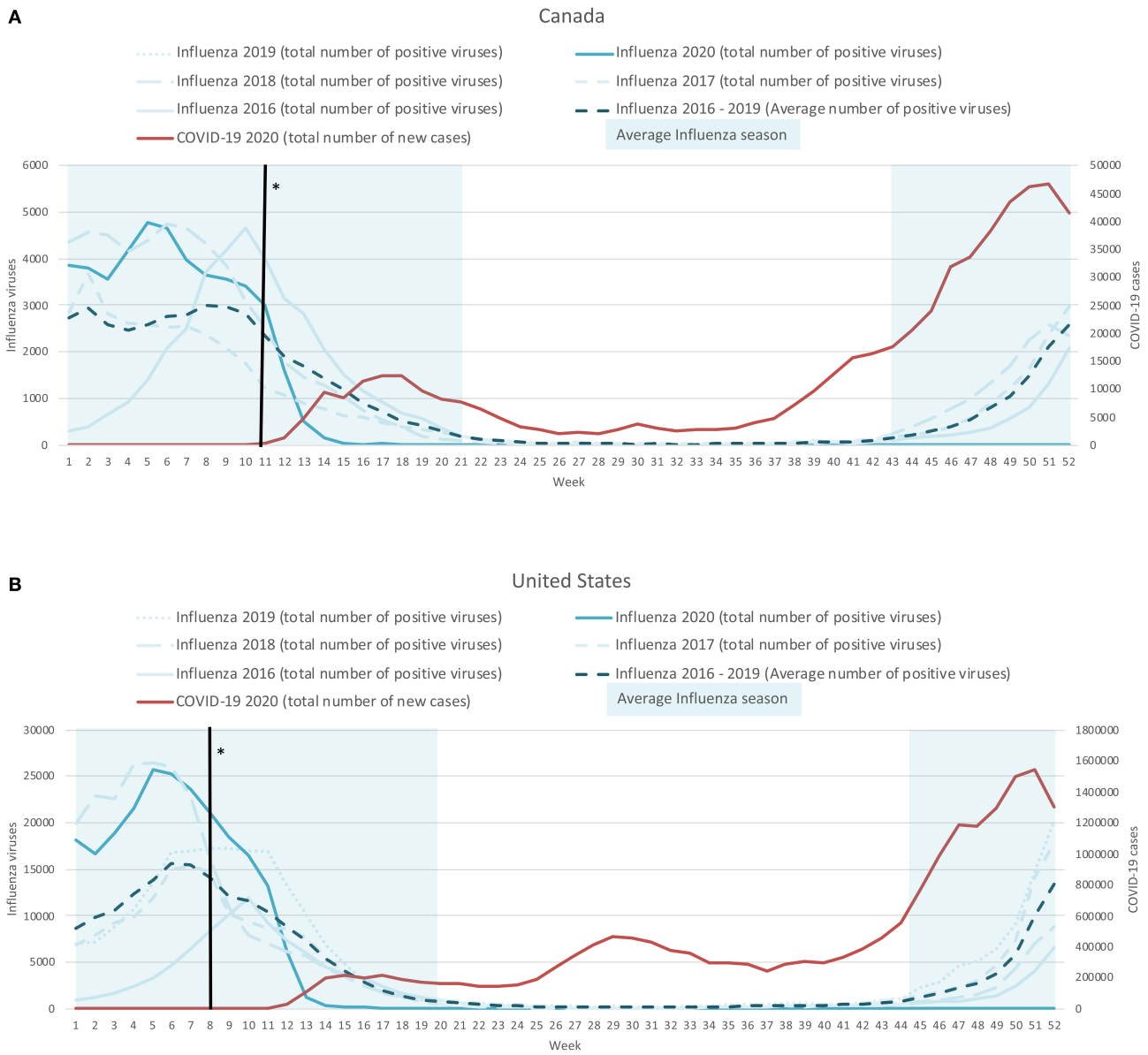

FIGURE 1 | Weekly time-series presentation of COVID-19 cases (for 2020; red line) and influenza cases (for the seasons 2016–2020; blue lines) across the northern hemisphere [Case of Canada **(A)** and the United States **(B)**]. *The week in which the country implemented policy restrictions is noted with a black vertical line.

visual analysis, including: (1) the COVID-19 behavioral mitigation measures appear to be having an unintended positive consequence on influenza spread; and (2) the fact that there were still COVID-19 cases after the introduction of mitigation measures, and in the absence of community spread of influenza, suggests that these viruses are not equally transmissible.

With regards to the first point, even though our analyses are descriptive in nature, our hypothesis that the introduction of government measures, such as the adoption of widespread behavioral changes worldwide with respect to isolation, hygiene and social distancing in response to COVID-19 would have reduced the trends in annual influenza cases, remains plausible (12–14). We observed consistent reductions in influenza activity across the globe, which was most notable once governments had introduced their first measures. It is also worth highlighting that the four countries introduced their measures at different times in the year, yet their impact on the incidence of influenza cases was comparably rapid. Once the world has overcome the current COVID-19 pandemic crisis, consideration of the potential role of more rigorous and widespread implementation of the COVID-19 behavior-based prevention measures to curb the transmission of the influenza virus, and its global mortality burden (15), is needed.

To further emphasize the role of the behavior-based measures is the fact that most countries, including the United States and Canada, did not run a flu vaccine campaign for 2020,

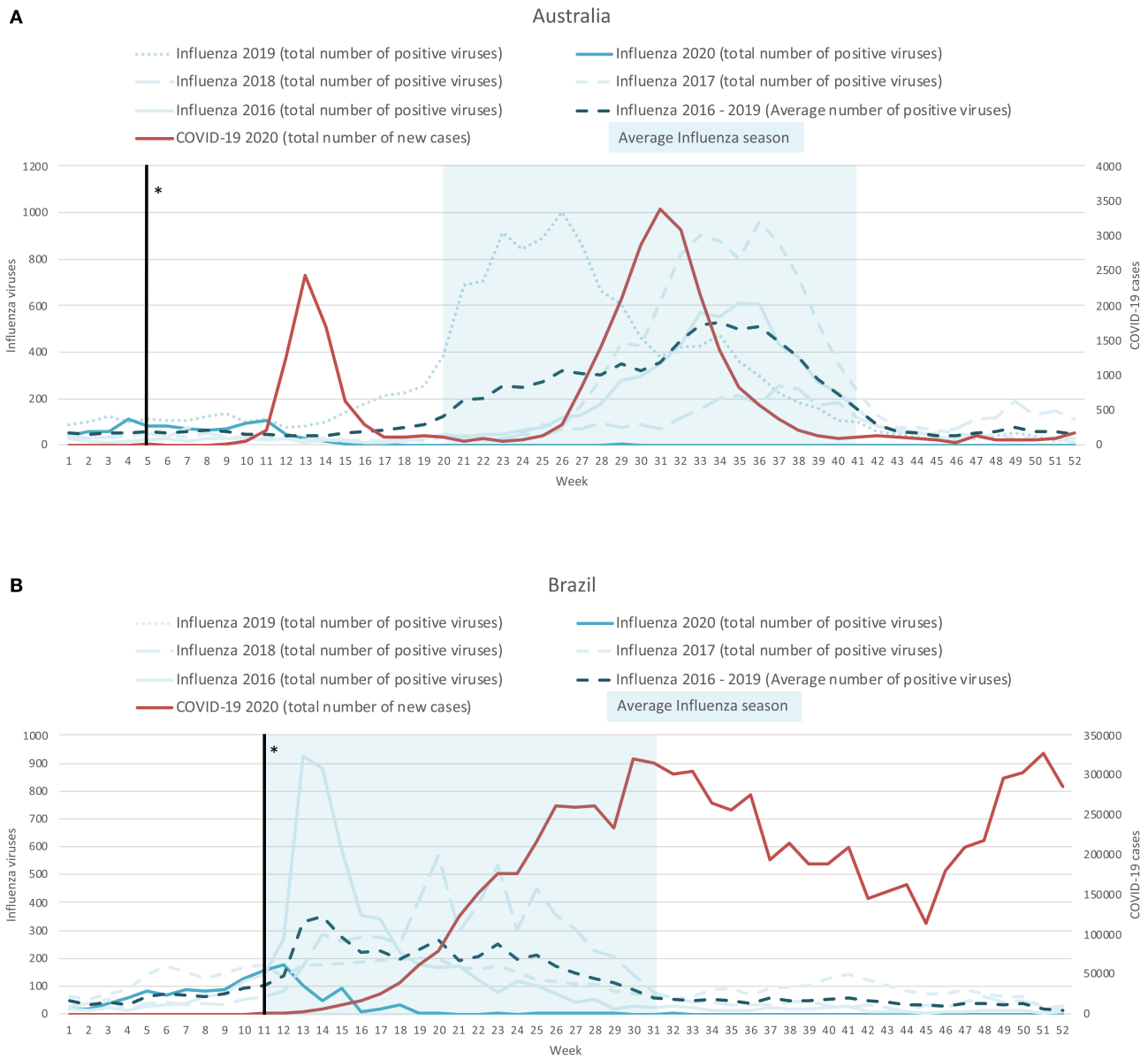

FIGURE 2 | Weekly time-series presentation of COVID-19 cases (for 2020; red line) and influenza cases (for the seasons 2016–2020; blue lines) across the southern hemisphere [Case of Australia **(A)** and Brazil **(B)**]. *The week in which the country implemented policy restrictions is noted with a black vertical line.

yet still managed to negate the usual incidence of influenza cases. This contrasts with Australia who ran an enhanced 2020 influenza vaccination campaign. When considering the impending distribution of the various COVID-19 vaccines, it might be possible for countries to drawn inspiration from this highly successful campaign, especially given the alarming increases in COVID-19 vaccine hesitancy (16). Elements of the Australian campaign included: continuous vaccination offer; aggressive public health messaging to inform the citizens, encourage vaccination and highlight the "*it's never too late to get the vaccine*" approach; increased advocacy for preventive behaviors; targeted messaging among at risk populations and healthcare workers; continuous surveillance and active monitoring (17). Importantly, it would seem that the development of public health policies and communication strategies aimed at increasing vaccine uptake might benefit from consultation with behavioral scientists, especially as the act of getting vaccinated is an important health behavior, whose insights have proven invaluable in the context of reducing COVID-19 transmission (18, 19).

Regarding our second implication, even though many of the parallels drawn between COVID-19 and influenza have already been discredited (20–26), it is often difficult to communicate this to the wider population. Our epidemiological mapping provides visually intuitive support to this difference which can help combat the misinformation that the impact of COVID-19 is

no different to influenza. The current data clearly show how the two differ with respect to their infectiousness. The fact that there continues to be increases in COVID-19 cases with concomitant reductions in influenza spread around the globe highlights how viral the SARS-CoV-2 virus is.

LIMITATIONS

Our study should be interpreted in consideration of some limitations. Firstly, our methodological approach included a simple graphical visualization strategy of raw data, and so causality cannot strictly be inferred from this descriptive analysis. Extending this, there could be some case misclassification with some COVID-19 cases actually being influenza cases. However, it should be noted that the data that we used was generally based on actual testing of both COVID-19 and influenza rather than just symptom reporting. Another limitation involves the nature of the recent influenza surveillance data. Case declines observed might be due to decreased testing over the course of the pandemic, as well as limited capacity for reporting in certain countries. This might be especially pertinent to low and middle-income countries where there is a historical lack of reliable estimates for influenza surveillance data. However, in the four countries highlighted in this paper this possible limitation might only apply to Brazil.

CONCLUSION

Our report provides descriptive evidence that the behavior-based COVID-19 mitigation measures are likely to be associated with an important reduction in the transmission and impact of influenza. In spite of this reduction in influenza, there was still community spread of COVID-19, highlighting that SARS-CoV-2 is markedly more contagious compared to influenza. These graphs, together with the higher global mortality rates of SARS-CoV-2 compared to influenza, provide clear evidence that the impact of COVID-19 is far greater than influenza. Finally, greater implementation of some of the key behavior-based COVID-19 mitigation measures (18, 19, 27) to reduce the mortality and burden of future influenza outbreaks should be considered by governments.

DATA AVAILABILITY STATEMENT

The data presented in this study come from publically available online repositories. The names of the repository/repositories and accession links can be found at: FluNet (www.who.int/flunet), Global Influenza Surveillance and Response System (GISRS) (available at: https://apps.who.int/flumart/Default?ReportNo=2); The Oxford COVID-19 Government Response Tracker (OxCGRT) (available at: https://github.com/OxCGRT/covid-policy-tracker/tree/master/data); Center for Systems Science and Engineering at Johns Hopkins University. Coronavirus COVID-19 (2019-nCoV) Dashboard (available at: https://github.com/CSSEGISandData/COVID-19).

AUTHOR CONTRIBUTIONS

JS: conception and design, data analysis, interpretation of data, drafting the article, article reviewing and critical revision. VB: conception and design, data acquisition and analysis, interpretation of data, drafting the article, article reviewing and critical revision. JB, JE, and KL: conception and design, interpretation of data, article reviewing and critical revision. SB: conception and design, interpretation of data, drafting the article, article reviewing and critical revision.

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ORIGINAL RESEARCH
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The Epidemiology of COVID 19 in the Amazon and the Guianas: Similarities, Differences, and International Comparisons

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Background: The COVID 19 epidemic submerged many health systems in the Amazon. The objective of the present study was to focus on the epidemic curves of the COVID 19 epidemic in different centers, and to look at testing and mortality data.

Methods: Publicly available datasets were used. The $\log_{10}$ of the daily cumulated number of cases starting from the day the territory reached 100 cumulated cases was plotted to compare the magnitude, shape and slope of the different curves. The maximum daily testing efforts were plotted for each territory in relation to the maximum daily number of diagnoses. The case fatality rate was computed by dividing the number of COVID 19 deaths by the number of confirmed cases.

Results: In the Amazonian regions in general the speed of growth was generally lower than in Europe or the USA, or Southern Brazil. Whereas, countries like South Korea or New Zealand "broke" the curve relatively rapidly the log linear trajectory seemed much longer with signs of a decline in growth rate as of early July 2020. After a very slow start, French Guiana had the lowest slope when compared to other Amazonian territories with significant epidemics. The Amazonian states of Roraima, Amazonas, Parà, and Amapà had among the highest number of cases and deaths per million inhabitants in the world. French Guiana had significantly fewer deaths relative to its number of confirmed cases than other Amazonian territories. French Guiana had a late epidemic surge with intense testing scale-up often exceeding 4,000 persons tested daily per million inhabitants. Brazil was an outlier with low daily testing levels in relation to the number of daily diagnoses.

Conclusions: There were marked heterogeneities mortality rates suggesting that socioeconomic, political factors, and perhaps ethnic vulnerability led to striking outcome differences in this Amazonian context.

Keywords: COVID 19, epidemiology, testing, mortality, Amazon, Guiana shield, vulnerable populations

INTRODUCTION

South America was affected by the COVID 19 epidemic after Asia, Europe, and North America, but the epidemic eventually caught up and overwhelmed the health system (1). This was perhaps best epitomized by the news images of the tragedy unfolding in Manaus, in the heart of the Amazon, as well as Guayaquil in Ecuador, or several cities in Peru (2, 3). Early in the epidemic, researchers studied the relation with climatic variables, and as for influenza (4–6), humidity and temperature were shown to have some impact on the reproductive number of COVID 19 (7–15). It has also been suggested that ultraviolet A and B radiation were associated with a reduction of the mortality of COVID 19 (16–18). Young populations, which is the case for the Amazonian region, are described as much less at risk of severe complications than older age groups (19). It was therefore somewhat unexpected to discover that Northern Brazil, with its hot and humid equatorial climate was one of the most affected regions in the world for COVID 19 (20). The Amazon basin and the Guiana shield are covered by a dense primary forest, roadways are therefore scarce and river or air travel are the main connecting routes between many villages and cities. Apart from the major cities, population density is very low, and poverty widespread notably in the favelas where the local population density is high and social distancing is difficult to implement in practice (21). The density of health professionals and hospital beds is also lower than in other parts of South America (22–24). Although, there are geographic commonalities, there are also differences in health expenditure per capita, differences in the organization of prevention testing and care, and differences in political leadership in confronting the crisis. Despite unprecedented research efforts and discoveries, the present pandemic is still incompletely understood, and its future uncertain. Describing and comparing trends and indicators between regions often yields instructive insights. In the present study, the objective was hence to focus on the dynamics of the COVID 19 epidemic in a singular region, the Amazon and the Guiana Shield, more specifically the Brazilian states of Amapa, Para, Roraima, and Amazonas, and in French Guiana and Suriname. A secondary objective was then to compare the Amazon and the Guiana shield to other regions of the world.

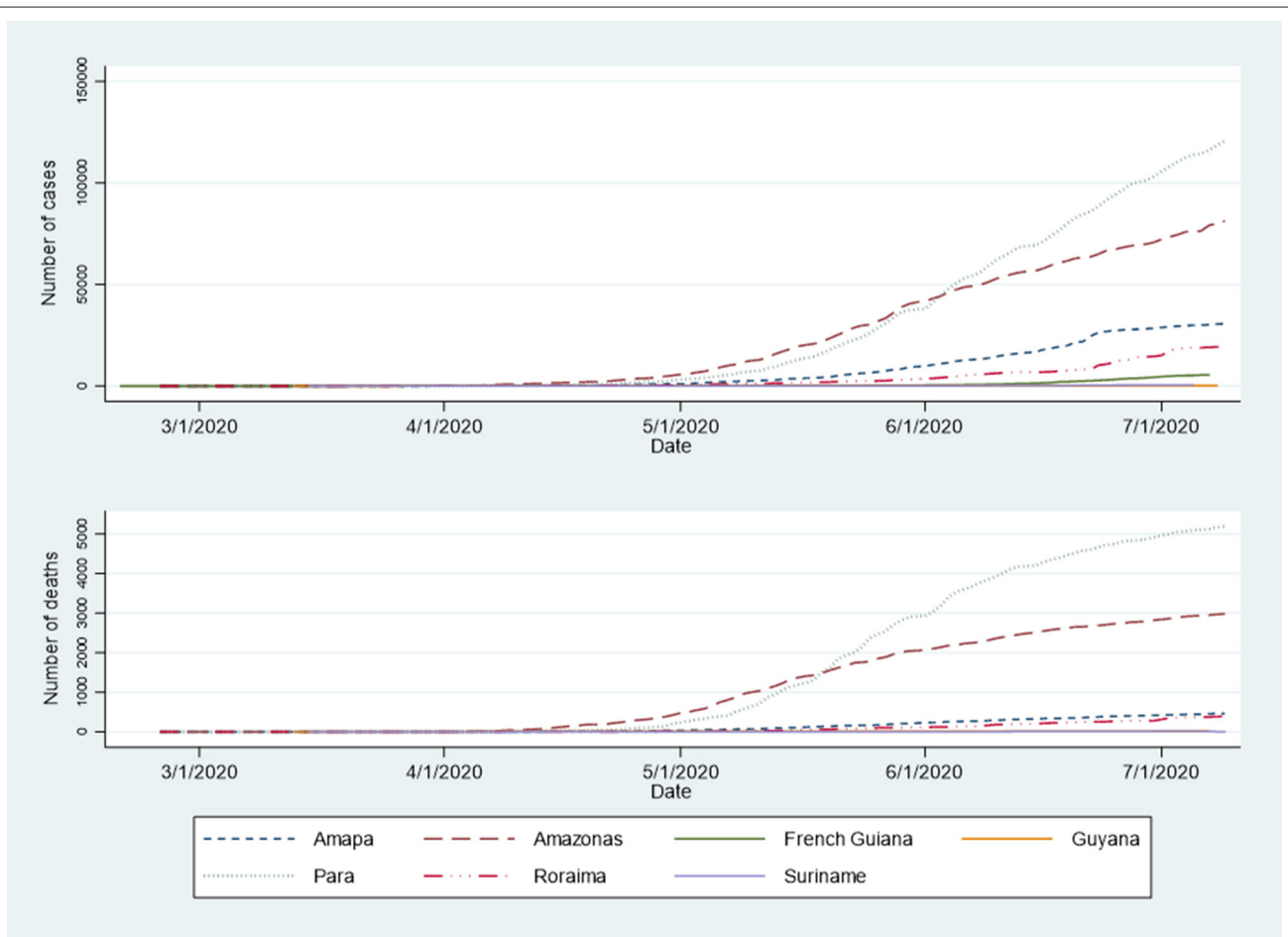

FIGURE 1 | Evolution of the number of COVID-19 cases and deaths in Amazonian territories.

METHODS

Data Sources

Data sets were downloaded from https://ourworldindata.org/coronavirus (1), which updates global data on COVID 19 (notably countries from continental Europe, North America, South America, East Asia, South East Asia…). Because the greater Amazonian area includes different countries or regions of countries, we use other data sources to obtain more detail, notably from the Brazilian ministry of health website (20), which entails state data. For French Guiana, a French overseas territory located on the Guiana shield, between Surinam an the Brazilian State of Amapá, the data was obtained from Santé Publique France (25), the French centers for disease control. The cumulated testing data was obtained from https://www.worldometers.info/coronavirus/ (26).

Comparisons

In order to compare the epidemic growth, we plotted the $\log_{10}$ of the daily cumulated number of cases starting from the day the territory reached 100 cumulated cases. This allowed to compare the magnitude, shape and slope of the different curves. The median R_t values were computed for Amazonian territories.

The magnitude of the maximum daily testing efforts in relation to the maximum daily number of diagnoses were also compared. Cumulated number of tests were also plotted in relation to the total number of COVID 19 deaths.

The case fatality rate was computed by dividing the number of COVID 19 deaths by the number of confirmed cases.

Data Analysis

Data was analyzed with STATA 15. In order to track the epidemic dynamics, we computed the effective reproduction number, which is the average number of new infections caused by a single infected individual at time t in the partially susceptible population. Indeed varying proportions of the population are immune to any given disease at any given time and social distancing may vary over time. The R_t values were calculated using the R EpiEstim package which computes R from daily new cases of COVID 19. Median values for each territory were plotted on a single graph.

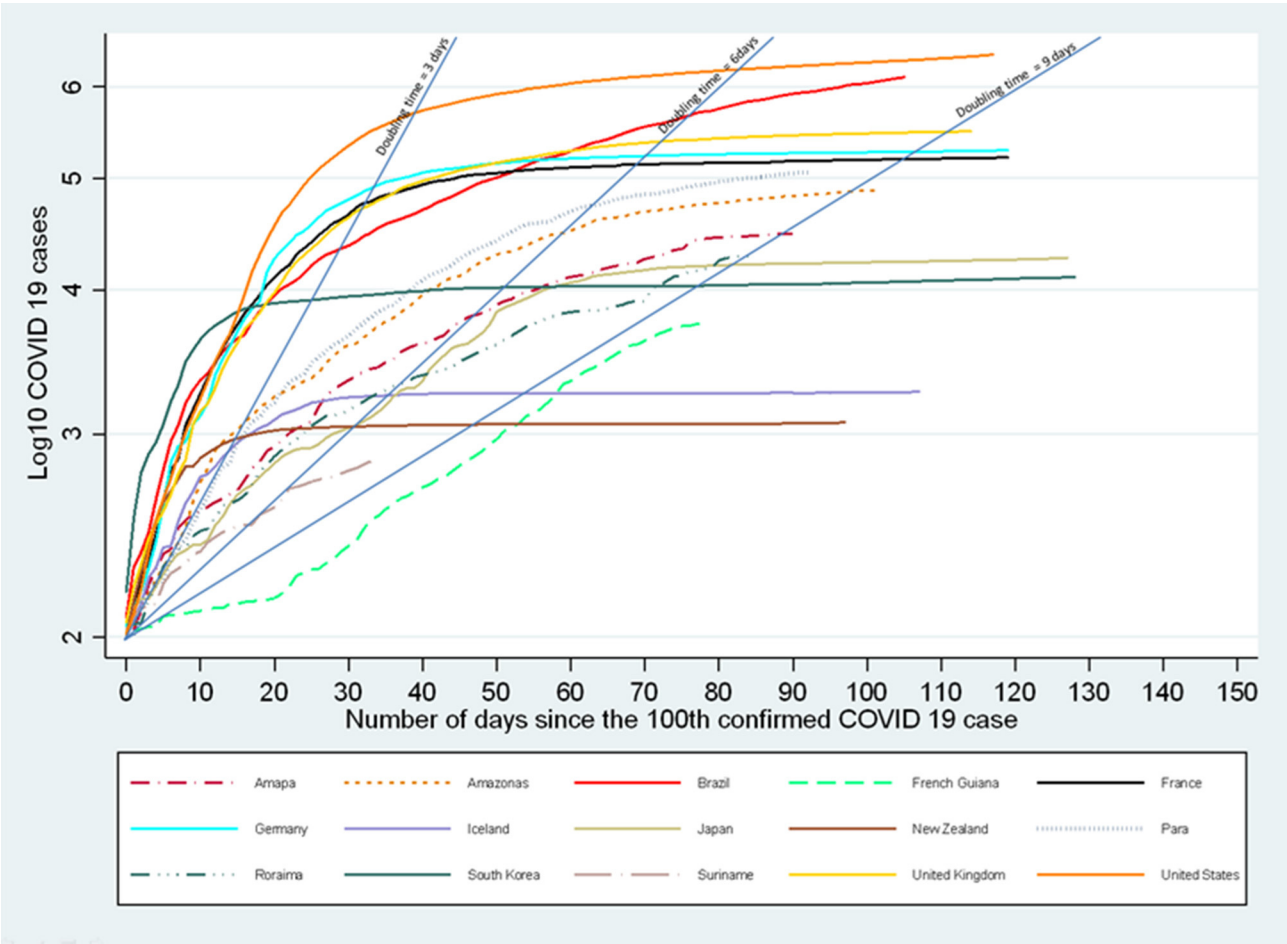

FIGURE 2 | Evolution of the number of COVID-19 cases in selected countries and Amazonian territories.

Regulatory and Ethical Considerations

The study used public anonymous aggregated data and did not require any ethical review according to French Authorities.

RESULTS

Comparison of Cumulated Case Numbers

Figure 1 shows the chronological growth of the number of cases and deaths in Amapa, Amazonas, Para, Roraima, French Guiana, Guyana, and Suriname. At the normal scale the Guiana shield territories are hardly visible given the disproportionately higher case and death numbers in Brazil. The much larger population in Brazil relative to the Guiana shield mostly concerns Amazonas (3,874,000) and Para (8,074,000) whereas Amapa (751,000) and Roraima (496,936) were closer in scale to the population of the Guiana Shield [French Guiana (290,000), Guyana (779,004), and Suriname (575,991)], yet there were still much greater numbers of COVID 19 cases and deaths.

Figure 2 shows the evolution of the $\log_{10}$ of the number of COVID-19 cases in selected countries. Regarding the Amazonian regions in general, the speed of growth was generally lower than in Continental Europe or the USA, or Southern Brazil. The slope was somewhat parallel to that of Japan. Whereas, countries like South Korea or New Zealand "broke" the curve relatively rapidly the loglinear trajectory seemed much longer with signs of a decline in growth rate as of early July 2020. After a very slow start, French Guiana had the lowest slope when compared to other Amazonian territories with significant epidemics (Guyana only declared 284 cases so far and was not plotted).

Plotting Number of Deaths and Cases per Territory

Figure 3 shows a scatterplot of the $\log_{10}$ of the number of deaths per million inhabitants and the $\log_{10}$ of the number of cases per million inhabitants. This shows the loose positive correlation of the number of deaths and the number of cases, and more importantly it shows that countries on the lower right side of the scatterplot have markedly less deaths that those on the upper left part of the plot. Hence among Amazonian territories **Figure 3** shows that the Amazonian states of Roraima, Amazonas, Pará, and Amapá had among the highest number of cases and deaths per million inhabitants in the world.

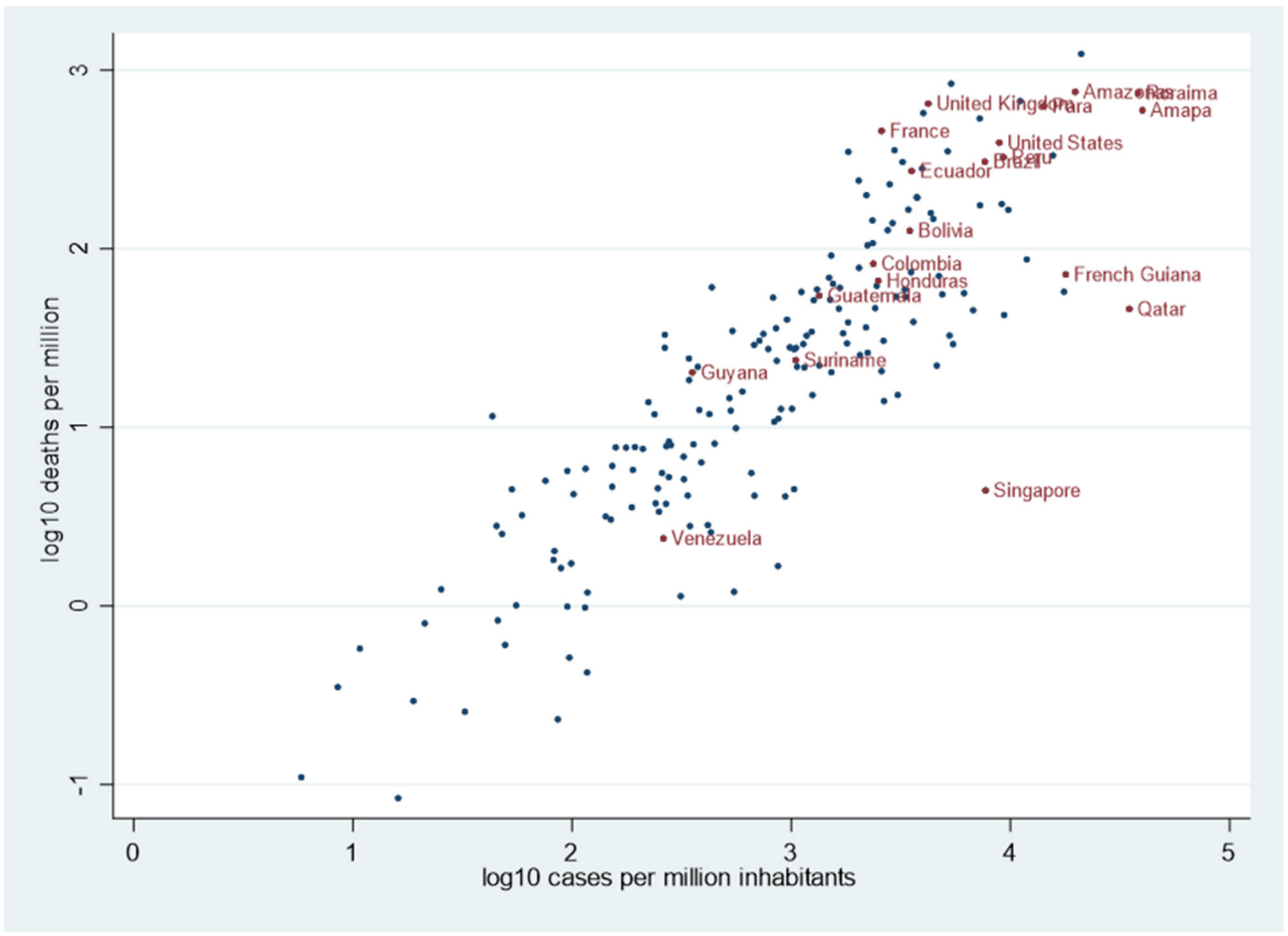

FIGURE 3 | Scatterplot of the $\log_{10}$ of the number of deaths and the $\log_{10}$ of the number of cases per million inhabitants in selected countries and Amazonian territories.

TABLE 1 | Comparison of total number of COVID-19 cases and deaths, per million inhabitants and respective proportion of deaths/cases (July 7th 2020).

Territory	Total cases	Total deaths	Cases per million population	Deaths per million population	Deaths per 100 cases
Amapa	30,004	449	39,952	598	1.50
Amazonas	76,424	2,938	19,727	758	3.84
Bolivia	40,509	1,476	3,470	126	3.64
Colombia	120,281	4,210	2,364	83	3.50
Ecuador	62,380	4,821	3,536	273	7.73
French Guiana	5,469	22	18,858	76	0.4
Guyana	278	16	353	20	5.76
Para	114,535	5,105	14,185	632	4.46
Peru	305,703	10,772	9,272	327	3.52
Roraima	18,948	371	38,129	746	1.96
Suriname	614	14	1,047	24	2.28
Venezuela	7,411	68	261	2	0.92

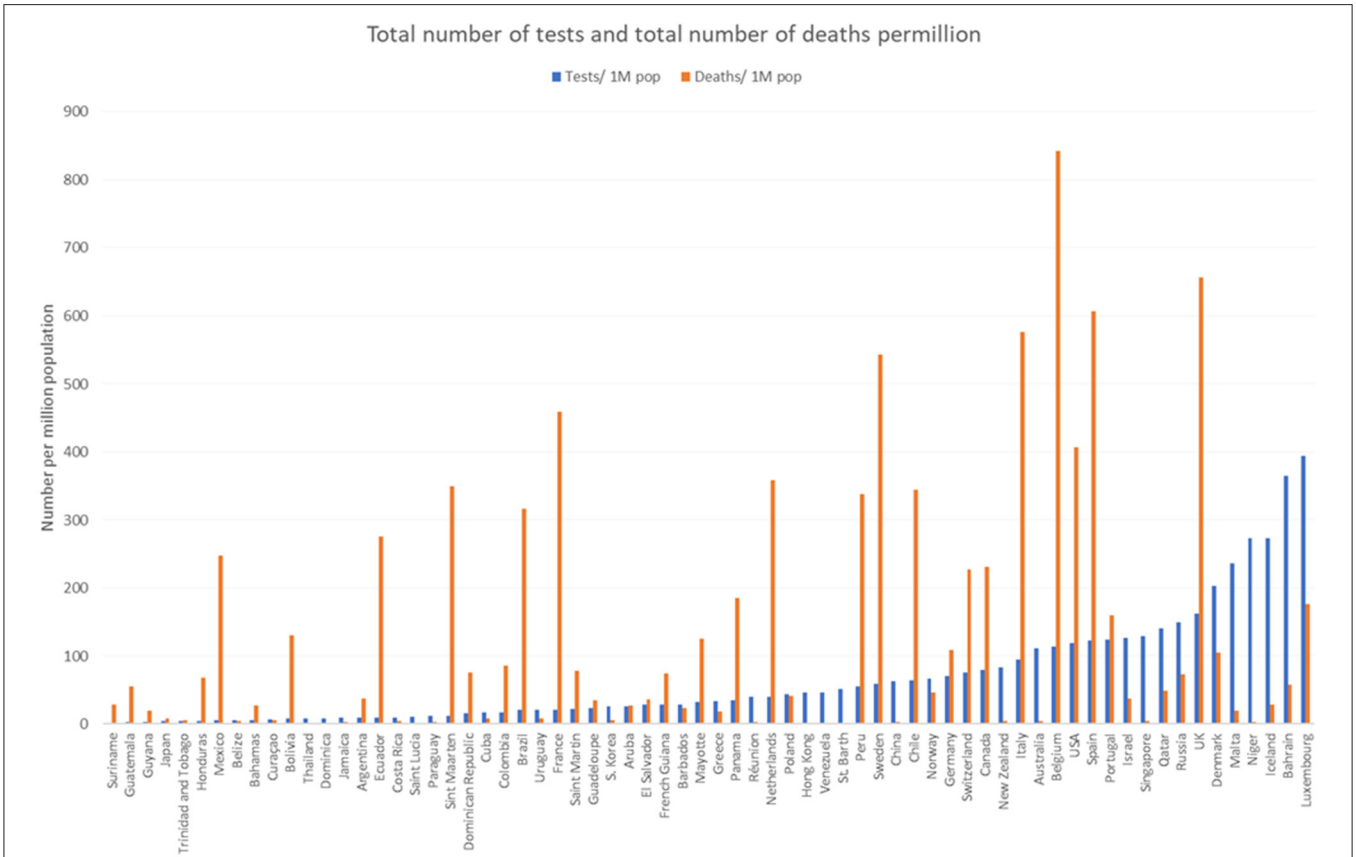

FIGURE 4 | Comparison of the total number of diagnostic tests and deaths per million inhabitants.

Other states with Amazonian territories (Colombia, Ecuador, Bolivia, Venezuela) are represented with pooled country data which does not allow to disaggregate the Amazonian population segment. Guyana despite a low number of confirmed cases had significant mortality and Suriname was in an intermediary position. French Guiana had significantly fewer deaths relative to its number of confirmed cases than other Amazonian territories.

Supplementary Figure 1 shows the relation between median age and case-fatality rate showing the relative youth of French Guiana and Amazonian territories.

Proportion of Confirmed Cases That Died

Table 1 represents the detailed data on July 7th and shows that the proportion of deaths per case ranged from 0.4% In French

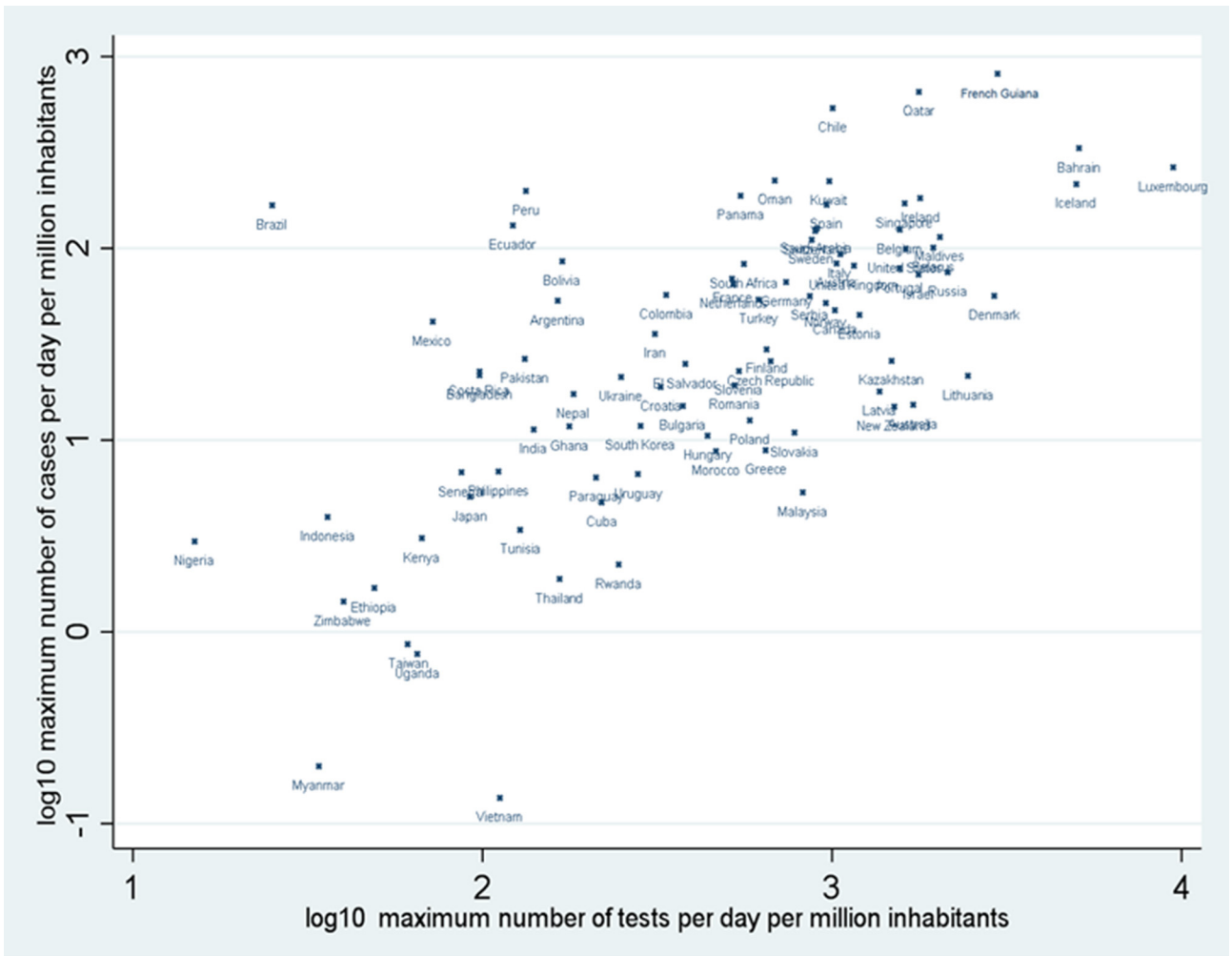

FIGURE 5 | Scatter plot of the $\log_{10}$ or the maximum number of cases per day per million inhabitants and the $\log_{10}$ of the number of persons tested per day millions inhabitants in selected countries and Amazonian territories.

Guiana to 7.7% in Ecuador, with among Amazonian territories Guyana with 5.7% mortality despite a low number of cases.

Cumulated Test Numbers and Deaths

Figure 4 does not include details on the total number of persons tested for Brazilian Amazonian states; it shows that in some countries the large number of cumulated tests was associated with a large number of deaths; in other countries the large number of tests was associated with few deaths, presumably reflecting different strategies. Brazil and continental France for example had relatively few tests but large numbers of deaths, whereas Iceland and Israel had intense testing with low mortality.

Maximum Number of Daily Tests and Diagnoses

Suriname and Guyana had relatively few cases and the cumulated number of tests was low; French Guiana had a late epidemic surge (passed the 100th case threshold on April 24th) with intense testing scale-up in May and June 2020 often exceeding 4,000 persons tested daily per million inhabitants, levels only surpassed by Bahrain, Iceland, and Luxembourg (**Figure 5**). **Figure 5** also shows that for countries with Amazonian territories with available daily testing data (Brazil, Peru, Bolivia, Colombia, and Ecuador) the maximum daily number of tests at the whole country level was low compared to the maximum number of cases, notably Brazil which was an outlier.

Median Daily R_t Values Over Time

Figure 6 shows the median daily R_t values for French Guiana, Amapá, Amazonas, Pará, and Roraima. Generally, apart from large daily fluctuation, the median values were between 1 and 1.5 and in their latest estimation were below 1.

DISCUSSION

The present results show that the spread of the COVID 19 epidemic in the Amazon was slower that in Europe, North America or Southern Brazil. Despite daily fluctuations median

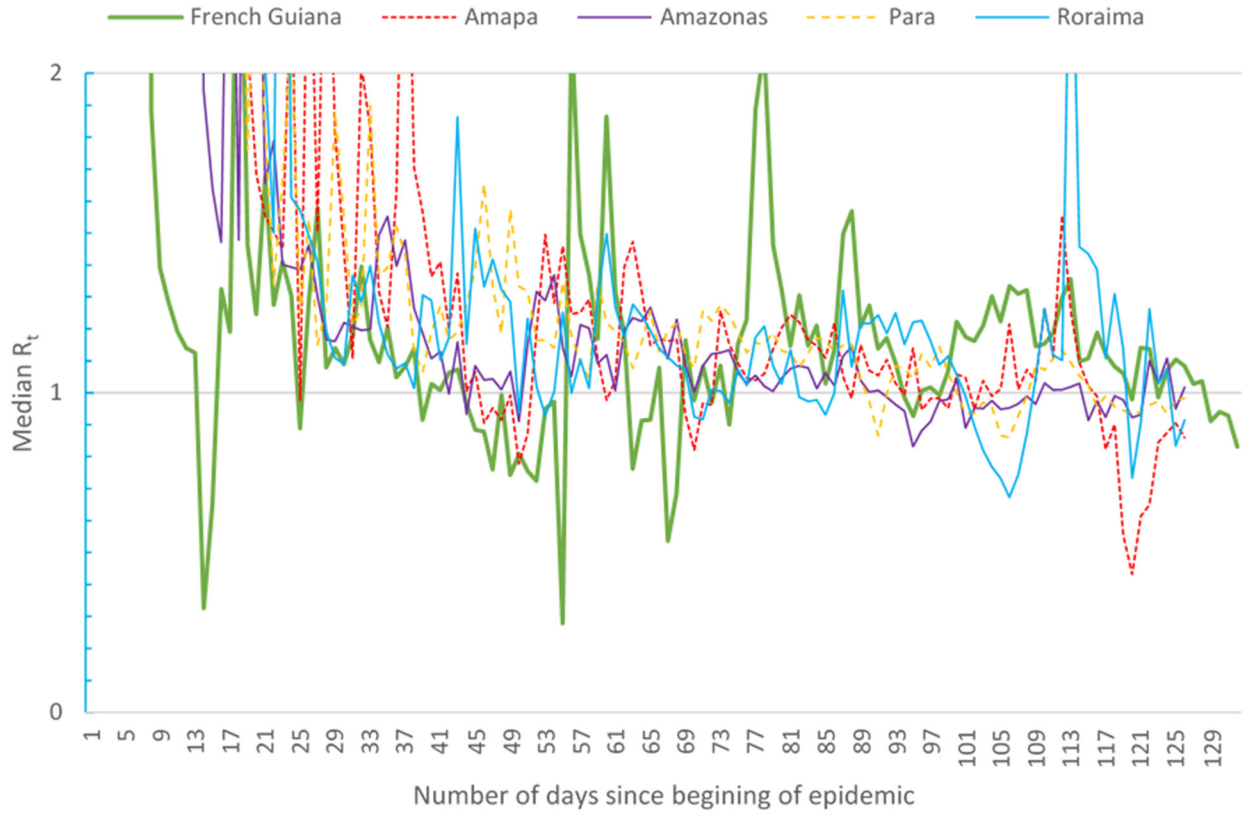

FIGURE 6 | Evolution of the median Rt in Amazon territories.

R_t values for territories with available data were generally slightly above 1 falling recently below 1. Amapa, the neighboring state with French Guiana seemed to be leading the trends observed in French Guiana. Perhaps lower population densities, scarce transport infrastructure, climatic factors, and perhaps the benefit of the knowledge of what had just happened in Asia and Europe slowed transmission. Nevertheless, transmission did occur and the curves seemed to take a longer time to "break" perhaps reflecting its gradual spread from community to community along the ramifications of social networks, often by boat (27–29). The heterogeneity between Amazonian territories is also a remarkable feature that reflects the scarce transport infrastructure, the very early lockdown of borders in early March, and obvious political commitment levels to tackle the problem. For Guyana, Suriname, and French Guiana, territories with few hospital and ICU beds the perspective of an overwhelming surge led early on to a lock down with interruption of air traffic, border closure, social distancing, confinement, quarantine, and isolation of patients. For Brazil, daily airline connection with major cities in southern Brazil presumably rapidly fueled the local epidemic in the North. In addition, there has been a great confusion between Federal, State and municipal levels reflected by the resignation of 2 health ministers, and the current absence of one to this date. It is very likely that the polarized political context has had a significant impact in the very high number of cases and deaths in the Amazonian States of Brazil, and beyond. For French Guiana and Suriname, after a first "phony war" period with very few cases imported from Europe which were easily controlled, the epidemic in the Amazonian states permeated across the border and with the lift of confinement, and perhaps the belief that there would be no epidemic after all, it gradually spread mostly across the most precarious communities, reaching all ethnic groups, despite uninterrupted contact tracing, and intensive community mobilization and testing.

Regarding the number of deaths, there was great heterogeneity. Studies have suggested that high ultraviolet radiation was associated with lower COVID 19 mortality (16–18) and that vitamin D deficiency (30), a frequent feature in Latin America, was associated with greater mortality. Although these factors may marginally affect mortality, it seems the explanation for such heterogeneity lies elsewhere. First despite the young age of the populations in the North, the magnitude of the number of cases in the Northern Brazilian states may have simply overwhelmed the health system and led to suboptimal

care; Although the statistical plots between confirmed cases and deaths suffers from the imprecision of not reflecting testing efforts, and testing seemed relatively low in Brazil. Presumably the real number of COVID 19 cases was much greater that the number of confirmed cases (31). However, the raw numbers of deaths and the sights of mass graves reported in much of the press demonstrate how massive the influx of severe patients was. Among of the particularities of the Northern states are the high levels of social deprivation, and its ethnic makeup which perhaps leads to greater levels of acquisition and mortality (32–39). The picture in French Guiana, however, was quite singular. There is a large poor immigrant population, many vulnerable ethnic groups, high levels of comorbidities such as diabetes, obesity, and hypertension, which were expected to increase the risk of severe forms and deaths (40). However, to this day the levels of mortality are very low. Among the potential explanations, we can cite the youth of the population (median 22 years), but it is of note that it is the same as that of Amapá, where case numbers per million were 2.1 times greater than in French Guiana and mortality per million was 7.8 times greater than in French Guiana. The intense scaling up of testing and contact tracing presumably led to a denominator that is close to the real number of cases in French Guiana. The surge of cases in French Guiana occurred between June and July and presumably care of patients benefitted from knowledge accumulated since the beginning of the epidemic: hence telemedicine for patients remaining at home and the aggressive search for any sign of silent hypoxia, anticoagulation, the use of steroids when pneumonia worsens, progress in ICU ventilation methods (such as nasal high flow therapy, prone position in intubated but also non-intubated COVID-19 patients), and early massive organizational efforts to expand hospital and ICU beds for COVID patients (41) all combined to reduce mortality in French Guiana.

The limitations of this study are that it relies of aggregated data from different information systems, with incomplete knowledge of policies, dates of implementation at different locations. Causal inferences are impossible with such data. Nevertheless, the strength of this study is that it attempts to focus on the Amazon basin and to compare between territories within the region and other countries with very different context, showing instructive contrasts.

In conclusion, despite a number of factors that were expected to slow down the spread of the virus, the epidemic spread widely in the Amazonian regions, and led to considerable mortality in Northern Brazil. Widespread poverty, low access to care in remote areas, ethnic factors, comorbidities, and political denial of the health crisis may have explained the tragic situation. By contrast French Guiana, which aggressively tackled the crisis early on observed a delayed epidemic with low mortality, showing as elsewhere that despite a number of challenges it is possible to significantly impact transmission and mortality and reduce the impact of an uncontrolled surge in severe COVID 19 infections. The present analysis also suggests that an integrated data analysis is possible at the regional level and that learning from comparing territories in this complex Amazonian context may help health authorities optimize the response to the massive crisis caused by this novel pathogen (42).

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found at: https://ourworldindata.org/, https://covid.saude.gov.br/.

AUTHOR CONTRIBUTIONS

MN analyzed data, wrote first draft, and validated final manuscript. CR, TS, and AAn collected data and edited manuscript. MG, CM, VS, MDo, RS, AAd, MDe, PA, LE, and FD data interpretation and manuscript edition.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.586299/full#supplementary-material

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frontiers in Medicine

OPINION
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COVID-19 Therapy: Could a Copper Derivative of Chlorophyll *a* Be Used to Treat Lymphopenia Associated With Severe Symptoms of SARS-CoV-2 Infection?

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Keywords: chlorophyll, therapy, lymphopenia, SARS-CoV-2, COVID-19, coronavirus, sodium copper chlorophyllin

INTRODUCTION

Chlorophyll *a* is a specific form of chlorophyll involved in oxygenic photosynthesis. It contains a magnesium ion surrounded by a large ring structure known as a chlorin. Four nitrogen atoms from the chlorin encase and bind the magnesium atom. The magnesium center uniquely defines the molecule as chlorophyll *a* (1). In order to harness the widely acknowledged therapeutic benefits of chlorophyll *a*, a chemical process known as re-greening must occur whereby the central atom is replaced with another metal yielding the same electrostatic charge, such as zinc or copper (2). Dietary chlorophyll, a formulation derived from sodium copper chlorophyllin (SCC), is a popular dietary supplement taken by health-conscious consumers (3, 4). Chlorophyll *a* derivatives including SCC are known to have a number of benefits when taken at therapeutic doses (3, 5). They are non-toxic, highly soluble compounds that are demonstrated to have higher uptake in human cell systems which likely triggers the chelation of ionic compounds (6, 7).

Several chlorophyll *a* derivatives have a profoundly cytotoxic effect *in vitro* and *in vivo* when compared to controls (5). Due to the antioxidant potential of chorophyllins double-blind placebo-controlled trials have revealed significant therapeutic outcomes, most notably in the prevention and treatment of cancers (5, 8–11). However, the therapeutic efficacy in treating numerous conditions within a broad range of clinical settings, particularly in countries like the US, UK, Canada and Australia, is largely neglected. For the recently emergent Corona Virus Disease-2019 (COVID-19) pandemic that is caused by infection with the novel human pathogen Severe Acute Respiratory Syndrome (SARS) Coronavirus (CoV)-2 (12) it was proposed that zinc chlorophyll may show potential as a therapeutic (13). This is because this tetrapyrrole derivative may aid the uptake and free ionization of zinc and thus potentially inhibit ribonucleic acid synthesis of SARS-CoV-2 in human epithelial lung tissue. Much like zinc chlorophyll, SCC is a non-toxic, water-soluble chlorophyll *a* derivative that may offer therapeutic benefits against SARS-CoV-2 but for entirely different reasons.

The therapeutic efficacy of SCC in both animals and humans via oral and parenteral routes, particularly intravenous infusions, is well-documented (3). Furthermore, its capacity to inhibit viral cytopathicity *in vitro* has been demonstrated (14). SSC exhibits significant anti-viral properties against a number of pathogenic viruses including the causative agents of highly infectious respiratory diseases such as influenza (14). A recent review suggested that in common with other copper-based compounds SCC could act as an anti-viral agent in the treatment of COVID-19 (15).

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Whilst the anti-viral property of zinc is established (16), vanishingly few studies consider the function that other metal ions may play in humoral immunity (17). In this context copper is an essential co-factor at the site of blood stem cell production and is thus instrumental in stimulating haematopoiesis (18–20).

As copper plays a key role in the production of leukocytes its deficiency has been linked to the condition of leukopenia, a reduced number of leukocytes in the peripheral blood, especially of neutrophils but also lymphocytes and granulocytes (21, 22). A limited number of reports have examined copper deficiency and the role it may play toward individual immunity and susceptibility to human diseases (23–25), while a few studies have investigated the importance of copper and copper-based compounds such as SCC in maintaining leukocyte homeostasis. Restoration of serum copper levels by intravenous infusion of patients led to a rise in peripheral blood leukocytes (18–20), highlighting the link between metabolic copper deficiency and susceptibility to disease. Similarly, likely due to the fact that it contains copper, SCC has also been shown to significantly increase leukocyte levels in a range of patients (10, 26–28), revealing the potential to treat those afflicted with leukopenia due to other diseases or disorders. Despite this cumulative clinical evidence, however, the therapeutic application of copper and chlorophyll *a* derivatives such as SCC, particularly with regard to acute leukopenia caused by viral infections, is rarely deliberated. Moreover, to date the use of SCC to treat aggressive lymphopenia [also called lymphocytopenia, an abnormally low concentration of lymphocytes in the peripheral blood, $\leq$ 1,100 cells/μL; (29)], a major feature of SARS-CoV-2 infection, has not been considered.

SARS-CoV-2 AND LYMPHOPENIA

At the time of writing there are over 109,140,000 confirmed cases of COVID-19 globally, of which close to 2,408,000 have proved fatal (30). Maintaining a satisfactory peripheral blood lymphocyte count (between 1,100 and 4,800 cells/μL in adults) is a key contributory factor in the survival of COVID-19 patients, such that lymphopenia is noted as an associated risk factor in COVID-19-related deaths (31–33). Hence, in the current absence of a regulatory authority-approved drug for use against severe cases of COVID-19 lymphopenia is a surrogate indicator of a patient's poor prognosis (29). This outcome is thought to be as a consequence of the overexpression of interleukin (IL)-6 (34). This activates unregulated proliferation of leukocytes, particularly first responders such as neutrophils, followed only later by macrophages and lymphocytes (32, 35). The cellular expansion triggers a pro-inflammatory cytokine storm that leads to excessive inflammation, destruction of epithelial tissue and pulmonary oedema (36–38), eventually resulting in cardiac arrest due to depletion of oxygen concentrations in the blood (39–41).

Abbreviations: COVID-19, Corona Virus Disease-2019; SARS, Severe Acute Respiratory Syndrome; SARS-CoV-2, Coronavirus (CoV)-2; IL, interleukin; SCC, sodium copper chlorophyllin.

Symptomatic COVID-19 patients show significant depletion of peripheral blood leukocytes, in particular presenting with lymphopenia when compared to asymptomatic or mild disease-presenting patients, suggesting greater disease severity correlates to a progressive reduction in lymphocytes (33, 41–43). In one corroborative study elevated neutrophil levels correlated to a significant reduction in the proportion of the total leukocyte population that comprised lymphocytes (31). This is likely due to a rapid innate immune response involving neutrophils (41, 43), since they are the most numerous and most abundant leukocyte early in infection. Also, when compared to lymphocytes, which take longer to mature and have a more specialized response (33), less metabolic expenditure is required to produce neutrophils. This may explain why as an apparent "last ditch" effort to survive some terminally ill COVID-19 patients overproduce neutrophils (31).

A symptom of many infectious diseases, leukopenia is contraindicated with life expectancy; thus, lower concentrations of peripheral blood leukocytes, in particular $CD4^+$ and $CD8^+$ T lymphocytes, are often surrogate indicators of disease severity (44). Perturbation of leukocyte homeostasis, specifically lymphopenia, predicts disease severity among symptomatic COVID-19 patients (38). In contrast, individuals who test positive for SARS-CoV-2 but are asymptomatic for COVID-19 do not present with low or reduced peripheral blood lymphocyte levels, which thus places them at a lower risk of life-threatening COVID-19-related complications (41). Immunocompromised persons are also at increased risk from COVID-19 due to their insufficient numbers of lymphocytes (42). This means that disease outcome is strongly associated with immunological response and thus increased risk is linked indirectly to perturbed haematopoiesis. An inadequate production of lymphocytes could be a direct result of the disease itself or due in part to pre-existing states such as old age and underlying immunocompromised conditions (31, 45). This suggests that maintaining adequate peripheral blood lymphocyte levels may control symptoms and disease severity of COVID-19 patients (40). This is achieved through preventing excessive production of neutrophils and overexpression of IL-6 (34), thereby averting the characteristic pro-inflammatory cytokine storm and potentially fatal pulmonary oedema that would otherwise ensue (40).

SODIUM COPPER CHLOROPHYLLIN AND COVID-19

Irrespective of whether it is pre-existing or triggered by exposure to SARS-CoV-2, peripheral blood lymphopenia predicts disease severity in COVID-19 patients (32). Finding ways to reduce as much as possible this critical immune-modulated deficit may improve treatment outcomes of symptomatic or at-risk individuals (40). In both humans and animals therapeutic doses of SCC significantly increase peripheral blood leukocyte levels (10, 46–48). Clinical trials in patients,

including children suffering from leukopenia due to cancer-related illness (28), demonstrated that an oral dose of SCC taken at 180 mg for adults and 40 mg for children three times daily significantly increased whole leukocyte concentrations, particularly neutrophils, compared to controls (26–28). Another study indicated this to be as effective as the standard treatment leucogen used to control neutropenia (10). Increases in leukocyte counts of $>30\%$ in 2 weeks and 82% after 1 month were reported. Restoration occurred for 85% of the subjects, with extremely minimal side effects and no noted toxicity. As participants in each of these trials were treated not for lymphopenia but instead neutropenia, unfortunately the concentration of lymphocytes was not recorded (10, 26–28). Overall, however, these findings suggest that similar therapy may be effective against SARS-CoV-2, as restorative activity of lymphocytes could occur before severe symptoms of COVID-19 appear.

Support for this proposal comes from murine models in which administration of SCC was demonstrated to significantly increase the peripheral blood concentration of lymphocytes (47, 49). It is therefore entirely possible that in human subjects a marked lymphocytosis induced by therapeutic doses of SCC, delivered orally or parenterally, may also be observed. Furthermore, SCC significantly suppresses IL-6, as shown by studies *in vitro* and *in vivo* (48, 50). For patients infected with SARS-CoV-2, SCC could be utilized to ameliorate aggressive immune-modulated outcomes by suppressing pro-inflammatory cytokine effects through blocking trans-signaling of IL-6, thereby leading to a reduction in lymphocytes and an overproduction of neutrophils (48, 50). Once a person is exposed to SARS-CoV-2 several days may elapse before they become symptomatic, if at all, and several more before severe symptoms develop (39). Hence, treatment with SCC at the time of onset of symptoms and/or at diagnosis, especially for immunocompromised patients, may control leukocyte levels contraindicated with disease severity. COVID-19 symptoms often start to worsen by days 10–12 after virus exposure while intensive care unit admission typically occurs from days 12–14 (36, 39). Therefore, taking SCC before the disease progresses to this point may prevent functional exhaustion of $CD4^+$ and $CD8^+$ T lymphocytes (33), thereby mitigating such outcomes as lymphopenia. This could also be applied over an extended duration to assist in the treatment of so-called COVID-19 "long haulers" (51). Moreover, the synergistic effect of inhibiting cytokine production and preventing destruction of T lymphocytes by increasing peripheral blood lymphocyte levels could also block overproduction of cytokines capable of suppressing an inflammatory reaction and the life-threatening pro-inflammatory cytokine storm (36). Additionally, as is known for copper and other chlorophyll *a* compounds, SCC may also act as an anti-viral agent (15). Therefore, maintaining homeostasis of the haematopoietic production of peripheral blood leukocytes, primarily of lymphocytes (46), may reduce the likelihood of disease progression to extreme severity.

DISCUSSION

Drugs to correct lymphopenia do exist but they are not widely available and must be administered under strictly controlled conditions (21). These experimental treatments carry the risk of unwanted side-effects, some of which are severe and even fatal (21). Such therapies can cause destruction of alveolar sacks and thus are unsuitable for the treatment of COVID-19 patients. In contrast, easily manufactured from fescue grass (*Festuca arundinacea*) as a green-black free-flowing powder, SCC is extremely well-tolerated in the diet of adults and children (2, 5, 10). In addition, this profile is unlikely to vary with therapeutic dose, as even at extremely high concentrations no toxicity is reported (8, 10). While indicated to be neither teratogenic nor embryo-lethal in a murine model (52), further research is needed to investigate the dose dependency of any effects (53), and thus to determine if SCC is safe to take when pregnant or breastfeeding. This proviso aside, the utmost consideration should be given to conducting clinical trials to treat COVID-19 patients in the convalescent phase using SCC. This is because it is evident that low peripheral blood leukocyte levels due to primary SARS-CoV-2 infection, and possibly reinfection (54), play a major role in compromising the recovery of individuals with symptomatic COVID-19.

CONCLUSION

Therapeutic doses of SCC have been demonstrated to provide an effective clinical treatment for leukopenia. On this basis, we propose that taking SCC at the onset of symptoms or, for immunocompromised patients, at the time of diagnosis, could reverse the lymphopenia observed during COVID-19. It is envisaged that in symptomatic individuals SCC treatment could control leukocyte homeostasis, specifically of lymphocytes, thereby preventing their progressive reduction that is associated with severe disease outcomes. By first restoring and then maintaining adequate peripheral blood levels of $CD4^+$ and $CD8^+$ T lymphocytes this would enable the immune system of an SCC-treated COVID-19 patient to respond appropriately to resolve SARS-CoV-2 infection. Additionally, it may produce a synergistic effect as SCC is known to block expression of the pro-inflammatory cytokine IL-6. Hence, importantly, such SCC therapy would avoid triggering the characteristically excessive inflammation that causes lasting lung epithelial cell damage and cytokine storm events which often precipitate a fatal outcome of COVID-19.

AUTHOR CONTRIBUTIONS

NC and AT-R each made substantial contributions to the conception of the work and to literature search, contributed significantly to writing the manuscript, revised it critically for important intellectual content, approved its final version, and agreed to its submission. Both authors contributed to the article and approved the submitted version.

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frontiers in Medicine

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SARS-CoV-2 Testing of 11,884 Healthcare Workers at an Acute NHS Hospital Trust in England: A Retrospective Analysis

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Healthcare workers (HCWs) are known to be at increased risk of infection with SARS-CoV-2, although whether these risks are equal across all roles is uncertain. Here we report a retrospective analysis of a large real-world dataset obtained from 10 March to 6 July 2020 in an NHS Foundation Trust in England with 17,126 employees. 3,338 HCWs underwent symptomatic PCR testing (14.4% positive, 2.8% of all staff) and 11,103 HCWs underwent serological testing for SARS-CoV-2 IgG (8.4% positive, 5.5% of all staff). Seropositivity was lower than other hospital settings in England but higher than community estimates. Increased test positivity rates were observed in HCWs from BAME backgrounds and residents in areas of higher social deprivation. A multiple logistic regression model adjusting for ethnicity and social deprivation confirmed statistically significant increases in the odds of testing positive in certain occupational groups, most notably domestic services staff, nurses, and health-care assistants. PCR testing of symptomatic HCWs appeared to underestimate overall infection levels, probably due to asymptomatic seroconversion. Clinical outcomes were reassuring, with only a small minority of HCWs with COVID-19 requiring hospitalization (2.3%) or ICU management (0.7%) and with no deaths. Despite a relatively low level of HCW infection compared to other UK cohorts, there were nevertheless important differences in test positivity rates between occupational groups, robust to adjustment for demographic factors such as ethnic background and social deprivation. Quantitative and qualitative studies are needed to better understand the factors contributing to this risk. Robust informatics solutions for HCW exposure data are essential to inform occupational monitoring.

Keywords: Healthcare workers (HCWs), SARS-CoV-2, COVID-19, nosocomial infection, occupational risk analysis and management

INTRODUCTION

The pandemic of SARS-CoV-2 serves to highlight the risk posed to healthcare workers (HCWs) by transmissible respiratory pathogens (1–7). As is the case for other highly pathogenic coronaviruses such as severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) coronaviruses, SARS-CoV-2 may also be transmitted in healthcare environments (8, 9). Protecting patients and HCWs from nosocomial novel coronavirus-19 disease (COVID-19) is a priority in the control of the SARS-CoV-2 pandemic (1, 10). There are multiple strands to this effort, including environmental controls, use of appropriate personal protective equipment (PPE), as well as rapid testing and the self-isolation at home of SARS-CoV-2 infected HCWs.

Approaches to HCW testing include: (i) PCR testing of those with symptoms (4, 11, 12) or (ii) universal PCR screening (13, 14), recognizing that up to 40% of infections may be asymptomatic (15). Each strategy has its limitations and the optimal approach remains to be determined. This decision must balance the risk to HCWs and patients with pragmatic concerns about resource allocation and maintaining safe levels of staffing. Antibody testing adds complementary, albeit retrospective, information about SARS-CoV-2 exposure. Together with PCR testing this provides a resource that can be analyzed to inform HCW infection risk.

Recent data suggest that HCWs from certain demographic backgrounds or occupational groups may have different risks of infection (2, 7, 16). To explore this further, we retrospectively analyzed a large real-world testing dataset obtained between 10 March and 6 July 2020 in an NHS Foundation Trust in England with 17,126 employees. In this setting, 3,338 HCWs underwent symptomatic PCR testing and 11,103 HCWs underwent antibody testing. The aims of the analysis were: (i) to describe the results of SARS-CoV-2 PCR and antibody testing in this population; (ii) to explore demographic and occupational factors associated with SARS-CoV-2 test positivity, thereby informing the approach to protecting HCWs against COVID-19 in preparation for the next stages of the SARS-CoV-2 pandemic.

METHODS

Ethics

As a study of healthcare-associated infections, this was exempt from ethical approval under Section 251 of the NHS Act 2006 and as a study of COVID-19 was also covered by Regulation 3(4) of the Health Service Control of Patient Information Regulations 2002 (March 2020). The study was registered as a clinical service evaluation with approval from the Medical Director. Data extraction and analysis was approved by the Caldicott Guardian (Reference No. 7566). A waiver was granted by the Newcastle and North Tyneside NHS Research Ethics Committee 1.

Setting

The Newcastle-upon-Tyne Hospitals (NUTH) National Health Service (NHS) Foundation Trust provides secondary care services to a local population of 302,820 (17) and is a tertiary referral center for the wider North East England and North Cumbria regions. During the period of analysis 17,126 staff were employed across two hospital sites, community sites as well as one offsite non-clinical hub with co-location of administrative, information technology, finance, and other support services. NUTH also contains one of two principal contact High Consequence Infectious Diseases (HCID) treatment centers and was the first HCID unit in the UK to manage patients with COVID-19 (18).

Hospital Infection Control

From January 2020 there was a focus in the UK on active case identification in people with epidemiological risk of SARS-CoV-2 exposure (contact with a confirmed case or travel to an area with widespread transmission). All suspected or confirmed cases were admitted to HCID units. By March it became clear from hospital admission data that widespread community transmission was occurring. Testing was restricted to hospitalized patients with compatible symptoms. During this period nationwide "lockdown" measures were implemented, including closure of schools, businesses, and travel restrictions for all but essential workers, including NHS workers, on 23 March 2020. Public Health England (PHE) issued regularly updated guidance on personal protective equipment (PPE) for HCWs in NHS hospitals and this guidance was followed in our organization for the entire study period (10th March – 6th July 2020). Briefly, "enhanced" or "level 2" PPE (FFP3 mask, eye protection (visor), hood, surgical gown, gloves, waterproof apron) was used for contact with all suspected or confirmed patients until 8 March. This was then downgraded to "level 1" PPE (surgical mask, risk-assessed eye protection, apron, gloves) for all patient contacts except those involving aerosol generating procedures (AGPs), which remained at level 2. From 1 April, level 1 PPE was mandated for all care episodes regardless of the patient's SARS-CoV-2 infection status, except for high-risk clinical areas (such as HDU/ICU) where level 2 PPE was used throughout. From 15 June 2020, surgical facemasks were mandated for all workers in NHS hospitals regardless of patient contact. In NUTH these guidelines were followed and implemented in real time, and PPE was made available to all staff members requiring it. Training was rolled out to all staff members across the Trust with particular attention given to staff members working in environments caring for patients with suspected COVID-19.

SARS-CoV-2 Testing Programme

The NUTH staff testing programme has been described elsewhere (19). Briefly, this was jointly developed by the NUTH Occupational Health and Infection Prevention and Control teams. PCR testing of a nasopharyngeal swab was offered to HCWs who were deemed to fulfill the PHE case definition for COVID-19 from 10 March 2020, with a view to early identification of SARS-CoV-2 infected HCWs and to reduce the need for HCWs to self-isolate without knowledge of their infection status. This was in line with the model recommended by NHS England on 12 April 2020. A local modification made by NUTH on 9 April was the inclusion in the case definition of loss

of sense of smell (anosmia) and/or taste (ageusia), predating the same change to national guidance on 18 May 2020. HCWs who developed COVID-19 symptoms were advised to immediately self-isolate, contact occupational health by email, and then undergo a nurse administered swab for PCR testing within 3 days (and not >5 days) of the onset of symptoms. Providing that the swab was negative and the HCW considered themselves sufficiently recovered they could return to work. Those who tested positive were advised to remain off work for at least 7 days and until their symptoms resolved (with the exception of a persistent cough or anosmia). As in other NHS settings, PCR testing was undertaken on PHE platforms, initially using the PHE RdRp PCR assay, switching to commercial platforms (Altona Diagnostics from 1 April 2020, with the addition of Roche cobas 6800 from 7 April 2020). In addition, from 29 May 2020, a programme of voluntary testing of SARS-CoV-2 antibody was offered to all NUTH employees. SARS-CoV-2 nucleocapsid IgG testing was undertaken on Roche (Elecsys Anti-SARS-CoV-2 serology assay, Roche Diagnostics) and Abbott (SARS-CoV-2 IgG assay, Abbott UK) platforms.

Data Collection

Data on all PCR and SARS-CoV-2 antibody (Ab) tests undertaken by the regional virology diagnostic laboratory during the period 10 Mar to 6 July 2020 were obtained from a prospectively maintained internal database. In addition, data from the NUTH Electronic Staff Record (ESR) were extracted to obtain demographic information (age, gender, ethnicity, staff role, postcode) of all HCWs employed by NUTH during the same period. Data for certain HCW groups not directly employed by NUTH were unavailable in ESR, therefore these groups were excluded. This included doctors at core and specialty trainee level who are employed by Health Education England North East, and North-East Ambulance Service staff. Data from ESR were matched to virology results data using surname and date of birth, with matching validated by first name, using a script written in Excel (Microsoft). Postcode data were used to obtain data on deprivation index from the Ministry of Housing, Communities and Local Government http://imd-by-postcode.opendatacommunities.org/imd/2019. Staff were assigned to 12 roles based on job title, clinical directorate and specific place of work (**Supplementary Table 1**). To investigate clinical outcomes of HCWs testing positive for SARS-CoV-2, we cross-referenced testing data with a retrospective database of COVID-19 inpatients managed in NUTH (20), and also searched for additional cases beyond the censor point of this analysis using the electronic inpatient record. Data on hospitalization, intensive care unit (ICU) admission, ventilation, and outcome were collected.

Data Analysis

Measures of central tendency and distribution were calculated using GraphPad Prism version 8.4.3 (GraphPad Software LLC, US). For the initial analysis of demographic factors, ethnicity data were categorized as either white (including white British, white Irish, white other) or black, Asian or any other minority ethnic background (BAME). Deprivation index was categorized into quartiles with the most deprived quartile taken as the reference group. Contingency tables and Chi^2 (χ^2) tests were used to compare positivity rates between groups. The ages of HCW with detectable or undetectable SARS-CoV-2 IgG antibody were compared using the Mann-Whitney test. Differences in positivity rates between staff roles were estimated using a multiple logistic regression model to adjust for the effects of age, ethnicity (BAME), gender, and deprivation (deciles were used for this analysis). Regression modeling was performed using the SAS JMP Pro Statistical Visualization Software (SAS Institute, UK). A dummy variable (phase) was created to assess for any interaction between staff roles and the proportions of HCWs presenting for antibody testing with and without a prior history of presentation for PCR testing. In addition, the robustness of the staff roles effect was examined by stepping candidate covariables in and out of the logistic regression supplemented by generalized linear regression. While the Ab positivity rates were higher for those presenting with a prior history of PCR testing, there was no statistically significant interaction between Staff Roles and Phase ($p = 0.6963$). The interaction term was dropped, and the odds ratios and 95% confidence intervals constructed for the comparison of each of the Staff Roles relative to the minimal exposure group (Administrative and Managerial).

RESULTS

PCR Testing

From 10 March to 6 July 2020, NUTH laboratories processed and provided SARS-CoV-2 PCR results on 44,781 combined nose/throat swabs. During this period, 3,721 PCR tests were undertaken on 3,338 HCWs who had contacted the symptomatic testing programme (representing 19.5% of all NUTH employees). The median (IQR) turnaround time from samples arriving in the laboratory to a result being available was 7.8 (6.5–10.5) h. In total 481/3,338 symptomatic HCWs tested positive for SARS-CoV-2 by PCR (14.4% [95% CI 13.3–15.6%] of those tested; 2.8% [2.6–3.1%] of all HCWs in the organization).

PCR Positivity Rates Varied Over Time

The number of HCWs presenting for testing and the rate of positive tests fluctuated during the study period, corresponding to the dynamics of SARS-CoV-2 transmission in the region (**Figure 1**). The number of tests performed per day ranged from three to 169 (**Figure 2A**). Most positive PCR tests (390/481, 80%) were returned in the 4 weeks between 23 March and 19 April, when around half of all PCR tests were done (1,959/3,721 [52.6%]). In this period the 7 day average per-test positivity rate peaked at 23.9%, before decreasing and becoming more variable as the number of tests performed on symptomatic staff reduced (**Figure 2B**). Per-test positivity rates (7-day average) in the last 4 weeks of the testing period were 0.8, 2.6, 0.0, and 0.0%, when there were only three positive tests in total.

COVID-19 Clinical Outcomes

To investigate clinical outcomes of HCWs symptomatically infected with SARS-CoV-2, we cross-referenced testing data with a separate database of COVID-19 inpatients managed in NUTH

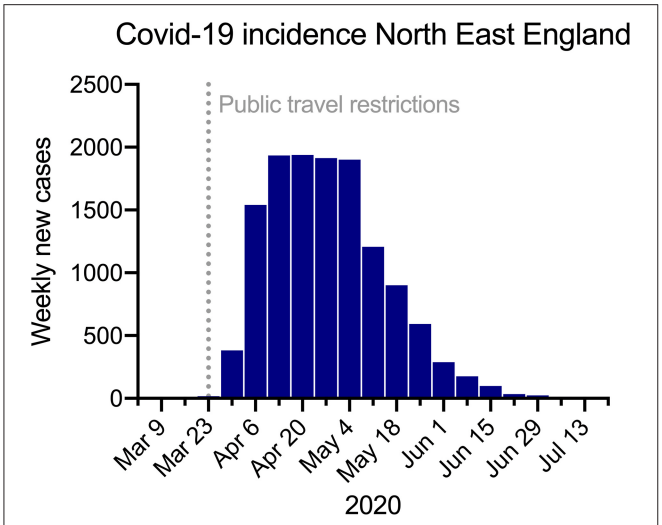

FIGURE 1 | Cases of COVID-19 in the North East England region during the study period.

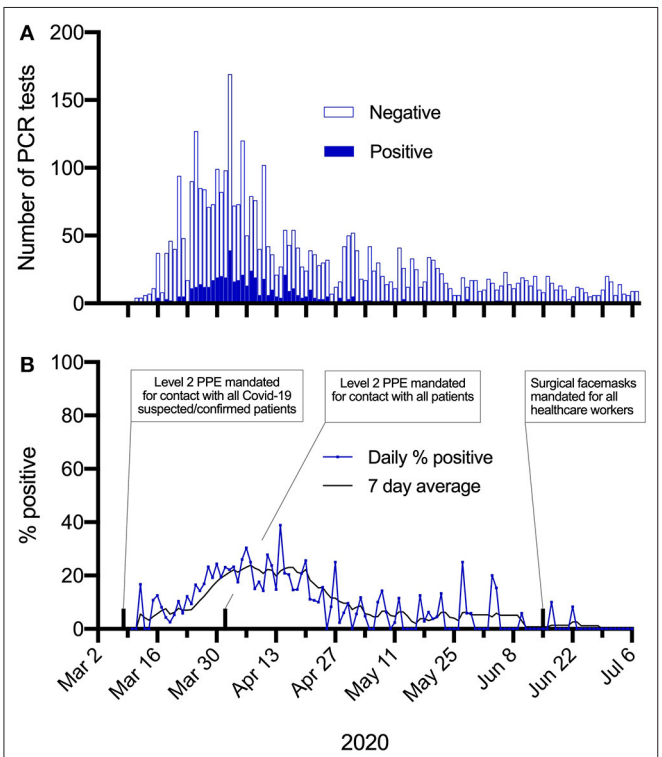

FIGURE 2 | Changes in PCR test positivity over time. **(A)** Total SARS-CoV-2 PCR tests in HCWs. Filled bars = positive tests. Open bars = negative tests. **(B)** Per test positivity (%), displayed as daily (blue line) or 7 day average (black line).

(20), and also searched hospital electronic patient records of PCR positive HCWs for additional cases beyond the censor date of this prior analysis. Seventeen of 481 (3.5%) HCWs testing positive were assessed in secondary care, and 10 (2.1%, 0.06% of all staff) required hospital admission. The median (IQR) [range] length of stay was 5 (3–8.5) [1–12] days. Three PCR-positive HCWs had severe disease on admission defined according to World Health Organization (WHO) criteria (oxygen saturations <90% without supplemental oxygen and/or respiratory rate > 30 breaths/min) and three (0.6%) were managed in critical care, two with non-invasive pressure support. No patients were intubated or required extracorporeal membrane oxygenation (ECMO). All survived to hospital discharge.

Antibody Testing

To complement the PCR analysis, data were analyzed from a voluntary seroprevalence survey which was available to all HCWs irrespective of role and/or prior PCR testing and widely advertised in the organization, from the 29 May. 11,103 of 17,126 HCWs (64.8%) came forward for antibody testing, including 2,557 HCWs who had previously undergone PCR testing (**Figure 3**). SARS-CoV-2 IgG was detected in 937/11,103 (8.4%) HCWs (5.5% of all staff). A gradient of seropositivity was observed, from 380/409 (92.9% [95% CI 90.0–95.0%]) of those testing positive by PCR, to 161/2,148 (7.5% [6.5–8.7%]) of those testing negative by PCR, and 396/8,546 (4.6% [4.2–5.1%]) of those who had not had a PCR test ($P < 0.001$, χ^2-test).

Demographic Factors Associated With Seropositivity

There was no difference in the median (IQR) age of HCWs with detectable or undetectable SARS-CoV-2 IgG antibody (median 43 [IQR: 30–54] and 43 [32–53] years, respectively, Mann-Whitney test $p = 0.7$). 734/8,549 (8.6% [95% CI 8.0–9.2]) females were seropositive compared to 150/2,037 (7.4% [6.3–8.6]) males (χ^2-test $p = 0.073$). Seropositivity in HCWs of white ethnicity was 774/9,500 (8.1% [95% CI 7.6–8.7] percent), compared to 95/894 (10.6% [8.8–12.8]) in those from BAME backgrounds (χ^2-test $p = 0.011$). Comparing deprivation data, seropositivity was noted in 301/2,926 (10.3% [95% CI 9.2–11.4]) of HCWs from the most deprived quartile, compared to 575/7,571 (7.6% [7.0–8.2]) of the less deprived three quartiles (χ^2-test $p < 0.001$).

Association of HCW Role With SARS-CoV-2 Infection

To explore associations between occupational role and the proportion of positive tests (defined as individuals with a positive test by PCR and/or antibody as a percentage of all those tested), HCWs were grouped into 12 categories based on roles recorded in ESR (as discussed in **Supplementary Table 1**). Logistic regression analysis was performed adjusting for the demographic factors described above (**Supplementary Table 1**). The administrative and managerial, non-patient facing group was used as the comparator for this analysis based on the fact that their role does not require close contact with patients or the hospital environment and that many of these staff work in an off-site location separate from the hospital sites.

Antibody Testing and PCR Testing

Following adjustment for age, sex, ethnicity, and deprivation decile there remained strong statistical evidence of differences in positivity rates across staff roles for both antibody and PCR testing ($p < 0.0001$). Most notably, the odds of having a positive antibody test were greater for domestic services staff, healthcare

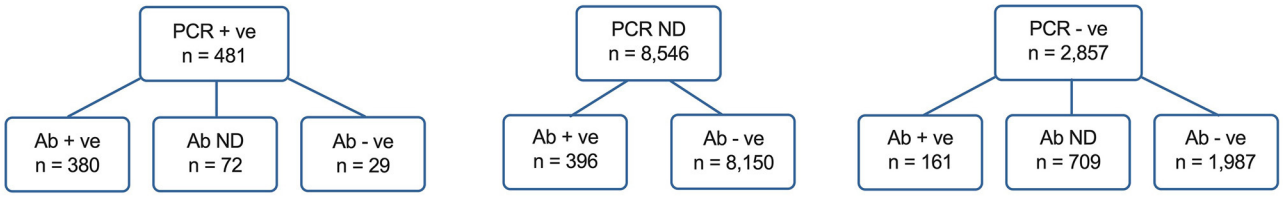

FIGURE 3 | Schematic demonstrating SARS-CoV-2 tests done in the study population. ND, not done.

assistants (HCA) and nurses, in addition to estates and catering and patient-facing clerical workers (**Figure 4A**). A similar pattern was observed for PCR testing with the odds of testing positive also being greater for domestic services staff, HCA, and nurses (**Figure 4B**). Adjusted odds ratios and 95% confidence intervals for antibody and PCR positivity for each of the roles relative to administrative and managerial workers (the reference group) are shown in **Figures 4A,B**. For reference, the raw data are included in **Supplementary Table 1**.

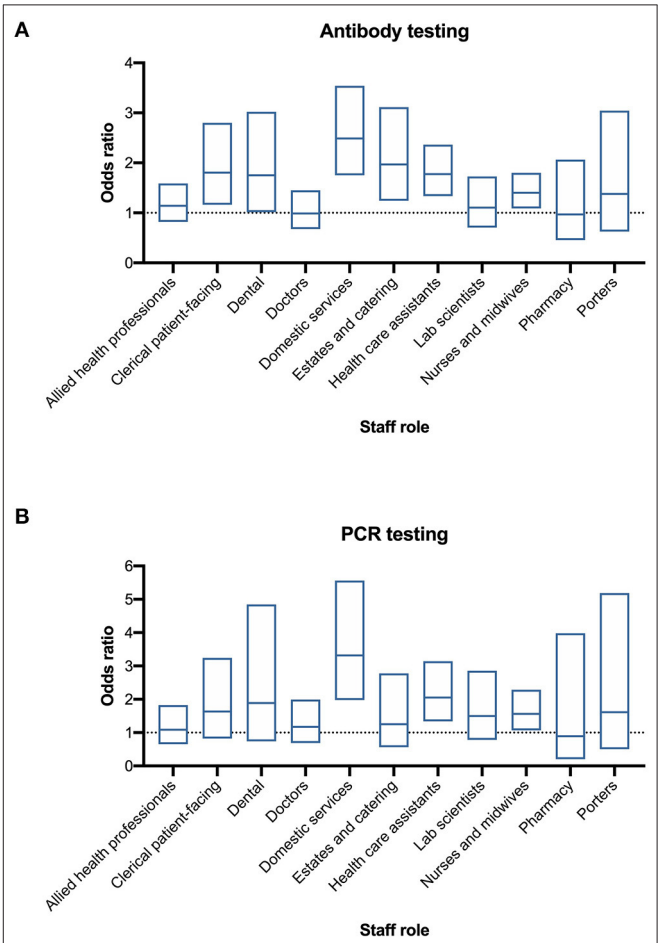

FIGURE 4 | Differential test positivity among HCW groups. **(A)** Adjusted odds ratio of a positive Ab test by staff category. **(B)** Adjusted odds ratio of a positive PCR test by staff category. Adjusted odds ratio (central line) and 95% confidence interval (box) calculated by logistic regression as described in text (compared to non-patient facing administrative and managerial workers).

DISCUSSION

The data we report here span the first wave of the SARS-CoV-2 epidemic in England and represent among the largest combined molecular and serological testing datasets in a HCW population. Nearly one in five employees in this large organization presented for PCR testing during the study period and 14.4% percent of those tested (2.8% of the workforce) had symptomatic SARS-CoV-2 infection detected by PCR. Over two thirds of the total workforce (over 10,000 HCWs) underwent antibody testing. 8.4% of those tested (5.5% of the workforce) were seropositive. This compares to seroprevalence estimates of 6.0% for England and 5.0% for the North East of England around the same period (21) and is consistent with increased exposure in HCWs.

These positivity rates are considerably lower than rates among HCWs in some areas of England, such as London (3), Birmingham (7), and in other parts of the North East (12), although are similar to other regions such as Oxford (2) and Cambridge (14). Factors determining the regional variation in HCW infection rates are unknown, although a relationship with the burden of inpatient cases is apparent (2, 3, 7). It was not possible to draw direct comparisons with community PCR positivity rates, due to the absence of community testing during this period in England. However, community transmission can be inferred from hospital admission data. We note that PCR-confirmed cases among HCWs fell during the study period, in parallel with the decline in community and hospital cases. This occurred despite the fact that most HCWs continued to commute to work and mix in the hospital environment. Similar observations were made at another NHS site (14, 22). No shortages of PPE were reported in our organization. This along with HCW training in donning and doffing PPE might have helped to reduce seroprevalence amongst our staff. These data suggest that the risk of sustained HCW-to-HCW transmission of SARS-CoV-2 can be mitigated in hospital environments (22), despite the recognized challenge of physical distancing in these and other healthcare settings (23).

In our analysis, baseline factors associated with seroconversion included being from black, Asian and minority ethnic (BAME) backgrounds, and living in areas of greater social deprivation, consistent with published data from both HCWs (2, 7) and the general population (21). Our analysis makes the

important additional contribution of showing that test positivity rates differ by occupational role, including after adjustment for contributing demographic factors. These occupational differences cut across clinical and non-clinical roles. Compared with the comparator group of administrative and management workers, nurses and midwives as well as healthcare assistants and dental hospital workers were more likely to test positive whereas doctors or allied health professionals did not, suggesting factors beyond patient contact may be involved.

Other interesting observations also emerged from the analysis. Among non-clinical HCWs exposed to the hospital environment, domestic services, and estates/catering workers were more likely to test positive, whereas laboratory workers handling potentially infectious specimens were not. Administrative staff working in the hospital environment (such as receptionists and ward clerks) had higher positivity rates than those outside it. The underlying reasons for differing rates among occupational groups are not known. An important limitation to the analysis was that details on individuals' contact with cases of COVID-19, either at home or in the workplace, was not collected routinely. This was in part due to how the HCW testing programme was developed, i.e., rapidly and under conditions of extremely high demand. In parallel there was also an unprecedented redeployment of HCWs to COVID-19 areas for clinical service provision throughout the organization. This change in activity was not captured in the ESR. The value of collecting this information was demonstrated recently in another UK study where similar differences in seroprevalence by occupation were noted, including increased seroprevalence rates in domestic services, porters, nurses and estates and catering staff, although only increased rates among domestic services staff and porters (as a combined group) remained significant after adjustment for exposure to COVID-19 (2). Other studies in the UK have not reported rates according to individual occupational roles (3, 7, 11), although did highlight an increased risk among "housekeeping" workers (7)—equivalent to domestic services workers in our dataset. Thus there is an emerging picture of higher seroprevalence rates among domestic services workers as well as those HCWs from BAME backgrounds (2, 7). Whilst the underlying reasons for this are likely to be multifactorial and to include economic and social factors, enhanced surveillance and/or targeted infection control measures are a priority in these groups.

So too are further studies to understand the relative contribution of risks. It is worth noting that within NUTH, domestic services staff used level 1 PPE from 8 March onwards. Our data also provide a signal of heightened risk in other occupational groups, notwithstanding the limitations described above. Analysis of the reproducibility of these observations in other datasets is justified. For example, some studies have shown nursing staff to be at increased risk of acquiring both SARS-CoV-2 (16, 24) and SARS-CoV (25), while others have not (26, 27). Duration of patient contact (16) and incorrect use of PPE (28–32) have also been cited as potential contributing factors in SARS-CoV-2 acquisition in health care settings. Whilst occupational risk is often the focus (3, 6, 13, 16), studies continue to highlight the contribution of community acquisition (2, 5, 33, 34). Until the underlying reasons for differential rates of positivity between occupational groups are established it will be important to continue to monitor infection rates in future waves of SARS-CoV-2 transmission to assess whether current risk mitigation strategies are sufficient. Our findings also highlight the urgent need for robust informatics solutions to allow for routine collection of exposure data at an organizational level.

This study has additional limitations. It is conceivable that the risk to HCW at NUTH, a tertiary center containing a High Consequence Infectious Diseases Unit, could be different than in other healthcare environments. Data were collected retrospectively, thus are more prone to bias. Testing and positivity rates varied throughout the study period and it is not possible to definitively rule out information biases related to the dynamics of the pandemic. Testing relied on HCWs presenting with symptoms or coming forward for antibody testing, therefore positives may have been missed in both cases, or alternatively this strategy may have selected for those at greater risk of testing positive. It was also not possible to account for a minority of HCWs who were shielding, and thus at much lower exposure risk, although this issue is likely to be shared across all occupational groups. Finally, small numbers made it necessary to pool some groups for analysis, resulting in relatively arbitrary staff categories (such as estates and catering or dental hospital workers).

A strength of this dataset, compared to other published studies, is the opportunity it provides to compare results of PCR and subsequent antibody testing in over 2,500 individual HCWs. Seropositivity was 93% in those with prior PCR-confirmed infection. These data are informative as there are few studies of seroconversion rates in HCWs or in people with mild COVID-19 confirmed by prior PCR testing. The results suggest that most patients with mild but symptomatic COVID-19 seroconvert, albeit with a notable minority (7%) who do not. Whether these individuals mount a T-cell response to SARS-CoV-2 is an open question. It is worth noting that in all cases antibody positivity was documented at a time after the positive PCR test, i.e., no PCR-confirmed re-infections occurred. Our data suggest that the ELISA assay is a broadly acceptable surrogate for SARS-CoV-2 exposure in studies of non-hospitalized populations. The observation that seropositivity was higher in those with a negative PCR test than those who had not undergone prior PCR testing is interesting and has been reported elsewhere (27). This is possibly explained by false negative SARS-CoV-2 PCR testing, which can arise through a number of practical (e.g., sampling technique) and methodological issues (e.g., assay design) (35). In mitigation, HCWs tasked with taking swabs underwent extensive training, only pooled nose and throat swabs were taken, and the most sensitive laboratory platforms were used once available.

The symptom-based testing approach we employed appears to have underestimated total HCW infections. The observation that around 4.6% of HCWs who did not present for symptomatic PCR testing were seropositive suggests that a considerable proportion of HCWs either experienced asymptomatic or pauci-symptomatic infection, or that they did not present for PCR testing despite experiencing symptoms. In support of the former hypothesis, a recent meta-analysis has indicated that between 4 and 41% of SARS-CoV-2 infections are asymptomatic (15). A large proportion of cases may be missed by a symptom-based

testing approach, consistent with our observations. Recent data in HCWs have confirmed that asymptomatic SARS-CoV-2 infection does occur (2, 3, 7, 13, 14) and this is central to the argument for asymptomatic screening (1). This is a reasonable approach in low incidence settings. However, important uncertainties to be balanced against asymptomatic HCW screening are the extent to which asymptomatic HCWs transmit SARS-CoV-2 (15), alongside more pragmatic considerations such as how frequently to screen and how to deal with the issue of prolonged asymptomatic shedding of SARS-CoV-2 RNA, which occurs in between a quarter (2) and a half (36) of HCWs, but is not thought to necessarily represent infectious virus (13, 37). Roll out of asymptomatic testing in healthcare settings is anticipated.

Despite an increased risk of SARS-CoV-2 infection, cumulative mortality rates appear lower in HCWs than in the general UK population (38). Our data demonstrate reassuringly low rates of both hospitalization and need for critical care. This may be due to the relative absence of risk factors for mortality in this population such as advanced age and comorbidities (20), coupled with earlier diagnosis and access to treatment. This pattern has also been reported in China (24) and the US (39).

In summary, the data reported here demonstrate that despite a relatively low level of infection compared to other UK HCW cohorts, there was an important differential risk of infection between occupational groups, robust to adjustment for other demographic factors such as BAME background and social deprivation. This finding adds to the growing evidence of differential risks among HCWs. In order to better understand the factors contributing to these risks, prospective quantitative and qualitative studies are a priority. In addition, robust informatics solutions to facilitate the routine collection of "real world" clinical data on HCW exposure and testing within the NHS are critical to inform risk assessment and monitoring.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: Subject to review by NHS Caldicott Guardian. Requests to access these datasets should be directed to Dr Ewan Hunter, ewan.hunter1@nhs.net.

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

DP, PM, KM, SC, PT, US, AE, JD, MS, LP-C, EH, EM, and YT: clinical care and service development. JC, SB-F, DS, JH, JS, YT, and BP: laboratory testing. AH, IS, EH, BP, and CD: study conception. AH, IS, KB, and BP: data collection. AH, DL, and CD: data analysis. DL: statistical analysis. AH and CD: manuscript writing. All authors: manuscript review.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.636160/full#supplementary-material

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Impact of Public Health Education Program on the Novel Coronavirus Outbreak in the United States

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The coronavirus outbreak in the United States continues to pose a serious threat to human lives. Public health measures to slow down the spread of the virus involve using a face mask, social-distancing, and frequent hand washing. Since the beginning of the pandemic, there has been a global campaign on the use of non-pharmaceutical interventions (NPIs) to curtail the spread of the virus. However, the number of cases, mortality, and hospitalization continue to rise globally, including in the United States. We developed a mathematical model to assess the impact of a public health education program on the coronavirus outbreak in the United States. Our simulation showed the prospect of an effective public health education program in reducing both the cumulative and daily mortality of the novel coronavirus. Finally, our result suggests the need to obey public health measures as loss of willingness would increase the cumulative and daily mortality in the United States.

Keywords: COVID-19, public health education, non-pharmaceutical intervention, face mask, social distancing

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INTRODUCTION

The novel coronavirus (COVID-19) pandemic caused by SARS-CoV-2 was first reported in Wuhan, China in December 2019 and later declared a pandemic by the World Health Organization (WHO) on March 11, 2020 (1–3). The emergence of the virus continues to cause devastating public health, and social-economic impact around the globe, including the United States (4, 5). The symptoms for COVID-19, which are similar to the common cold, though potentially more severe, include fever, cough, shortness of breath, fatigue, loss of taste or smell, sore throat, running nose, nausea, and diarrhea (6). As of December 12, 2020, there are over 71 million confirmed COVID-19 cases globally, resulting in over 1.6 million deaths (7). Within the United States, there have been over 16 million confirmed cases of coronavirus, with over 297,501 deaths (4).

The Centers for Disease Control and Prevention (CDC) on April 2, 2020, recommended the use of non-pharmaceutical interventions (NPIs) such as face masks in public (see **Figure 1**) and to practice social-distancing to curtail the spread of the virus (3, 5, 8–11). Non-pharmaceutical interventions have had a long history of preventing many infectious diseases such as the pandemic Influenza, Measles, and the Ebola Virus Disease (EVD) (12–16). Actions taken in the early stage of the coronavirus outbreak by the various state governments in the United States include declaring a

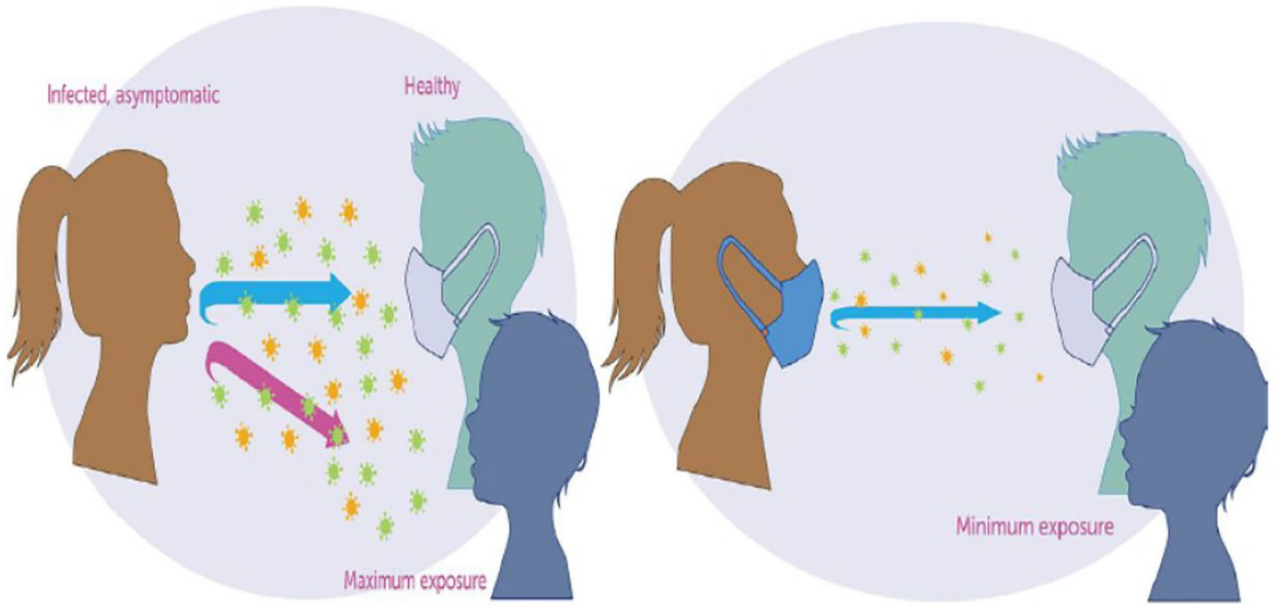

FIGURE 1 | The science of mask against COVID-19 (20).

state of emergency and issuing a state-wide shelter in place. The use of a face mask by the general public in the United States has been controversial as some state governors issued executive orders that voided face mask mandates within their jurisdiction (17).

Numerous mathematical models have been used to provide insights into public health measures for mitigating the spread of the novel coronavirus pandemic. Ferguson et al. (18) proposed an agent-based model to assess the impact of NPIs on COVID-19 mortality. In the absence of public health interventions, their model projected high mortality in the United States and the United Kingdom. Eikenberry et al. (3) developed a mathematical model to assess the impact of mask use by the general public on the transmission dynamics of the COVID-19 pandemic. Their results showed that broad adoption of even relatively ineffective face masks might reduce community transmission of COVID-19 and decrease peak hospitalizations and deaths. Recently, Ngonghala et al. (8) developed a mathematical model to assess the impact of NPIs on curtailing the public health burden of COVID-19 in the United States. Their study showed the effect of early implementation of face masks, lockdown, and lifting of social-distancing. Extending the duration of lockdown could reduce the daily cases, daily mortality in the United States. Mizumoto and Chowell (19) used a mathematical model to assess the potential for a coronavirus outbreak aboard the Diamond Princess cruise, which experienced a major COVID-19 outbreak during the months of January and February of 2020. Their study showed that the basic reproduction number of the model decreases with increasing the effectiveness of the quarantine and isolation measures implemented on the ship.

Despite public health campaigns regarding the use of a face masks and social-distancing in the United States, the local transmission of COVID-19 throughout different parts of the country continues to rise. While many people follow public health recommendations to the use of face mask and practice social-distance in public to limit the spread of the virus, others passionately fight against them. It is important to understand how educating the population on the importance of using a face mask and social-distancing could reduce the spread of the virus. The objective of this study is to use a mathematical model to assess the impact of public health education campaigns on the coronavirus outbreak in the United States.

MATERIALS AND METHODS

Model Formulation

The coronavirus model to be developed uses the natural history of the infection. The total human population at time t, denoted by $N(t)$, is sub-divided into mutually exclusive compartments of unwilling susceptible $[S_u(t)]$, willing susceptible $[S_e(t)]$, unwilling exposed $[E_u(t)]$, willing exposed $[E_e(t)]$, unwilling asymptomatic-infectious $[A_u(t)]$, willing asymptomatic-infectious $[A_e(t)]$, unwilling infectious with symptoms $[I_{us}(t)]$, willing infectious with symptoms $[I_{es}(t)]$, unwilling hospitalized or isolated at a health care facility $[H_u(t)]$, willing hospitalized or isolated at a health care facility $[H_e(t)]$, in intensive care units $[I_{cu}(t)]$, and recovered $[R(t)]$ individuals. Thus, the total population size N is given as

$$N(t) = S_u(t) + S_e(t) + E_u(t) + E_e(t) + I_{us}(t) + I_{es}(t) + A_u(t) + A_e(t) + H_u(t) + H_e(t) + I_{cu}(t) + R(t).$$

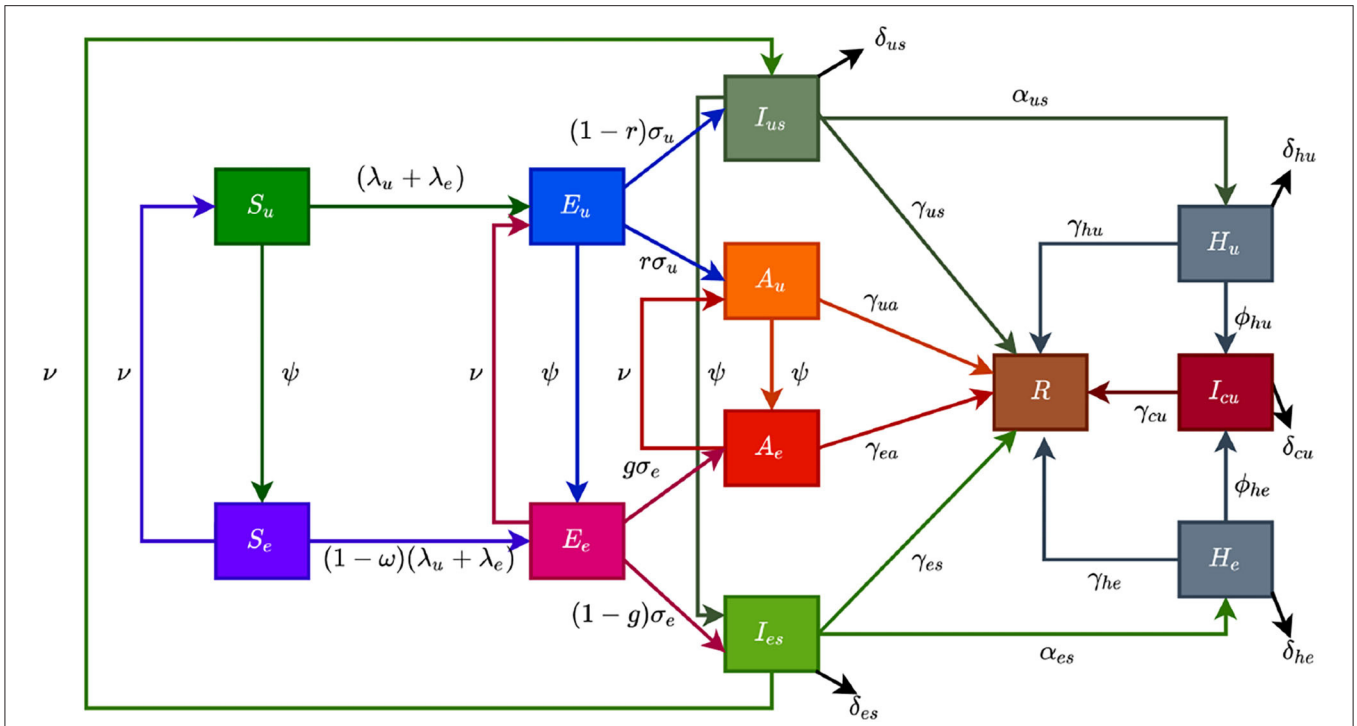

FIGURE 2 | Flow diagram of the model showing transitions from various compartments based on public health education.

TABLE 1 | Description of the state variables of the model (S1).

State variable	Description
S_u	Population of unwilling susceptible individuals
S_e	Population of willing susceptible individuals
E_u	Population of unwilling exposed individuals
E_e	Population of willing exposed individuals
I_{us}	Population of unwilling infectious individuals with severe clinical symptoms of COVID-19
I_{es}	Population of willing infectious individuals with severe clinical symptoms of COVID-19
A_u	Population of unwilling asymptomatic-Infectious individuals
A_e	Population of willing asymptomatic-Infectious individuals
H_u	Population of unwilling hospitalized individuals
H_e	Population of willing hospitalized individuals
I_{cu}	Population of individuals in ICU
R	Population of recovered individuals

TABLE 2 | Description of parameters of the model (S1).

Parameter	Description
β	Effective contact rates for willing(unwilling) individuals
ω	Efficacy of education in preventing COVID-19 infection ($0 < \omega \leq 1$)
$\eta_{A_u}(\eta_{A_e})(\eta_{H_u})(\eta_{H_e})$	Modification parameters ($0 < \eta_{A_u}(\eta_{A_e})(\eta_{H_u})(\eta_{H_e}) < 1$)
ψ	Education rate for individuals in S_u (E_u) (I_{us}) (A_u)
ν	Fatigue rate (loss of willingness to public health measures)
$\sigma_u(\sigma_e)$	Progression rates from $E_u(E_e)$ to $I_{us}(I_{es})$ or $A_e(A_e)$ class
$r(g)$	Proportion of individuals in $E_u(E_e)$ class who show clinical symptoms of COVID-19
$\alpha_{us}(\alpha_{es})$	Hospitalization rates for unwilling(willing) infectious individuals
$\phi_{hu}(\phi_{he})$	ICU admission rate for unwilling(willing) hospitalized individuals
$\gamma_{ua}(\gamma_{ea})(\gamma_{us})(\gamma_{es})(\gamma_h)(\gamma_{cu})$	Recovery rates for individuals in the $A(I_s)(H)(I_{cu})$ class
$\delta_{us}(\delta_{es})(\delta_h)(\delta_{cu})$	Disease-induced death rates for individuals in the $I_{us}(I_{es})(H)(I_{cu})$ class

The flow diagram of the model (S1) is depicted in **Figure 2** (the state variables and parameters of the model are described in **Tables 1**, **2**, respectively).

In model (S1), β is the effective infection rate for unwilling and willing individuals, while η_j, $(j \in \{A_k, H_k\}, k \in \{u, e\})$, is the modification parameters (where $0 < \eta_j < 1$) that accounts for a reduction in infectiousness of unwilling(willing) asymptomatic and hospitalized individuals compared to unwilling(willing) symptomatic individuals. Further, ψ represent the public health education rate for unwilling susceptible (S_u), exposed (E_u), symptomatic (I_{us}), and asymptomatic individuals (A_u),

respectively. It is assumed that public health education program toward the use of NPIs in preventing COVID-19 infection is imperfect (i.e., allowing willing susceptible individuals become infected with COVID-19), with an efficacy ω (where $0 < \omega \leq 1$). Furthermore, the parameters σ_j, $j = u, e$ represents the progression rates of unwilling (willing) exposed individuals. A proportion, $0 < r, g \leq 1$, of unwilling (willing) exposed individuals show clinical symptoms of COVID-19 and move to the class I_{js}, $j = u, e$, at the end of the incubation period. The remaining proportion, $(1 - r)$ and $(1 - g)$, show no clinical symptoms and move to the A_j, $j = u, e$, class. Further, ν represent the loss of willingness to wear a face mask, practice social-distancing in public, and frequently washing hands. The parameters α_{js}, $j = u, e$, is the hospitalization (or self-isolation) rates of unwilling(willing) individuals with clinical symptoms of COVID-19. Similarly, the parameters ϕ_{hu}, ϕ_{he} is the ICU admission rates. The parameters γ_{ja}, γ_{js}, γ_{hj}, γ_{cu}, $j = u, e$, represents the recovery rates for unwilling (willing) individuals in the A_j, I_{js}, H_j, I_{cu}, $j = u, e$ classes. Finally, the parameter δ_{js}, δ_{hj}, δ_{cu}, $j = u, e$ represents the COVID-induced mortality rate for individuals in the I_{js}, H_j, I_{cu}, $j = u, e$ classes. To formulate the model, we made the following assumptions:

(i) due to public health education, willing individuals wear face mask to prevent transmission, practise social-distancing and wash their hands while unwilling individuals do not.
(ii) public health education program is targeted at individuals who are unwilling to use a face mask or practice social-distance in public at rate (ψ).
(iii) to account for public health education saturation, we assume a willingness fatigue (i.e., loss of willingness to wear face mask, practise social-distancing, and frequent washing of hands),

The model (S1) is also an extension of the COVID-19 models in (3, 5, 8–10) by including compartments for individuals based on their willingness/unwillingness regarding the adherence to non-pharmaceutical interventions such as face mask, social-distancing, and hand washing to curtail the COVID-19 outbreak. Models of this type have been formulated for Influenza (12) and COVID-19.

RESULTS

Asymptotic Stability of Disease-Free Equilibria

The expression for the reproduction number ($\mathcal{R}_c$) for model with public health education program is given in the **Supplementary Material**.

Theorem 0.1. *The disease-free equilibrium (DFE) of the model (S1) is locally-asymptotically stable if $\mathcal{R}_c < 1$. If $\mathcal{R}_c > 1$, the epidemic grows rapidly, reaches a peak, and eventually declines to zero.*

The quantity $\mathcal{R}_c$ is the *reproduction number* of the model (S1). It measures the average number of new COVID-19 cases generated by a typical infectious individual introduced into a population where a certain fraction is protected.

Data Fitting and Parameter Estimation

Estimates for some of the parameters of the model (S1) were obtained from the literature (as indicated in **Table 3**). Other parameters, such as the effective infection rate parameters β, education rate ψ, education efficacy ω, and fatigue rate ν are obtained by fitting the model to the observed cumulative mortality data for the United States (21, 22). In particular, the United States Cumulative mortality data from January 22, 2020 (first index case) to December 8, 2020 were obtained from the John Hopkins Center for Systems Science and Engineering COVID-19 Dashboard (23). We fitted the model for three different time periods of the pandemic, with the first period from January 22, 2020 to July 5, 2020, second period from July 6, 2020 to September 30, 2020, and the third period from October 1, 2020 to December 8, 2020. This was done in order to correctly capture the trends observed in the daily mortality data (i.e., the COVID-19 waves observed). Hence, we obtained three set of values for the parameters to be estimated based on the different periods. Our choice of fitting the model to the mortality data is due to the fact that there is evidence of under-reporting and under-testing of COVID-19 cases in countries such as France, Italy, United States, Iran, and Spain. Hence, mortality data may provide a better indicator for COVID-19 case spread (8, 24). The data-fitting process involves implementing the standard nonlinear least squares approach using the *fmincon* Optimization Toolbox embedded in MATLAB. The estimated values of the unknown parameters are tabulated in **Table 4**. **Figures 3A–C** depicts the fitting of the observed and predicted cumulative mortality for the United States. Further, **Figures 3D,E** compares the simulations of the model using the fitted and fixed parameter in **Tables 3**, **4**. The results depicted in **Figure 3**, show that the model also captures the observed daily mortality data for each of the period considered. Thus, the parameter estimation of model (S1) shows that cumulative mortality data provides a very reliable calibration for coronavirus transmission dynamics. In **Figures 3A,D**, it is worth mentioning that the fit is not really good around mid March. This is not surprising since testing capacities have been ramped up around this time, leading to an increasing fraction of infections being detected.

Sensitivity Analysis

The model (S1) contains parameters, and uncertainty in their estimates are expected to arise. The effect of such uncertainties is assessed using uncertainty and sensitivity analysis (25–27). In particular Latin Hypercube Sampling (LHS) and Partial Rank Correlation Coefficients (PRCC) is used to identify model parameters that have the most influence on the model with the reproduction number ($\mathcal{R}_c$) as the response function. The purpose of this analysis is to determine effects of parameters on model outcomes (25–27). A highly sensitive parameter should be more carefully estimated, since a small change in that parameter can cause a large quantitative changes in the result (25–27). On the other hand, a parameter that is not sensitive does not require as much attempt to estimate, since a small change in that parameter will not cause a large variation to the quantity of interest (26). Parameters with large PRCC greater than +0.50 are said to be highly positively correlated with the response

function, while those <-0.50 are said to be highly negatively correlated with the response function (25–27). The parameters considered in the PRCCs analysis are the effective infection rate for unwilling (willing) individuals (β), education rates for unwilling (willing) individuals (ψ), education efficacy (ω), and fatigue rate (ν). We performed a PRCC analysis for the three different periods; however, the parameters have the same effect on the response function for the three periods. We chose to report one plot as displayed in **Figure 4**. The results show that the four parameters that mostly impact the response function ($\mathcal{R}_c$) are the effective infection rate (β), education rate (ψ), fatigue rate (ν), and education efficacy (ω). Based on the PRCC values, the transmission rate for unwilling individuals and the fatigue rate has a positive impact on $\mathcal{R}_c$, as an increase(decrease) in the transmission and fatigue parameter will increase(decrease) $\mathcal{R}_c$. In contrast, the education rate and efficacy have a negative impact on the $\mathcal{R}_c$, and an increase in these parameters will decrease the $\mathcal{R}_c$.

TABLE 3 | Baseline parameter values for the model (S1) drawn from the literature.

Fixed Parameter ($k = u, e$)	Value	References
σ_e, σ_u	1/2.5/day	(31, 32)
r, g	0.35	(33, 34)
η_{A_k}	1.5	Assumed
η_{H_k}	0.25	Assumed
α_{us}, α_{es}	1/6/day	(35)
ϕ_{hu}, ϕ_{he}	0.083/day	(36)
γ_{ua}, γ_{ea}	1/5/day	(35)
γ_{us}, γ_{es}	1/10/day	(18, 37)
γ_{hu}, γ_{he}	1/8/day	(18)
γ_{cu}	1/10/day	(18, 37)
δ_{ks}	0.015/day	(3, 5, 18)
δ_{hk}	0.015/day	(3, 5, 18)
δ_{cu}	0.0225/day	(3, 5, 18)

Numerical Simulation Results

To capture the trends observed in the daily mortality data obtained for the United States from January 22, 2020, to December 8, 2020, we considered three different periods of the pandemic with the first period from January 22, 2020, to July 5, 2020, second period from July 6, 2020 to September 30, 2020, and the third period from October 1, 2020 to December 8, 2020. First,

TABLE 4 | Estimated parameter values for the model (S1) using COVID-19 mortality data for the United States.

Estimated Parameters	1/22/2020–7/5/2020	7/6/2020–9/30/2020	10/1/2020–12/8/2020
β	0.8084	0.4369	0.2842
ψ	0.0279	0.0781	0.0249
ν	0.0011	0.0210	0.0461
ω	0.8982	0.8599	0.8896

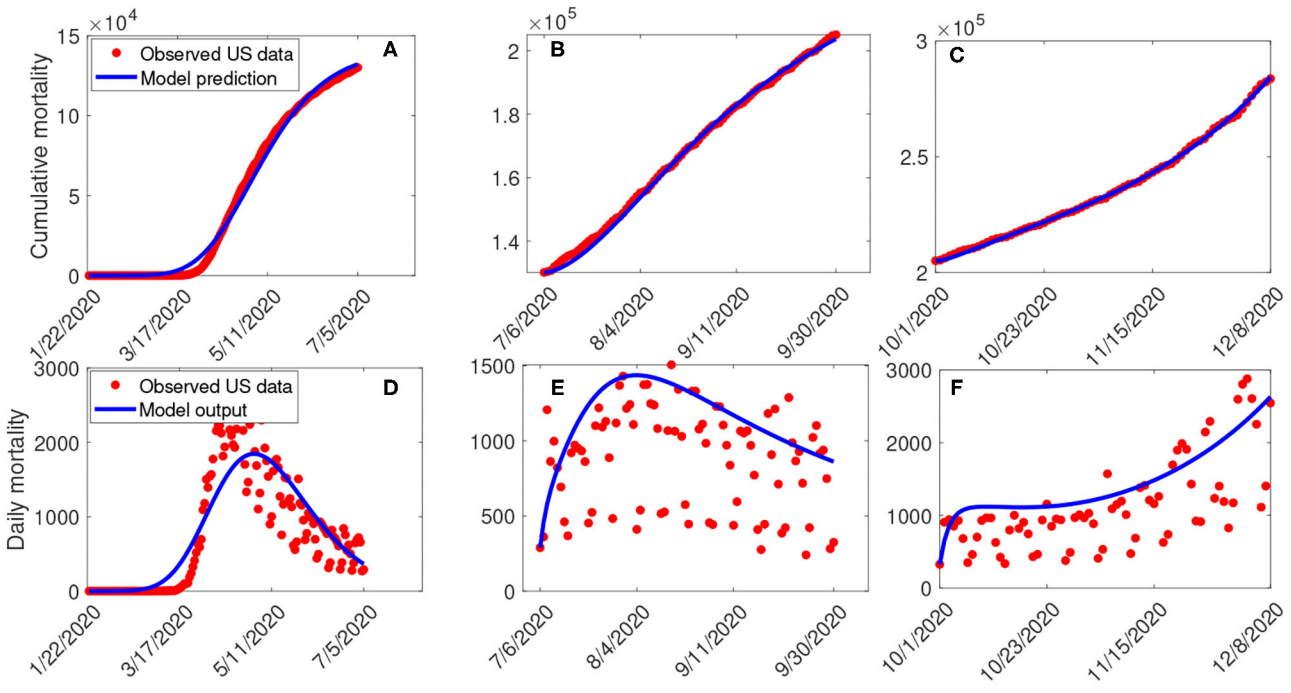

FIGURE 3 | (A–C) Data fitting of the model (S1) using the cumulative mortality data for the United States from January 22, 2020 to December 8, 2020. **(D–F)** Simulations of the model (S1) using the fixed and the fitted parameters from the cumulative mortality data for the United States in **Tables 3**, **4**.

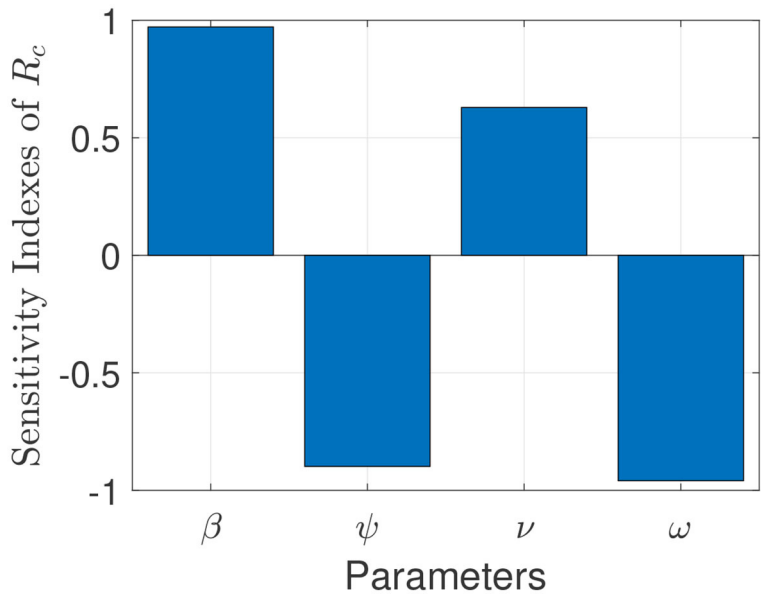

FIGURE 4 | Partial rank correlation coefficients (PRCCs) showing the impact of four model parameters on the reproduction number ($\mathcal{R}_c$) of the model. Parameter values used are as given in **Tables 3**, **4**.

we generated a contour plot of the reproduction number ($\mathcal{R}_c$) of the model (S1), as a function of education rate (ψ) and education efficacy (ω) (**Figure 5**). **Figure 5a** for the period January 22, 2020 to July 5, 2020, suggests that the control reproduction number $\mathcal{R}_c$ is practically independent of ψ (at least for $\psi \ll 1$). A similar trend is observed for the period July 6, 2020, to September 30, 2020, of the outbreak (**Figure 5b**). However, **Figure 5c** shows that for the period October 1, 2020, to December 8, 2020, as more people are being educated with high efficacy, the value of $\mathcal{R}_c$ decreases. It is worth mentioning that the value of $\mathcal{R}_c$ depends on the initial conditions, more precisely on the location of the specific DFE within the hyperplane of disease-free equilibria. Assuming that no individuals are educated at the beginning of the simulation, then the education efficacy (ω) will be irrelevant (sensitivity index close to zero) since in the beginning there are no individual that have already been educated. This impedes the immediate curtailment of the epidemic much more severely than too few individuals being in the process of being educated (this is exactly the reason, why ψ does barely affect $\mathcal{R}_c$: rather than the flux from uneducated to educated individuals the number of currently educated individuals acts on $\mathcal{R}_c$). As soon as a significant number of individuals is educated, the effect of the education efficacy on $\mathcal{R}_c$ will increase dramatically. Moreover, since $\mathcal{R}_c$ depends on the values of the initial conditions (S_u^* and S_e^*), it is expected that $\mathcal{R}_c$ decreases as more individuals are being educated over time.

Figure 6 depicts a contour plot of the reproduction number ($\mathcal{R}_c$) of the model (S1), as a function of the proportion of educated individuals among all susceptible $\left(\frac{S_e^*}{S_u^*+S_e^*}\right)$ and education efficacy (ω) for a fixed education rate (ψ). **Figure 6a** shows that for the period January 22, 2020 to July 5, 2020, with the baseline education efficacy, $\mathcal{R}_c$ can be brought to a value <1 if 90% among all susceptible individuals are educated. This result suggests that an incredibly high education rate (ψ) is necessary to curtail the outbreak effectively for the period January 22, 2020, to July 5, 2020. However, for the period July 6, 2020, to September 30, 2020, of the outbreak, with the baseline education efficacy, $\mathcal{R}_c$ can be brought to a value less than one if 76% among all susceptible individuals are educated (**Figure 6b**). **Figure 6c** shows that for the period October 1, 2020, to December 8, 2020, with the baseline education efficacy, $\mathcal{R}_c$ can be brought to a value less than one if 51% among all susceptible individuals are educated. This result further supports the need to educate more people if we are to effectively curtail the coronavirus outbreak, which is consistent with the results obtained in **Figure 5**.

Furthermore, we ran simulations of model (S1) using the parameter values in **Tables 4**, **5**, to assess the population-level impact of public health education program on the COVID-19 outbreak. The simulation result for the baseline scenario shows a projected 132,000 cumulative deaths by July 5, 2020, 205,600 by September 30, 2020, and 285,100 by December 8, 2020 (**Figure 7A**). Similarly, the projected peak daily mortality was 1,829 attained on April 28, 2020, 1,505 attained on August 3, 2020, and 2,808 attained by December 8, 2020 (**Figure 7B**). Further, with a 10% increase in education rate from the baseline value, **Figure 7A** shows a projected 44,400 cumulative mortality by July 5, 2020, 105,300 by September 30, 2020, and 178,000 by December 8, 2020. This result is approximately a 66.4% reduction in cumulative mortality by July 5, 2020, a 48.8% reduction in cumulative mortality by September 30, 2020, a 37.6% reduction in cumulative mortality by December 8, 2020, when compared to the baseline scenario. **Figure 7B** with a 10% increase in education

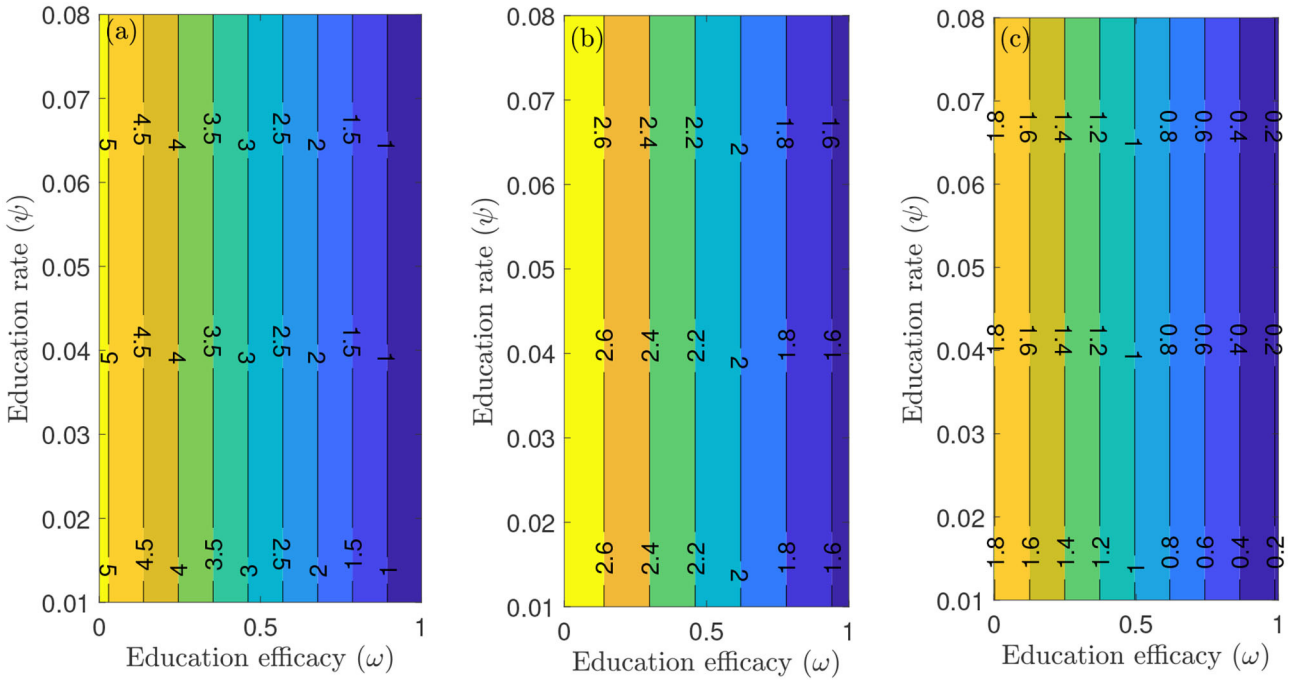

FIGURE 5 | Contour plot of the reproduction number ($\mathcal{R}_c$) of the model (S1), as a function of education rate (ψ) and education efficacy (ω). **(a)** First period from January 22, 2020 to July 5, 2020. **(b)** Second period from July 6, 2020 to September 30, 2020. **(c)** Third period from October 1, 2020 to December 8, 2020. Parameter values are as given in **Tables 3**, **4**.

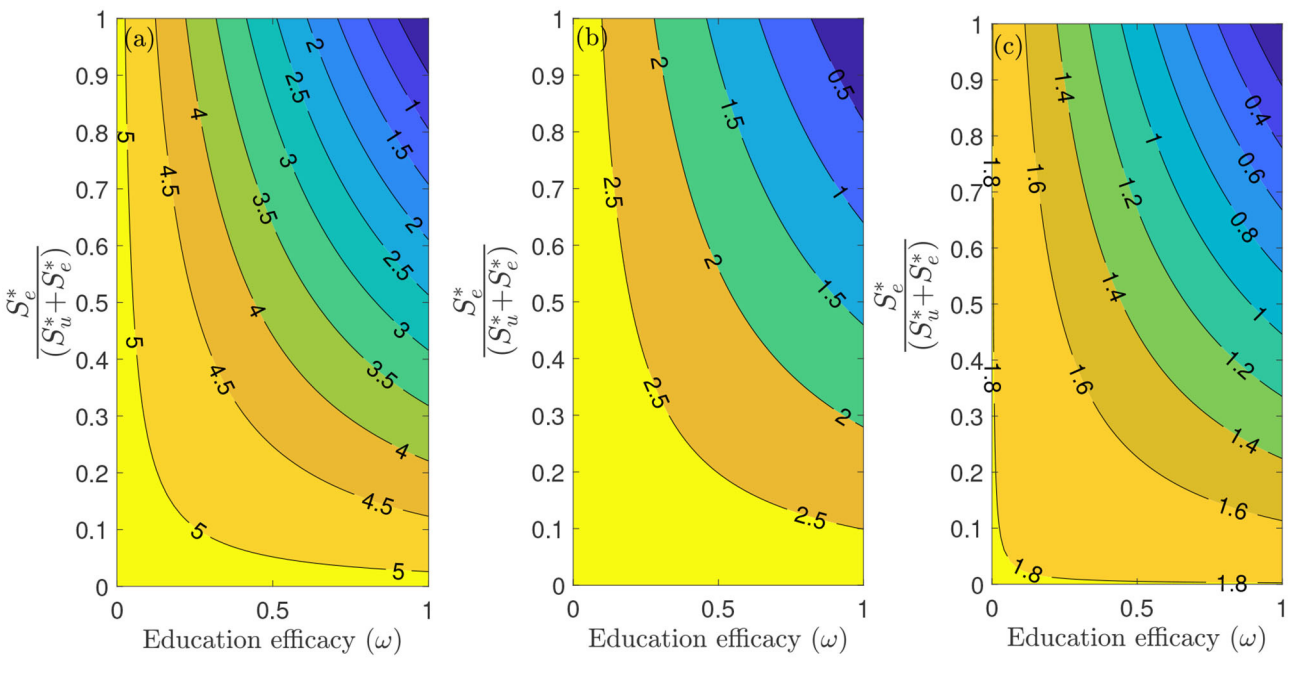

FIGURE 6 | Contour plot of the reproduction number ($\mathcal{R}_c$) of the model (S1), as a function of different ratios of $\left(\frac{S_e^*}{(S_u^*+S_e^*)}\right)$ and education efficacy (ω) with a fixed education rate ψ. **(a)** First period from January 22, 2020 to July 5, 2020. **(b)** Second period from July 6, 2020 to September 30, 2020. **(c)** Third period from October 1, 2020 to December 8, 2020. Parameter values are as given in **Tables 3**, **4**.

TABLE 5 | A summary of various increase in education rate.

	1/22/2020–7/5/2020		7/6/2020–8/30/2020		10/1/2020–12/8/2020	
Education rate	**cum. mort.**	**daily mort.**	**cum. mort.**	**daily mort.**	**cum. mort.**	**daily mort.**
Baseline	132,000	1,829	205,600	1,505	285,100	2,808
10% increase in ψ	44,400	617	105,300	1,103	178,000	2,359
20% increase in ψ	17,210	248	69,160	962	136,200	1,867
30% increase in ψ	7,676	115	53,060	887	115,200	1,526
40% increase in ψ	3,835	58	44,410	836	102,200	1,268

rate from the baseline value, shows projected 617 peak mortality by April 20, 2020, 1,103 by July 31, 2020, and 2,359 by December 8, 2020. This result is approximately a 66.3% reduction in peak daily mortality by April, 20, 2020, a 26.7% reduction in peak daily mortality by July 31, 2020, a 16% reduction in peak daily mortality by December 8, 2020 when compared to the baseline scenario. However, with a 40% increase in education rate from the baseline value, **Figure 7A** shows a projected 3,835 cumulative mortality by July 5, 2020, 44,410 by September 30, 2020, and 102,200 by December 8, 2020. This result is approximately a 97.1% reduction in cumulative mortality by July 5, 2020, a 78.4% reduction in cumulative mortality by September 30, 2020, a 64.2% reduction in cumulative mortality by December 8, 2020, when compared to the baseline scenario. **Figure 7B** with a 40% increase in education rate from the baseline value, shows projected 58 peak mortality by April 7, 2020, 836 by July 24, 2020, and 1,268 by December 8, 2020. This result is approximately a 96.8% reduction in peak daily mortality by April, 7, 2020, a 44.5% reduction in peak daily mortality by July 24, 2020, a 54.8% reduction in peak daily mortality by December 8, 2020, when compared to the baseline scenario. The result in **Figure 7** shows the need for an aggressive public health education program toward the use of NPIs to curtail the spread of the virus. A summary of the impact of various increase in education rate on cumulative mortality and peak daily mortality is tabulated in **Table 5**.

Figure 8 depicts the impact of the loss of willingness to public health measures on COVID-19 outbreak. The result shows that with a 10% increase in fatigue rate from the baseline value, **Figure 8A** projected 144,100 cumulative mortality by July 5, 2020, 228,800 by September 30, 2020, and 325,700 by December 8, 2020. This result is approximately a 9.2% increase in cumulative deaths by July 5, 2020, a 11.3% increase in cumulative deaths by September 30, 2020, and a 14.2% increase in cumulative deaths by December 8, 2020, when compared to the baseline scenario. **Figure 8B** with a 10% increase in fatigue rate from the baseline value, shows projected 1,955 peak mortality by May 1, 2020, 1,657 by August 3, 2020, and 4,451 by December 8, 2020. This result is approximately a 6.9% increase in peak daily mortality by May 1, 2020, a 10.1% increase in peak daily mortality by August 3, 2020, a 58.5% increase in peak daily mortality by December 8, 2020, when compared to the baseline scenario. However, with a 40% increase in fatigue rate from the baseline value, **Figure 8A** shows a projected 184,200 cumulative mortality by July 5, 2020, 313,200 by September 30, 2020, and 478,300 by December 8, 2020. This result is approximately a 39.5% increase in cumulative deaths by July 5, 2020, a 52.3% increase in cumulative deaths by September 30, 2020, and a 67.8% increase in cumulative deaths by December 8, 2020 when compared to the baseline scenario. **Figure 8B** with a 40% increase in fatigue rate from the baseline value, shows projected 2,412 peak mortality by May 3, 2020, 2,513 by August 23, 2020, and 9,935 by December 8, 2020. This result is approximately a 31.9% increase in peak daily mortality by April, 20, 2020, a 67% increase in peak daily mortality by July 27, 2020, a 254% increase in peak daily mortality by December 8, 2020, when compared to the baseline scenario. This result suggests the need to obey public health measures as loss of willingness would increase the cumulative and daily mortality in the United States. A summary of the impact of the various increase in fatigue rate on cumulative mortality and peak daily mortality is tabulated in **Table 6**.

DISCUSSION AND CONCLUSIONS

In this study, we developed a mathematical model for the transmission dynamics and control of COVID-19 in the United States by stratifying the total population into two subgroups of willing and unwilling individuals to the use of face masks, social-distancing in public, and proper/frequent hand washing. The model allows for the assessment of the impact of public health education programs on the coronavirus outbreak in the United States. The model was parameterized using cumulative mortality data for the United States from January 22, 2020, to December 8, 2020, to assess the population-level impact of public health education programs on the outbreak. In particular, we showed that the disease-free equilibrium of the model is locally-asymptotically stable whenever a certain epidemiological threshold, known as the reproduction number ($\mathcal{R}_c$) is less than one. The epidemiological implication of this result is that when $\mathcal{R}_c < 1$, a small COVID-infected individuals in the community will not lead to an outbreak.

We explored the sensitivity of the reproduction number with respect to public health education rate in the United States for three different periods of the outbreak. In particular, we showed that community transmission of COVID-19 could be significantly reduced with a very high education rate. In other words, our study shows that COVID-19 could have been effectively controlled if the public health education campaign has been intensified enough with high efficacy (and sustained) from the beginning of the pandemic. Furthermore, we also explored the sensitivity of the reproduction number with respect

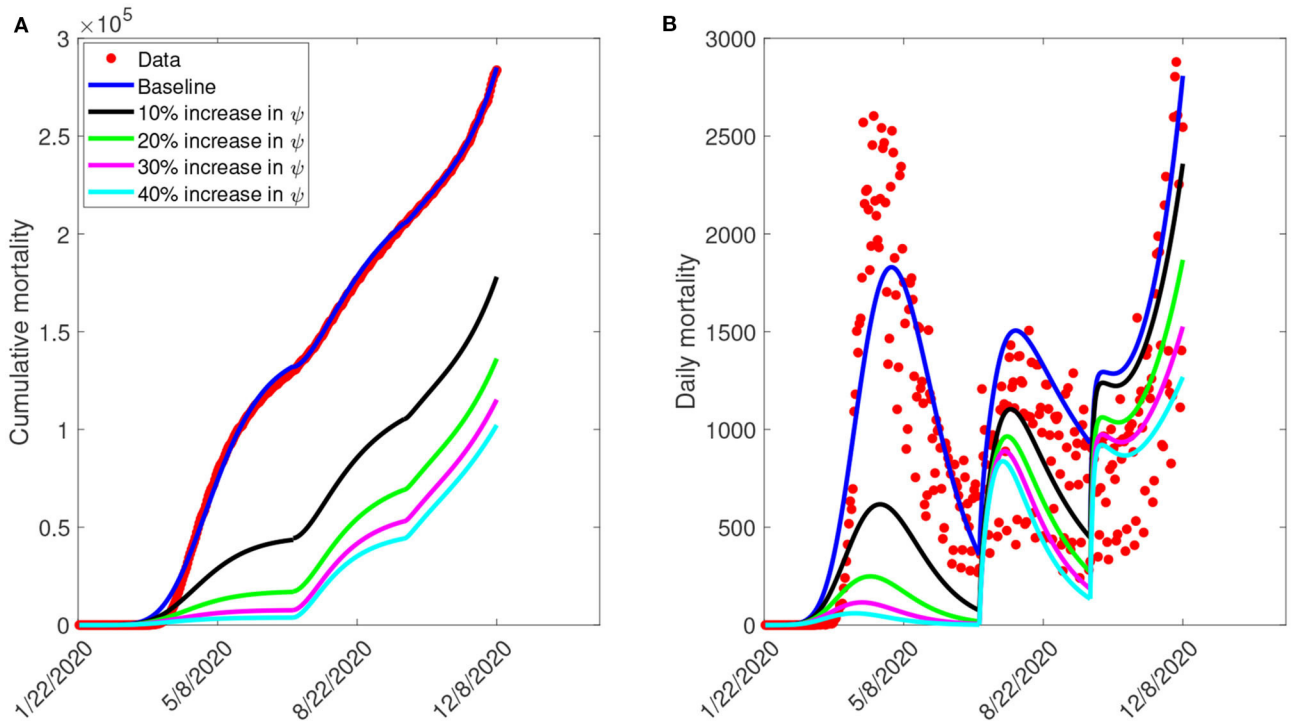

FIGURE 7 | Simulation of the model (S1), showing **(A)** cumulative mortality **(B)** daily deaths, as a function of time for various public health education rates in the United States from January 22, 2020 to December 8, 2020. Parameter values are as given in **Table 3**.

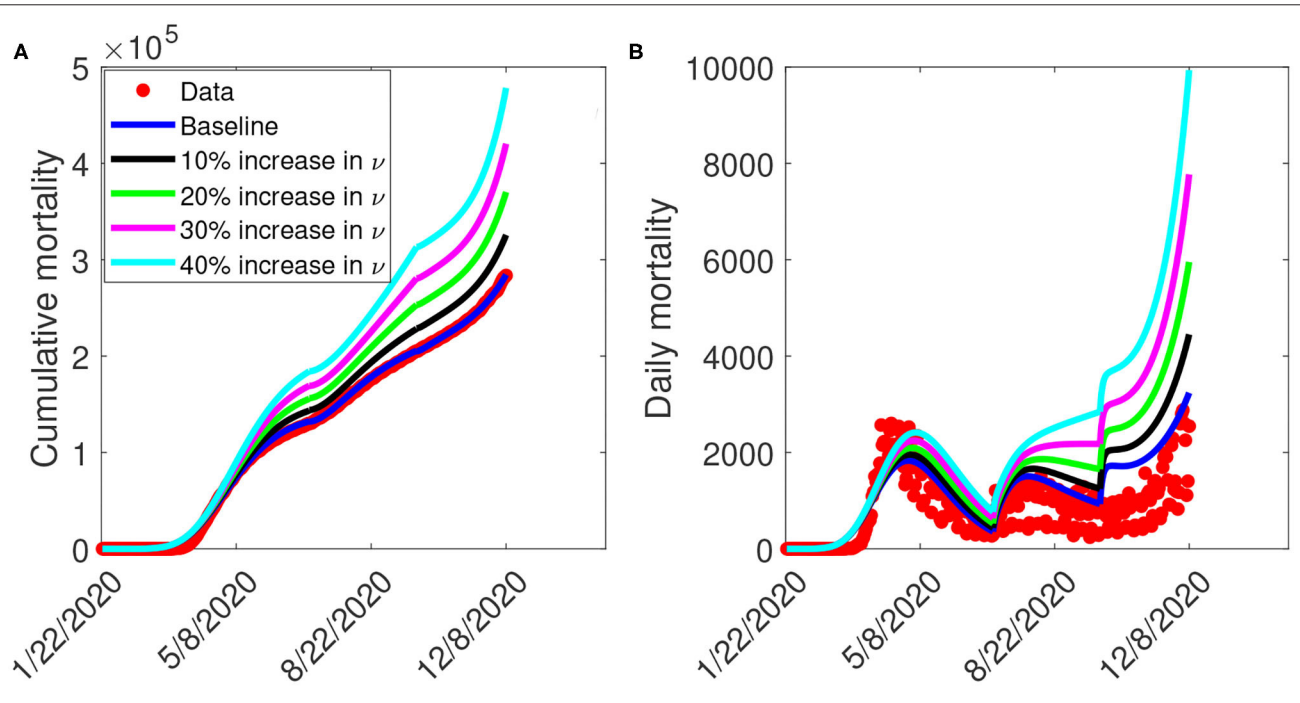

FIGURE 8 | Simulation of the model (S1), showing **(A)** cumulative mortality **(B)** daily deaths, as a function of time for various fatigue rates in the United States from January 22, 2020 to December 8, 2020. Parameter values are as given in **Table 3**.

TABLE 6 | A summary of various increase in fatigue rate.

	1/22/2020–7/5/2020		7/6/2020–8/30/2020		10/1/2020–12/8/2020	
Fatigue rate	**cum. mort.**	**daily mort.**	**cum. mort.**	**daily mort.**	**cum. mort.**	**daily mort.**
Baseline	132,000	1,829	205,600	1,505	285,100	2,808
10% increase in ν	144,100	1,955	228,800	1,657	325,700	4,451
20% increase in ν	157,400	2,094	253,200	1,860	370,300	5,953
30% increase in ν	169,100	2,247	280,600	2,139	420,400	7,771
40% increase in ν	184,200	2,412	313,200	2,513	478,300	9,935

to willingness fatigue rate in the United States for three different periods of the outbreak. Since the reproduction number $\mathcal{R}_c$ depends on the values of the initial conditions (S_u^* and S_e^*), our result shows that $\mathcal{R}_c$ can be brought to a value less than one (needed to effectively control the disease) as more individuals are being educated over time.

We also assessed the impact of public health education on the outbreak. Our simulation shows that the possibility of curtailing the spread of the virus (bringing $\mathcal{R}_c < 1$) in the United States is dependent on a very high education rate with high efficacy. The results obtained further showed the prospect of effective public health education programs in reducing both the cumulative and daily mortality of the novel coronavirus in the United States. In particular, a 10% increase in education rate from the baseline value reduces the peak mortality by 66.3% by April 20, 2020, 26.7% by July 31, 2020, and 16% by December 8, 2020, when compared to the baseline scenario. However, a 40% increase in education rate from the baseline value reduces the peak daily mortality by 96.8% by April 7, 2020, 44.5% by July 24, 2020, and 54.8% by December 8, 2020. This result is consistent with what was obtained in (3, 5, 8, 18), where the universal use of face masks greatly curtailed community transmission of COVID-19 and brought the pandemic under very effective control.

The Centers for Disease Control and Prevention (CDC) at the early stage of the pandemic recommended the use of a face mask, social-distancing in public, and proper/frequent hand washing to curtail the spread of the novel coronavirus caused by SARS-CoV-2 (3, 6, 28). Many state governments issued executive order mandating a face mask in public and restricting large gatherings of people. However, using a face mask and social-distancing in public places appears to be politicized in the United States (29). In particular, states like Georgia and Iowa barred Mayors and City Councils from introducing mask mandates, even as cases continues to rise in various counties in the state (30). While many people strictly adhere to public health measures, others passionately ignore them. We ran simulations to show the impact of loss of willingness (fatigue rate) on both the cumulative and peak daily mortality. The result indicates that non-compliance to public health measures would increase the cumulative and daily mortality in the United States. In particular, a 10% increase in fatigue rate from the baseline value increases the peak daily mortality by 6.9% by May 1, 2020, 10.1% by August 3, 2020, and 58.5% by December 8, 2020, when compared to the baseline scenario. However, a 40% increase in fatigue rate from the baseline value increases the peak daily mortality by 31.9% by April 20, 2020, 67% by July 27, 2020, and 254% by December 8, 2020, when compared to the baseline scenario. This result further supports the fact that states with less adherence to public health measures may experience more coronavirus cases and daily mortality than places where there is strict adherence (3, 5, 8).

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found at: John Hopkins University; https://github.com/CSSEGISandData/COVID-19.

AUTHOR CONTRIBUTIONS

EI, AR, RR, DI, JC, JH, MM, RP, and ZD conceived the study. EI and FA designed the model. EI collected and analyzed the data. EI and BO performed the numerical simulations. EI, AR, RR, DI, JC, JH, MM, RP, ZD, FA, BO, and LA drafted the manuscript. EI, AR, RR, DI, JC, JH, MM, RP, ZD, FA, BO, and LA revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.630974/full#supplementary-material

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MINI REVIEW
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The Unique Impact of COVID-19 on Human Gut Microbiome Research

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The coronavirus (COVID-19) pandemic has disrupted clinical trials globally, with unique implications for research into the human gut microbiome. In this mini-review, we explore the direct and indirect influences of the pandemic on the gut microbiome and how these can affect research and clinical trials. We explore the direct bidirectional relationships between the COVID-19 virus and the gut and lung microbiomes. We then consider the significant indirect effects of the pandemic, such as repeated lockdowns, increased hand hygiene, and changes to mood and diet, that could all lead to longstanding changes to the gut microbiome at an individual and a population level. Together, these changes may affect long term microbiome research, both in observational as well as in population studies, requiring urgent attention. Finally, we explore the unique implications for clinical trials using faecal microbiota transplants (FMT), which are increasingly investigated as potential treatments for a range of diseases. The pandemic introduces new barriers to participation in trials, while the direct and indirect effects laid out above can present a confounding factor. This affects recruitment and sample size, as well as study design and statistical analyses. Therefore, the potential impact of the pandemic on gut microbiome research is significant and needs to be specifically addressed by the research community and funders.

Keywords: COVID-19, gut microbiome, microbiome research, faecal microbiota transfer, clinical trials

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome (SARS) coronavirus 2 (SARS-CoV-2) and has resulted in a global pandemic, as well as subsequent restrictions of public and private life. While clinical trials worldwide have been challenged as a consequence, there are unique implications for the rapidly evolving gut microbiome research.

The gut microbiome is the vast diverse population of an estimated 100 million−100 trillion microorganisms and their genes that populate the gastrointestinal tract (1). Through complex pathways, they play essential roles in the immune and metabolic pathways, thereby influencing maintenance of health and the pathogenesis of disease (2). This complex system can be disturbed by disease or lifestyle changes, mechanisms that become highly relevant in the context of the COVID-19 pandemic.

We propose that there are direct interactions between the gut microbiome and COVID-19, as well as indirect associations through the lifestyle changes induced by lockdowns and increased hygiene (see **Figure 1**) that need to be considered for ongoing and future microbiome studies. These range from experimental and observational, to longitudinal and population studies, as well as clinical trials using Faecal Microbiota Transplantation, FMT. We highlight how recruitment to studies, representativeness of samples, as well as the collection, storage, and analysis of stool samples are affected and how these effects

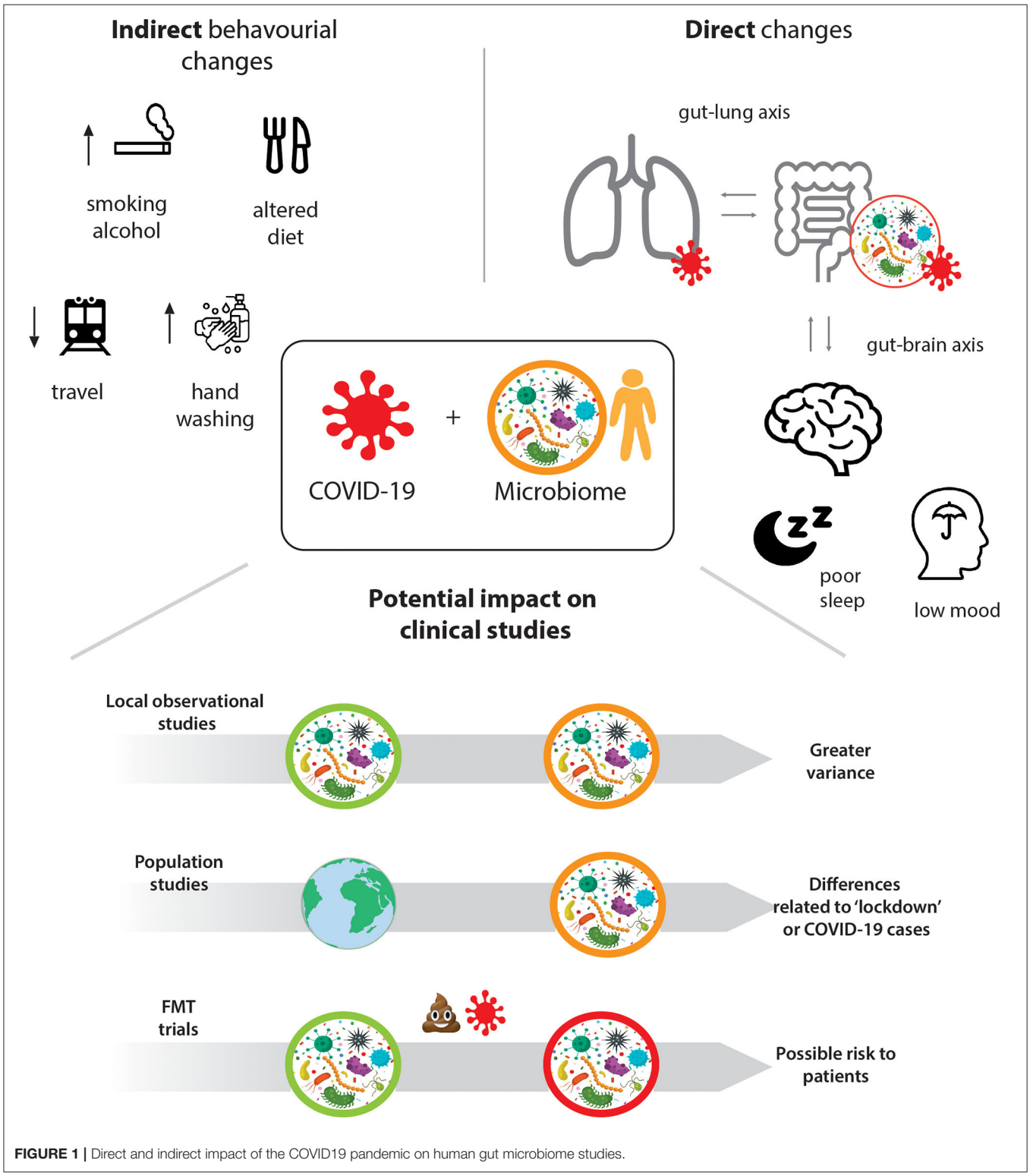

FIGURE 1 | Direct and indirect impact of the COVID19 pandemic on human gut microbiome studies.

can be mitigated through efficient study design, additional and rigorous statistical analysis, and collective effort.

DIRECT INTERACTIONS BETWEEN SARS-CoV-2 AND THE GUT MICROBIOME

Increasingly, evidence shows an interaction between COVID-19 and gut microbiota homeostasis. Interactions between a healthy host and microbiota are extensive. They involve regulation of the innate and adaptive immune system (3), as well as maintenance of gut immune homeostasis and have disease-modifying potential (4). Additionally, the role of the gut microbiota is implicated in several lung diseases, with an indication of bidirectional communication termed the "gut-lung axis" (5). While this literature is rapidly growing, we provide a high-level overview.

The gut microbiome appears to contribute to the course and severity of COVID-19. A disrupted gut microbiome (gut dysbiosis) can be understood in terms of loss of beneficial microbes, proliferation of potentially harmful microbes, and reduction of microbial diversity (6). This leads to epithelium breakdown and inflammation, which have been shown to increase levels of angiotensin-converting enzyme 2 (ACE2), the target of SARS-CoV-2. Additionally, increased gut permeability can lead to pro-inflammatory bacterial products to leak out and circulate systemically, triggering inflammatory cascades (7). A specific gut microbiota composition may predispose healthy individuals to severe COVID-19 infections; increased levels of pro-inflammatory bacterial species correlated with elevated levels of pro-inflammatory cytokines and increased disease severity. Disruptions to the bidirectional microbiome-immune system dialogue are thought to be the cause of chronic inflammatory conditions, such as ulcerative colitis, and acute systemic multi-organ dysfunction, often accompanied by abnormal cytokine production. Therefore, a disrupted gut microbiome may also contribute to increased pro-inflammatory cytokine production ("cytokine storm"), known to worsen severity of SARS-CoV-2 infection (8). Proteomic and metabolomic profiling has shown progression to severe COVID-19 infection can be predicted both in infected patients and in healthy individuals (9). Furthermore, elderly and immunocompromised populations are known to have reduced microbiota diversity. Since many of these vulnerable patients have had worse clinical outcomes for COVID-19, this strengthens the possibility that the gut microbiome is affecting clinical progression. Reduced gut microbiome diversity may therefore be useful as a predictive biomarker of COVID-19 severity (10).

SARS-CoV-2 ribonucleic acid (RNA) has been found in COVID-19 patients' faeces (11, 12), implying transmission of SARS-CoV-2 could include faecal-oral (13). Furthermore, a meta-analysis found gastrointestinal symptoms occurred in 17.6% of infected patients, and were more common in severe cases (14). Mechanisms underpinning gastrointestinal symptoms remain unclear but are thought to involve ACE2 receptors, which are highly expressed on intestinal epithelial cells (15), in particular the brush border membrane of small intestinal enterocytes. ACE2 gene expression has been shown to increase with age, potentially accounting for differential susceptibility to more severe disease (16). Xiao et al. reported significant infiltration of plasma cells and lymphocytes with interstitial oedema in a histological analysis of one patient's intestinal tract (17). ACE2 expression has been shown to be downregulated in SARS patients, leading to reduced absorption of tryptophan and decreased release of antimicrobial peptides (18). This can lead to gut dysbiosis and sustain virus survival (19). ACE2 modification of the microbiota may therefore account for the observed gastrointestinal symptoms (20).

Importantly, studies have shown that SARS-CoV-2 RNA can be detected from stool samples for up to 14 days following clinical resolution and a negative respiratory tract sample (11, 17, 21). These results align with articles reporting viral shedding in stool samples collected from patients suffering from infections caused by other human coronaviruses, such as SARS-CoV-1 and MERS-CoV (22). Although there are limitations to studies reporting SARS-CoV-2 viral shedding, including lack of detail on robustness of analytical methods implemented, and lack of clearly reported study designs, the results have clear potential implications for infection prevention control, as well as for the FMT field (see below). However, to what extent the viral RNA correlates with intact viral particles is currently unclear.

Regarding lung microbiota, only one study to date has found changes in microbiota composition in SARS-CoV-2 patients, finding more pathogenic bacterial strains compared to healthy controls (9). The SARS-CoV-2 microbial composition was similar to microbiome changes observed with other respiratory viruses such as influenza. It is not currently known how ecologically stable the gut microbiome is during COVID-19 progression. Evidence suggests an association between illness severity and microbiota diversity in mechanically ventilated patients (23); this may apply to severe COVID-19 cases requiring ventilation. Further microbiome disease-related changes have been found when considering complications of COVID-19, such as acute respiratory distress syndrome (ARDS). High-throughput sequencing of gut and lung microbiota indicate altered bacterial composition in ARDS patients (compared to patients without ARDS), these bacterial composition changes may correlate with COVID-19 outcomes (24).

INDIRECT EFFECTS OF PANDEMIC ON AN INDIVIDUAL OR POPULATIONS' MICROBIOME

The COVID-19 pandemic led many countries to enforce lockdowns and other measures to reduce virus transmission. Although these vary in form, they share the promotion of better hand hygiene, reduction in social interaction, travel limitations, and a shift towards working from home. There are several indirect effects of the pandemic that have the potential to impact the gut microbiome at a large scale.

A key message from governments across the world is the importance of hand hygiene. Indeed, there is now large-scale use of disinfectants and sanitisers across society. The contribution of the environment to the microbiome is recognised and the use of

sanitizers now and in the future may affect the microbiome of several ecological niches in humans, animals, and environments (25). While this may directly impact an individual's microbiome due to reduced exposure to environmental microbiota, its effect may also be seen at a population level. Health campaigns can result in long term behavioural changes (26), implying that the impact of regular hand sanitisation on the gut microbiome may endure long after the pandemic has resolved.

All aspects of travel have been severely restricted during the pandemic. Not only has national and international travel been reduced or even stopped, but there is a lack of household mixing. Overall, this will have lessened the exposure to a range of external environmental microbiota. Pre-pandemic, international travellers had a higher proportion of Escherichia species and increased antimicrobial resistance genes (27). While it is known that travel is a modifying factor for the adult microbiome (28), we do not know the effect of an absence of travel on the microbiome. These changes in travel habits may have impacted on the individual and population microbiome that could last into the future if international and national travel restrictions remain in place. The long-term increase in home working also needs to be carefully considered.

The impact of diet and lifestyle on the microbiome is unquestionable. The sudden lifestyle changes induced by the COVID-19 pandemic have been shown to alter eating habits, exercise and everyday behaviours (29). The increase in working from home, stockpiling groceries due to restrictions in shopping will have altered an individual's diet and therefore impacted the microbiome (30). Whether this results in greater or less diversity is unknown and may vary depending on the population itself. The psychological and emotional pandemic responses may have increased likelihood of dysfunctional or altered eating habits (31). Beyond modulation of host immunity, gut bacteria can also impact metabolic health, with specific gut bacteria changes and gut dysbiosis observed in metabolic disorders such as obesity and diabetes, known to be diet associated (32). Additionally, malnutrition is a massively concerning problem particularly for children in low and middle income countries, caused by pandemic-related financial straits, changes to food availability, and the interruption of school, healthcare, and social protection services (33, 34). Apart from the immediate increase of wasting syndrome, this will also have longer-term effects on physical and mental health through changes to the gut microbiome, setting off cascades of maladaptive metabolic responses and impaired immunity (35). Moreover, malnutrition has been suggested to negatively impact the course and outcome of a COVID-19 infection (36).

There are several non-dietary lifestyle changes that have occurred as a result of the pandemic. Exercise habits have changed both in the positive and negative manner. This is worthwhile considering since exercise can itself modulate the gut microbiome, orthogonally to changes induced by diet (37). The pandemic has increased alcohol consumption and smoking habits (38), in populations—both known to modulate the oral, lung, and gut microbiota (39, 40). A more unexpected result of the pandemic is the increase in pet ownership, which in itself can impact on the human gut microbiome (41).

The psychiatric and psychological burden of the pandemic is yet to be determined but early reports suggest a profound population level shift. The bidirectional microbiota-gut-brain axis has an active role in affecting mood and behaviour, research suggests population-level relationships between the microbiome and mental health (42). Social isolation, growing financial insecurity, and fear of the virus combined with unfamiliar social and lifestyle restrictions constitute major socioeconomic stressors that can potentially challenge individual and collective well-being and mental health, thus impacting the gut microbiome. The full psychiatric impact of the pandemic is not yet elucidated, but the implications are thought to be significant (31, 43). The pandemic has also been reported to alter sleeping patterns and quality (44), which in turn can negatively affect mood, stress, and anxiety. Additionally, the circadian rhythm is known to have a bidirectional relationship with the gut microbiome—disturbances in the gut microbiome can affect sleep regulation (41), and disturbances in circadian rhythms can alter the gut microbiome (45). This relationship has in fact been proposed as the mechanistic link between sleep disruption and metabolic syndrome, which can lead to diabetes, cardiovascular disease, and cancer (45). Therefore, the long-term consequences of COVID-19 on mental health should be considered in the light that this may implicate further microbiome interaction and additional negative health consequences for the host.

A recent review brings these changes together with the hygiene hypothesis (46), which refers to the current shift in the human microbiome composition towards lower diversity and loss of ancestral microbes that has been brought about by increased hygiene, antibiotics, and urban living (47). Taking these two processes together, the authors predict a substantial reduction of microbiome diversity which might not be able to be compensated for by the communal microbiome. We support this view and while the authors focus on opportunities for research into factors affecting the microbiome, we want to highlight the issues these changes present for ongoing and forthcoming microbiome research and clinical trials.

THE EFFECT OF COVID-19 ON MICROBIOME STUDIES

Experimental, Correlational, and Longitudinal Microbiome Studies

Due to wide-reaching effects of COVID-19 and its unique interaction with the microbiome, it is important to consider how representative of the target population participants have become. Characteristics of patients enrolled before, during and after the pandemic may now be systematically different (48). These characteristics extend at least to the microbiota composition and diversity, but there may be more subtle changes. Whilst it appears an elegant concept, the reality of defining pre-, during-, and post-pandemic periods may be prohibitively complex due to global variability in the timing of the pandemic. Additionally, national as well as individual adherence to specific measures to combat the pandemic, as outlined above, differed substantially which introduces potentially significant between-subject variability,

especially for studies recruiting globally. This could bias microbiome analysis and subsequent application of these results, particularly if studies are not designed to compare the pre-, intra-, and post-pandemic time points. There are further demographic and socioeconomic disparities to consider in light of the fact that COVID-19 is disproportionately affecting minority ethnicities and elderly populations, which already are lesser represented categories in any clinical trials (49). For longitudinal studies, for example, larger study populations may now be required due to the pandemic acting as a confounder, whereby more participants are lost to follow up due to COVID-19 infection.

Structural, clinical, physician, patient barriers to clinical trial participation during the pandemic have been already identified in the oncology field, accounting for most of patients' non-participation (50). Appraising these barriers from the perspective of microbiome trials, it is evident they can limit the resulting demographic of participants recruited. Structural barriers such as transportation, travel cost, availability of child and elderly care, changes in working patterns and employment opportunities have all been affected by the pandemic. Clinical barriers have increased during the pandemic due to narrower eligibility criteria and stricter safety concerns. There is also potential increased risk of selection bias and drop-out associated with personal aversion towards sample collection (51), due to individual awareness of the presence of viral RNA in faeces. Increased rate of comorbidities secondary to COVID-19 pandemic, together with the need to screen people for comorbidities in addition to asymptomatic infections, represents another issue.

Physician attitudes may also have changed as a result of the pandemic. Concerns about patient's safety may be heightened, meaning physicians might be hesitant to encourage enrolment in a new microbiome study. Moreover, time spent in clinical appointments for giving information about trials, discussing risks/benefits with the patient, and collecting informed consent is now severely affected due to restricted face-to-face interactions. Indeed, the generalised shift towards telemedicine for consultations may make recruitment to trials more difficult. It is also important to consider patients' and participants' attitudes may have changed, due to indirect effects of the lockdown, heightened concerns about sampling collection, and reluctance to attend clinical appointments and clinical trials in person.

Finally, sample collection and processing needs to be considered. The pandemic is unlikely to significantly interfere with most gut microbiome sample collection. The microbiome population can be investigated e.g., with 16S rRNA gene sequencing, quantitative PCR, or shotgun metagenomic sequencing. These investigative approaches analyse faecal samples; which rely on reproducible sample collection, storage and processing (52). However, there are technical issues, including safety concerns of shipping biological samples, according to the category of UN 3373 "Biological Substance, Category B." During lockdown, it is likely that sample transport, delivery, and storage have been delayed. It is advised that transportation time should be shortened as much as possible, to avoid undesirable microbial growth and decline of sample quality (53). Furthermore, faecal sample collection methods have been shown to affect the microbial community profile (52). If these have changed during the lockdown or have been adversely lengthened, this may have detrimental impacts on subsequent analysis and comparisons.

Interventional Faecal Microbiota Transfer Trials

There is growing scientific and clinical interest in the use of FMT to treat a diverse range of diseases in addition to *Clostridium difficle* infections; it is now trialled for inflammatory bowel disease, cancer and neurological disorders. FMT involves delivery or infusion of stool from a healthy donor to a patient with the disease of interest and presumed gut dysbiosis. In the UK, the MHRA has defined this as a pharmaceutical intervention and it is therefore subject to the same regulatory framework.

The presence of SARS-CoV-2 RNA in the stool of infected individuals raises the suggestion of virus transmission via FMT, the risks of which are currently unknown (13). It is also unclear if asymptomatic but serologically positive individuals can transmit the virus if viral particles are detected in faeces (54). The Food and Drug Administration (FDA) has subsequently advised additional safety protections for FMT are necessary, recommending stool used should have been originally donated before 1st December 2019. This clearly limits shelf-life of samples. Donor stool may have altered before, during and after COVID-19, which impacts trials ongoing or due to begin recruiting imminently. This suggests all stool samples should now be routinely tested and stringently screened for COVID-19 (55), which may lead to stricter exclusion criteria for stool donors. As the COVID-19 status of the donor may affect its recipient adversely, is it more acceptable to adopt a single donor approach instead of several pooled donors, who may collectively carry higher virus risk. The COVID-19 status of the recipient is also worth considering, as recipients may be rendered more susceptible if their donor is COVID-19 negative. Is it more advantageous for a donor to have had the virus previously and potentially confer immune protection via IMT, or does this conversely put the recipient more at risk of developing COVID-19? Unanswered questions remain.

In disease-focused microbiome trials, the impact of COVID-19 on the disease should be considered upon recruitment of participants receiving FMT. The disease population may now be atypical, due to COVID-19 disease interactions and interruptions of regular treatment, and likely constitute a vulnerable population. Indeed, clinical trials often focus on vulnerable populations, who are more at risk from COVID-19. Specifically, there is growing interest in bidirectional interactions of the gut microbiota and neurodegenerative diseases, which constitute a diverse population, likely to be more vulnerable. For example, patients with Multiple Sclerosis, a common patient group for FMT trials (56) and a high-risk group for COVID-19 (due to wide-spread use of immunosuppressant medication), may have shielded extensively or had reduced face-to-face check-ups due to reduction of clinical services. This altered environment may have subsequently changed their microbiome composition, raising the question of how representative the sample now is of the wider MS patient population independent

of the pandemic. The same applies to Motor Neurone Disease or Parkinson's Disease patients, whom are also increasingly the focus of FMT trials. Other risk factors for severe COVID-19 infection include hypertension, diabetes, and obesity. All are associated with changes to microbiota composition and diversity, posing the question of whether COVID-19 risk factors should be considered upon recruitment for microbiome trials.

Finally, FMT trials often utilise hazard ratios and primary endpoints, which may no longer be plausible if defined before the pandemic. Trials utilising imaging are likely to be delayed and restricted due to the pandemic. All are crucial considerations for future study analysis and interpretation. Possible mitigations include sophisticated covariate adjustments (57) for variable COVID-19 prognosis and trajectory. Double/debiased machine learning approaches may be indicated to distinguish primary outcomes and to perform formal statistical inference (58).

CONCLUSION

In summary, the COVID-19 pandemic may impact several aspects of microbiome studies that need to be explored further. The direct interaction between the gut microbiome and the severity of COVID-19 infection is a highly active research area and we look forward to the future publications in the area. Additionally, we have explored the indirect effects on individual and population microbiome composition. To reduce the impact of these changes on microbiome studies, pre-, intra-, and post-pandemic microbiome reference libraries may be necessary to exclude potential COVID-19-related confounders and to assess for stability across these fluctuating time points. Funders in this area may consider specific calls in this area and a UK or international gut microbiome consortium may be needed to coordinate efforts.

The impact for trials is an immediate concern. For trials already underway, this—in addition to the baseline shift of microbiome abundance—may mean the trial is no longer sufficiently powered. An open data policy is recommended to mitigate this, although funders should be open to additional studies being required. Finally, FMT studies must consider potential COVID-19 transmission, and may need to account for the pandemic-related microbiome compositional changes in the analysis. To avoid pandemic-related confounds when assessing microbiota interactions with non-COVID-19 diseases or interventions, large study populations will likely be most useful. Additional testing of stool donors (e.g., for COVID-19 infection or antibodies), potential confounds (e.g., shielding), and open microbiome data will undoubtedly be required. Again, additional funding may be required to specifically address these points.

In summary, "COVID-19 is with us for the long haul, a marathon that we will run for months or years to come" (59). Current studies and future work needs to specifically address and account for these potential sources of change. There are other emerging areas that need to be considered such as the effect of "Long COVID" and multiple COVID-19 vaccinations which may also impact the gut microbiome studies. We must maximise utility of data already collected and reconsider how future trials can be protected. Lessons can be learnt from rapid progress achieved by clinical trials designed to research COVID-19, exposing certain aspects of trials that can be improved universally to benefit patients, researchers and clinicians. The microbiome community must work with funders to perform the necessary research to establish the actual impact of the pandemic.

AUTHOR CONTRIBUTIONS

EB and NS devised the idea, conceived the figure and wrote and edited the manuscript. EL, EM, SB, JMc, and JMa contributed to the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: JMc holds shares in and is an employee of EnteroBiotix Limited. He is a named inventor on patents relating to the gut microbiome. JMa holds shares in Yaqrit Ltd.

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Evolution of Pathology Patterns in Persons Who Died From COVID-19 in Italy: A National Study Based on Death Certificates

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Background: In Italy, during the first epidemic wave of 2020, the peak of coronavirus disease 2019 (COVID-19) mortality was reached at the end of March. Afterward, a progressive reduction was observed until much lower figures were reached during the summer, resulting from the contained circulation of SARS-CoV-2. This study aimed to determine if and how the pathological patterns of the individuals deceased from COVID-19 changed during the phases of epidemic waves in terms of: (i) main cause of death, (ii) comorbidities, and (iii) complications related to death.

Methods: Death certificates of persons who died and tested positive for SARS-CoV-2, provided by the National Surveillance system, were coded according to ICD rev10. Deaths due to COVID-19 were defined as those in which COVID-19 was the underlying cause of death.

Results: The percentage of COVID-19 deaths varied over time. It decreased in the downward phase of the epidemic curve (76.6 vs. 88.7%). In February–April 2020, hypertensive heart disease was mentioned as a comorbidity in 18.5% of death certificates, followed by diabetes (15.9% of cases), ischemic heart disease (13.1%), and neoplasms (12.1%). In May–September, the most frequent comorbidity was neoplasms (17.3% of cases), followed by hypertensive heart disease (14.9%), diabetes (14.8%), and dementia/Alzheimer's disease (11.9%). The most mentioned complications in both periods were pneumonia and respiratory failure with a frequency far higher than any other condition (78.4% in February–April 2020 and 63.7% in May–September 2020).

Discussion: The age of patients dying from COVID-19 and their disease burden increased in the May–September 2020 period. A more serious disease burden was

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observed in this period, with a significantly higher frequency of chronic pathologies. Our study suggests better control of the virus' lethality in the second phase of the epidemic, when the health system was less burdened. Moreover, COVID-19 care protocols had been created in hospitals, and knowledge about the diagnosis and treatment of COVID-19 had improved, potentially leading to more accurate diagnosis and better treatment. All these factors may have improved survival in patients with COVID-19 and led to a shift in mortality to older, more vulnerable, and complex patients.

Keywords: SARS-CoV-2, mortality, cause of death, comorbidities, surveillance

INTRODUCTION

A key feature of the new pathogen SARS-CoV-2 is the causation of a severe disease (coronavirus disease 2019, COVID-19) characterized by a high rate of lethality. In Italy, the first ascertained COVID-19-related death was registered on February 21, 2020. Afterward, the number of deaths progressively increased, reaching a peak in March 2020 and then entering a descending phase until September 2020 with 35,457 total deaths, of which 84% occurred within May (1). From the beginning of the pandemic, Italy has been among the countries with the highest mortality from COVID-19 worldwide (2, 3).

As described elsewhere, (4) the national surveillance system managed by the Italian National Institute of Health registered all COVID-19 cases and collected death certificates of those who died, regardless of whether COVID-19 was the underlying or associated cause of death.

The first analysis of those death certificates, collected from the beginning of the pandemic until May 2020, pointed out that 88% of the recorded deaths had COVID-19 as the direct (underlying) cause of death, with slightly higher proportions among men and in the population aged 60–79 years (4).

Similar studies in other countries have reported that COVID-19 was a very significant cause of death in Europe during the first wave of the pandemic, e.g., in England, data from the Office for National Statistics showed that COVID-19 was to blame for one-quarter of all deaths in April 2020 ($n = 33{,}841$, 26.7% of the total deaths)[1] (5), and the role of comorbidities was also explored in the UK data (6).

In Italy, the peak of mortality from COVID-19 was reached on March 28 with 925 deaths. Afterward, a progressive reduction of the number of deaths was observed (7), until much lower figures were reached during summer (average of 14 deaths per day), resulting from contained virus circulation.

The present study aimed to determine, through the analysis of death certificates, whether and how the pathology patterns of individuals deceased from COVID-19 have changed during the phases of the epidemics in terms of (i) the main cause of death, (ii) co-morbidities, and (iii) complications related to death.

[1]Available online at: https://www.ons.gov.uk/peoplepopulationandcommunity/birthsdeathsandmarriages/deaths/bulletins/deathsinvolvingcovid19englandandwales/deathsoccurringinapril2020 (accessed February 18, 2020).

MATERIALS AND METHODS

The COVID-19 surveillance system managed by the Italian National Institute of Health (Istituto Superiore di Sanità; ISS) collects information on all SARS-CoV-2-positive individuals throughout the country (1, 3). In this framework, regions and autonomous provinces are required to provide death certificates of SARS-CoV-2-positive people. A joint group of researchers from the ISS and Italian National Institute of Statistics (Istat) was established to analyze these certificates.

This paper describes a comparison of the results of cause of death analysis during two different periods of the pandemic: February–April 2020, when the epidemic had a high impact on the Italian population, and May–September 2020, characterized by less effective viral circulation and reduced COVID-19 mortality.

Between February 21 and September 30, 2020, 35,457 deaths in SARS-CoV-2-positive patients were reported in Italy. Of that total, 35,440 were at least 30 years old, We focused on this age group since in the younger people the mortality is often due to other preexisting conditions.

The present analysis considered a sample of 5,662 death certificates corresponding to 16% of the above-mentioned 35,440 deaths occurring in the study periods. The sample selection is based of demographic and geographical distribution, trying to preserve a proportionality with respect to the total number of deaths. Death certificates had the following age distribution: 30–59 years: 287 in February–April and 40 May–September; 60–79: 1.850 and 214; 80 years and older 2.726 and 545. Age and sex distribution were similar to that of all COVID-19 deaths in both analyzed periods (**Figure 1**), and they were distributed all over the country.

The causes of death reported on death certificates were classified by Istat according to the International Classification of Diseases (ICD10) (8). For each death certificate, the underlying cause of death was identified, in line with the WHO definition, as "the condition initiating the train of morbid events directly leading to death." ICD10 coding was performed using the worldwide-used software Iris[2] and software's rejects were reviewed by expert coders.

All reported causes were then categorized according to their role in the death process as either of the following:

[2]Available online at: http://www.iris-institute.org (accessed February 18, 2020).

i) comorbidities: conditions "reported in the certificate different from" COVID-19 and "not caused by it." A pre-existing validated algorithm, developed for the study of multiple causes of death, was used to select comorbidities.
ii) complications of COVID-19: conditions reported by certifiers as "originating from" COVID-19.

The methodology used for selecting comorbidities and complications of COVID-19 was extensively described elsewhere (9). **Table 1** lists the analyzed conditions and the respective ICD10 codes.

Absolute and percent frequencies of certificates with Covid-19 as underlying cause, comorbidities and complications as

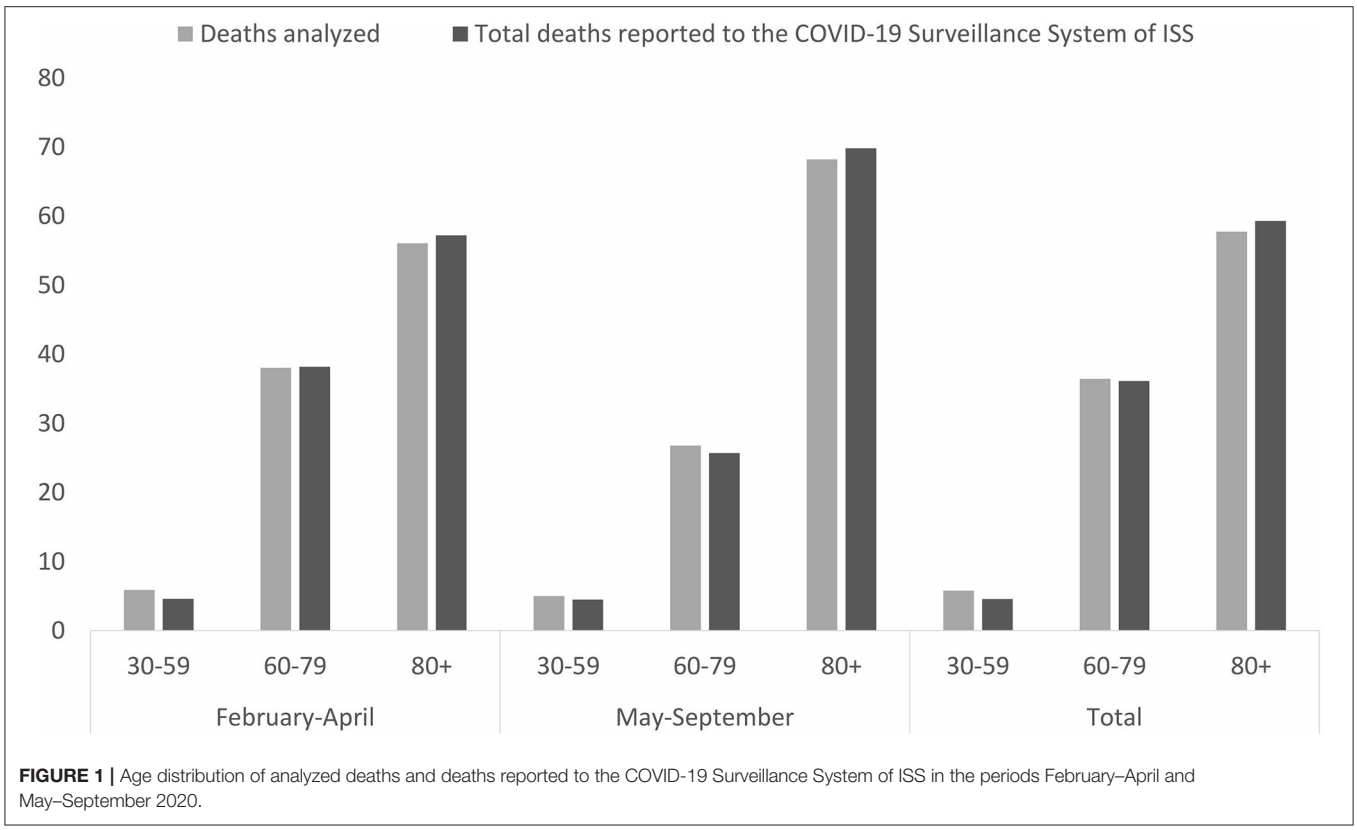

FIGURE 1 | Age distribution of analyzed deaths and deaths reported to the COVID-19 Surveillance System of ISS in the periods February–April and May–September 2020.

TABLE 1 | Comorbidities and complications of COVID-19 analyzed with ICD10 codes.

Comorbidities	ICD10 codes	Conditions reported as complications of COVID-19	ICD10 codes
Infectious and parasitic diseases	A00–B99	Sepsis, septic shock, and infections	A40–A41, A49, B25–B49, B99, R572
Neoplasms	C00–D48	Dehydration	E86
Diabetes	E10–E14	Encephalitis	G04, G93
Obesity	E66	Acute myocardial infarction	I21
Dementia and Alzheimer's	F01–F03, G30	Pulmonary embolism	I26
Hypertensive heart diseases	I10–I15	Heart complications	I50–I51
Ischemic heart disease	I20–I25	Cerebrovascular accidents	I60–I64
Cerebrovascular diseases	I60–I69	Respiratory distress and pulmonary edema	J80–J81
Other respiratory diseases	J00–J99	Intestinal complications	A00–A09, K50–K67
Other diseases of the circulatory system	I00–I09, I30–I51, I70–I99	Renal failure	N17, N19
Chronic lower respiratory diseases	J40–J47	Shock (cardiogenic)	R57 (excluding R572)
Chronic liver diseases	K70–KB		
Renal failure	N17–N19		
External causes	S00–T98		

well as the average number of comorbidities reported were computed. Logistic regression models were applied to identify which comorbidities and complications are mostly associated with the period of death (used as independent variable of the model). A separate age and sex adjusted model was performed for each comorbidity or complication. Odds ratios (ORs) with 95% confidence intervals (CIs) were computed using the period February-April as reference.

Ethical Issues

On February 27, 2020, the Italian Presidency of the Council of Ministers in compliance with the European General Data Protection Regulation (UE GDPR 2016/679) authorized the processing of personal data related to COVID-19 by the ISS and other public institutions for reasons of public interest in public health[3].

RESULTS

Of the 5,662 analyzed death certificates, 3,447 (60.9%) were for men and 2,215 (39.1%) for women; 327 (5.8%) deaths occurred in ages 30–59 years, 2,064 (36.4%) in ages 60–79 years, and 3,271 (57.8%) in ages 80 years or older. Most analyzed deaths (4,863 or 85.9% of the total) occurred in February–April 2020 (only 37 deaths occurred in February). In this period, males accounted for 63% of the total, whereas the percentage of males in May–September 2020 dropped to 48%. The age distribution was also slightly different in the two periods: average age was 79.2 (±0.1) and 81.9 (±0.4) in the first and second periods, respectively. Deceased who aged 80 years or older increased from 56% (the first period) to ∼70% (the second period) (**Figure 1**).

Table 2 shows some descriptive indicators concerning cause of death analysis. Overall, COVID-19 was the underlying cause of death in 87.2% of all deaths with differences in the two periods: 88.7 and 76.6% in the first and second periods, respectively.

The average number of comorbidities reported on death certificates was 1.28 and 1.52 in the first and second periods, and the percent of cases with comorbidities listed among causes of death increased from 71.6 to 81.6%, respectively.

Analysis of Comorbidities

Figure 2 shows the percentage of certificates reporting each comorbidity for the two periods, together with age and sex-adjusted ORs of the risk of being reported in May–September 2020 compared with those reported in February–April 2020.

The average number of comorbidities reported was 1.28 (±0.03 standard error) in the first period and 1.52 (±0.07) in the second period.

Neoplasms, hypertensive heart diseases, and diabetes were among the most frequently mentioned comorbidities with significant differences in the two periods. In February–April 2020, hypertensive heart disease was mentioned in 18.5% of death certificates, followed by diabetes (15.9% of cases), ischemic heart

. disease (13.1%), and neoplasms (12.1%). In May–September 2020, the most frequent comorbidity was neoplasms (17.3% of cases), followed by hypertensive heart disease (14.9%), diabetes (14.8%), and dementia/Alzheimer's disease (11.9%).

Also, age and sex-adjusted ORs showed that hypertensive heart diseases and obesity were significantly less frequently reported in May–September 2020 than in February–April 2020. OR was 0.75 (95% CI 0.61–0.93) for hypertensive heart diseases and 0.47 (95% CI 0.25–0.85) for obesity.

Comorbidities reported more frequently in May–September 2020 were neoplasms (OR = 1.69, 95% CI 1.37–2.08), dementia/Alzheimer's disease (OR = 1.62, 95% CI 1.26–2.09), cerebrovascular diseases (OR = 1.43, 95% CI 1.11–1.83), infectious and parasitic diseases (OR = 4.72, 95% CI 3.12–7.14), and chronic liver diseases (1.75, 95% CI 0.80–1.36).

As comorbidities more frequently observed in the second period seemed to be related to the older age of the decedents, ORs were estimated for the age stratum of 80 years and over, but no differences from the results obtained with the non-stratified models were observed.

Analysis of Complications

Figure 3 shows the percentage of each condition reported as a complication of COVID-19 together with age and sex-adjusted ORs for the association between complications and deaths occurring May–September 2020 compared with those in February–April 2020.

The most mentioned complications were pneumonia and respiratory failure in both periods with frequencies far higher than any other conditions.

Pneumonia was reported as a complication of COVID-19 in 78.4% of deaths in February–April 2020 and 63.7% in May–September 2020, with respiratory failure in 54.4 and 42.7% of cases, respectively. Moreover, these respiratory conditions are the only complications showing higher frequencies in the first period.

Among others, the following complications were found more frequently in May–September 2020: sepsis and infections unspecified (OR = 4.89, 95% CI 3.87–6.18), heart complications (OR = 1.78, 95% CI = 1.24–2.55), and pulmonary embolism (OR = 3.04, 95% CI = 1.58–5.85).

DISCUSSION

Excess mortality due to COVID-19 during the peak of the first epidemic period has been widely reported in the literature (10–14), whereas studies on the individual causes of death are scarce and based on small series. As reported by WHO, "death is defined for surveillance purposes as a death resulting from a clinically compatible illness in a probable or confirmed COVID-19 case"; however, this definition could lead to different interpretations. In fact, most countries have different approaches to determining the exact numbers of COVID-19 deaths, and few systems can produce cause of death statistics based on the underlying cause criteria in ICD10.

Our analysis performed on 5,662 death certificates has shown that among patients positive for SARS-CoV who died, the

[3]Available online at: https://www.gazzettaufficiale.it/eli/id/2020/02/28/20A01348/SG(accessed December 21, 2020)

TABLE 2 | Descriptive indicators of causes of death reported on death certificates.

	February–April	May–September	Total
Number of deaths analyzed	4,863	799	5,662
COVID-19 underlying cause of death (percentage of death certificates)	88.7	76.6	87.2
Non-COVID-19 underlying cause of death (percentage of death certificates)	11.3	23.4	12.8
Average number of comorbidities (± standard error)	1.28 ± 0.03	1.52 ± 0.07	1.31 ± 0.03
Certificates with comorbidities besides COVID19 (percentage)	71.6	81.6	73.0

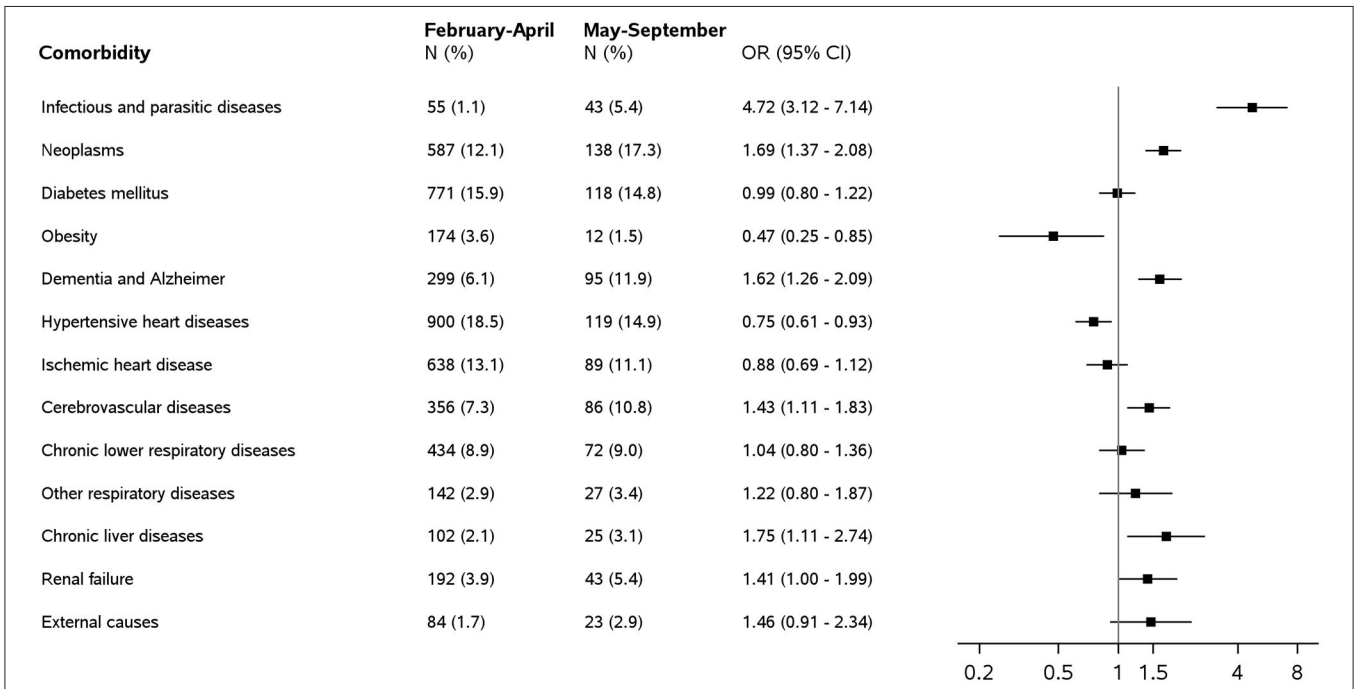

FIGURE 2 | Comorbidities on death certificates of people who tested positive for Sars-COV-2 by type and period: frequency (*N*), percentage of the total number of death certificates (%), and odds ratios (OR) with 95% confidence intervals (CIs) for the association between comorbidities and deaths occurring in May–September 2020 compared with those in February–April 2020. Analyses are adjusted by age and gender.

percentage of deaths presenting COVID-19 as the underlying cause varied over time. Particularly, it decreased in the downward phase of the epidemic curve (76.6 vs. 88.7%). Additionally, the age of patients dying with COVID-19 and their disease burden increased in the second epidemic period from May to September 2020. A more serious disease burden was observed in this period, with a significantly higher frequency of chronic pathologies such as dementia and Alzheimer's disease (15, 16), cerebrovascular diseases (17), diseases of the blood and hematopoietic system, diseases of the digestive system (18), and chronic liver diseases (19, 20).

These data suggest improved control of virus lethality or at least its mitigation in less fragile groups of the population and could be explained by different factors. First, there was less burden on the healthcare system in the second period of the epidemic. In the peak of the first period, emergency rooms, hospitals, and intensive care units were challenged by the need to simultaneously provide care to a high number of critically ill patients. Second, the organization of care improved in the second period of the epidemic. COVID-19 and non-COVID-19 care protocols and workflows were created in hospitals, community care approaches were developed, and specific diagnostic and therapeutic processes were implemented. Finally, knowledge of COVID-19 diagnosis and treatment improved over time, potentially leading to more accurate diagnosis and better treatment. All these factors may have improved survival in patients with COVID-19 and led to a shift of mortality toward older, more vulnerable, and complex patients (21).

The presence of some of these pathologies has already been dealt with in the literature, regarding COVID-19. The proportion of deaths without any contributing cause has decreased. Therefore, the reduced stress upon the national health system clearly seems to have played a major role in mitigating the impact of the pandemic. A separate focus

Complication	February-April N (%)	May-September N (%)	OR (95% CI)
Sepsis, septic shock and infections	249 (5.1)	140 (17.5)	4.89 (3.87 - 6.18)
Dehidration	20 (0.4)	15 (1.9)	4.59 (2.29 - 9.18)
Encephalitis	37 (0.8)	6 (0.8)	0.94 (0.39 - 2.26)
Acute myocardial infarction	31 (0.6)	6 (0.8)	1.39 (0.57 - 3.42)
Pulmonary embolism	33 (0.7)	14 (1.8)	3.04 (1.58 - 5.85)
Heart complications	156 (3.2)	42 (5.3)	1.78 (1.24 - 2.55)
Cerebrovascular accidents	25 (0.5)	11 (1.4)	3.10 (1.49 - 6.46)
Pneumonia	3,813 (78.4)	509 (63.7)	0.48 (0.38 - 0.59)
Respiratory distress and pulmonary edema	297 (6.1)	32 (4.0)	0.82 (0.56 - 1.20)
Respiratory failure, other respiratory symptoms	2,646 (54.4)	341 (42.7)	0.70 (0.59 - 0.83)
Intestinal complications	8 (0.2)	8 (1.0)	7.33 (2.67 - 20.17)
Renal failure	103 (2.1)	22 (2.8)	1.49 (0.92 - 2.40)
Shock (cardiogenic)	177 (3.6)	53 (6.6)	2.23 (1.61 - 3.10)

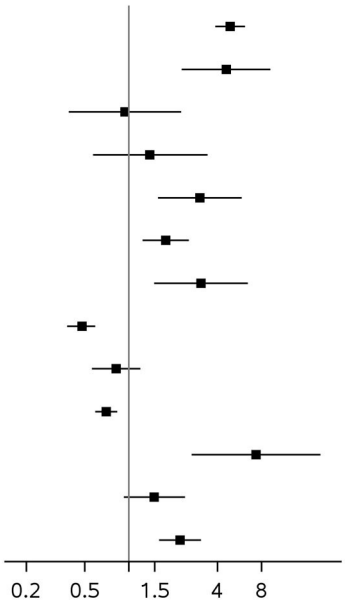

FIGURE 3 | Conditions reported as complications of COVID-19 on death certificates of people who tested positive for Sars-COV-2 by type and period: frequency (*N*), percentage of the total number of death certificates (%), and odds ratios (ORs) with 95% confidence intervals (CIs) for the association between complications and deaths occurring in May–September 2020 compared with those in February–April 2020.

should be put on infectious and parasitic diseases, which presented high odds in the second observational period. Such evidence is difficult to explain, although it may be associated with a more general organic decay in patients with severe forms of COVID-19, leading to a greater predisposition to develop infections.

Another relevant feature seems to regard the overall complications documented during the second period: during the epidemic peak, pneumonia and respiratory failure were the most relevant complications (4). These complications were significantly reduced when the outbreak was under control, thanks to prevention and mitigation progress (19). Moreover, the complications mentioned on death certificates collected during the second period were characterized by a high prevalence of sepsis, septic shock and infections, dehydration, and intestinal complications. These complications could be suggestive of a more systemic perspective of severity. Additionally, we can hypothesize that in the second epidemic period, typical COVID-19 respiratory conditions were better treated and managed, so death may have occurred when patients experienced additional non-respiratory complications that further worsened health status, leading to a negative prognosis.

A possible limitation of the present study relates to the generalizability of our findings to other countries. Italy has the oldest population in Europe and given the impact of age on the development of chronic conditions (comorbidities) it might be hypothesized that their occurrence in persons dying with COVID-19 might be higher than in other countries with a younger population. Also the older age of the Italian population can give reason to the higher COVID-19 mortality rate observed in Italy as compared with other countries. In addition, the organization of the health care systems (including the availability of hospital and intensive care unit beds) and its responsiveness to the epidemic might vary from other countries and this might explain differences among countries in mortality rate and in characteristics of persons dying with COVID-19.

Finally, the comparison between mortality observed during the ascending and descending phases of the epidemic curve has allowed us to confirm what was already observed. Mortality was strongly connected to SARS-CoV-2 circulation and, consequently, to a different pressure on the national health service.

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 on February 27, 2020, the Italian Presidency

of the Council of Ministers in compliance with the European General Data Protection Regulation (UE GDPR 2016/679) authorized the processing of personal data related to COVID-19 by the ISS and other public institutions for reasons of public interest in public health. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

FG, EG, and AM: contributed to the design of the study, performed the statistical analyses, and drafted the manuscript. SN, MP, SM, RC, LF, CO, and SS: contributed to the design of the study, and to the coding of mortality data. AC, GL, CLN, LP, and CD: contributed to the collection and management of mortality data. XA and AU: elaborated surveillance data. GO and GM: contributed to the conception and design of the study and revised the advanced draft of the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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BRIEF RESEARCH REPORT
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Validation of a Quick Flow Cytometry-Based Assay for Acute Infection Based on CD64 and CD169 Expression. New Tools for Early Diagnosis in COVID-19 Pandemic

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Objectives: Several parameters aid in deciphering between viral and bacterial infections; however, new tools should be investigated in order to reduce the time to results and proceed with an early target-therapy. Validation of a biomarker study, including CD64 and CD169 expression, was conducted.

Material and Methods: Patients with active SARS-CoV-2 infection (ACov-2), bacterial infection (ABI), healthy controls, and antiretroviral-controlled chronic HIV infection were assessed. Whole blood was stained and, after lysing no-wash protocol, acquired by flow cytometry. The median fluorescence intensity (MFI) of CD64 and CD169 was measured in granulocytes, monocytes, and lymphocytes. The CD64 MFI ratio granulocytes to lymphocytes (CD64N) and CD169 MFI ratio monocytes to lymphocytes (CD169Mo) were evaluated as biomarkers of acute bacterial and viral infection, respectively.

Results: A CD64N ratio higher than 3.3 identified patients with ABI with 83.3 and 85.9% sensitivity and specificity, with an area under the curve (AUC) of 83.5%. In contrast, other analytic or hematological parameters used in the clinic had lower AUC compared with the CD64N ratio. Moreover, a CD169Mo ratio higher than 3.3 was able to identify ACov-2 with 91.7 and 89.8 sensitivity and specificity, with the highest AUC (92.0%).

Conclusion: This work confirms the previous data of CD64N and CD169Mo ratios in an independent cohort, including controlled chronic viral HIV infection patients as biomarkers of acute bacterial and viral infections, respectively. Such an approach would benefit from quick pathogen identification for a direct-therapy with a clear application in different Health Care Units, especially during this COVID pandemic.

Keywords: biomarkers, flow cytometry, validation, CD169, CD64, SARS-CoV-2

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INTRODUCTION

The Siglec-1 or sialoadhesin (CD169) is constitutively expressed on macrophages and has been associated with anti-viral (1) and anti-tumor responses (2–4) and with regulatory function (5). The CD169 ligand is modified-sialic acid and has been involved in removing exosomes by subcapsular macrophages in lymph nodes (6). The expression of CD169 on monocytes is induced after the type-I interferon (IFN) treatment *in vitro* (7).

On the other side, the high-affinity Fc-IgG receptor (CD64) is expressed upon activation on neutrophils, macrophages, and some dendritic cell subsets (8), with different effector functions as opsonization and antibody-dependent cellular cytotoxicity (9). The IFN-gamma induces the CD64 expression on neutrophils *in vitro,* driving to a cellular immune response (7). The immune response against viral pathogens is based on recognizing both viral peptides and viral nucleic acids not present in the host. The receptors involved are Toll-like receptors, the retinoic acid-inducible gene I (RIG-I) receptor protein family, and cytoplasmic DNA receptors, with convergent pathways producing IL-1beta and type I-IFNs (IFN-alpha and IFN-beta, with anti-viral activity. By contrast, the anti-bacterial response induces a cellular response in which one of the main soluble factors is IFN-gamma.

Both CD markers were able to discern an acute bacterial from acute viral infection (10). Recently, an increased expression of CD169 on monocytes was confirmed in acute SARS-CoV-2 infection (11) that remained increased after 2 weeks from symptoms onset.

At present, in the context of the COVID-19 pandemic, one of the main problems is identifying those patients promptly with acute COVID-19 from other causes of infection, especially at admission into the hospital. Based on it, we propose the implementation of quick markers as those based on flow cytometry to classify patients. The present work aimed to validate the potential usefulness of these biomarkers in acute infectious processes, evaluate the ability to discern between acute and chronic viral infections, and assess their utility after a positive PCR to SARS-CoV-2 with time.

MATERIALS AND METHODS

A total of 83 samples from patients and healthy subjects were recruited at Marqués de Valdecilla Hospital in November 2020 after informed consent is given. The study was assessed by the regional ethic committee (CEIC, code 2020.167). Previously,

TABLE 1 | Demographic and analytical parameters in the different groups.

	HC ($n = 29$)	ABI* ($n = 12$)	HIV ($n = 18$)	ACov-2* ($n = 24$)	*p*-value
Patient sex					
Women	16 (55.2%)	8 (66.7%)	1 (5.56%)	12 (50%)	
Age (years)	60 (38–79)	81 (67–90.5)	54 (44–59)	84.5 (63.5–88.5)	$p < 0.001$
Biochemical parameters					
CRP (mg/dL)	0.4 (0.4–0.9)	11.9 (5.8–17)	0.4 (0.4–0.4)	5.4 (2.8–10.9)	$p < 0.001$
Ferritin (mg/dL)	202 (119–366)	330 (153–526)	317 (221–413)	687 (260–1,132)	NS ($p = 0.067$)
Hematological parameters					
Lymphocyte frequency (%)	23.6 (17.5–32.9)	13.4 (11.7–16.1)	38.7 (28–41.7)	13.8 (7.1–22.9)	$p < 0.001$
Neutrophil frequency (%)	64.2 (52.9–69.5)	78.3 (73.6–82.9)	50.6 (47.1–58.4)	77.7 (67.7–86.5)	$p < 0.001$
Monocyte frequency (%)	8.8 (7.5–11.7)	6.4 (4.9–8.0)	8.7 (7.9–9.7)	7.6 (4.1–9.9)	$p < 0.01$
Lymphocyte count (10^3 cells/ml)	1.4 (1.1–1.6)	1.2 (0.8–1.8)	1.9 (1.5–2.6)	0.8 (0.5–1.4)	$p < 0.001$
Neutrophil count (10^3 cells/ml)	3.3 (2.4–6.2)	5.7 (5.1–8.5)	3 (2.1–3.4)	3.9 (3.2–7.4)	$p < 0.01$
Monocyte count (10^3 cells/ml)	0.5 (0.4–0.8)	0.6 (0.4–0.7)	0.5 (0.4–0.6)	0.5 (0.2–0.6)	NS ($p = 0.320$)
Radiological test					
Pneumonia	NP	9 (75%)	NP	24 (100%)	
Microbiological analysis					
PCR SARS-CoV-2	NP	0 (0%)	NP	24 (100%)	
Legionella	NP	0 (0%)	NP	0 (0%)	
Streptococcus pneumoniae	NP	3 (25%)	NP	0 (0%)	
Staphylococcus lugdunensis	NP	1 (8.3%)	NP	0 (0%)	
Enterobacter cloacae	NP	1 (8.3%)	NP	0 (0%)	
Pseudomonas aeruginosa	NP	2 (16.7%)	NP	0 (0%)	
MRSA	NP	1 (8.3%)	NP	0 (0%)	
Staphylococcus haemolyticus	NP	1 (8.3%)	NP	0 (0%)	

The median and interquartile range (IQR) are shown. Kruskal–Wallis test has been used to determine differences between groups.

HC, Healthy Controls; ABI, Acute Bacterial Infection; HIV, Human Immunodeficiency Virus; ACov-2, Acute SARS-Cov-2 Infection; CRP, C-Reactive Protein; PCR, Polymerase Chain Reaction; MRSA, Methicillin Resistant Staphylococcus aureus; NS, Not Significant; NP, Not Performed.

ABI and ACov-2 groups details are summarized in **Supplementary Tables 1A,B.*

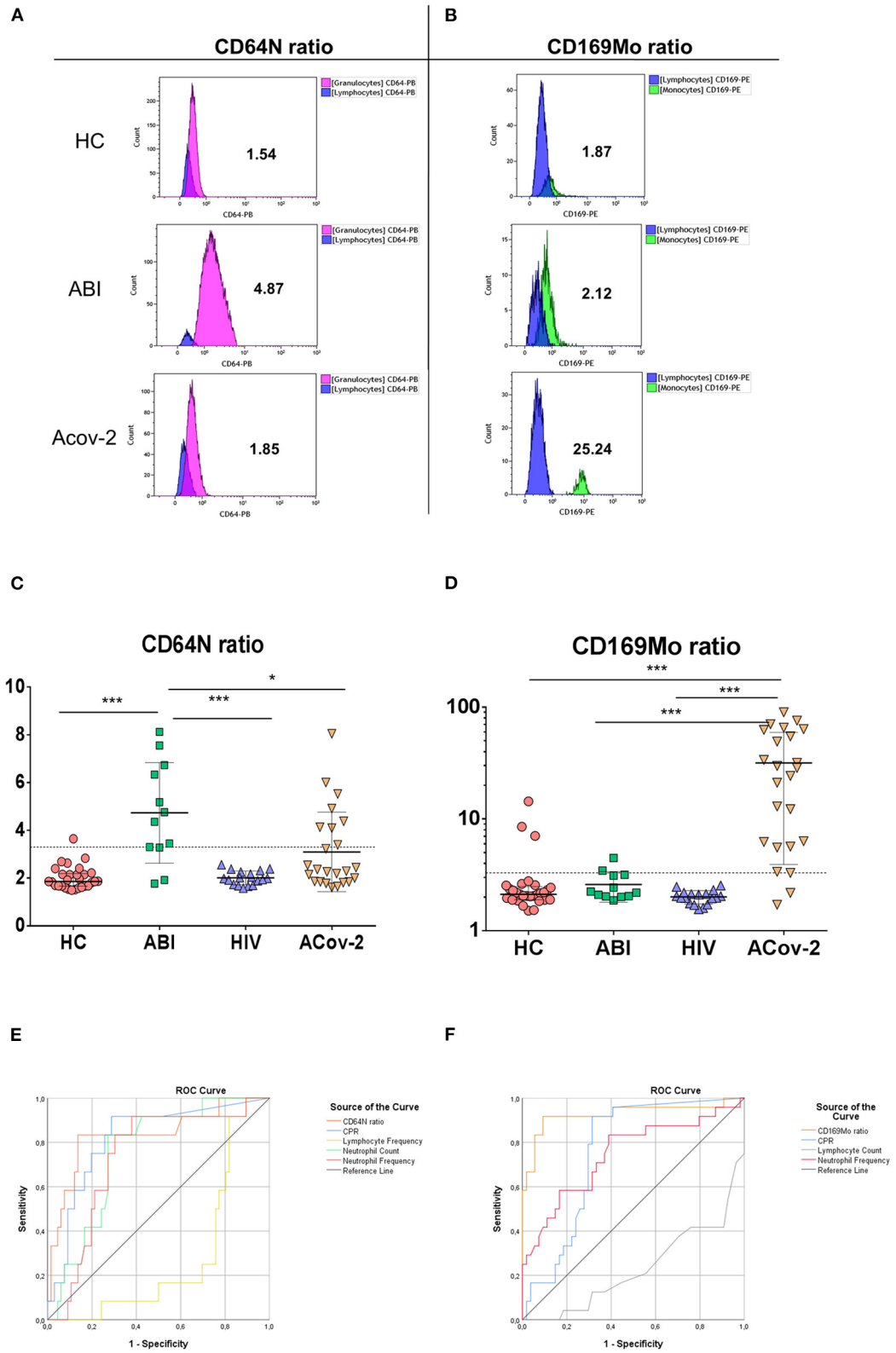

FIGURE 1 | Biomarkers of acute infection. Representative histograms of the median fluorescence intensity in neutrophil granulocytes (purple), lymphocytes (blue), and monocytes (green) of CD64 **(A)** and CD169 **(B)** expression in healthy control (HC), acute bacterial infection (ABI), and acute SARS-CoV-2 infection (ACov2) patient to

(Continued)

FIGURE 1 | calculate the ratio CD64 and CD169 as described in Material and Methods section. The ratios of CD64N **(A)** and CD169Mo are depicted **(B)**. The ratio CD64N **(C)** and CD169Mo **(D)** in HC (red light circles), ABI (green squares), chronically HIV infected patients (HIV, blue triangles), and acute SARS-CoV-2 (ACov-2, yellow triangles) are depicted. The dotted lines represent the cut-off value for calculating the Receiver Operational Characteristic (ROC) curve. The ROC curve of different parameters used to decipher acute infections are shown, C-reactive protein (blue line), neutrophil frequency (red line), absolute neutrophil count (green line), lymphocyte frequency (yellow line), lymphocyte count (gray line), CD64N ratio (orange line) **(E)**, and CD169Mo ratio (orange line) **(F)**. ***p <0.001 and *$p < 0.05$.

the utility of the CD64 and CD169 expression to differentiate between bacterial and viral infection at the emergency unit has been shown (12). In order to validate the assay in an independent cohort, several groups were established: firstly, a group of admitted patients with acute infection was recruited for the study, 12 with active bacterial infection (ABI) with confirmed bacterial pathogen isolation or recovery after antibiotic treatments and 24 patients with active SARS-CoV-2 infection (ACov-2), all the patients included in the group were tested for specific-SARS-CoV-2 polymerase chain reaction (PCR) prior admission; secondly, a group of 18 patients followed in the Infectious Disease Unit with antiretroviral-controlled chronic HIV infection (all of them without viral load at the moment of the assay) and finally, a group of healthy controls (HC) without evidence of infection were included ($n = 29$). The clinical and laboratory findings in each group are summarized in **Table 1**. The selection of HIV-infected patients with controlled infection with antiretrovirals was to determine the test's ability to discern between acute vs. controlled chronic viral infection.

All the samples were treated as potentially infectious following the current national guidelines and standard operating procedures to manage this kind of sample as suggested by World Health Organization (13).

One-step flow cytometry staining was performed. Briefly, 25 μL of EDTA whole blood sample was stained with 10 μL of the monoclonal antibody cocktail of CD169-phycoerythrin (PE)/HLA-DR-allophycocyanin (APC)/CD64-pacific blue (PB) and, simultaneously, added 500 μL of lysis buffer Optilyse® using a non-wash protocol (Beckman Coulter Inc, Brea, CA) during 15 min in the dark. The samples were acquired on 10-color flow cytometry (Navios EX) and analyzed by Kaluza Software (Beckman Coulter Inc, Brea, CA). The lymphocytes, monocytes, and neutrophils were gated based on forward and side scatter, and the median events in each population were 4,055, 1,015, and 8,944, respectively (**Supplementary Figure 1**). The median fluorescence intensity (MFI) in PE (CD169) and PB (CD64) channels was measured in each population. The MFI of CD64 expression on neutrophil to lymphocyte (CD64N) ratio and the MFI of CD169 expression on monocyte to lymphocyte (CD169Mo) ratio was calculated (**Figures 1A,B**).

Statistical Analysis

Statistical analysis was performed using SPSS software (Version 25.0, SPSS Inc, Chicago, IL, USA) and GraphPad Prism software version 6.0 (GraphPad Software, La Jolla, CA, USA). Descriptive data are presented as the median and interquartile range (IQR). Qualitative variables were shown as frequencies with percentages, and chi-square was performed to compare the data. The difference between groups was analyzed by Kruskal–Wallis analysis of ranks for non-parametric data following by the Mann–Whitney U-test and for parametric data t-Student test was used. Receiver operating characteristic (ROC) curves were used to select the optimal cut-off values of both biomarkers that better discriminate bacterial from acute viral infections. The optimal cut-off values were calculated using the Youden Index. Statistical significance was determined when $p < 0.05$ (the significant level was assigned as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

TABLE 2 | Correlation coefficients and p-values between biochemical and hematological parameters and biomarkers of acute infection.

	CD64N ratio	CD169Mo ratio
CRP	0.565***	0.359**
Ferritin	0.364*	ns
Lymphocyte frequency	−0.546***	ns
Neutrophil frequency	0.532***	0.223*
Monocyte frequency	−0.339**	ns
Lymphocyte count	−0.352**	−0.259*
Neutrophil count	0.431**	ns

*$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.*
CRP, C-Reactive Protein; ns, not significant.

RESULTS

The CD64N Ratio Is Increased in Acute Bacterial Infection

The median value of CD64N ratio was statistically increased in patients with acute bacterial infection (ABI) 4.56 IQR (3.29–6.53) vs. 1.86 (1.67–2.16) in HC group vs. 1.99 (1.74–2.26) in HIV group vs. 2.33 (1.85–4.11) inACov-2 group ($p < 0.001$, $p < 0.001$, $p = 0.024$), respectively (**Figure 1C**).

The CD64N ratio was statistically correlated with the different parameters used in clinic routine (**Table 2**) in the assessment of acute bacterial infections [WBC, frequency of neutrophils and lymphocytes, percentage of immature neutrophils, and C-reactive protein (CRP)] (for more details see **Supplementary Table 1A**).

In order to assess the best parameter to discriminate between acute bacterial infections, a receiver operating characteristic curve (ROC) for each parameter was calculated. Within all parameters studied, CD64N ratio had the highest area under the curve (AUC): 83.5% compared with 81.0, 75.5, 73.0, and 72.0% in C-Reactive protein, neutrophil counts, frequency of neutrophils, and frequency of lymphocytes (**Figure 1E**). A CD64N ratio of 3.3 was able to detect acute bacterial infection with 83.3

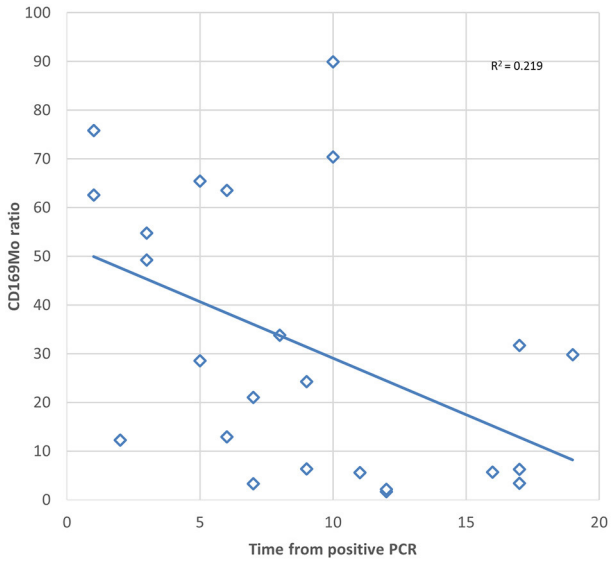

FIGURE 2 | Scatter plot showing the correlation between CD169Mo ratio and time in days from positive PCR of SARS-CoV-2.

and 85.9% sensitivity and specificity, respectively. Remarkably, seven patients included in the ACov-2 group also had an increased CD64N ratio, and two of them had a confirmed bacterial coinfection.

The CD169Mo Ratio Is Increased in Acute Viral Infection by SARS-CoV-2

The median value of CD169Mo ratio was statistically increased in patients with acute viral infection 26.41 (5.94–58.65) vs. 2.12 (1.9–2.43) in HC group vs. 2.01 (1.76–2.13) in HIV group vs. 2.22 (2.04–3.14) in ABI group ($p < 0.001$, $p < 0.001$, $p < 0.001$), respectively (**Figure 1D**). The CD169Mo ratio was significantly correlated with different clinic routine parameters in assessing acute viral infections (**Table 2**) (For more details, see **Supplementary Table 1B**).

In order to assess the best cut-off value to discriminate between acute viral infections, the receiver operator characteristic curve (ROC) for each parameter was also calculated. CD169Mo had the highest AUC 92.0%, within all parameters studied compared with 77.0, 75.8, 75.4, and 74.5% of lymphocyte counts, CRP, serum ferritin, and neutrophil frequency, respectively (**Figure 1F**). A ratio of CD169Mo of 3.3 was able to detect acute viral infection with 91.7 and 89.8 sensitivity and specificity, respectively (details of positive predictive value and negative predictive value and likelihood of all parameters associated with acute infection are summarized in **Supplementary Table 2**).

In order to assess the duration of the usefulness of the CD169Mo ratio after a positive PCR, a Spearman correlation was tested, and a significant negative association between the CD169Mo ratio and time from positive PCR was observed $p = 0.021$ (**Figure 2**).

DISCUSSION

The early identification of patients with acute SARS-CoV-2 infection at hospital admission is becoming of increased interest in order to identify those patients with COVID-19 disease. The PCR remains the gold standard test but with the pitfall of delayed time to results that usually takes no <4 h. This validation work confirms the one-step flow cytometry-based assay in <1 h as a suitable test to detect acute viral infection by SARS-CoV-2 and ABI. Both CD64N and CD169Mo ratios were able to detect ACov-2 and ABI better than the current biochemical parameters used in clinical routine to discern between acute viral or bacterial infection. The present work shows a cut-off value of 3.3 in both biomarkers, very similar to that described previously (14). However, this assay is not specific to ACov-2 infection since CD169Mo was increased in acute parainfluenza, human respiratory syncytial, and C-hepatitis virus infections at emergency units (12, 15).

Our validation cohort included a group of chronically infected HIV patients controlled by antiretroviral treatment and without HIV viral load at the assay moment. In this group, both biomarker values were comparable with the observed in the HC group, pointing to the exclusive role of CD169Mo ratio as a marker for acute viral infection, which normalizes after infection chronicity. This assay has the potential to detect acute viral and bacterial coinfection when both CD64N and CD169Mo are increased (12), as observed in two cases of ACov-2 in our cohort.

On the other hand, one patient of the ABI group had a CD64N ratio below the cut-off, and this value could be due to the prolonged time from the first bacterial isolation to the assay (18 days). However, this patient presented several different isolations during admission, with antibiotic treatment that could interfere with the CD64N ratio. In the same direction, two patients included in the ACov-2 group presented a low CD169Mo ratio, and in both cases, the positive PCR was detected 12 days before the assay. This observation fits with the decrease in the CD169Mo ratio after several days from positive PCR. Although this assay is intended for the acute phase of infection, further investigations should point to the dynamic of CD64N and CD169Mo expression with the clearance of pathogens to validate their usefulness in later steps of infections. In this study, a weak correlation was observed between the expression of CD169 from positive PCR, which may be due to lower activation of monocytes together with a greater clearance of the virus. A longitudinal study would be necessary to confirm this correlation.

An increased expression of CD169Mo in anti-tumoral response (16, 17) and children with active systemic lupus erythematosus has been described (18). Therefore, in these patient profiles, the CD169Mo ratio can be skewed and should be considered as potential confounders. In addition, the value of the CD169Mo ratio in patients with another autoimmune disease is still unknown.

These observations strongly suggest that the CD64N and CD169Mo ratio are robust biomarkers of acute infections, but the time after treatment reduces the capability to differentiate from bacterial or viral infection. In the current pandemic era, this assay could be used to identify patients with ACov-2 suspicion, but

further SARS-Cov-2 PCR is mandatory to confirm the pathogen involved in CD169Mo expression rise.

A limitation of this study is that the number of patients included with bacterial isolation is scarce. We have included one patient in whom the isolation was confirmed 6 days after the analysis, and 4 patients recovered after antibiotic treatment. For this reason, we did not perform further correlation analysis with the CD64N ratio. Recently, it has been demonstrated the potential role of CD64 expression in patients with sepsis in pediatric intensive care units (19, 20).

In conclusion, this study confirms the previous data of CD64N ratio and CD169Mo in an independent cohort and including controlled chronic viral HIV infection as a potential biomarker able to discern between acute bacterial and viral infections. These biomarkers would have a clear application in different Health Care Units and would benefit from a quick pathogen identification for a direct therapy.

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 CEIC, code 2020.167. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DS and ML conceptualization, supervision and review of the draft manuscript. AC-B and DS wrote the manuscript. ML and MCF-A searched for funding. AC-B, DS, and MG-L: statistical analysis. AR-B, SG, MR, EG, and JI: methodology and data management. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.655785/full#supplementary-material

Supplementary Figure 1 | Gating strategies are displayed. The median (range) of granulocytes events (pink) is 8,944 (1,229–49,726) of monocytes (green) are 1,015 (137–3,562) and 4,055 (7,45–234,450) for lymphocytes (blue).

Supplementary Table 1A | Main characteristics of ABI group patients. ID, identification; ABI, Acute Bacterial Infection; PCR, Polymerase Chain Reaction; CRP, C-Reactive Protein; N/L, neutrophils/lymphocytes; Mo/L, monocytes/lymphocytes; RTI, Respiratory Tract Infection; UTI, Urinary Tract Infection.

Supplementary Table 1B | Main characteristics of ACov2 group patients. ID, identification; ACov2, Acute SARS-CoV-2 Infection; PCR, Polymerase Chain Reaction; CRP, C-Reactive Protein; N/L, neutrophils/lymphocytes; Mo/L, monocytes/lymphocytes; Severity, mild (without oxygen-therapy), moderate (conventional oxygen therapy) and severe (high-flow nasal cannula oxygenation device).

Supplementary Table 2 | Evaluation indexes of diagnostic tools in ABI and ACoV2 groups. CRP, C-Reactive Protein; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; ABI, Acute Bacterial Infection; ACoV2, Acute SARS-CoV-2 Infection; ROC, Receiver Operational Characteristic (ROC). The variables included in **Supplementary Table 2** were those included in each ROC analysis (ABI and ACoV2).

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Comparison of Clinical Features and Outcomes of Medically Attended COVID-19 and Influenza Patients in a Defined Population in the 2020 Respiratory Virus Season

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The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-COV-2), which is causing the coronavirus disease-2019 (COVID-19) pandemic, poses a global health threat. However, it is easy to confuse COVID-19 with seasonal influenza in preliminary clinical diagnosis. In this study, the differences between influenza and COVID-19 in epidemiological features, clinical manifestations, comorbidities and pathogen biology were comprehensively compared and analyzed. SARS-CoV-2 causes a higher proportion of pneumonia (90.67 vs. 17.07%) and acute respiratory distress syndrome (12.00 vs. 0%) than influenza A virus. The proportion of leukopenia for influenza patients was 31.71% compared with 12.00% for COVID-19 patients ($P = 0.0096$). The creatinine and creatine kinase were significantly elevated when there were COVID-19 patients. The basic reproductive number (R_0) for SARS-CoV-2 is 2.38 compared with 1.28 for seasonal influenza A virus. The mutation rate of SARS-CoV-2 ranges from 1.12×10^{-3} to 6.25×10^{-3}, while seasonal influenza virus has a lower evolutionary rate ($0.60\text{-}2.00 \times 10^{-6}$). Overall, this study compared the clinical features and outcomes of medically attended COVID-19 and influenza patients. In addition, the S477N and N439K mutations on spike may affect the affinity with receptor ACE2. This study will contribute to COVID-19 control and epidemic surveillance in the future.

Keywords: influenza, COVID-19, epidemic, comparison, adaptive mutation

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INTRODUCTION

An increasing number of COVID-19 cases have caused a global health burden due to the rapid transmission throughout the human community. The pathogen SARS-COV-2 causes respiratory system and severe systemic symptoms through respiratory tract infection (1, 2). As of February 17th, 2021, more than 100 million COVID-19 cases were confirmed, and more than 2 million deaths had occurred (3). Although many countries are developing vaccines and racing to run clinical trials

(4), there are still many unresolved questions regarding viral invasion, pathogenesis and clinical features. In particular, the relationship between mutations and pathogenicity or transmissibility remains unknown.

The seasonal influenza epidemic is caused by strains of influenza virus, including two influenza A viruses (H1N1 and H3N2) and one influenza B virus. There have been four documented influenza pandemics in the past 100 years (in 1918, 1957, 1968, and 2009) (5). The influenza case fatality rates of the 1918 and 2009 H1N1 pandemics ranged from 0.1% to 2.5% (6, 7). The very important issue is the emergence of a new subtype or strain through uncontrollable and unpredictable mutations or antigenic drift and shift (8). The same situation may also occur with SARS-COV-2. Some studies have reported spike mutations and attempted to clarify the transmissibility change associated with new mutations (9, 10).

Currently, SARS-COV-2 remains a lasting threat to public health, causing mild respiratory system disease similar to that caused by seasonal influenza virus. To investigate the COVID-19 clinical progression, prognosis and SARS-COV-2 epidemic trends, we systematically contrasted the proportions or values of epidemiologic characteristics, clinical features, blood abnormalities, progressive symptoms, and hospitalization rates between influenza and COVID-19. Seasonal influenza was prevalent in winter, and COVID-19 also emerged in the last winter. The parameters of coinfection patients who presented with both COVID-19 and flu were also compared with those of COVID-19 patients. In addition, the mutants of influenza virus HA and SARS-COV-2 spike causing the epidemic were resolved and discussed. We expect that this comparative research will aid pandemic control and be beneficial to the clinical diagnosis of SARS-COV-2.

MATERIALS AND METHODS

Ethics

This study was approved by the Institutional Review Committee of Shiyan Renmin Hospital of Hubei University of Medicine, and no informed consent was required. This study was designed as a retrospective case analysis, with no patients directly involved in the study design, question setting, or outcome evaluation.

Study Population

From January 23, 2020 to February 27, 2020, the confirmed COVID-19 patients and influenza patients on outpatient visits and admission to Shiyan Renmin Hospital were uniformly collected. The clinical data of 75 COVID-19 patients, 41 influenza patients and 23 coinfection patients were retrospectively collected at the same time. All patients underwent chest computed tomography (CT) scanning at admission. Flu patients were infected with the seasonal influenza A or B virus, COVID-19 patients were infected with SARS-COV-2, and coinfection patients were infected with both SARS-COV-2 and influenza A or B virus or parainfluenza virus (PIV). The clinical subtype for COVID-19 was screened according to the Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia (Trial Version 7) which was released by the National Health Commission & National Administration of Traditional Chinese Medicine (11). Influenza diagnosis and treatment plan (2019 version) (12) was used for screening the flu patients.

Laboratory Examinations

After admission to the hospital, specimens from all patients were screened for SARS-COV-2 using throat swabs. The positive result of real-time reverse transcription-polymerase chain reaction (RT-PCR) confirmed the infection. Other respiratory pathogens were detected by indirect immunofluorescent assay using IgM antibodies, including *Legionella pneumophila*, *Coxiella burnetii*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, adenovirus (AdV), influenza A virus, influenza B virus, parainfluenza virus (PIV type 1+2+3), and respiratory syncytial virus (RSV). Sputum or body fluids were also examined at admission for other possible infections with bacteria or fungi.

Data Collection

The epidemiological data, symptoms, laboratory abnormalities on admission, clinical treatments, and outcomes were recorded and collected. The examination of WBC, influenza virus antigen, joint test of nine respiratory tract pathogens, procalcitonin (PCT), hypersensitive C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), blood gas analysis, and chest computed tomography (CT) were completed in the hospital. The above information was extracted from the case database of Shiyan Renmin Hospital.

Sequence Alignment and Mutation Analysis

The genome sequences of SARS-COV-2 were downloaded from the GISAID database (13). Multiple sequences were aligned using MAGE-X software (version 10.0.5), phylogenetic analysis was completed through multiple comparisons using neighbor-joining algorithms, and the number of bootstraps was 500. All spike mutations were referenced to the website of the SARS-COV-2 Sequence Analysis pipeline (14).

Dynamic Analysis of HA and Spike Structure

To analyze the dynamic model of the spike protein, we used the PDB file 6VXX. The model 1RUZ was used to validate the HA structure of influenza. PyMOL software (version 2.3.2) was used to map the S477N and N439K domain onto the 3D structure. MM/GBVI was used to calculate the binding free energy of each conformation with receptor ACE2 (15), ACE2 (PDB ID: 1R42) was used for computation.

Statistical Analysis

SPSS 22.0 software (SPSS, Inc. Chicago, USA) was used for statistical analysis of the obtained data, and the measurement data are shown as the medians and interquartile ranges (IQRs), which were compared with the Mann-Whitney *U*-test. The categorical variables are shown as numbers (%) and were compared with the χ^2 test or Fisher's exact test.

TABLE 1 | Demographic and epidemiologic characteristics of flu, COVID-19 and coinfection patients.

	Flu (Influenza A/B, $n = 41$)	COVID-19 ($n = 75$)	Coinfection (Influenza A/B, PIV $n = 23$)
Age	48.21 ± 10.30	47.93 ± 10.55	54.75 ± 9.24
Gender ratio (M/F)	1.05	1.27	1.3
Incubation period (days)	1.4 (1.3, 1.5)	5.1 (4.5, 5.8)	4.9 (4.1, 5.9)
Severity of illness	3 (7.32%)	9 (12.00%)	5 (21.74%)
Clear epidemiology history	10 (24.39%)	59 (78.67%)	19 (82.61%)

RESULTS

Epidemiological and Population Characteristics of COVID-19 and Influenza Patients

From January 23, 2020 to February 27, 2020, a total of 141 suspected patients were admitted to the isolation ward of our hospital, of which 75 were diagnosed with COVID-19 pneumonia, and 23 patients were determined to be coinfected with influenza A or B or parainfluenza virus. As shown in **Table 1**, the median age of COVID-19 patients was 47.93 years old, and there were 42 males and 33 females, which included 66 mild patients and 9 critically ill patients. The male/female ratio of influenza patients was 1.05, while that of COVID-19 patients was 1.27. COVID-19 patients with SARS-COV-2 infection had a longer incubation period of 5.1 days, while flu patients developed symptoms after 1.4 days. Perhaps the longer incubation period of SARS-COV-2 is adverse to the epidemiological investigations, as 18–21% of COVID-19 patients did not have a clear infection path. There may have been more asymptomatic patients among the flu patients, as only 24% of patients could be traced to an infection source. COVID-19 patients had a severe rate of 12%, and coinfection patients had a higher severe proportion (21.74%).

Clinical Characteristics and Laboratory Tests of Patients in Different Groups

The clinical characteristics of the patients are shown in **Table 2**. Some COVID-19 patients, flu patients and coinfection patients had the common manifestation of fever and cough. A total of 69.33 and 9.33% of COVID-19 patients had fever and nasal obstruction and rhinorrhea, respectively, which were all lower rates than those for the flu patients ($P = 0.0263$, $P = 0.0083$). Fatigue reported in 36% of COVID-19 patients was higher than the rate in flu patients ($P = 0.0023$). Patients coinfected with SARS-COV-2 and influenza virus or PIV had a lower cough rate than COVID-19 patients ($P = 0.0310$). Other symptoms included headache, nausea, vomiting, and diarrhea. For routine blood tests, a higher proportion of flu patients developed leukopenia than COVID-19 patients (31.71 vs. 12.00%, $P = 0.0096$), while coinfection patients had a decreased proportion (4.35%) of leukopenia relative to that of the COVID-19 patients, but there was no significant difference ($P = 0.2889$). A total of 70.67% of COVID-19 patients had increased CRP, sharing a median of 13.63 mg/L, which was higher than that of the flu group ($P = 0.0372$). The median of an increased proportion of ESR was not significantly different among the three groups. The increase of ESR (48.00 vs. 53.66%) and D-dimer (37.33 vs. 36.58%) was a common phenomenon in the COVID-19 and flu patients. The creatinine was significantly increased in the COVID-19 group than flu group ($P = 0.0239$), while creatine kinase was elevated when there were COVID-19 patients (16.00% or 30.43 vs. 2.44%).

Radiological Finding and Clinical Outcome Comparison

Among the 75 confirmed COVID-19 patients, 45 showed typical signs of viral pneumonia on chest CT. The other 23 patients showed lung infections but no typical signs on chest CT. In total, 90.67% of COVID-19 patients developed pneumonia, which is much higher than the proportion of influenza patients with pneumonia (17.07%, $P < 0.0001$) (**Table 3**), and this proportion increased to 95.65% in coinfection patients. The lesions for COVID-19 pneumonia were mostly in the subpleural area, with patchy or lumpy appearance (**Figure 1A**). The density of the lesions was commonly ground-glass opacities (GGOs), and there were real changes and thickened leaflet intervals. Influenza pneumonia showed GGOs with fewer solid components (**Figure 1B**). Twenty-one COVID-19 patients (28%) were diagnosed with underlying diseases (**Table 3**), and the top three were hypertension (13.33%), diabetes (8%), and coronary heart disease (5.33%). The proportion and order of underlying diseases in the flu group and coinfection group were consistent. A total of 18.67% of COVID-19 patients developed complications of liver injury, which was a higher rate than that of flu patients (4.88%, $P = 0.0395$). No flu patient had a complication of acute respiratory distress syndrome (ARDS), with a proportion of 12% in the COVID-19 group ($P = 0.0209$). In the coinfection group, ARDS was the most common complication (17.39%), followed by liver injury (13.04%) and kidney injury (8.70%).

Hospitalization and Treatment for Patients in Different Groups

The median hospitalization period of COVID-19 patients was 19 days, while flu patients after admission required only 4 days to discharge ($P < 0.0001$) (**Table 4**). According to the Diagnosis and Treatment Protocol for COVID-19 (11), all COVID-19 patients received broad-spectrum antiviral treatment, including interferon-α sprays, arbidol hydrochloride, or lopinavir and ritonavir (16). Antibiotics, including cephalosporins, carbapenem, quinolones and so on, and antifungal drugs were used when appropriate. By the end of the study period, all patients were being treated in the hospital, and 98.67% of COVID-19 patients were cured and survived; only one patient died (1.33%). All flu and coinfection patients were cured and discharged.

Pathogen Comparisons for Influenza Virus, SARS-COV and SARS-COV-2

Influenza virus belongs to the family *Orthomyxoviridae*, whose genome contains eight RNA segments. SARS-COV and

TABLE 2 | Clinical characteristics and selected laboratory abnormalities of flu, COVID-19 and coinfection patients.

	Influenza ($n = 41$)	COVID-19 ($n = 75$)[a]	Coinfection (Influenza A/B, $n = 23$)[b]	*P* value[a, b]
Clinical characteristics				
Fever(≥ 37.3°C)	36 (87.80%)	52 (69.33%)	15 (65.22%)	0.0263, 0.7989
Cough	21 (51.22%)	49 (65.33%)	9 (39.13%)	0.1662, 0.0310
Nasal obstruction and rhinorrhea	12 (29.27%)	7 (9.33%)	5 (21.74%)	0.0083, 0.1454
Sore throat	4 (9.76%)	14 (18.67%)	4 (17.39%)	0.2855, 1.0000
Shortness of breath and chest tightness	3 (7.32%)	9 (12.00%)	2 (8.70%)	0.5353, 1.0000
Fatigue	4 (9.76%)	27 (36.00%)	6 (26.09%)	0.0023, 0.3788
Diarrhea and vomiting	3 (7.32%)	7 (9.33%)	2 (8.70%)	1.0000, 1.0000
Blood routine				
WBC count($\times 10^9$/L)	5.18 (3.71, 8.12)	5.52 (4.19, 7.24)	5.34 (4.51, 6.23)	0.2112, 0.6358
($\leq 3.5 \times 10^9$/L)	13 (31.71%)	9 (12.00%)	1 (4.35%)	0.0096, 0.2889
Lymphocyte count ($\times 10^9$/L)	1.20 (0.83, 1.62)	1.23 (0.90, 1.59)	1.39 (1.05, 1.76)	0.9678, 0.0627
($\leq 1.1 \times 109$/L)	15 (36.59%)	20 (26.67%)	5 (21.74%)	0.2659, 0.6353
CRP (mg/L)	7.43 (4.26, 17.40)	13.63 (3.93, 26.60)	14.82 (4.87, 28.41)	0.0617, 0.2026
(≥5mg/L)	21 (51.22%)	53 (70.67%)	14 (60.87%)	0.0372, 0.3768
ESR(mm/h)	18 (7.50, 33.50)	17 (7.00, 31.50)	21.5 (9.50, 41.00)	0.4271, 0.5078
(≥15 mm/h)	22 (53.66%)	36 (48.00%)	12 (52.17%)	0.6979, 0.7261
(D-dimer mg/L)	0.17 (0.10, 0.28)	0.25 (0.14, 0.34)	0.28 (0.18, 0.37)	0.0046, 0.1246
(≥0.25 mg/L)	15 (36.58%)	28 (37.33%)	12 (52.17%)	0.4311, 0.0018
Creatine kinase (U/L)	104 (66.30, 149.60)	118 (78.50, 158.30)	115 (62.40, 175.20)	0.1157, 0.8316
(≥171 U/L)	1 (2.44%)	12 (16%)	7 (30.43%)	0.0013, 0.0027
BUN (mmol/L)	4.43 (3.69, 5.26)	4.38 (3.52, 5.21)	4.50 (3.76, 5.03)	0.9550, 0.5377
Creatinine (μmol/L)	86.32 (78.90, 92.90)	90.94 (74.70, 108.10)	97.99 (76.70, 118.04)	0.0008, 0.1659
(≥104μmol/L)	3 (7.32%)	18 (24%)	6 (26.09%)	0.0239, 0.5840

The data was shown as n (%) or median (IQR).
[a]*COVID-19 vs. Influenza group.*
[b]*Coinfection vs. COVID-19 group.*

TABLE 3 | Underlying diseases and progressive symptoms of flu, COVID-19 and coinfection patients.

	Influenza ($n = 41$)	COVID-19 ($n = 75$)[a]	Coinfection (Influenza A/B, $n = 23$)[b]	*P* value[a,b]
Underlying diseases	11 (26.83%)	21 (28.00%)	7 (30.43%)	1.0000, 0.7981
Hypertension	4 (9.76%)	10 (13.33%)	3 (13.04%)	0.7675, 1.0000
Diabetes	3 (7.32%)	6 (8.00%)	2 (8.70%)	1.0000, 1.0000
Coronary heart disease	3 (7.32%)	4 (5.33%)	1 (4.35%)	0.6965, 1.0000
Progressive symptoms				
Pneumonia	7 (17.07%)	68 (90.67%)	22 (95.65%)	< 0.0001, 0.4449
Acute respiratory distress syndrome	0	9 (12.00%)	4 (17.39%)	0.0209, 0.5049
Shock	0	1 (1.33%)	0	1.0000, 1.0000
Liver injury	2 (4.88%)	14 (18.67%)	3 (13.04%)	0.0395, 0.5333
Kidney injury	0	6 (8.00%)	2 (8.70%)	0.0629, 0.9151

The data was shown as n (%).
[a]*COVID-19 vs. Influenza group.*
[b]*Coinfection vs. COVID-19 group.*

SARS-COV-2 belong to the β-coronavirus (CoV) genus in the *Coronaviridae* family (17). SARS-COV-2 showed a high nucleotide sequence identity (79.5%) with SARS-COV (18). Recent reports have shown that SARS-COV-2 enters susceptible cells through binding with the receptor angiotensin-converting enzyme 2 (ACE2), which is the same as SARS-COV (19–21). All ages of people are susceptible to influenza A virus (22), while SARS-COV and SARS-COV-2 primarily infect adults (23).

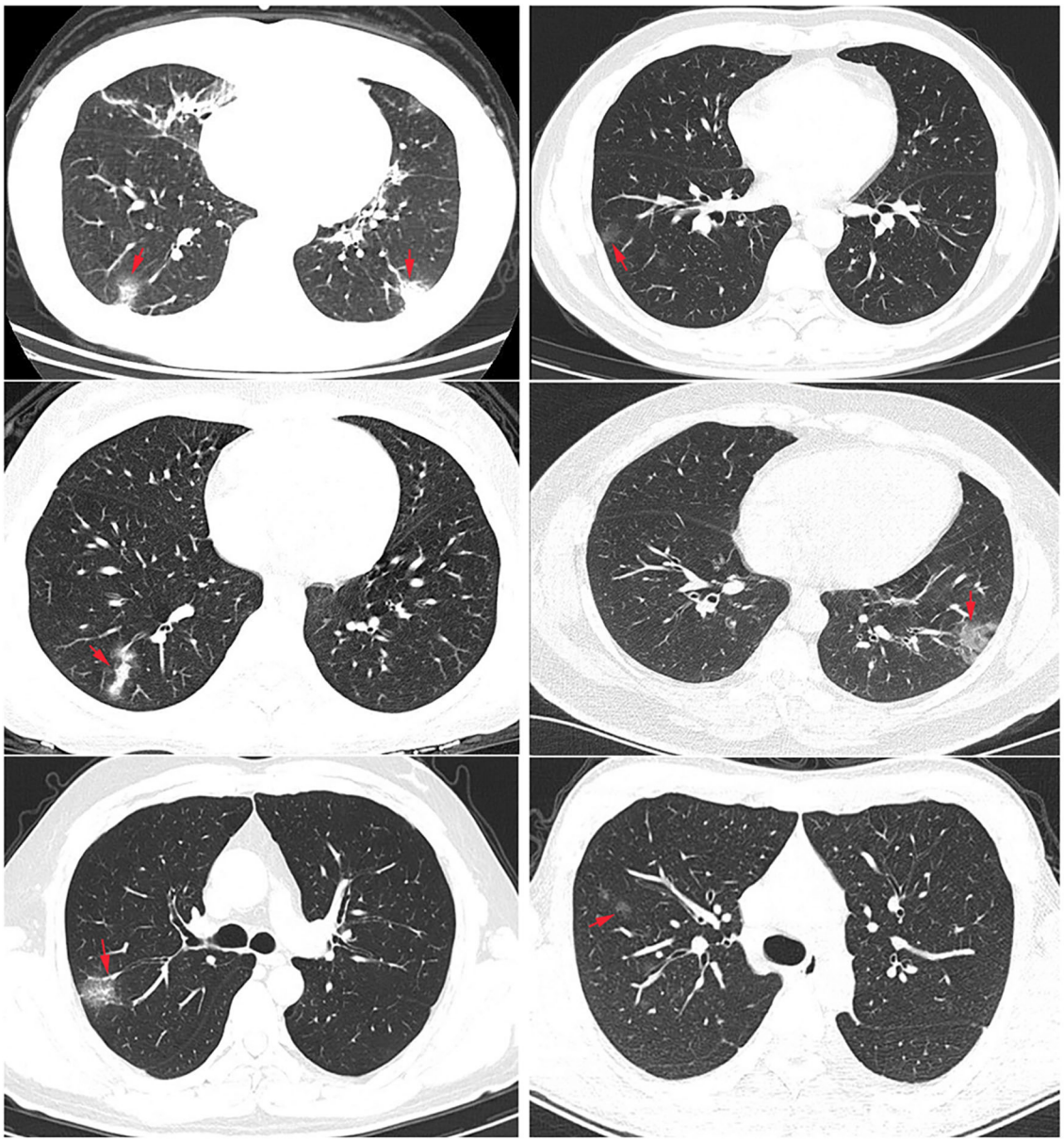

FIGURE 1 | Radiological findings: chest computer tomography (CT) images of COVID-19 and flu pneumonia at the same time after onset. **(A)** COVID-19 pneumonia showed multiple ground glass opacity with solid components in the bilateral subpleural area. The nucleic acid test for SARS-CoV-2 was positive. Arrows showed the lesions. **(B)** CT examination showed scattered ground glass opacity of both lungs in an influenza patient, mainly in the lung periphery with less solid components. SARS-CoV-2 nucleic acid test was negative for three times, and immunofluorescence test was positive for influenza A virus.

Because all three viruses can bind with receptors in the upper respiratory tract of humans, they are all easily transmitted by airborne droplets during coughing, sneezing or intimate conversation. The reproductive number (R_0) is defined as the average number of secondary cases generated per confirmed infectious case. The reported median R_0 for seasonal influenza virus is 1.28 (IQR: 1.19–1.37), except for during the 2009 pandemic (**Table 5**) (24). SARS-COV-2 had a median R_0 of 2.38 at the beginning of the epidemic (25), which is still controversial, but it is probably higher than the R_0 of SARS-COV (1.7-1.9) (26). Surveillance of genome mutation dynamics is critical for the effective control of diseases. To date, SARS-COV-2 seems to exhibit a higher mutation rate than influenza virus per site per year [$(1.12\text{-}6.25) \times 10^{-3}$ vs. $(0.60\text{-}2.00) \times 10^{-6}$] (27–29), which remains to be further determined. Relatively, SARS-COV has a similar evolution rate ($0.80\text{-}2.38 \times 10^{-3}$ per site per year) (30).

TABLE 4 | Hospitalization for flu, COVID-19 and coinfection patients.

	Influenza ($n = 41$)	COVID-19 ($n = 75$)[a]	Coinfection (Influenza A/B, $n = 23$)[b]	*P* value[a,b]
Hospitalization period (days)	3.95 (3, 5)	19.12 (11, 26)	19.48 (13, 25)	< 0.0001, 0.5424
Treatment	Oseltamivir, Peramivir	Following the guideline	Oseltamivir, Peramivir (for influenza A/B)	
Cure rate	41 (100%)	74 (98.67%)	23 (100%)	0.4577, 0.5778
Fatality rate	0	1 (1.33%)	0	0.4577, 0.5778

The data was shown as n (%).
[a]*COVID-19 vs. Influenza group.*
[b]*Coinfection vs. COVID-19 group.*

TABLE 5 | Pathogen comparisons for seasonal flu virus, SARS-COV-2 and SARS-COV.

	Seasonal influenza virus (H1N1, H3N2)	SARS-COV-2	SARS-COV
Family	*Orthomyxoviridae*	*Coronaviridae*	*Coronaviridae*
Susceptible crowd	Children and adults	Children and adults	Adults
Transmission	Droplets	Droplets	Droplets
R_0	1.28 (IQR: 1.19–1.37)	2.38 [95% (CI): 2.03–2.77]	1.7-1.9
Mutation (/site/year)	0.60-2.00 $\times$ 10^{-6}	1.12-6.25$\times$ 10^{-3}	0.80-2.38 $\times$ 10^{-3}

Adaptive Mutations of Influenza Virus and SARS-COV-2

Haemagglutinin (HA) on influenza virus and spike on SARS-COV-2 are both responsible for binding to the receptors on permissive cells. HA and spike are both homotrimers, where each monomer comprising two subunits, HA1 and HA2 or S1 and S2 (**Figures 2A,B**). Several important adaptive mutations occur in HA, including E190D and G225D for H1N1 and Q226L and G228S for H2N2 and H3N2 (31). Some mutations in the polymerase subunits PB1, PB2, and PA are critical for increasing polymerase activity and viral virulence (32). As of 2 June 2020, more than ten thousand mutant sequences of SARS-COV-2 were uploaded to the GISAID database (13). There were 18,539 nonsynonymous mutations on the spike protein, the D614G mutation was unusually enriched and present in more than 8,000 strains. Up to February 1st 2021, nearly 300,000 strains containing D614G variation, A222V and L18F were the second and third mutations (**Figure 2C**). N439K, S477N and N501Y which located in the receptor-binding domain may affect the immunogenicity or vaccination. Our result showed S477N and N439K mutations have the capability to enhance the affinity with receptor (**Figures 2D,E**). Additional studies are needed to elucidate the effect of mutations in which are not located in the receptor-binding domain (pocket) on influenza virus and SARS-COV-2 infection and their epidemiological outcomes.

DISCUSSION

To date, most epidemiological reports have clarified the case disparities in clinical manifestations, routine blood tests, and immunity factors of COVID-19 (33–35). However, the dual epidemics of COVID-19 and influenza makes the diagnosis, treatment, and vaccination face greater challenges, even though some studies have assessed the differences between influenza and COVID-19 in terms of clinical characteristics or outcomes (36, 37). Both are respiratory virus infections, and influenza and COVID-19 have many of the same symptoms, including respiratory system and gastrointestinal system symptoms. Severe cases also have loss of taste or smell, difficulty breathing, or shortness of breath (38). COVID-19 may cause gastrointestinal problems, such as diarrhea, vomiting, and abdominal pain (39). Both the SARS-COV-2 receptor ACE2 and cellular serine protease transmembrane protease serine 2 (TMPRSS2) are critical for the fusion of viral and cellular membranes, which are not only expressed in lung alveolar type 2 cells and gland cells but also highly expressed in the ileum and colon (40, 41), suggesting that the virus can invade the digestive tract and intestine, and viral RNA can be detected in patients' stool.

Certain risk factors predispose patients to increased morbidity and mortality following exposure to the influenza A virus or SARS-COV-2. Age is the most significant risk factor for influenza and COVID-19-related mortality (42, 43). Age may also have different effects on the results of influenza virus infection between males and females (44). For COVID-19, the sex difference is a significant factor for mortality. Recently reported data showed that the male mortality rate is 2.4 times that of females (70.3 vs. 29.7%) (43), and there were 1641 men among the 2248 confirmed COVID-19 patients, which was 2.6 times the proportion of women (73 vs. 27%) (45). Chronic respiratory diseases and cardiovascular diseases are the two major comorbidities for influenza patients (5), and other comorbidities associated with poor influenza outcomes include diabetes and obesity. In our study, the most common underlying diseases in both flu and COVID-19 patients were hypertension, diabetes, and coronary heart disease. There was a significant difference in the progressive symptoms and complications of pneumonia between influenza and COVID-19 illness, including concurrency and CT radiology features. Only 17% of flu patients developed pneumonia, while the number increased to 90.67% in COVID-19 patients. In the

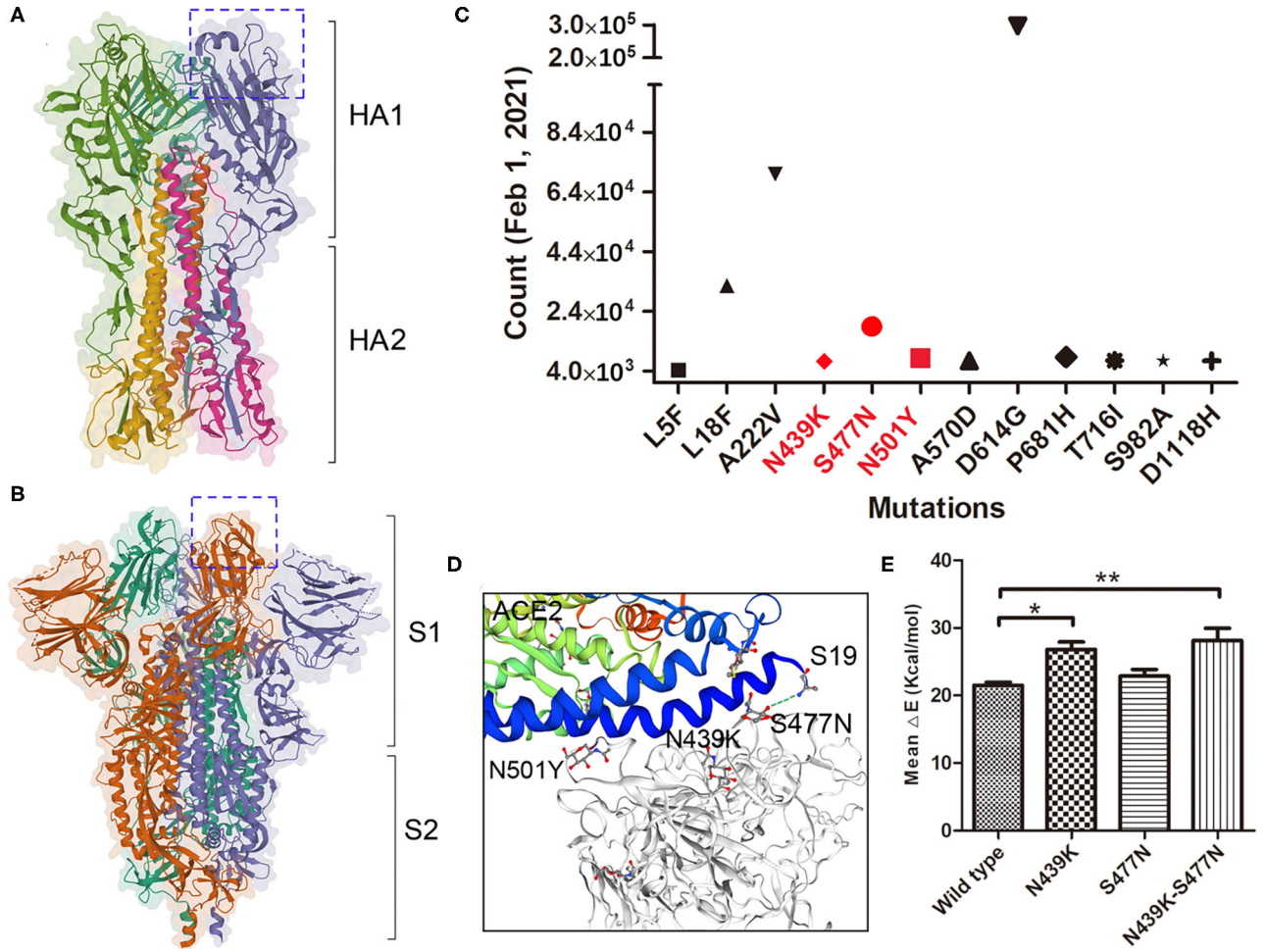

FIGURE 2 | Mutations on SARS-COV-2 spike protein. **(A)** The trimeric structure of influenza viral HA, each monomer is composed with HA1 and HA2 domain. The receptor-binding pocket were marked in box. **(B)** The trimeric structure of SARS-COV-2 spike protein, each monomer is composed with S1 and S2 domain. The receptor-binding domain (closed) were marked in box. **(C)** The major mutations on spike protein up to February 1st, 2021. Mutations in Red are located within the RBD. **(D)** The mutations binding with the receptor ACE2. Stimulation shown the residue S477N binding with ACE2 Sernine19. **(E)** The binding affinity of wild type and variants of spike with ACE2 was calculated by MM/GBVI. Data are the mean ± SD, n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the wild type (one-way ANOVA and Tukey's *post hoc* test).

early stage of flu pneumonia, X-ray imaging showed thickened and blurred lung texture and small patchy shadows. The advanced stage (onset 3-7 days) is dominated by GGOs and consolidation (46). COVID-19 pneumonia shows limited patchy shadows in the early stage, diffuse lung abnormalities, and multiple consolidations when the lesions are severe (47).

Dynamic changes in routine blood parameters refer to the evaluation of treatment or examination of the disease by observing the quantity change and shape distribution in blood cells. The total count of WBCs, neutrophils, lymphocytes, and monocytes progressively decreased, which may be related to the direct invasion of the virus into hematopoietic cells (48). The initial CRP of severe COVID-19 patients increased prior to CT findings (49), and the CRP value increased rapidly after admission, indicating a strong inflammatory response; the virus is prevalent in patients' bodies at this stage. ESR, elevated by the acute-phase response, can be used as an important indicator to distinguish patients with severe COVID-19 in the early stage (50). Severe COVID-19 illness is associated with a prominent elevation in ESR, which may provide additional information on disease progression. The BUN and blood creatinine levels rise rapidly before death in severe COVID-19 patients, this is consistent with our study (51). The inadequate sample size may limit the conclusion of our study, further studies are needed to analyze the dynamic changes in immune factors for COVID-19 progression and prognosis.

Several adaptation mutations have been identified in different segments of influenza A virus, and thoroughly researched mutations, including E627K and D701N on PB2 of avian flu virus and other subtypes, promote polymerase activity and adaption to act cooperatively with humans (52, 53). Adaptive mutations in SARS-COV-2 have also been reported, and the

most important mutation in spike is D614G, which has attracted global attention (54, 55). The D614G mutant began to spread in Europe in early February 2020 and became the mainstream strain worldwide by May 2020, accounting for nearly 70% of samples in Europe and North America (56). Zhang et al. (57) latest research showed that small genetic mutations in the SARS-COV-2 variants are prevalent throughout Europe and the United States, which may increase the number of spike proteins on virions, and it will greatly improve the viral infectivity by 9-10 times. The N439K variants can maintain fitness but can evade the antibody-mediated immunity (58), it is unclear what the S477N mutation will bring to the vaccines and epidemics. More studies are needed to compare the clinical outcomes and prevalence between adaptive mutants and wild type.

This study provides new insight into the differences in clinical outcomes, laboratory abnormalities, comorbidities and hospitalization between influenza and COVID-19 patients and discusses the relationship between viral adaptive mutations and protein function, which will provide a reference for clinical differential diagnosis and epidemic surveillance.

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

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR'S NOTE

COVID-19 remains one of the key threats to public health. SARS-COV-2 causes a mild respiratory system disease, which has symptoms similar to those of seasonal influenza virus. COVID-19 and seasonal influenza both emerged in the respiratory virus season. Comparison of clinical features and outcomes of medically attended COVID-19 and influenza A patients will help us to better diagnose and treat the infection caused by SARS-COV-2. In this study, we systematically contrasted the proportions or values of epidemiologic characteristics, clinical features, blood abnormalities, progressive symptoms, and hospitalization between influenza and COVID-19. The parameters of coinfection patients who presented with both COVID-19 and flu were also compared with the COVID-19 cases. In addition, mutants of influenza virus HA and SARS-COV-2 spike causing the epidemic were resolved and discussed according to the literature or computation. This comparative research aims to aid pandemic control and be beneficial to clinical diagnosis.

AUTHOR CONTRIBUTIONS

LL, FZ, JY, and ZxL contributed to the design of experiments. LL, FZ, JR, SY, MJ, XuL, ZjL, XiL, WD, YL, and HT contributed to the conduction of experiments. LL, FZ, JR, SY, MJ, and ZjL contributed to the reagents. LL, FZ, JR, SY, MJ, XuL, ZjL, XiL, JL, JZ, and ZxL contributed to the analyses of the data. LL and ZxL contributed to the writing the paper. JY and ZxL contributed to the editing the paper. All authors contributed to the article and approved the submitted version.

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REVIEW
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COVID-19 Pandemic: Advances in Diagnosis, Treatment, Organoid Applications and Impacts on Cancer Patient Management

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Coronavirus disease 2019 (COVID-19) caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread globally and rapidly developed into a worldwide pandemic. The sudden outburst and rapid dissemination of SARS-CoV-2, with overwhelming public health and economic burdens, highlight an urgent need to develop effective strategies for the diagnosis and treatment of infected patients. In this review, we focus on the current advances in the diagnostics and treatment for SARS-CoV-2 infection. Notably, we also summarize some antineoplastic drugs repurposed for COVID-19 treatment and address the diagnostic and therapeutic challenges for oncologists to manage cancer patients in this COVID-19 era. In addition, we emphasize the importance of organoid technology as a valuable experimental virology platform to better understand the pathogenesis of COVID-19 and assist rapid screening of drugs against COVID-19.

Keywords: COVID-19, SARS-CoV-2, diagnostics, treatment, cancer patient, organoid

INTRODUCTION

In December 2019, coronavirus disease 2019 (COVID-19) caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged as a new world pandemic (1, 2). As of 9 January 2021, more than 88.9 million cases and 1.91 million deaths have been reported across 188 countries (3), indicating that the SARS-CoV-2 outbreak has become a serious public health emergency of international concern. Coronaviruses, including four genera (Alpha-, Beta-, Gamma-, and Deltacoronavirus), are enveloped, positive-sense, single-stranded RNA viruses that cause infectious diseases in humans and mammals (4). According to phylogenetic analysis of viral genomes, SARS-CoV-2 is a new member of the Beta coronavirus genus, which also includes severe acute respiratory syndrome coronavirus (SARS-CoV). Viral entry into target cells is facilitated by interactions between the spike (S) protein of coronaviruses and the host cell receptor angiotensin-converting enzyme 2 (ACE2) (1, 5–7). Following receptor engagement, the SARS-CoV-2 S protein is primed by cellular serine protease transmembrane protease serine 2 (TMPRSS2) before fusion of the viral and cellular membranes, which is a critical step for the entry and spread of SARS-CoV-2 into host cells (5, 8) (**Figure 1**).

Since accumulation of SARS-CoV-2 in the respiratory tract is the most serious manifestation, fever and respiratory symptoms, such as cough, shortness of breath, sore throat, etc., are the most common initial symptoms of COVID-19 (9). The impact of COVID-19 goes well beyond the respiratory system to influence the heart and vessels. Several clinical studies showed the correlation between COVID-19 and cardiovascular disease (10, 11). The presence of pre-existing cardiovascular disease is associated with worse prognosis and increased mortality in COVID-19 patients (9, 11, 12). COVID-19 can result in cardiac and vascular complications including acute cardiac injury, myocardial injury, arrhythmia and venous thromboembolism (12, 13). A growing concern over the potential drug-disease interactions in patients with cardiovascular diseases and COVID-19 remains to be solved (14, 15). In addition, SAR-CoV-2 also influences other tissues and organs, such as the brain, eyes, nose, liver, kidneys and intestines (16, 17) (**Figure 1**). The damage to these organs may manifest specific symptoms, such as seizure, stroke and brain damage, conjunctivitis, diarrhea, hematuria, and oliguria (9).

Given the vast majority of people are still vulnerable to SARS-CoV-2, the development of strategies to diagnose and treat patients with COVID-19 is urgently needed. In this review, we aim to summarize the clinical manifestations of COVID-19 patients, current advances in diagnostic methods and treatment strategies, and organoid applications to fight against COVID-19. Of note, we focus on some repurposing of antineoplastic drugs for COVID-19 and the diagnostic and therapeutic challenges in the management of cancer patients during the current COVID-19 pandemic.

DIAGNOSTIC STRATEGIES FOR SARS-CoV-2 INFECTION

Fever and respiratory symptoms are the most common onset symptoms of COVID-19 (9, 18). After screening clinical symptoms and epidemiological history, the highly suspected group required laboratory testing or imaging tests to confirm the COVID-19 diagnosis (19).

After the nucleotide sequence of SARS-CoV-2 was identified from patients' respiratory tract samples by Chinese facilities via deep sequencing analysis (20), a series of detection products based on RT-PCR were obtained. The general process was to sample RNA from the upper respiratory tract, extract RNA, and determine whether it was positive after PCR with a specific primer. There are also serological-based tests. In China, some experts proposed the application of CT imaging to diagnose typical cases in epidemic areas (21), but chest CT screening is not suggested for populations with low infection rates because of its low positive predictive value (22) but may be considered a primary tool for the current COVID-19 detection in epidemic areas (23). In addition to nucleic acid PCR testing and serological testing, there are also tests based on other principles, such as antigen-based testing (24), CRISPR-based methods (25), and physics-based methods (26). One of the main advantages of antigen detection is the fast detection speed. However, antigen detection is very specific to viruses but not as sensitive as molecular PCR tests. SHERLOCK SARS-CoV-2 is short for Specific High-sensitivity Enzymatic Reporter unLOCKing and is based on Cas13a protease and a guide RNA (gRNA) used to recognize a specific new coronavirus genomic sequence (27). No instrument is required, and a simple test similar to a pregnancy test can quickly detect the presence of a new coronavirus RNA sequence using a Sherlock CRISPR SARS-CoV-2 Kit (27, 28). At present, the most widely used detection method is the combination of nasopharyngeal swab nucleic acid PCR and serological IgG/IgM detection (29). Nucleic acid PCR test results are still the gold standard for COVID-19 diagnosis, and serological tests can be used as a supplement (30).

In nucleic acid detection, the sampling site is also critical. The virus can be detected in respiratory, stool, serum (31), urine (32), and sperm samples (33). Saliva or nasopharyngeal swabs are the most convenient to obtain. Doctors use bronchoscopy to sample the lower respiratory tract (34). However, this procedure increases the patient's pain and reduces the efficiency of the test. The kits developed later were mostly nasopharyngeal swabs. At present, there are studies that show that the accuracy of oropharyngeal swab sampling detection may be higher than that of nasopharynx sampling, which further reduces the difficulty of sampling and the patient's pain (35).

As the pandemic began, the requirements for detection time and accuracy were greatly improved. As of 11 May 2020, the FDA had issued 67 individual emergency use authorizations (EUAs) for test kit manufacturers and laboratories for three types of testing (PCR-based testing, serologic testing and antigen testing) (36). The testing time for ID NOW COVID-19 provided by Abbott Laboratories is the shortest at present. Here, we list several typical FDA-approved testing kits and new testing methods in the laboratory stage (**Table 1**).

Abbreviations: COVID-19, Coronavirus disease 2019; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; SARS-CoV, Severe acute respiratory syndrome coronavirus; S protein, Spike protein; ACE2, Angiotensin-converting enzyme 2; TMPRSS2, Transmembrane protease serine 2; MERS, Middle East Respiratory Syndrome; ARDS, Acute respiratory distress syndrome; ICU, Intensive care unit; SHERLOCK, Specific high-sensitivity enzymatic reporter unlocking; gRNA, Guide RNA; EUA, Emergency use authorizations; CQ, Chloroquine; HCQ, Hydroxychloroquine; HIV, Immunodeficiency virus; SARS, Severe acute respiratory syndrome; LPV/r, Lopinavir/ritonavir; RBD, Receptor binding domain; hrsACE2, Human recombinant soluble ACE2; CM, Camostat mesylate; AAK1, AP2-associated protein kinase 1; JAK, Janus kinase; IL-6, Interleukin-6; CML, Chronic myelogenous leukemia; GIST, Gastrointestinal stromal tumor; DFSPs, Dermatofibrosarcoma protuberans; ALL, Acute lymphoblastic leukemia; PDGFR, Platelet-derived growth factor receptor; VEGF, Vascular endothelial growth factor; ALI, Acute lung injury; CEA, Carcinoembryonic antigen; CA, Carbohydrate antigens; SCCA, Squamous cell carcinoma antigen; CYFRA21-1, Cytokeratin-19 fragment; HuNoV, Human noroviruses; ECM, extracellular matrix; ACTT-1, Adaptive Covid-19 Treatment Trial; FDA, Food and Drug Administration; ECMO, extra-corporal membranous oxygenation; RECOVERY, randomized evaluation of COVID-19 therapy; Ad26, adenovirus type 26; IDSA, Infectious Diseases Society of America; CDC, Centers for Disease Control and Prevention.

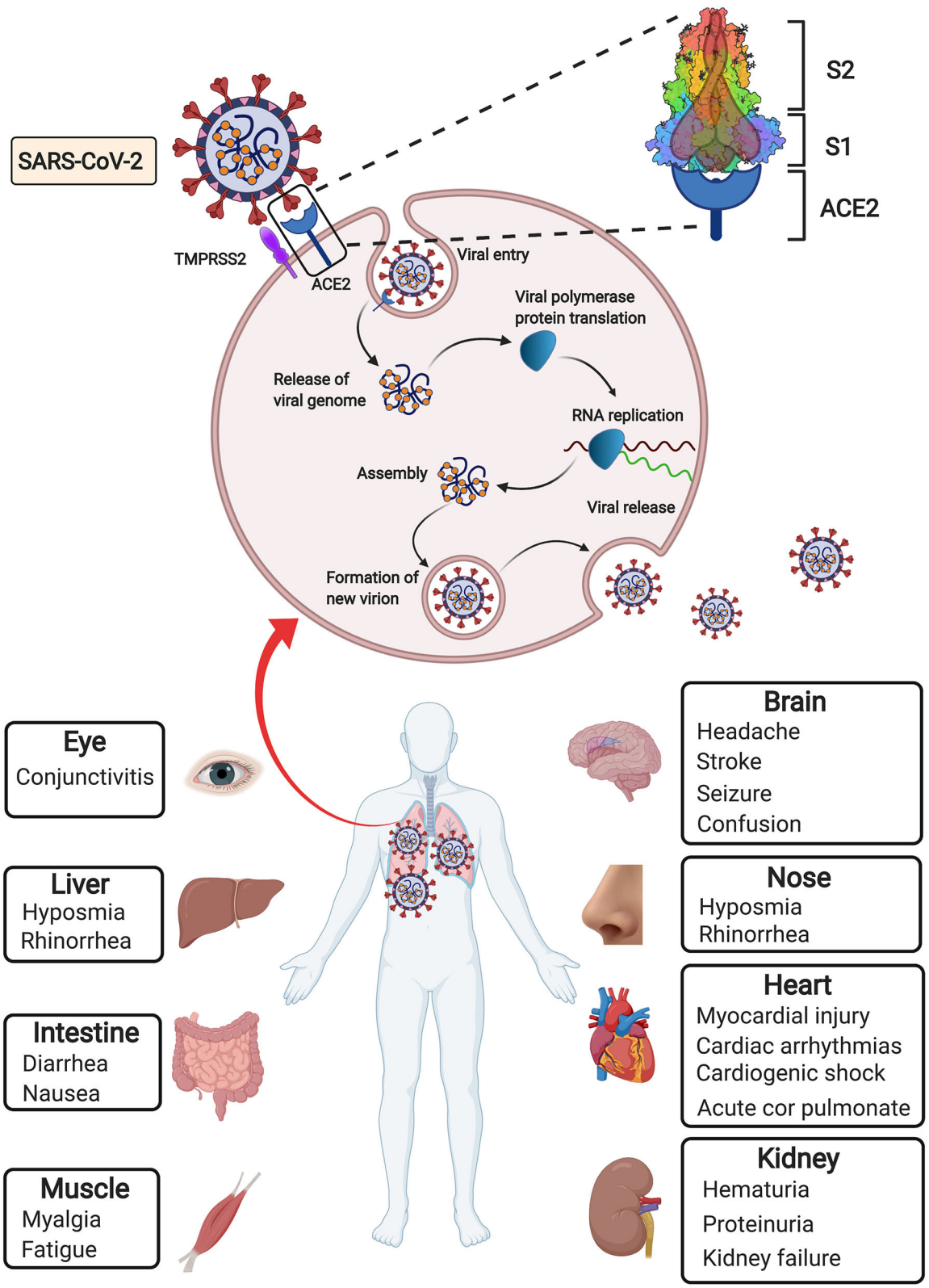

FIGURE 1 | Simplified depiction of SARS-CoV-2 lifecycle and extrapulmonary manifestations of COVID-19. SARS-CoV-2 enters host cells through interaction of its surface spike protein with the ACE2 receptor on the membranes of host cells in the presence of TMPRSS2, which mediates virus–cell membrane fusion and following viral entry. Then viral genomic RNA is released and translated into viral polymerase proteins. Viral RNA is assembled to form mature virions, followed by release of the new virions from the host cells. In addition to the most common pulmonary manifestation of COVID-19, extrapulmonary manifestations derived from many other injured organs have been observed.

TABLE 1 | Diagnostic methods for COVID-19.

	FDA approved	Institution	Specimen	Testing time	Notes
Virus RNA test					
TaqPath SARS-CoV-2 Assay	YES	Rutgers Clinical Genomics Laboratory (USA)	Oropharyngeal, nasopharyngeal, anterior nasal, mid-turbinate nasal swab, saliva	n. r	RT-PCR, can detect saliva specimen
TaqPath™COVID-19 Combo kit	YES	Thermo Fisher Scientific (USA)	Nasopharynx swab	4 h	RT-PCR
Pixel	YES	Labcorp (USA)	Nasopharynx swab	n. r	RT-PCR, the only home collection kit
Cobas® SARS-CoV-2	YES	Roche (USA)	Nasopharynx swab	3.5 h	RT-PCR
Xpert Xpress SARS-CoV-2	YES	Cepheid (USA)	Nasopharynx swab, nasal wash or aspiratory specimen	45 min	RT-PCR, can run up to 2,000 samples per day
ID NOW COVID-19	YES	Abbott Laboratories (USA)	Nasopharynx swab throat swabs	13 min	ID NOW Instrument based
Bio-Rad SARS-CoV-2 ddPCR Test	YES	Bio-Rad Laboratories (USA)	Nasopharynx swab	5.5 h	RT-ddPCR
BioFire Respiratory Panel 2.1 (RP2.1)	YES	BioFire Diagnostics (USA)	Nasopharynx swab	45 min	Nested multiplex PCR, a multiplexed nucleic acid test
iLACO (isothermal LAMP based method for COVID-19)	NO	Shenyang University (China)	n. r	20 min	RT-LAMP
Sherlock CRISPR SARS-CoV-2 Kit	YES	Sherlock BioSciences, Inc. (USA)	Upper respiratory specimens	<1 h	RT-LAMP+ CRISPR-Cas13 based
CRISPR-based DETECTR assay	NO	Mammoth Biosciences (USA)	Respiratory swab	<40 min	CRISPR-Cas12-based, PPV: 95%, NPV: 100%
Dual-Functional Plasmonic Photothermal Biosensors	NO	Institute of Environmental Engineering (Switzerland)	Respiratory swab	≈17 min	Plasmonic photothermal biosensor based
Serological test					
Serology Test qSARS-CoV-2 IgG/IgM Rapid Test	YES	Cellex (Japan)	Serum and plasma	15–20 min	IgG/IgM The first serological test authorized under EUA.
Platelia SARS-CoV-2 Total Ab assay	YES	Bio-Rad Laboratories (USA)	Serum and plasma	n. r	IgM/IgA/IgG specificity> 99%, sensitivity 98%
SARS-CoV-2 IgG assay	YES	Abbott Laboratories (USA)	Serum and plasma	29 min	IgG
Elecsys®Anti-SARS-CoV-2	YES	Roche (USA)	Serum and plasma	18 min	IgG Specificity> 99.8%, sensitivity 100%
Antigen					
Sofia 2 SARS Antigen FIA	YES	Quidel Corporation (USA)	Nasopharynx swab	<15 min	Test nucleocapsid protein antigen

n. r, not reported; RT-LAMP, reverse transcriptional loop-mediated isothermal amplification; PPV, Positive predictive value; NPV, Negative predictive value.

THERAPEUTIC STRATEGIES AGAINST COVID-19

Given the time-consuming process to develop new drugs starting from scratch, several FDA-approved drugs indicated for other diseases have been repurposed to treat COVID-19 because of their antiviral properties. Notably, some antineoplastic medications have also shown capacities for severe COVID-19 by mitigating hyperactive immune responses and are now being investigated in ongoing clinical trials (**Table 2**). Here, we summarize the ongoing therapeutic choices, including antiviral drugs, convalescent plasma therapy, and repurposing antineoplastic medications, that are promising to help us fight against COVID-19.

TABLE 2 | FDA-approved antineoplastic drugs repurposed for COVID-19 treatment.

Antineoplastic drugs	Mechanism of action	FDA approved cancer-specific indications	COVID-19 clinical trial identifier
Tocilizumab	Binds soluble and membrane bound IL-6 receptors, preventing IL-6 mediated pro-inflammatory effect	Cytokine release syndrome	NCT04361552, NCT04331795
Siltuximab	Prevents the binding of IL-6 to both soluble and membrane- bound IL-6 receptors	Multicentric Castleman's disease	NCT04329650, NCT04330638
Imatinib	Multiple tyrosine kinase inhibitor	CML; DFSPs; GIST; ALL; MDS	NCT04357613, NCT04346147
Thalidomide	Immunomodulatory and antiangiogenic effect, suppression of tumor necrosis factor-α	Multiple myeloma	NCT04273529, NCT04273581
Bevacizumab	Monoclonal antibody inhibits the binding of VEGF to its cell surface receptors	Colorectal cancer; Non-squamous non-small cell lung cancer; Glioblastoma; cervical cancer; Renal cell carcinoma	NCT04305106, NCT04275414

CML, Chronic myelogenous leukemia; DFSPs, Dermatofibrosarcoma protuberans; GIST, Gastrointestinal stromal tumor; ALL, Acute lymphoblastic leukemia; MDS, Myelodysplastic syndrome; VEGF, Vascular endothelial growth factor.

REMDESIVIR

Remdesivir (GS-5734) is a nucleotide analog prodrug that blocks viral replication by inhibiting viral RNA polymerase (37). The therapeutic effectiveness of remdesivir was first evaluated in both cell-based assays and a rhesus monkey model against Ebola virus, in which remdesivir exhibits potent suppression of viral replication and protection from lethal disease (38). However, the efficacy of remdesivir treatment failed to be proven in a randomized controlled human clinical trial in response to a recent Ebola outbreak in the Democratic Republic of Congo (39). Interestingly, a recent *in vitro* study indicated that remdesivir has antiviral activity against SARS-CoV-2 (40). In the case report of the first patient with confirmed COVID-19 in the United States, the patient was intravenously administered remdesivir on hospital day 7 based on the patient's worsening clinical status, including persistent fevers and severe pneumonia. On the 8th day, the patient's clinical condition improved without any adverse events related to remdesivir treatment (41). In a small cohort study of patients with severe COVID-19 who underwent compassionate-use remdesivir treatment, improved clinical outcomes were observed in 36 of 53 patients (68%). However, one clinical trial (ClinicalTrials.gov: NCT04257656) indicated that remdesivir did not exhibit statistically significant clinical benefits compared with those of a placebo (42). But this trial was underpowered due to incomplete full enrollment of eligible patients. The most recent Adaptive Covid-19 Treatment Trial (ACTT-1) was a double-blind, randomized, placebo-controlled trial administrating intravenous remdesivir in 1,062 hospitalized COVID-19 patients (43). The result of this trial showed that remdesivir significantly shortened the time to recovery in COVID-19 patients compared with placebo. However, remdesivir is not routinely recommended in mechanically ventilated COVID-19 patients. Recently, the FDA has approved remdesivir for the treatment of Covid-19 patients requiring hospitalization (44). Because remdesivir alone fails to improve survival rates of COVID-19 patients, several ongoing trials are still awaited to confirm the efficacy and safety of remdesivir combined with modifiers of the immune response for patients with COVID-19 (43, 45).

CHLOROQUINE AND HYDROXYCHLOROQUINE

Chloroquine (CQ) and hydroxychloroquine (HCQ) (an analog of chloroquine) are two well-known medications used for treating malaria and autoimmune diseases, such as rheumatoid arthritis and lupus (46, 47). Both CQ and HCQ are able to exhibit broad-spectrum antiviral effects by elevating the endosomal/lysosomal pH essential for virus and host cell fusion (47, 48). CQ could also suppress SARS-CoV entry by interfering with the glycosylation of the ACE2 receptor (47, 49, 50). HCQ is typically preferred over CQ due to its better clinical safety during long-term usage, allowance for higher daily dose, and lower potential for drug-drug interaction (51, 52).

Recent *in vitro* studies showed that both CQ and HCQ can effectively control SARS-CoV-2 infection (40, 53). However, in the early stage of the COVID-19 pandemic, there were not enough medical evidence to prove the efficacy of CQ and HCQ treatment for COVID-19, and the results from different small sample studies were controversial (54). Some studies have gained much attention, indicating that HCQ is effective in the treatment of COVID-19 (55, 56). A small open-label non-randomized clinical study from France reported that patients who received 600 mg of HCQ daily had a significant reduction in the viral load. The efficacy of HCQ was reinforced in combination with azithromycin for virus elimination (56). However, the limitations of this study are that comparisons were made between patients at different clinical centers, and six patients (23%) among the 26 HCQ-treated patients were lost to follow-up due to early cessation of treatment, which weakened the conclusion. The same research group later published another study evaluating the effectiveness of HCQ and azithromycin combination therapy in 80 patients. The results showed that 93% of treated patients were negative in nasopharyngeal viral load testing after 8 days

(55). However, this study failed to include a control group. Thus, it is unclear whether patients who did not receive HCQ and azithromycin combination therapy would have similar results. It is noteworthy that a prospective study from France failed to obtain any evidence of obvious clinical benefits or strong antiviral effects upon the combination treatment of HCQ and azithromycin for hospitalized patients with severe COVID-19 (57). In their study, 11 patients received the combination therapy of HCQ and azithromycin. However, eight of 10 patients (one patient was not tested due to death) were still positive for SARS-CoV-2 after 6 days. Two patients were transferred to the ICU, and one had to discontinue treatment due to adverse cardiac effects. This study also did not have a control group. Eight of 11 patients had severe comorbidities, including obesity, solid cancer, hematological cancer, and HIV infection, which could be potential confounding effects to influence the results. Similarly, a retrospective study from the U.S. revealed that there was no evidence that therapy with HCQ, either with or without azithromycin, reduced the risk of mechanical ventilation. An association of increased overall death rates was found in patients treated with HCQ alone (58). However, the patients enrolled in this study were all male and over 65 years old (median age), which could introduce bias in this study. In addition, a multicenter, open-label randomized controlled trial including 150 patients in China also concluded that the administration of HCQ did not improve the condition of patients, with a higher negative conversion rate (59). Although the U.S. FDA issued an EUA for the use of HCQ to treat COVID-19 in the United States, the FDA also cautioned against the use of HCQ or CQ for COVID-19 outside of the hospital setting or a clinical trial due to the risk of heart rhythm problems raised by a recent study (60). Therefore, larger high-quality randomized controlled trials are needed to provide a definitive answer regarding the efficacy and safety of this combination. Recently, the controlled, open-label Randomized Evaluation of COVID-19 Therapy (RECOVERY) trial compared the effects between HCQ and usual care in patients hospitalized for COVID-19 (61). Unfortunately, patients who received HCQ treatment did not have better clinical outcomes than those who received usual care. The WHO SOLIDARITY trial also released preliminary results on the efficacy of HCQ in hospitalized patients for COVID-19, and the results were in accordance with the ones from the RECOVERY trial (61). Therefore, HCQ is not an effective treatment for hospitalized patients for COVID-19. The living WHO guideline development panel made a strong recommendation against the use of HCQ for people who are not COVID-19 positive (62). But it remains unclear whether HCQ or CQ could be used in mild-to-moderate COVID-19 cases.

LOPINAVIR/RITONAVIR

Lopinavir, a human immunodeficiency virus (HIV) type 1 aspartate protease inhibitor, was identified as having an *in vitro* inhibitory effect against SARS-CoV-1 by screening approved drugs for treating severe acute respiratory syndrome (SARS) (63–65). Lopinavir is administered in a fixed-dose combination with ritonavir, a potent CYP3A4 inhibitor, to increase the plasma concentration of lopinavir through the inhibition of cytochrome P450 (64, 66). In an open-label clinical study, treatment with a combination of lopinavir/ritonavir and ribavirin reduced the risk of adverse clinical outcomes (ARDS or death) and viral load among patients with SARS compared with that in a historical control group treated with ribavirin only (64). However, the efficacy of lopinavir/ritonavir was difficult to interpret in that study due to lack of randomization and a contemporary control group and the concomitant use of ribavirin and corticosteroid. Lopinavir was also found to have anti-MERS-coronavirus (CoV) activity both *in vitro* (67) and in a non-human primate animal model (68). Although several clinical case reports indicated that lopinavir/ritonavir (LPV/r)-based combination therapy with ribavirin and interferon alpha led to virological clearance and clinical resolution of infection (69–71), more convincing clinical trial data about the efficacy of this combined therapeutic strategy are needed (71). Therefore, a randomized controlled clinical trial of LPV/r and recombinant interferon-β1b vs. placebo for MERS is currently under way (ClinicalTrials.gov: NCT02845843) (72). Intriguingly, recent research showed that SARS-CoV-2 leveraged species-specific interferon-driven upregulation of angiotensin-converting enzyme 2 (ACE2) to promote infection (the SARS-CoV-2 receptor ACE2 is an interferon-stimulated gene in human airway epithelial cells and is detected in specific cell subsets across tissues). Thus, treatment involving interferon could enhance SARS-CoV-2 infection instead, and caution should be applied in the clinical treatment of patients with COVID-19. For the treatment of severe COVID-19, an open-label, randomized, controlled trial comparing lopinavir/ritonavir (400/100 mg twice daily) ($n = 99$) to standard care ($n = 100$) was performed. The results revealed that lopinavir/ritonavir treatment failed to significantly promote throat viral clearance, facilitate clinical improvement, or reduce mortality in severe COVID-19 patients (66). In addition, one recent study systematically evaluated the clinical characteristics of COVID-19 in patients with liver test abnormalities and found that the use of lopinavir/ritonavir resulted in 4-fold enhanced risk of liver injury (73). The RECOVERY trial is the first large-scale randomized clinical trial to show the effects of lopinavir/ritonavir in patients hospitalized for COVID-19 (74). The result indicated that lopinavir/ritonavir treatment did not reduce duration of hospital stay, risk of progression to invasive mechanical ventilation, or 28-day mortality rate. The interim results of the WHO SOLIDARITY trial also reported that lopinavir–ritonavir did not improve clinical outcomes for COVID-19 patients who require hospitalization (74). Based on the results of recent high quality randomized clinical trials, lopinavir–ritonavir monotherapy is not recommended for patients admitted to hospital with COVID−19.

APN01

ACE2 has been identified as the key receptor for SARS-CoV both *in vitro* and *in vivo* (75, 76). ACE2 not only acts as the entry receptor of SARS-CoV but also protects

against acute lung injury by reducing destructive inflammatory reactions (77). The receptor-binding domain (RBD) of the spike protein of SARS-CoV-2 is very similar to the RBD of SARS-CoV, indicating that both viruses possibly use the common host cell receptor ACE2. Recent studies confirmed that the spike protein of SARS-CoV-2 directly contacts ACE2 to enter cells, and SARS-CoV-2 recognizes human ACE2 even more efficiently than SARS-CoV, suggesting an increased capacity of person-to-person SARS-CoV-2 transmission (6, 78, 79). Treatment with human recombinant soluble ACE2 (hrsACE2) has been proposed to suppress SARS-CoV-2 infections because excessive ACE2 can not only competitively bind with SARS-CoV-2 to block the virus from entering the host cells but also protect the lung from injury by recovering cellular ACE2 activity (80). hrsACE2 could effectively inhibit SARS-CoV-2 replication in Vero cells, engineered human blood vessels and kidney organoids (77). Thus, APN01 (hrsACE2) developed by Apeiron Biologics has undergone a placebo-controlled, double-blinded, phase II clinical trial to evaluate its clinical efficacy and safety in the treatment of COVID-19 patients (ClinicalTrials.gov: NCT04335136).

CAMOSTAT MESYLATE

Camostat mesylate (CM), a serine protease inhibitor of TMPRSS2, was developed in Japan primarily for chronic pancreatitis and postoperative reflux esophagitis (81). Since TMPRSS2 is a serine protease that cleaves and activates the spike protein of SARS-CoV-2, which is vital for SARS-CoV-2 entry and viral transmission through interaction with ACE2, CM has become a potential drug candidate for treating COVID-19 (5). Camostat mesylate was validated to inhibit SARS-CoV-2 infection of lung cells, indicating that the host cell entry of SARS-CoV-2 can be effectively inhibited by the clinically proven inhibitor CM. CM is currently undergoing randomized clinical trials (ClinicalTrials.gov: NCT04374019, NCT04355052) that aim to assess whether CM reduces viral entry of SARS-CoV-2 and improves clinical outcomes of patients with COVID-19.

BARICITINIB

Most viruses enter cells through receptor-mediated endocytosis. One of the pivotal regulators of endocytosis is AP2-associated protein kinase 1 (AAK1) (82). Richardson et al. found, using the BenevolentAI machine learning method, a group of AAK1 inhibitors that could suppress clathrin-mediated endocytosis and thereby impair the ability of the virus to infect cells (83). In this study, baricitinib, a Janus kinase (JAK) inhibitor indicated for the treatment of rheumatoid arthritis (RA) (84), was identified with a particularly high affinity for AAK1. Unlike other AAK1 inhibitors, such as the oncology drugs sunitinib and erlotinib, which have serious side effects at the high doses required to inhibit AAK1 effectively, baricitinib can be administered with once-daily oral dosing and trivial side effects (83, 85). In addition, baricitinib has the potential for combination therapy with direct-acting antivirals, such as lopinavir/ritonavir or remdesivir, currently being used and investigated during the COVID-19 pandemic because of its minimal interaction with the relevant cytochrome P450 (CYP) drug-metabolizing enzymes (85). Cantini et al. conducted a pilot study on the safety and clinical efficacy of baricitinib treatment combined with lopinavir-ritonavir in patients with moderate COVID-19 pneumonia (86). However, the limitations of this study, including its open-label, non-randomized feature, lack of properly designed control group, and limited patient number treated with baricitinib, require larger randomized controlled trials to further demonstrate the efficacy of baricitinib treatment.

CONVALESCENT PLASMA THERAPY

As a classic passive immunotherapy, convalescent plasma therapy has been used to prevent and treat many infectious diseases since the 1890s (87). Convalescent plasma therapy was successfully applied to the treatment of SARS, H5N1 influenza, 2009 H1N1 pandemic, and MERS, with improved clinical conditions and reduced mortality (88–91). However, in the Ebola virus disease setting, convalescent plasma therapy failed to achieve significant survival improvement (92). Since SARS, MERS, and COVID-19 share similar clinical and virological features (93), convalescent plasma therapy could be a potential treatment alternative for COVID-19 patients (94). One recent laboratory study indicated that sera from several patients can neutralize the COVID-19 virus isolated from the bronchoalveolar lavage fluid of a critically ill patient (1). A systematic review (95) was conducted to assess the clinical efficacy of convalescent plasma therapy for patients with COVID-19. Based on five available clinical studies (87, 96–99), convalescent plasma therapy seems to be promising, with reduced mortality, improved clinical status, and virus clearance. Several randomized clinical trials have been conducted to evaluate the potential benefits of convalescent plasma therapy. Li et al. found convalescent plasma therapy added to standard treatment failed to result in statistically significant improvement in the time to hospital discharge and clinical improvement within 28 days compared with standard treatment in severe or life-threatening COVID-19 patients (100). Another randomized trial in COVID-19 patients with severe pneumonia also observed no significant differences in clinical conditions or overall mortality rates between groups treated with convalescent plasma and placebo (101). But it remains unclear whether convalescent plasma treatment works as a treatment for certain COVID-19 patients incuding mild-to-moderate COVID-19 cases. The RECOVERY trial (Clinical Trials.gov: NCT04381936), the world's largest trial of convalescent plasma is still recruiting COVID-19 patients who do not require invasive mechanical ventilation or extra-corporal membranous oxygenation (ECMO). The completion of RECOVERY trial may provide further evidence about the effectiveness and safety of convalescent plasma treatment.

REPURPOSING ANTICANCER MEDICATIONS FOR COVID-19 TREATMENT

IL-6 or IL-6 Receptor Inhibitors

Interleukin-6 (IL-6) is upregulated in various solid tumors or hematopoietic malignancies and plays a key role in the initiation and progression of many cancers via the IL-6/JAK/STAT3 pathway (102). Inhibitors targeting IL-6 or the IL-6 receptor have already been used for treating cancers, such as ovarian cancer and metastatic renal cell carcinoma (103, 104). In addition, overwhelmingly elevated IL-6 also plays a central role in cytokine release syndrome (CRS), which can progress quickly to ARDS (105–108). Emerging data indicate that up to 20% of COVID-19 cases develop into ARDS, which is the main cause of mortality in critical patients with COVID-19 (109, 110). Several studies reported that increased serum IL-6 levels were detected in patients with COVID-19 (9, 18) and could serve as an indicator for COVID-19 severity and in-hospital mortality (19, 111, 112). Thus, targeting the IL-6 signaling pathway is a potential therapeutic strategy to control CRS in COVID-19 patients. Tocilizumab, a humanized monoclonal antibody against the IL-6 receptor is currently being used for treating COVID-19 cases with CRS. In one retrospective study of 21 severe and critical COVID-19 patients, tocilizumab effectively improved clinical symptoms and reduced patient mortality without obvious adverse reactions (113). In another study of 100 consecutive patients with COVID-19 pneumonia and ARDS, tocilizumab produced rapid antihyperinflammatory efficacy and remarkable clinical improvement (114). However, the effectiveness of tocilizumab against CRS in the COVID-19 patient setting still needs additional evidence from large randomized, controlled clinical trials. Another humanized anti-human IL-6 receptor monoclonal antibody, sarilumab, and siltuximab, a chimeric antibody targeting IL-6, are currently being evaluated for treating COVID-19 patients with cytokine storm (110). In conclusion, a therapeutic strategy of blocking IL-6 or the IL-6 receptor may be considered a promising choice for the treatment of severe COVID-19 pneumonia and respiratory failure (**Table 2**).

Imatinib

Imatinib is an oral anticancer medication used for treating chronic myelogenous leukemia (CML), gastrointestinal stromal tumor (GIST), dermatofibrosarcoma protuberans (DFSPs), and acute lymphoblastic leukemia (ALL) (115). Imatinib plays an inhibitory role in some tyrosine kinase activities, including the oncogenic fusion protein BCR-ABL1 (whose overactivation can result in CML), c-kit (whose mutations are involved in GIST formation), platelet-derived growth factor receptor (PDGFR), and ABL1 kinase (116). In addition, imatinib also displays *in vitro* antiviral capacities against SARS-CoV and MERS-CoV, which are phylogenetically related to SARS-CoV-2 (20, 117). Therefore, imatinib has been postulated to possibly have antiviral function against SARS-CoV-2. In fact, a recent study showed that imatinib binds to the receptor-binding domain (RBD) of SARS-CoV-2 spike protein and inhibits virus replication *in vitro*, indicating imatinib as a potential repurposed drug candidate for COVID-19 treatment (118). In a clinical case report, a patient with COVID-19 pneumonia displayed clinical improvement after receiving imatinib treatment, whereas the clinical condition deteriorated upon hydroxychloroquine (HCQ) and lopinavir/ritonavir (LPV/r) therapy (119). Currently, several ongoing clinical trials are testing the value of imatinib as a promising treatment option for COVID-19 (**Table 2**). One clinical trial from France (ClinicalTrials.gov: NCT04357613) aims to assess the use of imatinib in aged hospitalized patients with COVID-19. One randomized double-blind trial from the United States (ClinicalTrials.gov: NCT04357613) is evaluating the safety and efficacy of imatinib compared with placebo for the treatment of hospitalized COVID-19 patients. Another randomized, double-blind, placebo controlled, clinical trial from Netherlands (EudraCT2020-001236-10) tries to investigate whether imatinib prevents pulmonary vascular leak in patients with Covid19.

Thalidomide

Thalidomide was originally given to expectant mothers to alleviate morning sickness between 1958 and 1962 but was later removed from the market due to its serious teratogenicity (120). However, research on the efficacy of thalidomide in other conditions, including cancer, continued, and thalidomide was recently approved by the FDA for treating multiple myeloma (121, 122). In addition, preclinical animal studies showed that thalidomide could alleviate lung injury, with reduced inflammation status and improved survival in mouse models of H1N1 influenza virus infection, indicating the potential therapeutic merit of thalidomide in viral infection (123). Intriguingly, a case report revealed that thalidomide presented an antiviral effect on one patient with COVID-19 (124). The patient with severe COVID-19 received oral thalidomide and low-dose methylprednisolone due to deteriorated clinical manifestations and limited response to other therapies. The patient achieved significant clinical improvement within 1 week of thalidomide treatment (124). However, since this is a single case report, additional clinical studies are needed to confirm the effectiveness of thalidomide and rule out any relevant severe side effects. One clinical trial (ClinicalTrials.gov: NCT04273581) aims to evaluate the efficacy and safety of thalidomide use in combination with low-dose hormones in the treatment of severe COVID-19. Another clinical trial (ClinicalTrials.gov: NCT04273529) is investigating the use of thalidomide in the treatment of patients with moderate COVID-19 pneumonia. Currently, these two clinical trials are still underway evaluating thalidomide therapy in patients with moderate or severe COVID-19 (**Table 2**).

Bevacizumab

Vascular endothelial growth factor (VEGF) has been identified as a key molecule in the process of endothelial injury and increases microvascular permeability (125). Higher VEGF levels were observed in COVID-19 patients with ARDS than in healthy people (126). Therefore, VEGF is considered a potential therapeutic target in COVID-19 patients with acute lung injury

(ALI) and ARDS. Bevacizumab, a recombinant humanized anti-VEGF monoclonal antibody, is widely used to treat a number of types of solid malignancies, including lung cancer, colon cancer, glioblastoma, and renal-cell carcinoma (127), and is now being evaluated for treating severe or critical patients with COVID-19 pneumonia (**Table 2**). The result of one clinical trial (ClinicalTrials.gov: NCT04275414) indicated that bevacizumab plus standard care showed remarkable efficacy for treating severe COVID-19 patients (128).

CURRENT DIAGNOSTIC AND THERAPEUTIC CHALLENGES IN CANCER PATIENT CARE DURING THE COVID-19 PANDEMIC

Due to the current COVID-19 pandemic, healthcare professionals are facing the overwhelming challenges of rapidly increasing new infection cases, not only to effectively cope with the COVID-19 crisis but also to do so without overlooking the care of patients with other diseases, such as cancer. Cancer patients are more vulnerable to COVID-19 infection and more likely to develop serious events than non-cancer COVID-19 patients due to the immunosuppressive state caused by the cancer itself and anticancer treatments (129–131). Specifically, the rates of severe events in COVID-19-infected patients with hematologic cancer, lung cancer, and metastatic cancers were higher than those in patients without cancer (130). Cancer patients who received surgical or chemotherapy treatments exhibited higher mortality rates and a higher possibility of developing critical symptoms (129, 130). Thus, it is important for oncologists to determine how to properly diagnose and treat cancer patients in this COVID-19 era.

It can be challenging to diagnose whether cancer patients are infected with COVID-19 because some common symptoms of SARS-CoV-2 infection, including fever, dry cough, and shortness of breath, may also be caused by various kinds of cancer. Patients with central-type lung cancer or multiple lung metastases can develop respiratory distress, which often occurs in severe and critical COVID-19 patients (132, 133). Notably, interstitial infiltrate pneumonia displayed by cancer patients who underwent radiotherapy or immune-checkpoint inhibitor treatment could overlap with the symptoms and CT scan characteristics of COVID-19 patients (134–136). Intriguingly, recent studies showed that the levels of some cancer markers, including carcinoembryonic antigen (CEA), carbohydrate antigens (CA) 125 and 153, squamous cell carcinoma antigen (SCCA), and cytokeratin-19 fragment (CYFRA21-1), were elevated in COVID-19 patients and were correlated with the severity of COVID-19 (137, 138).

During the COVID-19 epidemic, medical resources focused on combating COVID-19, fear of nosocomial infection and social distancing led to delay of the daily treatment for cancer patients. For uninfected cancer patients, most nonemergency surgery, intravenous chemotherapy and radiotherapy have been suspended (139). Nonetheless, it is pivotal to maintain medical and surgical treatments for cancer patients (140). Modified management including thorough COVID-19 screening for every cancer patient scheduled for operations, reduced hospital stay, and establishment of virtual connection between patients and their relatives can help reduce cross infection and facilitate safe surgical treatments (140). Many oncologists also use online follow-ups, and switch to oral chemotherapy rather than intravenous administration (141). For elective cancer surgery, COVID-19-free surgical pathways were related with lower pulmonary complication rates, SARS-CoV-2 infection rates, and mortality rates compared with no defined pathway (142). The establishment of COVID-19-free surgical pathways, which provides elective surgery, critical care, and inpatient ward care with no shared areas with COVID-19 patients, is paramount during COVID-19 pandemic (142). Of note, Silvia Fiorelli et al. highlighted the importance that lung cancer patients should continue to receive prompt surgical treatment, and upgraded management strategy is needed for the surgical treatment, patient selection and perioperative management (143). Based on appropriate patient screening and improved precautions, no COVID-19 positive cases were recorded among the medical staff or the hospitalized patients during their hospital stay. Their high-volume thoracic surgery center has successfully maintained safe surgical treatment for lung cancer patients (143). For cancer patients with COVID-19 coinfection, whether to continue antitumor therapy is still controversial. A stable lung cancer patient died rapidly with a history of long exposure to nivolumab immunotherapy (144), but it has also been reported that it is safe to continue targeting in mild cases (145). However, because antitumor therapy will further weaken the immune system and the short-term risk brought by COVID-19 is much higher than the risk of tumors, antitumor therapy for COVID-19-positive cancer patients still needs to be very cautious.

APPLICATIONS OF ORGANOID TECHNOLOGY IN COVID-19

Organoids are 3D structures that can be generated from adult tissue-specific stem cells, embryonic stem cells, or induced pluripotent stem cells and recapitulate pivotal features of original tissues (146, 147). Organoids provide unique opportunities for modeling and studying human diseases, including congenital and acquired conditions, to establish paradigms for pathogenesis research, high-throughput drug screening, and living organoid biobanks of specific diseases, facilitating personalized treatments (148–150). Cancer patient-derived organoids have been widely used to investigate the mechanism of tumorigenesis and for personalized medicine approaches (151). More importantly, organoids have proven to be ideal models to investigate infectious diseases and the related pathogenic mechanisms (148). Ettayebi et al. successfully modeled human norovirus (HuNoV) infection and propagation using human small intestinal organoids and identified that bile acts as a critical factor for HuNoV replication (152). Similarly, intestinal, lung, gastric, and brain organoids have been applied to model infectious diseases, including Cryptosporidium (153), Middle East respiratory syndrome coronavirus (154), *Helicobacter pylori* (155, 156), influenza virus

(157), and Zika virus (158, 159) infections, enabling a better understanding of virus-host interactions, virus pathogenesis and virus transmission.

Currently, limited knowledge of SARS-CoV-2 pathogenesis and transmission is mainly based on clinical features, bioinformatic analysis, and rare autopsy reports (9, 160, 161), in part due to the lack of appropriate *in vitro* cell research models that faithfully resemble host tissues. Therefore, human organoids have been recently adopted by several research groups to investigate the mechanisms of SARS-CoV-2 infection and virus-induced tissue damage (17, 77, 161, 162). Human liver ductal organoids were employed to investigate the infection and liver damage of SARS-CoV-2 and have enabled the identification of liver damage caused directly by viral infection (161). Along the same lines, it has been proven that SARS-CoV-2 can readily infect human intestinal enterocytes, and the host cell membrane-bound serine proteases TMPRSS2 and TMPRSS4 promote the infection process, which indicates that human small intestinal organoids serve as a faithful experimental model for the study of SARS-CoV-2 infection and relevant biology, facilitating future drug testing (17, 162–164). Remarkably, SARS-CoV-2 has been shown to directly infect engineered human blood vessel organoids and kidney organoids, which can be blocked by human recombinant soluble ACE2 (hrsACE2) at early stages of SARS-CoV-2 infection (77).

Since SARS-CoV-2 was reported to affect multiple human organs and the underlying mechanisms are still unclear (16), human organoids of the intestinal, lung, kidney, liver, stomach, retinal, brain, and cardiac systems can be leveraged to study pathogenesis in an organ-specific manner (146, 165). In addition, organoid platforms have facilitated personalized drug screening for cancer (146, 166, 167); hence, organoids can also be applied for high-throughput drug screening to discover potential candidates against COVID-19 (**Figure 2**). Recently, several groups have used organoid-pathogen-immune cell coculture systems to study host–pathogen interactions (168, 169). Organoids were infected with microorganisms (viral or bacterial) before culturing together with immune cells in the triple coculture system (170). In this setting, organoids provide great opportunities to probe the interaction between the epithelium, immune system and SARS-CoV-2 and enable potentially new therapeutic targets for treatment. Further organoid studies for dissecting the pathogenesis of COVID-19 are bound to enable improved understanding and potential drug discoveries (**Figure 2**).

COVID-19 VACCINES

Vaccination can efficiently elicit human immunity to prevent infection and disease dissemination, thus helping restrain the SARS-CoV-2 crisis. Multiple methods have been used to generate clinical vaccine candidates for SARS-COV-2, including mRNA vaccines, DNA vaccines, viral vector vaccines, and inactivated virus vaccines (171). Several studies have shown promising immune response inductions and no adverse safety events in Phase III clinical trials (172–175). Currently, over sixty COVID-19 vaccines are being tested in clinical trials, with eleven approved for at least limited use (176). Food and Drug Administration (FDA) have granted three highly effective COVID-19 vaccines for EUAs, including two mRNA vaccines from Pfizer-BioNtech and Moderna, and one adenovirus type 26 (Ad26) vaccine from Johnson & Johnson (177, 178). The two mRNA vaccines require two doses, and second dose should be given within 3 weeks of the first dose for the Pfizer-BioNtech vaccine and within 4 weeks for the Moderna vaccine. Both two mRNA vaccines require ultracold storage, making it harder to distribute. The Ad26 vaccine from Johnson & Johnson is the first single-dose COVID-19 vaccine, and has the advantage of being stable at refrigeration temperature (178). Nonetheless, it still takes time for most people to receive the COVID-19 vaccines. And questions also arise around the safety and effectiveness of COVID-19 vaccines in the setting of cancer patients and elderly population. More researches addressing these unclear issues are needed to identify whether cancer patients and elderly people could benefit from COVID vaccines.

LIMITATIONS OF THIS REVIEW

Several limitations also exist in this review. Firstly, we have cited some preprints in the references, because these papers are still under review or awaiting for publication in official journals. Since these preprints have not been peer reviewed, some interpretations and conclusions from them may need further validation. Secondly, we only discussed the diagnostic and therapeutic challenges in cancer patient care in the COVID-19 era. But some patients with autoimmune diseases or organ transplants are also more vulnerable than healthy people. The diagnostic and therapeutic management of these patients is also noteworthy. Lastly, although there are a great number of important papers, ongoing clinical studies and trials, we can only refer to the most important ones in this review based on our limited knowledge.

CONCLUSIONS AND FUTURE PERSPECTIVES

How to appropriately manage patients with COVID-19 remains a rapidly evolving preventative and therapeutic challenge. And the efficacy and safety of vaccination in cancer patients or elderly people remain unclear. Therefore, doctors are still urgently seeking existing drugs repurposed for treating COVID-19. Although several therapeutic agents mentioned above in this review are encouraging for treating patients with COVID-19, the clinical trials evaluating definite efficacy and risk of adverse events are still underway. Several guidelines of COVID-19 including IDSA (Infectious Diseases Society of America) guidelines, WHO living guidance, COVID-19 rapid guideline, and CDC (Centers for Disease Control and Prevention) guidelines are important references in terms of diagnosis, treatment, prevention of COVID-19 (62, 179–181). In addition, clinical doctors should continually monitor and

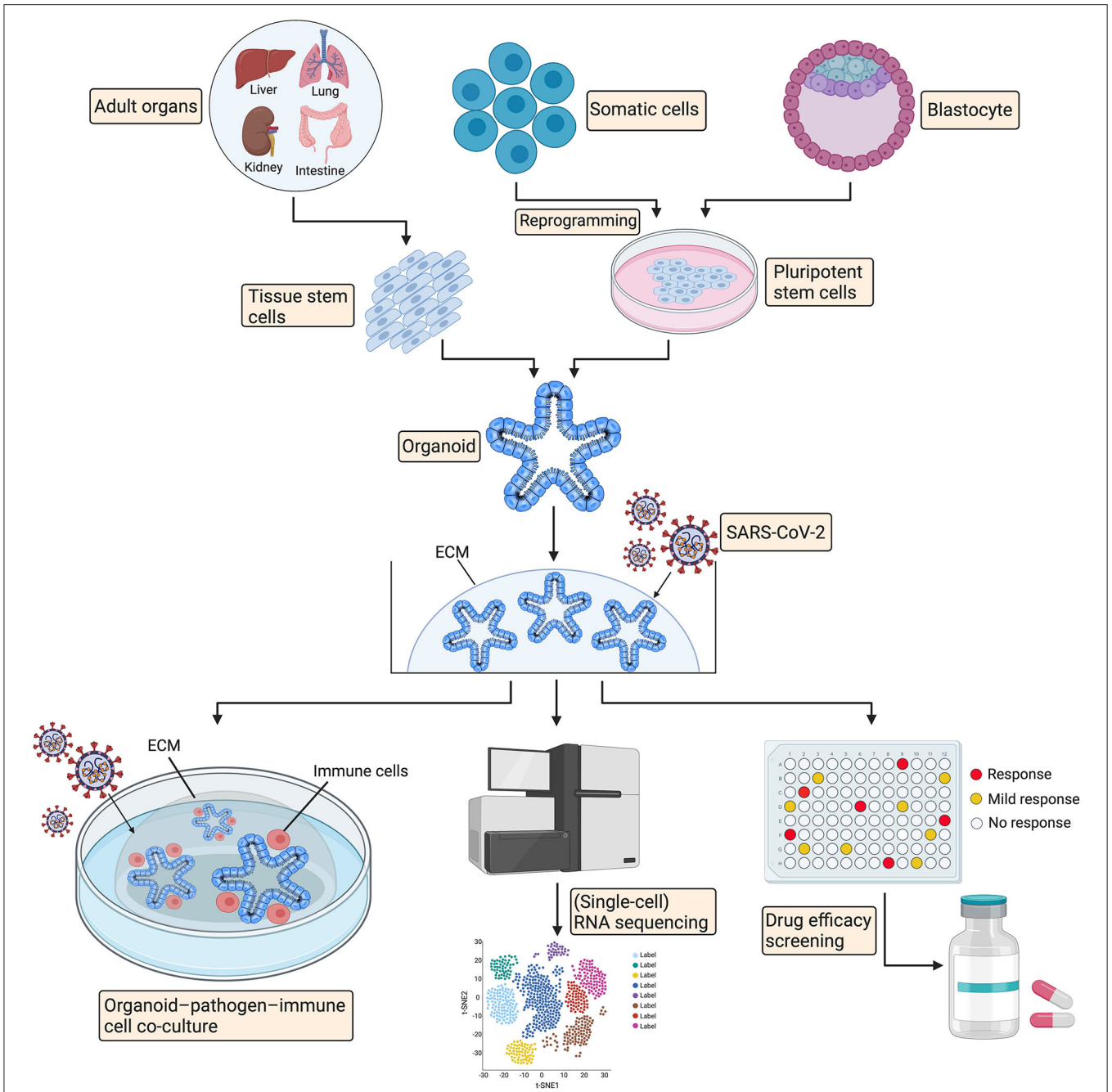

FIGURE 2 | Promising applications of organoid technology in COVID-19. Organoids can be established from adult tissue stem cells, induced pluripotent stem cells, or alternatively, from embryonic stem cells. Organoids can be utilized for pathogenesis investigation via organoid-pathogen-immune cell coculture system and RNA sequencing. Organoids can also facilitate high-throughput drug screening for COVID-19 treatment. ECM, extracellular matrix.

adjust management strategies as new literature becomes available. However, caution should be taken when interpreting the available clinical data, since many studies are uncontrolled and have not been peer reviewed.

The COVID-19 outbreak challenges oncologists to properly protect cancer patients, who are assumed to be vulnerable to SARS-CoV-2 infection, without jeopardizing the management of cancer treatment. However, there are still multiple unknowns about how to manage cancer patients who might be exposed to potential infection or may have been infected with SARS-CoV-2. It is important to determine whether COVID-19 would negatively influence active cancer therapies and whether antineoplastic treatments might prevent the cytokine storm caused by SARS-CoV-2. Additionally, data about whether tumor

stages and disease status have an impact on COVID-19's interactions with cancer and cancer treatments are lacking. Thus, well-designed, multicentered, prospective cohort studies are required to solve these complex COVID-19 puzzles for cancer patients.

Management of highly contagious and potentially fatal COVID-19 has underscored the urgent need to develop efficient diagnosis methods, specific antiviral therapies or vaccines to fight against SARS-CoV-2. In the current era in which cutting-edge technological methods are available, it is pivotal for us to make collaborative efforts to translate basic and innovative science into the discovery of optimal diagnostic and therapeutic options for clinical applications.

AUTHOR CONTRIBUTIONS

CY, LQ, and JW conceived this review and collected the literature. CY and JW drew the schematic diagram. CY and LQ prepared the tables and wrote the manuscript. SZ conducted the study supervision and revised the manuscript. All authors read and approved the final manuscript.

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BRIEF RESEARCH REPORT
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The Association Between Alpha-1 Adrenergic Receptor Antagonists and In-Hospital Mortality From COVID-19

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Effective therapies for coronavirus disease 2019 (COVID-19) are urgently needed, and pre-clinical data suggest alpha-1 adrenergic receptor antagonists (α_1-AR antagonists) may be effective in reducing mortality related to hyperinflammation independent of etiology. Using a retrospective cohort design with patients in the Department of Veterans Affairs healthcare system, we use doubly robust regression and matching to estimate the association between baseline use of α_1-AR antagonists and likelihood of death due to COVID-19 during hospitalization. Having an active prescription for any α_1-AR antagonist (tamsulosin, silodosin, prazosin, terazosin, doxazosin, or alfuzosin) at the time of admission had a significant negative association with in-hospital mortality (relative risk reduction 18%; odds ratio 0.73; 95% CI 0.63–0.85; $p \leq 0.001$) and death within 28 days of admission (relative risk reduction 17%; odds ratio 0.74; 95% CI 0.65–0.84; $p \leq 0.001$). In a subset of patients on doxazosin specifically, an inhibitor of all three alpha-1 adrenergic receptors, we observed a relative risk reduction for death of 74% (odds ratio 0.23; 95% CI 0.03–0.94; $p = 0.028$) compared to matched controls not on any α_1-AR antagonist at the time of admission. These findings suggest that use of α_1-AR antagonists may reduce mortality in COVID-19, supporting the need for randomized, placebo-controlled clinical trials in patients with early symptomatic infection.

Keywords: COVID-19, coronavirus disease, alpha-1-adrenergic receptor antagonist, infectious disease, off-label drug use

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INTRODUCTION

The viral replication phase in Coronavirus disease 2019 (COVID-19) can be followed by a hyperinflammatory host immune response, hereafter referred to as COVID-19-associated hyperinflammation, which can lead to acute respiratory distress syndrome (ARDS), multiorgan dysfunction, and death despite maximal supportive care (1–4). While dexamethasone and other immunosuppressive strategies have shown some promise in improving outcomes in patients with severe COVID-19, they have not shown benefit (and may be detrimental) when given to patients with less advanced disease (5–7). To date, immunomodulatory therapeutic strategies that prevent the development of hyperinflammation and thereby halt progression to severe COVID-19 do not exist.

Catecholamines (adrenaline, noradrenaline, and dopamine) are monoamine hormones that signal through adrenergic receptors (ARs) expressed on tissues including cells of the immune system (8–10). Cells of the innate and adaptive immune system (phagocytes, lymphocytes) are capable of producing catecholamines *de novo* and signal in an autocrine/paracrine self-regulatory fashion (9, 11). Beyond their well-established role in neurotransmission and physiological fight-or-flight responses, catecholamines have been shown to amplify immune responses and enhance acute inflammatory injury *in vitro* and *in vivo* by increasing cytokine production in immune cells (e.g., IL-6, TNF-α, MIP-2) (8, 10–12). In animal models of hyperinflammation, prophylactic treatment with an alpha-1 adrenergic receptor (α_1-AR) antagonist that inhibits all three receptor subtypes (α_{1A}-,α_{1D}-, and α_{1B}-AR) can prevent cytokine storm and death by blocking deleterious catecholamine signaling and immune responses (11). In a retrospective analysis of patients hospitalized with acute respiratory distress, patients incidentally taking any α_1-AR antagonist had a 34% relative risk reduction of being mechanically ventilated and dying ($n = 16{,}801$, odds ratio 0.70) compared to non-users (13). Similarly, the risk of progression to mechanical ventilation and death was significantly reduced in a retrospective analysis of >300,000 patients hospitalized with pneumonia who were prescribed α_1-AR antagonists prior to their index admission, suggesting that baseline inhibition of catecholamine signaling may improve clinical outcomes in acute lower respiratory tract infection or inflammation (13). We therefore hypothesized that early treatment with α_1-AR antagonists can improve mortality and ameliorate disease in patients with symptomatic SARS-CoV-2 infection (14), but data demonstrating the efficacy of α_1-AR antagonists in COVID-19 specifically is lacking.

The objective of this study was to examine the association of use of α_1-AR antagonists with in-hospital mortality in patients with COVID-19. Here, we analyzed a large cohort of patients hospitalized at Veterans Health Administration (VA) hospitals, in whom α_1-AR antagonists are commonly used to treat unrelated diseases such as benign prostatic hyperplasia (BPH), post-traumatic stress disorder (PTSD), or arterial hypertension (15). We hypothesized that patients with COVID-19 taking α_1-AR antagonists at the time of hospital admission would be less likely to die during their hospitalization.

METHODS

Study Population and Variables

We included all patients admitted to a VA hospital between February 20, 2020, and October 7, 2020 with a confirmed COVID-19 diagnosis (**Figure 1**). Diagnosis codes for COVID-19 were identified from the Centers for Disease Control and Prevention (CDC) coding guidelines for COVID-19 (16, 17). The VA COVID-19 Shared Data Resource was used to identify VA patients with a SARS-CoV-2 laboratory test result (18). This data resource combines VA-specific lab results with non-VA lab results using text extraction from patient medical records. Because over 90% of α_1-AR antagonist users in the analysis were older men, we excluded women to reduce unmeasured confounding unrelated to COVID-19, specifically with respect to respiratory conditions. We also excluded patients under age 45 and patients over age 85 given the strong relationship between the severity of COVID-19 and age.

An expanded sample included all patients with laboratory-confirmed, "suspected positive," or "possible positive" COVID-19 according to National COVID Cohort Collaborative (N3C) criteria (19). This Suspected COVID-19 sample excluded patients who tested negative for SARS-CoV-2. To the extent we can measure COVID-19 severity at time of admission, we find that this cohort was not operationally different from the main cohort based on vital signs at time of admission (**Supplementary Figure 1**).

The primary outcomes were death during the index hospitalization and death within 28 days of admission. The primary exposure variable was the use of α_1-AR antagonists at the time of admission for the index hospitalization. Active prescriptions of α_1-AR antagonists (tamsulosin, silodosin, prazosin, doxazosin, alfuzosin, and terazosin) were identified and defined by the patient having medication on hand on the day of the index admission, regardless of dosage. Secondary analyses examined the effect of tamsulosin (the most commonly prescribed α_1-AR antagonist with selective antagonism on α_{1A}- and α_{1D}-, but not α_{1B}-ARs) and doxazosin (a non-selective antagonist acting on all three α_1-ARs) individually. Finally, with in-hospital therapies evolving during the pandemic, we repeated the analysis by week and VA hospital to ensure results were not driven by any particular time or location.

We obtained data on patient demographics, vital signs, and prescription drugs from the VA's corporate data warehouse (CDW). Patient comorbidities were captured based on the International Classification of Diseases, Version 10 codes from VA care in the year prior to index admission. Other physiologic variables, including oxygen saturation and temperature, were defined at time of inpatient admission.

Analysis

Analyses followed the methodology of a companion paper examining patients with acute respiratory distress and pneumonia (13). Unadjusted analysis compared patients with α_1-AR antagonist prescriptions to all other patients with COVID-19 using Fisher's exact-test. We then estimated propensity scores and trimmed the sample to ensure overlap in

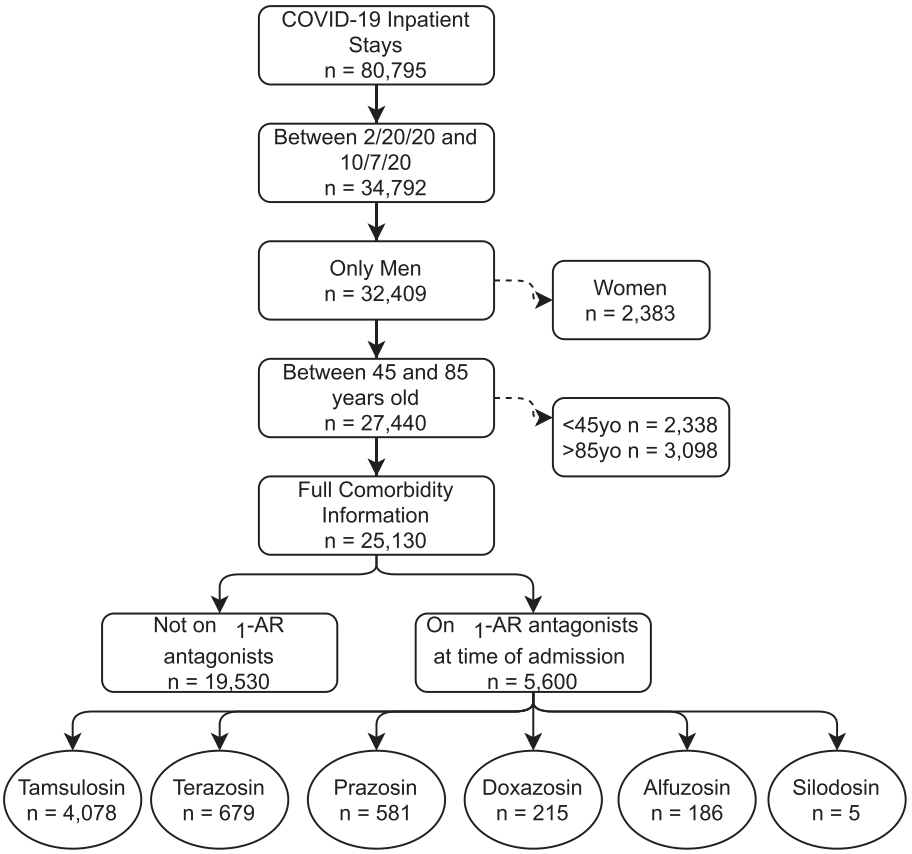

FIGURE 1 | CONSORT Flow Diagram. Consort diagram. Note that the bottom row of medications are not mutually exclusive, with a small number of patients having more than one on hand at time of admission.

the propensity score distributions of the exposed and unexposed groups. On this reduced sample, the adjusted analysis used inverse propensity-weighted logistic regression adjusting for patient age at admission (input as a demeaned cubic polynomial to allow a non-linear relationship), calendar week, location of hospitalization, and comorbidities diagnosed any time in the two years prior to the index inpatient stay. This approach is "doubly robust" in that it uses the observed confounders in both the calculation of the propensity score and the odds ratios. Comorbidities included in the matching procedure were diabetes mellitus, arterial hypertension, heart failure, ischemic heart disease, acute myocardial infarction, chronic obstructive pulmonary disease (COPD), end-stage renal disease (ESRD), and PTSD. We also included an indicator variable for oxygen saturation under 94 percent on the day of admission.

All of the control variables reflect information on patients prior to admission with COVID-19. As noted above, we controlled for secular changes in COVID-19 care using calendar week, starting with February 20, 2020. We chose not to examine endpoints during the hospital stay, such as use of a ventilator or admission to the ICU, given this is based on physician coding or data structures that we cannot assure were handled uniformly, especially during surges. We also chose not to control for processes of care during the stay given this could introduce bias in the analysis.

We then conducted a 5:1 matched analysis using the same covariates as the adjusted model (10). This approach assigns each exposed patient to a set of five unexposed patients most similar on observed characteristics and does not make assumptions about the functional form of the potential relationship between confounders and the outcome. Matches were selected using a greedy, nearest-neighbor approach based on Mahalanobis distance (11). The matched analysis used the Cochran-Mantel-Haenszel test to obtain odds ratios, confidence intervals, and *p*-values. We also present relative risk reductions (RRR) for the matched cohorts, and the pre- and post-matching balance of covariates is shown in **Figure 2**.

RESULTS

Sample Characteristics

The sample contained 25,130 patients with COVID-19, with 5,600 patients taking any α_1-AR antagonist at time of admission. Of those taking α_1-AR antagonists, 73% of patients were on tamsulosin (n = 4,078), 12% on terazosin (n = 679), 10% on prazosin (n = 581), 4% on doxazosin (n = 215), 3% on

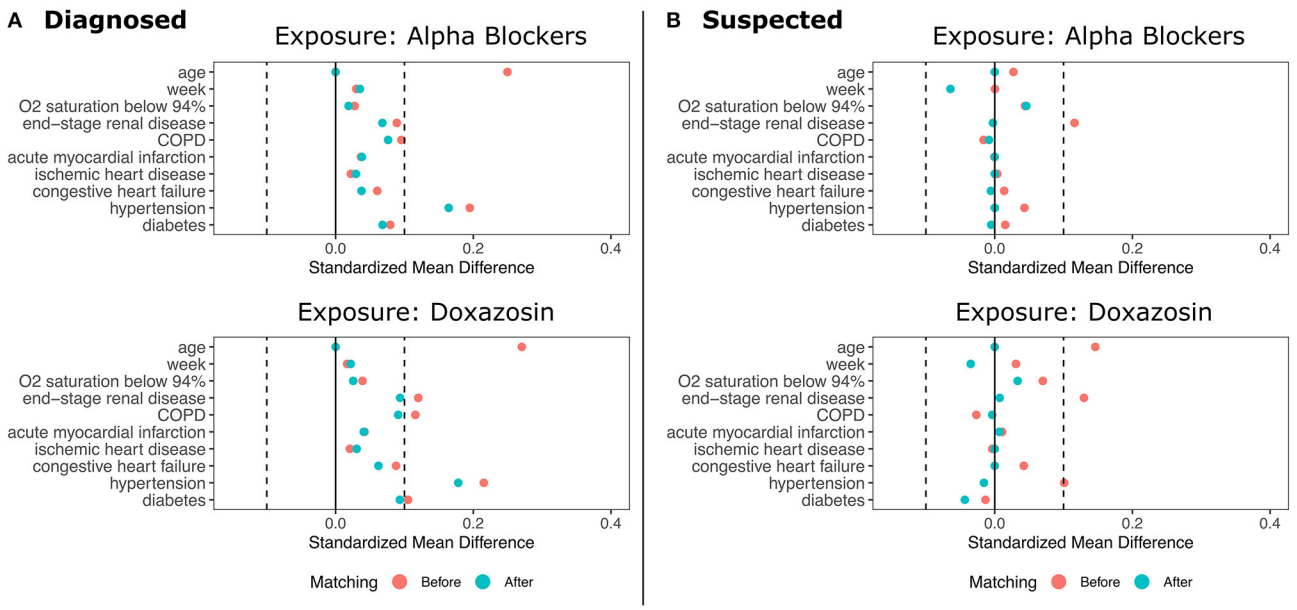

FIGURE 2 | Standardized Mean Differences in Patient Characteristics Before and After Matching. **(A)** shows the results for patients diagnosed with COVID-19. **(B)** shows the results for patients diagnosed with COVID-19 plus suspected COVID-19 patients. Top panel show data for any α_1-AR antagonists; bottom panels show data for doxazosin.

alfuzosin (n = 186), and <1% were on silodosin (n = 5) (**Figure 1**). One hundred and seventy-seven patients had active prescriptions for more than one α_1-AR antagonist at the time of admission. Demographic characteristics, medical comorbidities, and Charlson Comorbidity Index for patient groups prior to matching are shown in **Table 1**. The differences in sample characteristics after matching are summarized in **Figure 2**.

Risk of In-Hospital and 28-Day Mortality

For all patients admitted to VA hospitals between February 20, 2020, and October 7, 2020, the overall in-hospital mortality rate was 2.5%. Among hospitalized patients with confirmed COVID-19 (8.9% of all admissions), in-hospital mortality was 6% overall and 5.5% in our sample. Patients with confirmed COVID-19 taking any α_1-AR antagonist, compared to non-users, had an 18% relative risk reduction for death during their hospitalization (243/5,309 = 4.6% in matched treatment group vs. 984/17,538 = 5.6% in matched control group, $p \leq 0.001$, **Figure 3**) and a 17% relative risk reduction for death within 28 days from the date of admission (331/5,309 = 6.2% in matched treatment group vs. 1,318/17,538 = 7.5% in matched control group, $p \leq 0.001$, **Figure 3**).

The top panel of **Figure 3** shows the unadjusted, propensity score adjusted, and matched odds ratios among patients diagnosed with COVID-19 (n = 25,130). The bottom panel expands the denominator to also include patients with suspected COVID-19 (n = 32,016). The dark green odds ratios in **Figure 3** represent all α_1-AR antagonists, while the lighter green represent doxazosin. Results were similar for the suspected COVID-19 sample. Patients taking any α_1-AR antagonists, compared to non-users, had an 20% relative risk reduction for death ($p \leq 0.001$) in this cohort (**Figure 3**).

The use of doxazosin, a non-selective α_1-AR antagonist targeting all three α_1-AR subtypes, resulted in a 74% relative risk reduction for death in hospitalized patients with COVID-19 during the index admission (2/155 = 1.3% in matched treatment group vs. 39/775 = 5.0% in matched control group, odds ratio for death 0.23; p = 0.028, **Figure 3**). Use of tamsulosin, the most commonly prescribed α_1-AR antagonist in this cohort with selectivity for α_{1A}- and α_{1D}-ARs, was associated with a 18% relative risk reduction for death during the inpatient stay (odds ratio for death 0.77; p = 0.002, **Supplementary Figure 2**). Even though COVID-19 has affected different parts of the United States at different times, we found no evidence that these results were driven by any particular time period or location (**Supplementary Figures 3**, **4**).

DISCUSSION

In this retrospective analysis of patients with COVID-19, we found a significant negative association between the use of α_1-AR antagonists and in-hospital or 28-day mortality. These results are consistent with findings from a recent retrospective study of >300,000 patients hospitalized with pneumonia or ARDS unrelated to SARS-CoV-2 infection that identified a significant risk reduction for the progression to mechanical ventilation and death in individuals who were receiving any α_1-AR antagonists as compared to non-users (5), suggesting that the benefits of α_1-AR inhibition for mortality may be independent of etiology in patients with lower respiratory tract infection or inflammation.

Interestingly, we found much larger effect sizes in reducing mortality for patients treated with doxazosin, an antagonist on all three α_1-AR subtypes (α_{1A}-, α_{1D}-, and α_{1B}-AR), than for a

TABLE 1 | Patient and sample characteristics at time of admission.

	Control (n = 19,316)	Any 1α-AR antagonist(n = 5,600)	Overall (n = 25,130)
Age			
Mean (SD)	67.4 (9.02)	70.4 (7.83)	68.1 (8.85)
Median (Min, Max)	69.0 (45.0, 85.0)	72.0 (45.0, 85.0)	70.0 (45.0, 85.0)
Comorbidities in the prior year			
Hypertension: *n* (%)	15,603 (79.9%)	4,955 (88.5%)	20,558 (81.8%)
CAD: *n* (%)	776 (4.0%)	283 (5.1%)	1,059 (4.2%)
CHF: *n* (%)	5,611 (28.7%)	1,866 (33.3%)	7,477 (29.7%)
COPD: *n* (%)	6,495 (33.2%)	2,284 (40.8%)	8,779 (34.9%)
Diabetes: *n* (%)	9,695 (49.6%)	3,076 (54.9%)	12,771 (50.8%)
MI: *n* (%)	1,347 (6.9%)	448 (8.0%)	1,795 (7.1%)
BPH: *n* (%)	4,989 (25.5%)	4,412 (78.8%)	9,401 (37.4%)
PTSD: *n* (%)	4,199 (21.5%)	1,661 (29.7%)	5,860 (23.3%)
ESRD: *n* (%)	5,902 (30.4%)	2,063 (37.1%)	7,965 (31.9%)
Charlson Comorbidity Index: mean (SD)	4.00 (3.45)	4.87 (3.53)	4.47 (3.48)
SpO_2 <94%: *n* (%)	5,770 (29.5%)	1,706 (30.5%)	7,476 (29.7%)
VA Hospital			
508 (Atlanta, GA)	390 (2.0%)	113 (2.0%)	503 (2.0%)
528 (VA Upstate New York, NY)	346 (1.8%)	100 (1.8%)	446 (1.8%)
541 (Cleveland, OH)	335 (1.7%)	91 (1.6%)	426 (1.7%)
549 (Dallas, TX)	393 (2.0%)	136 (2.4%)	527 (2.1%)
573 (Gainesville, FL)	384 (2.0%)	134 (2.4%)	518 (2.1%)
580 (Houston, TX)	525 (2.7%)	175 (3.1%)	700 (2.8%)
589 (Kansas City, MO)	421 (2.2%)	171 (3.1%)	592 (2.3%)
614 (Memphis, TN)	827 (4.2%)	264 (4.7%)	1,092 (4.3%)
626 (Nashville, TN)	471 (2.4%)	131 (2.4%)	602 (2.4%)
630 (VA New York Harbor, NY)	458 (2.3%)	104 (1.9%)	562 (2.2%)
636 (Omaha, NE)	286 (1.4%)	88 (1.6%)	374 (1.5%)
644 (Phoenix, AZ)	413 (2.1%)	105 (1.9%)	518 (2.1%)
657 (St Louis, MO)	388 (2.0%)	94 (1.7%)	482 (1.9%)
671 (San Antonio, TX)	509 (2.6%)	119 (2.1%)	628 (2.5%)
673 (Tampa, FL)	413 (2.1%)	117 (2.1%)	530 (2.1%)
Other VA hospitals	12,973 (66.4%)	3,657 (65.2%)	16,630 (66.1%)

CAD, coronary artery disease; CHF, congestive heart failure; COPD, chronic obstructive pulmonary disease; MI, acute myocardial infarction; BPH, benign prostatic hyperplasia; PTSD, post-traumatic stress disorder; ESRD, end-stage renal disease. SpO_2 < 94% refers to an oxygen saturation reading below 94% on admission. PTSD was excluded from the adjusted analysis due to collinearity with other comorbidities. Listed VA hospitals had the most COVID-19 inpatient hospitalizations during the study period.

pooled population of patients treated with any α_1-AR antagonist in whom tamsulosin was the most common drug (72%). This was similarly true for patients treated exclusively with tamsulosin, a "uroselective" α_1-AR antagonist on α_{1A}- and α_{1D}-ARs without clinically relevant inhibition of α_{1B}-ARs expressed by immune cells and the peripheral vasculature (20). In patients with test-confirmed COVID-19, baseline use of doxazosin was associated with significantly reduced in-hospital and 28-day mortality compared to controls (odds ratio for death during admission 0.19 in adjusted cohort; odds ratio and relative risk reduction for death 0.23 and 74% in matched cohort, respectively). Baseline use of tamsulosin in patients with confirmed COVID-19, by comparison, was associated with significant, but less pronounced reductions in mortality. A similar trend was previously observed in patients with pneumonia in whom use of doxazosin was associated with lower risk of mechanical ventilation and death than tamsulosin (13). These observed differences in effect size are biologically plausible and may reflect the distinct pharmacological selectivity of doxazosin and tamsulosin for α_1-AR subtypes.

Immune cells can induce expression of all three α_1-AR subtypes (i.e., α_{1A}-, α_{1D}-, and α_{1B}-ARs (21), and catecholamine signaling through these individual receptors may be highly redundant (12). As such, α_1-AR antagonists acting on all three receptor subtypes (i.e., doxazosin, prazosin, alfuzosin, terazosin) may be required to effectively interrupt autocrine and paracrine catecholamine signaling in monocytes and other immune cells that enhance inflammatory injury (14, 20). Indeed, pre-clinical data suggests that non-selective α_1-AR antagonists are effective in preventing hyperinflammation and death in animal models of cytokine storm syndrome (11). The markedly improved survival in patients on doxazosin as compared to

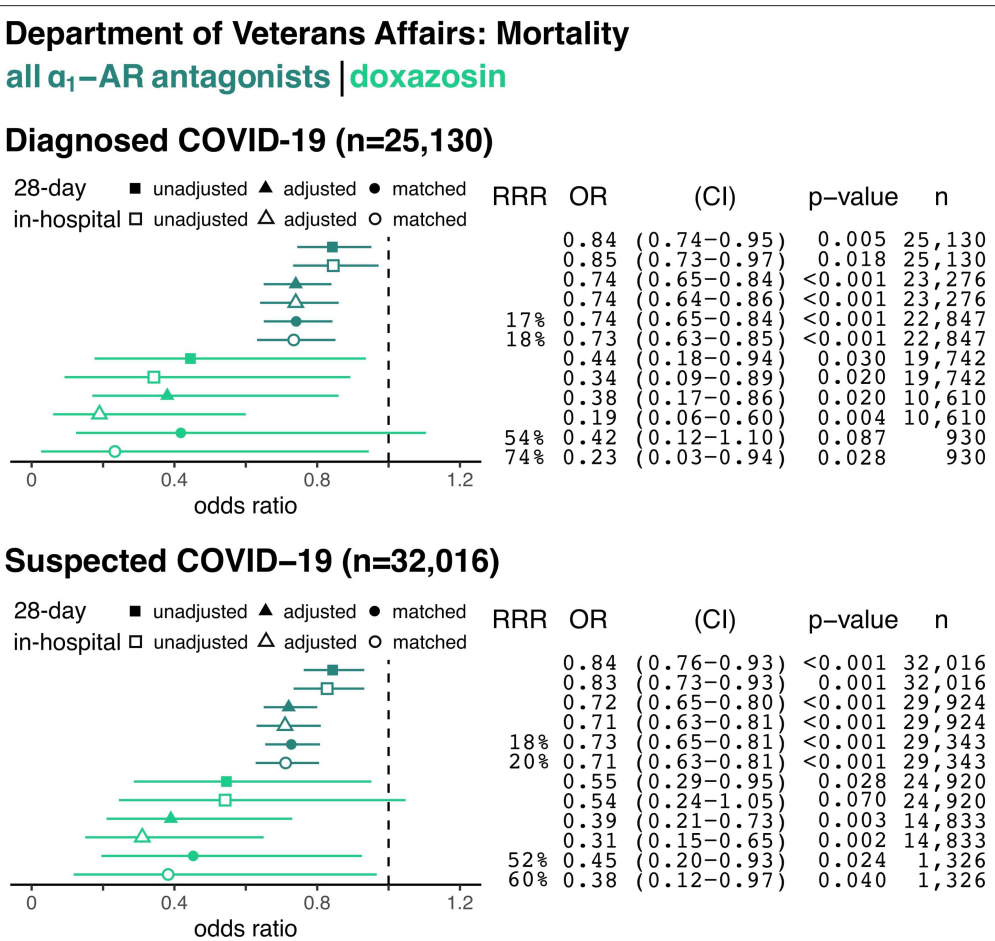

FIGURE 3 | The Association Between Alpha-1 Adrenergic Receptor Antagonists and In-Hospital and 28-Day Mortality from COVID-19. Data are shown for hospitalized patients with confirmed COVID-19 (top panel) and with confirmed plus suspected COVID-19 (e.g., no confirmatory testing available, bottom panel). Forest plots show the odds ratios (OR) for in-hospital mortality based on prior use of any α_1-AR antagonists (i.e., tamsulosin, silodosin, prazosin, terazosin, doxazosin, or alfuzosin; dark green) or only doxazosin (light green) in each panel. Unadjusted (square), adjusted model (triangle), and matched model (circle) analyses are shown for each sample group. Filled symbols reflect the odds of death within 28 days from index hospital admission (including deaths after discharge), whereas empty symbols reflect odds of death during the index admission. Relative risk reduction (RRR), odds ratios (ORs) for death, 95% confidence intervals (CI), *p*-values, and sample size (*n*) for each analysis are shown on the right.

tamsulosin or any α_1-AR antagonist (a cohort highly enriched in tamsulosin use) may therefore be consistent with a redundancy in catecholamine signaling pathways which are globally inhibited by doxazosin, whereas tamsulosin allows for continued signaling through the α_{1B}-AR. These findings have practical implications for the selection of α_1-AR antagonists for the prevention of inflammatory injury and suggest that the immunomodulatory benefits may not be uncoupled from inhibition of α_{1B}-ARs expressed on the peripheral vasculature.

Additional studies have explored the efficacy of α_1-AR blockade in the prevention of inflammatory and autoimmune injury. In a model of encephalitis, early α_1-AR inhibition reversed neutrophil infiltration in lungs and prevented hemorrhagic pulmonary edema (22). The non-selective α_1-AR antagonist prazosin has been shown to ameliorate experimental autoimmune encephalomyelitis (23). In a pre-clinical model of ischemia-reperfusion injury, prazosin administration resulted in decreased expression of IL-6, TNF-α, IL-10, and IL-1, and prevented mortality (24). Finally, α_1-AR antagonism has been shown to block cytokine production in human peripheral blood mononuclear cells from patients with juvenile polyarticular arthritis, and treatment with doxazosin abrogated catecholamine-augmented secretion of IL-6 (25). These studies suggest a role of catecholamine-associated augmentation of injurious cytokine responses beyond cytokine release syndrome and acute lung infection and highlights the potential of α_1-AR antagonists across various inflammatory diseases.

One concern with observational analysis is confounding by indication, especially if medications given during a hospital stay are correlated with disease severity. To avoid confounding by indication, this analysis examined the use of α_1-AR antagonists prior to index hospitalization. This class of medications is primarily used to manage chronic diseases such as arterial hypertension, PTSD, or BPH. As such, prescribing practices would not be biased by the severity of COVID-19. In addition, our results were not driven by a specific location or time period.

This study has important strengths and weaknesses. We have focused on mortality as a definitive clinical outcome, thereby avoiding process measures, such as use of mechanical ventilators or admission to an ICU, that are subject to local and individual practice patterns and would be biased if clinicians or hospitals changed their practices in unobserved ways. Another strength is our use of information prior to the COVID-19 admission for risk adjustment. One limitation in this study was the exclusion of women which was required due to limitations in samples size since α_1-AR antagonists are most commonly used to treat benign prostatic hyperplasia and 90% of patients in the VA system are men (26). A second limitation, best addressed in prospective clinical trials, was our inability to examine dose effects given our sample size.

Our results suggest that inhibition of catecholamine signaling with doxazosin (and other α_1-AR antagonists) may reduce in-hospital and 28-day mortality in patients with COVID-19 and highlight the need for randomized placebo-controlled clinical trials to examine the efficacy of α_1-AR antagonists for improving survival and preventing adverse outcomes from COVID-19. Importantly, α_1-AR antagonists are inexpensive, administered orally, do not require refrigeration, and have a well-established safety profile. Thus, if trials confirm these results, α_1-AR antagonists could be widely deployed to reduce mortality from inflammatory injury. Importantly, α_1-AR antagonists are immunomodulatory, but not immunosuppressive drugs. Long-term use of doxazosin does not appear to be associated with the development of opportunistic infection in human studies (27). Indeed, some studies suggest an overall decreased risk of urinary tract infection compared to placebo as may be expected based on its effect on dynamic prostate and bladder function (28). The absence of serious infectious complications may be explained by the unique mechanism of action of α_1-AR antagonists compared to immunosuppressive drugs currently employed in the treatment of severe COVID-19 (e.g., dexamethasone, baricitinib, tocilizumab) which confer an increased risk of opportunistic infection.

In summary, patients hospitalized with COVID-19 had lower odds of in-hospital and 28-day death if they had an active prescription for any α_1-AR antagonist (tamsulosin, silodosin, prazosin, terazosin, doxazosin, or alfuzosin) at the time of admission. Among different α_1-AR antagonists, doxazosin was associated with a 74% relative risk reduction for death, while tamsulosin had a more modest 18% relative risk reduction for death. A clinical trial testing the efficacy and safety of α_1-AR antagonists such as doxazosin to prevent hyperinflammation and reduce mortality in COVID-19 would appear warranted.

DATA AVAILABILITY STATEMENT

The original contributions generated in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Stanford IRB. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JV, BV, CB, and MK conceived of the idea. LR, LG, AK, MP, and RX conducted statistical analyses and results presentation. AK, MP, RX, ZS, and SA developed the methodology. LR, MK, JV, and TW wrote the manuscript with input from all authors. MK and TW are co-senior authors. All authors reviewed the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.637647/full#supplementary-material

Supplementary Figure 1 | Vital Signs at Time of Admission. The diagrams show vital signs for patients diagnosed with COVID-19 (red line) and an expanded cohort of patients with suspected COVID-19 (blue line). Smoothed lines are from a LOESS model with 95% confidence intervals shown (gray ribbons).

Supplementary Figure 2 | In-hospital and 28-Day Mortality by Use of Tamsulosin at Time of Hospital Admission with COVID-19. Data are shown for hospitalized patients diagnosed with confirmed COVID-19 (top panel) and with confirmed plus suspected COVID-19 (bottom panel). Forest plots showing odds ratios (OR) of

in-hospital mortality based on prior use of any alpha-1 adrenergic receptor antagonists (dark green) or tamsulosin (light blue) in each panel. Relative risk reduction (RRR), odds ratios (ORs) for death, 95% confidence intervals (CI), and *p*-values (for unadjusted, adjusted, and matched models), and sample size (*n*) for each analysis are shown on the right.

Supplementary Figure 3 | Adjusted Odds of In-hospital Mortality and Use of α_1-AR Antagonists by Week. Top panel shows adjusted odds ratios of in-hospital mortality and use of α_1-AR antagonists by week of admission. Top panel truncated between 0 and 2 to aid visualization. Bottom panel shows number of new admissions by week and use of α_1-AR antagonists (bottom).

Supplementary Figure 4 | Adjusted Odds of In-hospital Mortality and Use of α_1-AR Antagonists by VA Station. Top panel shows adjusted odds ratios of in-hospital mortality in patients taking α_1-AR antagonists by VA station. Top panel truncated between 0 and 2 to aid visualization. Bottom panel shows number of new admissions and use of α_1-AR antagonists by VA station (bottom). For other VA stations, the number of admissions of patients not using α_1-AR antagonists was 7,645 and number of admissions of patients using α_1-AR antagonists was 1,845. VA stations shown: 508 = Atlanta, 549 = Dallas, 573 = Gainesville, 580 = Houston, 589 = Kansas City, 614 = Memphis, 630 = New York Harbor, 644 = Phoenix, 671 = San Antonio, 673 = Tampa.

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Conflict of Interest: In 2017, The Johns Hopkins University (JHU) filed a patent application on the use of various drugs to prevent cytokine release syndromes, on which BV and KK are listed as inventors. JHU will not assert patent rights from this filing for treatment related to COVID-19. MK received personal fees from Bristol-Myers Squibb and Celltrion, unrelated to the manuscript. BV and KK are founders of and hold equity in Thrive Earlier Detection. KK is a consultant to and is on the Board of Directors of Thrive Earlier Detection. BV and KK are founders of, hold equity in, and serve as consultants to Personal Genome

Diagnostics. KK and BV are consultants to Sysmex, Eisai, and CAGE Pharma and hold equity in CAGE Pharma. BV is also a consultant to Nexus. KK and BV are consultants to and hold equity in NeoPhore. CB is a consultant to Depuy-Synthes and Bionaut Pharmaceuticals. CB, BV, and KK are also inventors on technologies unrelated or indirectly related to the work described in this article. Licenses to these technologies are or will be associated with equity or royalty payments to the inventors, as well as to JHU. The terms of all these arrangements are being managed by JHU in accordance with its conflict of interest policies. SA is an advisor and holds an equity stake in two private companies, Prealize (Palo Alto, California, USA) and Dr. Consulta (Brazil). Prealize is a healthcare analytics company, and Dr. Consulta operates a chain of low-cost medical clinics in Brazil.

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.

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SARS-CoV-2 Diagnostic Tests: Algorithm and Field Evaluation From the Near Patient Testing to the Automated Diagnostic Platform

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Introduction: Since the first wave of COVID-19 in Europe, new diagnostic tools using antigen detection and rapid molecular techniques have been developed. Our objective was to elaborate a diagnostic algorithm combining antigen rapid diagnostic tests, automated antigen dosing and rapid molecular tests and to assess its performance under routine conditions.

Methods: An analytical performance evaluation of four antigen rapid tests, one automated antigen dosing and one molecular point-of-care test was performed on samples sent to our laboratory for a SARS-CoV-2 reverse transcription PCR. We then established a diagnostic algorithm by approaching median viral loads in target populations and evaluated the limit of detection of each test using the PCR cycle threshold values. A field performance evaluation including a clinical validation and a user-friendliness assessment was then conducted on the antigen rapid tests in point-of-care settings (general practitioners and emergency rooms) for outpatients who were symptomatic for <7 days. Automated antigen dosing was trialed for the screening of asymptomatic inpatients.

Results: Our diagnostic algorithm proposed to test recently symptomatic patients using rapid antigen tests, asymptomatic patients using automated tests, and patients requiring immediate admission using molecular point-of-care tests. Accordingly, the conventional reverse transcription PCR was kept as a second line tool. In this setting, antigen rapid tests yielded an overall sensitivity of 83.3% (not significantly different between the four assays) while the use of automated antigen dosing would have spared 93.5% of asymptomatic inpatient screening PCRs.

Conclusion: Using tests not considered the "gold standard" for COVID-19 diagnosis on well-defined target populations allowed for the optimization of their intrinsic performances, widening the scale of our testing arsenal while sparing molecular resources for more seriously ill patients.

Keywords: COVID-19, SARS-CoV-2, immunoassay, diagnostic, antigen, PCR, point-of-care, NAAT

INTRODUCTION

At the time of writing (January 7, 2021), Belgium is emerging from a second wave of COVID-19 epidemic. The World Health Organization (WHO) recommended mass use of reverse transcription real-time PCR (RT-PCR) to detect active SARS-CoV-2 infections (1). However, the unprecedented high volume of samples reaching laboratories led to global scarcities of reagents and delays making prolonged containment measures less acceptable by the population (2). Since then, a new set of diagnostic tools have been developed, such as antigen detection immunoassays or molecular point-of-care tests. These tools could allow diversification of testing strategies and decrease shortages and overflows.

Thanks to their high sensitivity, ranging from 73.9 to 89.5% for high viral load samples [10^5-10^7 RNA copies/swab (3)], and their overall specificity (4, 5), antigen-detection rapid diagnostic tests have been integrated in several countries' testing strategies (6–10)[1,2]. Both Centers for Disease Control and Prevention (CDC) (11) WHO (12) and European Center for Disease Control and Prevention (ECDC) (13) have issued guidelines for their use. However, practical considerations are still lacking (including the best target populations). Meanwhile, several manufacturers have developed molecular point-of-care tests, most of which additionally target influenza and/or RSV (14, 15) while others offer wider respiratory syndromic panel (16).

In addition, high throughput antigen-dosing systems based on chemiluminescence enzyme immunoassay represent an interesting alternative (17). This solution, recently deployed in German airports, is a striking example of delocalized laboratory medicine (18).

Following this expansion of available diagnostic tools, a deeper reflection has come to light on the best use of these various testing solutions according to their sensitivity, their turnaround time, the context in which the result will be used (patient vs. population-centered approach), the kinetics of the epidemic and the availability of reagents and consumables (19).

All of the above may partly explain the apparent confusion we are currently witnessing in the deployment of antigen rapid diagnostic tests and/or molecular point-of-care tests in most industrialized countries, either in terms of choosing the most appropriate diagnostic tests or the target population to apply these tests to. We would like to share here the results of evaluations we performed on four antigen rapid diagnostic tests, one automated antigen dosing assay and one molecular point-of-care test for the diagnosis of COVID-19, not only from an analytical "laboratory" point-of-view but also through their field implementation during the second Belgian COVID-19 wave. Using different techniques at different levels in a multi-step, integrated, and adaptive diagnostic algorithm helped us to diversify and increase our overall testing capacity.

[1]Bundesministerium für Soziales Gesundheit Pflege und Konsumentenschutz. Österreichische Teststrategie SARS-CoV-2.

[2]Robert Koch Institut. Hinweise zur Testung von Patienten auf Infektion mit dem neuartigen Coronavirus SARS-CoV-2.

METHODS

Population

LHUB-ULB (Laboratoire Hospitalier Universitaire de Bruxelles—Universitair Laboratorium Brussel) is a clinical laboratory serving five university hospitals (containing a capacity of around 3,000 beds) as well as a network of general practitioners in Brussels, Belgium. LHUB-ULB's service area covers 700,000 inhabitants (20). From July to September 2020, patients undergoing a SARS-CoV-2 RT-PCR were retrospectively categorized through a structured algorithm into four categories according to the information provided on the orders: symptomatic outpatients, hospital admissions (symptomatic or not), asymptomatic high-risk contacts, or mandatory screenings. The RT-PCR median C_T values from these four groups were compared using the Tukey-Kramer method.

Symptomatic Cases Definition

We used the case definition provided by the Belgian national health institute (Sciensano) for COVID-19 (21). The acute apparition of one major symptom, the presence of two minor symptoms, or the aggravation of chronic respiratory symptoms without any other obvious cause was defined as a possible case (**Supplementary Table 1**). A confirmed case was a person with a SARS-CoV-2 positive sample.

Diagnostic Tests

Antigen Rapid Diagnostic Tests

Four lateral-flow immunoassays were evaluated: Panbio™ COVID-19 Ag Rapid Test Device (Abbott Rapid Diagnostics, Germany), BD Veritor™ SARS-CoV-2 (Becton-Dickinson and Company, USA), COVID-19 Ag Respi-Strip (Coris BioConcept, Belgium) and SARS-CoV-2 Rapid Antigen Test (SD Biosensor, Republic of Korea). Reading was performed by trained operators except for the BD Veritor™ for which an automated reader (BD Veritor™ System) was used.

An analytical performance study was performed using nasopharyngeal swabs. The swabs preserved in universal transport media (UTM) were sent to our laboratory for a SARS-CoV-2 RT-PCR, and then kept refrigerated overnight after

the RT-PCR was performed. The four assays were performed at the same time by two trained operators. The amount of UTM engaged was according to the recommendations by each manufacturer for evaluation purposes but not for clinical use.

After the performance study, antigen rapid diagnostic tests were done in point-of-care settings, either a practice within our network of general practitioners, or in the emergency room of the Saint-Pierre university hospital. Each possible COVID-19 outpatient, who was within 7 days of symptoms onset, was offered an antigen rapid diagnostic test and informed that a negative result would require an additional sampling for RT-PCR as recommended at the time (21). Each antigen rapid diagnostic test sampling and test procedure was performed according manufacturer instructions (**Supplementary Table 2**).

The user-friendliness of each antigen rapid diagnostic test was assessed with a four-part questionnaire adapted from the Scandinavian evaluation of laboratory equipment for point-of-care testing SKUP/2008/114 evaluation (22).

Molecular Point-of-Care Test

To assess the analytical performance of the Cobas® Liat SARS-CoV-2 & Influenza A/B nucleic acid test (Roche Molecular Systems, USA), nasopharyngeal swabs, which were sent to our laboratory for a SARS-CoV-2 RT-PCR and tested positive, were kept refrigerated overnight before testing. In addition, frozen samples from February 2020 which underwent at that time a Cobas® Liat Influenza A/B & RSV RT-PCR assay were also tested.

Automated Antigen Dosing Assay

Antigen dosing was performed using the Lumipulse® *G* SARS-CoV-2 Ag (Fujirebio, Japan) assay, expressing the dosage in pg/mL. For biosafety consideration, a viral-deactivation step (56°C heating for 30 min) was added to the manufacturer's instructions protocol (23).

The analytical performance study was performed on UTM swabs kept refrigerated overnight after a SARS-CoV-2 RT-PCR. All available positive samples were selected. Negative samples were randomly selected to obtain a positive/negative ratio around 2:1.

In the second part of the evaluation, we evaluated the Lumipulse® performance on UTM samples sent to our laboratory for SARS-CoV-2 RT-PCR for patients who required scheduled hospital admission, COVID-19 contacts, or for healthcare workers.

Gold Standard and Statistical Analysis

Analytical performance study of antigen rapid diagnostic tests, molecular point-of-care test and automated antigen dosing were carried out on three different sets of samples.

SARS-CoV-2 RT-PCR was considered as the gold-standard. Except for some antigen rapid diagnostic tests, for which negative results were controlled by various other RT-PCR protocols, samples underwent the RealTi*m*e SARS-CoV-2 assay (Abbott Molecular, USA) on our *m*2000 platform. As detection of both targeted genes (RdRp and N) is performed using the same fluorophore, the C_T values of this assay are observed up to 32 cycles, and not comparable with C_T values of other RT-PCR assays. Consequently, only the C_T values obtained using the RealTi*m*e SARS-CoV-2 assay were considered.

Statistical analyses and receiver operating characteristic (ROC) curves were performed using Analyse-it® for Microsoft Excel v3.80.

RESULTS

Trends of C_T Value in the Different Populations

LHUB-ULB performed 31,397 SARS-CoV-2 RT-PCR including 1,708 positive nasopharyngeal samples (5.4%) from 1,568 patients. 1,169 SARS-CoV-2 infected patients were categorized as follows: 580 symptomatic outpatients (49.6%), 318 admissions (27.2%), 178 contacts (15.2%), and 93 screenings (7.9%). The median C_T for symptomatic outpatients (13.8/32) was significantly lower than for any other group (**Figure 1**). The median C_T for contacts (17.4/32) was significantly lower than for admissions (20.8/32, $p = 0.0044$) and for screenings (23.2/32, $p = 0.0002$). Hence, antigen rapid diagnostic test was considered for symptomatic outpatients, automated antigen dosing for screenings and molecular point-of-care tests for hospital admissions.

Analytical Performance Studies

Antigen Rapid Diagnostic Tests

Ninety-nine UTM samples including 61 positive (C_T ranging from 3.86/32 to 30.94/32) were selected. In this frame, the sensitivities of each antigen rapid diagnostic test were ranging from 36.1 to 49.2% (**Table 1**). The latest C_T detected antigen rapid diagnostic tests was 18.06/32. No false positive result was observed.

Molecular Point-of-Care Test

The agreement of the Cobas® Liat with the *m*2000 system for SARS-CoV-2 diagnostic was of 90.9% (50/55) for positive samples. C_T values correlation between instruments was good ($R^2 = 0.931$). The Cobas® Liat yielded positive results for all positive samples presenting a C_T value below 27.29/32 and yielded positive results for samples with C_T of up to 29.11/32. Eighteen of the 19 frozen Influenza A positive samples and 5 of the 6 frozen influenza B positive samples yielded coherent positive results. Agreements for negative samples were of 100% for each parameter.

Automated Antigen Dosing Assay

Two hundred fourteen samples were selected including 136 positive samples. ROC curve analysis yielded an area under the curve (AUC) of 0.893±0.021 (**Supplementary Figure 1**). The highest Youden Index was at a threshold of 13.75 pg/mL (sensitivity 67.7%, specificity 97.1%). At a threshold set at 1.32 pg/mL [similar to a previous study (17) and to the manufacturer proposed cut-off at 1.34 pg/mL (24)], sensitivity was 78.9% and specificity of 73.9%. To exclude any false positive, the threshold had to be set at 20.27 pg/mL (sensitivity 63.9%). Finally, using a $C_T < 20/32$ as a judgement criterion, the AUC of the ROC curve was 0.984 ± 0.007 (**Supplementary Figure 2**) with an optimal

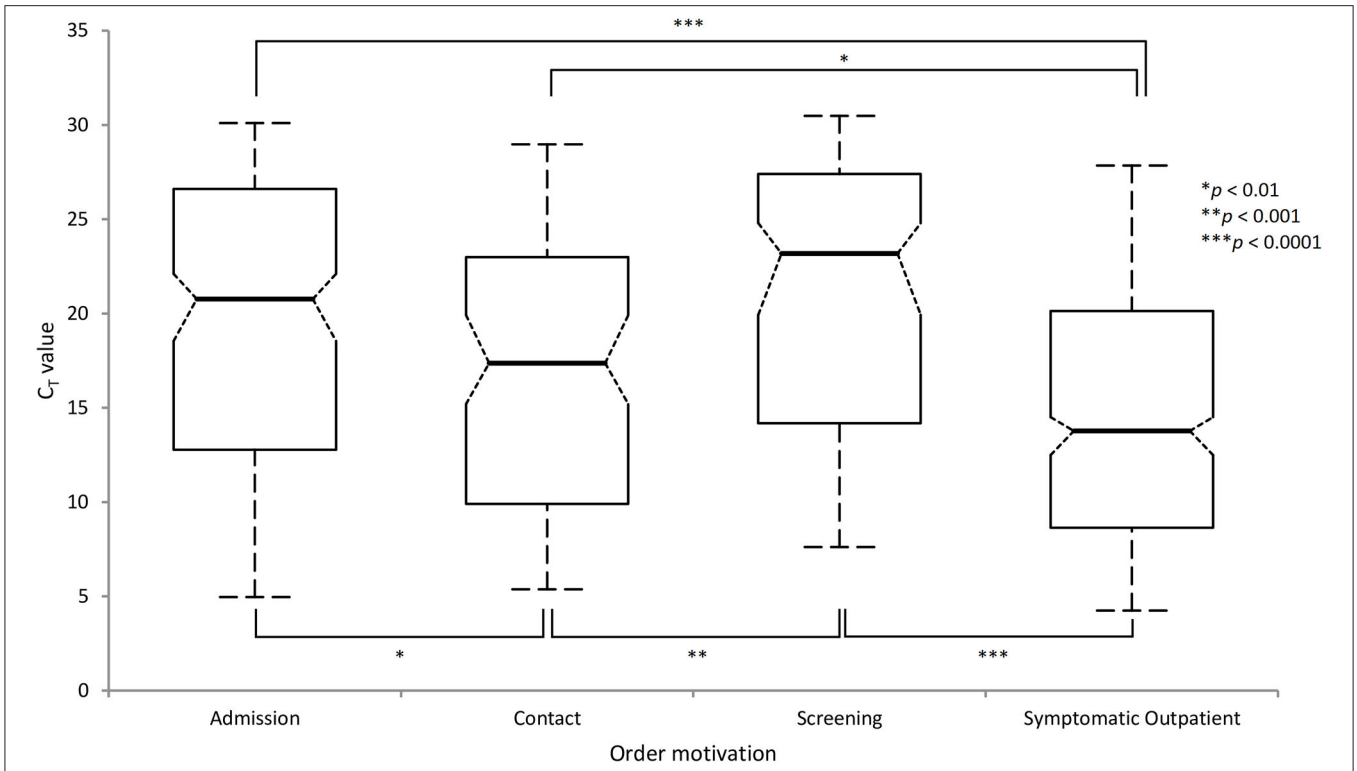

FIGURE 1 | Distribution and comparison of C_T values in target populations according to the motivation of the order using Abbott RealTi*m*e SARS-CoV-2 solution (Comparisons using the Tukey-Kramer method).

TABLE 1 | Compared analytical performances of four SARS-CoV-2 antigen rapid diagnostic tests using 99 nasopharyngeal swabs preserved in universal transport media as proxy vs. Abbott RealTi*m*e SARS-CoV-2 assay.

	Buffer dilution factor	Sensitivity (IC_{95})	Specificity	Last C_T detected
Panbio™ COVID-19	1/2	45.9% (34.0–58.3%)	100%	18.06/32
Coris COVID-19 Ag Respi-Strip	1/2	39.3% (28.1–51.9%)	100%	13.31/32
SD Biosensor™ SARS-CoV-2	1/2	49.2% (37.1–61.4%)	100%	18.06/32
BD Veritor™ SARS-CoV-2	1/6	36.1% (25.2–48.8%)	100%	13.9/32

Youden index at a threshold of 20.27 pg/mL (sensitivity 87.4%, specificity 98.1%).

Elaboration of the Diagnostic Algorithm

Following these results, we elaborated the algorithm described in **Figure 2**: whereas the diagnosis of outpatients was mainly based on point-of-care antigen rapid diagnostic tests, the hospital algorithm combined antigen rapid diagnostic tests, molecular point-of-care tests and conventional RT-PCR in an integrative diagnostic strategy. Four clinical situations were further identified: screening of asymptomatic patients, patients requiring immediate admission, symptomatic outpatients with symptoms lasting for less or more than 5 days.

Field Performance Evaluation

Antigen Rapid Diagnostic Tests

Four hundred ninety-four symptomatic outpatients underwent an antigen rapid diagnostic test. Two hundred and nine (42.3%) were positive. Sixteen negative antigen rapid diagnostic tests were excluded due to missing RT-PCR results. Overall sensitivity was 83.3% (95% confidence interval (IC_{95}): 78.2–87.4%—**Table 2**). Taken individually, each assay's sensitivity was not significantly different from the others, ranging from 78.3 to 87.7%. Only the BD Veritor™ was conducted on a sufficient number of patients to allow a meaningful comparison between the emergency room (sensitivity: 88.2%, IC_{95}: 76.6–94.5%) and the general practitioners (sensitivity of 87.3%, IC_{95}: 76.0–93.7%), yielding no significant difference. Sensitivity according to days since onset of symptoms (DSO), dropped significantly from 86.9% (IC_{95}: 81.6–90.8%) for up to 4 DSO to 63.6% (IC_{95}: 46.6–77.8%) from 5 DSO (*t*-test, $p < 0.001$). False negative antigen rapid diagnostic tests had C_T ranging from 4.93/32 to 29.02/32.

The user-friendliness was satisfactory for all four antigen rapid diagnostic tests tested (**Table 3**). The Coris COVID-19 Ag Respi-strip had a less satisfactory rating. The main practical issue was its readiness: its "strip-in-a-tube" format was considered by

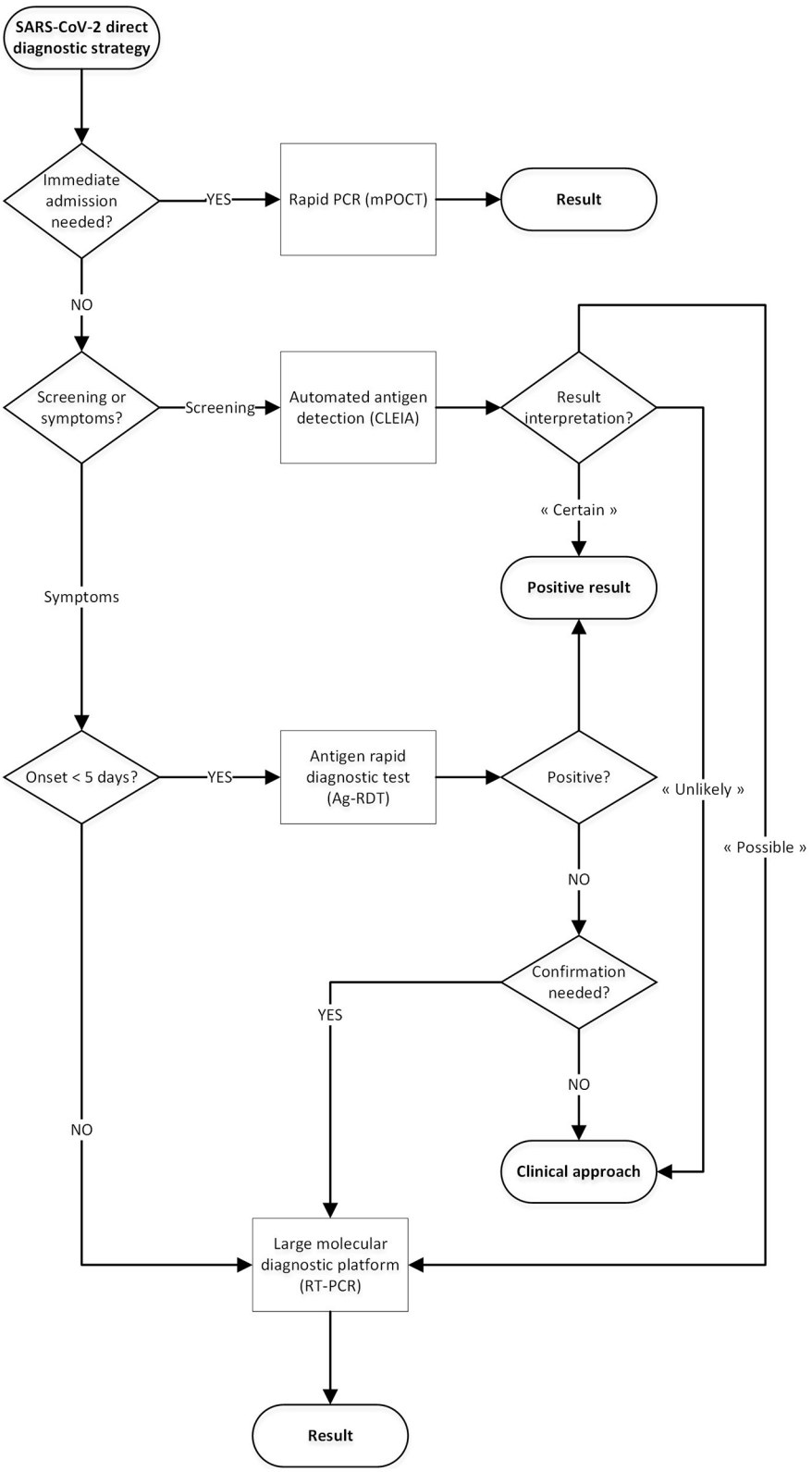

FIGURE 2 | Proposal for a SARS-CoV-2 direct diagnostic decision algorithm.

TABLE 2 | Compared analytical performances of four SARS-CoV-2 antigen rapid diagnostic tests used in a point-of-care setting at the emergency room of Saint-Pierre University Hospital (Brussels, Belgium) and at a general practitioner consultation.

	N	Sensitivity (IC_{95})	False negative median C_T (range)
Overall	478	83.3% (78.2–87.4%)	17.60 (4.93–29.02)
Manufacturer			
BD Veritor™ SARS-CoV-2	177	87.7% (80.1–92.7%)	15.46 (4.93–18.54)
- At the general practitioner consultation	110	87.3% (76.0–93.7%)	
- At the emergency room	67	88.2% (76.6–94.5%)	
Panbio™ COVID-19 Ag Rapid Test Device	101	80.8% (68.1–89.2%)	18.32 (10.29–23.68)
Coris COVID-19 Ag Respi-Strip	135	80.0% (69.2–87.7%)	21.56 (15.52–29.02)
SD Biosensor™ SARS-CoV-2 Rapid Antigen Test	65	78.3% (58.1–90.3%)	15.53 (14.92–16.15)
DSO			
<5 DSO	395	86.9%* (81.6–90.8%)	18.38 (10.90–29.02)
- 0–1 DSO	97	89.1% (78.2–94.9%)	
- 2 DSO	118	90.3% (80.5–95.5%)	
- 3 DSO	118	80.3% (68.7–88.4%)	
- 4 DSO	62	89.3% (72.8–96.3%)	
≥5 DSO	53	63.6%* (46.6–77.8%)	15.46 (4.93–27.02)

DSO, days since symptoms onset; N, number of performed tests; IC_{95}, 95% confidence interval.
**p-value < 0.001 (Student's t-test).*

operators as non-practical and leading to a potential biosafety hazard when the reading is difficult. Notably, SD Biosensor™ and Coris BioConcept did not provide any internal control in their kit. BD Veritor™ was the only kit offering nasal swabbing and automated reading.

Automated Antigen Dosing Assay

Two hundred seventy-nine patients (including 93 asymptomatic patients screened for a scheduled hospitalization) were tested. Their SARS-CoV-2 carriage status was categorized as "unlikely" if dosing below 1.32 pg/mL ($n = 219$, 78.5%), "possible" if dosing from 1.32 to 20.27 pg/mL ($n = 46$, 16.5%) and "certain" if dosing higher than 20.27 pg/mL ($n = 14$, 5.0%). All patients with "certain" results had a positive RT-PCR. Seven patients out of 46 (15.2%) with a "possible" result and five out of 219 (2.3%) with an "unlikely" result were tested positive according to RT-PCR, respectively (**Table 4**). Thus, the overall sensitivity for asymptomatic patients was of 86.7% (13/15). Hence, using this assay for the pre-admission screening of these 93 patients would have spared 87 RT-PCR (93.5%) for the cost of one missed low-positive ($C_T = 26.04/32$).

DISCUSSION

In most industrialized countries, the large scale use of RT-PCR to detect active SARS-CoV-2 infections has shown limits in its capacity to broadly screen the population while providing timely and therefore meaningful results for optimized prevention and treatment. To fill this gap, SARS-CoV-2 antigen rapid diagnostic tests and molecular point-of-care tests are now considered as an adjunct to the RT-PCRs performed on large automated platforms (25).

Our results provide substantial evidence that no current antigen rapid diagnostic test is sensitive enough to be performed on UTM specimen (i.e., at the laboratory). During the first wave in Europe, we proposed a strategy combining antigen rapid diagnostic tests and RT-PCR, both performed in the laboratory (26). We stopped using antigen rapid diagnostic tests in the laboratory during the declining phase of the epidemic, not because of their low sensitivity [as stated by colleagues (27)], but because the proportion of samples from recently infected patient dropped, impairing these tests' usefulness (28). Regular follow-up of the positivity rate could allow adaptations of antigen rapid diagnostic test strategy as proposed by CDC (11) and ECDC (13). Here, we demonstrate the added-value of antigen rapid diagnostic tests at the point-of-care level for <5-days symptomatic outpatient thanks to their ease-of-use, rapid time-to-result, and low cost.

Our results show slightly lower sensitivity than previously reported (25). Indeed, part of the false negative results observed is likely due to variability in the adherence to protocol regarding sampling, incubation time and DSO. Sensitivity and specificity of such antigen rapid diagnostic tests strongly depend on their good execution and reading which are harder to achieve at the frontline where the expertise of personnel can vary; especially in this time of pandemic when the turn-over is higher than usual. This was confirmed by other recently published studies targeting the same population, with sensitivity ranging from 70.0 to 80.4% (29–31).

The absence of significant difference between antigen rapid diagnostic tests clinical performances highlights the need to assess their user-friendliness as a main criterion of choice. Our analysis underlined the need to consider very practical aspects such as opening caps while wearing gloves, ensuring biosafety outside a laboratory (see **Figure 3**) and instructions targeting non-laboratory operators, as recently discussed for low-resource settings (32). Besides, an immediate, in-person communication of a positive result likely allowed a stronger message and a better adhesion regarding quarantine, hygiene and contact-tracing than if done through virtual means, days after the consultation.

TABLE 3 | User friendliness assessment of four COVID-19 antigen rapid diagnostic tests, adapted from SKUP/2018/114 protocol.

Operation facilities	Mean of $N = 3$ questioned operators			
	BD Veritor™ SARS-CoV-2	**Coris COVID-19 Ag Respi-strip**	**Panbio™ COVID-19 Ag rapid test device**	**SD Biosensor™ SARS-CoV-2 Rapid Antigen Test**
To prepare the test	Intermediate (1S 2I)[a]	Intermediate[b]	Intermediate (1S 2I)[b]	Satisfactory (2S 1I)[a]
To prepare the sample	Satisfactory	Satisfactory	Satisfactory	Satisfactory
Application of specimen	Satisfactory	Satisfactory	Satisfactory	Satisfactory
Number of procedure step	Satisfactory	Satisfactory	Satisfactory	Satisfactory
Test design	Satisfactory	Unsatisfactory (2U, 1I)[c]	Satisfactory	Satisfactory
Reading of the result	Satisfactory	Difficult[d]	Satisfactory	Satisfactory
Sources of errors	Satisfactory	Intermediate[d]	Satisfactory	Satisfactory
Hygiene when using the test	Satisfactory	Unsatisfactory[e]	Satisfactory	Satisfactory
Size and weight of the package	Satisfactory (2S 1I)[f]	Satisfactory	Satisfactory	Satisfactory
Storage conditions for tests, unopened package*	15–30°C	15–30°C	15–30°C	15–30°C
Storage conditions for tests, opened package*	15–30°C	15–30°C	15–30°C	15–30°C
Environmental aspects: waste handling*	Special precautions	Special precautions	Special precautions	Special precautions
Intended users*	Health care personnel	Health care personnel	Health care personnel	Health care personnel
Information in instruction in the insert				
Preparations/Pre-analytic procedure	Satisfactory	Satisfactory	Satisfactory	Satisfactory
Specimen collection	Satisfactory	Intermediate[g]	Satisfactory	Satisfactory
Measurement procedure	Satisfactory	Satisfactory	Satisfactory	Satisfactory
Reading of result	Satisfactory	Satisfactory	Satisfactory	Satisfactory
Description of the sources of error	Satisfactory	Satisfactory	Satisfactory	Satisfactory
Help for troubleshooting	Satisfactory	Satisfactory	Satisfactory	Satisfactory
Readability/clarity of presentation	Satisfactory	Intermediate[h]	Satisfactory	Satisfactory (1I 2S)[j]
General impression	Satisfactory	Intermediate[i]	Satisfactory	Satisfactory (1I 2S)[j]
Measurement principle*	Satisfactory	Satisfactory	Satisfactory	Satisfactory
Available insert in ENG + FR + NL*	Partly[k]	Partly[k]	Partly[l]	Partly[k]
Time factors*				
Required training time	<2 h	<2 h	<2 h	<2 h
Duration of preparations/Pre-analytical time	<6 min	<6 min	<6 min	<6 min
Duration of analysis	10–20 min	>20 min	10–20 min	10–20 min
Stability of test, unopened package	>5 months	>5 months	>5 months	>5 months
Stability of test, opened package	>30 days or disposable	>30 days or disposable	>30 days or disposable	>30 days or disposable
Stability of quality control material unopened	>5 months	No QC provided	>5 months	No QC provided
Analytical quality control*				
Reading of the internal quality control	Satisfactory	Unsatisfactory[m]	Satisfactory	Unsatisfactory[m]
Usefulness of the internal quality control	Satisfactory	Unsatisfactory[m]	Satisfactory	Unsatisfactory[m]

** Objective informational items were filled by the principal investigator.*
[a] Caps of the buffer tubes difficult to manipulate.
[b] Requires a tube rack.
[c] The use of a strip in a closed tube with a very difficult capping was not considered practical for the operators.
[d] Difficult reading through a closed tube although transparent.
[e] Operators were forced to open the tubes to extract the strip in case of a doubt with the reading causing biosafety concern.
[f] Oversized packaging compared to the number of test.
[g] Lack of precise instruction.
[h] Lack of clarity.
[i] A quick reference guide would have been appreciated.
[j] Small typo and dense content.
[k] Only available in English.
[l] Not available in Dutch.
[m] Not provided.

TABLE 4 | Analytical performances of the Lumipulse® *G* SARS-CoV-2 Ag on target populations in the detection of SARS-CoV-2 using a categorization of the risk system.

		Lumipulse® automated antigen detection									
		Certain (>20.27 pg/mL)		Possible (1.32–20.27pg/mL)				Unlikely (≤1.32pg/mL)			
PCR result	**N**	**Positive**	**Lowest C_T**	**N**	**Negative**	**Positive**	**Lowest C_T**	**N**	**Negative**	**Positive**	**Lowest C_T**
Overall	279	14	3.89	46	39	7	15.7	219	214	5	22.61
Scheduled hospitalizations	93	1	12.73	6	4	2	15.7	86	85	1	26.04
Contacts	67	4	6.98	13	12	1	31.23	50	49	1	22.9
Health workers	119	9	3.89	27	23	4	19.92	83	80	3	22.61
- With symptoms	67	8	3.89	13	13	-	-	46	43	3	22.61

FIGURE 3 | Diagnostic center set outside under a tent by a general practitioners group in Uccle, Belgium (October 22, 2020).

The Cobas® Liat yielded stunning performances for a 20-min triplex molecular point-of-care test compared to our RT-PCR. However, invalid results were experienced with viscous samples. The addition of a molecular point-of-care test for patients attending the emergency room and needing hospitalization, regardless of the suspicion of COVID-19, allowed a faster management of inpatients avoiding the admission of asymptomatic SARS-CoV-2 carrier in "COVID-free" units, or the admission of SARS-CoV-2-negative patients in COVID-19 units pending their RT-PCR results. Furthermore, influenza and SARS-CoV-2 co-detection allows a better surveillance at a time where the potential co-circulation of the influenza and SARS-CoV-2 is still unknown. The costs of these molecular point-of-care tests stay high and their availability low. Hence, their use should be considered by targeting the best population with regards to the reduction of global costs related to isolation, use of protective equipment and prevention of nosocomial clusters.

In the present study, the Lumipulse® *G* SARS-CoV-2 Ag showed an overall good analytical performance compared to RT-PCR; and more specifically, to exclude negative and low positive samples using different criteria and cut-off values than the ones proposed by the manufacturer. These cut-offs need to be adapted and chosen regarding the local epidemiology and the objectives of the screening. Our cut-off values diverged from the one proposed in a previous study (17). However, despite the fact we added a viral deactivation by heating, our results yielded a better AUC of the ROC curve. In case of limited access to RT-PCR, such technique can allow testing people who would be otherwise not tested. Its higher throughput and

sensitivity than antigen rapid diagnostic tests and its faster time-to-result than RT-PCR make it an interesting intermediary tool. Its low costs and its probable good assessment of infectiousness allow a relevant periodic testing in terms of infection control. Therefore, using antigen dosing could be the best solution to repeatedly test high number of high risk contacts while sparing RT-PCR resources. However, their biosafety must be carefully considered and viral neutralization applied if needed; viscous samples may cause pipetting errors and specific interpretation algorithm should be elaborated.

Our study presents some limitations. We did not consider alternative specimens for SARS-CoV-2 detection such as saliva, the use of serology or broad molecular "syndromic" respiratory panels that could be of use in a larger diagnostic algorithms (33). The emergence of new variants should not impact the value of our algorithm due to the different targets of the assays. However a careful follow-up of their performances over time should be implemented.

CONCLUSION

In conclusion, our study underlines the importance of shifting our attention from a narrow focus on the sole analytical performances of the diagnostic tools available (especially when these are similar) to an integrated approach taking into account (i) practical consideration such as time-to-result, field ease-of-use, availability of reagents (ii) target populations (iii) intended use of produced results, and (iv) kinetic of the epidemic. Hence, we elaborated here a diagnostic algorithm based on these considerations to optimize the use of the newly extended arsenal of SARS-CoV-2 direct diagnostic tools, from the decentralized setting to the automated lab, to ensure clinical microbiologists enough ammunition for a reliable and meaningful COVID-19 diagnostic.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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 from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements. 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

NY, CDe, MD, F-ZB, FP, MW, and CDu did the investigations. NY and MH contributed to literature review and the writing of the initial draft. NY, CM, FP, HD, ND, M-LD, and SM contributed to manuscript revision, data compilation, and figure presentation. All authors provided critical review and commentary. NY, ND, FC, MB, and MH contributed to study design, manuscript preparation, literature review, revision, and project administration. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.650581/full#supplementary-material

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Assessment of COVID-19 Pandemic in Nepal: A Lockdown Scenario Analysis

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The Government of Nepal issued a nationwide lockdown from 24 March to 21 July 2020, prohibiting domestic and international travels, closure of the border and non-essential services. There were only two confirmed cases from 610 Reverse Transcription Polymerase Chain Reaction (RT-PCR) tests and no fatalities when the government introduced nationwide lockdown. This study aimed to explore the overall scenario of COVID-19 including spatial distribution of cases; government efforts, and impact on public health, socio-economy, and education during the lockdown in Nepal. We collated and analyzed data using official figures from the Nepalese Ministry of Health and Population. Nepal had performed 7,791 RT-PCR tests for COVID-19, the highest number of tests during the lockdown. It has recorded its highest daily rise in coronavirus infections with a total of 740 new cases from the total of 4,483 RT-PCR tests performed on a single day. Nepal had reported a total of 17,994 positive cases and 40 deaths at the end of lockdown. The spatial distribution clearly shows that the cases were rapidly spreading from the southern part of the country where most points of entry and exit from India are located. To contain the spread of the virus, the government has also initiated various preventive measures and strategies during the lockdown. The Government of Nepal needs to allocate more resources, increase its capacity to test and trace, establish dedicated isolation and quarantine facility and impose local restrictions such as a local lockdown based on risk assessment rather than a nationwide lockdown.

Keywords: COVID-19, coronavirus, public health, Nepal, spatial distribution analysis, lockdown, impacts, challenges

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INTRODUCTION

Coronavirus disease (COVID-19) outbreak originating from Wuhan, China in late 2019 has spread worldwide claiming more than 2.5 million lives all over the world as of 01 March 2021 (1). On 11 March 2020, the World Health Organization (WHO) declared it as a pandemic (1). Since the outbreak of the disease WHO through its guidelines has prioritized the actions for responding to the virus; urged the government to maintain health facilities, raise public awareness, and stock up on medical supplies (2).

Several modeling studies have been conducted during the early phases of the outbreak to predict the epidemic and effectiveness of multiple population-wide strategies, including lockdown, social distancing, quarantine, testing and contact tracing, and media-related awareness among others to

mitigate the spread of COVID-19 (3–9). The strict lockdown was enforced to limit the spread of COVID-19 in countries such as Italy, Spain, France, the UK after the steady rise in cases whereas Nepal introduced lockdown during the early phase of the pandemic (10). Lockdown is the blanket approach that buys time to prepare the healthcare system (active case finding through testing and tracing, case management, for example, quarantine, isolation and treatment, and availability of protective equipment) to confine the virus and its spread. The Government of Nepal issued a nationwide lockdown from 24 March to 21 July 2020, prohibiting domestic and international travels, closure of border and non-essential services in the first stage, which was later eased on 11 June 2020.

The basic reproduction number (R0) which measures the potential transmission of an infectious disease is a fundamental metric to determine if an outbreak is expected to continue. In general, the disease is expected to spread and become epidemic if R0 is more than one and to decline and ultimately end if R0 is <1. The R0 value of the COVID-19 outbreak was not available for Nepal when the government was preparing for the lockdown. However, in neighboring countries, the estimated R0 value of coronavirus was above 2.0 in India (4) and between 2.2 to 3.5 in China (11), indicating a potential to cause an outbreak. There were only two confirmed cases from 610 Reverse Transcription Polymerase Chain Reaction (RT-PCR) tests and no fatalities before lockdown (12). The indexed case was found on 23 January 2020 in Kathmandu on a person who had traveled from Wuhan, China (13). The second case was confirmed 2 months later on 23 March who had traveled to Nepal from France *via* Qatar (14).

This study aimed to assess the overall scenario of COVID-19 during the lockdown (positive cases, RT-PCR test performed, recoveries, total active and deaths cases including case fatality ratio) including spatial distribution of the cases, government measures to manage the pandemic; and impacts on public health, socio-economy, and education. Finally, we offer helpful suggestions to address the challenges brought by the impact of COVID-19.

METHODS

Study Design

This was a descriptive study to assess the multiple scenarios of the COVID-19 pandemic in Nepal during the lockdown period. Statistical and spatial presentations of data, government efforts, impacts on public health, socio-economy, and education were discussed to summarize the situation of Nepal during the lockdown period. The main focus of the study was for the lockdown period, however, data was updated and discussed to reflect the post lockdown scenario.

Study Area

Nepal is a lower-middle-income country in South Asia between India (in the south, east, and west) and China (in the North). It has a population of around 30 million. The new constitution promulgated on 20 September 2015 made Nepal a federal democratic republic and is now divided into seven provinces, 77 districts, and 753 local units (municipalities and rural municipalities). It is divided into three physiographic regions: Mountain region (Great Himalayan Range in the northern part), Hilly region, and Terai region (low land region at the Indian border in the southern part).

Data Collection

We analyzed results from the COVID-19 situation reports prepared by the Nepalese Ministry of Health and Population (MOHP). For this study, we downloaded the COVID-19 situation reports from the openly available online resources on the web portal (covid19.mohp.gov.np). We collated the data for the lockdown period (from 24 March to 21 July 2020). The MOHP has defined a confirmed COVID-19 case as any person with laboratory confirmation of COVID-19 infection, irrespective of clinical signs and symptoms, that is, person who had RT-PCR tested positive for Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2). Municipal level data has been taken from the data prepared for *COVIRA*, a COVID-19 risk assessment tool (15).

Statistical Analysis

Daily positive cases reported in local administrative units (municipality or rural municipality) were analyzed along with total daily data for the country. Data were analyzed descriptively in a Microsoft Excel 2019 Version 16.0 (Microsoft Corporation, Redmond, Washington, USA). The number of COVID-19 cases, daily RT-PCR tests performed and the number of recoveries were used to assess the COVID-19 pandemic situation. The number of deaths and Case Fatality Rate (CFR) was presented by different age groups. CFR was calculated as the proportion of confirmed deaths among identified confirmed cases. Everyday data of COVID-19 positive cases were presented in local level units. The maps used to show the spatial distribution of COVID-19 cases in Nepal were created using the QGIS Version 3.14 (www.qgis.org).

RESULTS

Figure 1 shows the daily and weekly average of tests and positive cases during the lockdown period. On 29 June, Nepal had performed 7,791 tests for COVID-19, the highest number of tests during the lockdown. It has recorded its highest daily rise in coronavirus infections with a total of 740 new cases from the total of 4,483 test performed on a single day on 3 July. The number of daily cases was decreasing after its peak on 3 July but subsequently, the number of tests was also decreasing.

Figure 2 shows COVID-19 cumulative cases, recoveries, and active cases during the lockdown. As per the figure, the number of people recovered from the coronavirus in Nepal increased during the lockdown. However, the number of active cases decreased. On 1 July there were 10,390 active cases in the country which was continuously declining till the end of the lockdown period. Lack of proper management of quarantine and isolation centers may have caused a significant increase in cases 2-months after lockdown started as most of the cases were imported from India, and were later transmitted to the community. Data show that the bending of the curve had started before the government decided

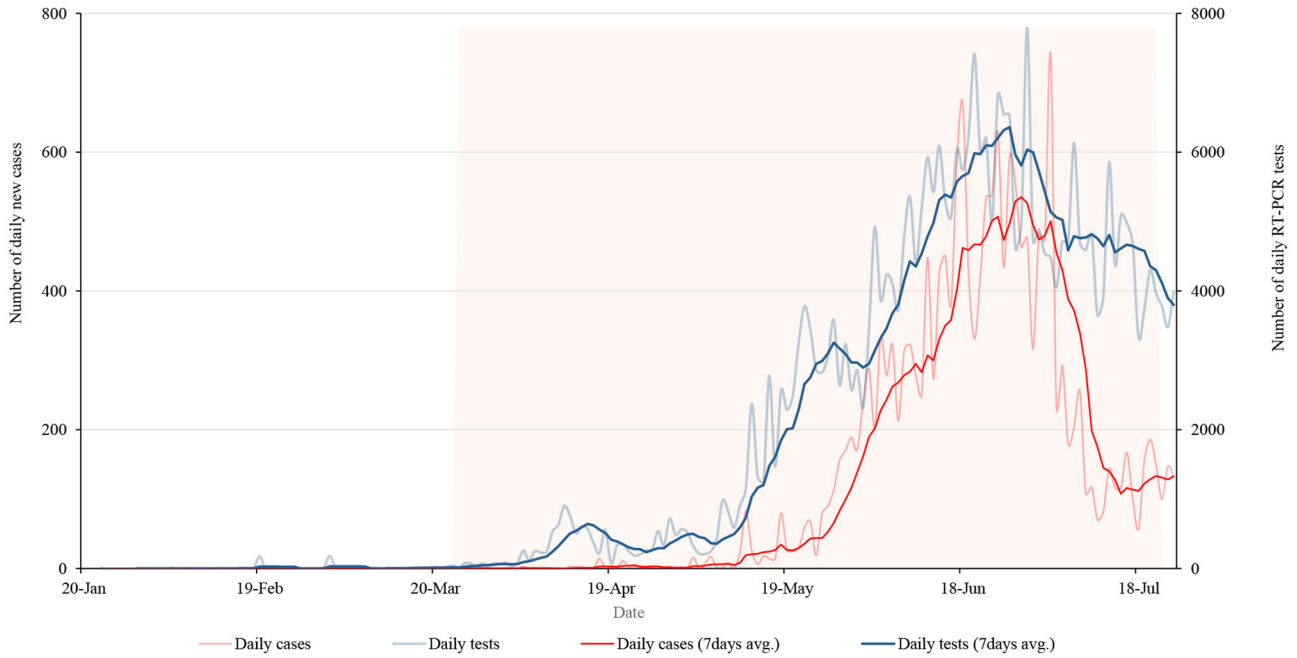

FIGURE 1 | Daily new cases and RT-PCR tests along with 7 days average of cases and tests during the lockdown period.

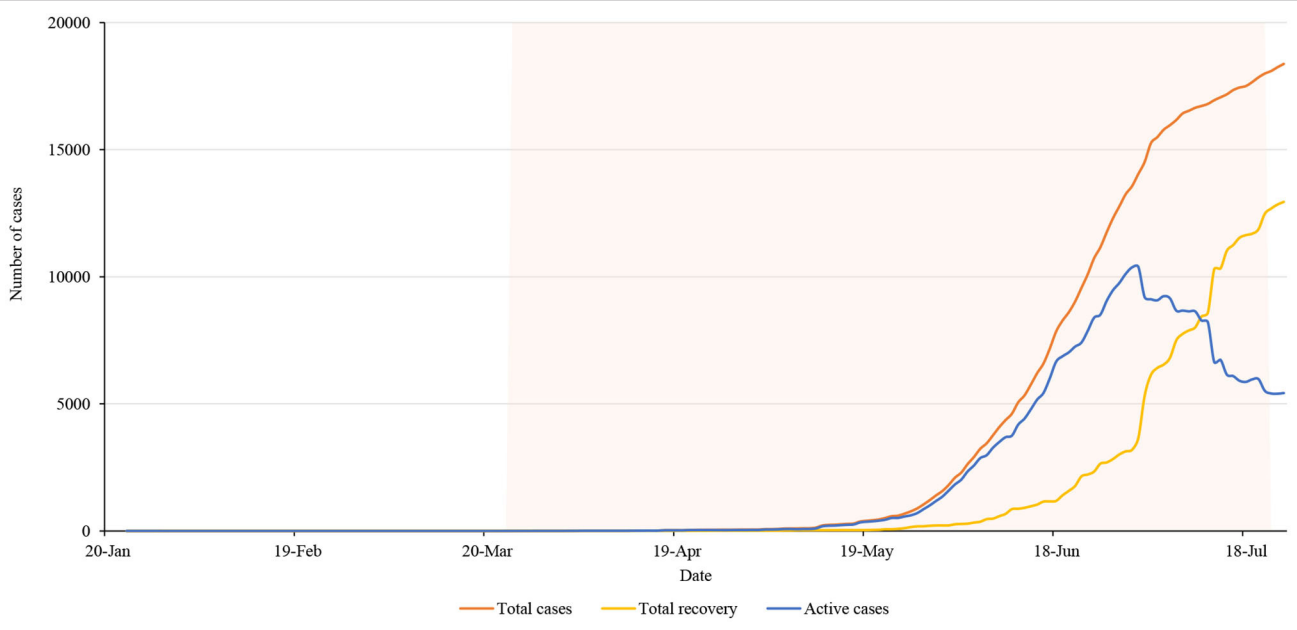

FIGURE 2 | Total cases, recovery and active cases in Nepal during the lockdown period.

to lift the nationwide lockdown however the risks of further transmission in the community were prevailing.

Death and Case Fatality Ratio

Nepal had reported a total of 17,994 positive cases and 40 deaths on the last date of nationwide lockdown. **Figures 3A,B** shows the distribution of cases and a death toll, respectively, in age and gender groups. It clearly shows that the younger population of age group 21–30 years is more infected, and most importantly the fraction of female who got infected is relatively smaller. The main reason behind such distribution is most of the cases were imported by young immigrants who went abroad for work. The

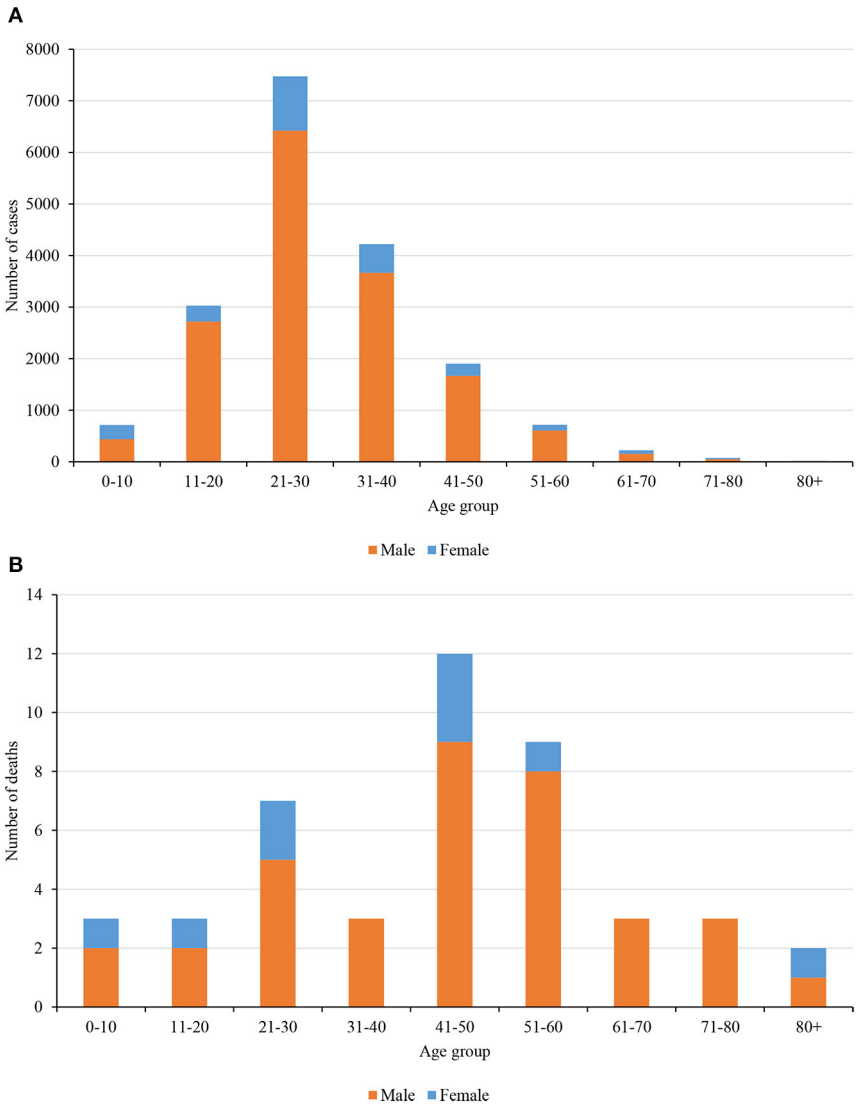

FIGURE 3 | (A) COVID-19 cases distribution among age and gender groups during the lockdown period. **(B)**. COVID-19 deaths distribution among age and gender groups during the lockdown period.

number of deaths and the corresponding overall death rate is very small (0.22%) however the relative death rate curve tells a different story (**Figure 4**).

CFR in Nepal shows a similar trend to the *COVIRA* risk model (15) which is the risk model based on early-stage pandemic data. **Figure 4** shows the comparison of the *COVIRA* risk model and Nepal data (relative percentage of CFR). The correlation between CFR in age groups and the *COVIRA* model is 0.986 which shows very good agreement of data. The trend of CFR by the age group shows an exponential relation with an r-square value of 0.714. Hence, one of the alarming points for Nepal is, if the infection spread in the community and the older people get infected, the death toll would rapidly increase.

Spatial Distribution

Figure 5 shows the spatial distribution of total cases on the first and the 15th days of the months over a lockdown period. These maps clearly show that the cases were rapidly spreading from the southern part of the country where most points of entry and exit from India are located.

The government lifted the lockdown when the curve of daily cases was flattening, however, the situation in the country was not totally under control. There was a high possibility of spreading the virus if an infected person was exposed to others until around 2 weeks of infection. Mapping the report of last active cases across the country was important to look at the prevailing risk zones. **Figure 6** shows the number of days since the last case was reported on the day when the lockdown was lifted.

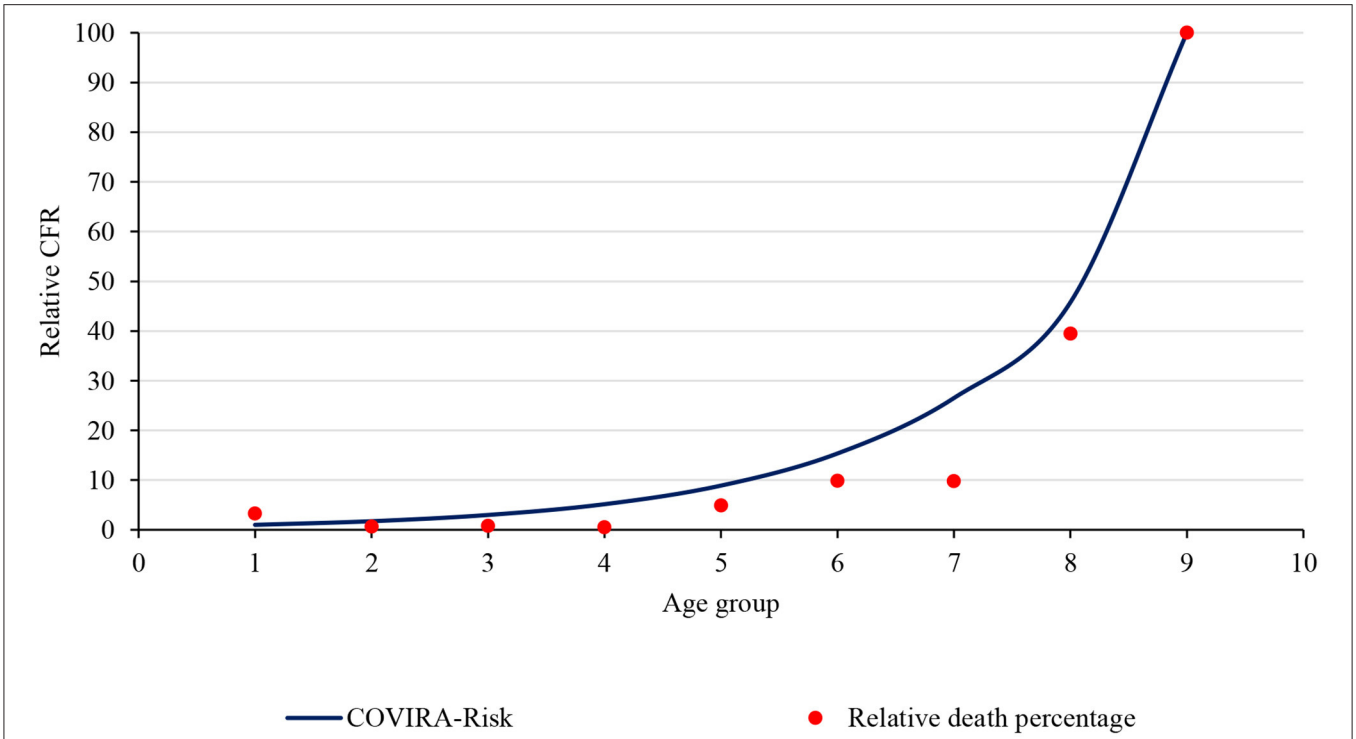

FIGURE 4 | Relative case fatality ratio and comparison with the COVIRA risk model.

Government Efforts on Managing the Pandemic During the Lockdown

The Government of Nepal has initiated various preventive measures and strategies during the lockdown as shown in the list below (16).

- Guidelines issued for the management and handling of quarantine.
- Dissemination of information, education and communication materials on social distancing, handwashing, proper use of masks and hand sanitizers, mass awareness *via* television, radio, social media and pamphlets.
- Launch of mobile application (*Hamro Swasthya*), the web portal (covid19.mohp.gov.np).
- Two toll-free call centers to provide counseling on COVID-19 prevention and treatment.
- Daily briefings by the MOHP to update about the current situation.
- Travel restriction, testing, and tracing.
- Health sector emergency response plan for COVID-19 pandemic which includes strategies to deal quarantine management, case investigation, contact tracing, community-level screening and testing, strengthening laboratory capacity etc.
- Protocol on the safe management of dead bodies.
- Guidelines issued for the management of isolation of COVID-19 cases.
- National testing guidelines for COVID-19.
- Public health standards to be followed by people and institutions during the COVID-19 pandemic and lockdown.
- Allowed private laboratories to perform RT-PCR test.
- Availability of RT-PCR tests in all seven provinces of Nepal.
- Health standards for isolation of COVID-19 cases.
- Training of trainers on case investigation and contact tracing.
- MOHP endorsed the standards for home quarantine.
- The number of hospitals for the management of COVID-19 (as of 21 July 2020):
  - Hospitals with COVID-19 clinics: 125
  - Level 1 COVID Hospitals (for management of positive cases with mild symptoms): 16
  - Level 2 COVID Hospitals (for management of positive cases with moderate or severe symptoms): 16
  - Level 3 COVID Hospitals (for management of COVID positive cases who needs multi-speciality services): 4
- Total laboratories established capable of doings RT-PCR: 28
- Total intensive care unit beds allocated for COVID-19 cases: 942 of total 2,600 ICU beds available in the country.
- Total ventilators allocated for COVID-19 cases: 496 of total 900 ventilators available in the country.

DISCUSSION

Nepal had performed 7,791 tests for COVID-19, the highest number of tests during the lockdown. It has recorded its highest daily rise in coronavirus infections with a total of 740 new

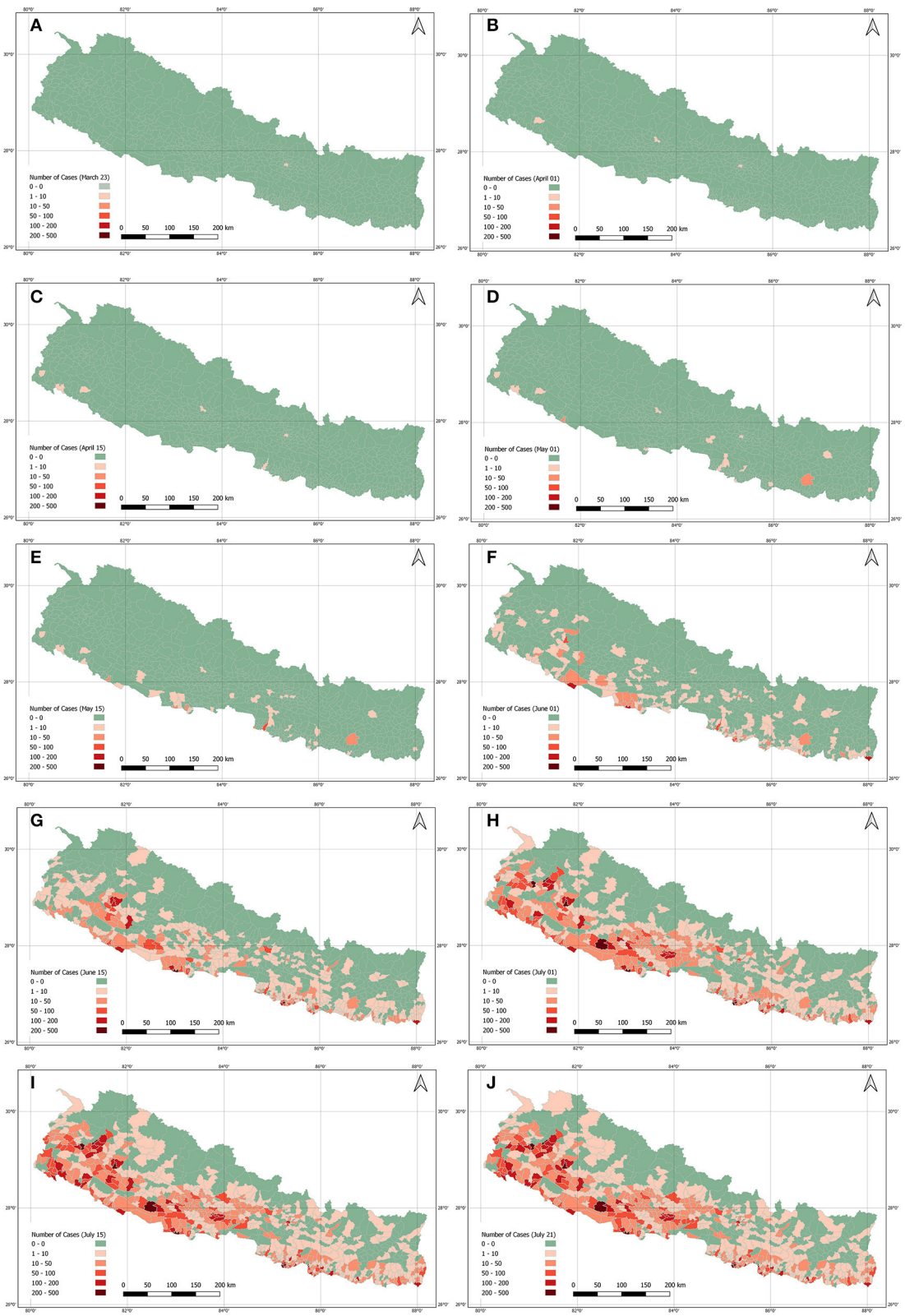

FIGURE 5 | Total positive COVID-19 cases across the country by date during lockdown period. **(A)**: March 23. **(B)**: April 01. **(C)**: April 15. **(D)**: May 01. **(E)**: May 15. **(F)**: June 1. **(G)**: June 15. **(H)**: July 1. **(I)**: July 15. **(J)**: July 21.

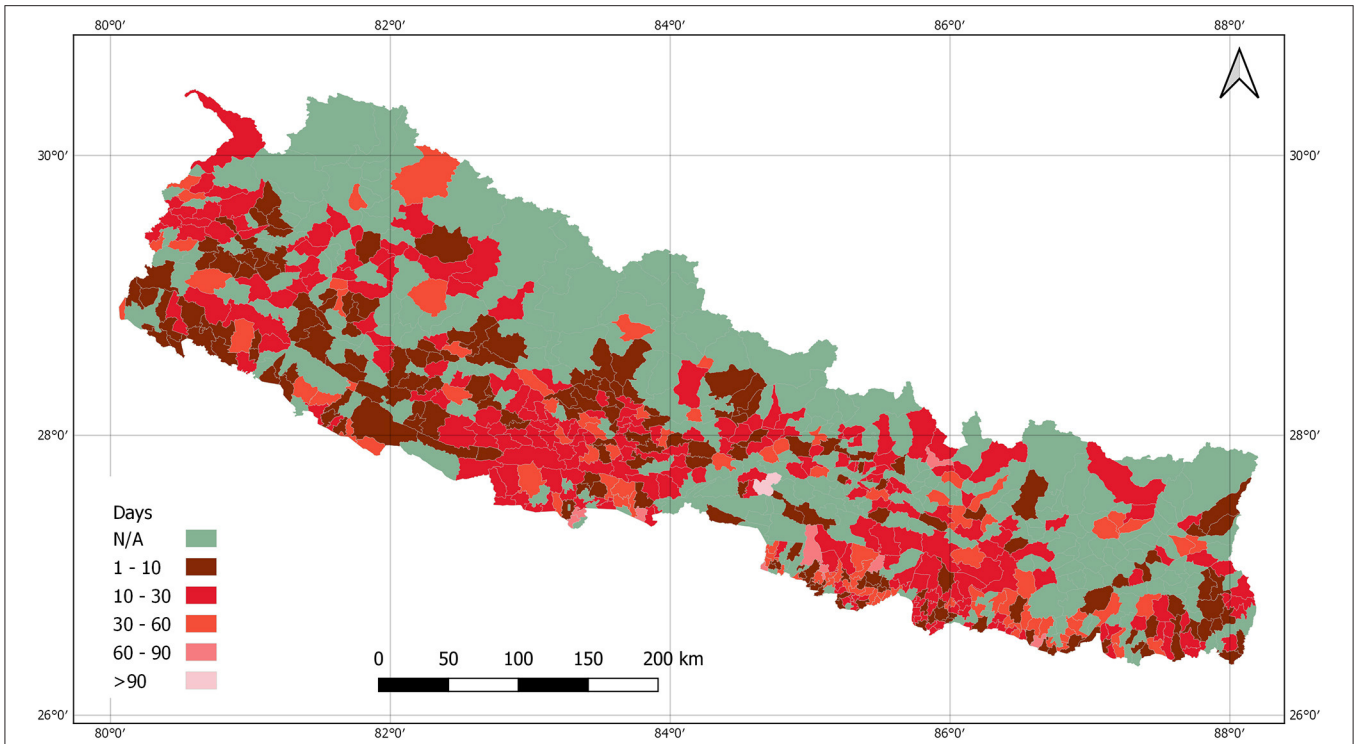

FIGURE 6 | Number of days since the last case reported on the day of lockdown lifted.

cases from the total of 4,483 tests performed on a single day. The number of people recovered from the coronavirus in Nepal increased during the lockdown. Nepal had reported a total of 17,994 positive cases and 40 deaths on the last date of nationwide lockdown. The spatial distribution clearly shows that the cases were rapidly spreading from the southern part of the country where most points of entry and exit from India are located.

Socioeconomic Impact

Nationwide lockdown restricted the socioeconomic activities all over the country, where very few essential services were run throughout the period. Multidimensional impacts of lockdown have been found in society, many people lost their jobs, and businesses along with other health care were impacted. It disrupted the supply chain, shut many informal and small enterprises, and pushed more vulnerable people into poverty (17). The tourism industry hit hard in Nepal where it fell below 10%, resulting in more than 13,000 job loss of trekkers and guides (18). There are 1.6 to 2 million jobs at risk due to the COVID-19 crisis where 80.8% of total jobs in the country are informal (19). A household survey on the impact of COVID-19 on food security and vulnerability conducted by the World Food Programme, Nepal and the Ministry of Agriculture and Livestock Development showed that of the 4,416 households from across the country, only 42% had 1 month worth of food stocks (20).

Healthcare Impact

The lockdown has affected the health of individuals and disrupted healthcare services, particularly emergency and regular health services. During the lockdown, at the individual level, one of the most notable impacts was on psychological health. Quarantine, social isolation, and travel restrictions had negatively impacted the mental health of people who have COVID-19 and their families. A few preliminary studies have shown psychological issues such as stress, anxiety, depression, insomnia among the general population (21–23) as well as frontline health workers (24, 25). A study by Gupta and colleagues conducted among 150 health workers showed that 38 % of the healthcare workers on COVID-19 duty in Nepal suffered anxiety and/or depression (24). Another online survey conducted among 475 health workers showed that 41.9% of health workers had symptoms of anxiety, 37.5% had depression symptoms and 33.9% had symptoms of insomnia (25). Incidents of stigmatization and social discrimination of healthcare workers, people who have COVID-19 and their families were also reported in Nepal during the lockdown (26, 27).

These effects of lockdown on psychological health are in line with evidence from other countries. Studies from these countries show that lockdown, quarantine, and isolations have increased social isolation, frustration, loneliness, boredom, inadequate supplies, financial insecurity, and stigma which are associated with increased risk of depression, stress, anxiety, confusion, fear, emotional disturbance, insomnia, grief and irritability (28–30). A

study conducted in India shows that the prevalence of depression and anxiety has increased by eight to ten-fold among the adult population during lockdown (31). Women in general suffered more from lockdown reporting increased depression, anxiety, stress, and insomnia (32, 33).

Nepal Police record shows that during the lockdown, the number of suicide cases has increased. Within 74 days of lockdown, a total of 1,227 people committed suicide, which is more than 15 suicidal death per month compared to the previous year (34). Although reasons for what had caused suicide and suicidal thoughts are still unknown in Nepal, they could be linked to the uncertainty about the pandemic, self-isolation, financial burden, loss of family members, stigma as evident in previous disasters and epidemic (35–37). In addition to suicide, domestic violence, sexual abuse, and rape were being perpetrated during the lockdown in Nepal (38).

The government's priority to combat COVID-19 and the lockdown adopted to contain its transmission put vulnerable populations such as pregnant women, children, the elderly and people with non-COVID diseases at risk by impacting their ability to access essential healthcare services. For example, pregnant women faced barriers to accessing regular antenatal care and delivery services (39) and patients with non-communicable diseases faced barriers to access long-term care and medicines (40) during the lockdown periods. Millions of children aged between 6 months and 5 years missed measles and rubella mass immunization, vitamin A, and deworming tablets because the Government of Nepal postponed these national-level

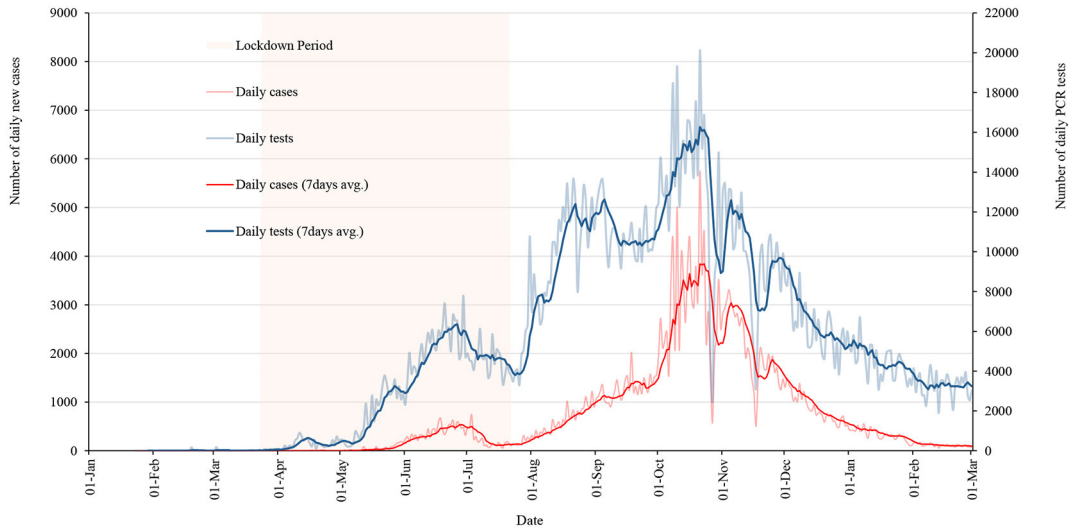

FIGURE 7 | Daily new cases and RT-PCR tests along with 7 days average of cases and tests as of 01 March 2021.

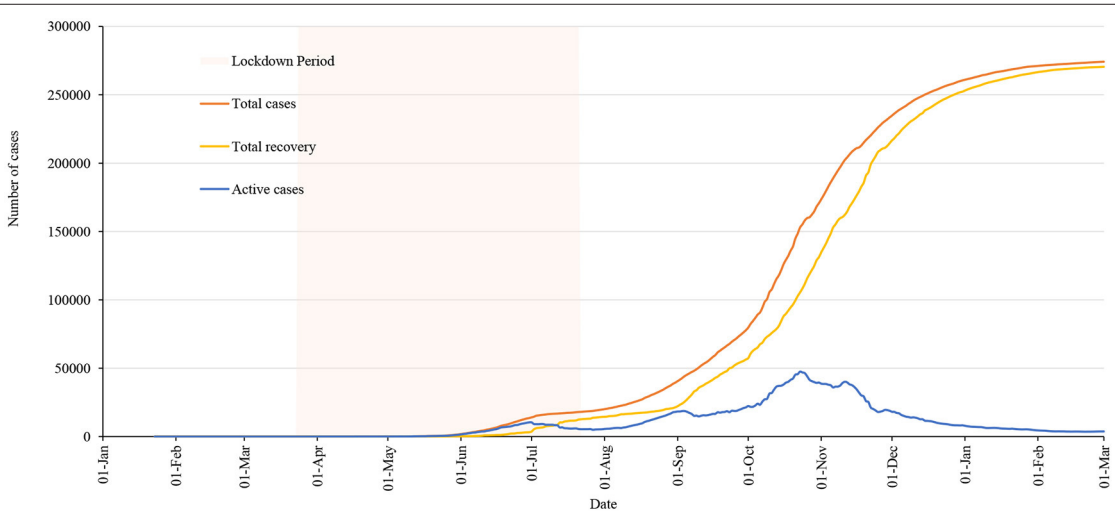

FIGURE 8 | Total cases, recovery, and active cases in Nepal as of 01 March 2021.

campaigns (41). Limited ability to access such essential and routine health care services poses an urgent threat to the nation's health and could reverse some of the achievements in reducing maternal, newborn, and child deaths.

Challenges

Future COVID-19 cases in Nepal will depend on the situation in India where the cases are increasing rapidly. Nepal shares an open border with India and there may be an increase in the number of Nepali workers returning from India who remain stranded in different parts of India due to the lockdowns in both countries. It is estimated that 600,000 migrant workers will return to Nepal within a few weeks of lifting the nationwide lockdown restrictions (42). This flow of migrant workers could increase the number of cases as the government has not been able to utilize the lockdown time efficiently to prepare and ready for responding to COVID-19. The challenge will be to test and trace these people for COVID-19. Before the lockdown, thousands of migrant Nepali workers returned to Nepal without proper screening from the Indian states of Maharashtra, Delhi and Gujarat, where the R0 value is more than one indicating an outbreak of COVID-19 in these states (4).

The testing capacity of the country has not increased due to a shortage of RT-PCR test kits, personal protective equipment, trained workers, and medical supplies. With limited testing capacity, it is challenging to monitor the transmission of the virus in Nepal because the suspected cases continue to transmit the virus while awaiting the COVID-19 test. Some COVID-19 cases remain asymptomatic, so it is difficult to predict the severity of the outbreak. There are only a few health facilities capable of treating and managing the cases with some degree of preparedness and readiness to provide the services (43, 44). If the number of cases becomes higher than the capacity of these health facilities to cope with the increased demand it would be more challenging to contain the virus.

Another challenge is to control other communicable and non-communicable diseases amidst the COVID-19 pandemic. Non-communicable diseases such as cancer, hypertension, cardiovascular disease, diabetes, chronic respiratory diseases, mental illness are already a major public health problem in Nepal and accounted for about 71% of the total annual deaths in 2019 (45). These diseases will exacerbate and the impact could be much higher than the COVID-19 if they are left behind in the fight against COVID-19.

Strengths and Limitations

The study strength included the use of openly available official figures from the MOHP web portal to provide an overall scenario of the COVID-19 pandemic during the lockdown. The findings would be applicable to compare the post-lockdown situation of the COVID-19 pandemic in Nepal and to guide more effective measures to contain the spread of the coronavirus. One of the limitations with openly available data was individual-level patient details were not accessible to perform detail epidemiological analysis. The number of people who had COVID-19 represents only a reflection of those who were tested rather than the actual figure. Also, manual gathering and submitting data from hospitals to the central government means can result in a delay and the loss of some information in reporting the number of deaths or cases so may not be a true reflection of the daily case counts. Further, the MoHP data exclude deaths from COVID-19 outside hospitals, such as those in the home. In the daily situation reports shared by the MoHP, there was inconsistency in the details provided. Some daily situation reports of the COVID-19 had information on gender while others did not contain such key information.

Post-lockdown Scenario

This paper was mainly designed to present and discuss the lockdown scenario, however considering the publication date,

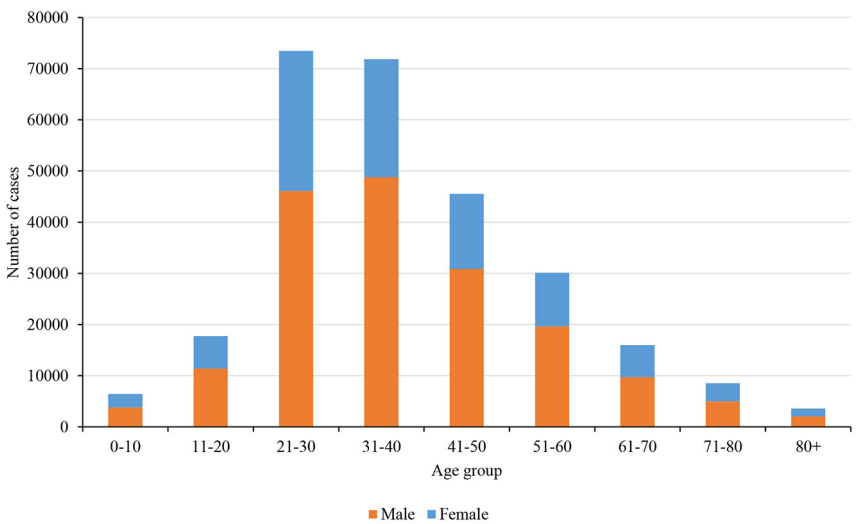

FIGURE 9 | COVID-19 cases distribution among age group and gender as of 01 March 2021.

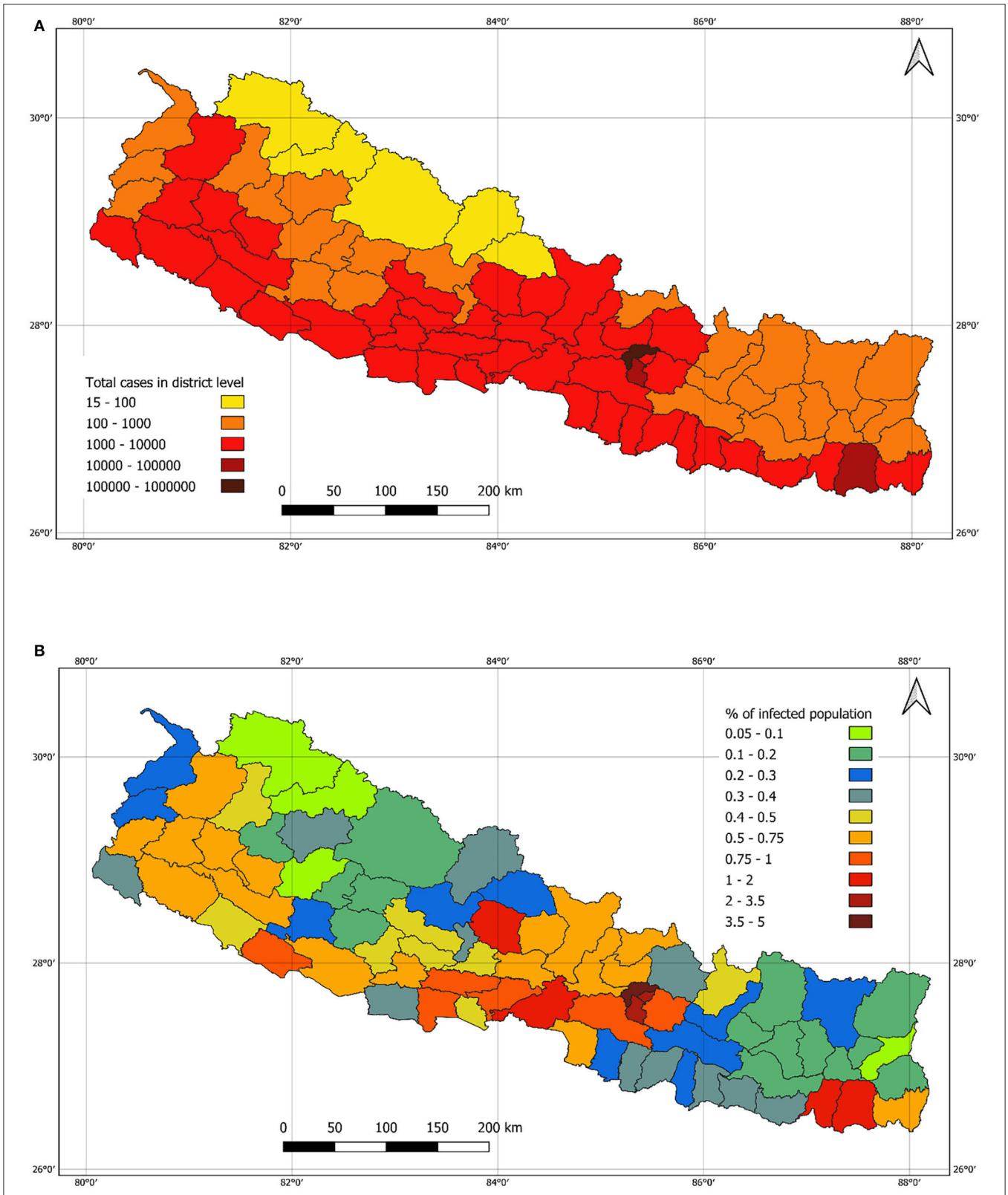

FIGURE 10 | (A): Number of cases in district level as of 01 March 2021. **(B)**: Population infection rate in district level as of 01 March 2021.

data are updated and discussed for post-lockdown scenario. **Figure 7** shows the RT-PCR tests including cases and **Figure 8** shows the total cases, recoveries, and active cases as of 01 March 2021. The number of COVID-19 cases had increased until 21 October 2020 and after that the number of cases reported and number of tests conducted had declined (**Figure 7**). The two dips in **Figure 7**, that is, on 27 October 2020 and 17 November 2020, were the festival days where less tests were performed and consequently less cases were identified.

Figure 9 shows the distribution of COVID-19 cases among the age group and gender as of 01 March 2021. It shows the community transmission of the coronavirus in the post-lockdown period contributed to the increase of cases among higher age groups and in the female. As discussed previously, one of the challenges is having a higher mortality rate when the virus spread among the older generation, which has been the case in recent days. As of 01 March 2021, mortality rate was above 1%.

Figure 10A shows the number of cases in the districts. Compared with the lockdown period, major cities including the capital city Kathmandu have a relatively higher number of cases, which resembles the overall risk scenario reported (15). **Figure 10B** shows the infected population percentage at the district level across the country. Mugu had a lowest (0.05%) infected population whereas Kathmandu had a maximum of 4.5% infected population.

CONCLUSION AND RECOMMENDATION

This study provides an overall scenario of the COVID-19 pandemic during the lockdown in Nepal. The capacity of the health system to quickly test to find out if anyone develops symptoms of COVID-19 and tracing and testing of close contacts of those who test positive for COVID-19 must be increased. The government needs to allocate resources, such as the necessary public health workforce, availability of personal protective equipment, expansion of intensive care unit beds, and purchase of extra ventilators. Other actions to stop spread include managed isolation in a designated setting for people who cannot afford to self-isolate or in a dedicated quarantine facility who can't self-quarantine.

Another approach the government could take to manage local COVID-19 outbreaks is to impose local restrictions. This could be in the form of local lockdown based on risk assessment rather than the nationwide lockdown. As the restrictions of COVID-19 lockdown are eased, there would be more flow of people which leads to more exposure so preventive measures should be established in shopping centers, cities, shops and workplaces. Citizen and institutional/governance awareness is always a key factor in disaster risk reduction and managing pandemic, that often lacking in the developing world (46). The government should continue disseminating information on following social distancing, hand hygiene, and face covering. Data from the post lockdown period also shows the ways to tackle future pandemics considering the socioeconomic condition of the community.

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

KS, AB, and RP conceptualized the study and prepared the first draft of the paper. KS and RP produced most of the figures, analyzed, and interpreted the data. All authors have approved the final version.

ACKNOWLEDGMENTS

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Epidemiological and Clinical Characteristics of COVID-19: A Retrospective Multi-Center Study in Pakistan

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The emergence of a pathogen responsible for a mysterious respiratory disease was identified in China and later called a novel coronavirus. This disease was named COVID-19. The present study seeks to determine the epidemiological and clinical characteristics of COVID-19 in Pakistan. This report will exhibit a linkage between epidemiology and clinical aspects which in turn can be helpful to prevent the transmission of the virus in Pakistan. A retrospective, multiple center study was performed by collecting the data from patients' with their demographics, epidemiological status, history of co-morbid conditions, and clinical manifestations of the disease. The data was collected from 31 public-sector and 2 private hospitals across Pakistan by on-field healthcare workers. A Chi-square test was applied to assess the relationship between categorical data entries. A total of 194 medical records were examined. The median age of these patients was found to be 34 years. A total of 53.6% active cases were present including 41.2% males and 12.4% females till the end of the study. Adults accounted for most of the cases (94.3%) of COVID-19. Fever (86.60%), cough (85.05%), fatigue (36.60%), dyspnea (24.74%), and gastrointestinal discomfort (10.31%) were among the most frequently reported signs and symptoms by the patients. However, 4.12% of the total patient population remained asymptomatic. The median duration of hospital stay was found to be 14 (0–19) days. The earliest source of the spread of the virus may be linked to the foreigners traveling to Pakistan. Spread among men was more as compared to women. A few cases were found to be positive, due to the direct contact with pets or livestock. Hypertension (7.73%), diabetes (4.64%), cardiovascular conditions (2.58%) were the most common co-morbidities. The percentage mortality was 2.50% with the highest mortality among elders.

Keywords: epidemiology, COVID-19, clinical characteristics, diagnosis, treatment, Pakistan

INTRODUCTION

In December 2019, an event of respiratory disease due to an unknown cause with similarities to that of pneumonia was identified in China (1). Later, the World Health Organization (WHO) acknowledged it to be the sixth emergency service of public health on January 30, 2020 (2) and declared it as a global pandemic in March 2020 (3, 4). On February 11, 2020, the WHO named this viral pneumonia as Corona Virus Disease-19 (COVID-19) (5). The metagenomics analysis was performed through the samples of bronchoalveolar lavage taken from the infected patients (6) and the newly identified pathogen was named as 2019 novel coronavirus (2019-nCoV) by the United States Center for Disease Control and Prevention (CDC) (7). The COVID-19 had almost 88% genetic resemblance to the severe acute respiratory syndrome (SARS). Two SARS viruses were bat-derived coronaviruses bat-SL-CoVZXC21 and bat-SL-CoVZC45 (8). The receptor for the COVID-19 virus is the same as that of SARS-CoV, i.e., angiotensin-converting enzyme-2, ACE-2 receptor (9). The novel corona virus is now listed as the 7th member of the coronavirus family (10).

Multiple epidemiological studies reported that the COVID-19 is identified in Wuhan, China on December 8, 2019 (2, 11–13). This disease later spread worldwide including Iran, Europe, India, United Kingdom (UK), and Pakistan, and officially became a pandemic on March 11, 2020 (13, 14). In Pakistan, the first incidence of this disease was identified at the end of February 2020 (15, 16). COVID-19 is extremely contagious and its spread takes place *via* human-to-human transmissions (17). As of February 15, 2021, the total reported cases in Pakistan were 564,077, while total deaths were around 12,333 and the total recovered were 525,277, as per the data released by the Government of Pakistan (https://covid.gov.pk/).

The coronavirus is encased with an exceptionally huge positive-sense strand of the RNA genome, which mutates very rapidly due to errors in the RNA (10, 18). Pertaining to its continuous mutation, it is highly contagious and may be identified in several animals (19–21). In one of the Indian analysis, the prediction was floated that the cases of COVID-19 will keep on increasing with higher transmission rates as well as with seasonal occurrences (22, 23). Several mathematical models have suggested that the spread of the virus may be retarded by taking precautionary measures including social distancing, isolation, and contact tracing (24, 25). In humans, some patients may remain asymptomatic or may be a carrier of the disease (26–29).

In Pakistan, some patients were reported to be asymptomatic which may serve as a carrier to other people, if not managed properly (30, 31). The purpose of this study is to assess heedfully the epidemiological and clinical characteristics of COVID-19 in Pakistan. This study will exhibit a linkage between epidemiology and clinical aspects which in turn can be helpful to prevent the transmission of the virus in Pakistan.

MATERIALS AND METHODS

A retrospective, multiple center study was performed by collecting the patient's demographics, epidemiological status, history of co-morbid conditions, and assessment of clinical manifestations. The data were collected from 33 hospitals (31 public sector hospitals and 2 private sector hospitals) with the help of in-field healthcare workers i.e., doctors, nurses, or pharmacists of the respective hospitals involved in the medical care of these patients. The diagnosis of COVID-19 was made either by taking a specimen from a throat swab and then performing a Real Time-Polymerase Chain Reaction (RT-PCR) in a laboratory setting or by evaluating the clinical symptoms to ascertain the diagnosis. There were 189 lab-confirmed cases; on the other hand, five patients were diagnosed with definitive travel history, signs, and symptoms of COVID-19.

Data collection was initiated on March 16, 2020, and the follow-up of the study was made on April 14th, 2020. Whereas, the data of a few patients were also collected by the end of April 2020. The confirmed 194 cases of the disease through either RT-PCR or clinical diagnosis were considered. A total of 192 cases were confirmed by RT-PCR whereas 2 cases were diagnosed based on clinical manifestations.

Ethical Approval was conferred from Riphah International University, Lahore, Pakistan (Letter No. RCVETS-701). Efforts were made to ensure data collection from different provinces of Pakistan including Punjab, Sindh, Khyber Pakhtunkhwa, Gilgit Baltistan, Islamabad, and Azad Jammu and Kashmir (AJK), a self-governing state under the constitution of Pakistan.

Statistical Analysis

The data were analyzed by using Statistical Package for Social Sciences version 21 (SPSSv21). Frequency, percentages, median, ranges, and interquartile ranges were used to display data. Mann-Whitney *U*-test was used for comparison across the groups. A Chi-square test was applied to assess the relationship between categorical data entries.

RESULTS

As of April 14, 2020, epidemiological data of 194 patients were collected including 10 (5.15%) patients from the Sindh province, 157 (80.93%) from Punjab, 2 (1.03%) from Islamabad, 3 (1.55%) patients from Gilgit-Baltistan (GB), 6 (3.09%) patients from Khyber Pakhtunkhwa (KPK), and 16 (8.25%) from AJK. The team was unable to collect any data from the province of Baluchistan, the least populated province, due to limited resources. **Figure 1A** depicts the locations and the amount of patient data collected from that facility. On the other hand, **Figure 1B** represents the official data by the Government of Pakistan (http://covid.gov.pk/stats/pakistan) of all the patients throughout the country as of April 13, 2020, at 0530 h.

A total of 194 patients' medical records were examined. The median age of these patients was found to be 34 years with an interquartile range (IQR) of 27–48 years. The youngest patient was 6 months old, whereas the oldest one was 87 years of age.

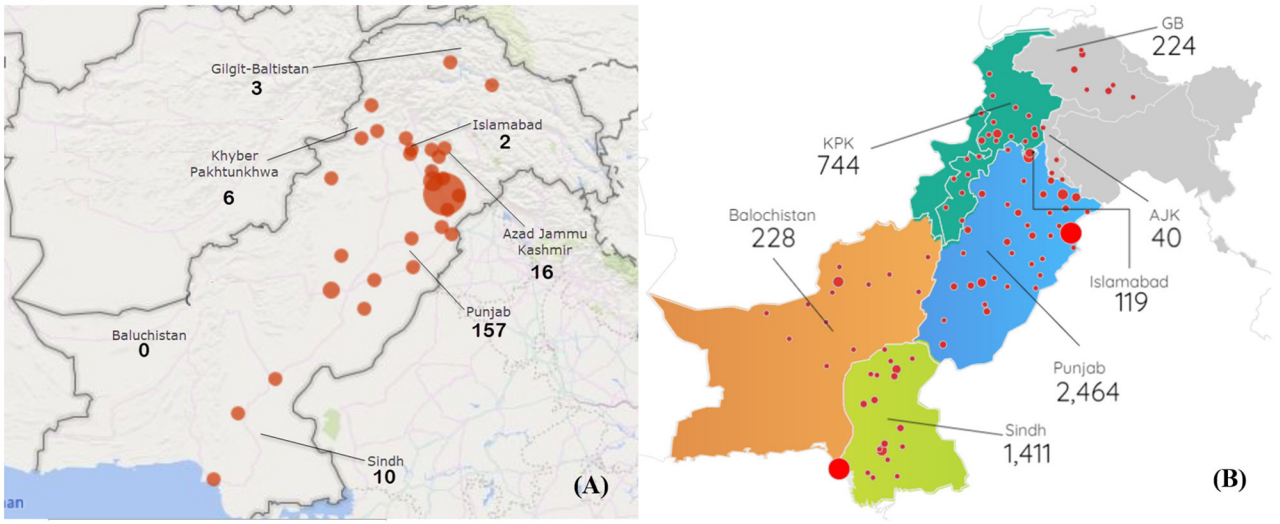

FIGURE 1 | (A) Depicts the sample data distribution throughout the country. **(B)** Represents the population data generated by Government of Pakistan. The size red dots represents the number of the patient data collected from each district in **(A)** whereas, the red dots in **(B)** represents the number of active patients. The values below each province name represents the number of patients.

Adults accounted for most of the active cases of COVID-19 with 55 (28.4%) patients in 18–29 years of age, 49 (25.3%), 31 (16.0%), and 21 (10.8%) patients were found to be in the age ranges of 30–39, 40–49, and 50–59 years, respectively. The study also included 27 older patients altogether as per locally accepted criterion of old aged individuals. 11 (5.7%) of young patients were also infected.

The distribution of infected males and females were found to be 157 (80.9%) and 37 (19.1%), respectively (**Table 1**). One of the females was pregnant and tested positive for COVID-19 although, she remained asymptomatic with no reported complications.

The earliest hospital admission of our sample dates was back on February 26, 2020, of a patient who had a recent prior visit to Iran. However, this patient presented to the hospital after 20 days of arrival in Pakistan. Most of the earlier cases were found to be amongst the foreigners. A total of 72 (37.1%) patients had a recent travel history abroad and local transmission comprised 122 patients (62.9%) in this study. Among patients with travel history, 20 (10.3%) patients were from Spain, 17 (8.8%) from the United Kingdom, 10 (5.0%) from Iran, and 7 (3.6%) were from the Kingdom of Saudi Arabia. For most of the patients, the transmission was found to be of unknown origin (37.6%) since they did not have a substantial travel history and were unaware of any contact that could have infected them. 34 (17.5%) individuals had direct contact with the already infected patients of COVID-19. Paramedical staff and doctors are at great risk due to a lack of proper PPE and safety equipment. Among the data collected from different hospitals, most of the patients got infected by direct contact from healthcare providers, including 12 physicians and five paramedical staff. Besides, it was observed that individuals with more public exposure were part of our study, including an epidemiologist, a religious scholar, a lawyer, and a news reporter.

Hypertension was observed to be the most prevalent co-morbidity affecting 15 (7.73%) patients of the total sample. This was followed by diabetes (4.64%), heart conditions (2.58%), asthma (1.55%), and other minor co-morbidities (2.58%) (**Table 1**).

Clinical Manifestations

The signs and symptoms of the patients were recorded, and 168 (86.60%) patients exhibited fever. The cough was the second most frequent sign and was experienced by 165 (85.05%) of the patients. 71 (36.60%) patients' complaints of having fatigue. Dyspnea or shortness of breath was the next most occurring symptom (24.74%). Some of the patients also (10.31%) reported gastrointestinal discomfort. 17 (8.76%) patients had the flu, whereas six patients had a cold. Surprisingly, a considerable number of patients (6.70%) also reported a loss of sense of smell and taste. Myalgia, nausea, anorexia, and sore throat were reported by 10 (5.15%), 8 (4.12%), 5 (2.58%), and 3 (1.55%) of patients, respectively. Redness of eyes, dizziness, and anxiety was also observed in 0.52% of the patients. Conversely, 8 (4.12%) patients were asymptomatic. Two patients were put on ventilators; however, both patients expired.

The median duration of hospitalization for COVID-19 patients was found to be 14 days with a stay range of as low as 0 days and as high as 43 days. Paracetamol was the most prescribed medicine (4.64%), followed by chloroquine (1.55%) and cetirizine (1.03%).

Clinical outcomes were evaluated in the last section of data collection. As of April 14, 2020, a total of 70 (36.1%) patients were recovered and discharged. On the other hand, 20 (10.3%) of the deaths were reported. The rest of the patients were still in the hospital, 76 (39.2%) of them were stable and more likely to be discharged in a few days, while 28 (14.4%) patients were still

TABLE 1 | Demographical and epidemiological status of the patients ($N = 194$).

	All patients ($N = 194$)	Male ($n = 157$)	Female ($n = 37$)
Median age in years (Interquartile range)	**34 (27–48)**	**33 (27–48)**	**35 (27–50)**
<18	11 (5.7)	7 (4.5)	4 (10.8)
18–29	55 (28.4)	46 (29.3)	9 (24.3)
30–39	49 (25.3)	42 (26.8)	7 (18.9)
40–49	31 (16.0)	24 (15.3)	7 (18.9)
50–59	21 (10.8)	16 (10.2)	5 (13.5)
60–69	20 (10.3)	16 (10.2)	4 (10.8)
≥70	7 (3.6)	6 (3.8)	1 (2.7)
Gender			
Female		37 (19.1)	
Male		157 (80.9)	
Epidemiological status			
Local	**122 (62.9)**	**97 (61.8)**	**25 (67.6)**
Direct contact	34 (17.5)	9 (5.7)	1 (2.7)
Religious congregation	11 (5.7)	11 (7.0)	0 (0.0)
Infected family member	4 (2.1)	0 (0.0)	4 (10.8)
Unknown origin	73 (37.6)	77 (49.0)	20 (54.1)
Foreign	**72 (37.1)**	**60 (38.2)**	**12 (32.4)**
Spain	20 (10.3)	17 (10.8)	3 (8.1)
United Kingdom	17 (8.8)	12 (7.6)	5 (13.5)
Iran	10 (5.2)	10 (6.4)	0 (0.0)
Kingdom of Saudi Arabia	7 (3.6)	4 (2.5)	3 (8.1)
Italy	4 (2.1)	4 (2.5)	0 (0.0)
Turkey	4 (2.1)	4 (2.5)	0 (0.0)
Other countries	10 (5.2)	8 (5.1)	1 (2.7)
Arrival from abroad to hospital admission median time in days (Interquartile range)	**4 (2–6)**	**4 (2.75–6.25)**	**3 (2–3.75)**
History of chronic medical conditions			
Hypertension	15 (7.73)	13 (8.3)	2 (5.4)
Diabetes	9 (4.64)	7 (4.5)	2 (5.4)
Heart conditions	5 (2.58)	5 (3.2)	0 (0.0)
Asthma	3 (1.55)	1 (0.6)	2 (5.4)
Others	5 (2.58)	0 (0.0)	1 (2.7)

The values represent the number of patients along with percentages [n (%)] where no unit is mentioned. The median age is represented in years along with interquartile range [age (IQR)]. Median time in days along with interquartile range [time (IQR)].

TABLE 2 | Clinical features, signs, symptoms, methods of diagnosis, medications, and outcomes.

Signs and symptoms	All patients ($N = 194$)	Male ($n = 157$)	Female ($n = 37$)
Fever	168 (86.60)	136 (86.6)	32 (86.5)
Cough	165 (85.05)	134 (85.4)	31 (83.8)
Fatigue	71 (36.60)	60 (38.2)	11 (29.7)
Shortness of breath	48 (24.74)	35 (22.3)	13 (35.1)
Gastrointestinal discomfort	20 (10.31)	17 (10.8)	3 (8.1)
Flu	17 (8.76)	15 (9.6)	2 (5.4)
Loss of sense of taste and smell	13 (6.70)	10 (6.4)	3 (8.1)
Myalgia	10 (5.15)	7 (4.5)	3 (8.1)
Nausea	8 (4.12)	7 (4.5)	1 (2.7)
Cold	6 (3.09)	6 (3.8)	0 (0.0)
Anorexia	5 (2.58)	4 (2.5)	1 (2.7)
Sore throat	3 (1.55)	3 (1.9)	0 (0.0)
Dizziness	1 (0.52)	1 (0.6)	0 (0.0)
Redness of the eye	1 (0.52)	1 (0.6)	0 (0.0)
Anxiety	1 (0.52)	1 (0.6)	0 (0.0)
Asymptomatic patients	8 (4.12)	7 (4.5)	1 (2.7)
Patients on ventilators	2 (1.03)	1 (0.6)	1 (2.7)
Methods of diagnosis			
Specimen by throat swab for RT-PCR laboratory-confirmed	189 (97.4)	154 (98.1)	35 (94.6)
Clinical-confirmed	5 (2.6)	3 (1.9)	2 (5.4)
Median duration of hospital stay in days (range)	14 (0–19)	14 (0–19)	14 (0–15)
Treatment/medications administered			
Paracetamol	9 (4.64)	7 (4.5)	2 (5.4)
Chloroquine	3 (1.55)	3 (1.9)	0 (0.0)
Cetirizine	2 (1.03)	2 (1.3)	0 (0.0)
Clinical outcomes			
Recovered and discharged	70 (36.1)	64 (40.8)	6 (16.2)
In hospital and stable	76 (39.2)	59 (37.6)	17 (45.9)
In hospital and under observation	28 (14.4)	21 (13.4)	7 (18.9)
Expired	20 (10.3)	13 (8.3)	7 (18.9)

The values represent the number of patients along with percentages [n (%)] where no unit is mentioned. Median duration of hospital stay in days along with range [duration (Range)].

under observation out of which 23 (11.8%) patients recovered and 5 (2.5%) died as per data collected by the end of April 2020. A total of 53.6% active cases were present including 41.2% males and 12.4% females till the end of the study (**Table 2**).

Mann-Whitney *U*-test was applied to evaluate the clinical outcomes of the disease against the patients' age. It was observed that the patients who were recovered had an average age of 33.66 years. The patients who were kept in hospital but were stable had an average age of 37.51 years; those under observation had an average age of 38.61 years. Patients who expired were having an average age of 55.30 years ($P < 0.05$). The highest recovery percentage (72.73%) was among young patients with ages <18 years whereas; the highest mortality percentage was among older patients with an age range of 60–69 years (**Table 3**).

DISCUSSION

As of April 11, 2020, the COVID-19 attained over 1.6 million cases as per WHO, claiming nearly a hundred thousand lives (32). Recent data presents that COVID-19 cases have been increased and crossed over 80 million globally by February 2021, according to WHO. Pakistan is also amongst the countries affected most by this pandemic with estimated cases of over 0.5 million by February 2021 and a mortality rate of 1.7% (33).

The median age of infected individuals was 34 years. The adult age group (19–59 years) was more affected by the infection. The population demographics of the country, according to the 1998

TABLE 3 | Disease outcome status with categorical age-wise distribution.

Age categories	Disease outcome *n* (%)			
	Recovered	Stable	Under observation	Expired
<18	8 (72.73)	1 (9.09)	2 (18.18)	0 (0.00)
18–29	22 (40.00)	24 (43.64)	7 (12.73)	2 (3.64)
30–39	18 (36.73)	20 (40.82)	9 (18.37)	2 (4.08)
40–49	12 (38.71)	15 (48.39)	3 (9.68)	1 (3.23)
50–59	3 (14.29)	10 (47.62)	3 (14.29)	5 (23.81)
60–69	6 (30.00)	6 (30.00)	2 (10.00)	6 (30.00)
≥70	1 (14.29)	0 (0.00)	2 (28.57)	4 (14.29)

census, suggesting that nearly 40% of the country's population comprises adults whereas, 53% of the total population is under 19 years of age. 5.54% of the total population is above 60 years of age (34). The occurrence of the infection in females (19.1%) is less as compared to the male (80.9%) population. Our results bear similarity to a recent study in China, where the percentage of the infected females was lesser as compared to the males (26, 35). A previous study also suggested that male mice were more susceptible to the SARS-CoV and MERS-CoV as compared to the female mice (36). Currently, there is no reliable evidence regarding the influence of sex on the susceptibility of the infection. Hence, further studies are required to ascertain this behavior.

The earliest source of the spread of the virus may be linked to the foreigners entering Pakistan from Iran. The disease outbreak in Iran was reported in late January 2020, but the first cases of COVID-19 were identified in late February 2020 (37). Therefore, the dissemination of the virus in Pakistan may be firstly linked to Iran. The travelers from Spain contributed to the highest number of infected patients. According to our data, the local transmission of the virus was massive in the province of Punjab, which is one of the most populated provinces of Pakistan (38). There is already strong evidence of human-to-human transmission of the disease (39). Our study also confirms that individuals with more public exposure are at a higher risk of acquiring the disease. Furthermore, the religious congregations held in March also led to an increased number of cases. Therefore, social distancing must be encouraged to avoid the exponential dissemination of the disease (40, 41).

Another alarming situation observed in our study was the fact that 17 (8.76%) healthcare workers including physicians and paramedics were found to be infected (42, 43). They were also affected by stress and anxiety during the pandemic (44, 45). During the recent coronavirus outbreak in China, a substantial number of healthcare workers acquired the infection (43, 46). A study from China reported that 3.8% of healthcare workers were affected by the disease (47). Another publication discussed the mortality of 23 healthcare workers along with two physicians in China bringing in light the risk these health workers deal with within their daily routine (48). However, in our study, this percentage is quite higher as compared to the reported studies. The lack of personal protective equipment (PPE), prolonged exposure to patients, and inadequate knowledge of the disease transmission among the healthcare providers may have increased such incidents (49, 50). Increased awareness of self-protection, adequate supplies of PPE, and a prompt response may aid in decreased susceptibility of infection among healthcare workers (46, 51, 52).

The most prevalent comorbidities in our study were hypertension, diabetes, cardiovascular conditions, and asthma. A similar pattern was also found in different studies where hypertension was the most prevalent co-morbidity followed by diabetes, heart diseases, and respiratory illnesses (53–55). Some other studies revealed the same pattern as mentioned earlier, with hypertension, diabetes, and cardiovascular diseases as the prime co-morbidities (56, 57).

Fever, cough, fatigue, dyspnea, and gastrointestinal discomfort were among the most frequently reported signs and symptoms by the patients (58). The clinical symptoms of 100 patients admitted to a hospital in Karachi included dry cough, fever, lethargy, fatigue, dyspnea, myalgia, vomiting, nausea, and diarrhea (59). A single-center study from Pakistan has depicted a similar trend of clinical symptoms as our study (60). Other studies have reported a similar set of signs and symptoms (61–63). However, 4.12% of the total patient population remained asymptomatic. This trend is also like the other studies (62, 64, 65). Surprisingly, 6.70% of the patients reported a loss of sense of smell and taste which along with other symptoms, is a strong predictor of COVID-19 infection (66–69). Moreover, flu, myalgia, nausea, cold, anorexia, sore throat, dizziness, redness of the eyes, and anxiety were also reported (45, 63, 70).

Paracetamol, chloroquine, and cetirizine were the most frequently prescribed medicines during the early days of the COVID-19 pandemic in Pakistan (71–74). However, the current data is insufficient to assess the effect of medications on the outcome of the disease. Paracetamol was the most prescribed medicine as it is the safest drug for managing the COVID-19 symptoms in place of ibuprofen (75), followed by chloroquine which is being imagined as a miracle drug (76). Altogether, 20 deaths were reported in our study 104 (53.61%) patients were still hospitalized, with 76 patients in stable condition, and the rest 28 patients were still under observation. Complete recovered patients 70 (36.1%) and were discharged from the hospital. Out of 28 patients, 23 (11.8%) patients recovered and 5 (2.5%) died by the end of April 2020.

There might be a link between COVID-19 with the human population and animals. Some zoo animals were also reported positive for SARS-CoV-2, however, under experimental conditions, chicken and ducks were not affected with COVID-19 (77). Inter-specie transmission of COVID-19 was very recent and must be addressed after conducting different research studies. Different experimental trials suggest that pets (cats, dogs) might also be susceptible to SARS-CoV-2 from humans (19, 78).

The present study has some limitations since 53.61% of the sample patients were still hospitalized, and the recovery of these patients was not ascertained. The data collected was not evenly distributed throughout the country. Moreover, remained unable to investigate more clinical indicators such

as complete blood count, CT scans, or chest X-rays since there were a limited number of tests performed by the hospitals. The non-availability of data such as the date of onset of symptoms had prevented us from evaluating more factors such as the incubation period of the virus. The current study is amongst one of the first studies to portray the epidemiological picture of COVID-19 in Pakistan. Being a lower-middle-income country, Pakistan is facing many challenges from inadequate health facilities to poor socioeconomic conditions. Our study may help in identifying and developing a response that may alleviate the rapid onset of disease.

CONCLUSIONS

The earliest source of the spread of the virus may be linked to the foreigners traveling Pakistan. Spread among men was more as compared to females. Fever, cough, fatigue, dyspnea are the most common symptoms. A few positive cases were found to be directly in contact with pets or livestock. Hypertension, diabetes, cardiovascular conditions are the most common co-morbidities. The percentage mortality was 2.50% with the highest mortality among elders.

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 Institutional Ethics Committee (IEC) of Riphah College of Veterinary Sciences. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MA and BB conceived the idea and did a write-up of the manuscript. SR did the critical appraisal of findings with literature search. SA, AM, and RM did the acquisition of patient data and drafting of the article. SM did the analysis and interpretation of the data. MH and MR did general supervision of the research group and final approval of the version to be published. All authors contributed to the article and approved the submitted version.

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REVIEW
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Research and Management of Rare Diseases in the COVID-19 Pandemic Era: Challenges and Countermeasures

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The ongoing coronavirus disease 2019 (COVID-19) pandemic has disrupted every aspect of our life. The need to provide high-level care for an enormous number of patients with COVID-19 infection during this pandemic has impacted resourcing for and restricted the routine care of all non-COVID-19 conditions. Since the beginning of the pandemic, the people living with rare disorders, who represent a marginalized group of the population even in a normal world, have not received enough attention that they deserve. Due to the pandemic situation, they have experienced (and experiencing) an extreme inadequacy of regular clinical services, counseling, and therapies they need, which have made their life more vulnerable and feel more marginalized. Besides, the clinicians, researchers, and scientists working on rare genetic diseases face extra challenges due to the pandemic. Many ongoing research projects and clinical trials for rare and genetic diseases were stalled to avoid patients' and research staff's transmission to COVID-19. Still, with all the odds, telehealth and virtual consultations for rare disease patients have shown hope. The clinical, organizational, and economic challenges faced by institutions, patients, their families, and the caregivers during the pandemic indicate the importance of ensuring continuity of care in managing rare diseases, including adequate diagnostics and priority management strategies for emergencies. In this review, we endeavored to shed light on the issues the rare disease community faces during the pandemic and the adaptations that could help the rare disease community to better sustain in the coming days.

Keywords: COVID-19, rare disease, clinical management, counseling, telemedicine

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BACKGROUNDS

The coronavirus disease 2019 (COVID-19) pandemic remains an enormous global challenge due to its persistent spread and unpredictable disease course. As of February 2021, the disease has caused ∼110 million confirmed cases and ∼2.5 million deaths (1). Current understanding of the COVID-19 pathobiology indicates that infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiologic cause of COVID-19, results in an impaired adaptive host inflammatory response, causing excessive activation of innate pathways to generate a cytokine storm and edema leading to pulmonary fibrosis and severe pathology (2, 3). Risk factors for adverse outcomes include old age, male sex, and comorbidities (4, 5). Also, people with weakened immune systems face a

higher risk. With the great efforts of clinicians, researchers, and academicians worldwide, vaccines have rolled out for mass vaccination in some countries, and other countries are also in the process of starting vaccination programs. The world is hoping to get back to a "normal" world soon. However, there is still uncertainty of management strategies for the patients who require critical care and effective treatment. Researchers and clinicians have so far recorded only a dearth of reports of infected patients with rare diseases. In the literature and our own experience, few patients with rare diseases have presented COVID-19, perhaps because of their awareness of risks and preventive measures (6, 7). As a result, only a few small cohort studies and case reports on the effects of COVID-19 on people with rare diseases, e.g., thalassemia, are available (8–10). Because of the insufficient clinical evidence, any comment on the relationship between certain rare diseases and COVID-19 may be regarded as mere theories; however, they should not be ignored.

The COVID-19 pandemic has heightened uncertainty over all aspects of our life, including family and community life, economies, and healthcare, and none more so than the most vulnerable of us—individuals with rare diseases. There are between five to eight thousand rare diseases, most of them with a genetic basis, affecting ∼400 million people worldwide (11–13). Even in the best of times, people with rare diseases and their caregivers report significant care inadequacies and unmet clinical needs. Besides, the difficulty and expense of assembling large cohorts of affected individuals for study and garnering research funding is already a concern for researchers. Along with the general anxieties about health concerns everyone else has, people with rare diseases have a double burden of challenges due to the pandemic. They also face uncertainty about the supply of medications and the accessibility of essential occupational therapies they need regularly.

The COVID-19 pandemic has also impacted clinical and health research severely. It caused stall many translational, clinical, and basic science research (14), thus influencing every medical practice aspect. It has also led to a sudden rift in the medical research on diseases other than COVID-19, making the rare disease research more challenging and slower. Numerous experiments and clinical trials have been abandoned, suspended, or post-poned (15, 16). Many have paused on their ongoing clinical research to focus on SARS-CoV-2 related research or made substantial modifications to ensure safe clinical care in the hospital. As a result, the research and development on other diseases, e.g., cancer, cardiovascular conditions, and rare diseases, may experience (and already is experiencing) disruption—potentially causing the people living with these diseases to suffer delayed access to new drugs and/or management strategies (17). While combatting the pandemic mainly focusing on the general people, collaborations between the patient, scientific communities, government, diagnostic service providers, and rare disease research need prioritization to ensure proper management of rare diseases. Persisting needs include dissemination of specific knowledge regarding optimal care and research to prevent, treat, and cure disease.

This review discusses the difficulties and struggles of rare disease patients, caregivers, and researchers studying such diseases, amidst COVID-19 and even after the pandemic is over. Also, it focuses on how to manage these challenges better in a world free of COVID-19.

IMPACT OF COVID-19 ON RARE DISEASE COMMUNITIES

The far-reaching impacts of the COVID-19 pandemic on rare disease communities were reflected in a recent report by the U.S. National Organization for Rare Disorders (NORD) (18). The report suggests that almost all respondents (∼98%) were overwhelmingly concerned and worried about the pandemic due to several reasons (**Figure 1**). Among them, 95% of families had been directly influenced by COVID-19, with more than 50% having medical appointments replaced with a telephone or video call. Besides, three out of every five respondents expressed concerns about a potential shortage of medication and medical supplies.

The COVID-19 pandemic led to a loss of jobs, whether temporarily or permanently, for over one-fourth of the respondents. Over 10% of these job losses resulted in a loss of their health insurance (24). As many individuals with rare diseases require continuous treatment support, which needs the families' financial stability, job loss due to pandemic has directly impacted their routine management.

The European Organization for Rare Diseases (EURORDIS) found a similar impact of the COVID-19 pandemic on people living with rare diseases. It reported that nine out of ten Europeans living with a rare disease had faced pause or interruptions in their regular health care since the beginning of the pandemic, and most of them were worried that this pause could be life-threatening. Most pre-scheduled surgeries, transplants, and rehabilitation therapies, e.g., speech and physical therapies, have been canceled or postponed (19). As the pandemic persists, some hospitals have temporarily closed rare disease units, and as a result, patients who used to receive treatments in these units are experiencing difficulties.

For the time being, most consultants are now trying to provide support and services to people with rare diseases by telephone, videoconferencing. It was reported that nearly half of the respondents had received telemedicine service as in-person consultations are now not recommended. In addition, according to the survey of EURORDIS, most of the respondents have no or limited access to medical therapies such as chemotherapy, infusions, and hormonal treatment. Moreover, diagnosis assessments, e.g., blood or cardiac tests and medical imaging are important parts of daily care for such individuals; however, more than half of the respondents no longer have access to diagnostic facilities due to lockdown and fear of virus transmission. Else, appointments, follow-up meetings are mostly on post-pone, regular therapy schedules are interrupted, and urgent visits are hindered.

The pandemic had significant and enormous repercussions on the healthcare systems as they went through a drastic reorganization to respond to this health emergency (25). Rare disease communities worldwide are particularly impacted due

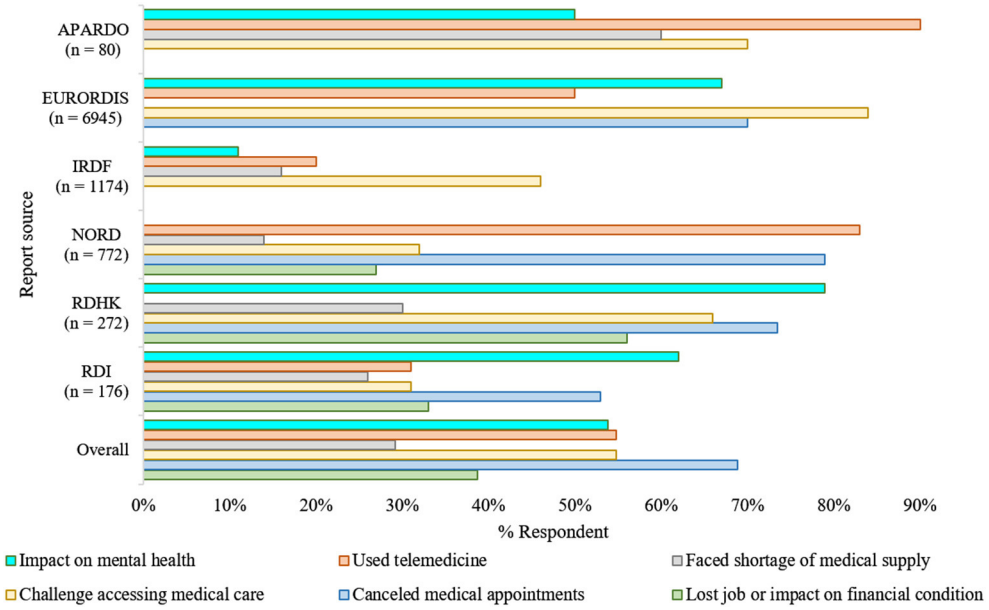

FIGURE 1 | The impacts of COVID-19 in the rare disease community (7, 18–22). Overall, as adjusted estimation from multiple surveys reflects, the pandemic's unexpected emergence had a substantial repercussion on the regular healthcare, mental health, and financial arrangements of the rare disease communities. The consequences were asymmetric in various aspects; however, they were analogous irrespective of the geographical region where the surveys took place (7, 18–23). NORD, National Organization of Rare Disease; EURORDIS, European Organization for Rare Diseases; RDHK, Rare Disease Hong Kong; IRDF, Italian Rare Disease Foundation; RDI, Rare Disease Ireland; APARDO, Asia Pacific Alliance of Rare Disease Organizations. The surveys included were independent of each other and not all focused on the same parameters.

to these reorganizations, especially in terms of their regular healthcare (**Figure 1**). Studies led by UNIAMO Italian Rare Disease Foundation (UNIAMO–Federazione Italiana Malattie Rare onlus) and Rare Disease Ireland report similar outcomes (20, 21). Ninety five percentage ($N = 1{,}174$) of the respondents from Italy reported having rare conditions, 14% of whom had two or more pathological conditions, and ∼1% had a condition without confirmed diagnoses (20). Over half of the participants (52%) from Italy, one of the hardest-hit countries by the pandemic, indicated that they had given up hospital treatment to help limit their infection exposure (20). Another half (46%) faced problems in continuing their ongoing medication/therapies, as the government forced the outpatient facilities to ramp down in their service to operate only for life-saving and urgent interventions (20). In the Rare Disease Ireland survey, 53% of the participants reported cancellation of a scheduled medical appointment at a cost to the immediate and long-term health and well-being of those living with a rare condition (21). Also, 26% of these respondents reported difficulties in accessing medicines and other medical supplies. Besides, 62% believe that COVID-19 is hurting their mental health. Similar findings were reported by the Rare Disease Hong Kong (RDHK); more than 50% of the study cohort, consisting of 272 participants with 89 distinct rare conditions, opined that their medical treatment was interrupted by pandemic (7). Many participants also complained about deficits in the healthcare provision, shortage of medical supplies, and mental instability during this period (7, 26). Studying participant responses from 10 different countries affected differently by the pandemic, the report from Asia Pacific Alliance of Rare Disease Organizations (APARDO) almost recapitulated the surveys from Italy, Ireland, and Hong Kong (22).

CHALLENGES TO PATIENTS

The novel coronavirus disease COVID-19 possesses challenges for millions of people with rare diseases, from possible increased anxiety and stress to potentially reduced access to necessary medical treatment. Besides, some pathologies lead to the greater fragility of the rare disease patient, such as immune deficiencies, complex congenital syndromes, chronic lung diseases, congenital heart disease, and hereditary metabolic pathologies at risk of acute decompensation. Therefore, many patients with rare diseases generally require ongoing assistance, from drug therapies to rehabilitation treatments to medical devices, often lifesaving.

Most of the rare disease patients have specific pathologies linked to increased perception of the risk of possible side effects following SARS-CoV-2 infection. Favism, for example, is a rare disease caused by Glucose-6-phosphate dehydrogenase (G6PD) deficiency and G6PD deficient cells are more vulnerable to SARS-CoV-2 infection. G6PD enzyme is sensitive to oxidative action on red blood cells, potentially triggering hemolytic crises. Among the administered drugs to deal with the pandemic from SARS-CoV-2, chloroquine and hydroxychloroquine have oxidative properties, triggering severe hemolysis in favism patients (27, 28). However, data from multiple rare connective tissue disorder patient registries suggest that anti-rheumatic drugs, e.g., hydroxychloroquine is impartial, prolonged use

of corticosteroids at moderate to high could be deleterious and the use of some specific TNF inhibitors could produce protective outcomes (29, 30). In addition, many autoimmune or neuromuscular diseases can be treated with cortisone or immunosuppressants that determine an increased risk, both in terms of morbidity and mortality, in case of respiratory virus infection, such as SARS-CoV-2 infection (31). Interestingly, rare connective tissue disorders and immune-compromised rheumatic disease patients were not found to be at a higher risk for SARS-CoV-2 infection (6, 31, 32).

However, the challenges in the management of rare diseases are three-fold compared to diagnosing and treating common diseases (33). They may struggle to find appropriate physicians knowledgeable about the disease's pathophysiology, the natural course of the disease, and epidemiological information to manage them (12). Also, many of the individuals with rare diseases may struggle to receive an early diagnosis and suffer the consequences. For instance, a newborn with a rare condition may experience proper and delayed diagnosis under the current situation, which may significantly add to its sufferings in the coming days. Besides, this can potentially result in a rise in the cost needed for disease-specific treatment (34, 35).

The National Institutes of Health (NIH) estimates that only 5% of rare diseases have approved treatments (36, 37), while many therapies presumably work only at the young age of the patients, and if the disease is in primary stage (38). Many rare diseases are progressive, and the clinical condition deteriorates over time (39). As an immediate response to the pandemic, most pharma industries and researchers concentrate on therapy development for COVID-19, and it is causing a halt in the development of therapeutics for diseases other than COVID-19, including rare diseases. Since the outbreak, they are fighting without proper palliative care, presumably letting them down while fighting a progressive disease (40). Thus, for rare disease patients, such a pause in development is effectively a regression in progress.

CHALLENGES TO INVESTIGATORS STUDYING RARE DISEASES

Investigators wishing to study the clinical progressions, pathomechanism, and natural history of rare diseases face significantly more obstacles than common disease researchers (33). For example, constituting a cohort of adequate size for a clinical study is a lot more difficult for rare disease investigators. It often requires international or multi-institutional collaboration. The COVID-19 pandemic situation has added to the impediments to gather such cohorts as effective collaborations have become tougher to develop.

Besides, funding support for rare disease research is usually limited (41). Since the pandemic began, scientists working on preclinical studies hoping that human trials could be launched by the coming year(s) had to shut down most of their experiments (42). Many of the rare disease researchers had to switch gear to facilitate more robust research focusing on COVID-19 (43). However, delays in producing a treatment could mean the forever loss of some people, maybe kids, who live with rare diseases, and some may progress to a non-recoverable or non-manageable state from where they could be treated.

CHALLENGES RELATED TO FUNDING

The impact of COVID-19 on the currently ongoing research projects and funding was so crippling and will undoubtedly be long-lasting. Many organizations that usually fund research on rare diseases are now facing financial crises (44). NIH (45), Patient-Centered Outcomes Research Institute (PCORI) (46), and other major funders took prompt measures for making the proper guideline on proposal submission and fund distribution that allows grant personnel to be paid in a relaxed timeline. Research institutions prioritize COVID-19 related research proposals while other proposals are delayed or postponed (47). Also, governments are spending a considerable portion of their out-of-pocket budget to manage the COVID-19 situation. Many organizations are moving their money to start COVID-19 related works (48, 49).

The genetic sequence of SARS-CoV-2 was released in early January 2020, just weeks after the first reported cases, significantly accelerated research and therapeutic development on COVID-19. As of March 14th 2021, over 5,017 clinical trial studies related to COVID-19 are registered on ClinicalTrials.gov (50). After almost 5 months since the genetic sequence release, 148 studies associated with hydroxychloroquine, 13 with remdesivir, 50 with vaccines, and 100 with diagnostic testing were registered (51). Another 3,733 different studies are registered on the World Health Organization's International Clinical Trials Registry Platform (WHO ICTRP) (52).

Furthermore, as the world has recently seen a huge blow due to an infectious disease, we may observe a flow of money toward infectious disease research from non-communicable and rare disease research in the coming future (25, 53). In the long run, the pandemic will possibly force the reallocation of research grants at the expense of research areas funded before the pandemic.

SUPPLY OF MEDICAL EQUIPMENT AND THERAPIES

Few human-derived rare disease therapies such as plasma, blood factors, and cell therapies are being studied as treatments for COVID-19 (54). Thus, they may be facing the risk of shortages. For example, Immunoglobulin (Ig), derived from human plasma, has a complex supply chain and is used to treat primary immune deficiency and others (55), has faced shortages in the US and some other parts of the world for some time (56). Some essential medical supplies have also faced dramatic price hikes during this period (57). Additionally, blood donations have significantly been reduced due to social distancing and heightened infection concerns (58). Those who are willing to donate blood are being screened strictly to avoid transmission and ensure safety protocols (59), which is also putting pressure on the already over-stretched systems.

In early 2020, hydroxychloroquine, a well-known drug for autoimmune disorders, e.g., lupus and rheumatoid arthritis, had gained some focus as a potential COVID-19 treatment

(60), resulting in its place in the FDA's (Food and Drug Administration) shortage list for months. Similarly, as few companies are trying to develop plasma COVID-19 therapies (61), it is expected to put pressure on plasma supply. The FDA is working proactively to evaluate the entire supply chain, including active pharmaceutical ingredients, finished dose forms, and other components that may be impacted in any supply chain area due to the COVID-19 outbreak, along with pharmaceutical companies and manufacturers, including those for rare disease therapies.

CLINICAL TRIALS DURING COVID-19

The effect of COVID-19 on clinical trial research has been enormous, with thousands of trials—around 80% of non-COVID-19 trials—being stopped or interrupted (62). The major difficulty for clinical trials lies in the in-person visits to hospitals or clinics for either follow-up or therapeutic administration. The rare disease patients have a higher risk of contracting the virus if the hospitals do not have separate areas for COVID-19 patients. Thus, many companies postponed or canceled new clinical trials and pushed back trial visits for existing ones (42). The National Cancer Center Singapore faced difficulties with more than 200 ongoing clinical trials due to travel restrictions from different countries, as many participants come from the South Asian region (42). In a report from Spain, the La Paz University Hospital had 59 hemophilia-related clinical trials and registries active in the Thrombosis and Homeostasis Unit, which was interrupted due to a nationwide lockdown (63). However, they tried to mitigate this situation through a telemedicine program, which eventually proved to be partly able to replace in-person patient care (63, 64).

Moreover, clinical investigators responsible for clinical trials are being reallocated to manage a significantly higher number of COVID-19 patients. Many clinicians, scientists, research administrators, clinical trial-related officials were pulled away from working on clinical trials to work in emergency medical care, especially during the first months of the pandemic (62). They are, in most cases, yet to resume from where they stopped. Moreover, clinical research administrators responsible for clinical trials are being reallocated to manage a significantly higher number of COVID-19 patients in clinical setups and COVID-19 related clinical trial programs. These represent significant challenges in maintaining clinical trial continuity in the coming future. In addition, the ramp down or cancellation of trials will have a superfluous effect on early career researchers, and even those who may be able to work from home—biostatisticians and epidemiologists—suffer the equivalent challenges that many have in maintaining work-life balance, which is especially true for those with kids (62).

COVID-19 VACCINATION AND RARE DISEASE COMMUNITY

The FDA granted emergency use authorization of two COVID-19 vaccines, the Pfizer/BioNTech vaccine and the Moderna vaccine (65), last December 2020. To date, millions of people worldwide have been receiving the vaccine doses (66). Scepticisms over the vaccines' efficacy due to emergency use authorizations are on the discussion; this concern is heightened among the rare disease communities as there were not enough rare disease individuals for the clinical trial. Some are also hoping to get genetic therapy after getting vaccinated, putting them into concerns over the effects of vaccination. Nevertheless, the officials of the regulatory boards have denied such speculations (67).

The Pfizer/BioNTech vaccine, which showed 95% efficacy (68) against COVID-19, had 43,548 people in the phase III trial (69), consisting of more than 2,900 people with chronic pulmonary disease (70). Still, none of the participants showed pulmonary hypertension (a rare condition). In comparison, the Moderna vaccine showed 94.1% efficacy in phase III clinical trial, which enrolled ∼5% of the 30,000 participants with significant cardiac disease and pulmonary hypertension (70, 71). In addition, the COVID-19 mRNA vaccines exclusively target the SARS-CoV-2 virus and are unable to alter the recipient's genetic information (72, 73).

Also, people with rare diseases undergoing or expecting gene therapies are concerned if the vaccines are compatible with the therapy. Some gene therapies for rare diseases are based on adeno-associated viruses (AAV); however, that is a different virus that shares little similarity with coronavirus or vaccines. Some vaccines, e.g., the Oxford-AstraZeneca and CanSino vaccines, use adenovirus; however, these are completely different viruses from the AAV used for the gene therapies, despite the similar name (74). Nevertheless, rare disease patients undergoing or awaiting gene therapies, immunosuppressant drugs, blood-thinning medicines, or immunocompromised individuals are recommended to discuss with their clinicians to determine whether/when a vaccine is permitted.

EMERGING COMPLICATIONS DURING THE PANDEMIC

As COVID-19 continued to spread, clinicians' concern was complications associated with SARS-CoV-2 in rare disease patients. Verdoni et al. reported ten cases of a Kawasaki-like disease in young boys and girls in Bergamo, Italy (75) from February 18 to April 20, 2020, i.e., during the peak of the pandemic in the country. It is a rare acute vasculitis that affects children under 5 years of age, and the coronary artery inflames throughout the body (76). Among the ten cases, two children had a positive PCR swab, and eight had a positive serology test for SARS-CoV-2. However, these tests' clinical relevance is unclear as they were not done at the same time. Most Kawasaki disease patients respond well to intravenous immunoglobulin, though 10–20% need supplementary anti-inflammatory treatment (77). In this cohort, eight children among ten received corticosteroids in high dose, in addition to intravenous immunoglobulin. These differences raised confusion, whether the cohort has Kawasaki disease with SARS-CoV-2 or an emerging Kawasaki-like disease is characterized by multisystem inflammation. Moreover, researchers have reported clusters of similar cases across Europe (78). In addition, patients with rare hematological disorders (79), especially sickle cell disease patients, are at higher

risk of bacterial infections partly due to asplenic conditions (80). There is a chance that such bacterial infection may be misdiagnosed as COVID-19 infection and can delay access to life-saving antibiotics due to unnecessary isolation and panic (81). A study on 211 non-ICU COVID-19 patients showed that preexisting pulmonary hypertension (PH) and right ventricular dysfunction (RVD) were associated with severe outcomes in COVID-19 (82). Also, COVID-19 can result in neurological complications, e.g., rare encephalitis diseases and Creutzfeldt-Jakob disease, as the virus was reported to be identifiable in the cerebrospinal fluid (CSF) (23, 83).

While these rare and sporadic incidences may reflect pure coincidence, these undoubtedly bring extra concerns for the people living with rare diseases. Similarly, patients with cancer face severe bacterial infection risk due to vulnerable physical conditions (84). Late diagnosis of such conditions in the first pandemic wave shows how rare and difficult it is to recognize the disease in a deficient or malfunctioning healthcare system, which should be reorganized to deal with future pandemics. Moreover, studying the association between COVID-19 and rare diseases potentially provide important insights into physiological conditions that can be extended to understanding rare diseases and other relevant conditions.

ADAPTATION OF RARE DISEASE RESEARCH WITH THE NEW NORMAL

As the pandemic continues, the world has seen some ups and downs in terms of cases and fatality rates. The easing of public restrictions has resulted in a second wave. New cases are increasing since early August, which may carry with a lot of newer restrictions (17). Thus, countries need to be prepared for what is coming in the next winter. The government should engage vulnerable patients, including the rare disease patients, widely for essential health services. The unprecedented impacts of COVID-19 on the people living with rare diseases, their family and caregivers, researchers, and stakeholders (**Figure 2**) should be considered to avoid further damage.

COVID-19 pandemic has resulted in an unexpected economic downturn, affecting emerging biotech companies to survive and thrive amidst new safety guidelines and restructured core business strategies. They are applying for emergency capital to maintain continuity and push forward. While maintaining social distancing guidelines, biotech businesses transfer all medical research to exempt commercial collaborators from closed academic labs. Moreover, works that can be done using digital facilities, like conducting online meetings, data entry, and online data analysis, are being done online to reduce transmission.

The decentralization programs have been prioritized for uninterrupted clinical trials during the pandemic. Orphan drug developers and their partners are reshaping clinical trial administration, either entirely virtual or hybrid approaches (85), to adapt to the new normal. The FDA has also prepared flexible guidelines for clinical trials that allow the research to introduce virtual interviews or visits, self-administration, and remote monitoring. These changes will take time to cope up with the patients, caregivers, and even the clinicians. To make sure this works, companies will need to work with disease stakeholders, regulators, and everyone else in the health sector to design functional trials with successful results and ensure robust and standardized data collection (54).

Nonprofits rely heavily on in-person engagements to ensure continuity and must rethink how they raise money to maintain their work. Their fundraising strategies have been significantly restructured overnight. Without the in-person fundraiser events, non-profit companies emphasize funding from the virtual realm and corporate sources to keep going in a post-COVID-19 world. Once society returns to the new normal, research and development should move forward with creative and insightful ideas.

After the pandemic is over, our lives may not be the old normal again for an extended time. The general medical practice will be changed for an extended period, with social distancing and work from home. For rare disease patients, this would be a more crucial period than ever. Governments should focus on telemedicine services at this time to maintain social distancing. Typically, a fragile, rare disease patient may require pharmacological care and personalized motion, communication support, rehabilitation support, and behavioral therapy. A telemedicine service, in that case, should be personalized. For example, Rare Bone Disease (RBD) patients have several comorbidities associated with other body systems, which requires constant attentive care and cautious multidisciplinary follow-up. However, as we are in the middle of a pandemic, most healthcare workers are busy handling COVID-19 cases in the front line. To manage this emergency, the European Reference Network on Rare Bone Diseases (ERN BOND) brought together 78 experts on RBD, and along with Italian RBD, healthcare professionals created the "COVID-19 Helpline for Rare Bone Diseases" (25, 86). This 24/7 helpline provides high-quality information and recommendations on RBD remotely to patients and healthcare professionals by the RBD experts working in intensive care units or COVID-19 units. Given the convenience of remote consultation, telemedicine can meet people's daily healthcare needs, like cold and fever, without creating pressure at hospitals and timely relieve tensions about the disease.

Telemedicine may not serve the best for the patients if professional and technical characteristics are not maintained. For example, healthcare professionals should answer calls and handle at least 5 years of experience. Even though online services are nowadays app-based, direct telephone communication will allow the patients, especially elderly patients, to communicate rapidly and directly. Most importantly, the service should be in the local language and be available 24/7. According to the local laws, there are different policies and regulations on telemedicine (**Table 1**) and digitalization in different regions. The concept is new for many of the patients and still evolving by itself as an alternative health consultancy system; both the care providers and the patients need to be aware of their roles and responsibilities to maintain privacy and confidentiality and provide effective feedback to help improve the system. Also, the patients must have the full authority in choosing to participate or to change decisions on whether to continue or not to continue with the

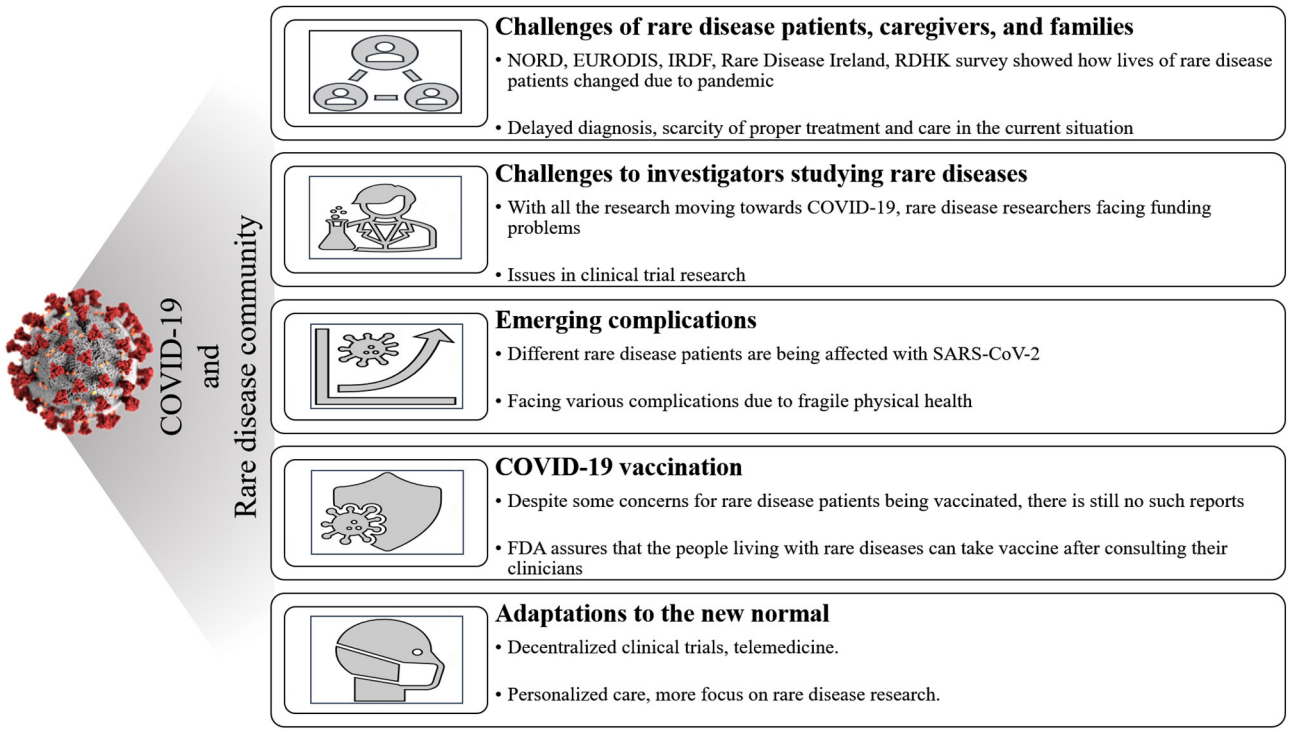

FIGURE 2 | Summary of the impacts of the COVID-19 pandemic on the rare disease communities. The patients, stakeholders, investigators are under serious strain owing to the required care for patients with COVID-19, which has had a knock-on effect on the management of other patients, including the patients living with rare diseases. The pandemic interrupted the regular healthcare for people living with rare diseases and research focusing on rare diseases, mostly because of the extraordinary restrictions imposed to prevent and control SARS-CoV-2 transmission. Besides, many rare disease patients with fragile health are affected by SARS-CoV-2, putting them in a more challenging state. Besides, the fear of possible adverse effects of the vaccines on the management and treatment of rare diseases has been raised. The adaptations to the "new normal" and changes to clinical protocols to help prevent or control SARS-CoV-2 transmission (and any other outbreaks coming in the future) have also been a concern among the rare disease communities and are a point of discussion.

TABLE 1 | Some regulatory and ethical implications of telemedicine in different countries*.

Regulatory and ethical implications	Narrative/description
Legislation and licensing of telehealth products	The telemedicine act from Malaysia and the healthcare services act of Singapore focus on patient safety through proper licensing of medical institutions and healthcare professionals providing telemedicine services (87, 88)
Informed consent and options to choose	Most telemedicine guidelines necessitate the individual's consent, and the patient can change their decision at any time (89)
Privacy, confidentiality, and data security	To ensure data security and confidentiality, Indonesia and Vietnam only allow internet-based registered health facilities for telemedicine service. Indonesia, Malaysia, and Thailand have policies that utilize government information networks for data management and data security. However, according to most guidelines, individual telemedicine providers are responsible for data security (90–94)
Feedback and evaluation	National Telemedicine Guideline of Singapore prioritizes quality improvement activities, cost, accessibility of care, and patient satisfaction. Telemedicine guidelines from Malaysia and Indonesia emphasize communication between doctors and patients to avoid medicolegal consequences (88, 95–97)
Cross border telemedicine	European Union (EU) acts state the right to access to medical treatment in another Member State (Article 1) of European Union (EU), right to access one's written or electronic medical record, (Article 4/2/f), right to be informed about the treatment received, availability, quality, and safety of the service used (Article 4/2/b) (98)
Licensing and qualifications of healthcare professionals	Each national entity in charge of medical practice regulation regulates the qualifications and other legal or ethical aspects of healthcare providers based on its region, including those involved in cross-border telemedicine (98)

**This table contains only the key data on the regulatory and ethical implications of telemedicine implementation that are most relevant to the COVID-19-related contexts; the references were systemically identified by searching Global Regulations, PubMed, and Google Scholar.*

service. Moreover, rare disease communities require specialized health professionals to understand better and diagnose their condition promptly.

In a post-pandemic era, the lower-middle-income countries should focus on strengthening the primary health care systems, including trained health professionals who can monitor disease patterns and be alert about the potential outbreaks. Besides that, an instantly accessible trained personnel database and a disease database are also required. For maintaining further emergencies, a predictably safe platform needs to be made where regulatory reviews can be done faster, and massive scale production of therapies, medical supply, and vaccines can be possible. An organized system is necessary for antivirals to screen existing treatments and candidate drugs in a standardized manner. When we return to normal, we must apply what we learned from this pandemic and plan precisely for a dynamic and robust genetic care system for rare disease patients.

CONCLUSION

The impact of the COVID-19 pandemic on rare disease communities is asymmetrical in different contexts. While even in a "normal" world, they often face isolation and anxiety due to their uncertain condition and must navigate through several clinicians to obtain the care they need, the additional anxiety due to COVID-19, triggered by the worldwide emergency health protocol and the loaded pressure on health systems, research, development, and the pharma industry, has made the challenges more extravagant than ever. While facing the current challenges, it is essential to keep in mind that access to therapies and continued government and private funding of drug development, translational research, and basic research is crucial to saving rare disease patients' lives. The COVID-19 pandemic experience regarding health emergencies and rare disease management represents the basis for establishing healthcare policies to ensure preparedness for providing adequate care for people with rare diseases.

AUTHOR CONTRIBUTIONS

SA conceived the study. SC and SA designed the study, reviewed, and revised the manuscript. SC wrote the draft manuscript. SS helped SC in drafting the manuscript. SA, SC, and SS approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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