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

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REVIEW
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COVID-19: In the Eye of the Cytokine Storm

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The dysregulated release of cytokines has been identified as one of the key factors behind poorer outcomes in COVID-19. This "cytokine storm" produces an excessive inflammatory and immune response, especially in the lungs, leading to acute respiratory distress (ARDS), pulmonary edema and multi-organ failure. Alleviating this inflammatory state is crucial to improve prognosis. Pro-inflammatory factors play a central role in COVID-19 severity, especially in patients with comorbidities. In these situations, an overactive, untreated immune response can be deadly, suggesting that mortality in COVID-19 cases is likely due to this virally driven hyperinflammation. Administering immunomodulators has not yielded conclusive improvements in other pathologies characterized by dysregulated inflammation such as sepsis, SARS-CoV-1, and MERS. The success of these drugs at reducing COVID-19-driven inflammation is still anecdotal and comes with serious risks. It is also imperative to screen the elderly for risk factors that predispose them to severe COVID-19. Immunosenescence and comorbidities should be taken into consideration. In this review, we summarize the latest data available about the role of the cytokine storm in COVID-19 disease severity as well as potential therapeutic approaches to ameliorate it. We also examine the role of inflammation in other diseases and conditions often comorbid with COVID-19, such as aging, sepsis, and pulmonary disorders. Finally, we identify gaps in our knowledge and suggest priorities for future research aimed at stratifying patients according to risk as well as personalizing therapies in the context of COVID19-driven hyperinflammation.

Keywords: COVID-19, immunosenecence, inflammation, SARS-CoV-2, sepsis, aging

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INTRODUCTION

Accumulating evidence suggests that patients with severe COVID-19 develop a dysregulated release of cytokines also known as a "cytokine storm" or "cytokine storm syndrome." The cytokine storm produces an excessive inflammatory and immune response, especially in the lungs, leading to acute respiratory distress (ARDS), pulmonary edema and multi-organ failure. In these situations, an overactive, untreated immune response can be deadly, suggesting that mortality in COVID-19 cases is likely due to this virally driven hyperinflammation. While the risk factors and phenotype profiles that cause otherwise healthy individuals to become critically ill still remain unknown, preliminary evidence suggests that other inflammatory processes such as aging or permanent lung damage may make one predisposed to a poorer prognosis. In this review, we summarize the latest data available about the role of the cytokine storm in COVID-19 disease severity as well as potential therapeutic approaches to ameliorate it. We also examine the role of inflammation in

other diseases or conditions often comorbid with COVID-19, such as aging, sepsis, and pulmonary disorders. Finally, we identify gaps in our knowledge and suggest priorities for future research aimed at stratifying patients according to risk as well as personalizing therapies in the context of COVID19-driven hyperinflammation.

INFLAMMATION IN PATHOLOGY AND DISEASE

Inflammation is a vital phenomenon of a healthy immune response. However, dysregulated inflammation can result in severe damage, multisystemic organ dysfunction or even death. Rampant inflammation plays a central role in several pathologies such as sepsis, rheumatoid arthritis, respiratory diseases, cancer, and aging. Below we briefly review the role of inflammatory responses in several of these pathologies or conditions, which have been found to be aggravating factors in COVID-19.

Inflammation and Aging

As we age the effectiveness of the innate and adaptative immune response declines, and this results in reduced protection against external pathogens, decreased ability to vaccination and increased susceptibility to infection, and limited repair capacity of damaged cells and tissues. This process is called "immunosenescence" (1) and it likely plays a central role in the age-related severity of COVID-19. Immunosenescence makes the innate immune response become more active, increasing the number of natural killer cells (NK) and releasing pro-inflammatory cytokines, such as Interleukin 6 (IL-6), Tumor Necrosis Alpha (TNFα), and C-reactive protein (CRP). In turn, this results in a chronic, low-grade inflammation, a phenomenon that has been termed as "inflammaging" (2). This chronic inflammation might contribute to biological aging and is a significant risk factor for age-related diseases, such as type 2 diabetes, Alzheimer's disease, hypertension, atherosclerosis, arthritis, hypertension, and cancer (2, 3). "Inflammaging" is a highly significant risk factor for both morbidity and mortality in the elderly. For example, Fabbri et al. (4) have shown that older people with a high baseline of IL-6 levels in combination with a faster increase in IL-6 levels over time have a significantly higher number of chronic diseases or multimorbidity as compared to those with high baseline levels but with a slower increase in IL-6 over time. IL-6 is a proinflammatory cytokine secreted by macrophages during the initial, acute phase of an inflammatory response. During this acute phase response, another downstream inflammatory marker, CRP is released in response to IL-6 (5). Both proteins are markers of systemic inflammation and predictors of mortality in older adults as well as in people with community-acquired pneumonia (6) which indicates that they could be used to identify individuals at higher risk of developing severe COVID-19 as well. In older people, muscle tissue has higher levels of these inflammatory cytokines, that together with a lack of physical activity, malnutrition, and hormonal dysregulation among other factors, may lead to the development of sarcopenia, the age-related loss of muscle mass and function that is one of the hallmarks of aging (3). This suggests that Clinical Frailty Indexes, which are rapid and already available in clinical practice, could be useful to screen for patients at risk of severe COVID-19.

Inflammation and Sepsis

Sepsis is a life-threatening clinical process characterized by the dysregulation of homeostasis and the presence of a systemic inflammatory response syndrome. Sepsis is caused by an infection (from different types of pathogens, from bacteria to fungi and viruses) and leads to multiorgan dysfunction (7). Consequently, the dysregulated release of cytokines plays a central role in the syndrome's pathophysiology (8). Sepsis is strongly time-dependent, and it is known that the levels of inflammation biomarkers are prone to change abruptly. This makes it challenging to characterize cytokine profiles since they evolve rapidly as sepsis progresses. For example, it has been shown that IL-6, TNFα, and IL-10 levels peak within the first 2 h of the syndrome and then progressively decrease with time (9). Indeed, therapies based on blocking the proinflammatory action of cytokines such as TNFα and IL-1 have failed to improve sepsis outcomes in human trials because of the highly dynamic nature of these biomarkers, which have rapidly changing kinetic profiles (10, 11). Another confounding factor in characterizing sepsis is the heterogenous presentation of the syndrome, which varies depending on the type of infection (bacterial vs. viral, gram-negative vs. gram-positive), genetic polymorphisms and comorbidities. Recent advances in machine learning are overcoming this problem by enabling the simultaneous evaluation of extremely large volumes of data. For example, a recent article showed that sepsis patients can be categorized within 4 different phenotypes according to age and type of organ dysfunction, which could help stratify patients, predict their prognosis, and fine-tune therapeutic approaches in the near future (12).

Sepsis survivors are immunosuppressed, which makes them easy targets for viral infections such as COVID-19 (13). Likewise, nosocomial infections by polyresistant bacteria are a real threat for the critical COVID-19 patient, especially for those that require mechanical ventilation. Immunomodulatory therapies aimed at alleviating the cytokine storm originated by COVID-19 should be carefully designed in order to avoid putting these patients at a higher risk of bacterial or fungal sepsis.

Inflammation and Respiratory Disease

Inflammation also plays a central role in respiratory diseases such as chronic obstructive pulmonary disease (COPD), asthma, and pulmonary fibrosis. In allergic asthma, epithelial cells respond to the presence of the allergen by producing cytokines such as IL-25 and IL-33 (14). In turn, these activate the TH2 response mediated by IL-4, IL-13, and IL-5 (15). IL-4 and IL-13 are important for regulating the production of IgE and the activation of macrophages (14), while IL-5 mediates eosinophilic inflammation (15, 16). COPD is a chronic inflammation of the lungs, which is usually triggered by long-term exposure to particulate matter or smoke. Consequently, consistently high levels of proinflammatory cytokines such as TNFα (17), IL-1 (18), or IL-6 (19) can be found in the sera of COPD patients. COPD

may overlap with other respiratory conditions such as asthma or pneumonia. Exacerbation episodes due to environmental factors (pollen and pollution air levels) or respiratory viruses may cause an acute inflammatory response (20). For example IL-6 can be found at higher levels in the serum of patients going through an acute exacerbation episode (21). The accumulation of scar or fibrotic tissue in the lung may result in idiopathic pulmonary fibrosis. The process is largely mediated by TGF-beta (22). Extracellular matrix deposition in pulmonary fibrosis is parenchymal, and it has been proposed that it is the result of a "profibrotic cytokine storm" (14).

CYTOKINE STORM IN CORONAVIRUS DISEASES

Much can be learned about the role of inflammation in the course of the infections from previous respiratory coronaviruses. SARS-CoV-2, the severe acute respiratory disease coronavirus (SARS-CoV-1) and the Middle Eastern Respiratory Syndrome coronavirus (MERS-CoV) infect the lower respiratory airways and can cause severe pneumonia. During the first 9 days the patient shows flu-like symptoms such as cough and fever, often accompanied by diarrhea (23). During this phase, the virus replicates very fast and produces several proteins that are known to block interferon (IFN) responses (24). Histology studies show acute phase diffuse alveolar damage accompanied by edema, inflammatory infiltrate, and the formation of hyaline membrane (25). Shortly afterwards, viral titers in nasopharyngeal aspirates reach a peak and start decreasing. During this time, patients experience hypoxemia and high fever. By the third week about 20% will develop acute respiratory distress syndrome (ARDS) (23).

The production of chemokines by immune cells plays a central role in coronavirus-related hyper-inflammation. For example, infection of human monocyte-derived macrophages with SARS-CoV resulted in a very low production of IFN-γ but successfully induced the expression of CXCL10/IFN-inducible protein 10 and CCL2/monocyte chemotactic protein 1 (26). Chemokine upregulation was also observed after infection in dendritic cells (27). In serum, higher levels of pro-inflammatory cytokines and chemokines, through activation of Th1 cell-mediated immunity and hyper innate inflammatory responses, have been correlated to disease severity in SARS-CoV infections (28). Similarly, the "cytokine storm" responsible for the poor prognosis of MERS-CoV is controlled by T helpers 1 (Th1) mediators, and involves high levels of IFN as well as proinflammatory factors such as IL-1beta, IL-6, and IL-8, which are generated by airway epithelial cells (29). High serum levels of these cytokines are also indicative of severe MERS-CoV (30).

The origin of the dysregulated release of cytokines in these infections has been ascribed to diverse factors (31). It is assumed that the rapid viral replication in the first stages of the infection results in high proinflammatory responses. Furthermore, the virus generates high levels of proteins that are known to attenuate and delay IFN responses, which provokes an accumulation of

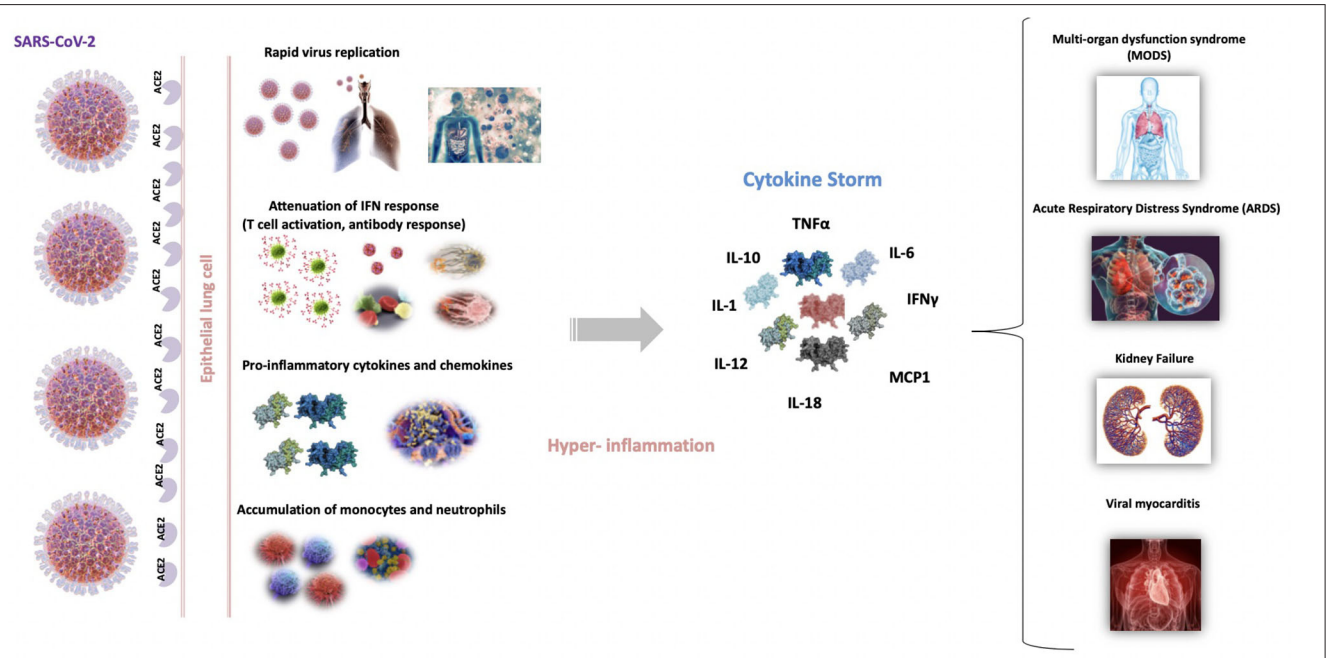


FIGURE 1 | Schematic representation of the origin and repercussions of COVID-19 cytokine storm. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) into entry epithelial lung cells, binding to angiotensin-converting enzyme 2 receptor (ACE2). Th rapid viral replication in the first stages of the infection results in high proinflammatory state that attenuate and delay the IFN responses, which provokes an accumulation of pathogenic inflammatory macrophages. This, in turn, results in an even higher production of cytokines. This cytokine storm produces an excessive inflammatory and immune response, especially in the lungs, leading to ARDS, pulmonary edema, apoptosis of epithelial cells, vascular damage and multi-organ failure.

TABLE 1 | Summary of the clinical studies with inflammatory markers in COVID19 patients.

Population	Location	*Age, Sex	Laboratory markers	Cytokines and Chemokines	Treatment	References
N = 8 (3 critically ill vs. 5 severe)	China	5 (2 mo−15) 75% (male)	↑WCC, ALT, PCT, CRP, D-dimer, LDH ↓LC =AST, CK	↑IL6, IFNγ = IL2, IL4	-Antiviral (100%) (oseltamivir virazole and/or interferon) -Antibiotic (62.5%) -Glucocorticoids (62.5%) -Traditional Chinese Medicine (50%) -Plasma infusion (25%) -IV immunoglobin therapy (50%)	(38)
N = 11 ICU severe patients	China	58 (26-72) 83% (male)	↑CRP, D-Dimer, LDH ↓LC = ALT, WBCC. CK	↑IL6, IL10 = IFNγ, IL2, IL4	-Antiviral (100%) -Antibiotic (100%) -Antifungal (91%) -Glucocorticoids (82%) -IV immunoglobin therapy (9.1%)	(39)
N = 21 (11 severe vs. 10 moderate)	China	56 (50-65) 81% (male)	↑WBCC, NC, AST, ALT. CK, LDH, D-Dimer, PCT, CRP, Ferritin ↓Albumin, LC = PC	↑IL6, IL10, IL2-R, TNFα = IL8	-Antiviral (82%) (oseltamivir and/or ganciclovir) -Antibiotic (100%) (moxifloxacin and/or cephalosporin) -Glucocorticoids (100%) (methylprednisolone)	(40)
N = 41 (13 ICU vs. 28 non-ICU)	China	49 73% (male)	↑WBCC, NC, Prothrombin Time, D-dimer, AST, ALT, Cardiac troponin I, PCT (initially normal, increased ICU with infection), CK, LDH ↓LC, Albumin, PC (not data on CRP)	↑IL1B, IL1RA, IL6, IL7, IL8, IL9, IL10, basic FGF, GCSF, GMCSF, IFNγ, IP10, MCP1, MIP1A, MIP1B, PDGF, TNFα, VEGF = IL5, IL12p70, IL15, Eotaxin, RANTES	-Antiviral (93%) (oseltamivir) -Antibiotic (100%) -Corticosteroids (22%)	(41)
N = 43 (15 severe vs. 28 mild)	China	54 (19-70) 60% (male)	↑CRP, Fibrinogen, ↑D-Dimer =WBCC, LC, AST, ALT, CK	↑ IL6	[Not available]	(42)
N = 48 (21 mild, 10 severe, 17 critically ill)	China	65 (47-83) 77% (male)	↑WBCC, Cardiac troponin I (mild and severe), AST (higher in critically ill and mild), ALT (higher in critically ill and mild), CK (higher in critically ill and mild), PCT ↓ LC, Cardiac troponin I (critically ill)	↑ IL6 (critically ill and mild)	[Not available]	(43)
N = 53 (34 severe vs. 19 moderate and) *N* = 8 healthy controls	China	#62 (22-78)	= WBCC, AST, ALT, CK ↓LC ↑ CRP, LDH, NC	↑IL1RA, IL6, IL10, IL18, CTACK, MIG, IFNγ, IP10 = IL2RA, MCP3, HGF, MIP1A, MCSF ↑IP10, MCP3, IL1RA (specially higher in severe vs moderate)	-Antiviral (38%) -Corticosteroids (30%)	(44)
N = 91 (9 severe vs. 82 mild)	China	50 41% (male)	↓WBCC, LC ↑D-Dimer, CK, CRP = AST, ALT, PCT	[Not available]	[Not available]	(45)
N = 94 (8 mild, 75 moderate, 11 severe)	China	#40 (1-78) 45% (male)	↑WBCC, NC, CRP, CK, LDH ↓ LC	↑ IL6	-Antiviral (49%) (IFN-α + lopinavir/ritonavir) -Antiviral (22%) (IFN-α + lopinavir/ritonavir+ ribavirin	(46)
N = 123 (21 severe vs. 102 mild)	China	52 (30-76) 66% (male)	↓ LC	↑IL6, IL10 =IL4, IL17, TNFα, IFNγ	[Not available]	(47)

(Continued)

TABLE 1 | Continued

Population	Location	*Age, Sex	Laboratory markers	Cytokines and Chemokines	Treatment	References
N = 138 (36 ICU vs. 102 non-ICU)	China	56 (42-68) 54% (male)	↑WBCC, D-dimer, AST, ALT, CK, LDH, PCT, Cardiac troponin I ↓LC = PC	[No data available]	-Antiviral (90%) (oseltamivir) Antibiotic (100%) (moxifloxacin [64.4%]; ceftriaxone [24.6%]; azithromycin, [18.1%]) -Glucocorticoids (45%)	(48)
N = 150 (68 death vs. 82 discharged)	China	58.5 (15-81) 68.5% (male)	↑WBCC, MB, AST, ALT, BUN, CK, LDH, Cardiac troponin I, CRP, Ferritin ↓ LC, PC	↑ IL6	-Antiviral (58%) -Antibiotic (96%) -Antifungal (12%) -Glucocorticoids (37%)	(47)
N = 452 (286 severe vs. 166 non severe)	China	58 (47-67) 52% (male)	↑Leukocytes, NLR, PCT, ESR, Ferritin, CRP ↓ LC	↑ IL6, IL2-R, IL8, IL10, TNFα	[No data available]	(49)
N = 1,099 (173 severe vs. 926 non-severe)	China	47 (35-58) 58% (male)	↑CRP ↓WBCC, LC = D-Dimer, AST, ALT	[Not available]	-Antiviral (36%) (oseltamivir) -Antibiotic (58%) -Antifungal (3%) -Glucocorticoids (19%)	(50)

WBCC, White blood cell count; ALT, Alanine aminotransferase; PCT, procalcitonin; CRP, C-reactive protein; LDH, lactate dehydrogenase; LC, lymphocyte count; AST, Aspartate aminotransferase; CK, creatine kinase; IV, intravenous; PC, platelet count; NC, neutrophil count; MB, myoglobin; BUN, blood urea nitrogen; ESR, Erythrocyte sedimentation rate; NLR, Neutrophil-to-lymphocyte ratio.

pathogenic inflammatory monocyte-macrophages (24, 32). This, in turn, results in an even higher production of cytokines in the lungs (33). The consequences of this hyper-inflammation are also diverse, ranging from the dampening of T-cell responses, which leads to an even less controlled inflammatory response, to the apoptosis of epithelial cells, vascular damage, and ARDS (31) (**Figure 1**).

The dynamic interplay of factors involved in the cytokine storm generated by coronavirus infections makes it complicated to design therapies to halt its progression. For example, treatment with corticosteroids has been found to be mildly beneficial, not beneficial at all, or even deleterious in different studies (34–36). These disparate results show the complexity of the problem and the need to personalize timing and dosage for each particular case. Similarly, studies in macaques have shown that administering pegylated IFN-γ protects type 1 pneumocytes against SARS-Cov-1 infection when administered in the early stages of the infection. However, the same treatment had no effect on patients who were diagnosed at later stages, and therefore that had already progressed to severe MERS (37). These studies highlight the relevance of closely monitoring disease progression in order to maximize the benefits of therapies aimed at ameliorating coronavirus-induced hyperinflammation.

Cytokine Storm in COVID-19

Table 1 summarizes the main findings published at the beginning of the pandemic (February 2020–April 2020) about COVID-19 and cytokine storm. The mortality of critically ill Chinese patients with SARS-CoV-2 pneumonia was between 50 and 62% (51, 52). The duration of terminal cases was usually 1–2 weeks after intensive care unit (ICU) admission. Older patients (>65 years) with higher SOFA score and ARDS were at increased risk of death (52). Several studies from countries that were first affected by the pandemic reported an increased prevalence of dysregulated immune responses in patients with COVID-19. This dysregulation is frequently accompanied by higher levels of inflammation or "hyperinflammation" and it is more likely to occur in elderly people with comorbidities, who have weaker immune functions and chronic inflammation as hypothesized above (53). It has been shown that aberrant pathogenic T cells and inflammatory monocytes are rapidly activated and produce a large number of cytokines, thus inducing this inflammatory storm (54) (**Figure 1**).

A recent paper published in Lancet was the first to report the epidemiological, clinical, laboratory, and radiological characteristics, treatment, and clinical outcomes of 41 laboratory-confirmed cases infected with SARS-CoV-2 (41). In this study the authors showed that severe COVID-19 patients who developed ARDS due to higher inflammation were more likely to die. Both, Th1 pro-inflammatory cytokines and Th2 anti-inflammatory cytokines were higher in COVID-19 patients. Of note, those with higher levels of Th1 cytokines required ICU admission, suggesting that the cytokine storm was associated with disease severity and consequently with a worse prognosis.

Interestingly Quin et al. (49) found that the severe group with COVID-19 had higher neutrophil count and a lower number of lymphocytes, inducing a cytokine storm in the body and damage

in the lungs and heart, among other organs. It is known that the increase of neutrophil-to-lymphocyte ratio (NLR), is a marker of systemic inflammation and infection that could be used as a predictor of bacterial infection, including pneumonia. The increase of NLR in this study is consistent with the findings from Wang et al. (48). These authors also found a relevant increase in the levels of procalcitonin (PCT), another marker of infection, used regularly in the clinic as a marker to aid in the diagnosis of bacterial infections and to guide antibiotic therapy (55, 56). These findings are also consistent with the recent report from Huang et al. (41).

Chen et al. (40) found that the SARS-CoV-2 infection induced a cytokine storm (increase in IL-6, IL-2R, IL-10, and TNFα) and lymphopenia, a decrease in $CD4^+$ and $CD8^+$T cells, as well as suppressed IFN-γ production by $CD4^+$T cells, which might be correlated with disease severity in COVID-19.

Zheng et al. (57) found no statistical differences in IL-6 and TNF-α plasma concentrations in mild or severe COVID-19 patients. However, they showed that elevated exhaustion levels and reduced functional diversity of T cells in peripheral blood may also predict severe progression in COVID-19 patients. A study in 30 COVID-19 patients found that the platelet to lymphocyte ratio (PLR) was associated with a poorer prognosis and a longer than average hospitalization time. The authors suggest that the PLR of patients could be a proxy of the cytokine storm, since the recruitment of neutrophils and other inflammatory cells to the site of injury plays a crucial role in the inflammatory response, providing a new inflammation index to monitor patients with COVID-19 (58).

Another recent study reported that the serum SARS-CoV-2 viral load (RNAaemia) is strongly associated with the levels of IL-6 in COVID-19 patients (43). The levels of IL-6 were 10-fold higher in critically ill patients compared to severe patients. This study strongly suggests that IL-6 is a promising prognosis biomarker and therapeutic target in critically ill COVID-19 patients. Similarly, another study found that the combination of IL-6 and D-Dimer measurements had the highest specificity and sensitivity for early prediction of the severity of COVID-19 patients (∼94%), and that they were also useful to track pneumonia development (42). Levels of lactate dehydrogenase (LDH) and creatine kinase (CK) have been associated with viral mRNA elimination, suggesting that a constitutive decrease of LDH or CK levels probably predict a favorable recovery response for COVID-19 patients (46).

The serial detection of IFN-γ-induced protein 10 (IP-10), monocyte chemotactic protein-3 (MCP-3) and IL-1ra in 14 severe cases showed that the continuous high levels of these cytokines were associated with disease deterioration and fatal outcome (44). The authors suggest that the combination of these cytokines are independent predictors for the progression of COVID-19.

Several studies have also reported that the activation of coagulation pathways (i.e., increased D-dimer, a marker of thromboembolism), during the immune response to COVID-19 might also lead to an overproduction of proinflammatory cytokines leading to multiorgan injury and death (59).

In summary, early reports show a correlation between the cytokine storm syndrome and severity leading to a poor prognosis in COVID-19.

TABLE 2 | Potential immunomodulators used for COVID-19 disease at the beginning of the pandemic.

Drug	Clinical use
Azitromicine	Antibacterial, Immunomodulator
Steroids	Anti-inflammatory, Septic Shock ARDS
IFN-β 1b	Rheumatic Diseases Hepatitis C
Tocilizumab	Anti-IL6R Rheumatic Diseases, Cytokine Storm
Sarilumab	Release Syndrome in CAR-T cells
Baricitinib	JAK inhibitor, Rheumatic Diseases
Anankinra	IL-1R antagonist Autoimmune Diseases
Convalescent Serum	Treatment of SARS and MERS
Immunoglobulins	Autoimmune Diseases
Nitric oxide	ARDS Lung Hypertension
Remdesivir, Favipiravir, Lopinavir/Ritonavir	Antiviral treatment

ARDS, acute respiratory distress syndrome; JAK, Janus tyrosine kinase; IL-1R, interleukin 1 receptor; IL-6R, interleukin 6 receptor; CAR-T cells, Chimeric antigen receptor T.

Below we also review early therapeutic attempts at relieving this hyperinflammatory state with available drugs at the time of the pandemic.

RE-PURPOSED FDA DRUGS THAT HAVE BEEN USED TO TREAT CYTOKINE STORM IN COVID-19 PATIENTS

Currently, the preferred therapeutic approach to COVID-19 seems to be a multimodal treatment combining antibiotics, antiviral (i.e., remdesivir, favipiravir, lopinavir/ritonavir, etc.), and anti-inflammatory drugs with support therapies for the respective organic failures (60, 61). Antiviral treatment is important to decrease viral load and replication, decreasing RNAemia and consequently the inflammatory stimulus. The main immunomodulatory agents used for COVID-19 at the beginning of the pandemic are summarized in **Table 2**. Later on, some of these drugs have demonstrated to have no effect on survival and prognosis of the disease.

Emerging evidence indicates that there are potential benefits when managing the cytokine storm in COVID-19 patients by administering steroids, IL-6/IL-6-receptor (IL-6R) blocking antibodies, TNF inhibitors, IL-1 antagonists, and Janus kinase inhibitor (JAK) inhibitors (62). Steroids inhibit the synthesis of various cytokines (IL1-8, TNFα, IFNg, GM-CSF) produced by an array of different cells (macrophages, monocytes, lymphocytes, endothelial, or epithelial). Steroids act through a variety of mechanisms, including the inhibition of transcription, by preventing protein translation and destroying mRNA, by inhibiting the synthesis of cytokine receptors and activating

transcriptional factors such as AP-1 or NF-kB, and by genetic dispersion of adhesion molecules (ICAM-1). Glucocorticoids may also modulate inflammation-mediated lung injury. Recently, a controlled, open-label trial evaluating the potential use of oral or intravenous dexamethasone for critically ill COVID-19 showed a decrease in mortality among those who were receiving either invasive mechanical ventilation or oxygen alone at randomization but not among those receiving no respiratory support (63).

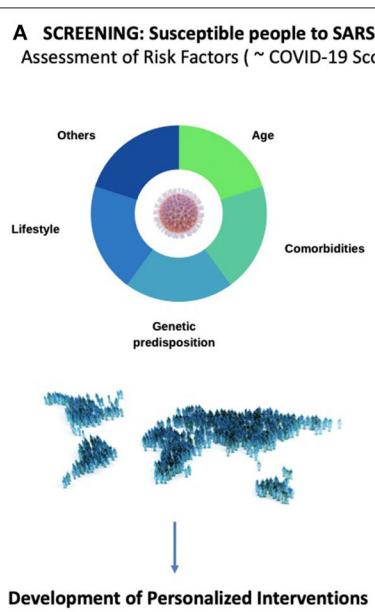
Tocilizumab is an FDA-approved immunosuppressive drug targeting IL-6R that is commonly used for the treatment of rheumatoid arthritis (RA) (64). Tocilizumab has also been used to manage cytokine release syndrome (CRS) in patients receiving chimeric antigen receptor (CAR) T cell therapy (65). In COVID-19 patients, Tocilizumab has been shown to improve the clinical symptoms by reducing the inflammation and decreasing severity (54). Several drugs such as Sarilumab (IL-6R antagonist), anakinra (IL-1 receptor antagonist), Baricitinib, Fedratinib, and ruxolitinib (JAK inhibitors) are under study at the moment in several phase II/III clinical trials around the world.

Wu and Yan (66) suggest that in COVID-19 patients the FDA approved JAK2 inhibitor Fedratinib, in combination with antiviral drugs, could be used to reduce the mortality associated with hyperinflammation by suppressing the production of several Th17 cytokines (i.e., IL1b and TNFalpha, IL21,IL22, IL17) and the formation of pulmonary edema. It has also been suggested that modulators targeting the cytokine IP-10 are a promising therapeutic strategy in the treatment of the acute phase of ARDS since they could ameliorate acute lung injury (44).

Hydroxychloroquine (HCQ), an anti-malarial drug which exhibits an antiviral effect similar to that of chloroquine, could mitigate the severe progression of COVID-19, inhibiting the cytokine storm by suppressing T cell activation (67). HCQ has been shown to decrease the production of IFN, TNF, IL-6, and IL-1 and promote autophagic inhibition. However, recent results from a clinical trial have shown that the use of hydroxychloroquine, alone or with azithromycin, did not improve clinical status of COVID-19 patients compared to standard care (68).

Azithromycin is an antibacterial agent (macrolide) that is also being used to treat COVID-19 patients in combination with other drugs such as hydroxychloroquine. It has a well-known immunomodulatory effect and could be an important element of multimodal treatments, even in seriously ill patients (69).

Blood purification, a treatment in which a patient's blood is passed through a device to remove waste products, and toxins, is a promising therapy in reducing the cytokine storm that occurs as a late complication of critically ill COVID-19 patients, although the small cohort ($n = 3$) merits more investigation (70). Administering convalescent plasma is a promising alternative that has been used with notable success in SARS-Cov1, MERS, or Ebola patients. The plasma must be collected from recovered patients who can donate blood, do not show any symptoms for more than 14 days and have yielded negative results on Covid-19 tests. The small clinical experience with using this method for treating COVID-19 is encouraging, although it will require a thorough examination and more contrasted data through clinical trials (71). Another alternative is the use of therapeutic plasma exchange for fulminant COVID-19 patients. The main



B COVID-19 Classification and patient management

CLASSIFICATION[1]	COVID-19 type 0 (asymptomatic)	COVID-19 type I (mild)	COVID-19 type II (severe)*	COVID-19 type III (critically ill)*
Comorbidities	YES/NO	NO	YES	
Pneumonia	NO	YES	YES	*Patient type I and type II that didn't respond to treatment*
Other symptoms (fever, cough, throat pain, loss of taste or smell, diarrhea, etc)	NO	YES	YES	
Treatment	[not applicable] previous comorbidities treatment	Antiviral Antibiotic Oxygen therapy Glucocorticoids Others	Monitoring previous comorbidities treatment Antiviral Antibiotic Oxygen therapy Glucocorticoids Others	Monitoring previous comorbidities treatment Antiviral Antibiotic Oxygen therapy Glucocorticoids Others

[1]SARS-CoV-2 positive testing (RT-PCR, antigen, antibodies, etc)
*Age is a risk factors in these patients

FIGURE 2 | Prevention and management COVID-19. **(A)** There is an urgent need to improve our understanding on the phenotype profiles behind the progress from mild to severe or critical COVID-19. This includes analyzing the different risk factors as well as circulating biomarkers. In turn, this could lead to personalized therapies with reduced side effects. It is also imperative to screen the elderly for risk factors that predispose them to severe COVID-19. Immunosenescence and comorbidities should be taken into consideration **(B)** An operative classification to screen COVID-19 patients is showed in this panel. Age is a risk factor specially in the severe and critically ill patients.

arguments for using this treatment would be to removing cytokines, stabilizing endothelial membranes and resetting the hypercoagulable state.

While therapeutic outcomes are rather anecdotal at the moment, several ongoing large-scale clinical trials will surely shed more light into the validity of these and other therapeutic approaches aimed at ameliorating the cytokine storm in COVID 19 patients. However, judging from the results obtained from previous SARS cases, the success of these therapies will likely be intimately tied to timing and dosage, due to the highly dynamic nature of the inflammatory process. No proven benefits of immunomodulatory treatments in severe COVID-19 infections are known at the moment. Additional risks such as a higher incidence of opportunistic infections or reactivations of infections such as hepatitis b under treatment with Tociluzimab should be taken into account. Therefore, the use of these immunomodulators in COVID-19 must be evaluated very critically. Finally, by the time this manuscript was written, only the treatment with Remdesivir has shown some clinical improvement (i.e., shortening the time to recovery) in COVID-19 patients (72, 73).

CONCLUSION

To date, no available FDA drug or therapy has demonstrated 100% efficacy for patients with COVID-19. The high percentage of critically ill patients in the COVID-19 pandemic has forced some ICUs to take desperate measures. In this context, it is imperative to identify biomarkers for predicting disease severity and prognosis in order to make more efficient choices regarding the use of limited resources in ICUs as to avoid their oversaturation. According to early reports depicted in **Table 1**, increased levels of inflammatory markers such as D-dimer, CRP, IL-6, and CK, together with a reduction in lymphocyte counts, and increased ferritin levels are common in COVID-19 patients and have been associated with severe stages of COVID-19. Close monitoring of these biomarkers could reveal the evolution from mild to severe COVID-19 and avoid poor outcomes in future cases.

There is a need to better understand the disease, its pathophysiology, temporal evolution, prognostic clinical and analytical parameters, the immunity (or not) generated, and the real impact of the proposed treatments that are being evaluated in different clinical trials at this moment. Lessons learnt from other hyperinflammatory syndromes such as sepsis and the cytokine storm responsible for the poor outcomes in SARS and MERS show a strong time dependence between cytokine levels and disease progression. We propose that measuring inflammation biomarkers frequently over time is the best strategy to characterize the evolution of the cytokine storm, since it provides a personalized biomarker profile for each patient. In turn, this could be used for guiding the timing and dosage of anti-inflammatory treatments, as well as to assess their effectiveness. Point-of-care diagnostic devices will likely be crucial to enable these kinetic biomarker measurements without collapsing central diagnosis laboratories (74–77).

Young individuals or people with no associated morbidities typically have mild symptoms or remain asymptomatic, while the elderly experience substantially more severe symptoms and lethality. Prevention and screening strategies should be implemented to manage COVID-19 disease (**Figure 2**). Older patients (>60 years) with comorbidities or chronic medical conditions and ARDS are at increased risk of death from COVID-19. This is likely due to immunosenescence and increased frailty associated with aging, that lead to a loss of function and fitness. It is noteworthy that even after a SARS-CoV-2 vaccine is developed and available as a prevention strategy, it might not provide full protection to the elderly due to this decline in immune function. Evidence from other diseases such as H1N1 has shown that the effectiveness of the influenza vaccine differs annually due to mutations in the virus. Similar scenarios can be expected with the COVID-19 pandemic, which makes it prescient to study other preventive strategies such as screening the most vulnerable population. Clinical frailty indexes could provide a simple method to identify these patients. This could be supplemented with measurements of inflammation biomarkers such as IL-6, which are, to date, the most consistent and reliable risk markers for adverse health outcomes in older people (3). Also, older COVID-19 patients who become critically ill often have cardiovascular, metabolic and/or kidney diseases and/or cancer, among other complications. Therefore, close monitoring of previous (multi)morbidities during hospitalization is of vital importance in order to discern their role in COVID-19 disease progression. Recent advances in Artificial Intelligence (AI) could make possible a holistic view of the COVID-19 disease, as the AI can combine and analyze all the patient's clinical history data with biomarker measurements, frailty scores, and therapeutic interventions, all within seconds. Once trained, the AI could also be used to identify patients at risk of severe or critical COVID-19 that require special care.

Finally, there is an urgent need to develop rehabilitation treatments for COVID19 survivors. Patients with respiratory symptoms who survive the virus might be probably left with chronic problems, such as lung fibrosis, lower lung function, and kidney damage (78, 79). Interventions such as oxygen therapy, physical activity and others focused on the mitigation of these chronic conditions is crucial as well. Similarly, a long-term study of inflammation markers may shed new light on the impact of the cytokine storm in COVID19 survivors.

AUTHOR CONTRIBUTIONS

RR, MB and MG-F contributed to develop the content of the manuscript and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SYSTEMATIC REVIEW
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Association of Peripheral Lymphocyte and the Subset Levels With the Progression and Mortality of COVID-19: A Systematic Review and Meta-Analysis

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Current evidence is controversial in the association between peripheral lymphocyte levels and the progression and mortality of Corona Virus Disease 2019 (COVID-19), and this meta-analysis aimed to clarify the association. A systematic search was conducted in public databases to identify all relevant studies, and the study-specific odds ratio (OR) and 95% confidence intervals (CI) were pooled. Finally, 16 studies were identified with a total of 1,873 progressive COVID-19 cases and 5,177 stable COVID-19 cases. In COVID-19 progression, lymphocyte levels showed a significant negative correlation (OR: 0.68, 95% CI: 0.51–0.89), but it was not significant in the subsets of CD3+ T cells (OR: 0.97, 95% CI: 0.93–1.02), CD4+ T cells (OR: 0.93, 95% CI: 0.80–1.08), CD8+ T cells (OR: 0.96, 95% CI: 0.92–1.00), B cells (OR: 0.98, 95% CI: 0.92–1.04), or NK cells (OR: 0.80, 95% CI: 0.61–1.04). In COVID-19 mortality, lymphocyte levels showed a significant negative correlation (OR: 0.41, 95% CI: 0.20–0.85), but it was not significant in the subsets of CD3+ T cells (OR: 0.95, 95% CI: 0.86–1.05), CD4+ T cells (OR: 1.06, 95% CI: 0.86–1.31), CD8+ T cells (OR: 0.38, 95% CI: 0.14–1.01), B cells (OR: 0.98, 95% CI: 0.92–1.04), or NK cells (OR: 0.80, 95% CI: 0.61–1.04). In conclusion, current evidence suggests a significant negative association of peripheral lymphocyte levels with COVID-19 progression and mortality, but it was not significant in the subsets of CD3+ T cells, CD4+ T cells, CD8+ T cells, B cells, and NK cells.

Keywords: COVID-19, lymphocytes, progression, mortality, meta-analysis

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INTRODUCTION

In December 2019, an outbreak of pneumonia of unknown cause occurred in Wuhan, and rapidly spread throughout the world (1). The pathogen was confirmed to be a distinct clade of the β-coronavirus associated with human severe acute respiratory syndrome (SARS) (2). The novel virus was officially named SARS-CoV-2, with the disease termed COVID-19. Epidemiological data demonstrated high infectivity in SARS-CoV-2 and high mortality in multiple cohorts. Thus, it was important to identify laboratory parameters capable of discriminating the COVID-19 patients at high risk of progression or mortality, which would help physicians to provide timely intervention and improve the patients' prognosis.

Lymphocytes and the subsets of T cells, B cells, and NK cells play a key role in the maintenance of immune system function. After SARS-CoV-2 infection, the patients were characterized by a significant decrease of peripheral lymphocytes and the subsets (3). However, current studies are considered controversial on the association between peripheral lymphocyte levels at baseline and the progression and mortality of COVID-19, and no meta-analyses have focused on this. Thus, we conducted a systematic review and meta-analysis to clarify the association.

METHODS

Literature Search

The databases of PubMed, China Wanfang Database, China Knowledge Resource Integrated Database (CNKI) were searched from inception to July 15, 2020, using key words including: ("COVID-19" OR "Corona Virus Disease 2019" OR "SARS-CoV-2" OR "2019-nCoV" OR "2019 novel coronavirus") AND ("fatality" OR "mortality" OR "survivor" OR "non-survivor" OR "decease" OR "death" OR "prognosis" OR "progression" OR "outcome" OR "risk factor" OR "efficacy" OR "recovery"). Studies in languages other than English or Chinese were excluded. Moreover, we also reviewed the references of related studies and reviews for undetected studies. This study was approved by the ethics committee of Shanghai University of Traditional Chinese Medicine.

Study Selection and Exclusion

We selected the studies with full texts available. The studies were included if they met the following criteria: (i) all hospitalized patients discussed had a definite diagnosis of COVID-19; (ii) the patients discussed were divided into the progressive group [e.g., admission to an intensive care unit (ICU), the use of mechanical ventilation, or death] or the stable group during the hospitalization; (iii) the study evaluated the association of the baseline lymphocytes levels or the main subtypes of CD3+ T cells, CD4+ T cells, CD8+ T cells, B cells, or NK cells (measured by multiple-color flow cytometry with human monoclonal antibodies) with the COVID-19 progression or mortality; (iv) presented relative risk (RR), odds ratio (OR), or hazard ratio (HR) estimates with 95% confidence intervals (CI). The exclusion criteria were as follows: abstracts without full texts, reviews, and case reports.

Data Extraction and Quality Assessment

Two authors extracted the data by a standardized collection form. All differences were resolved by discussion. In each study, the following information was extracted: first author, publication year, study area, diagnostic criteria, clinical outcomes, number of cases per group, lymphocyte types, effect sizes with 95% CI, and adjusted factors. The Newcastle-Ottawa Scale (NOS) was used to assess the methodological quality of the included studies.

Statistical Analysis

To compute a summary OR with its 95% CI, we used the study-specific most adjusted OR or HR and its 95% CI in all analyses. The heterogeneity among studies was estimated by the Q-test and I^2 statistic. $I^2 > 50\%$ represented substantial heterogeneity, and the summary estimate was analyzed by a random-effects model. Otherwise, a fixed-effects model was applied. Publication bias was assessed by using funnel plots and Egger's test. All statistical analyses were performed using the software STATA version 11.0 (StataCorp LP, College Station, TX, USA), and all tests were sided with a significance level of 0.05.

RESULTS

Characteristics of the Included Studies

The search strategy identified 7,385 records: 6,528 from PubMed, 465 from CNKI, 322 from Wangfan, and 70 from other sources (**Figure 1**). After excluding duplicated and irrelevant records, 16 studies were included in this meta-analysis with a total of 10,624 COVID-19 cases (**Table 1**) (4–19). Thirteen studies derived from China, while one study came from the USA, one from Spain and one from India. Fourteen studies were case-control designed, and the studies by Du et al. and Petrilli et al. were prospective designed. During the hospitalization, the cases were divided into the progressive group (n = 1,873) and the stable group (n = 5,177). Ten studies were adjusted by multivariable analysis. In quality assessment, the NOS scores ranged from 6 to 8, with an average of 7.13.

Lymphocytes and COVID-19 Progression

Fourteen studies investigated the association between baseline lymphocyte levels and COVID-19 progression, with a total of 1,651 progressive cases and 4,089 stable cases (**Figure 2**). The meta-analysis indicated a significant negative association (OR: 0.68, 95% CI: 0.51–0.89, $P = 0.006$; $I^2 = 77.6\%$, $P < 0.001$). Egger's test detected no significant publication bias ($P = 0.707$).

Lymphocyte Subsets and COVID-19 Progression

Three studies focused on the association between CD3+ T cells and COVID-19 progression (207 progressive cases and 695 stable cases), while four studies on CD4+ T cells (231 progressive cases and 663 stable cases), seven studies on CD8+ T cells (461 progressive cases and 1,827 stable cases), one study on B cells (102 progressive cases and 307 stable cases), and one study on NK cells (102 progressive cases and 307 stable cases) (**Figure 2**). The meta-analysis indicated no obvious association in CD3+ T cells (OR: 0.97, 95% CI: 0.93–1.02, $P = 0.190$; $I^2 = 76.3\%$, $P = 0.015$), CD4+ T cells (OR: 0.93, 95% CI: 0.80–1.08, $P = 0.345$; $I^2 = 80.8\%$, $P = 0.001$), CD8+ T cells (OR: 0.96, 95% CI: 0.92–1.00, $P = 0.061$; $I^2 = 92.7\%$, $P < 0.001$), B cells (OR: 0.98, 95% CI: 0.92–1.04, $P = 0.482$), or NK cells (OR: 0.80, 95% CI: 0.61–1.04, $P = 0.092$). Egger's test detected no significant publication bias in CD8+ T cells ($P = 0.053$).

Lymphocytes and COVID-19 Mortality

Eight studies investigated the association between baseline lymphocyte levels and COVID-19 mortality, with a total of 914 non-survivors and 3,294 survivors (**Figure 3**). The

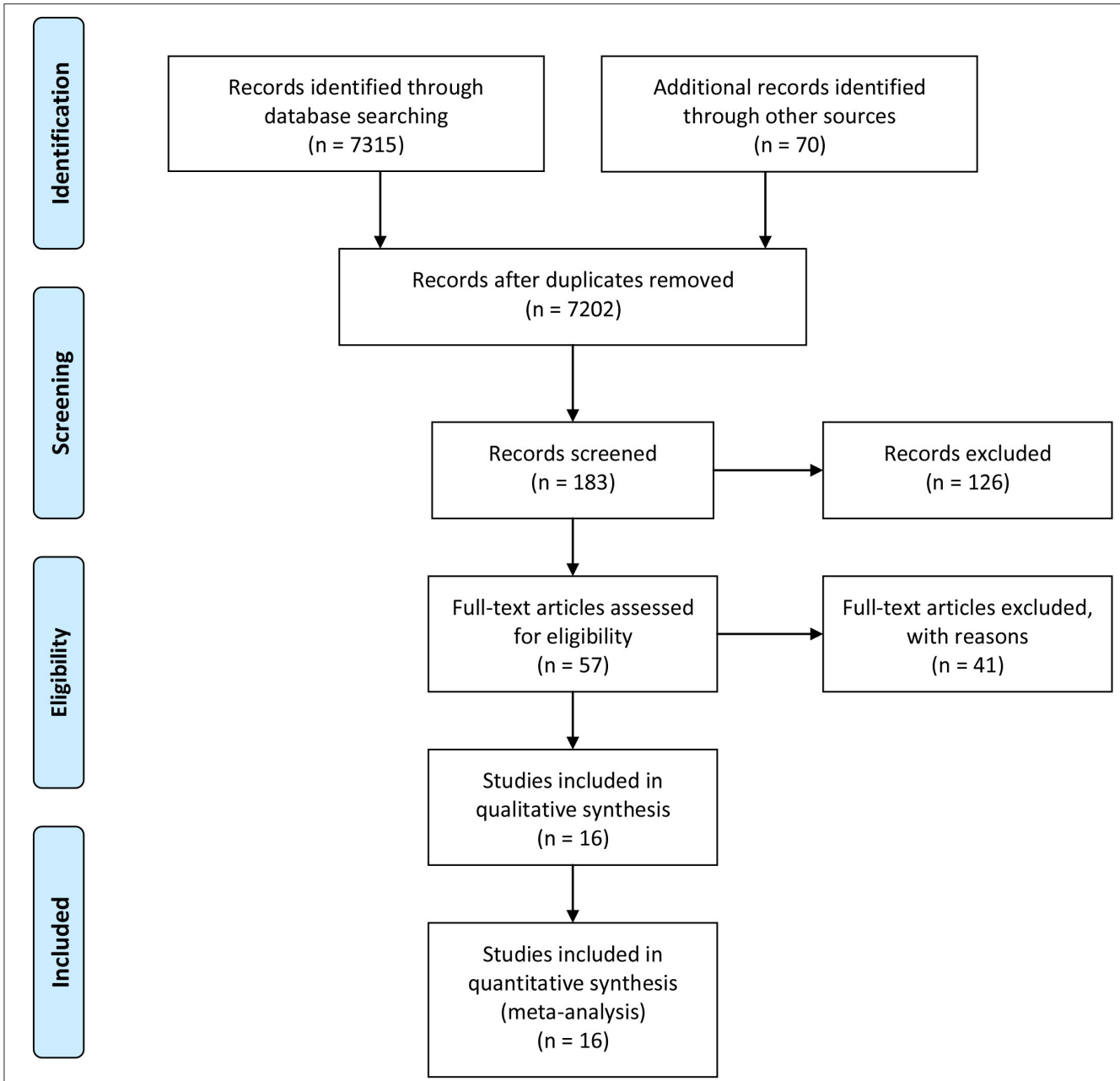

FIGURE 1 | Flowchart of literature search.

meta-analysis indicated a significant negative association (OR: 0.41, 95% CI: 0.20–0.85, $P = 0.016$; $I^2 = 43.3\%$, $P = 0.090$). Egger's test detected no significant publication bias ($P = 0.445$).

Lymphocyte Subsets and COVID-19 Mortality

Two studies focused on the association between CD3+ T cells and COVID-19 mortality (146 non-survivors and 347 survivors), while two studies on CD4+ T cells (146 non-survivors and 347 survivors), four studies on CD8+ T cells (368 non-survivors and 1,322 survivors), one study on B cells (102 progressive cases and 307 stable cases), and one study on NK cells (102 progressive cases and 307 stable cases) (**Figure 3**). The meta-analysis indicated no obvious association in CD3+ T cells (OR: 0.95, 95% CI: 0.86–1.05, $P = 0.345$; $I^2 = 17.0\%$, $P = 0.272$), CD4+ T cells (OR: 1.06, 95% CI: 0.86–1.31, $P = 0.579$; $I^2 = 25.1\%$, $P = 0.248$), CD8+ T cells (OR: 0.38, 95% CI: 0.14–1.01, $P = 0.052$; $I^2 = 93.3\%$, $P < 0.001$), B cells (OR: 0.98, 95% CI: 0.92–1.04, $P = 0.482$), or NK cells (OR: 0.80, 95% CI: 0.61–1.04, $P = 0.092$).

TABLE 1 | Characteristics of included studies.

References	Area	Diagnostic criteria	Clinical outcomes	Sample size	Incident cases	Indicators	Effect sizes*	Adjustment
Aggarwal et al. (4)	India	WHO interim guidance	ICU admission, mechanical ventilation, death	32	12	Lymphocytes	0.47 (0.08–2.81)	-
Chen et al. (5)	China	Chinese interim guidance; WHO interim guidance	ICU admission	249	22	Lymphocytes	4.05 (0.89–18.50)	Age, male, comorbidity, WBC, CRP, albumin, AST, LDH, eGFR
						CD4+ T cells	0.55 (0.33–0.92)	
Du et al. (6)	China	WHO interim guidance	Death	179	21	Lymphocytes	0.273 (0.061–13.415)	-
						CD8+ T cells	0.251 (0.071–0.883)	Age, hypertension, cardiovascular or cerebrovascular diseases, dyspnea, fatigue, sputum production, headache, WBC, neutrophils, cTnI, myoglobin, creatinine, D-dimer, blood pressure
Guan et al. (7)	China	WHO interim guidance	ICU admission, mechanical ventilation, death	879	54	Lymphocytes	0.38 (0.13–1.06)	-
Yun et al. (8)	China	Chinese interim guidance (5th edition)	ICU admission	292	21	CD3+ T cells	0.996 (0.991–1.000)	Neutrophils, ALT, AST, albumin, LDH, creatinine, cystatin-C, transferrin, CRP, procalcitonin, D-dimer, creatine kinase, CK-MB, NT-proBNP, cTnI, myoglobin
						CD8+ T cells	1.006 (1.001–1.010)	
Liu et al. (9)	China	Chinese interim guidance (4th edition)	Disease progression, death	78	11	Lymphocytes	0.625 (0.428–65.868)	-
Liu et al. (10)	China	WHO interim guidance	Death	245	33	Lymphocytes	0.86 (0.34–2.15)	Age, sex, BMI, hypertension, chronic liver disease, HIV infection, COPD, smoking, respiratory rate, ALT, creatinine, PT, D-dimer
Luo et al. (11)	China	WHO interim guidance	Death	1,018	201	CD8+ T cells	0.169 (0.105–0.272)	Age, sex, hypertension, CHD, diabetes, pulmonary diseases
Pan et al. (12)	China	Chinese interim guidance; WHO interim guidance	Death	124	89	Lymphocytes	0.249 (0.090–7.550)	Sex, SpO2, breath rate, diastolic pressure, neutrophil, CRP, PCT, LDH, D-dimer
Petrilli et al. (13)	USA	WHO interim guidance	ICU admission, mechanical ventilation, discharge to hospice, death	2,725	990	Lymphocytes	0.57 (0.40–8.73)	Time, age, sex, race, smoking, BMI, underlying diseases, temperature, SpO2, ALT, AST, CRP, D-dimer, ferritin, PCT, TnI
			Death	2,737	424	Lymphocytes	0.72 (0.55–10.48)	
Urra et al. (14)	Spain	WHO interim guidance	ICU admission	172	27	Lymphocytes	0.769 (0.687–0.861)	-
						CD8+ T cells	0.380 (0.152–0.950)	
Wu et al. (15)	China	WHO interim guidance	ARDS	201	84	Lymphocytes	0.37 (0.21–0.63)	-
						CD3+ T cells	0.83 (0.72–0.96)	
						CD4+ T cells	0.74 (0.59–0.93)	
						CD8+ T cells	0.74 (0.53–1.04)	

(Continued)

TABLE 1 | Continued

References	Area	Diagnostic criteria	Clinical outcomes	Sample size	Incident cases	Indicators	Effect sizes*	Adjustment
			Death	84	44	Lymphocytes	0.51 (0.22–1.17)	-
						CD3+ T cells	0.81 (0.59–1.11)	
						CD4+ T cells	0.83 (0.51–1.35)	
						CD8+ T cells	0.51 (0.24–1.09)	
Xu et al. (16)	China	Chinese interim guidance (6th edition)	Death	239	147	Lymphocytes	0.81 (0.58–12.5)	Age, malignancy, platelet, ARDS, acute cardiac injury, AKI, liver dysfunction, coagulopathy
Zhang and Han (17)	China	WHO interim guidance	Death	409	102	Lymphocytes	0.012 (0.001–0.128)	Age, sex, diarrhea, WBC, neutrophil
						CD3+ T cells	0.968 (0.933–1.004)	-
						CD4+ T cells	1.114 (0.997–1.244)	
						CD8+ T cells	0.835 (0.745–0.937)	
						B cells	0.979 (0.923–1.039)	
						NK cells	0.796 (0.611–1.039)	
Zhou et al. (18)	China	WHO interim guidance	Death	191	54	Lymphocytes	0.19 (0.02–1.62)	Age, D-dimer, SOFA score, coronary heart disease
Zhou et al. (19)	China	Chinese interim guidance (5th edition)	Aggravation	17	5	Lymphocytes	0.997 (0.993–0.999)	WBC, CRP, albumin, LDH, D-dimer
						CD4+ T cells	0.995 (0.989–1.000)	
						CD8+ T cells	0.993 (0.984–1.002)	

WHO, World Health Organization; ARDS, acute respiratory distress syndrome; WBC, white blood cells; CRP, C-reactive protein; PCT, procalcitonin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; eGFR, estimated glomerular filtration rate; cTnI, cardiac Troponin I; BMI, body mass index; COPD, chronic obstructive pulmonary disease; CHD, coronary heart disease; ARDS, acute respiratory distress syndrome; AKI, acute kidney injury; PT, prothrombin time; NT-proBNP, N terminal pro B type natriuretic peptide; SOFA, Sequential Organ Failure Assessment.

**Effect sizes for the lowest vs. highest lymphocyte levels were adjusted to the highest vs. the lowest.*

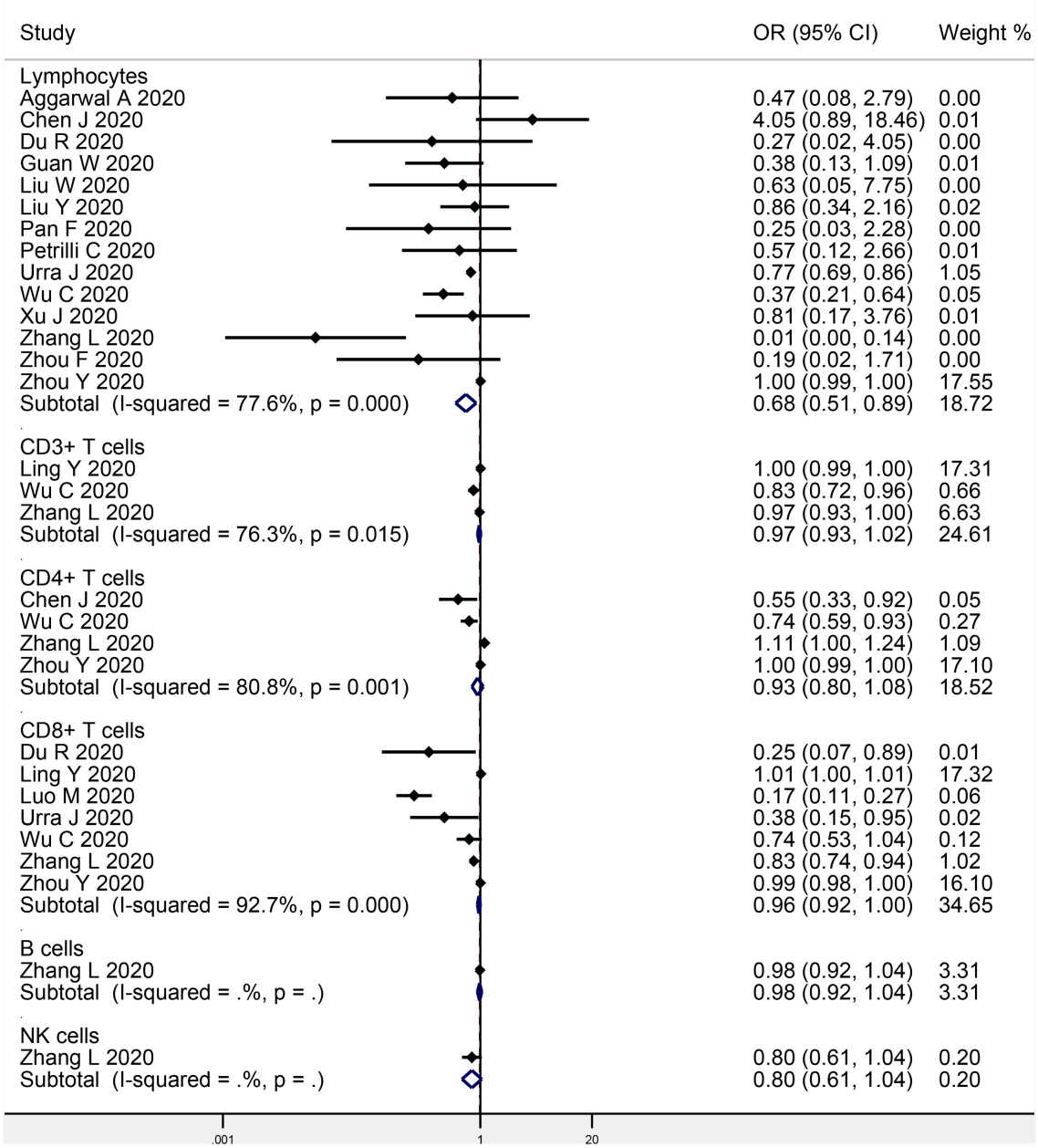

FIGURE 2 | Meta-analysis of the association of peripheral lymphocytes or the subsets with COVID-19 progression.

DISCUSSION

As with SARS and MERS, lymphopenia was common in COVID-19 patients, suggesting an impairment of the immune system in the pathogenesis of the SARS-CoV-2 infection. In subsets, CD4+ T cells, CD8+ T cells, B cells, and NK cells were found with a decrease in COVID-19 patients (3). On admission, severe cases had a lower level of lymphocytes, CD4+ T cells, CD8+ T cells, and B cells than mild cases, which was similar in SARS (20, 21). Thus, it was thought that lymphopenia was associated with not only COVID-19 severity but also its prognosis. However, the multivariable analyses in several studies found no significant association of lymphocytes with the COVID-19 progression or mortality (4, 5, 10, 12, 13, 16, 18, 19). This aroused our attention on whether peripheral lymphocytes or the subsets could be a potential predictor for the COVID-19 prognosis.

Finally, our meta-analysis found a significant negative association of peripheral lymphocyte levels with COVID-19 progression or mortality. Lymphopenia was commonly reported in patients with COVID-19 (72%), indicating an impairment of the immune system during the course of the SARS-CoV-2 infection. This might be caused by direct attachment of

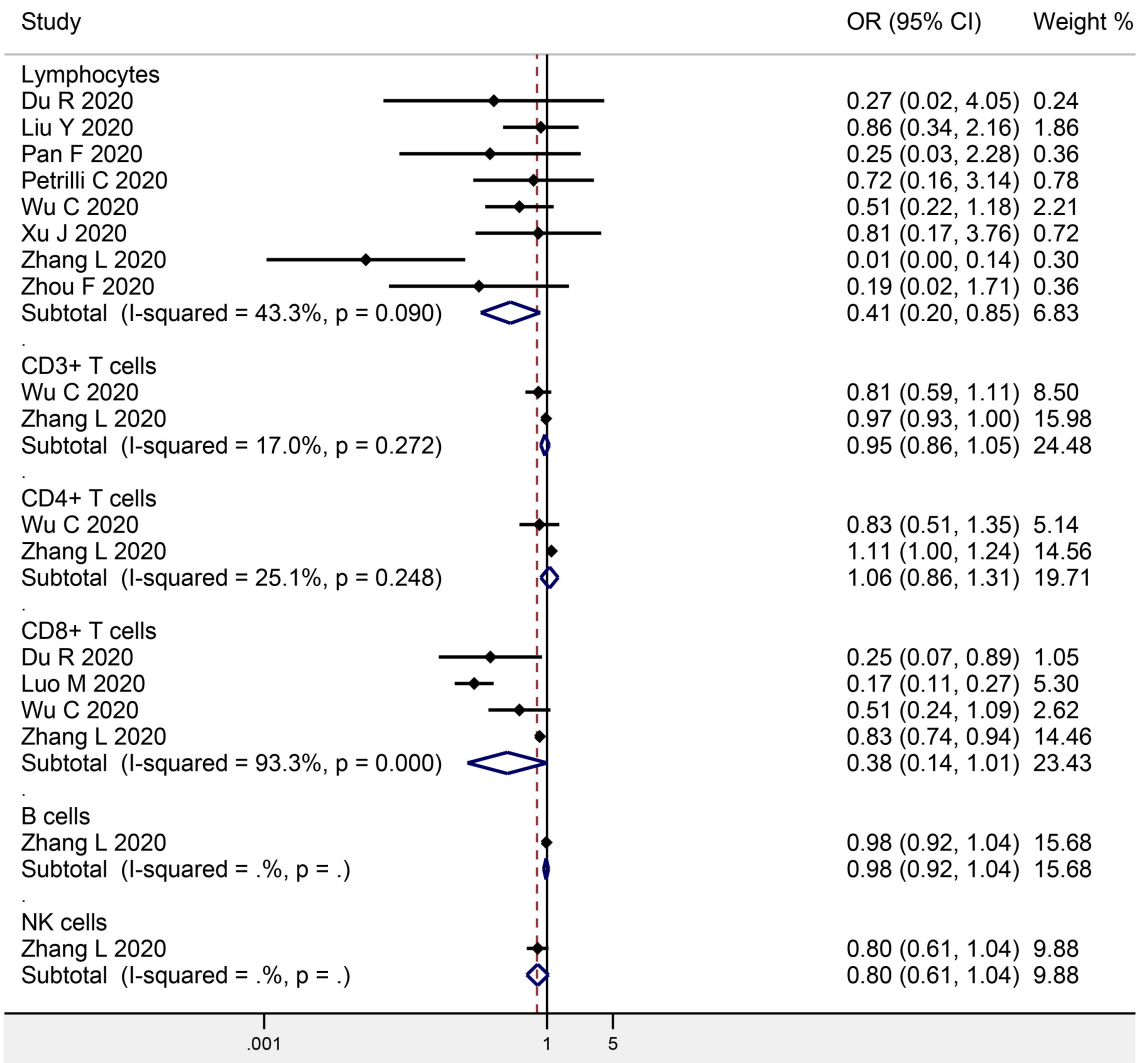

FIGURE 3 | Meta-analysis of the association of peripheral lymphocytes or the subsets with COVID-19 mortality.

SARS-CoV-2 or indirect immune injuries from inflammatory responses. On the other hand, the infiltration of peripheral lymphocytes into the inflamed lung tissues could also result in the decrease in lymphopenia. Thus, the baseline and post-treatment alteration of peripheral lymphocyte levels were thought to be reliable indicators of COVID-19 progression or mortality.

However, no subsets showed a significant association with COVID-19 progression or mortality. These findings were similar to the results in the Wang et al. study (3). In their study, the subsets of CD4+ T cells, CD8+ T cells, B cells, and NK cells decreased in COVID-19 patients, and severe cases had a lower level than mild cases. However, the baseline levels of the subsets showed no association with the clinical outcomes after one-week of treatment. Nevertheless, post-treatment decrease of CD8+ T cells and B cells and increase of CD4+/CD8+ ratio were independent predictors for poor efficacy. This might contribute to the ubiquity of lymphopenia in COVID-19, which reduced its specificity in prognostic prediction. Secondly, the patients with lower lymphocyte levels tended to have a severe infection, which caused a general inflammatory status and a more severe disease evaluation. For those patients, anti-inflammatory treatment showed a good efficacy, and the patients with a slow or meager response to the inflammation were more likely to development refractory diseases. This could also explain the findings by the Mo et al. study that the patients without fever on admission were at high risk of poor efficacy (22). Thus, it was not the baseline levels of the subsets but the post-treatment alteration that might be reliable indictors for the prediction of COVID-19 progression or mortality. On the other hand, we only investigated the subsets of T cells, CD4+ T cells, CD8+ T cells, B cells, and NK cells, and the other subsets might have an obvious change, involving naïve, central memory, effector memory, and terminally differentiated cells, as well as regulatory T cells and $PD1^{+}CD57^{+}$ exhausted T cells (23, 24).

This meta-analysis had several strengths. Firstly, to the best of our knowledge, this was the first meta-analysis to evaluate the association between peripheral lymphocyte levels and the progression and mortality of COVID-19. Secondly, we included the subsets of CD3+ T cells, CD4+ T cells, CD8+ T cells, B cells, and NK cells, and investigated not only the association with COVID-19 progression but also with the mortality. However, several limitations in this study should be considered. Firstly, the number of cases and controls in some studies was relatively small. Secondly, the obvious heterogeneity between studies was observed. Thirdly, the analyses of some subsets were limited in the number of included studies.

In conclusion, current evidence suggests a significant negative association of peripheral lymphocyte levels with COVID-19 progression and mortality, but it was not significant in the subsets of CD3+ T cells, CD4+ T cells, CD8+ T cells, B cells, and NK cells.

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

QL and PZ designed the study and wrote the manuscript. QL and ZW collected the data. YY, YZ, and PT analyzed the data. All authors contributed to the article and approved the submitted version.

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SARS-CoV-2 Infections in the World: An Estimation of the Infected Population and a Measure of How Higher Detection Rates Save Lives

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This paper provides an estimation of the accumulated detection rates and the accumulated number of infected individuals by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Worldwide, on July 20, it has been estimated above 160 million individuals infected by SARS-CoV-2. Moreover, it is found that only about 1 out of 11 infected individuals are detected. In an information context in which population-based seroepidemiological studies are not frequently available, this study shows a parsimonious alternative to provide estimates of the number of SARS-CoV-2 infected individuals. By comparing our estimates with those provided by the population-based seroepidemiological ENE-COVID study in Spain, we confirm the utility of our approach. Then, using a cross-country regression, we investigated if differences in detection rates are associated with differences in the cumulative number of deaths. The hypothesis investigated in this study is that higher levels of detection of SARS-CoV-2 infections can reduce the risk exposure of the susceptible population with a relatively higher risk of death. Our results show that, on average, detecting 5 instead of 35 percent of the infections is associated with multiplying the number of deaths by a factor of about 6. Using this result, we estimated that 120 days after the pandemic outbreak, if the US would have tested with the same intensity as South Korea, about 85,000 out of their 126,000 reported deaths could have been avoided.

Keywords: infection fatality ratio, infection detection ratio, estimates of SARS-CoV-2 infections, asymptomatic SARS-CoV-2 population, multiple linear regression

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INTRODUCTION

Governments and policymakers dealing with the COVID-19 pandemic will fail in their objectives if their actions are guided by misleading data or subsequent misinformation. The authorities should have reliable estimations of the number of SARS-CoV-2 infected individuals. However, there are few attempts to estimate the total amount of infections (1–5). Consequently, health systems face enormous challenges since an unknown and probably a high proportion of all SARS-CoV-2 infections remains undetected. Moreover, data suggest that infected individuals can be highly contagious before the onset of symptoms and SARS-CoV-2 can be also highly contagious in individuals who will never develop any symptoms (6–10).

Undetected infections are dangerous because infectious individuals spread the coronavirus in unpredictable ways. Undetected infections consist of non-PCR-tested individuals with symptoms and asymptomatic individuals (non-COVID-19 patients) that are likely to remain undetected over all phases of the infection. However, non-PCR-tested individuals with symptoms would tend to auto-select themselves, depending on the severity of their symptoms (from mild to severe), toward treatment and late detection. For this reason, it is important to know the proportion of the infected population which is asymptomatic or has such mild symptoms that self-select them into the group of non-PCR-tested individuals (11–15). Here, regarding the estimation of the number of infections, and for purposes of public health, I advocate the view by Amartya Sen and Martha Nussbaum that is preferable to be vaguely right than precisely wrong.

The public health problem is that undetected asymptomatic individuals, as well as late-detected SARS-CoV-2 infected individuals, increase the risk for vulnerable groups[1]. Since there is a transmission channel between the level of detection and the number of deaths, the early detection of asymptomatic infections, pre-symptomatic, and mild COVID-19 cases is a public health concern.

Moreover, undetected cases also are responsible for the collapse of the health system by numerous aggravated and sometimes unexpected COVID-19 patients requiring treatment in a short period. Overwhelmed health care systems reduce the recovery prospects of patients by the lack of treatment, undertreatment, increased risk of mistreatment of all patients, including those with COVID-19, and also put at unnecessarily risk the health workforce (21, 22).

The problem is that many governments formulate their strategies and responses to the pandemic based on figures that they can control. This problem of reverse causality produces contra-productive incentives for governments since public opinion tends to negatively react to the report of the cumulative and the marginal numbers of detected (reported) cases. The contradiction is that something good, such as the increase in the testing efforts by governments can be perceived by the public opinion as something bad (due to the increase in detections). Worldwide, the media communicates confirmed cases and deaths as the relevant parameters to take into consideration when assessing the evolution of the pandemic. This is a mistake since this emphasis discourages governments from decidedly pushing for mass testing with the obvious consequence of an increased number of detected cases (although, as shown in this paper, there is a theoretical mechanism relating more testing with saving lives). More sophisticated observers would use the crude and adjusted case fatality ratios to assess the pandemic evolution. However, international comparisons show that crude and adjusted case fatality ratios are highly heterogeneous and their use can be misleading (23, 24). For instance, the simple division of the cumulative number of deaths by the cumulative number of confirmed cases underestimated the true case fatality ratio in past epidemics (24, 25). Although nowadays many case fatality ratios have been estimated in this pandemic correcting many of the observed past biases (26–28), they are still depending on testing efforts made by countries.

The problem with heterogeneous case fatality ratios (different proportions of all cases that will end in death due to methodological differences on the denominator) is that they are not anchored at any exogenous information that allows researchers to perform international or territorial comparisons based on credible, and transparent assumptions. Consequently, to rely on the number of confirmed cases makes international comparations impossible since governments have shown to implement highly heterogeneous SARS-CoV-2 testing strategies ending up in different levels of location-based under-ascertainment.

In an attempt to solve the mentioned problem, we anchor our analysis in the cumulative number of deaths, which is a statistic much more difficult to alter, in free societies, than the number of SARS-CoV-2 tests[2].

We use this information together with the newest and sound estimates of the age-stratified infection fatality ratios (IFRs) provided in the recent SARS-CoV-2 related literature. In particular, we base our analysis on the IFR of 0.657% reported in Verity et al. (26). This IFR is very close to the 0.75% reported in a meta-analysis of 13 IFR estimates from a wide range of countries, and that were published between February and April of 2020 (30). We also assume orthogonal attack rates of the infection which is also supported by recent literature (16). By weighting the age-stratified IFRs by the country population age-groups shares in each country, it is possible to obtain country-specific IFRs.

The relevance of this study is 3-fold: Firstly, the estimation of the true number of infections includes not only confirmed cases but COVID-19 undetected cases, as well as SARS-CoV-2-infected individuals without the disease, or in a pre-symptomatic stage. Therefore, to provide an estimation of the true number of SARS-CoV-2 infections is of more utility than to be only informed about the number of confirmed infections. This is because confirmed cases depend on the testing efforts that can be altered or even manipulated by governments. Moreover, one can compare the true estimate of infections with the number of COVID-19 patients that require hospitalization. Such ratios can contribute to predicting, with exogenous-to-government information, shortages of the health systems.

[1]Some evidence has been found claiming that elderly and male individuals are in higher relative risk since the consequences of COVID-19 are more severe amongst them (16–20).

[2]Death-related statistics are nor free of problems. It is recognized that not all deaths due to COVID-19 in all countries are reported following WHO international norms and standards for medical certificates of COVID-19 cause of death and International Classification of Diseases (ICD) mortality coding (29). Moreover, in many countries, there is controversy over whether the COVID-19 death figures are reliable or not (for instance in Spain, Chile, and the UK), especially when these figures are compared against those from the number of excess deaths during the pandemic. More generally, it is a matter of concern that the official accumulated death figures show significant breaks responding probably to counting issues rather than to real deaths' dynamics. In our data, these breaks can be found in Spain, Chile, China, Ecuador, the Philippines, and the United Kingdom.

Secondly, the estimation of the true number of SARS-CoV-2 infections allows us to estimate the detection rate of the infection, which is a measure of the performance of health systems and governments while facing the pandemic. One can expect that higher levels of detection of SARS-CoV-2 infections, which includes asymptomatic population, and those in their early stages of the infection (which are more infectious) can reduce the risk exposure of the susceptible population with relatively a high risk of death, that is, the elderly and those individuals with preexisting conditions (17). Accordingly, a highly neglected statistic, such as the detection rate should be considered highly relevant from the public health point of view. Thirdly, in this paper, we test the hypothesis that higher detection rates can save lives while providing a measure of this impact (having in mind that is preferable to be vaguely right than precisely wrong). Thus, this study aims to quantify the importance of testing while providing empirical support to the utility of implementing massive SARS-CoV-2 tests.

Overall, this study argues that it is crucial to compute the evolution of the cumulative number of estimated SARS-CoV-2 infected individuals, and subsequently, the cumulative detection rates. This information would provide public health managers and governments the incentives to improve detection rates, rather than to the opposite. Moreover, the identification strategy can be used at lower levels of aggregation, such as regions, provinces, and municipalities to improve responses to the pandemic, including the planning of selective lockdowns or spatial-selective enhancements of the installed critical care units.

In summary, this study proposes a baseline estimation of the number of SARS-CoV-2 infections and detection rates based on current information and transparent assumptions. However, the assumptions discussed later in this paper can be later modified to match the current scientific available evidence and country-specific developments and contexts.

DATA AND METHODS

Data

For this research, we use the cumulative number of deaths and confirmed cases in the world and by country, published by OurWorldInData.org, a project of the Global Change Data Lab with the collaboration of the Oxford Martin Programme on Global Development at the University of Oxford[3]. Age-stratified demographic proportions of the population were obtained from the UN population data[4]. The age-stratified IFRs are those reported in Verity et al. (26)[5]. Our method also requires to know the distribution of the number of days between infection and death. Since this number is unknown, we approach to this number using the sum of the median incubation period as reported in Lauer et al. (31), and the mean number of days between the onset of symptoms and death as reported in Verity et al. (26). For our empirical exercise, we rely on World Development data by the World Bank (GDP per capita and health expenditure as a share of the GDP)[6] and in World Health Organization data for BCG vaccination[7].

In this study, our regression analysis relies on data for 91 countries covering above 86% of the world population. The remaining countries were excluded because they either do not have significant mortality figures (for instance Uruguay, Monaco, Bermuda, etc.), or full data.

Methods

Estimation Strategy

In this study, we rely on a very simple rationale. At a given point in time, the cumulative number of deaths should be a proportion of the cumulative number of infections somewhat in the past. But how many days in the past? The answer lies in the sum of the number of days of incubation and the number of days between the onset of symptoms and death. This rationale follows a report focusing on the 40 most-affected countries by the pandemic in the world (32). However, in this paper, we deviated from the mentioned report by using the key parameters in a different way, which translated into a different estimation of the number of infected individuals.

On average, deaths occur ∼18 days (17.8 days with 95% credible interval [CrI] 16.9–19.2) after the onset of COVID-19 symptoms (26), while the incubation period of COVID-19 has been estimated in about 5 days (5.1 days with 95% CI, 4.5–5.8) as reported in Lauer et al. (31). Thus, by comparing the cumulative number of deaths at time t in country i ($cdeaths_{(i,t)}$) with the country-specific infection fatality ratio (ifr_i), which is assumed constant over time, it is possible to obtain a rough approximation of the cumulative number of SARS-CoV-2 infections 23 days (18 days + 5 days) in the past ($cinfected_{(i,t_{-23})}$)[8].

$$cinfected_{(i,t_{-23})} = \frac{cdeaths_{(i,t)}}{ifr_i} \quad (1)$$

[3]Available online at: https://ourworldindata.org/coronavirus-source-data

[4]United Nations, Department of Economic and Social Affairs, Population Division. *World Population Prospects 2019*, Online Edition. Rev. 1. United Nations (2020). Available online at: https://population.un.org/wpp/Download/Standard/Population/ (accessed April 19, 2020).

[5]Following Verity et al. (26), the estimated IFRs correct for many types of bias. The infection fatality ratios were obtained after combining adjusted case fatality ratios with data on infection prevalence amongst individuals returning home from Wuhan in repatriation flights.

[6]https://data.worldbank.org/indicator/ (accessed April 24, 2020).

[7]https://apps.who.int/gho/data/node.main.A830?lang=en (accessed April 24, 2020).

[8]Differently to Bommer and Vollmer (32), we include the incubation period while avoiding the subtraction of the number of days between the onset of symptoms and detection to the relevant lag period. These differences explain the discrepancies between both set of estimates. Moreover, by combining the cumulative distribution function of the SARS-CoV-2 incubation period as reported in Lauer et al. (31) and an approximation of the Gamma distribution with correction for epidemic growth of the days between the onset of symptoms to death as reported in Verity et al. (26), one can calculate a vector of probabilities to weight the cumulative number of deaths required in equation 1. The weighting vector goes from t_{-2} (representing the proportion of deaths of those who experienced 1 day between infection and the onset of symptoms, plus one day from the onset of symptoms to death) to t_{-72} (representing the proportion of deaths of those who experienced 12 days between infection and the onset of symptoms and 60 days between the onset of symptoms to death). The smoothed approach produces almost an identical estimation of the cumulative number of infected individuals. Given that and for the sake of simplicity, we prefer to use the non-smoothing approach.

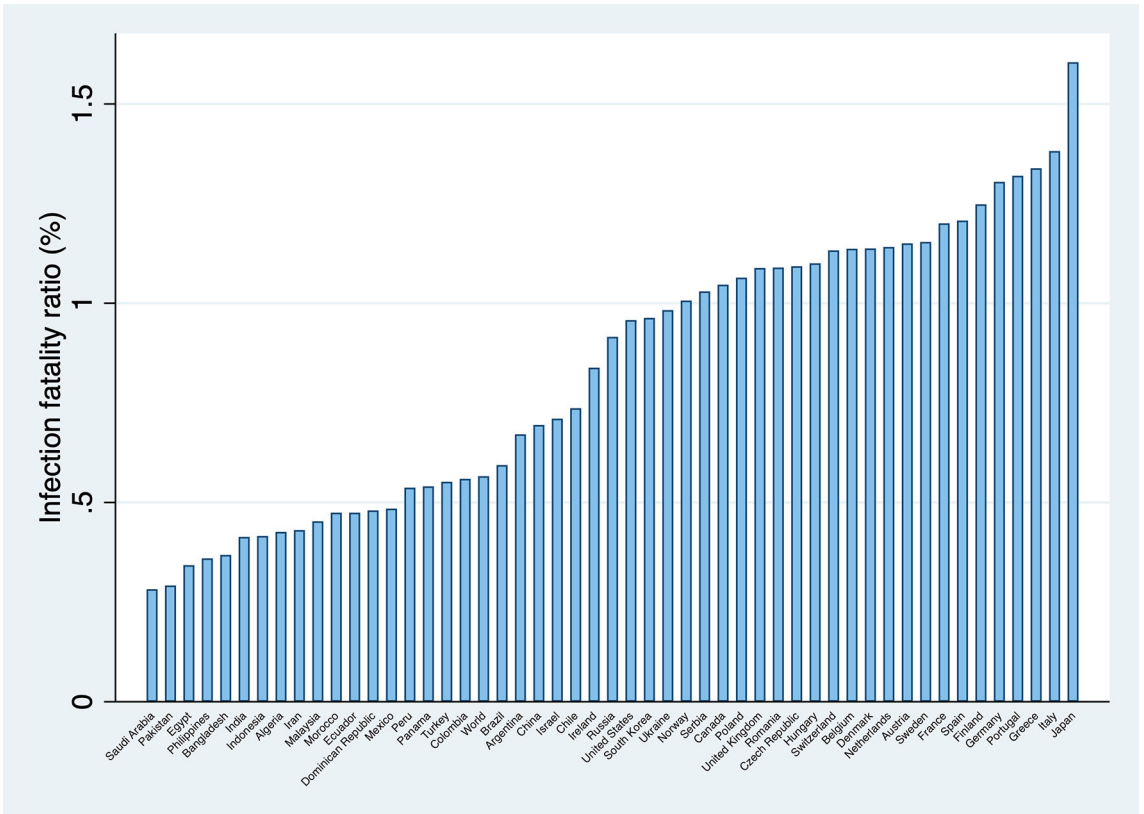

FIGURE 1 | Infection fatality ratios (selected countries, in percentage). Source: Own elaboration.

Additionally, we use the ratio between the cumulative number confirmed (detected) cases at time t_{-23} in country i ($cconfirmed_{(i,t_{-23})}$) and the cumulative number of infected individuals ($cinfected_{(i,t_{-23})}$) at time t_{-23} in country i as a rough measure of the cumulative rate of detection of SARS-CoV-2 infections at time $t_{-\ 23}$.

$$detection\ rate_{(i,t_{-23})} = \frac{cconfirmed_{(i,t_{-23})}}{cinfected_{(i,t_{-23})}} \tag{2}$$

Infection Fatality Ratio

In order to estimate the country-specific infection fatality ratio for country i used in equation 1, we weight the age-stratified infection fatality ratios reported in Verity et al. (26), by the age-group population shares of country i. The calculation of the age-stratified infection fatality ratios relies on two assumptions that can be modified when producing point estimates of the number of individuals affected by a SARS-CoV-2 infection. Firstly, it assumes that there are no cross-country differences in the average overall health status of the population, comorbidity, or in the soundness of the different health systems. In absence of standardized country-specific information of these variables, this assumption is convenient although, at first sight, it can be considered a restrictive one. However, it is quite the opposite since, in richer countries with higher proportions of elderly populations, the estimated infection mortality ratios are likely to be overestimated. If so, our estimates of the infected population represent a lower limit of the true number of infections. The second assumption is that the attack rate of the coronavirus is unrelated to the age and sex of susceptible individuals. This is in concordance with the evidence in respiratory infections in previous pandemic processes (26, 33). Then, the distribution of IFRs across countries reflects the "fixed" lethality of the virus associated to a varying demographic structure of the population across the world.

Figure 1 presents the calculated infection fatality ratios for the world, and for 50 countries in which the lethality of the pandemic has been more significant.

Recently, a cross-sectional epidemiological study with a super-spreading event in the county of Heinsberg in Germany offered the opportunity to estimate the infection fatality ratio in the community (34). The estimated infection fatality ratio was 0.36%. Although this number is surprisingly low when compared with other estimations, for instance, the used in this study for Germany (1.3%), it is not evident that the true infection fatality ratio is closer to 0.36% rather than 1.3%. This is because there can be local factors that explain the discrepancy as pointed out in the Heinsberg study. Amongst these factors, it might be mentioned

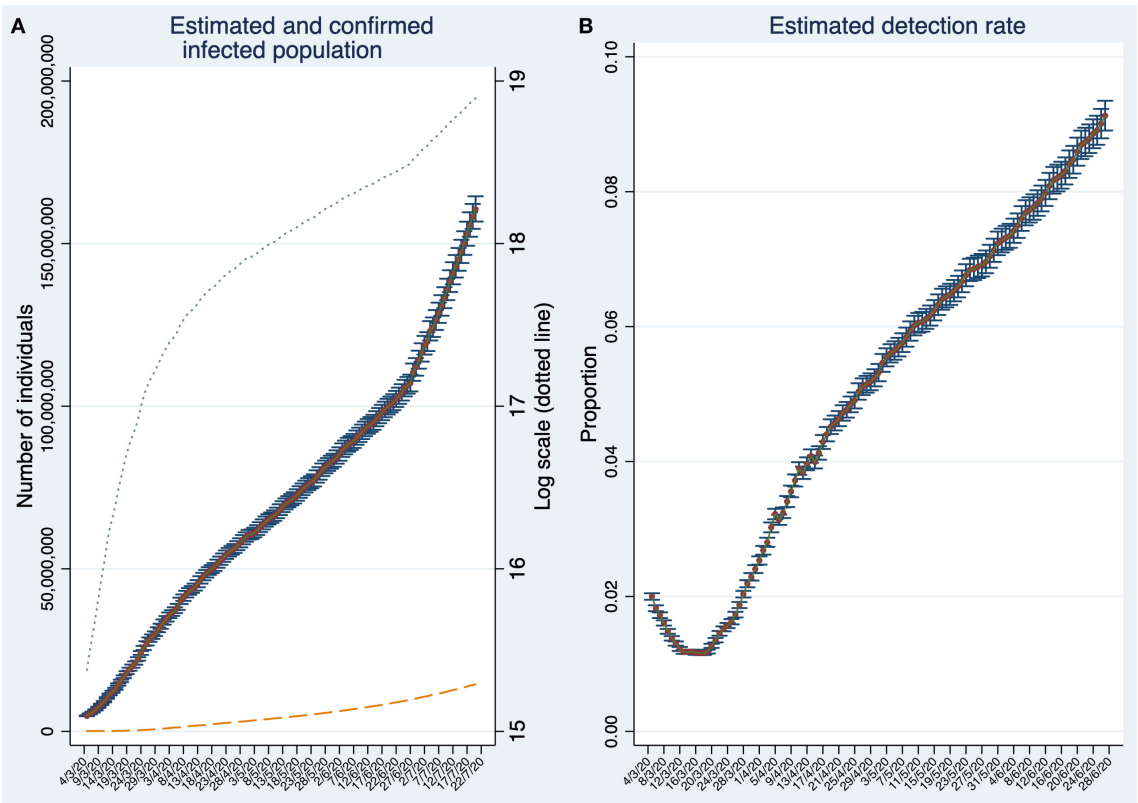

FIGURE 2 | Estimates of the number of SARS-CoV-2 infections and the estimated detection rate in the World. **(A)** Estimated and confirmed infected population. **(B)** Estimated Global detection rate. Note that **(B)** depicts the detection rates until t_{-23}. Both panels display 95% confidence intervals. Source: Own elaboration.

comorbidity gaps, ethnic differences, the quality and coverage of the health systems, climatic differences, immunization levels, etc.[9].

Consequently, it might be necessary to assess the consequences of using an overestimated infection fatality ratio (that is, an IFR closer to the one reported in the Heinsberg study, or others inferred from seroprevalence data (36). The answer is that the number of infections would be underestimated, and that detection rates would be overestimated (since the infection fatality ratio is on the denominator). An overestimation of the detection rates reduces the validity of international rankings based on this figure. However, from the public health point of view, this would be irrelevant since, as discussed later, all countries should increase their detection rates of SARS-CoV-2 infections as much as possible.

Regression Analysis

To investigate whether improving the detection rates of SARS-CoV-2 infections is potentially associated to save lives, we use a parsimonious synchronic cross-country multiple linear regression[10]. That is, we use the information reported 15, 60, and 105 days after the confirmation of the first 100 SARS-CoV-2 infections, which corresponds to the pandemic outbreak (PO). At a given pandemic phase, we regress the natural logarithm of the cumulative number of deaths in country i, $\ln\left(deaths_i\right)$, on their estimated detection rates (DR_i) and its squared to assess whether there is a non-linear relationship of this conditional correlation[11].

The four parsimonious regressions have a demographic control that corresponds to the estimated country-specific infection fatality ratio (ifr_i). This is a non-endogenous control since it only captures the impact of demography (population shares by age-groups) on the number of deaths and not the reverse. The regressions control for the population size of the country i in its natural logarithmic form $\ln(pop_i)$. This control is necessary because the share of the susceptible population remains persistently at relatively higher levels in more populated countries when compared with the less populated ones. We also include the natural logarithm of the number of confirmed SARS-CoV-2 infections in each country $\ln(confirmed_i)$. This is a measure of the persistence of the mortality process while controlling for cross-country differences in their absolute testing

[9]For instance, the reported IFRs for a group of 9,496 Danish blood donors with no comorbidity aged 17–69 reached 0.082% (35).

[10]In the context of the pandemic, a synchronic estimation refers to the use of information of countries in the same pandemic phase, that is, after the same number of days since the pandemic outbreak. On the contrary, a non-synchronic estimation neglects the pandemic phases but considers as reference period the calendar day.

[11]Output tables without the square of the detection rates are available in the **Supplementary Material**.

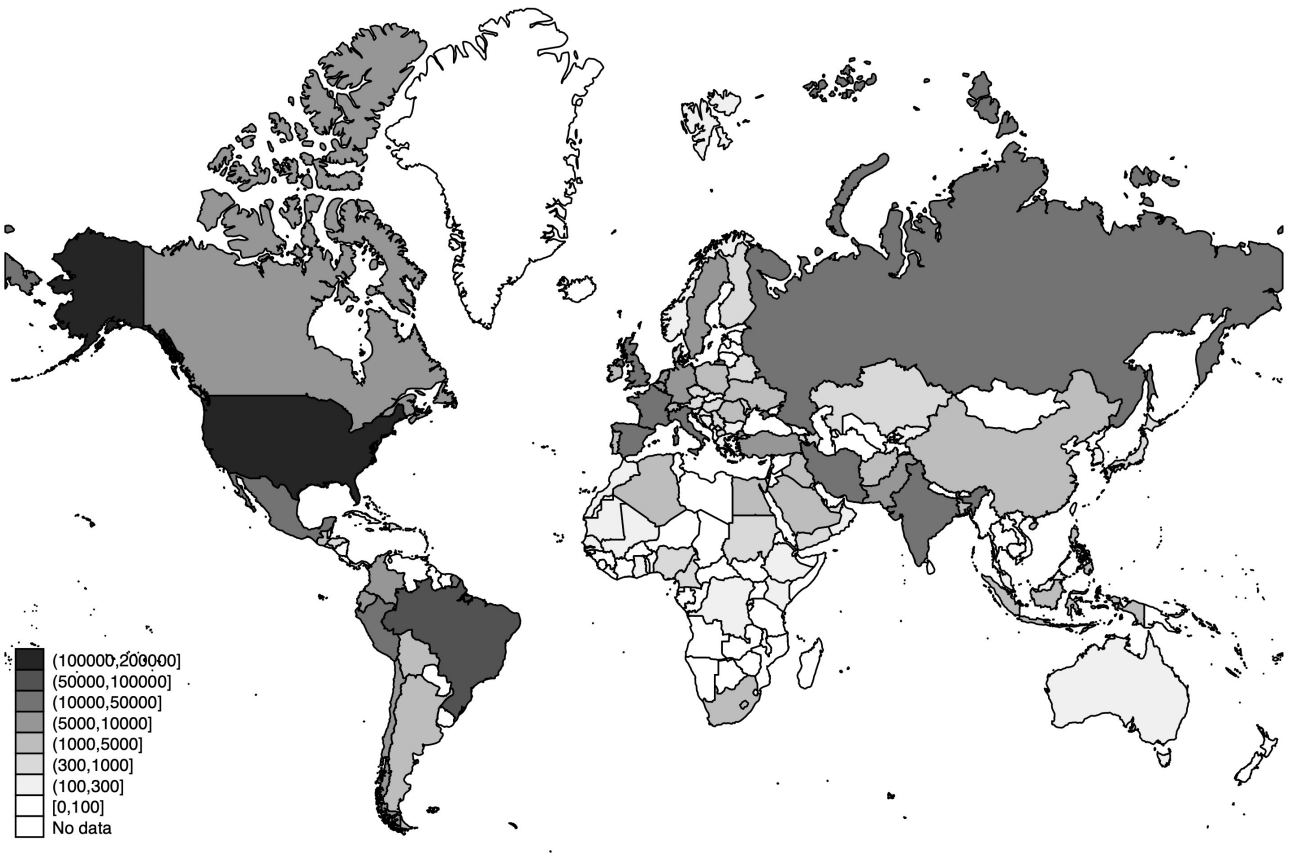

FIGURE 3 | World distribution of deaths as of 20 July 2020. Source: Own Elaboration.

performances. The regressions also control for the economic performance of a country by means of the natural logarithm of the per capita gross domestic product $\ln(gdppc_i)$[12]. We also include the current health expenditure as share of GDP in 2017 ($healthshare_i$). This control is needed to account for relative resource-dependent differences in the coverage/quality of the health systems around the globe. Finally, we use available data to explore a possible association between BCG vaccination and aggravated cases of COVID-19, and deaths [a relationship which is being investigated in some clinical trials (37)][13]. The evidence is still inconclusive because the argued existence of uncontrolled confounders (38–42). However, if these confounders exist, they can bias the relationship between SARS-CoV-2 detections rates and the cumulative number of deaths. Based on this argument, we include a raw of dummies capturing the degree of BCG vaccination coverage as follows: *BGC group 1*: no mandatory vaccination (up to 49.9% coverage), *BGC group 2*: 50 to 79.9% coverage, *BGC group 3*: 80 to 89.9%, *BGC group 4*: 90 to 98.9%, and *BGC group 5*: 99 to 100%. The reference category is *BCG group 1*.

$$\begin{aligned}\ln\left(deaths_i\right) &= \alpha + \beta_1 DR_i + \beta_2 DR_i^2 + \beta_3 ifr_i + \beta_4 \ln(pop_i) \\ &+ \beta_5 \ln(confirmed_i) + \beta_6 \ln\left(gdppc_i\right) \\ &+ \beta_7 healthshare_i + \beta_8 bcg_{2i} + \beta_9 bcg_{3i} + \beta_{10} bcg_{4i} \\ &+ \beta_{11} bcg_{5i} + \mu_i \quad \forall\, i = 1, \ldots, 91 \end{aligned} \tag{3}$$

Robustness

An alternative approach is used to indirectly investigate the conditional association between detection rates and SARS-CoV-2 related deaths. Instead of using the detection rates and its square, we use the natural logarithm of the estimated number of infections $\ln(infections_i)$ while dropping from the equation the natural logarithm of the number of confirmed (detected) SARS-CoV-2 infections as follows:

$$\begin{aligned}\ln\left(deaths_i\right) &= \alpha + \beta_1 \ln(infections_i) + \beta_2 ifr_i + \beta_3 \ln(pop_i) \\ &+ \beta_4 \ln(gdppc_i) + \beta_5 healthshare_i \\ &+ \beta_6 bcg_{2i} + \beta_7 bcg_{3i} + \beta_8 bcg_{4i} + \beta_9 bcg_{5i} + \mu_i \\ &\forall\, i = 1, \ldots, 91 \end{aligned} \tag{4}$$

Regarding the statistical inference, significance tests rely on a heteroscedasticity consistent covariance matrix (HCCM) type

[12] In constant 2017 international dollars with the same purchase power.

[13] https://apps.who.int/gho/data/node.main.A830?lang=en (accessed April 24, 2020).

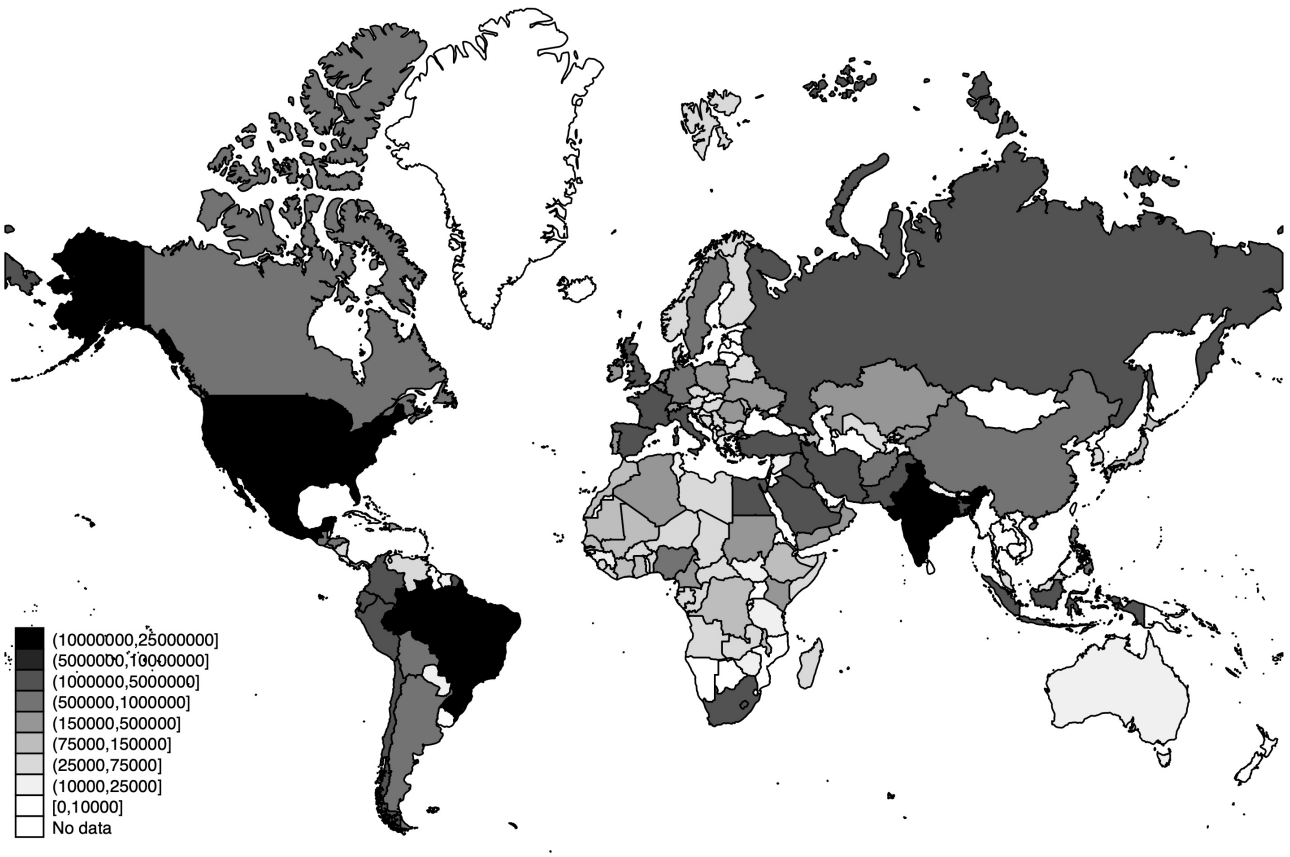

FIGURE 4 | World distribution of the estimated number of SARS-CoV-2 infections as of 20 July 2020. Source: Own Elaboration.

HC3 which is suitable when the number of observations is small (43). Although in the presence of heteroscedasticity of unknown form, Ordinary Least Square estimates are unbiased, the inference can be misleading due to the fact that the usual tests of significance are generally inappropriate (43).

Additionally, we estimate the same set of equations (the main specification and the robustness specification 15, 60, and 105 days after the pandemic outbreak) using robust regressions. We do this because we have the concern that parameter estimates may be biased if, in some countries (outliers), the report of the cumulative number of deaths has been involuntarily altered or even manipulated. Robust regression resists the effect of such outliers, providing better than OLS efficiency when heavy-tailored error distributions exist as it can be likely the case (44).

RESULTS

Descriptive Analysis

On July 20, the estimated infected population reaches about 160 million individuals (**Figure 2A**). This number is about 19 times larger than the reported number of confirmed cases (about 8.6 million represented by the dashed line). Note that the number of infections is estimated based on detection rates calculated 23 days in the past. Thus, for the period t_{-23} to t, the number of SARS-CoV-2 infected individuals are estimated using the estimation rate as in t_{-23}. Therefore, the estimation of SARS-CoV-2 infected individuals can be biased if detection rates deteriorate or improve considerably within this time span.

The accuracy of our estimations can be assessed by contrasting them against to those provided by population-based seroepidemiological studies. There are some studies of this type focusing on restricted geographical areas, for instance, in Germany and Switzerland (34, 45). However, to the best of our knowledge, there is only one country level and large scale population-based seroepidemiological study performed in Spain (46). The ENE-COVID study in Spain finds that, on 11 May, 5% of the population would test IgG positive against SARS-CoV-2. It implies that about 2.35 million individuals were infected by SARS-CoV-2. Similarly, in our study we estimated on 11 May an infected population of about 2.25 million individuals. This evidence suggests that our method can be a suitable alternative when population-based seroepidemiological studies are not available, which is frequently the case. Here, it is important to recognize that, from the public health point of view, it is preferable to be vaguely right than precisely wrong. On 11 May, Spain confirmed only 246,504 cases (about 10% of all estimated infections). At that time, it would have been

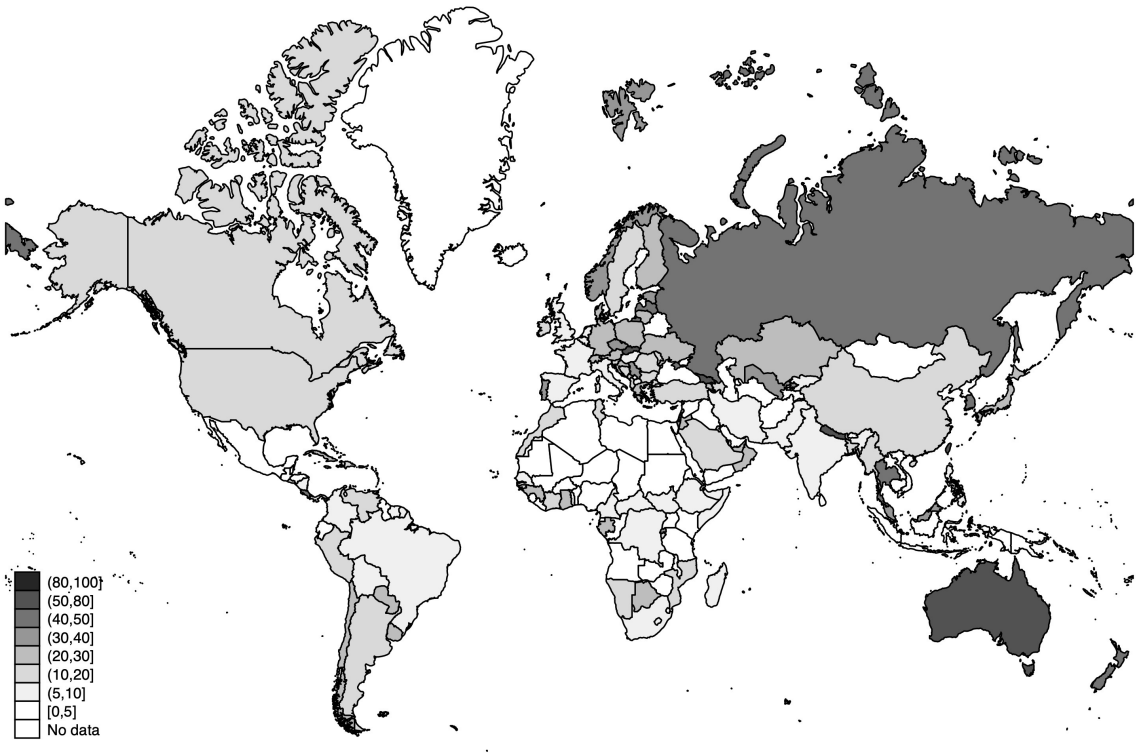

FIGURE 5 | World distribution of the estimated detection rates of SARS-CoV-2 infections as of 20 July 2020 (in percentage). Source: Own Elaboration.

convenient that public health authorities and the public opinion would have the information that, for each confirmed case, there were significantly much more individuals spreading the infection in unpredictable ways.

Back to the global estimates, by comparing the cumulative number of estimated infections with the cumulative number of confirmed (detected) cases, we obtain, at the end of June 2020, a global detection rate of about 9% (**Figure 2B**). The global detection rate curve shows an U-shape with a minimum at the beginning of the third week of March reaching only 1.1%. The last data suggest that detection rates are steadily increasing. Moreover, the semi-logarithmic plot in **Figure 2A** suggests that the infection stopped spreading at its maximum pace approximately during the third week of March, but unfortunately, it increased its speed again around the last week of June.

The world distribution of the number of deaths, the estimated number of SARS-CoV-2 infections, and the detection rates of SARS-CoV-2 infections across the world are displayed in **Figures 3**–**5**, respectively.

Since the global estimates are no more than an aggregation of the trajectories made by the different countries in the world, we investigate how heterogeneous the detection rates across countries are. **Table 1** presents this information in a synchronic way. The rankings compare countries in the same phase of their respective pandemic processes, that is after 15, 30, 45, 60, 75, and 90 days after the confirmation of the first 100 SARS-CoV-2 infections (pandemic outbreak). This approach allows us to perform such an international comparison.

At a first sight, it is noteworthy the fact that each of the first 24 countries ranked on the top by the initial detection rate (15 days after the beginning of the pandemic outbreak) does not accumulate more than 500 deaths 45 days after initiating their pandemic processes. Thus, it seems to exist a strong correlation between detection rates and the cumulative number of deaths for a given stage of the pandemic process. Countries with high counts of deaths ranked very badly in their initial detection rates. For example, the US, Spain, Italy, UK, France, and Belgium ranked in place 90, 82, 81, 89, 87, and 85, out of 91 countries listed in the ranking.

A second conclusion is that the relative improvement of detection rates over time, that is, 30, 45, 60, 75, and 90 days after the beginning of the pandemic processes, does not alter the fact that those countries are still ranked the worst in terms of deaths. That is, improving detection over time has declining returns to scale when comes to save lives.

The depicted relationship between detection rates and the cumulative number of deaths remains almost unchanged when using non-synchronic data as of 20 May in **Table 2**. This table mixes information of countries at different stages from their pandemic processes. So, it must be interpreted with caution. Although efforts to increase detection have been significative in

TABLE 1A | Synchronic descriptive statistics (15, 30, and 45 days after the pandemic outbreak).

Country/Days since the first 100 cases were confirmed	Detection rankings			Confirmed Cases (in thousands)			Estimated Cases (in thousands)			Estimated detection rate (Percentage)			Number of deaths (Count)		
	15	30	45	15	30	45	15	30	45	15	30	45	15	30	45
South Korea	1	2	3	6.3	8.8	10.2	15.8	22.5	25.3	39.8	39.1	40.4	42	103	183
Australia	2	1	1	1.8	6.0	6.7	6.7	9.7	10.4	27.3	61.1	64.1	7	45	74
Luxembourg	3	6	7	2.2	3.4	3.8	9.3	11.2	12.3	23.3	30.0	30.9	23	69	90
Thailand	4	3	2	1.4	2.6	2.9	6.0	6.9	7.0	23.0	37.8	41.7	7	41	54
Lithuania	5	5	12	0.8	1.3	1.4	3.4	4.1	5.4	23.0	32.4	26.3	9	36	46
Croatia	6	12	13	1.0	1.8	2.1	4.5	7.4	8.3	22.6	24.4	25.3	7	36	77
Estonia	7	8	8	0.6	1.3	1.6	3.4	4.7	5.5	18.7	27.9	30.1	1	25	50
Norway	8	7	6	1.7	5.5	7.1	10.2	19.2	22.7	17.0	28.7	31.2	7	50	154
Finland	9	24	23	1.0	2.8	4.5	7.2	18.4	24.0	13.3	15.0	18.6	4	48	186
Israel	10	4	5	3.0	10.7	15.4	24.2	33.1	39.0	12.5	32.5	39.5	10	101	199
Czech R.	11	10	11	2.1	5.7	7.4	16.6	22.7	27.2	12.4	25.3	27.1	9	119	218
Japan	12	21	30	0.4	1.0	3.7	3.4	6.8	24.2	12.1	15.4	15.1	6	36	73
Greece	13	15	18	0.9	2.0	2.5	7.8	10.8	12.3	11.4	18.7	20.3	26	90	130
Chile	14	13	24	2.4	7.9	14.9	21.7	38.7	85.5	11.3	20.5	17.4	8	92	216
Austria	15	11	10	3.6	12.3	14.8	33.4	50.4	54.4	10.9	24.4	27.3	16	220	463
Bosnia & H.	16	31	33	0.7	1.3	1.9	6.1	11.9	15.1	10.8	11.0	12.9	24	48	79
Albania	17	18	17	0.4	0.6	0.8	3.6	3.6	3.9	10.4	16.8	21.6	22	26	31
Slovenia	18	25	28	0.6	1.2	1.4	6.3	8.2	8.8	10.1	14.5	16.0	9	50	82
Bulgaria	19	30	31	0.5	0.8	1.6	4.7	7.7	11.0	9.8	11.0	14.5	10	41	72
Puerto Rico	20	26	22	0.8	1.4	2.3	8.1	10.3	11.6	9.8	13.3	19.4	42	84	113
Cuba	21	19	19	0.6	1.4	1.8	7.3	8.4	8.8	8.5	16.3	20.2	16	54	77
Malaysia	22	16	16	1.5	4.0	5.5	18.3	22.5	24.7	8.3	17.6	22.4	14	63	93
Tunisia	23	29	37	0.6	0.9	1.0	7.2	8.1	8.7	8.2	11.1	11.8	22	38	44
Serbia	24	9	4	1.2	5.7	9.4	14.7	20.9	23.2	8.0	27.3	40.3	31	110	189
Portugal	25	14	14	4.3	16.0	23.9	54.1	80.5	94.5	7.9	19.8	25.3	76	470	903
Suriname	26	–	–	0.3	–	–	3.9	–	–	7.8	–	–	8	–	–
Switzerland	27	17	20	4.8	20.2	27.7	75.8	119.4	137.7	6.4	16.9	20.1	43	540	1,134
Moldova	28	35	39	1.0	2.6	4.5	15.4	26.9	39.9	6.3	9.7	11.2	19	73	143
South Africa	29	51	58	1.4	2.6	6.0	22.3	52.4	121.0	6.2	5.0	4.9	5	48	116
Ukraine	30	23	21	1.7	7.6	14.2	28.4	50.6	72.0	5.9	15.1	19.7	52	193	361
Nicaragua	31	39	–	1.1	2.0	–	19.8	24.4	–	5.6	8.3	–	46	64	–
Macedonia	32	33	42	0.5	1.2	1.5	8.7	11.6	14.9	5.6	10.6	10.2	17	54	86
Denmark	33	28	26	1.5	5.1	7.9	27.2	39.7	47.2	5.4	12.8	16.8	24	203	384
El Salvador	34	41	34	0.2	0.7	1.7	4.7	8.7	14.0	5.0	8.0	12.3	8	15	33
Libya	35	53	–	0.4	0.7	–	8.3	16.5	–	4.7	4.2	–	5	18	–
Panama	36	36	36	1.3	4.0	6.7	28.5	43.9	56.6	4.6	9.2	11.9	32	109	192
Poland	37	34	32	1.6	6.7	11.9	35.7	67.3	90.4	4.6	9.9	13.2	18	232	562
Argentina	38	47	51	1.1	2.7	4.7	27.6	44.7	69.6	4.1	5.9	6.7	34	122	237
Bangladesh	39	43	45	2.9	10.9	26.7	73.0	151.7	297.2	4.0	7.2	9.0	101	183	386
Russia	40	27	9	2.3	24.5	106.5	60.6	188.2	370.0	3.9	13.0	28.8	17	198	1,073
Guatemala	41	66	79	0.4	0.9	3.1	10.2	33.4	123.4	3.8	2.7	2.5	11	24	55
China	42	20	25	14.4	70.6	80.3	383.7	455.1	473.8	3.8	15.5	16.9	304	1,771	2,946
Romania	43	38	40	1.5	6.3	11.3	41.4	75.9	104.7	3.5	8.3	10.8	29	306	631
Turkey	44	32	29	15.7	74.2	122.4	471.0	677.4	786.3	3.3	11.0	15.6	277	1,643	3,258
Saudi Arabia	45	44	27	1.2	4.9	20.1	38.6	74.0	124.3	3.2	6.7	16.2	8	65	152
Germany	46	22	15	3.8	57.3	125.1	123.2	374.0	545.7	3.1	15.3	22.9	8	455	2,969
Haiti	47	37	38	0.5	2.5	4.7	17.5	29.0	40.8	3.0	8.6	11.5	21	48	82
Ireland	48	46	41	2.4	9.7	19.6	81.9	159.7	187.4	2.9	6.0	10.5	36	334	1,102

(Continued)

TABLE 1A | Continued

Country/Days since the first 100 cases were confirmed	Detection rankings			Confirmed Cases (in thousands)			Estimated Cases (in thousands)			Estimated detection rate (Percentage)			Number of deaths (Count)		
	15	30	45	15	30	45	15	30	45	15	30	45	15	30	45
Morocco	49	42	35	1.0	3.0	5.2	34.8	39.6	42.6	2.9	7.7	12.3	70	143	191
South Sudan	50	40	43	0.3	1.3	1.9	12.0	16.4	18.7	2.8	8.0	10.1	6	14	34
Dominican R.	51	49	46	1.6	4.7	8.2	57.9	83.7	98.7	2.7	5.6	8.3	77	226	346
Canada	52	45	48	3.4	20.7	43.9	125.1	340.8	552.5	2.7	6.1	7.9	35	509	2,302
Hungary	53	52	53	0.7	1.9	3.0	25.5	38.6	45.9	2.7	5.0	6.6	32	189	351
Colombia	54	56	55	1.1	3.2	7.0	40.2	79.5	130.0	2.6	4.1	5.4	17	144	314
Niger	55	77	81	0.6	0.8	0.9	26.7	36.6	37.7	2.4	2.1	2.4	19	36	55
Pakistan	56	64	62	1.6	6.0	15.8	71.6	203.6	377.3	2.3	2.9	4.2	18	107	346
U. Arab E.	57	48	44	0.7	5.4	12.5	29.5	91.6	128.4	2.3	5.9	9.7	6	33	105
Sweden	58	60	56	1.6	6.4	14.4	77.9	197.1	287.1	2.1	3.3	5.0	16	373	1,540
Ecuador	59	74	64	2.3	7.9	24.9	118.0	359.2	652.6	2.0	2.2	3.8	79	388	900
Somalia	60	61	61	0.6	1.4	2.0	31.8	43.8	46.9	1.9	3.1	4.2	28	55	78
Bolivia	61	71	68	0.4	1.1	3.1	19.7	46.5	92.4	1.8	2.3	3.4	28	55	142
Burkina Faso	62	76	80	0.4	0.6	0.7	25.5	29.5	30.7	1.6	2.1	2.4	23	41	48
Honduras	63	87	76	0.4	0.7	2.0	24.8	44.4	70.7	1.6	1.5	2.8	25	61	116
Iraq	64	62	73	0.5	1.4	1.8	36.3	44.6	61.2	1.5	3.1	3.0	42	78	88
Sierra Leone	65	63	67	0.3	0.8	1.1	23.0	26.0	30.4	1.5	3.0	3.6	20	45	50
Kenya	66	86	85	0.2	0.4	0.8	16.1	27.4	45.7	1.5	1.5	1.8	11	21	50
Cameroon	67	73	77	0.8	1.8	2.8	57.1	82.5	107.3	1.4	2.2	2.6	12	59	136
D. R. Congo	68	79	71	0.3	0.6	1.4	19.3	31.1	42.0	1.4	1.8	3.3	22	31	61
Algeria	69	72	70	1.3	2.7	4.8	102.5	122.5	147.8	1.3	2.2	3.3	152	384	470
Mauritania	70	59	65	0.7	2.2	4.5	53.4	64.6	120.3	1.3	3.4	3.7	31	95	129
Netherlands	71	54	50	3.0	16.6	32.7	239.8	395.9	482.8	1.2	4.2	6.8	106	1,651	3,684
Iran	72	68	59	9.0	29.4	68.2	733.6	1,167.9	1,440.0	1.2	2.5	4.7	354	2,234	4,232
Mali	73	85	86	0.4	0.7	1.1	30.9	46.3	63.5	1.2	1.5	1.7	21	38	67
Chad	74	80	84	0.5	0.8	0.9	41.2	43.5	43.5	1.2	1.8	2.0	50	65	73
Afghanistan	75	83	74	0.6	1.5	4.7	48.8	96.5	159.4	1.1	1.6	2.9	18	57	122
Peru	76	58	54	1.1	11.5	37.0	106.5	319.1	627.9	1.0	3.6	5.9	30	254	1,051
Sudan	77	84	82	0.8	2.7	5.5	80.1	177.8	234.9	1.0	1.5	2.3	45	111	314
Philippines	78	67	69	1.1	4.6	7.8	118.9	177.0	234.0	0.9	2.6	3.3	68	297	511
Brazil	79	81	83	3.9	22.2	66.5	433.6	1,333.9	3,175.9	0.9	1.7	2.1	114	1,223	4,543
Indonesia	80	75	72	1.3	4.6	9.5	148.1	215.2	307.2	0.9	2.1	3.1	114	399	773
Italy	81	55	52	7.4	63.9	135.6	899.6	1,566.6	2,024.0	0.8	4.1	6.7	366	6,077	17,129
Spain	82	50	47	9.2	94.4	181.5	1,205.5	1,865.5	2,182.4	0.8	5.1	8.3	309	8,189	18,893
India	83	69	66	1.3	11.4	33.1	164.6	456.0	899.4	0.8	2.5	3.7	32	377	1,074
Egypt	84	82	78	0.6	2.2	5.0	77.0	136.9	203.1	0.7	1.6	2.5	36	164	359
Belgium	85	65	57	2.3	18.4	38.5	316.7	634.0	770.7	0.7	2.9	5.0	37	1,283	5,683
Nigeria	86	88	75	0.3	1.5	5.0	54.2	111.0	175.1	0.6	1.4	2.8	10	44	164
France	87	70	60	4.5	40.2	98.1	742.3	1,732.2	2,149.8	0.6	2.3	4.6	91	2,606	14,967
Mexico	88	89	87	1.4	6.3	20.7	251.9	691.8	1,525.6	0.5	0.9	1.4	37	486	1,972
U.K.	89	78	63	3.3	38.2	114.2	907.2	1,904.7	2,945.9	0.4	2.0	3.9	144	3,605	15,464
U.S.	90	57	49	4.7	189.6	639.7	1,547.1	5,216.8	8,058.6	0.3	3.6	7.9	85	4,079	30,985
Yemen	91	90	88	0.3	0.7	1.1	126.8	170.9	242.1	0.2	0.4	0.5	66	160	302

This ranking is made up of all countries with more than 30 deaths due to COVID-19 40 days after the pandemic outbreak. Countries are ranked by their detection rates 15 days after the pandemic outbreak. Missing values in this table indicate that the country has not reached the requested number of days after its pandemic outbreak. Source: Own elaboration.

TABLE 1B | Synchronic descriptive statistics (60, 75, and 90 days after the pandemic outbreak).

Country/Days since the first 100 cases were confirmed	Detection rankings			Confirmed Cases (in thousands)			Estimated Cases (in thousands)			Estimated detection rate (Percentage)			Number of deaths (Count)		
	60	75	90	60	75	90	60	75	90	60	75	90	60	75	90
South Korea	6	7	6	10.7	10.8	11.1	26.9	27.9	28.8	39.7	38.7	38.6	236	254	263
Australia	1	1	1	6.9	7.1	7.3	10.8	10.8	11.0	63.8	65.7	65.9	97	101	102
Luxembourg	8	9	10	3.9	4.0	4.1	12.4	12.4	12.4	31.7	32.5	32.9	104	110	110
Thailand	3	4	4	3.0	3.1	3.1	7.3	7.3	7.3	41.4	42.0	42.9	56	57	58
Lithuania	14	16	15	1.6	1.7	1.8	6.1	6.4	6.5	25.8	26.4	27.6	60	71	76
Croatia	16	18	22	2.2	2.2	2.3	8.8	8.8	9.5	25.4	25.4	23.7	95	103	107
Estonia	9	10	9	1.7	1.8	2.0	5.9	5.9	5.9	29.6	31.2	33.4	61	66	69
Norway	7	8	8	7.8	8.3	8.4	23.3	24.0	24.7	33.6	34.3	34.1	208	233	237
Finland	19	19	17	6.0	6.6	7.0	25.6	26.1	26.3	23.3	25.3	26.7	267	308	324
Israel	5	6	5	16.5	16.8	18.4	41.0	42.7	45.9	40.3	39.3	40.0	258	281	299
Czech R.	10	11	12	8.1	9.0	9.8	29.6	30.5	32.1	27.5	29.5	30.4	280	317	328
Japan	13	12	14	11.1	15.4	16.4	42.8	54.5	57.6	26.0	28.2	28.5	186	543	777
Greece	24	25	24	2.7	2.9	3.1	13.4	14.0	14.3	20.3	20.6	21.3	151	172	183
Chile	27	33	27	37.0	90.6	167.4	209.2	608.1	866.7	17.7	14.9	19.3	358	944	3,101
Austria	12	13	16	15.7	16.3	16.8	58.1	58.9	61.0	26.9	27.7	27.5	608	633	672
Bosnia & H.	35	34	36	2.3	2.6	3.3	16.0	17.6	21.7	14.6	14.7	15.1	135	158	168
Albania	20	26	31	1.0	1.2	1.9	4.2	6.4	11.1	23.0	18.9	17.0	31	33	43
Slovenia	31	32	33	1.5	1.5	1.5	9.1	9.2	9.4	16.0	16.0	15.9	102	106	109
Bulgaria	33	35	34	2.2	2.5	3.5	14.1	17.7	22.2	15.8	14.2	15.6	110	144	181
Puerto Rico	11	5	3	3.3	5.3	6.9	12.2	12.8	15.3	27.2	41.7	44.9	129	143	151
Cuba	22	20	19	2.0	2.2	2.3	9.0	9.1	9.3	21.7	24.2	24.9	82	83	85
Malaysia	15	15	11	6.5	7.1	8.3	25.4	26.7	26.7	25.5	26.7	31.1	107	115	117
Tunisia	41	43	42	1.0	1.1	1.2	8.9	9.0	9.1	11.8	12.0	12.7	47	49	50
Serbia	2	2	7	10.6	11.4	12.4	24.3	25.5	34.2	43.7	44.8	36.4	230	244	256
Portugal	17	14	13	27.7	31.0	35.6	109.6	115.5	121.1	25.2	26.9	29.4	1,144	1,342	1,495
Switzerland	23	24	25	29.9	30.5	30.8	145.4	147.8	148.4	20.6	20.7	20.8	1,476	1,613	1,659
Moldova	39	38	35	6.7	9.2	14.0	55.1	73.4	89.8	12.2	12.6	15.5	233	323	464
South Africa	64	60	55	14.4	32.7	73.5	304.5	592.7	1,015.7	4.7	5.5	7.2	261	683	1,568
Ukraine	21	21	21	20.6	27.0	37.2	91.7	119.4	151.1	22.4	22.6	24.6	605	788	1,012
Macedonia	48	51	47	1.9	2.6	4.8	20.9	33.8	46.6	8.9	7.7	10.3	110	147	222
Denmark	25	22	23	10.1	11.2	11.9	50.2	52.5	53.2	20.1	21.4	22.4	514	561	587
El Salvador	46	49	–	2.9	4.6	–	31.1	53.3	–	9.4	8.7	–	53	107	–
Panama	37	37	37	9.4	13.5	21.4	73.6	99.2	151.6	12.8	13.6	14.1	269	336	448
Poland	30	29	26	16.9	22.5	28.2	105.0	125.4	142.1	16.1	17.9	19.8	839	1,028	1,215
Argentina	52	45	40	7.8	17.4	34.1	106.9	161.7	254.4	7.3	10.8	13.4	366	556	878
Bangladesh	38	31	32	57.6	105.5	159.7	460.1	638.5	965.1	12.5	16.5	16.5	781	1,388	1,997
Russia	4	3	2	262.8	396.6	529.0	639.9	896.2	1,146.1	41.1	44.2	46.2	2,418	4,555	6,948
Guatemala	78	76	–	7.1	13.8	–	239.1	417.0	–	3.0	3.3	–	252	547	–
China	29	40	43	81.1	82.4	83.8	480.8	667.4	667.6	16.9	12.3	12.5	3,241	3,316	4,636
Romania	36	36	41	15.8	18.6	21.2	119.2	136.2	158.9	13.2	13.7	13.3	1,002	1,219	1,369
Turkey	28	28	28	148.1	163.9	179.8	853.5	906.0	956.9	17.3	18.1	18.8	4,096	4,540	4,825
Saudi Arabia	26	27	30	44.8	80.2	119.9	227.4	435.6	678.5	19.7	18.4	17.7	273	441	893
Germany	18	17	18	157.6	172.2	180.5	626.6	662.7	680.8	25.2	26.0	26.5	6,115	7,723	8,450
Haiti	40	–	–	6.1	–	–	51.6	–	–	11.8	–	–	110	–	–
Ireland	42	42	44	23.2	24.8	25.2	198.5	204.4	207.7	11.7	12.1	12.2	1,488	1,631	1,703
Morocco	32	30	29	7.1	8.0	9.6	44.7	46.4	52.7	16.0	17.3	18.2	194	208	213
Dominican R.	43	41	39	13.2	18.0	24.6	116.8	148.3	183.3	11.3	12.2	13.4	441	516	635

(Continued)

TABLE 1B | Continued

Country/Days since the first 100 cases were confirmed	Detection rankings			Confirmed Cases (in thousands)			Estimated Cases (in thousands)			Estimated detection rate (Percentage)			Number of deaths (Count)		
	60	75	90	60	75	90	60	75	90	60	75	90	60	75	90
Canada	45	44	45	67.7	84.7	96.2	700.1	784.8	820.9	9.7	10.8	11.7	4,693	6,424	7,835
Hungary	53	52	53	3.6	3.9	4.1	50.1	52.4	53.9	7.1	7.5	7.6	467	534	568
Colombia	54	54	60	14.9	29.4	53.1	233.8	429.7	809.2	6.4	6.8	6.6	562	939	1,726
Niger	81	80	–	1.0	1.0	–	38.9	39.5	–	2.5	2.6	–	65	67	–
Pakistan	60	62	52	37.2	66.5	139.2	686.1	1,230.3	1,658.4	5.4	5.4	8.4	803	1,395	2,632
U. Arab E.	34	23	20	21.8	33.9	42.3	145.2	159.4	171.5	15.0	21.3	24.7	210	262	289
Sweden	55	53	50	22.7	30.8	40.8	365.8	417.2	457.6	6.2	7.4	8.9	2,769	3,743	4,542
Ecuador	70	71	71	31.5	38.6	46.8	763.3	890.2	1,027.2	4.1	4.3	4.6	2,594	3,334	3,896
Somalia	57	59	–	2.6	2.9	–	48.0	51.8	–	5.5	5.7	–	88	90	–
Bolivia	62	61	65	8.4	16.9	30.7	160.1	310.3	534.0	5.2	5.5	5.7	293	559	970
Burkina Faso	80	78	74	0.8	0.9	0.9	30.7	30.7	30.9	2.7	2.9	2.9	52	53	53
Honduras	67	74	68	4.4	7.4	15.4	103.6	182.8	316.5	4.2	4.0	4.9	188	290	426
Iraq	82	82	76	3.0	5.5	17.8	124.6	442.8	1,081.1	2.4	1.2	1.6	115	179	496
Sierra Leone	68	–	–	1.4	–	–	33.3	–	–	4.2	–	–	59	–	–
Kenya	79	73	72	2.0	3.7	6.4	68.7	90.8	147.6	2.9	4.1	4.3	64	104	148
Cameroon	73	64	64	5.4	9.2	12.6	158.6	173.4	215.9	3.4	5.3	5.8	177	273	313
D. R. Congo	65	63	63	3.0	4.8	6.9	66.6	89.8	117.8	4.5	5.3	5.9	69	106	167
Algeria	66	69	69	7.5	9.8	11.5	176.2	209.3	237.2	4.3	4.7	4.9	568	681	825
Netherlands	51	50	51	40.8	44.2	46.7	514.4	529.6	534.8	7.9	8.4	8.7	5,082	5,715	5,977
Iran	59	57	61	89.3	107.6	137.7	1,638.3	1,843.7	2,132.3	5.5	5.8	6.5	5,650	6,640	7,451
Mali	83	79	–	1.6	2.0	–	69.4	74.8	–	2.3	2.7	–	94	112	–
Chad	84	–	–	0.9	–	–	44.4	–	–	2.0	–	–	74	–	–
Afghanistan	71	67	66	11.8	22.1	30.2	296.0	443.9	591.6	4.0	5.0	5.1	220	405	675
Peru	49	46	46	84.5	155.7	229.7	1,017.4	1,497.7	2,038.9	8.3	10.4	11.3	2,393	4,371	6,688
Sudan	75	77	–	8.3	9.7	–	266.9	311.7	–	3.1	3.1	–	506	604	–
Philippines	69	68	58	11.4	15.0	24.2	273.5	314.0	358.5	4.2	4.8	6.7	751	904	1,036
Brazil	76	66	54	177.6	411.8	802.8	5,729.2	8,243.9	10,800.0	3.1	5.0	7.4	12,400	25,598	40,919
Indonesia	72	72	70	15.4	24.5	36.4	425.5	583.9	762.3	3.6	4.2	4.8	1,028	1,496	2,048
Italy	50	48	49	187.3	215.9	228.7	2,287.7	2,412.9	2,485.6	8.2	8.9	9.2	25,085	29,958	32,616
Spain	47	47	48	215.2	230.7	239.4	2,381.4	2,247.5	2,345.9	9.0	10.3	10.2	24,824	27,563	27,127
India	63	65	59	82.0	173.8	320.9	1,675.2	3,312.0	4,874.1	4.9	5.2	6.6	2,649	4,971	9,195
Egypt	77	75	73	10.4	20.8	41.3	340.3	614.6	975.5	3.1	3.4	4.2	556	845	1,422
Belgium	56	55	57	50.3	55.8	58.7	823.7	847.7	857.3	6.1	6.6	6.8	7,924	9,108	9,522
Nigeria	74	70	67	8.9	15.2	24.1	266.1	339.2	486.8	3.4	4.5	4.9	259	399	558
France	61	58	62	126.8	140.7	149.1	2,350.2	2,424.9	2,468.3	5.4	5.8	6.0	23,660	27,074	28,662
Mexico	85	81	75	47.1	90.7	150.3	2,899.6	4,823.5	6,766.9	1.6	1.9	2.2	5,045	9,930	17,580
U.K.	58	56	56	186.6	246.4	278.0	3,403.7	3,778.5	3,971.6	5.5	6.5	7.0	28,446	34,796	39,369
U.S.	44	39	38	1,069.8	1,443.4	1,770.4	10,137.1	11,539.1	12,571.5	10.6	12.5	14.1	63,006	87,568	103,781

This ranking is made up of all countries with more than 30 deaths due to COVID-19 40 days after the pandemic outbreak. Countries are ranked by their detection rates 15 days after the pandemic outbreak. Missing values in this table indicate that the country has not reached the requested number of days after its pandemic outbreak. Source: Own elaboration.

the above-mentioned countries, none of them is still ranked on the top part of the ranking with 91 countries for which we have full data (US in ranking 36, Spain 45, Italy 46, Belgium 55, UK 56, and France in place 58). Similarly, in this non-synchronic ranking, with the exception of Russia, none of the first 10 countries accumulated more than 500 deaths on May 20.

In **Table 3**, we present the non-synchronic ranking as of 22 June. The US is in place 35, Spain 49, Italy 53, Belgium 63, UK 61, and France 67. It is noteworthy that, except for Russia, none of the first 16 countries in this ranking have accumulated more than 2,000 fatalities on 22 June. More importantly and despite the incredible efforts to increase the tests amongst the more developed countries, none of them were able to detect more than 16% of the estimated infections (the US detected 15.7% on 22 June). It implies that testing efforts need to be deployed at the first stages of the pandemic process due to its cumulative nature.

TABLE 2 | Non-synchronic descriptive statistics as of 20 May.

Country	Detection rate ranking	Confirmed Cases	Estimated Cases	Number of Deaths	Detection rate (Percentage)	Country	Detection rate ranking	Confirmed Cases	Estimated Cases	Number of Deaths	Detection rate (Percentage)
Australia	1	7,068	10,803	99	65.4	Macedonia	47	1,839	20,629	106	8.9
Serbia	2	10,733	24,472	234	43.9	Bangladesh	48	25,121	284,758	370	8.8
Russia	3	299,941	713,396	2,837	42.0	Peru	49	99,483	1,137,309	2,914	8.7
Thailand	4	3,034	7,293	56	41.6	Netherlands	50	44,249	529,581	5,715	8.4
Israel	5	16,650	42,239	277	39.4	Argentina	51	8,796	114,004	393	7.7
South Korea	6	11,110	28,757	263	38.6	Sweden	52	30,799	417,234	3,743	7.4
Norway	7	8,257	24,041	233	34.3	Hungary	53	3,598	50,265	470	7.2
Luxembourg	8	3,958	12,368	109	32.0	Colombia	54	16,935	256,149	613	6.6
Estonia	9	1,791	5,891	64	30.4	Belgium	55	55,791	847,676	9,108	6.6
Czech R.	10	8,647	30,105	302	28.7	U.K.	56	248,818	3,792,371	35,341	6.6
Japan	11	16,385	57,455	771	28.5	Iran	57	124,603	1,992,740	7,119	6.3
Austria	12	16,257	58,599	632	27.7	France	58	143,427	2,444,441	28,022	5.9
Malaysia	13	6,978	26,056	114	26.8	Pakistan	59	45,898	844,102	985	5.4
Germany	14	176,007	671,716	8,090	26.2	India	60	106,750	2,054,585	3,303	5.2
Portugal	15	29,432	113,959	1,247	25.8	South Africa	61	17,200	363,475	312	4.7
Lithuania	16	1,562	6,060	60	25.8	Philippines	62	12,942	287,923	837	4.5
Croatia	17	2,232	8,763	96	25.5	Libya	63	68	1,544	3	4.4
Finland	18	6,399	26,036	301	24.6	Ecuador	64	34,151	784,137	2,839	4.4
Puerto Rico	19	2,805	11,951	124	23.5	Algeria	65	7,377	173,847	561	4.2
Albania	20	949	4,088	31	23.2	Brazil	66	271,628	6,890,826	17,971	3.9
Ukraine	21	18,876	86,905	548	21.7	Bolivia	67	4,481	115,342	189	3.9
Denmark	22	11,044	52,134	551	21.2	Indonesia	68	18,496	480,800	1,221	3.8
Cuba	23	1,887	8,926	79	21.1	D. R. Congo	69	1,731	47,886	61	3.6
Greece	24	2,840	13,671	165	20.8	Somalia	70	1,502	44,338	59	3.4
Switzerland	25	30,535	147,794	1,613	20.7	South Sudan	71	285	8,421	6	3.4
Saudi Arabia	26	59,854	303,501	329	19.7	Egypt	72	13,484	401,828	659	3.4
Turkey	27	151,615	862,911	4,199	17.6	Honduras	73	2,955	88,824	147	3.3
Poland	28	19,268	114,170	948	16.9	Afghanistan	74	7,653	230,911	178	3.3
U. Arab E.	29	25,063	150,496	227	16.7	Nigeria	75	6,401	203,510	192	3.1
Bulgaria	30	2,292	14,227	116	16.1	Cameroon	76	3,529	113,118	140	3.1
Slovenia	31	1,467	9,206	104	15.9	Haiti	77	596	19,339	22	3.1
Morocco	32	7,023	44,481	193	15.8	Burkina Faso	78	806	30,682	52	2.6
Bosnia & H.	33	2,319	15,813	133	14.7	Suriname	79	11	429	1	2.6
Chile	34	49,579	359,514	509	13.8	Niger	80	914	37,746	55	2.4
Romania	35	17,191	125,640	1,126	13.7	Sierra Leone	81	534	24,508	33	2.2
U.S.	36	1,528,568	11,884,244	91,921	12.9	Guatemala	82	2,133	103,309	43	2.1
Panama	37	9,867	77,349	281	12.8	Kenya	83	963	49,412	50	1.9
China	38	84,065	667,702	4,638	12.6	Iraq	84	3,611	199,779	131	1.8
Moldova	39	6,340	51,912	221	12.2	Mexico	85	54,346	3,289,790	5,666	1.7
Ireland	40	24,251	203,128	1,561	11.9	Mali	86	901	57,558	53	1.6
Tunisia	41	1,044	8,865	47	11.8	Sudan	87	2,591	169,575	105	1.5
El Salvador	42	1,498	12,905	31	11.6	Chad	88	545	42,352	56	1.3
Dominican R.	43	13,223	116,836	441	11.3	Mauritania	89	81	27,361	4	0.3
Canada	44	79,101	763,889	5,912	10.4	Yemen	90	167	67,359	28	0.2
Spain	45	232,555	2,247,533	27,888	10.3	Nicaragua	91	25	14,739	8	0.2
Italy	46	226,699	2,472,703	32,169	9.2						

Countries are ranked by the detection rates of SARS-CoV-2 infections as of 20 May. Source: Own elaboration.

TABLE 3 | Non-synchronic descriptive statistics as of 22 June.

Country	Detection rate ranking	Confirmed Cases	Estimated Cases	Number of Deaths	Detection rate (Percentage)	Country	Detection rate ranking	Confirmed Cases	Estimated Cases	Number of Deaths	Detection rate (Percentage)
Australia	1	7,461	11,478	102	65.0	Sweden	47	56,043	479,232	5,053	11.7
Russia	2	584,680	1,271,052	8,111	46.0	Peru	48	254,936	2,272,406	8,045	11.2
Puerto Rico	3	6,525	14,521	149	44.9	Spain	49	246,504	2,357,978	28,324	10.5
Thailand	4	3,148	7,301	58	43.1	Macedonia	50	5,106	49,133	238	10.4
South Korea	5	12,438	30,176	280	41.2	South Sudan	51	1,882	18,688	34	10.1
Israel	6	20,778	51,835	306	40.1	Pakistan	52	181,088	1,908,427	3,590	9.5
Norway	7	8,708	25,098	244	34.7	Italy	53	238,499	2,533,481	34,634	9.4
Estonia	8	1,981	5,896	69	33.6	Netherlands	54	49,593	538,868	6,090	9.2
Serbia	9	12,894	38,735	261	33.3	El Salvador	55	4,626	53,327	107	8.7
Luxembourg	10	4,120	12,508	110	32.9	Brazil	56	1,085,038	12,484,118	50,617	8.4
Czech R.	11	10,498	32,666	336	32.1	Nicaragua	57	2,014	24,386	64	8.3
Malaysia	12	8,572	27,040	121	31.7	South Africa	58	97,302	1,269,375	1,930	7.7
Portugal	13	39,133	127,864	1,530	30.6	Hungary	59	4,102	54,281	572	7.6
Japan	14	17,916	61,665	953	29.1	Suriname	60	314	4,170	8	7.5
Austria	15	17,285	61,817	690	28.0	U.K.	61	304,331	4,151,851	42,632	7.3
Lithuania	16	1,798	6,497	76	27.7	India	62	425,282	6,033,057	13,699	7.0
Germany	17	190,359	699,154	8,885	27.2	Belgium	63	60,550	861,976	9,696	7.0
Finland	18	7,143	26,402	326	27.1	Philippines	64	30,052	438,038	1,169	6.9
U. Arab E.	19	44,925	178,155	302	25.2	Iran	65	204,952	3,050,048	9,623	6.7
Cuba	20	2,312	9,281	85	24.9	Colombia	66	68,652	1,030,695	2,237	6.7
Ukraine	21	36,560	148,376	1,002	24.6	France	67	160,377	2,515,344	29,640	6.4
Chile	22	242,355	991,336	4,479	24.4	D. R. Congo	68	5,826	98,916	130	5.9
Croatia	23	2,317	9,957	107	23.3	Cameroon	69	11,610	199,080	301	5.8
Denmark	24	12,391	53,638	600	23.1	Bolivia	70	24,388	424,522	773	5.7
Greece	25	3,266	14,533	190	22.5	Somalia	71	2,779	49,186	90	5.7
Poland	26	31,931	150,582	1,356	21.2	Afghanistan	72	28,833	565,246	581	5.1
Switzerland	27	31,209	148,912	1,680	21.0	Indonesia	73	45,891	905,257	2,465	5.1
Saudi Arabia	28	157,612	823,639	1,267	19.1	Nigeria	74	20,244	409,327	518	4.9
Turkey	29	187,685	984,358	4,950	19.1	Honduras	75	12,769	263,032	363	4.9
Morocco	30	9,977	55,386	214	18.0	Algeria	76	11,771	242,645	845	4.9
Albania	31	1,927	11,300	44	17.1	Egypt	77	55,233	1,182,338	2,193	4.7
Bulgaria	32	3,905	23,586	199	16.6	Ecuador	78	50,640	1,084,641	4,223	4.7
Bangladesh	33	112,306	678,767	1,464	16.5	Kenya	79	4,738	109,829	123	4.3
Slovenia	34	1,520	9,410	109	16.2	Libya	80	544	12,846	10	4.2
U.S.	35	2,280,912	14,248,772	119,975	15.7	Sierra Leone	81	1,327	31,692	55	4.2
Moldova	36	14,200	93,470	473	15.2	Mauritania	82	2,813	75,687	108	3.7
Bosnia & H.	37	3,354	22,225	169	15.1	Guatemala	83	13,145	398,078	531	3.3
Panama	38	26,030	180,819	501	14.4	Sudan	84	8,580	276,728	521	3.1
Argentina	39	42,772	303,341	1,011	14.1	Burkina Faso	85	903	30,773	53	2.9
Romania	40	24,045	177,775	1,512	13.5	Mali	86	1,961	73,306	111	2.7
Dominican R.	41	26,677	197,251	662	13.5	Niger	87	1,036	39,912	67	2.6
Tunisia	42	1,157	9,144	50	12.7	Mexico	88	180,545	7,666,945	21,825	2.4
China	43	84,572	668,564	4,639	12.6	Chad	89	858	43,988	74	2.0
Ireland	44	25,379	208,366	1,715	12.2	Iraq	90	30,868	1,582,972	1,100	2.0
Canada	45	101,326	845,149	8,430	12.0	Yemen	91	941	203,732	256	0.5
Haiti	46	5,211	44,065	88	11.8						

Countries are ranked by the detection rates of SARS-CoV-2 infections as of 22 June. Source: Own elaboration.

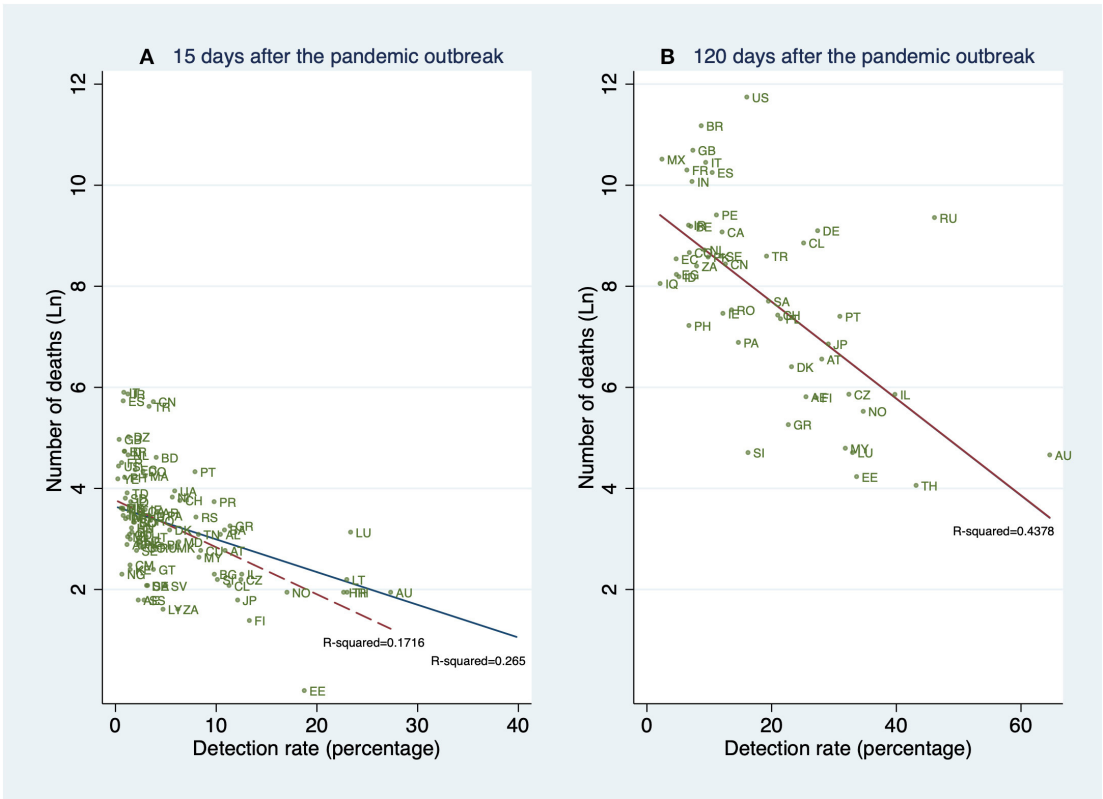

FIGURE 6 | Linear prediction for the natural logarithm of the cumulative number of deaths from a linear regression of ln ($deaths_i$) on the detection rates (DR_i) 15 and 120 days after the pandemic outbreak (PO). **(A)** Detection rates and deaths 15 days after the PO. **(B)** Detection rates and deaths 120 days after the PO. **(A)** contains all 91 countries (in **Table 1A**). **(B)** contains all 61 countries (in **Table 1A**) whose pandemic processes have more than 120 days since the PO. The dashed fitted line excludes South Korea (KR). Source: Own elaboration.

Tables 2, **3** show that moving over time from relatively low to relatively high cumulative detection rates is unlikely and probably very expensive. This is due to the over proportional efforts needed to expand testing relative to the exponentially growing infections at the early stages of the pandemic. Consequently, from the public health point of view, it is much more advantageous, technically, and economically feasible, to implement mass testing from the very beginning of the pandemic process. To achieve this goal, health authorities and governments would require understanding the linkages between the cumulative detection rates and the minimization of the pandemic related fatalities and economic damage.

Unconditional Analysis

In this analysis, we show the unconditional relationship between detection rates and deaths. The fitted lines in **Figure 6** are obtained after regressing the natural logarithm of the cumulative number of deaths in the country *i* on their estimated cumulative detection rates (DR_i). The results strongly suggest a negative relationship between detection rates and the cumulative number of deaths. This strong negative slope is in concordance with the hypothesis that, by detecting a higher proportion of the SARS-CoV-2 infected population, many lives can be saved, in particular, the lives of the elderly and those individuals with preexisting conditions.

The strong association between the number of deaths and the estimated cumulative detection rates remains significant 15, and 120 days after the PO. These associations are shown in **Figures 6A,B**, respectively.

Figure 7 shows the relationship between detection rates (15 and 120 days after the PO) and deaths 120 days after the PO. This descriptive result is of interest since it suggests that, unconditionally, early detection is associated with death outcomes 120 days after the PO to a greater extent than the contemporary detection rates, that is, 120 days after the PO.

Although this information suggests the existence of a strong relationship between detection rates and the cumulative number of deaths, this slope may be confounded by the variables mentioned before. Thus, in the next section, we show the results of our conditional analysis as described earlier.

Multivariate Regression Analysis

Our results in **Table 4** show that higher detection rates are associated with a reduction in the number of deaths after controlling for demography (age-structure of the population and population size), economic performance (GDP per capita), and

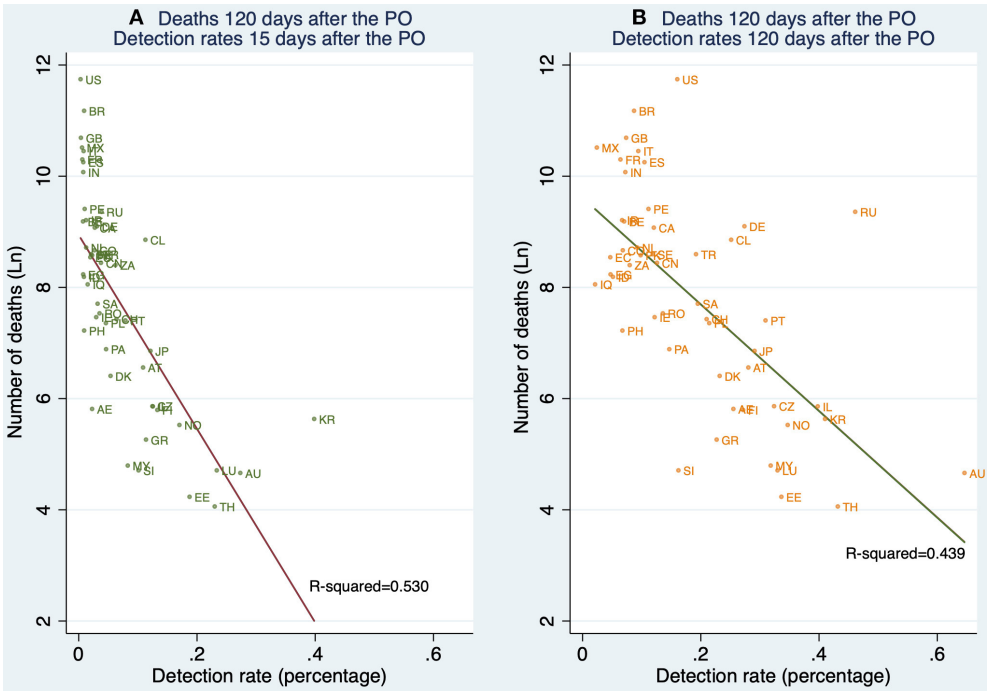

FIGURE 7 | Linear prediction for the natural logarithm of the cumulative number of deaths 120 days after the pandemic outbreak (PO) from a linear regression of ln ($deaths_i$) on the detection rates (DR_i) 15 and 120 days after the PO. **(A)** Deaths reported 120 days after the PO, and detection rates estimated 15 days after the PO. **(B)** Deaths reported 120 days after the PO, and detection rates 120 days after the PO. Note: This figure contains all 61 countries (in **Table 1A**) whose pandemic processes have more than 120 days since the pandemic outbreak. Source: Own elaboration.

the relative resources that the economies devote to their health systems. Over time, the cross-sectional regressions increase in explanatory power, from a R-squared of 0.71 in model 2 to 0.95 in model 8.

Based on these results, **Figure 8** shows a strong conditional gradient between detection rates and the cumulative number of deaths. For instance, for a hypothetical country with average and constant endowments, the cost in terms of deaths of detecting 5% vs. 35% is about 1.81 natural logarithm points which corresponds to $exp^{1.81} = 6.13$. That is, the average country detecting 5% is associated with a number of deaths about 6.1 times higher when compared with the same country detecting 35% of all SARS-CoV-2 infections.

To put this result in perspective, let us simulate what would be the number of deaths in the U.S., if instead of detecting 16.02% 120 days after the pandemic outbreak, the country would have detected with the same intensity as South Korea (41.01%). Evaluating the number of deaths at the endowments of the U.S, the country would have fewer deaths by 1.14 natural logarithm points. It means that the current U.S deaths are now 3.13 times higher than they would be if the country would have tested with similar intensity as South Korea. Since the number of deaths 120 days after the pandemic outbreak reached 126,140, detecting at the rate of South Korea would have saved about 85,794 lives in the U.S. at that time.

Finally, looking at the regression coefficients in **Table 3**, it is noteworthy the fact that during the pandemic outbreak, a 1% higher detection rate is associated with more lives saved than a 1% increase in the health expenditure over the GDP. Our results also suggest that the number of deaths, rather than depending on the relative solvency of the health system, could depend in a greater extent on the size and opportunity of the testing efforts.

The conclusion is the more tests the better. Although in this study we employed an economics inspired approach to figure out the importance of testing, our findings are also endorsed by recent medical literature on coronavirus as well as by another economics inspired models providing support to a causal relationship between detection and saving lives (47–50).

Robustness of the Results

Robust regressions provide estimates that are close to the ones reported in **Table 4**. Consequently, it is unlikely that the results reported in this study are outlier driven. Additionally, results are robust to heteroscedasticity of unknown form for small samples. Nevertheless, results should be interpreted with caution. The few observations available for the regressions and lack of data does not allow to rule out the possibility that there are omitted variables that have the potential to bias the results.

It is important to keep in mind that results can be biased if omitted variable problem exists. That is, there are variables that are correlated with the explained outcome but at the same time they are also correlated with the explanatory variables of interest. For instance, one can think in countries implementing lockdowns because lower detection rates

TABLE 4 | Synchronic multiple linear regression of the natural logarithm of the cumulative number of deaths on the estimated detections rates.

Dependent Variable: Ln(deaths)/Explanatory Variables	**Days since the first 100 cases were confirmed**							
	15		**60**			**120**		
	Model (1)	**Model (2)**	**Model (3)**	**Model (4)**	**Model (5)**	**Model (6)**	**Model (7)**	**Model (8)**
Estimated detection rate	−0.193***	−0.225***	−0.120***	−0.118***	–	−0.100***	−0.0976***	–
	(0.0358)	(0.0269)	(0.0368)	(0.0435)	–	(0.0220)	(0.0217)	–
Estimated detection rate (Squared)	0.00410***	0.00497***	0.00135	0.00132	–	0.000948*	0.000931*	–
	(0.00127)	(0.000698)	(0.000919)	(0.00107)	–	(0.000501)	(0.000484)	–
Estimated detection rate 15 days after PO	–	–	–	–	−22.30***	–	–	−16.78***
	–	–	–	–	(6.266)	–	–	(5.479)
Estimated detection rate 15 days after PO (squared)	–	–	–	–	47.64*	–	–	35.63
	–	–	–	–	(28.54)	–	–	(24.01)
Infection fatality rate	0.960***	0.922***	1.586***	1.512***	1.396***	1.525***	1.506***	1.439***
	(0.333)	(0.328)	(0.267)	(0.270)	(0.370)	(0.172)	(0.179)	(0.329)
Population size (Ln)	−0.150**	−0.146**	−0.0285	−0.0179	0.0105	0.0699**	0.0649*	0.0267
	(0.0656)	(0.0688)	(0.0856)	(0.0780)	(0.0787)	(0.0345)	(0.0356)	(0.0518)
Confirmed cases (Ln)	0.860***	0.773***	0.943***	0.910***	0.705***	0.931***	0.929***	0.849***
	(0.0995)	(0.108)	(0.0696)	(0.0640)	(0.0796)	(0.0324)	(0.0334)	(0.0639)
GDP per capita (Ln)	−0.446***	−0.417***	0.0399	0.0570	0.0742	0.181***	0.168**	−0.0194
	(0.108)	(0.103)	(0.0842)	(0.0913)	(0.138)	(0.0600)	(0.0642)	(0.154)
Health spending as % of GDP	−0.0570*	−0.0552	−0.0147	−0.0270	−0.000955	0.00186	−0.0115	0.00210
	(0.0330)	(0.0354)	(0.0231)	(0.0260)	(0.0277)	(0.0143)	(0.0163)	(0.0312)
BCG group 2	–	−0.441	–	0.175	0.124	–	0.185*	0.196
	–	(0.323)	–	(0.161)	(0.206)	–	(0.101)	(0.233)
BCG group 3	–	−0.396	–	0.0449	−0.0185	–	0.185	0.197
	–	(0.284)	–	(0.235)	(0.305)	–	(0.165)	(0.332)
BCG group 4	–	−0.704***	–	−0.193	−0.205	–	−0.0324	−0.131
	–	(0.220)	–	(0.184)	(0.209)	–	(0.102)	(0.213)
BCG group 5	–	−0.411*	–	−0.172	−0.175	–	0.0200	0.0653
	–	(0.210)	–	(0.152)	(0.176)	–	(0.0856)	(0.178)
Constant	4.530***	5.355***	−2.365	−2.204	−1.313	−5.437***	−5.174***	−2.425*
	(1.391)	(1.434)	(1.431)	(1.442)	(1.629)	(0.679)	(0.776)	(1.362)
Observations	87	87	84	84	84	74	74	74
R-squared	0.672	0.708	0.950	0.954	0.934	0.984	0.985	0.954
R-squared adjusted	0.643	0.666	0.945	0.947	0.924	0.983	0.983	0.946
F-test	26	21.86	342.3	274.9	110.5	594.5	404.1	137.8

*Standard errors in parentheses. Significance levels: ***p < 0.01, **p < 0.05, *p < 0.1. Source: Own elaboration.*

(Argentina), or relaxed social distancing rules because higher detection rates (Australia). Nevertheless, these non-observed variables yield to an underestimation of the true association between detection rates and the cumulative number of deaths. Thus, detection matters.

DISCUSSION

In this study, we have proposed a method to estimate the number of SARS-CoV-2 infections for the globe and also for all 91 major countries covering more than 86% of the world population. On June 22, we find that, worldwide, about 160 million individuals have been infected by SARS-CoV-2. Moreover, only about 1 out of 11 these infections have been detected. We find that detection rates are very unequally distributed across the globe and that they also increased over time from about 1% during the second and third weeks of March to about 9% on June 22. In an information context in which population-based seroepidemiological studies are not available, this study shows a parsimonious alternative to provide estimates of the number of SARS-CoV-2 infected individuals. By comparing our estimates with those provided by the ENE-COVID study in Spain, we confirm the utility of our approach keeping in mind that from the public health point of view, it is preferable to be vaguely right than precisely wrong.

In order to provide reliable estimates of the number of SARS-CoV-2 infections and of the cumulative detection rates, it is necessary that governments provide real-time information about

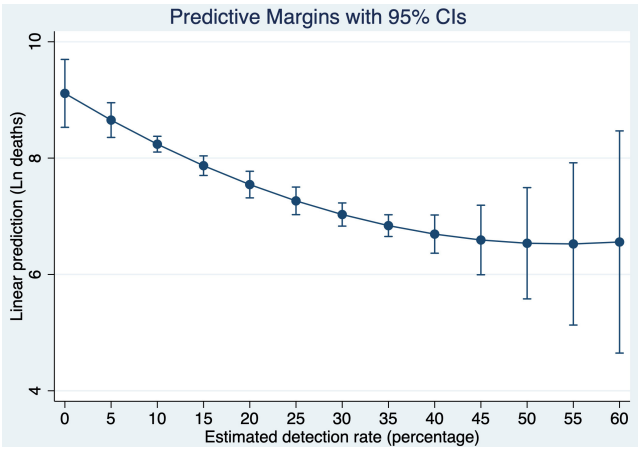

FIGURE 8 | Predictive Margins of the cumulative number of deaths at different detection rates of SARS-CoV-2 infections after 120 days of the pandemic outbreak. Source: Own elaboration.

the number of COVID-19 deaths. This study supports the view that an accurate communication of the fatality cases can have consequences on the development of the pandemic itself. Thus, it is also a call for allowing international comparison following WHO international norms and standards for medical certificates of COVID-19 cause of death and International Classification of Diseases (ICD) mortality coding.

Additionally, in our empirical analysis, we have presented parsimonious evidence, that higher detection rates are associated with saving lives. Our conditional analysis shows, for example, that if the US would have had the same detection rate trajectory as South Korea, about two-thirds of the reported deaths could have been avoided (about 85,000 lives).

We find that detection rates at the very early stages of the pandemic seem to explain the great divergence in terms of deaths between countries. Moreover, we showed evidence that moving from relatively low to high cumulative detection rates (and thus saving lives) is unlikely and difficult. This is probably due to the high level of efforts needed to expand testing relative to the exponentially growing infections at the early and middle stages of the pandemic. Thus, from the public health point of view, it is better to deploy testing efforts at the first stages of the pandemic process. To do this would be much more advantageous, in terms of saved lives, but also it would be technically, and economically feasible.

Already, many developed countries with well-developed health sectors were not able to avoid unnecessary deaths by their inaction in terms of promoting mass testing to counter the pandemic outbreak at early stages.

To achieve the goal of implementing mass testing from the very beginning of the pandemic outbreak, governments need to understand the consequences of not doing that. Thus, the evidence presented in this paper offers a rigorous macro-level linkage between detection rates and the cumulative number of deaths which may be useful in future pandemics. This evidence also supports the implementation of mass testing in the likely coming secondary pandemic outbreak (so-called second waves).

Further research should be devoted to understanding why the detection capacity in many advanced countries was too weak, late, and also so weakly correlated (if correlated) with the income levels. In this paper, we claim that governments have incentives against test because the public opinion tends to primarily react to the report of the cumulative and the marginal numbers of detected (reported) cases. The contradiction is that something good, such as the increase in the testing efforts by governments, can be perceived by the general public as something negative (due to the increase in detections). In consequence, are low detection rates in developed countries simply a management failure, or are there long-run incentives that promoted this behavior among many rich countries? It is clear that during the ongoing pandemic, improving detection rates is a race against time, but are there institutional and/or technological constraints that hamper detection improvements that can save lives? All these questions are relevant for this and future pandemics. This study claims that all countries in the world should be able to respond to a pandemic outbreak with massive testing in the very short run. This would be an efficient approach since it is also likely that higher detection rates are also associated with a lesser impact of the pandemic on the economy.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

CV conceived this research, performed the background work, collected the data, performed all statistical analyses, and wrote the paper.

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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.2020.00489/full#supplementary-material

Supplementary Set of Figures. Estimated number of SARS-CoV-2 infections by country.

Supplementary Table 1 | Synchronic multiple linear regression of the natural logarithm of the cumulative number of deaths on the estimated detections rates (linear specification).

Supplementary Table 2 | Synchronic multiple linear regression of the natural logarithm of the cumulative number of deaths on the estimated number of SARS-CoV-2 infections.

Supplementary Table 3 | Synchronic robust multiple linear regression of the natural logarithm of the cumulative number of deaths on the estimated detections rates (linear specification).

Supplementary Table 4 | Synchronic robust multiple linear regression of the natural logarithm of the cumulative number of deaths on the estimated detections rates (non-linear specification).

Supplementary Table 5 | Synchronic multiple linear regression of the natural logarithm of the cumulative number of deaths on the estimated number of SARS-CoV-2 infections.

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Genomic Modeling as an Approach to Identify Surrogates for Use in Experimental Validation of SARS-CoV-2 and HuNoV Inactivation by UV-C Treatment

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Severe Acute Respiratory Syndrome coronavirus-2 (SARS-CoV-2) is responsible for the COVID-19 pandemic that continues to pose significant public health concerns. While research to deliver vaccines and antivirals are being pursued, various effective technologies to control its environmental spread are also being targeted. Ultraviolet light (UV-C) technologies are effective against a broad spectrum of microorganisms when used even on large surface areas. In this study, we developed a pyrimidine dinucleotide frequency based genomic model to predict the sensitivity of select enveloped and non-enveloped viruses to UV-C treatments in order to identify potential SARS-CoV-2 and human norovirus surrogates. The results revealed that this model was best fitted using linear regression with r^2 = 0.90. The predicted UV-C sensitivity (D_{90} – dose for 90% inactivation) for SARS-CoV-2 and MERS-CoV was found to be 21.5 and 28 J/m^2, respectively (with an estimated 18 J/m^2 obtained from published experimental data for SARS-CoV-1), suggesting that coronaviruses are highly sensitive to UV-C light compared to other ssRNA viruses used in this modeling study. Murine hepatitis virus (MHV) A59 strain with a D_{90} of 21 J/m^2 close to that of SARS-CoV-2 was identified as a suitable surrogate to validate SARS-CoV-2 inactivation by UV-C treatment. Furthermore, the non-enveloped human noroviruses (HuNoVs), had predicted D_{90} values of 69.1, 89, and 77.6 J/m^2 for genogroups GI, GII, and GIV, respectively. Murine norovirus (MNV-1) of GV with a D_{90} = 100 J/m^2 was identified as a potential conservative surrogate for UV-C inactivation of these HuNoVs. This study provides useful insights for the identification of potential non-pathogenic (to humans) surrogates to understand inactivation kinetics and their use in experimental validation of UV-C disinfection systems. This approach can be used to narrow the number of surrogates used in testing UV-C inactivation of other human and animal ssRNA viral pathogens for experimental validation that can save cost, labor and time.

Keywords: genomic modeling, UV-C inactivation, viruses, SARS-CoV-2 (2019-nCoV), norovirus (NoV), surrogates

INTRODUCTION

Coronaviruses belong to the family of *Coronaviridae*, comprising of 26 to 30 kb, positive-sense, single-stranded RNA, in an enveloped capsid (Woo et al., 2010). Coronaviruses can cause severe infectious diseases in human and vertebrates, being fatal in some cases. Severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV-1), a β-coronavirus emerged in Guangdong, southern China, in November, 2002 (Guan et al., 2003), and the Middle East respiratory syndrome (MERS) coronavirus (MERS-CoV), was first detected in Saudi Arabia in 2012 (Alagaili et al., 2014). Since late December 2019, a novel β-coronavirus (2019-nCoV or SARS-CoV-2) has been responsible for the pandemic coronavirus disease (COVID-19) with >7.2 million confirmed cases throughout the world, and a fatality rate of approximately 5.7% as of 11 June, 2020 (World Health Organization [WHO], 2020a). This 2019-nCoV is thought to have originated from a seafood market of Wuhan city, Hubei province, China, and has spread rapidly to other provinces of China and other countries (Zhu et al., 2020).

According to current evidence documented by the World Health Organization [WHO] (2020a,b), SARS-CoV-2 virus (2019-nCoV) is transmitted between humans through respiratory droplets and contact (person-to-person, fomites, etc.) routes (World Health Organization [WHO], 2020b). van Doremalen et al. (2020) reported that SARS-CoV-2 remained viable in aerosols throughout the 3 h duration of the experiment and more stable on plastic and stainless steel than on copper and cardboard, and virus was detected up to 72 h after the application to these surfaces at 21–23°C and 40% relative humidity. Given the ability of these viruses to survive in the environment, appropriate treatment strategies are needed to inactivate SARS-CoV-2. As per WHO recommendations, SARS-CoV-2 may be inactivated using chemical disinfectants. As of 07 April, 2020, the United States Environmental Protection Agency [USEPA] (2020) has announced a list of 428 registered chemical disinfectants that have been approved for use against SARS-CoV-2 (United States Environmental Protection Agency [USEPA], 2020). On the other hand, physical disinfection method "ultraviolet light (UV) treatment" (with germicidal UV-C at wavelengths from 100 to 280 nm) can be an effective approach to inactivate SARS-CoV-2 on surface areas and in the air. UV inactivates a broad spectrum of microorganisms by damaging the DNA or RNA and thereby prevents and/or alters cellular functions and replication (Patras et al., 2020). UV-C inactivation of various microorganisms such as pathogenic bacteria, spores, protozoa, algae and viruses has been reported (Malayeri et al., 2016; Bhullar et al., 2019; Gopisetty et al., 2019; Pendyala et al., 2019, 2020; Patras et al., 2020). Because UV inactivation studies with SARS-CoV-2 requires specifically trained and skilled personnel working under biosafety level 3 (BSL-3) laboratory containment conditions, the use of surrogate coronaviruses has the potential to cross these hurdles for experimental validation of designed UV systems. Based on the biophysical properties and genomic structure, literature studies on testing the efficacy of disinfectants against coronaviruses used the following surrogates; murine hepatitis virus (MHV), Human coronavirus 229 E, transmissible gastroenteritis virus (TGEV), and feline infectious peritonitis virus (FIPV) (Kumar et al., 2020). However, the selection of potential surrogates to SARS-CoV-2 requires a comparative evaluation of UV-C sensitivity between these viruses. As of date, the precise experimental UV-C susceptibility (D_{90} value) of SARS-CoV/SARS-CoV-2 is not reported.

Human noroviruses (HuNoVs) cause >80% of global non-bacterial gastroenteritis that can be spread through contamination of food, water, fomites, or direct contact, and also via aerosolization (Fankhauser et al., 2002; Widdowson et al., 2005; Godoy et al., 2006). HuNoVs are also single-stranded RNA viruses that are small 27 to 32 nM in size that belong to the *Caliciviridae* family. However, HuNoVs are enclosed in a non-enveloped capsid, unlike SARS-CoV-2 that is enveloped. UV-C inactivation data on the HuNoV genogroups is limited due to the lack of available cultivation methods to obtain high infectious titers (Doultree et al., 1999; Ettayebi et al., 2016; Estes et al., 2019). Thus, reverse transcription quantitative polymerase chain reaction (RT-qPCR) is widely used for assessing survivor populations of HuNoVs after treatment. However, research studies showed overestimation of survivors with RT-qPCR in comparison to virus infectivity plaque assays (Rönnqvist et al., 2014; Wang and Tian, 2013; Walker et al., 2019). As an alternative, cultivable animal viruses [caliciviruses, echoviruses and murine norovirus (MNV)] have been used as surrogates to determine UV-C inactivation of HuNoVs (Thurston-Enriquez et al., 2003; de Roda Husman et al., 2004; Lee et al., 2008; Park et al., 2011), but proper selection of surrogates which mimic the UV-C inactivation characteristics of HuNoVs is required to evaluate kinetics and scale up validation studies.

Furthermore, it is well known that microorganisms respond to UV exposure at rates defined in terms of UV rate constants (Patras et al., 2020). The slope of the logarithmic decay curve is defined by the rate constant, which is designated as k. The UV rate constant k has units of cm^2/mJ or m^2/J and is also known as the UV susceptibility. It can be also defined as D_{90} or D_{10} [dose for 90% inactivation or 10% survival] as the primary indicator of UV susceptibility. UV dose is expressed as J/m^2 or mJ/cm^2 (Patras et al., 2020). The varied microbial sensitivity to ultraviolet light (UV) among species of microbes, is due to several intrinsic factors including physical size, presence of chromophores or UV absorbers, presence of repair enzymes or dark/light repair mechanisms, hydrophilic surface properties, relative index of refraction, specific UV spectrum (broad band UVC/UVB versus narrow band UVC), genome based parameters; molecular weight of nucleic acids, DNA conformation (A or B), G+C%, and % of potential pyrimidine or purine dimerization (Kowalski et al., 2009).

The physical size of a virus bears no clear relationship with UV susceptibility, except that for the largest viruses, as size increases, the UV rate constant tends to decrease slightly (which is likely the result of UV scattering) (Kowalski et al., 2009). There is no thorough literature available on the above-mentioned optical parameters, hydrophilic surface properties and repair mechanisms relating to UV sensitivity. On the other hand, genome sequences of UV susceptibility can be easily retrieved from genome databases and the development of genomic models

based on the above mentioned genome-based parameters is feasible to predict the UV susceptibility of ssRNA viruses, which include human pathogenic novel viruses (such as SARS-CoV-2) and cultivation-challenging HuNoVs.

Our hypothesis is that predicting UV-C inactivation based on genomic modeling, will enable the determination of surrogates to be used in UV-C validation studies. In the present study, we attempted to develop a genomic model to predict and compare the UV sensitivity of enveloped SARS-CoV-2 and non-enveloped HuNoVs and to determine their suitable surrogates for use in UV-C process validation.

MATERIALS AND METHODS

Collection of Reported ssRNA Viruses UV_{254} Sensitivity (D_{90} Values)

We collected UV-C sensitivity of ssRNA viruses form published studies and carefully selected D_{90} values (**Table 1**). The selection was based on the careful assessment of methods that were used to determine UV-C sensitivity. The selected UV-C sensitivity of an ssRNA virus is determined via the standard method (Bolton and Linden, 2003), with the $\log_{10}$ survivors as a function of UV dose and represented as D_{90}.

TABLE 1 | Reported UV sensitivity (D_{90}) data for ssRNA viruses.

Virus	Average D_{90} (J/m^2)	Reference source
Murine sarcoma virus	190	Kelloff et al., 1970; Nomura et al., 1972
Bacteriophage MS2	183[a]	Malayeri et al., 2016
Moloney murine leukemia virus	115	Nomura et al., 1972
Murine norovirus	100	Lee et al., 2008; Park et al., 2011
Coxsackievirus	79	Battigelli et al., 1993; Gerba et al., 2002; Shin et al., 2005
Human parechovirus	75	Gerba et al., 2002
Polio virus	73	Gerba et al., 2002; Thompson et al., 2003; Lazarova and Savoye, 2004; Shin et al., 2005; Simonet and Gantzer, 2006
Canine calicivirus (CCV)	67	de Roda Husman et al., 2004
Feline calicivirus (FCV)	60	Thurston-Enriquez et al., 2003; de Roda Husman et al., 2004; Park et al., 2011
Sindbis virus	55	Zavadova and Libikova, 1975; von Brodorotti and Mahnel, 1982
Venezuelan equine encephalitis virus	55	Smirnov et al., 1992
Western equine encephalomyelitis virus	54	Dubinin et al., 1975
Hepatitis A virus	51	Wilson et al., 1992; Battigelli et al., 1993; Wiedenmann et al., 1993
Semliki forest virus	25	Weiss and Horzinek, 1986
Measles virus	22	Stefano et al., 1976
SARS-CoV-1	18[b]	Kariwa et al., 2006

Average D_{90} values refer average of reference source studies.
[a]Average value of all (45) MS2 reports.
[b]Estimated value from initial linear kinetics of data and considering 90% of light transmission through test fluid.

Determination of Genomic Parameters; Genome Size, and Pyrimidine Dinucleotide Frequency Value (PyNNFV)

The molecular size and nucleotide sequences of genomes used in this study were directly obtained from available NCBI genome database (**Table 2** and **Table 5**). PyNNFV model was developed based on the frequency of each type of pyrimidine dinucleotides (TT, TC, CT, and CC) which varies based on genome sequences. Pyrimidines are almost 10 times more susceptible to photoreaction (Smithyman and Hanawalt, 1969), while strand breaks, inter-strand cross links and DNA-protein cross links form with less frequency (1:1000 of the number of dimers and hydrates) (Setlow and Carrier, 1966). Three simple rules were formulated for sequence-dependent dimerization (Becker and Wang, 1989); "(i) When two or more pyrimidines are neighboring to one another, photoreactions are observed at both pyrimidines, (ii) Non-adjacent pyrimidines exhibit little or no photoreactivity, and (iii) Purines form UV photoproducts when they are flanked at 5′ side by two or more adjacent pyrimidine residues." Therefore, we considered 100% probability of formation of photoreaction products when PyNN are flanked by pyrimidines on both sides and 50% probability when PyNN are flanked by purine on either side. The individual PyNNs were counted by the exclusive method (each pyrimidine considered in one PyNN combination only). Research studies showed the proportion of photoreaction products in the order of TT > TC > CT > CC (Douki, 2013), thus same sequence was followed in counting individual PyNNs. **Table 3** shows the method used for PyNNFV calculation in this study. A mathematical function was written to calculate PyNNFV from the potential PyNNs to exist in the genome of RNA (Eq. 1).

$$\text{PyNNFV} = \frac{(\text{TT\%})(\text{TC\%})(\text{CT\%})(\text{CC\%})}{\text{genome bp}} \quad (1)$$

The PyNNFVs from complete genome sequences of 16 ssRNA viruses and corresponding reported D_{90} values were used to plot a model graph. Then, the correlation between PyNNFVs and D_{90} values was analyzed by fitting the appropriate regression model (linear regression).

RESULTS AND DISCUSSION

Table 1 shows the median D_{90} values collected from UV-C inactivation studies of various ssRNA viruses. The data was selected from the studies conducted with uniform viral suspensions in transparent medium (water or phosphate buffer saline), followed standard method for UV dose calculation (Bolton and Linden, 2003). The D_{90} values reported for ssRNA viruses ranged from 18 J/m^2 for SARS-CoV-1 to 190 J/m^2 for murine sarcoma virus. Genomic parameters; genome size,

TABLE 2 | Genome size and identified pyrimidine dinucleotide values for collected ssRNA viruses.

Virus	NCBI Accession #	Genome (bp)	PyNNFV[a]
Bacteriophage MS2	NC_001417.2	3569	0.00804
Murine sarcoma virus	NC_001502.1	5833	0.00807
Human parechovirus	NC_001897.1	7348	0.00210
Murine norovirus	NC_008311.1	7382	0.00570
Coxsackievirus	KX595291.1	7410	0.00314
Polio virus	NC_002058.3	7440	0.00263
Hepatitis A virus	KP879217.1	7476	0.00209
Feline calicivirus	NC_001481.2	7683	0.00363
Moloney murine leukemia virus	NC_001501.1	8332	0.00598
Canine Calicivirus	NC_004542.1	8513	0.00345
Semliki forest virus	NC_003215.1	11442	0.00141
Venezuelan equine encephalitis virus	NC_001449.1	11444	0.00153
Western equine encephalomyelitis virus	NC_003908.1	11484	0.00151
Sindbis virus	NC_001547.1	11703	0.00149
Measles virus	NC_001498.1	15894	0.00134
SARS-CoV-1	NC_004718.3	29751	0.00067

[a] *Pyrimidine dinucleotide frequency value.*

PyNNFVs of respective viruses were shown in **Table 2**. The values are in the range of 3569 bp to 29751 bp for genomic size; 0.00067–0.00807 for PyNNFV.

Genomic Models to Predict UV-C Sensitivity of ssRNA Viruses

To determine the relationship between genome size and UV-C sensitivity, the D_{90} values were plotted against the genome size of various ssRNA viruses (**Figure 1**). The data were best fitted to log linear regression model with $r^2 = 0.63$. The results revealed that there was a decisive relationship between genome size and UV sensitivity across the range of 3569–29751 bp.

Further to evaluate the influence of base composition and sequence along with genome size on UV-C sensitivity, the D_{90} values were plotted versus PyNNFV (**Figure 2**). Linear regression model was best fitted with $r^2 = 0.90$. Therefore, based on the value of r squared a moderate positive relationship was found between PyNNFV and UV-C sensitivity of the virus. The following linear regression equation shows the correlation between D_{90} values and PyNNFV.

$$y = 19984x + 10.409 \quad (2)$$

Also, to predict the distribution of UV-C sensitivities and estimates of the true population mean using this model, 95% prediction and confidence intervals were shown in **Figure 2**. To confirm the adequacy of the fitted model, studentized residuals versus run order were tested and the residuals were observed to be scattered randomly, suggesting that the variance was constant. It can be indicated from **Figure 3** that predicted values were in close agreement with the experimental values and were found to be not significantly different at $p > 0.05$ using a paired t-test. Despite some variations, results obtained predicted model and actual experimental values showed that the established models reliably predicted the D_{90} value. Therefore, the predictive performance of the established model can be considered acceptable. The applicability of the models was also quantitatively evaluated by comparing the bias and accuracy factors (**Table 4** and Eqs 3 and 4).

TABLE 3 | Calculation of PyNNFV value for SARS-CoV-2.

Parameter	TT	TC	CT	CC
PyNNs[a]	2454	1020	881	535
PyNNs flanked with purine[a]	773 (ATT) 412 (TTA) 530 (GTT) 230 (TTG)	324 (ATC) 250 (TCA) 174 (GTC) 37 (TCG)	298 (ACT) 244 (CTA) 187 (GCT) 91 (CTG)	281 (ACC) 90 (CCA) 84 (GCC) 10 (CCG)
Total PyNNs flanked with purine	1945	785	820	472
PyNNs flanked without purine	509	235	61	63
Probability of each PyNN[b]	1481.5	627.5	471	299
PyNNs(%)[c]	4.956341	2.099294	1.575725	1.000301
Genome size	29891			
PyNNFV	0.000555			

[a] *Values are counted using exclusive method (once one doublet or triplet is located in the genome, it is excluded from participating in other dimers).*
[b] *Overall probability of each PyNN is calculated by considering 50% probability (0.5) for PyNNs flanked with purine and 100% probability (1.0) for PyNNs flanked without purine.*
[c] *PyNNs% was determined by calculating the% of probability of PyNNs in total genome.*

$$AF = 10^{\frac{\sum \log\left|V_P/V_E\right|}{n_e}} \quad (3)$$

$$BF = 10^{\frac{\sum \log\left(V_P/V_E\right)}{n_e}} \quad (4)$$

$$E(\%) = \frac{1}{n_e}\sum_{i=1}^{n}\left|\left|\frac{V_E - V_P}{V_E}\right|\right| \times 100 \quad (5)$$

The average mean deviation (E%) were used to determine the fitting accuracy of data (Eq. 5). Where, n_e is the number of experimental data, V_E is the experimental value and V_P is the predicted value.

In most cases, as shown in **Table 4**, the accuracy factor (AF) values for the genomic model were close to 1.00, except for Measles virus (0.83), Semliki forest virus (0.86). The bias factor (BF) values for the predicted models were also close to 1.00, ranging from 1.02 to 1.21 for the parameter studied. These results clearly indicate that there was a good agreement between predicted and observed D_{90} values. Ross et al. (2000) stated that predictive models ideally would have an AF = BF = 1.00, indicating a perfect model fit where the predicted and actual response values are equal. However, typically, the AF of a fitted model will increase by 0.10–0.15 units for each predictive variable in the model (Ross et al., 2000). Genomic model, as in this study, that forecasts a response may be expected to have AF and BF

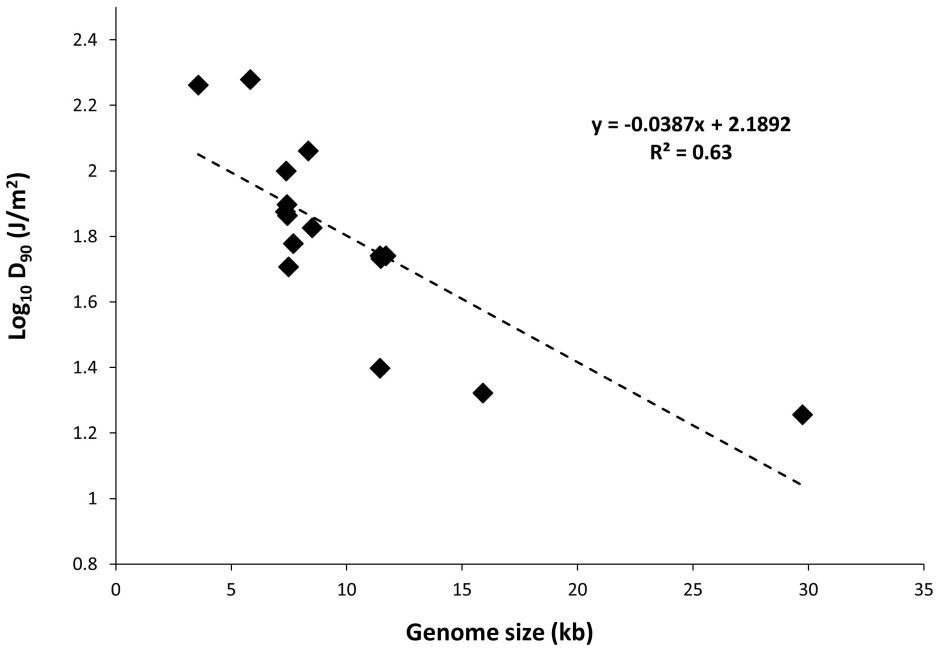

FIGURE 1 | Plot of genome size versus UV-C sensitivity of ssRNA viruses.

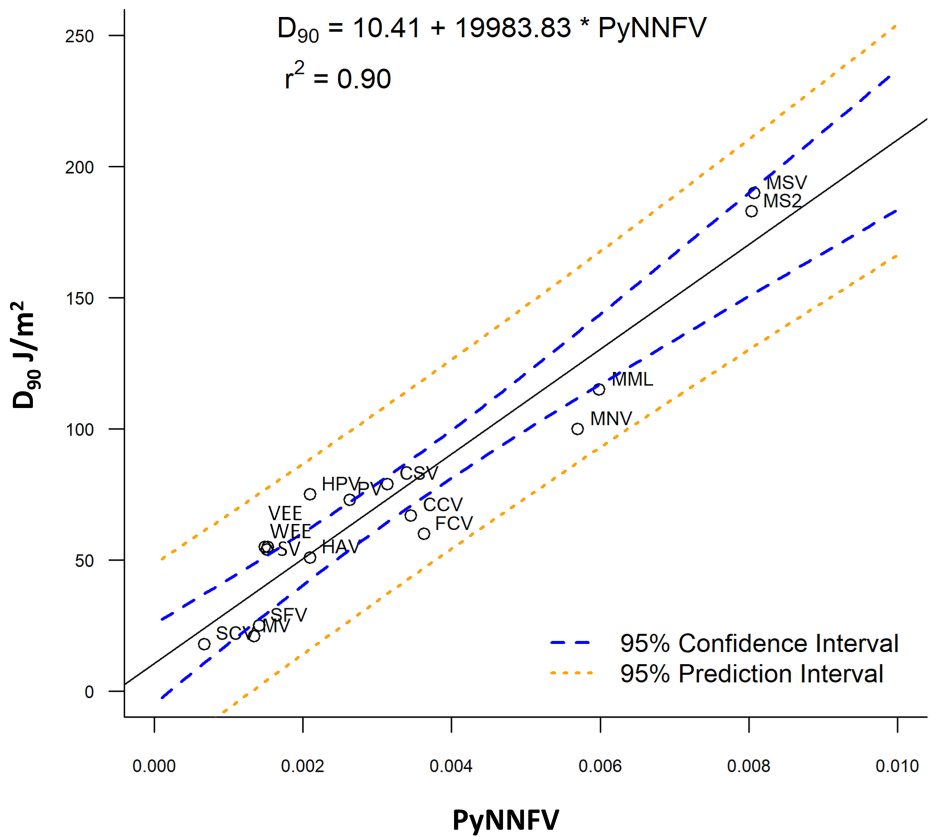

FIGURE 2 | Plot of PyNNFV versus UV-C sensitivity of ssRNA viruses. MSV, murine sarcoma virus; MS2, bacteriophage MS2; MML, moloney murine leukemia virus; CSV, coxsackie virus; HPV, human parechovirus; PV, polio virus; CCV, canine calcivirus; FCV, feline calcivirus; HAV, hepatitis A virus; SV, sindbis virus; VEE, venezuelan equine encephalitis virus; WEE, western equine encephalomyelitis virus; SFV, semliki forest virus; MV, measles virus; SCV, SARS-CoV-1.

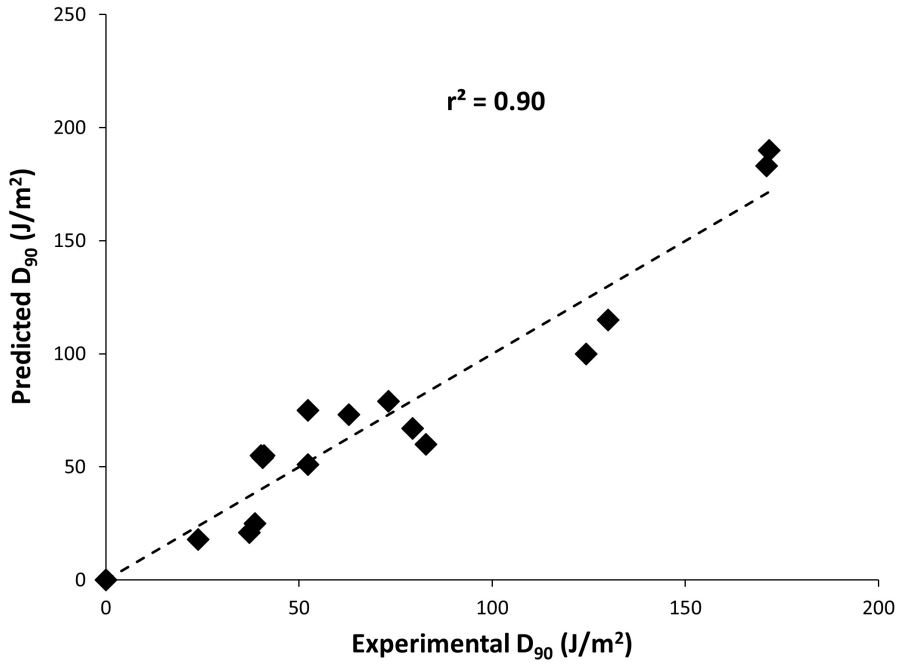

FIGURE 3 | Plot of model predicted D_{90} values versus Experimental D_{90} values.

TABLE 4 | Accuracy factors (AF) and Bias factors (BF) for D_{90} values in the regression analysis.

Virus	AF	BF	E (%)[a]
Bacteriophage MS2	1.02	1.02	2.18
Feline calicivirus	0.9	1.11	12.71
Coxsackievirus	1.03	1.03	2.48
Canine calicivirus	0.94	1.06	6.17
Semliki forest virus	0.86	1.16	18.18
Murine sarcoma	1.03	1.03	3.22
Measles virus	0.83	1.21	25.67
SARS-CoV-1	0.91	1.1	10.88
Murine norovirus	0.93	1.08	8.09
Moloney murine leukemia virus	0.96	1.04	4.33
Human parechovirus	1.13	1.13	10.09
Western equine encephalomyelitis virus	1.1	1.1	8.25
Venezuelan equine encephalitis virus	1.1	1.1	8.55
Sindbis virus	1.11	1.11	9.03
Hepatitis A virus	0.99	1.01	0.82
Polio virus	1.05	1.05	4.62

[a]*Average mean deviation.*

values ranging from 0.83 to 1.21 or an equivalent percentage error range of 0.82–25.67%.

Prediction of UV Sensitivity of Various Corona Viruses and Human Noroviruses

Owing to good model fitting, the PyNNFV genomic model was used to predict UV sensitivity of coronaviruses including SARS-CoV-2 and different HuNoV genogroups. PyNNFV values of target viruses were calculated from genomic sequences obtained from the NCBI database. The UV sensitivities were predicted by substituting PyNNFV value in Eq. 2. **Table 5** shows PyNNFV values and corresponding predicted D_{90} values of target viruses. Predicted D_{90} of SARS-CoV-2 virus (21.5 J/m^2) (**Table 5**) is closer to the estimated D_{90} of SARS-CoV-1 (18 J/m^2) from the experimental study (**Table 1**). Kariwa et al. (2006) irradiated 2 mL of SARS-CoV-1 in 3-cm petri dishes without stirring UV-C light at 134 μW/cm^2 for 15 min, and observed reduction in infectivity from 3.8×10^7 to 180 $TCID_{50}$/mL with equivalent to D_{90} value of 226 J/m^2. In contrast, Darnell et al. (2004) showed 4 log reduction of SARS-CoV-1 at UV-C exposure of 4016 μW/cm^2 for 6 min which is equivalent to D_{90} value of 3610 J/m^2. The authors conducted the experiment in a 24 well plate containing 2 mL virus aliquots without mixing. These two studies neither calculate the average irradiance nor provide conditions for uniform UV-C dose distribution throughout the test fluid and thereby reported higher values. The model predicted D_{90} value of MERS-CoV (28.1 J/m^2) that is found to be higher than SARS-COV-2, whereas murine hepatitis coronavirus (MHV) strains showed similar UV-C sensitivity (D_{90} values = 20.3 to 21 J/m^2). For α- and γ-coronaviruses, the predicted D_{90} values (17.8 to 18.3 J/m^2) were lower than the β-coronaviruses (**Table 5**). Saknimit et al. (1988) demonstrated the efficiency of UV-C irradiation on the inactivation of MHV and CCV coronaviruses using 15 W UV-C lamp at a distance of 1 m and reported efficient UV-C inactivation after 15 min treatment. From this data, the estimated D_{90} values for MHV and CCV (γ-coronavirus) were 17 and 15 J/m^2, respectively, and observed to be slightly lower (∼20%) than the model predicted values (**Table 5**). Overall the results show

TABLE 5 | Predicted of UV sensitivity with respect to dimerization values of target ssRNA viruses.

Virus	NCBI Accession#	Genome (bp)	PyNNF values	Predicted D_{90} Values (J/m^2)
α-coronaviruses				
Transmissible gastroenteritis virus	KX499468.1	28614	0.000391	18.2
Canine coronavirus	KP981644.1	29278	0.000379	18.0 (15.0)
Feline infectious peritonitis virus	KC461237.1	29357	0.000393	18.3
Human coronavirus 229E	KF514433.1	27165	0.000489	20.2
β-coronaviruses				
SARS-CoV-2	MT192772.1	29891	0.000549	21.5
MERS-CoV	MH734115.1	30033	0.000883	28.1
Murine hepatitis virus strain A59	MF618252.1	29947	0.000532	21.0 (17.0)
Murine hepatitis virus strain S	GU593319.1	31147	0.000515	20.7 (17.0)
Murine coronavirus MHV-1	FJ647223.1	31386	0.000526	20.9 (17.0)
Rat coronavirus	JF792617.1	31274	0.000494	20.3
Bat coronavirus BM48-31	NC_014470.1	29276	0.000603	22.5
Bat coronavirus HKU9-1	NC_009021.1	29114	0.000465	19.7
Bat coronavirus HKU4-1	NC_009019.1	30286	0.000580	22.0
Bat Hp-betacoronavirus	NC_025217.1	31491	0.000691	24.2
SARS coronavirus A022 (Civet)	AY686863.1	29499	0.0006401	23.2
SARS coronavirus B039 (Civet)	AY686864.1	29525	0.0006402	23.2
γ-coronavirus				
Avian infectious bronchitis virus	NC_001451.1	27608	0.000371	17.8
Human noroviruses (non-enveloped)				
Norovirus GI	NC_001959.2	7654	0.002936	69.1
Norovirus GII	KF712510.1	7509	0.003934	89.0
Norovirus GIV	JF781268.1	7839	0.00336	77.6

Values in parenthesis denote estimated D_{90} values from experimental study (Saknimit et al., 1988).

that coronaviruses are highly sensitive to UV-C light than other ssRNA viruses reported in **Table 1**. From the UV sensitivity data obtained using the genomic model, it was observed that UV doses ranging from 90 to 141 J/m^2 are required for 5 log reduction of human pathogenic coronaviruses (SARS-CoV-1, MERS-CoV, 2019-nCoV). Here we demonstrate an example of UV exposure using a low-pressure mercury lamp. If the UV-C lamp source provides an average irradiance of 0.4 mW/cm^2 or 4 W/m^2 (under uniform dose distribution conditions), a mere 35 s treatment is adequate to inactivate β-coronaviruses (99.999% or 5 log reduction). Since the developed model relies on total PyNNFV (not on specific gene sequences), slight viral mutations should not cause significant variations in UV sensitivity. For instance, if the PyNNFV value of SARS-CoV-2 changes up to ±10%, the model predicted UV sensitivity (D_{90} value) ranges from 20.4 to 22.6 J/m^2 with the change of just ±2.6%.

The predicted D_{90} values of HuNoVs are 69.1, 89, and 77.6 J/m^2 for genogroups, GI, GII, and GIV, respectively (**Table 5**). The results revealed that the UV-C sensitivity of GII was lower with higher predicted D_{90} value in comparison to GI and GIV. To the best of our knowledge, limited experimental data is currently available on UV-C sensitivity of HuNoVs. Some research studies used RT-qPCR method to estimate MNV survivors and validated with virus infectivity assay (Wang and Tian, 2013; Rönnqvist et al., 2014; Walker et al., 2019). The reported validation results showed that the values obtained with RT-qPCR method are overestimated compared to standard virus infectivity assays (Wang and Tian, 2013; Rönnqvist et al., 2014; Walker et al., 2019). For instance, Rönnqvist et al. (2014) reported 4-log reduction of MNV at a UV dose of 60 mJ/cm^2 with the infectivity assay, whereas just 2-log decline of MNV and HuNoV RNA levels was found at a UV dose of 150 mJ/cm^2 by the RT-qPCR method. The experimental D_{90} values of conservative surrogates (MNV, echovirus and caliciviruses) obtained via viability assay are reported to be in the range of 60–100 J/m^2 (**Table 1**).

Identification of Potential Surrogates for UV-C Inactivation

Validation of the UV-C inactivation kinetics of specific pathogens such as SARS-CoV-2 is not possible (without the use of appropriate surrogates) because of the need for sophisticated biosafety level (BSL)-3 containment, and to protect the researchers, and the public from health risk in environmental settings. For HuNoV, research on reproducible cultivable systems that obtain high titers are still on-going. Hence, criteria for the selection and application of surrogates are required to ensure that the surrogates mimic the behavior of the SARS-CoV-2 or HuNoVs under specific treatment conditions, while ensuring safety of personnel and also decreasing labor, cost and time. Also, surrogates are useful in process validation studies

at scale up that can reduce the uncertainties linked with UV-C dose measurement.

As seen from **Table 5**, the model predicted D_{90} value (∼21.5 J/m^2) of SARS-CoV-2 was comparable to MHV strains (non-pathogenic to humans) of the β-coronavirus group (∼21 J/m^2), higher than α-coronaviruses (TGEV, CCV, and FIPV) and γ-coronavirus (AIBV) (∼18 J/m^2). Also, since both SARS-CoV-2 and MHV are β-coronaviruses, MHV-strain A59 may show similar behavior under various culture conditions making it a potential surrogate for SARS-CoV-2 for UV-C inactivation kinetics and validation studies.

For HuNoVs, the predicted D_{90} values of all genogroups (69–89 J/m^2) were higher than D_{90} values of the reported caliciviruses (60–67 J/m^2) in our study, echoviruses (75 J/m^2), except being lower than MNV-1 (100 J/m^2) (**Tables 1**, **5**). Use of surrogates that exhibit similar or slightly higher D_{90} values to target pathogens can avoid the risk associated with improper inactivation, hence our results indicate that MNV-1 is the better choice (though conservative) to validate UV-C inactivation of all HuNoVs under laboratory experimental setup conditions.

In conclusion, a predictive genomic-modeling method was developed for estimating the UV sensitivity of SARS-CoV-2 and HuNoVs. Results of the model validation showed that the developed model had acceptable predictive performance, as assessed by mathematical and graphical model performance indices. We predicted the $\boldsymbol{D}_{90}$ values by conducting extensive genomic modeling. Although the parameters reported here may suffice to estimate the UV sensitivity, experimental research directed to address various knowledge gaps identified in this study is required to maximize the accuracy of predicted models. Additional parameters will be computed to the predictive model as needed, including terms for the presence of chromophores or UV absorbers and for possible UV scattering.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

BPe and AP conceived of the presented idea and wrote the manuscript. BPe developed the theory and performed the computations. BPo contributed to statistical analysis. BPe, AP, and DD'S contributed to the interpretation and discussion of the results. All authors contributed to the article and approved the submitted version.

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OPINION
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Corona Pandemic: Assisted Isolation and Care to Protect Vulnerable Populations May Allow Us to Shorten the Universal Lock-Down and Gradually Re-open Society

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INTRODUCTION

Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), the virus that causes coronavirus disease 2019 (COVID-19), has emerged as a major threat to mankind. The proportion of those dying from COVID-19 is highest in the elderly population and those with pre-existing co-morbidities (such as severe obesity, hypertension, diabetes, cancer, chronic respiratory, renal, or cardiovascular disease) (1–4). As individuals can spread the virus rapidly without exhibiting any symptoms, multiple countries have taken steps to shut down schools, universities, whole companies and businesses, and entire villages, towns, and countries have been isolated. These drastic measures will have enormous economic, public health, and psychological consequences (5–9).

It is likely that we are significantly underestimating the prevalence of COVID-19. The proportion of asymptomatic infected individuals has been estimated between 17.9% (10) and 51% (11), but could be as high as 80% (12). It has not been established whether transmission can occur before symptoms appear, but the virus has been detected in the stool of an asymptomatic child (13) and there are indications of transmission from asymptomatic carriers (14). In some countries, it may be too late to sufficiently "flatten the curve" (15) based on a universal lock-down strategy. Coercive measures could be counterproductive and erode public trust and cooperation (16). Moreover, it is of great concern that large-scale lock-down of society has many additional negative impacts. It is of critical importance therefore that more refined strategies are considered, which may help containing the pandemic whilst minimizing significant societal disruption, and help allocate resources in the most effective ways. Others have pointed out that, despite the breadth and allure of travel bans and mandatory quarantine, an effective response to SARS-CoV-2 requires newer, more creative legal tools but without clear recommendations on how to achieve this (17).

While certain countries start to re-open their societies and borders, most remain on lockdown measures to different extents. We suggest here that more selective assisted isolation of vulnerable populations would reduce the predictable increase in hospital admissions and more rapidly alleviate the fallout from total lockdown measures.

ALTERNATIVE APPROACH TO COMPLETE LOCK-DOWN

SARS-CoV-2 infection rarely leads to symptoms in people below 20 years of age (18) and usually causes mild symptoms in people up to the age of 50 years (19). Additional risk factors (2, 4, 20) negatively impact the outcome, and may potentially include high dose exposure in health care settings. Even though COVID19 sometimes leads to need for treatment at intensive care units (ICU) also for younger individuals, the virus appears most dangerous for a selected group of the most vulnerable people. In several countries, the average age of the deceased patients is around or above 80 years. We must consider diverting our major efforts to protect the vulnerable—elderly and patients with pre-existing comorbidities—by providing safe and assisted isolation and care; not least now that lockdown rules start to be relaxed. The vulnerable have to receive the necessary support to stay home, isolated, until it is safe again for them to return to normal life with social and physical contacts.

1. Identify and provide uninfected caregivers who do not spread the virus. Preferentially and ideally, these people will already have had COVID19 and have cleared the infection. With blood tests that measure antibodies against SARS-CoV-2 (21) we now have the tool to identify a majority of individuals that have had and cleared the infection. In some individuals the SARS-CoV-2 antibody response may be too be too low to be detected and before tests assessing T cell immunity become available for routine use all subjects with negative tests must be assured to be non-immune and tested by RNA-based tests that can detect SARS-Co-V-2 RNA in respiratory specimens. These tests must be performed routinely and regularly in people who assist isolated people. The ability to test large numbers of individuals, rapidly, repeatedly and effectively, for the presence of the virus, and for the existence of immunity is a cornerstone of this strategy. Certification of tests should be fast-tracked, as time is of essence here.
2. Educate caregivers on how to avoid spreading the virus, including hygiene rules and provision of personal protective equipment, including respiratory protective and risk mitigation measures.
3. Establish programs for home delivery of food, medications, and other essential items, to avoid unnecessary exposure, especially of the vulnerable populations. Clear protocols for handling and cleaning the delivered goods must also be established.
4. Provide shelter for those infected, isolating them from family members, and to those who are already in the proximity of infected family members to avoid transmission from asymptomatic family members to vulnerable ones.
5. In parallel, isolation of individuals with symptoms, diagnosed cases, and their contacts should continue (22).

These measures are per se not easy to accomplish but might be more efficient than the universal lockdown that is being pursued in different countries to different degrees.

HOW CAN WE MOST SAFELY AND RAPIDLY REVERT TO "NORMAL"?

Here, we face two main options and some potentially risky and tough choices.

A. The prolonged complete lockdown is not sustainable for an extended period of time due to its drastic and increasing economic and societal fallouts.
   - Even if successful, universal curfews would have to be implemented over many months, with unforeseeable consequences on society in many ways. Still, there are then billions of virus-naïve people who could potentially support new outbreaks.
   - The economic collapse with mass unemployment will have deleterious effects on health, including increasing mortality also in younger age groups. As an example, the much less severe economic turbulence of 2009 was calculated to cause the death of 260,000 individuals just by cancer (5), and the negative effects on health in the developing countries was very large (6).
   - In addition, these measures will, over time, destabilize society, not only through tremendous economic losses, but also through the risk of increasing social unrest and the psychological consequences of social isolation (7, 9). Selective damage to people with vulnerable job categories, particularly in countries without adequate social network safety, will put them in desperate situations and soon left without options (8).
   - Lastly, one could argue that a functioning economy and intact supply chains will better enable us to protect the more vulnerable and limit severe outcomes. We will then have enough hospital/ICU beds to take care of those who will need them.

B. Protect the vulnerable and then progressively ease overall restrictions. We must carefully consider and follow the emerging epidemiological data, especially the number of infections with severe outcome in younger individuals and those without preexisting conditions, before coming to premature conclusions. However:
   - the intensification of measures to protect the vulnerable must be implemented in priority.
   - Once these measures established, day-care centers, schools, and colleges could re-open, to care for young children whose parents are unable to provide full-time care due to

essential professions to prevent them from being cared for by grandparents, who need protection, not exposure.
- In the meantime, the general population of less vulnerable and mostly younger people should still respect physical distancing and hand hygiene standards. This includes having sufficient sanitizers provided in public places, e.g., in stores at checkout lines, or public transport. Anyone in this group who exhibits any potential COVID-19 symptoms should immediately follow the recommended self-isolation procedures and not return to society until after having been symptom-free for 2 days. Otherwise, they should live near normally, work, go to school, go to shops, and consume to prevent economic downturns.
- Economic support from governments and banks should be provided, especially for those industries/small businesses that experience a shortfall or have been forced to close down (i.e., travel, hotels, restaurants, cruises, artists, concert halls, etc.), because the vulnerable must stay isolated. Urgent funding is also required for hospitals, laboratories, and researchers to enable the fastest possible development of diagnostic assays and new therapies.
- Gradually then, a substantial proportion of the less endangered population will become infected by SARS-CoV-2 and develop immunity, leading to a gradual end of the epidemic (something that might already be happening in hotspots). More universal testing for active virus and anti-viral antibodies could then be used to determine when it would be time to advise the vulnerable to resume a regular and normal life again. We hypothesize that such a well-controlled shorter bubble could work in our favor, allowing resumption of societal functioning and resource generation until clinically proven treatment options for the critically ill and vaccines become available.

DISCUSSION

There have been several pandemics in recent decades such as the Asian Flew, the Honkong flew, The Swine flew, etc., with great losses of lives, but without the dramatic influence on societies as the present pandemic. The approach to contrast the COVID19 (or SARS-CoV-2) pandemic varies greatly among countries. Intensive testing coupled with tracking and isolation has at least so far indeed bent the curve so far in South Korea and New Zealand, possibly, also in Singapore, Hong Kong, and China (the latter with rather drastic containment measures). However, these measures have isolated subjects at risk, but have not increased immunization of the population with so called herd immunity through the transient infection of the less vulnerable. Hence, they still leave plenty of risk for re-emerging outbreaks, as increasingly reported. The strategy we propose is more sustainable in the long term, protecting the vulnerable population while we wait for herd immunity to be established, either through natural infection among the lower-risk population or a vaccine. Otherwise, society would be forced to remain closed, or return to lockdown.

Sweden has used a policy rather similar to our recommendations to protect vulnerable groups, without a

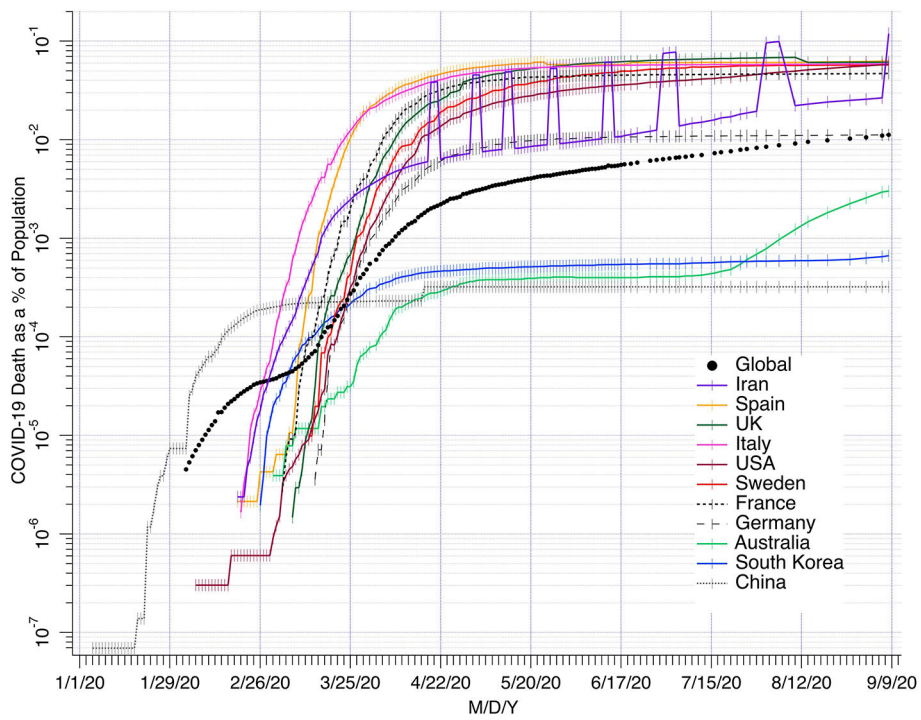

FIGURE 1 | COVID19 deaths as % of the population (vertical axis). Source: Center for Disease Control and Prevention CDC USA. The seven raised data points for Iran are from ncr-iran.org.

total lock-down of the society. Day-care centers and schools, shops, and even restaurants have remained open, while maintaining hygiene and physical distancing recommendations to slow down spreading of the disease. The COVID-19 curve has been flattened enough to maintain 20–30% of ICU capacity available (23). Difficulties to keep elderly completely isolated has caused loss of many lives, but mainly among people >80 years (median age 84 years for those who have died) with co-morbidities and limited life expectancy. Yet, the death rate has remained similar or sometimes even lower than in several other European countries hit by the epidemic at the same time (**Figure 1**). All curves tend to a slower rate over 8 months irrespective of the degree of lockdown measures implemented. One explanation for the rather similar death rates caused by the pandemic could be that in every country there are many undocumented mild cases that spread the disease and overall a degree of herd immunity is developing. Australia, due to strict lockdown now has an increase, and the numbers of new cases with Covid19 are increasing in several European countries with previous strict lock-down.

Although some economic depression has been unavoidable because of both decreased consumption and the dependence on global economy, according to the European Union the economy is expected to be less negatively impacted in Sweden than in countries with total lock-down measures.

In conclusion, we here offer some considerations on possible paths to ease out of restrictions with a focus on protecting the vulnerable, decreasing the load on hospital, health force and caregivers, and promoting immunity in the population to reduce the risk of future epidemics.

Politicians will have to face the natural unease accompanied with releasing restrictions under such measured conditions. Still, it is key to balance restrictions with the stage of the epidemic in certain areas and with the long-term impacts that broad and severe restrictions will have. Once we emerge from the acute phase of this tragedy, we will have to divert much of our resources to preventive measures to avert future impacts of emerging viral and bacterial infections.

AUTHOR CONTRIBUTIONS

JL and MvH wrote the first draft. DH, DS, and MF added some references. All authors revised the manuscript and approved the final version.

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The COVID-19 Pandemic and Outbreak Inequality: Mainstream Reporting of Singapore's Migrant Workers in the Margins

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Singapore saw a majority of COVID-19 infections plaguing low-skilled migrant construction workers by late April 2020. In the initial phases of the outbreak, mainstream frames were quick to highlight the gathering of low-skilled workers in open areas as sites to be surveilled, shaping the divisive practices of othering in early frames on migrant worker behaviors. These reports were manufactured as Singaporeans continued to gather in public spaces in large groups during the outbreak. Mainstream reports were quick to inform audiences of the surveillance and control on display for disciplining migrant workers. As the crisis developed to impact migrant workers predominantly, the erasures are recovered with mainstream press reporting worker vulnerabilities and discussing the structural implications of managing migrant labor as neoliberal subjects. The structural conditions of migrant labor were, in fact, centered in mainstream narratives, and dormitories as public health threats were extensively discussed, shedding light on discourses relating to outbreak inequality. The rights framework, however, remained largely absent in mainstream news frames about migrant workers.

Keywords: COVID-19, migrant workers, outbreak inequality, mainstream press, voices, culture-centered analysis

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INTRODUCTION

The media is a crucial source of information about migrants and migration, influencing how they are portrayed and constructed. Studying the mainstream media for representing or limiting the enactment of subaltern agency informs us the role of the media in shaping migrant narratives. According to Bleich et al. (2015), the media creates openings for migrants and minorities to engage in the public sphere that allows them to bring forward their representations, interests, and identities. This paper interrogates how the mainstream media shaped frames about low-wage migrant workers and the COVID-19 pandemic in Singapore.

In Singapore, despite the early onset and governance of the COVID-19 outbreak in January 2020, the nation-state came under significant scrutiny by international media for outbreak inequality among its migrant construction worker (MCW) community. By May 2020, MCWs made up 90% of infections in the country (Ng and Ong, 2020; Yea, 2020). In discussing the context of migrant workers and the COVID-19 infections in Singapore, Dutta (2020a) critiques the pernicious use of neoliberal techniques in the control and management of low-wage migrant workers. He argues that these techniques of labor management led to the rapid and exponential infections among this vulnerable group of workers.

Defined as extreme neoliberalism, the authoritarian management and governmentality of low wage migrant workers in Singapore (control, surveillance, and management) exacerbated infection rates within these community of workers (Dutta, 2020a). Through the adoption of neoliberal governmentality, the various stakeholders extract from the subaltern body maximum economic benefits, with limited freedoms to organize and activate for themselves. These techniques of management include policy frameworks that govern low-waged migrant workers (MCWs and MDWs). Regulations tied to work permits that bind them to a single sponsor (Parreñas et al., 2020), silencing dissidence from workers that protest these conditions, and disciplining their labor through strict controls via work permit terms, tight visa restrictions, and health surveillance (disease screening) are examples (Bal, 2015; Dutta and Kaur-Gill, 2018; Kaur-Gill and Dutta, 2020). Dutta (2020a) argues that this model of governmentality is exported to Asia as a gateway for transnational capital. The poor housing standards (cramped, unhygienic, unsanitary) such as dormitories (Hamid and Tutt, 2019; Dutta, 2020b) and the absence of a labor rights frameworks to protect migrant workers from exploitative conditions are other ways we see the techniques of neoliberalism exercised in the management of low-wage migrant workers in Singapore. Such neoliberal techniques of management create the rife opportunity for precarities and im(mobilities) for low-wage temporary migrant workers working in the city-state (Dutta and Kaur-Gill, 2018). These neoliberal tailored strategies of labor-management leave vulnerable workers to several exploitative conditions.

This paper, therefore, studies mainstream narratives in its discussion of migrants and their health during the COVID-19 outbreak in Singapore, theorizing the operating logics of the mainstream press in an authoritarian context of news production (George, 2007, 2012; Ortmann and Thompson, 2020). Wald (2008) positions the outbreak narrative during a pandemic as the curation of a myth by architectural scripts of science, in which journalistic stories impact and transform a new global order. Wald's (2008) points to the accumulation of contradictions that begin appearing in an outbreak, "…obsolescence and tenacity of borders, the attraction and threat of strangers, and especially the destructive and formative power of contagion" (p. 33), where belonging and citizenship are weaponized. The weaponizing of belonging and citizenship during a crisis have health impacts for those that are typically othered in society, due to their vulnerable social positions.

THE COVID-19 PANDEMIC AND LOW-WAGE MIGRANT WORKERS

Research during the COVID-19 pandemic caution how healthcare systems may remain neglectful of international migrant workers (Liem et al., 2020). For these workers, health services already remain difficult to access. Furthermore, pre-COVID-19 pandemic, international migrant workers already suffer a lower quality of health that is potentially further heightened during a pandemic (Liem et al., 2020). MCWs and MDWs make up ∼9.9% of the population in Singapore. At 555,100 workers as of December 2019 (Ministry of Manpower, 2020), in a population of 5.6 million, low-skilled migrant workers make up a significant portion of Singapore society. The literature on migrant worker mobility continues to indicate jarring disparities faced by migrant workers during the migratory process (Dutta, 2017a; Yeoh et al., 2017; Hamid and Tutt, 2019).

In Singapore, MCWs are hired in large numbers to partake in what is known as 3(D) labor, dirty, dangerous, and difficult (Dutta, 2017b), mostly working in the construction sector (Kaur et al., 2016). MDWs, on the other hand, come from various parts of South East Asia and South Asia, migrating to participate in caregiving work within the confines of Singaporean households (Dutta et al., 2018). Both MCWs and MDWs partake in precarious work with restrictive conditions. Ordinarily, MCWs partake in long working hours, dangerous work, are at high risk to workplace fatality, and reside in poor living conditions that create serious health concerns for this migrant community (Dutta, 2017a,b). Rubdy and McKay (2013) describe,

> their living conditions are invariably the most basic, as they are packed in overcrowded, squalid and unsafe premises that violate fire safety and land use laws, and that are located on the edge of the island's built up area, among derelict or industrial land, poorly served by public transport links (p. 160).

Living conditions of MCWs have been a site of extensive debate pre-COVID-19 crisis and further amplified during the COVID-19 pandemic. Dutta (2017a) laid out a range of structural conditions tied to labor that remain precarious to migrant worker health. During the COVID-19 pandemic, the structural conditions of migrant workers were amplified, with 90% of all infections in Singapore disproportionately afflicting the MCW community locally. Dutta's (2017a) findings "point toward how lived experiences with workplace safety are situated amidst the shifting contexts of work, attending to the overarching socio-cultural contexts of workplaces as well as spaces of everyday living" (p. 10). During the COVID-19 pandemic in Singapore, MCWs bore the brunt of the infections in the city-state, leading to a spotlight on the inequitable living conditions FCWs inhabit in Singapore (Yeung and Yee, 2020).

In providing a framework to make sense of the rise in infections, Dutta (2020a) surveyed $n = 100$ surveys and $n = 45$ interviews with MCWs living in dormitories. The findings located that most workers described the structural barriers faced in containing the infectious disease spread. MCWs discussed accommodation standards concerning how it afflicted workers from practicing social distancing. The acquiring of sanitizer and soap remained challenging to access. Finally, fear and anxiety of depressed wages and the infectious disease spread were conveyed as mental health concerns. Lee et al. (2014) study on health information seeking behaviors among the migrant workers living in dormitories, indicated that while a majority of MCWs were accessing healthcare services, there were challenges with limited knowledge on insurance plans and delays in accessing health facilities when required. To add, a

population of workers surveyed in the study highlighted that they would not seek treatment for serious health concerns. Harrigan et al. (2017) study on MCWs shared the relationship between migration and mental health in conversation with deportation threats made by employers when conducting construction work. These studies on MCW health make known to us the various ways health disparities are magnified for these communities of workers to the conditions of their labor. Baey and Yeoh (2018) further reinforce precarity and precariousness of migrant labor where a "growing vein of scholarship on migrant workers undertaking low paid, insecure and irregular jobs have shown how experiences of precariousness are inextricably linked to broader patterns of intensifying neoliberalism, labor market deregulation and the flexibilization of labor in post-industrialist societies" (p. 268).

For MDWs, unequal power relations such as living and working with their employers, create ambiguous and uncertain conditions for rest hours, privacy concerns, confinement and surveillance, mental health issues, food insecurity, and abuse (Dutta et al., 2018). Precarious and im(mobile) work conditions amplify health disparities for these workers. During the COVID-19 pandemic where Singapore rolled out its circuit breaker measures (Singapore's framing of a lockdown), NGOs such as the Humanitarian Organization for Migration Economics (HOME) saw a 20% increase in distress calls from MDWs during the circuit breaker relating to being overworked, not receiving enough rest and the sustaining of verbal abuse (Humanitarian Organisation for Migration Economics, 2020).

OTHERING OF MIGRANT WORKERS IN MAINSTREAM DISCOURSE

Mainstream reports are often the early storytellers of an outbreak and fundamentally shape the outbreak narrative. When a pandemic threatens a global population, (Wald, 2008; Mason, 2012) the new fear of a floating population that moves as mobile migrants, passing mobile germs, fuels the media practices of boundary building of nation-states. In the context of globalized neoliberal economies, the racialization of the COVID-19 pandemic moves beyond just race alone, but interplays within the context of mobility and migration, where the movement of racial people as transnational citizens are centered in the discussion of epidemics (Briggs, 2003; Mason, 2015). With disparities in migration and mobility, pandemics become moments of governance of biosecurity threats by nation-states, rendering unequal health effects when the outbreak manifest (Briggs and Hallin, 2016; Sanford et al., 2016).

For example, Maunula's (2017) discourse analysis of the Canadian media on the H1N1 outbreak revealed, "expansion of "risk space" makes possible a particular kind of "pandemic subject" which operates as a neo-liberal bio-citizen" (p. ii), referring to risk space as the expansion of H1N1 spread by mobile citizens. In a study by Warren et al. (2010), media frames of the H1N1 pandemic reveal the role of the traveling body as a central actor in media discourse. Othering practices surfaced in discourses that centered the mobile person, where the frames of the "airport as a site for control and the ethics of the treatment of the traveler as a potential transmitter of disease" (Warren et al., 2010, p. 727). Dutta (2016) suggests that the militaristic global operation to contain the SARS virus amplified racist depictions of the Chinese cultural practices in health communication messages. Lee et al. (2005) inform how media portrayals of geographic borne spread contribute to the stigma of specific peoples in a way that is not proportionate to the risk as pandemics arouse stories of the omnipresent and its mysteries. Specifically, discussing the structural conditions of migrant labor in Singapore during the COVID-19 crisis, Dutta (2020a) argues that the "extreme neoliberal model of pandemic management" coupled with the authoritarian tools of disciplining and surveilling migrant labor, intensified the COVID-19 infections disproportionately for this marginalized population.

Not only are the structural conditions of labor for MCWs and MDWs impact health outcomes of workers, but migrant workers also remain erased from mainstream society by mainstream discourses. In discussing, public health and migrant workers, Grove and Zwi (2006), pay attention to how othering theory contributes to the subjugation of migrants in dominant discourse. The techniques of othering are meant to keep out subjects to not belong to the mainstream, naming the other, and mark the difference to oneself (Dervin, 2012). The creation of difference is aimed at stigmatizing the other as divergent and peculiar, "to reinforce notions of our own "normality," and to set up the difference of others as a point of deviance" (Grove and Zwi, 2006, p. 1933). Grove and Zwi (2006) argue that the implications for othering migrants and reinforcing their marginal social position have downstream effects for public health. These include erasures of voice in mainstream discourses, where their stories remain unheard (Breen et al., 2006; Grove and Zwi, 2006). Tan's (2014) research on stereotypes in conversation with the integration of migrant workers in Singapore revealed that the mainstream press disseminated both positive and negative stereotypes of migrant workers. Negative stereotypes included "migrant workers as dangerous people" (p. 174) and "migrant workers as people with low integrity" (p. 174), threatening local livelihoods. Positive stereotypes circulated in the mainstream press suggested that migrants were necessary labor for the local economy.

Cheng's (2016) study of migrant workers in broad media entities in Taiwan often rendered workers as mere numerical entities, "…that treats this group as a faceless collective existing for the market need" (p. 2514). The absence of worker voices amplifies the degrading portrayal of migrant workers via the media through negative representations (Magpanthong and McDaniel, 2016; Mintarsih, 2019). Mintarsih (2019) suggested a lack of space for domestic workers both in their host and destination countries to form their narratives in dominant discursive spaces. The transience of migrant workers in state rhetoric contributed to how they are discursively constructed as the other in the media (Hamid, 2015; Kaur et al., 2016). Rubdy

and McKay (2013) in studying language ideology and migrant work in Singapore suggests that the circulation of the nation as house metaphor,

> lends logic to the portrayal of immigrants as "filth" that needs to be swept out for the house to be kept clean, strongly reminiscent of the colonial's derogatory references to the subaltern native as "dirty," "filthy," "degraded," and "debased" (p. 165).

The social position of migrant workers in discourse and everyday lived experiences cannot be divorced from the poor structural conditions and health disparities faced by migrant workers.

Media Frames and Migrant Workers

Kaur et al. (2016) studied the role of the mainstream press in reporting the Little India Riot. It refers to a conflict that took place between MCWs in Singapore and the police on 8th December 2013, after a fatal bus accident killed a MCW. This led to a confrontation between workers and the police in the Little India area where MCWs congregate on their off days. The mainstream press centered voices of state actors in delivering a cultural explanation regarding the cause of the riot. Migrant worker voices remained predominantly absent in framing of the riot in dominant discourse, where voices of rioters were "violently silenced," according to (Tan, 2016, p. 9) by mainstream narratives. The mainstream press re-circulated cultural tropes of South Asian FCWs that reinforced policies that sought to surveil, discipline, and re-arrange migrant worker bodies in various peripheral spaces across Singapore (Goh, 2019). Goh (2019) goes on to "argue that the 2013 riot by migrant workers accelerated the production of dormitory space to exclude migrant workers from access to the city and reproduce their physical needs" (p. 356). These dormitories become a dominant point of discussion during the COVID-19 outbreak concerning MCWs. Kaur et al. (2016) concluded that mainstream narratives "left absent in the frames were the alternative narratives grounded in the voices of the FCW (Foreign Construction Workers)" (p. 27).

Kaur-Gill et al. (2019) study on the media representations of MDWs revealed once again the absence of migrant worker voices in the mainstream press, "…voices of FDWs in the reporting of the news are often rendered absent, and when they are represented, they are often quoted to reinforce FDW policies set by the state" (p. 12). In discussing these representations located in The Straits Times, the dominant discursive space renders FDW voices peripheral, with civil society actors acting as proxies for FDW advocacy. While discussion on migrant labor and domestic workers were centered, the margins as sites for speaking remained erased. The othering of migrant workers in Singapore as outgroups are rooted in its post-colonial identity (Goh, 2008; Anderson, 2013; Dirksmeier, 2020). Bal (2017) discussed media representations of migrant workers as fraught, as victims, or as threats to Singapore society. The criminal representation of migrant workers surfaced throughout the 1990s (Bal, 2017), similar to Cheng's (2016) findings of migrant workers in Taiwan as represented through criminality. However, in Singapore, these representations were shifted when state voice actively dismissed these stereotypes due to the state's reliance on migrant workers to develop its local infrastructural economy (Bal, 2017).

Migrant worker rights in dominant discourse have been mostly absent. Goh et al. (2017) posit the limitations of a rights discourse in illiberal contexts, illiberal contexts referring to authoritarian governance that limit freedoms and coercively subdues dissent through institutional, state, and legal entities. According to Goh et al. (2017), the human rights discourse has limited appeal in changing state behavior toward the treatment of vulnerable migrants in places like Singapore. In studying the framing of the day-off campaign in Singapore by civil society actors, they purport the success of the campaign because it used a "cultural mediation strategy of vernacularization" (p. 89), where rights claims were framed in a manner that fit "with the institutional logics and cultural repertoire of Singapore society" (p. 89). However, (Goh et al., 2017) conclude that migrant workers must ultimately participate in advocacy about them, centering migrant voices in mediating for themselves. Where migrant worker voices were activated in advocacy efforts, the rights discourse framed the central tenets of advocacy (Dutta et al., 2018). Dutta and Kaur-Gill (2018) argue that without a rights framework in dominant mainstream discourse, the seductive lure of neoliberal practices will continue to locate itself in migrant labor management and governmentality.

THE PRESS IN SINGAPORE

The press in Singapore broadly falls under the Singapore Press Holdings (SPH) and the broadcasting wing under Mediacorp. While not government-owned, the SPH is primarily influenced by the dominant political leadership in the country (George, 2012). Mediacorp, the broadcasting arm of the news, operates through grants and subsidies provided by the state (George, 2012). While SPH runs the Straits Times (largest English language newspaper) and The New Paper (packaged as a tabloid press), Mediacorp runs the Today newspaper (George, 2012).

Ranked at 158th on the International Press Freedom Index (Reporters without Borders, 2020), the mainstream media in Singapore is said to operate through various steps of control by the state (Tey, 2008), exercised through "political and punitive coercion" (p. 884). George (2012) conceptualizes the press as managed through calibrated coercion, where "Singapore can be seen as a textbook case of a state that has adopted a long-term view of power, deliberately reining in its use of force in order to build ideological consent" (p. 96). Tey (2008) discusses the state of the press in Singapore as pragmatic, where news is manufactured to pivot nation-building ideologies. Fundamentally, the press remains in harmony with the state, often working in consensus with state ideology (Tey, 2008; Kenyon, 2010; George, 2012). Therefore, the production of news is said to be deeply rooted in the machinery of the state. Critics and academics suggest that it remains challenging for journalists to operate through the Fourth Estate with limited freedoms of information laws, and "…a monolithic and cohesive state machinery that is not prone to leaks" (George and Venkiteswaran, 2019, p. 23). Out of bound (OB) markers

determine what can and cannot be reported in the mainstream press (George, 2012). OB markers in Singapore (George, 2012) refer "… to the boundaries of political acceptability (p. 65)" in journalistic reporting and remains a central aspect of what is considered acceptable and responsible reporting by the state. OB markers are present because of the state's belief that journalism should be about shaping a core national identity and setting societal norms and agendas (George, 2012). The management of journalism in Singapore, according to George (2012), is sophisticated and calibrated through ideological control, rather than the use of overt coercion. George (2012) concludes that we cannot "overlook the power of ideology, and especially economic incentives, as tools of cooptation and control" (p. 25) when studying the news media in Singapore.

In an authoritarian context, the press takes a leading role in conveying public health messages (Schwartz, 2012; Basnyat and Lee, 2015). In Singapore, (Deurenberg-Yap et al., 2005) revealed that there was high trust in the state and its institutions in the management and knowledge transfers of the epidemic, despite limited knowledge about control measures and the SARS virus itself by Singaporeans. Kleinman and Watson's (2006) study of SARS in China, discussed the role of the media in a contentious relationship with the state, where issues of transparency and information control remained central in heightening the outbreak, revealing detrimental impact for news reporting. Leung and Huang's (2007) study of western led English media (including The Straits Times) on the coverage of SARS by China found that the ritual of othering was entrenched in Western media representations, reflecting the power dynamic present even as journalistic responses should remain balanced and accurate. The study found that the post-communist state was labeled inaccurately and unfairly in the handling of the SARS crisis, othering its political structure negatively.

FRAMING

Entman (1993) attempted to conceptualize framing amid a fractured understanding of the theoretical concept. How texts are communicated in the transfer of information by the media requires systematic analysis. Framing refers to how texts are selected and arranged for salience to communicate a "problem definition, causal interpretation, moral evaluation, and/or treatment recommendation for the item described" (p. 52). Entman (1993) states that the arrangement of text that both center sources of information, pose issues as problems, articulate stereotypical positions, and reinforce keywords tell us how frames are produced. Similarly, the rendering absent of positions also frame for readers schemas that inform how and what to think about specific issues. For example, Poirier et al. (2020) adopted framing analysis to make sense of how the Canadian media framed the COVID-19 pandemic. The study revealed that there were different key frames in the framing of the pandemic by anglophone media (Chinese outbreak frame) vs. francophone media (economic crisis frame).

Health communication frames by the press have been critiqued by Seale (2002) and Dutta (2008) for not merely portraying the known facts of viruses, but producing knowledge that centers specific racialized actors and engaging in information injections that politicize the health crisis. During the COVID-19 outbreak, Atlani-Duault et al. (2020) have called for health communication researchers to understand how perceptions in online discourses in shaping virus blame must be studied to mete out more robust health communication responses. Studying frames of mainstream reports give us rich insight into how invisible viruses conjure public health emergencies as racialized threats. In interrogating the role of the media in shaping discourses about migrant workers and public health, these research questions are posed, how did the mainstream media frame migrant workers during the COVID-19 pandemic? Whose voices were anchored in the discussion about migrant workers? How was migrant health discussed in the context of media as structure?

CULTURE-CENTERED MEDIA ANALYSIS

The culture-centered approach (CCA) studies the interactions of culture, structure, and agency to unpack issues of health marginality. It is predominantly offered as a meta-theoretical framework to study marginalized populations through ethnographic methods (Dutta-Bergman, 2004). However, the intersections of culture, structure, and agency have theoretically guided textually centered data in the analysis of discourses as well (e.g., Sastry and Dutta, 2012). For this study, the CCA provides theoretical input on the way communicative inequalities play out in the media's talk about the health of a subaltern group. With the CCA's roots deeply etched in the critical project of health in the margins (Sastry et al., 2019), the CCA is expressly concerned with identifying and mitigating asymmetries of power and control within spaces of knowledge production that are related to "health inequalities, the loss of health status, and the concomitant erasure of the voices and agendas of marginalized communities across the globe" (p. 2). The ontological commitments of the CCA theory are in unpacking how health is spoken about and defined, how health is discussed, and expressed by structural actors that limit the input of subaltern voices (Dutta, 2008).

The CCA provides a theoretical lens in making sense of how communicative inequalities play out in media infrastructures in the discussion of health about subaltern groups (Dutta, 2016). Several studies have adopted the theoretical tenets of the CCA to explore textual data relating to health, including media frames. Sastry and Dutta (2011), for example, critically interrogate media frames in the US mainstream news media in the construction of the Indian context of HIV/AIDS. Similarly, Khan (2014) studied media messages of public service campaigns about HIV/AIDS, adopting a textual analysis to unpack the discourse critically. Khan's (2014) study indicates the critical impulse required to show how messages reinforce the domination of subordinated groups. The CCA pays attention to how subalternity is created,

perpetuated, and supported by the mainstream health discourses (Dutta, 2008). Mainstream media entities are one of the ways power structures determine the shaping of dominant health discourses in society. With the CCA providing a framework for a critical project, the mainstream is studied for how it reproduces absences, calling for us to pay attention to subaltern erasures in the circulation of dominant discourses about them.

In using this approach, this paper studies how mainstream discourses in the mainstream press reproduce communicative inequalities about migrant workers and their health disparities in Singapore during the COVID-19 pandemic. The mainstream media infrastructure is read and interpreted as structural, where "discourse constitutes material inequities, justifying the poverty and silences at the margins of contemporary mainstream political economies" (Dutta, 2011, p. 14). Mainstream media is constituted as a medium of power, a shifting, dynamic, and a contested space where different actors in society clamor for spaces of representation (Dutta, 2011).

The mainstream press in Singapore serves as a conduit that circulates discourses entrenched in nation-building ideologies (Bokhorst-Heng, 2002). On migrant workers, the authoritarian neoliberal management of migrant labor is key to manufacturing the profitabilities to Singapore's economy in dominant discourse; hence, their surveillance (Kaur et al., 2016). A CCA critique would position the mainstream media as occupying a dominant state voice in its media messages, rendering absent the margins (migrant workers) in which it seeks to discipline and control. The critical bent of the CCA serves to critique the role of such mass media entities that are built into the logic of circulating dominant ideologies of health and social change. In using this alternative lens to make sense of media infrastructures, the paper employs a culture-centered media analysis that critically pays attention to how the media deploy erasures and absences in their reporting of migrant workers. Also, how agentic articulations of the margins are activated, represented, and portrayed by local media infrastructures. By theorizing media as structure, the CCA acts as a lens for how the relationships of power are sustained and re-circulated via media messages. The CCA reads a Marxist interpretation of structures, as institutions in our social system that create functions of inequity both materially and discursively for the vulnerable, and as sites that hold significant institutional power, erasing subaltern sectors from conditions of impoverishment (Dutta, 2011). Therefore, the CCA theorizes media institutions' as structural entities. Power is a central point of understanding the organization of media entities with the media structure co-opted by the state to serve its agendas. Thus, the hegemon of the mainstream media in Singapore is a potent site of erasure regarding discourses concerning the most voiceless and marginalized in Singapore society, leaving the margins intact when they remain unheard. How the othering practices of the mainstream media create further vulnerabilities for the margins require critical interrogation when dominant discourses translate into material disadvantages for the margins. With mainstream press as structure, are there openings for creating spaces for structural transformation by narrativizing the margins to be represented? How does the media organize structures in their storytelling of migrant labor? Communicative inequalities in the CCA refer to the absence or erasure of discourses from the margins, but also co-constructing discourses in the margins by prying open dialogic spaces, envisioning erased sites where communicative social change can occur (Dutta, 2011).

METHOD

The study adopted a grounded theory analysis of $n = 390$ articles from three English-language mainstream newspapers in Singapore (The Straits Times, Today, and The New Paper). The article corpus was located through a search via a Factiva database using the terms "Wuhan Virus," "Coronavirus," "COVID-19," and "migrant workers" from 5th January 2020 to 9th May 2020. The search paused on 9th May as episodic frames (infection clusters), and thematic articles (e.g., food, accommodation, treatment, structural conditions, treatment, voices) about migrant workers reached saturation with no novel categories emerging. Removed from the analysis were articles that did not centralize migrant workers in the Singapore context. This study specifically looked at low-skilled migrants (domestic workers and construction workers). Therefore, all articles that did not pertain to these two groups of migrant workers were removed from the search criteria. Other redundancies include articles related to migrant workers not located in Singapore or when migrant workers were mentioned for peripheral reasons (e.g., in an upcoming story).

The purpose of the study was to interrogate how migrant workers were constructed in the context of the COVID-19 crisis and public health in Singapore. Articles included forum letters, opinion pieces, and letters by the editors were all considered for analysis as they are central in the manufacture and production of dominant discourses about migrant work in Singapore.

DATA ANALYSIS

With the evolving COVID-19 crisis, it remained integral to study how the news shaped discourses about migrant workers to unearth new themes and frames positioned by the media. In adopting grounded theory to analyze news articles for theme development, this research was able to move beyond the already established categories on migrant worker frames (e.g., Hilsdon, 2003; Kaur et al., 2016; Kaur-Gill et al., 2019). Grounded theory analysis assisted with establishing inductive insight without pre-conception on themes and allowing for more significant discovery of how subaltern voice was erased or represented (Charmaz and Belgrave, 2007). By immersing, paragraph by paragraph, in examining the media content, I was able to detail both the patterns of voice were represented, along with the erasures. Erasures were coded for each article (absence, presence, and salience) when migrant worker voices were quoted in the news article (e.g., quotes, soundbites, names, visibility). In **Table A1**, I provide an illustration of the coding process.

In connecting the open and axial (meanings to categories), conceptual ideas begin concretizing, developing the selective codes in the analysis (Charmaz, 2006). The axial stage involved studying carefully why certain concepts were discussed or positioned in particular ways, and how were specific meanings of interactions applied to the text. Part of the process included making sense of relational categories. The process allowed me to analyze the dialectics of discourses, representations, noting how the erasures of subaltern voice played out. At the selective coding stage, core categories remain clear in centralizing the discussions and tensions that appear (Bryant, 2017). The illustration of the coding process is reflected in **Figure A1**. A culture-centered analysis takes place at the level of selective coding conversation with grounded theory foregrounds the interplays of culture, structure, and agency in the construction of margins. Specifically, in the context of migrant health and well-being, a culture-centered analysis teases out the workings of structure, culture, and agency in the constructions of migrant health, where the analysis foregrounds how voice (agentic articulations) play out, attending to how migrant voices emerge and are centered in stories.

Three key themes are reported in the findings, Theme 1: Reporting the Other as Public Health Threat, Theme 2: Othering Cultures and Habits, and Theme 3: The Other Speaks Back. Emergent themes were discussed in conversation with CCA theorizing of communicative inequalities as structural inequalities, making sense of voice as agentic enactment in how mainstream discourses addressed quotations from various actors in its stories. The emergent themes were theorized using CCA's culture, structure, agency nexus on communicative absences, inequalities, and voice. Saturation of the conceptual categories were achieved when the media continued centering structural explanations in dominant stories about migrant workers and their health during COVID-19.

FINDINGS

Theme 1: Reporting the Other as Public Health Threat

Migrant worker frames surfaced increasingly in media reporting during the COVID-19 crisis due to infections primarily afflicting the MCW community in the months of late March, April, and May 2020. MCWs were discussed as infection victims in mainstream reports. The structural conditions of migrant labor were surfaced in light of the surge in infection rates by the MCW community. The structural conditions of labor were only discussed when infection rates surged exponentially, crippling Singapore's international reputation as a model nation in the management of the COVID-19 pandemic in the earlier months. In late April and early May 2020, headlines regarding the structural conditions of migrant labor begin appearing, "Workers' dorms step up measures after 3 clusters emerge" (Yang, 2020) discussing their living conditions as a public health concern. Food received by migrant workers were another point of conversation, "Over 10 m meals served to foreign workers confined to dorms" (Tan and Ang, 2020). Finally, the crisis of infections among the MCW community took up mainstream news cycles at length. The conditions of migrant workers surfaced; specifically, the positioning of structural factors relating to migrant labor is attributable to the competing voices leveraged by the media in the championing for better treatment of MCWs discussed in Theme 3.

Separate categories on the rate of infections were reported for MCWs in the mainstream press. Community cases included all other categories of residents. The mainstream media obediently picked up these categorizations after state actors positioned for the press the discourse on two outbreaks, "Singapore facing two separate outbreaks: in the community and foreign worker dormitories" (Khalik, 2020). Reiterated in multiple reports:

> Mr. Wong added that Singapore is "dealing with two separate infections" – one happening in dormitories where numbers are rising sharply, and another in the general population where "numbers are more stable for now" (Phua and Ang, 2020, para 6).

The separation of MCW infections from the general population were conferred to be epidemiologically necessary with an academic expert cited to clarify the separation:

> Dr. Jeremy Lim, co-director of global health at the NUS Saw Swee Hock School of Public Health, said on Wednesday (May 6) the Government's framing of Covid-19 as two separate outbreaks—one in foreign worker dormitories and the other in the community—was a "defensible" one from a public health perspective (Ho, 2020, para 2).

The argument positioned in the mainstream press revealed that due to the different strategies employed in managing workers' residing in dormitories, the infection categorizations as separate for MCWs versus residents were deemed unavoidable. The separation of categories, however, led to the discursive constructions of COVID-19 infections as largely othered in mainstream frames. Mainstream press centered state voice in positioning why the outbreak required the narrative of two curves, contributing to how MCWs were othered. As subjects for exclusion, workers remain relegated to the peripheries of the mainstream discourse even during the COVID-19 outbreak. The press re-circulated the techniques of othering by reinforcing the dual reporting of the infections instead of presenting datasets as national figures. The press did not critically interrogate how risk discourse is political, where limiting the communication of risk to the general public becomes a political strategy. Therefore, the communication of epidemiological insight is political, where the segmenting of a population to mark out difference during an outbreak is politically strategic (Lupton, 1993). How science is deployed for communication remains politically situated during a pandemic. Nevertheless, the mainstream press continued to reinforce the duality as necessary, carving out the peripheries of infections for its readers.

Information inequality among migrant workers was another frame that emerged in the early reporting of the pandemic and infections among MCWs. An article frames the narrative of information inequality specifically relating to health information seeking as being fraught for migrant workers. Citing an

NGO spokesperson that "the language barrier makes it difficult for migrant workers to follow news developments about the outbreak. Many of them get their information secondhand, not always accurately" (Ho, 2020). The press begins cautioning readers about the challenges faced by migrants in health information translation. The othering practices in the representation of migrant workers as targets of misinformation or misguided information are selected. The mainstream press was also quick to report migrant workers as targets of fake news and misinformation:

> With fake news spreading faster than the coronavirus itself among foreign workers, the Migrant Workers' Centre (MWC) has been relying on its network of volunteers who are also workers themselves to disseminate accurate information on the ground in a timely manner (Zhuo, 2020b, para 2).

Disciplining the Migrant Other

Early reporting also focused on MDWs singling out the community for congregating on their off day, "Maids, employers get coronavirus advisory amid reports of foreign domestic workers denied rest days" (Menon, 2020). The surveilling and disciplining of MDW bodies were picked up by the mainstream press in anchoring state voice in the distribution of advisories. In the early onset of the outbreak, the frame of disciplining the migrant other emerged in mainstream news. The frame remained the earliest shaping of the news regarding migrant workers relating to the COVID-19 outbreak. In tracing the discourse, mainstream press, specifically, The Straits Times begin circulating news on the surveilling of FDWs on their off day "Maids, employers get coronavirus advisory amid reports of foreign domestic workers denied rest days" (Menon, 2020) and "Advisory issued to maids, employers on rest-day arrangements" (Menon, 2020). Before the majority of infections were found to disproportionately afflict the MCWs (Ng and Ong, 2020), early media reports focused their attention on MDWs off days:

> The advisory said households who have young children, the elderly or those with illnesses or special needs at home, may be concerned with the risk of transmission should their FDWs become infected while out on their rest day (Menon, 2020, para 5).

In this report, the mainstream press picked up on a state advisory cautioning against MDWs spending their day off in outdoor spaces. The day-off remains a contentious issue for MDWs. Only in 2013 were FDWs granted a mandatory day off, and this continues to be a site of negotiation for MDWs with their employers (Dutta et al., 2018). These advisories were followed up with notices to not go out on their off days, with Ministry of Manpower (MOM) officers breaking up migrant worker congregations, "Coronavirus: MOM triples ground efforts to disperse migrant workers who gather on Sunday" (Zhou, 2020b) and "Coronavirus: MOM will revoke work passes of migrant workers in large gatherings if they refuse to disperse" (Zhou, 2020a). This frame strongly cautions against migrant worker gatherings, urging "On Wednesday, MOM advised foreign workers to remain in their residence on rest days, and said their employers and dormitory operators should "educate" them on this" (Today, 2020). Disciplining the migrant other was seen in how the mainstream press, more specifically The Straits Times, remained unquestioning in their reports about why FDWs were subjected to such disciplining and surveillance when local Singaporeans were not. Two days later, on 11th February, a headline by Today, "Despite MOM's advisory, some employers don't intend to keep their domestic workers at home" (Today, 2020), begin critically engaging with various voices on these advisories. The article centered migrant voices as following social distancing rules yet were told to disperse by officers:

> Ms. Ana Liza Dazo, 38, from the Philippines, was at the Kallang field to have lunch with her friend after remitting money nearby. "It's our day off and our employer said we could head out while taking care to observe the precautions," said. These precautions included eating separately, not sharing utensils or drinks, and sitting more than 1 m apart. But this did not stop officers from asking her to pack up and go home (Zhou, 2020b, para 15).

Mainstream press while quick to report these advisories issued by MOM, centered alternative voices that touted such advisories as otherizing FDWs:

> One employer Rose Awang, 57, told TODAY that it would not be morally right to restrict her domestic worker from going out even during this public health crisis. We go out very often. Children go to school every day. Why can't she go out," the real estate professional asked" (Lim, 2020, para 9).

Here the article refers to a lack of restrictions on gatherings for residents, but advisories were issued to domestic workers not to head out or congregate on their rest days. The above quote positioned in the article signals to readers through employer voice how these contradictions surfaced. In othering workers, the disciplinary mechanisms issued by the state remained salient in the discussion of migrant workers in the mainstream press during the outbreak. These disciplinary strategies that adopt the language of health surveillance is a tacit strategy that has been employed to surveil the bodies of migrant workers that perpetuate their im(mobilities) in the host country (Dutta and Kaur-Gill, 2018).

Benevolent Actors and the Grateful Other

With migrant workers centering news articles in more pronounced ways by April 2020 due to the surge in the number of workers testing positive for COVID-19, othering techniques framed the role of the state and the migrant worker in mainstream reports. Reports included "Minister shares migrant worker's note of heartfelt thanks" (Cheong, 2020), where media reports shared the voice of state actors in highlighting worker gratefulness for aid received during the COVID-19 outbreak,

> A Bangladeshi worker's heartfelt note thanking the Singapore authorities and those helping migrant workers deal with the Covid-19 outbreak was read out in Parliament yesterday by Manpower Minister Josephine Teo. Mrs Teo had highlighted the worker's gratitude after pointing out efforts by the Government,

> community groups and companies to help migrant workers here (Cheong, 2020, para 1).

State benevolence as frames in the mainstream press reflected the state's magnanimity for workers suffering in the peripheries of the city-state. Such frames were articulated periodically in the mainstream press. For example, the mainstream press presented recovering workers in stark contrast to dormitory conditions, "there are en suite toilets, in-cabin dining and strict infection control and safe distancing measure aboard the ship, as well as Wi-Fi, in-cabin entertainment and scheduled outdoor time" (Phua and Ang, 2020). These articles parlayed how the state managed and looked after MCWs during the recovery phase from COVID-19 on cruise ships. Mainstream frames were also quick to balm the conversations on the mediocre to poor quality of food received by MCWs. State voice were centered in frames about food such as the provision of food for migrant workers during isolation and confinement:

> He (state actor) said there are 34 professional caterers providing meals to about 200,000 workers—akin to catering for the whole of Ang Mo Kio GRC. The Government is footing the bill for all meals in purpose-built dorms. It is not clear how much the caterers are charging but one of them, Neo Group, said it charges only for ingredients and labour costs (Tan and Ang, 2020, para 3).

NGOs and volunteers were highlighted for their acts of benevolence shown to workers,

> Foreign workers have been the focus of this year's May Day celebrations as they make up a disproportionately large group of Covid-19 patients, and government leaders have assured them their health and other needs will be taken care of. Public agencies are also working with non-governmental organisations on these efforts (Yeoh et al., 2020, para 5).

> Singaporeans from all walks of life also stepped forward to help, like Project Belanja, a community project to provide meals to migrant workers. "It is this resilience and cohesive spirit of our Singapore society—individuals, volunteers, front-line workers, businesses and many others—that gives me the confidence that we will overcome Covid-19 and emerge stronger" (Lim, 2020, para 10).

A variety of articles centered the efforts of NGOs and the local community in differing efforts to assist the disenfranchised migrant, "More than 35,000 masks have been sewn and will be distributed to workers through the Migrant Workers' Centre from this week" (Teng, 2020, para 17). Articles continued to portray the benevolence of the local community in stepping up for migrant workers, implicitly portraying migrant worker subjects as the "helpless other". These frames of benevolence failed to interrogate the inequities faced by MCWs. The wearing of masks became mandatory by 14th April 2020 (Tay, 2020). The mainstream media had reported the provision of masks MCWs on 19th April 2020 in framing benevolent actors. These frames were manufactured to include how local corporate entities were stepping up for migrant workers, "A total of 400,000 migrant workers and 250,000 domestic helpers will receive face masks as part of efforts to improve the safety of foreign workers, said Temasek Foundation on Sunday (Apr 19)" (Today, 2020), without interrogating the discrepancies of mask distribution in the onset of the outbreak. Corporate and NGO entities stepping in to provide for the helpless other continue to circulate frames that remain uncritical of the state's management and response toward MCWs. This meant also centering advocacy efforts within the scope of service delivery, rather than narratives of structural overhaul. The free masks distribution exercise for residents were completed on 12th April 2020 with the eligibility criteria clearly stated as "Every resident with a registered home address can collect one (1) Reusable Mask each from collection points at designated Community Club/Centres (CCs) and Residents' Committee (RC) Centres" (Gov.sg, 2020). The mainstream media did not clarify if these residents included with dormitory addresses.

Separately, on 22nd March 2020, Today centered another story on migrant worker NGOs, titled "Covid-19: Sharp decline in volunteers for non-profit clinic, but migrant workers have reason for cheer" (Today, 2020), pointing to how grateful the workers were to be able to access healthcare through telemedicine, "Whether they get to see a doctor in person or not, migrant workers TODAY spoke to are simply thankful that they could still come to the clinic" (Today, 2020). Workers are depicted as grateful subjects, play to how the devices of othering operate in shaping outsiders and therefore, grateful subjects to the host country. The grateful subject is located within voices by state, NGO, and local actors.

It is noteworthy to mention that even when operating tropes of the "grateful other," migrant worker conditions remained salient throughout news reports discussing benevolence. For example, despite re-narrating state voice on ensuring worker conditions improve, the mainstream press also framed the narrative of the grateful other concerning the poor conditions faced:

> The Government has been criticised recently for the quality, quantity and type of food served to workers under lockdown. Several hundred thousand foreign workers have been confined to their dorms or other places of residence as part of efforts to curb the outbreak (Cheong, 2020, para 3).

The Straits Times reporting reintroduces frames on the poor conditions faced by workers even when centering frames of the helpless subject as the grateful other. Here the press re-circulated the continuing issues consistently on the questionable quality food received by MCWs. In reports about migrant workers, mainstream press shared, "The recent spike in coronavirus infections among migrant workers in Singapore has put a spotlight on the living conditions of a foreign workforce long invisible to many" (Ho, 2020). Thus, while mainstream press continued to uphold the state's efforts in combating the crisis with migrant workers and the COVID-19 outbreak, mainstream press,

also pushed the OB markers, by constantly and consistently re-visiting frames regarding the structural conditions of labor via NGO voice or expert voice:

> In a May Day statement, Home called for a close examination of the systemic issues affecting migrant workers and to take concrete steps to better their working and living conditions, and change biases and attitudes toward them (Yeoh et al., 2020, para 11).

> Dr. Imran Tajudeen, from the Department of Architecture at the National University of Singapore, cited the lack of social integration of migrant workers, who may be housed in dormitories on the peripheries of society (Yeoh et al., 2020, para 14).

Frames on structural conditions surfaced and re-surfaced even in the presentation of the state and society's benevolence to the migrant subject. As previously discussed by Goh et al. (2017), these structural conditions were often always parlayed in tandem and not antagonism to state voice. Hence, the focus on their benevolence. The production of benevolence on frames about structural conditions operate to cooperate with the institutional logics of migrant worker advocacy. A rights framework remained absent in frames about migrant workers and the COVID-19 pandemic. Even when fundamentally advocating for a rights framework through expert voice, "Legalising a framework for worker-led and worker-owned unions and groups that represent the needs of low-wage migrant workers" (Wong, 2020), the direct pronouncement of a rights discourse were absent.

Theme 2: Othering Culture and Habits

The mainstream press published forum articles that singled out MDWs as subjects for containment, with these letters activating "dirty foreigner" tropes:

> When meeting their friends, these foreign domestic workers take food and beverages that they have prepared at home to consume together at these gatherings. When they leave, empty food packs and drink cans are strewn at these places (Chin, 2020, para 2).

In engaging with such cultural tropes, the mainstream press published counter-narratives that sought to oppose such perspectives, including an NGO response denouncing such tropes, "it is unfair to target domestic workers and unnecessary to restrict their gatherings in public places at this point in time" (Kumar, 2020). Here, the mainstream press both created spaces for such tropes to play out, while also positioning alternative voices that referenced such letters as problematic.

As the pandemic manifested to afflict the MCW community significantly by April 2020, the mainstream press continued to discuss migrant workers in the context of cultures and habits. With a controversy erupting over the publication of a forum letter in the Chinese language mainstream press (a xenophobic letter about MCWs). The letter only led to further discussion on the cultures and habits of migrant workers in discursive constructions and circulation of them in mainstream news. The Straits Times picked up on the letter when a state actor cautioned that the forum letter was xenophobic:

> A forum letter published in Chinese daily Lianhe Zaobao that linked the Covid-19 outbreak in dormitories to foreign workers' personal hygiene and living habits showed racism and deep insensitivity, Home Affairs and Law Minister K. Shanmugam said yesterday (Lim, 2020, para 1).

In furthering the frame, a Straits Times forum piece was published, denouncing the letter, "Forum: Culturally insensitive to refer to workers' eating habits as unhygienic" (Hoe, 2020). At no point, migrant worker voices were activated to respond directly to how dirty foreigner tropes about them were represented. Today, solely relied on state voice throughout its article to emphasize the xenophobic and racist nature of the letter, and to reprimand the letter writer for airing such views:

> "I think the letter reveals some underlying racism... Because it typecasts an entire group—several hundred thousand of them—as lacking in personal hygiene, on the basis of their background, because they all come from backward countries," he said in an interview with Lianhe Zaobao on Friday (Apr 17) (Mahmud, 2020, para 2).

While state voice emphasized the lack of understanding regarding transmissions among migrant workers, state voice was strategically framed to depict the letter as xenophobic:

> Mr Shanmugam also said that the letter is xenophobic and deeply insensitive, and reflected a "lack of understanding of why we have this transmission of COVID-19 amongst our foreign worker population" (Mahmud, 2020, para 2).

NGO voice had begun cautioning against COVID-19 infections and stigmatization concerning migrant workers early. Even though a majority of infections became visible by late April 2020, early reports citing NGO voice begin framing for the reader, how migrant workers were potential targets of stigma relating to the COVID-19 virus:

> Home executive director Catherine James is concerned that foreign workers may end up bearing the brunt of stigmatisation, given the intense paranoia about the disease, which was first reported in the Chinese city of Wuhan in December. In one case she encountered, a Chinese worker stranded in Singapore due to a salary claim was unable to find a dormitory that would take him in and wound up sleeping in an Internet cafe until Home found him shelter (Ho, 2020, para 26).

NGO voice was utilized in mainstream reports to sound the alarm on the plight of migrant workers. We see the outbreak inequality narrative forming by 3rd April 2020 (Yang, 2020). Frames of culture and habits were played up in the narratives that discussed the possibilities and impossibilities of integration of migrant workers with the local community by NGO and expert voices:

> Ultimately, said MWC's Mr. Menon, it takes two hands to clap and society must be more accepting. "We have tried to rally the migrant workers to interact and hopefully integrate with Singaporeans, but learned quite a painful lesson over time that integration is a two-way street" (Ho, 2020, para 2).

The mainstream media presented for the readers how the tools and techniques of othering about migrants were pivoted in mainstream discourse. Here, a state-affiliated NGO voice discussed the challenges of integration of migrant workers by speaking to how Singaporeans exclude MCWs, "If we don't have an equal number of Singaporeans willing to welcome and accept them, it's very difficult" (Ho, 2020). Other articles pushed further, by directly addressing the prejudices of culture and habits in comparison to the lives of Singaporeans as not being too different,

> Never mind the fact that they gather under trees or on fields because there are often no accessible or affordable places for these workers to take a breather after a hard week's work, in the same way that Singaporeans kick back with beers with friends in kopitiams, bars or at home (Yuen, 2020, para 13).

Here Yuen (2020) does not only seek to address xenophobia toward migrants but provides a structural explanation rather than a cultural one in tackling unfounded fears about their behaviors by Singaporeans. In Kaur et al. (2016) previous research on migrant worker portrayals, the mainstream press connected cultural explanations in shaping the constructions of MCWs in conflict with state voices. Here we see a shift in how MCWs are discussed more specifically as victims of structural injustices rather than adopting a cultural explanation for their behaviors.

Dormitories as Spread and Contagion

Central to mainstream reporting about migrant workers were dormitories as a site of spread and contagion, "Migrant workers living in dormitories continue to be the most severely impacted demographic, comprising the majority of the remaining cases" (Yang, 2020). The discursive construction of dormitories as spread and contagion sought to provoke discussions on the responsibility of various actors for the poor treatment migrant workers in the city-state. One the one hand, press reports centralized the roles of dormitories as sites where social distancing was required, "Coronavirus pandemic; Stricter rules at foreign worker dormitories to enforce safe distancing" (Tan, 2020), with another article discussing strategies of containment:

> Dormitory operators should also monitor the health of residents in blocks, limit their movements and prevent mixing of workers between blocks. They must stagger timings for kitchen and shower use, and limit the number of people in recreational rooms and minimarts. Operators must also put up signs telling workers not to gather at common areas (Tan, 2020, para 6).

Keeping workers within dormitories as a strategy for containment as problematic was reported by The Straits Times, framing the conditions of the dormitories that make social distancing challenging, "Coronavirus: Workers describe crowded, cramped living conditions at dormitory gazetted as isolation area" (Lim, 2020). Mainstream press emphasized the conditions of dormitories as sites of spread, framing dormitories as a threat to public health for workers,

> At least six workers at the S11 Dormitory @ Punggol, where there are 63 confirmed cases of Covid-19, told The Straits Times that the rooms are infested with cockroaches and toilets are overflowing. Workers have to queue for food with no social distancing measures to keep them apart (Lim, 2020, para 2).

The article discusses the dehumanizing conditions of the dormitory and the locking down of workers in these spaces,

> Mr. Venkate said officers from the Ministry of Manpower (MOM) visited the dormitory last Saturday night and, at around 9 p.m., one announced that the dormitory would be fully locked down, with no one allowed to leave the premises (Lim, 2020, para 2).

Dormitories thus, were both discussed as a site where the spread of COVID-19 infections took place rapidly due to the poor conditions that enabled the surge in infections:

> His comments came as the foreign worker community here has been hit hard by the c coronavirus pandemic, with more than 2,600 workers in large dormitories infected. There are about 200,000 migrant workers in these purpose-built dormitories (Wong, 2020, para 6).

In the quote above, a spokesperson from a state-affiliated NGO cites the scale and spread of infections in dormitories. These sites also became the space where illegal confinement and poor treatment of workers were amplified. NGO groups sounded the alarm on the confinement of 20 workers, "A dormitory operator who forcibly confined 20 workers in a locked room had been given a stern warning from the Ministry of Manpower (MOM)" (Zhuo, 2020a) that was picked up by mainstream media. With a lack of space in dormitories and a positive Covid-19 worker, "the reason given by the operator was to prevent them from moving around after a close contact was confirmed positive for Covid-19," said the MOM (Zhuo, 2020a).

The reporting of dormitories by the mainstream press, presented competing voices regarding the state of dormitory conditions. These competing voices called out the severe structural reasons for the spread of infectious disease, as well as the limitations of these sites for containment and isolation of workers. The inmate treatment in the design of dormitories (Rubdy and McKay, 2013) and peripheral locations of these dormitories are reported extensively. An article by Today published a study on worker treatment during the COVID-19 crisis. The article anchored academic voice to critically interrogate the othering practices embedded in the ideological construction of these dormitories in peripheral and marginal ways, "the power held by the employers and dormitory owners, accompanied by the absence of transparent and safe infrastructures for raising complaints translates into unhealthy structures remaining intact," he wrote in the white paper (Today, 2020). The article presented the interest of various actors that amplified the public health threat within the dormitories of MCWs.

The dormitory as news frame remained in the spotlight in the discussion of migrant workers, where the dialectics of spread and contagion remain absent in the analysis. The structural

conditions of the dormitories also continued to occupy media narratives, discursively engaging with the concept of peripheral dormitories and migrant workers. Headlines in May 2020 reinforcing "structural and mindset changes needed to improve wages and living conditions of foreign workers, say analysts" (Ho, 2020). Expert and academic voices were referenced in the presentation of dormitories as underlying structural conditions that remained a public health threat for workers in the short-term and long-term:

> "These infrastructures are not adequate as there are often long queues and the facilities remain unclean," Prof. Dutta wrote. All in all, the responses do point to the existence of "unhealthy structures" which "most likely" contributed to the further spread of Covid-19 in the foreign worker community, Prof Dutta said (Ho, 2020, para 27).

> Improving the wages and living conditions of foreign workers in Singapore requires a whole-of-society effort. Not only must the Government take the lead in making structural changes, but Singaporeans, too, must change their us-versus-them mindset, said analysts (Ho, 2020, para 1).

Dormitories, dormitory operators, employers, and the dormitory regulators were discussed as key structural actors in the management and spread of the virus among MCWs. While mainstream press did not interrogate the dialectics of spread and contagion, the press extensively discussed the role of dormitories as unsanitary, inhuman, and a public health risk for migrant workers (Tan, 2020).

Theme 3: The Other Speaks Back

While in other previous literature, migrant worker voice remained in the margins of mainstream discourse (Kaur et al., 2016; Tan, 2016), worker voices were spotlighted in moments of this health crisis as rupture points. These voices were anchored in a few ways. In spotlighting their structural conditions, foreign worker voice appeared and re-appeared in moments of heightened surveillance of their conditions during COVID-19 as a public health threat. Voices were centered in the discussion of their living conditions:

> "There are many cockroaches in the kitchen and also in our rooms. The urinals in the toilets are overflowing with urine and the workers step on the urine and then walk to their rooms," said Indian national Venkate S.H., 34 (Lim, 2020, para 11).

Worker voices were anchored in the discussion of fear of spread and contagion in dormitory settings,

> "On Sunday, most of us woke up at 8 a.m. and were waiting for our breakfast, which arrived at about 10 a.m. Everybody queued together to get the food. There was no social distancing. We also did not have masks. Only a few workers had their own masks" (Lim, 2020, para 17).

Voices of workers were used to deploy serious public health issues that threatened the rapid spread of infections in the migrant worker dormitories when reports of infections begin surging in the city-state and the abysmal absence of information regarding the lockdown:

> Said Mr. Venkate: "This happened suddenly. We did not stock up on food. I can't go out to buy my coffee. But some people have food and they were cooking in the kitchen because dinner was still not here at 8 p.m." (Lim, 2020, para 21).

Migrant worker voices were deployed to break the news about the conditions of the dormitories, but were also activated to humanize workers,

> We want our writing to change locals' views of migrants and also migrants' views of themselves, "he says. All everyone thinks is that we do dirty, dangerous and difficult work. But we can also be poets, photographers, film-makers. We can be inspired by what we do" (Ho, 2020, para 12).

The Straits Times piece created space for the margins to activate for themselves by speaking back to Singaporeans about who they are beyond just the label of MCWs. The article fronted how MCWs were also volunteers,

> In the days before he fell ill, he was volunteering with grassroots initiatives to rally donations for workers restricted from leaving their dormitories during circuit breaker measures and organise the distribution of supplies, such as masks and sanitisers, to them (Ho, 2020, para 15).

In humanizing workers by creating space for their voices to speak back to tropes about them, mainstream press can be seen pushing the OB markers for critical voices from the margins to be heard. Even in Today's reporting of MDW movements during their off days, mainstream press anchored MDW voice to disrupt the single thread of state voice regarding the advisories, "it's also status quo for Ms. Cristina Mandoza Fayco, a 57 year-old domestic worker." "My boss never said anything to me like, "Don't go out" or "Don't do this or that"," she said (Today, 2020). Mainstream press configured reporting practices as radical moments for the margins, centering competing actors as voice, while also in moments of crisis voices of the margins for more considerable change and advocacy.

Humanizing the Other: Structural Conditions of Labor

As the COVID-19 situation develops in Singapore with the number of cases crossing 25,000 by early-mid May 2020, the media actively centers and discusses migrant worker health with the bulk of infections afflicting the MCW community. With headlines such as "Covid-19 outbreak brings migrant workers from margin to centre of Singapore's attention" (Yuen, 2020), suggests the mainstream media was attentive to migrant worker treatment in Singapore. Articles such as Yuen's (2020) points the reader's attention by dispelling prejudices about migrant workers, concluding for the readers

> Migrant workers are part of our community. Covid-19 has brought them front and centre into our lives. It is time we

> stopped pushing them to the margins and started the hard work of integrating them better into our society (Yuen, 2020, para 52).

While the discussion of the margins remained the center of this story, the agentic voices of migrant workers remained absent even when articles chose to position the margins as a frame for readers. The structural conditions of labor are highlighted and reinforced throughout the article to unpack the very conditions of labor that limit migrant worker health,

> Most Singaporeans turn a blind eye to this invisible class of workers, who are out of sight and out of mind. Many locals are unaware of the structural issues they face in terms of housing or welfare. The issues in the past—the lack of trust and interaction between the different groups—remain buried (Yuen, 2020, para 36).

The voices of multiple experts were presented in news articles in discussing structural conditions of labour, including academics and NGOs. Headlines in May 2020 focused on "Structural and mindset changes needed to improve wages and living conditions of foreign workers, say analysts" (Ho, 2020), "Solving Singapore's foreign workers problem requires serious soul searching, from top to bottom" (Ng and Ong, 2020), and "Migrant worker housing: How Singapore got here" (Ng, 2020). These articles fronted quotes from academic experts and NGO voices in discussing better treatment and solutioning through systemic changes in the management of their labor. These articles predominantly surfaced in late April-early May, where the majority of infection clusters were located, sites where migrant workers reside and work. Citing academic studies, a Today article, for example, cited surveys conducted with migrant workers on their current needs, "Almost eight in 10 workers also find it a challenge to maintain a 1 m distance from others due to "cramped conditions" at their dormitories, the survey found" (Wong, 2020). On the discussion of structural conditions, the mainstream media highlighted dormitory conditions, worker wages, the economic model for the management of migrant labor, ethical, and moral obligations in the treatment of migrant worker rights, and health conditions. For example, Today reports,

> With Singapore now facing what has been touted as "a crisis of a generation," some, like Assoc Prof Theseira and fellow NMP Anthea Ong, have called for a committee of inquiry into the foreign worker dormitory outbreak to work out the structural changes that Singapore sorely needs (Ng and Ong, 2020, para 111).

This quote was presented under a sub-heading titled "Tweaking Singapore's Economic Model" (Ng and Ong, 2020) in the article, highlighting the need to review the current system of labor management. A Straits Times piece captures a quote from an expert,

> But a whole-of-society mindset change is needed for the support to be sustained, said Dr. Lim. "The mental model we have traditionally taken is that foreign workers are part of the community but separate; we accept that there should be different standards (for them)" (Ho, 2020, para 7).

In centering such voices of change, these articles articulate radical shifts in how migrant workers are treated in Singapore. Ng and Ong (2020) humanized workers by including their narratives and centered their lived experiences in the report:

> Mr Liton has reason to be worried—two of his friends, Asit and Zakir, who live in the larger purpose-built dormitories, have been diagnosed with the disease and hospitalised. We all want to go home in good health … My wife miss(es) me more and more, he said (Ng and Ong, 2020, para 5).

> While Mr Liton ponders over the future, his host country—Singapore—will also have to reassess its whole relationship with migrant workers like him, especially its "addiction" to cheap migrant labour, and examine whether the lessons learnt from the explosion of COVID-19 cases in the workers' dormitories could be used to implement meaningful changes (Ng and Ong, 2020, para 10).

Efforts by Ng and Ong (2020) in reporting through voices of the vulnerable shift the lens in which migrant workers are relegated to the margins for audiences in mainstream discourse.

DISCUSSION

The mainstream press in Singapore is theorized as a structural entity in close affiliation to the state's reprimand (George, 2012; Dutta et al., 2019). However, during the COVID-19 crisis, the role of mainstream media in reporting the margins revealed moments of ruptures where the media created openings for an in-depth discussion on low-wage migrants and their structural conditions that require change. From a CCA perspective, the discussion of structural conditions of migrant workers points to critical transformative openings by the media that are anchored in structural changes on the systems of hire of migrant labor (Kaur-Gill and Dutta, 2020). In pandemic response, mainstream media created openings in the discussion of these possibilities in dominant communicative spaces.

By centering competing voices that curated a variety of different threads on migrant workers and the COVID-19 crisis, structurally-centered articles about their treatment in Singapore society were located by early May 2020. Migrant worker voices were activated in shedding light on the hazardous labor conditions discussed extensively in mainstream reports in April and early May 2020. Conflicting and contending positions articulated by expert voices (civil society and academic experts) created avenues for structural factors to emerge as salient in discussing the underlying conditions the exacerbated infections among the migrant worker community.

Mainstream Media, the COVID-19 Pandemic and Transformative Openings

The mainstream press in Singapore has been rebuked for the presentation and selection of frames that propel narratives in participation with the state's public relations performance, limiting room for frames that provide alternative rationalities and realities of the margins (George, 2012). In the reporting of the COVID-19 crisis and migrant workers, the hegemonic tropes are laid out for consumption, while also pushing for dissident discourses on migrant worker issues. Reports by both Today and the Straits Times pushed the OB markers by challenging the status quo of worker labor conditions, where competing voices did not fall in line with state narratives on the discussion of migrant workers as in the past. During the COVID-19 crisis, we see how the media structure in speaking for the agendas of the powerful were disrupted. Transformative openings, where agencies of the vulnerable are not just discussed, but activated. Worker voices were presented but also activated in agentic ways in news reporting in the mainstream press. While on the one hand, the media structure continued to perpetuate the status quo, as the state's mouthpiece on issues on migrant workers and their health. Under conditions of this pandemic, moments were located where the media structure created openings for the discussion of the margins in transformative ways where the media structure reflected sites of transformative openings in its discussion of the margins. The media created space for advocacy tied to the structural conditions of labor, highlighting systemic changes required for the better treatment of migrant workers, but also discourses that centered a need for moral and ethical change in the treatment of migrant workers in Singapore. This finding might potentially indicate a shift in the frames of discourse on migrant worker advocacy as culturally mediated vernacular reported in Koh et al. (2017) study. The timeline of how the crisis played out provides perspective on how shifting discourses on migrant worker health were discussed at different stages of news reporting. When expert and NGO voices dialogued about these issues on various forums, the mainstream press was quick to anchor these voices on articles about structural conditions of migrant workers in Singapore by citing these forums and webinars.

Another point where we see the OB markers shifting is when the press produced news about the racializing of the neoliberal migrant subject in pandemic response. It is worthwhile noting that George (2012) posited that a key OB marker in Singapore related to sensitives regarding race and religion. However, when it came to the reporting of migrant workers as racialized sick subjects, the media created space for the discussion of xenophobic and racist discourses that contributed to the othering of workers. George (2012) positions that "OB markers discourage the media from initiating debates on matters that could stir ethnic passions" (p. 66). However, these exclusions of ethnicity concerning migratory figures were not limited in mainstream news reporting. Reports included themes that othered cultures and habits discussed in the findings, while also reflecting on how such discursive constructions of the migrant other occupied problematic assumptions in society.

Voices Framing Outbreak Inequality

Discourses on dormitories in mainstream news revealed the implications the COVID-19 infections had on the state's management of a public health crisis among those residing in the margins. The dormitories that housed migrant workers revealed significant implications for infectious disease threats. The media did not overtly position the migrant worker crisis in Singapore as outbreak inequality, however, the narratives of migrant worker infections through April and May 2020 point to the jarring disparities migrant workers faced during the COVID-19 pandemic. These frames also primarily occupied media narratives that temporally compressed migrant worker narratives as dominant protagonists and victims in news reports. Furthermore, the disorderliness of the dialectics of spread and contagion in the reporting of the dormitories are tensions that are deeply anchored in how the crisis was managed in real-time, creating ambiguity and uncertainty about the conflicting crisis emerging out of spaces of marginality. Where the media focused on the transnational migrant worker community as racialized sick subjects in this pandemic, competing voices emerged in the discussion of their current health crisis.

Competing voices included academic/expert voices, NGO voices, state voice, migrant worker voices, voices of employers/dormitory operators, and voices of journalists in opinion pieces about migrant worker health, and local voices. Local voices were often used by mainstream reports to facilitate contentious viewpoints about local perspectives on migrant workers during the crisis. While local voices occupied spaces in forum letters on migrant workers, migrant worker voices remained absent in the discussion about them. What role does the mainstream press, therefore, play, in leaving the margins absent from speaking back to tropes that circulate about them via mainstream discourse? Where migrant workers are left absent from the engagement in the discursive constructions about them, the mainstream press continues to act as a conduit that anchors discourses for the dominant, providing limited entry points for the margins to speak back. NGO voices were present to fill that role and typically articulated the ethical and moral obligations regarding the treatment of migrant workers via both forum letters and as actives quotes in news reports. By late April, early May, these voices actively called for systemic structural changes of their conditions of labor alongside expert views. The mainstream press did not centralize state voice in discussing the structural conditions of labor and instead used competing voices to push for social change in the better treatment of migrants. Goh et al. (2017) argued that civil society actors did not pivot rights centered discourses in illiberal contexts. This study informs us that in political sites that are illiberal, controversial social issues can be pivoted through credible voices that center opposing or unpopular perspectives.

Margins and Voice

The study located that migrants worker voices were activated in media reports to discuss their health crisis, they still remained in the peripheries of reporting. However migrant worker voices were not absent or largely erased as discussed in previous

research (Kaur et al., 2016; Tan, 2016; Goh et al., 2017; Kaur-Gill et al., 2019). A rights discourse, however, remained inconsiderable in mainstream news about migrant workers and their health. Migrant worker voices were present in various news reports in dialectical ways, humanizing workers while also framing them as victims of an exploitative system. Part of such discursive constructions, both in humanizing and victimizing, the constitution of the "migrant other" remained etched in news frames.

On investigative reports on dormitory conditions, specific articles created openings for migrant voices to position their conditions and voice out about the treatment received. While migrant worker voices were activated, they were also constructed as the "helpless other"; as victims to the systemic exploitation of their vulnerabilities. In framing the migrant worker as victims of outbreak inequality, it suggests the margins as a site for mediation, circulating hegemonic discourses of migrant workers and their dormitories as sites for discipline, surveillance, and management. However, it is noteworthy to mention that with competing voices came competing threads on the discussion of migrant workers and their health. Thus, while state voice remained central in narratives on the Little India Riot episode (Kaur et al., 2016), with Ahmed et al. (2019) citing that the mainstream press reported the riot without diving into the cause and reasons behind the conflict, attributing this to the stringent press control that promotes the state's position.

In this study, we see how competing voices diversify and complexify migrant worker perils. The dialectics on migrant workers, their health, their treatment, and management of labor, indicated both emerging advocacy in mainstream journalistic rituals, while also pandering to state rhetoric on disciplining and surveilling of worker bodies in early reports. Scholars discussing migrant workers and civil society in politically illiberal spaces have indicated that when advocating for migrant workers, locals were averse to a right-centered frame as it tipped "dominant social and institutional logics" (Goh et al., 2017, p. 90)—therefore re-framing a rights-centered discourse to a moral and economic one through culturally-mediated vernacularization. Nevertheless, (Goh et al., 2017) conclude that, ultimately, migrant workers should center their advocacy, which remains in line with the CCA's position of migrant worker voices entering dominant discourses and re-framing them. Similarly, Tan (2016) shares that the denial of voice and the discourse failing to move toward structural transformation limits the reimaginations of change for the margins.

While the mainstream press created dialogic moments for frames on structural remediation to emerge, unlike in previous studies (e.g., Tan, 2016; Goh et al., 2017; Ahmed et al., 2019; Kaur-Gill et al., 2019), the press also continued to center sensationalist articles that evoked streams of xenophobia. The various opposing dialectics play out in mainstream reporting. The linearity of the press room as a mouthpiece of the state (George, 2012; Whitten-Woodring and James, 2012; Kaur et al., 2016), requires more significant interrogation, where moments of crisis, points to possibilities of negotiation for more significant representational spaces by the margins in media representation. We see competing voices shifting the hegemonic discourses on migrant labor and health, where alternative voices, including subaltern voice progressing the discourse toward a structural exploration of the conditions experienced. In centering competing voices, the mainstream press creates entry points for alternative possibilities of social change. Narratives are grounded in the lived realities of worker conditions, despite the illiberal management of the mainstream media structure. However, there remain better opportunities for the media to create entry points for centering the margins, with Sastry and Dutta (2011) suggesting that "when we start listening to these subaltern voices in mainstream platforms of knowledge, policymaking, and intervention development, alternative rationalities emerge in the discursive space that question the implicit assumptions of the dominant articulations within neoliberal frameworks of organizing health care" (p. 531).

FUTURE STUDIES AND LIMITATIONS

There are several limitations, such as the timeline and selection criteria of the articles that included collating articles within a specific period. This limited a longitudinal analysis on how frames about migrant workers play out in the mainstream press throughout the crisis. Furthermore, the coding process adopted a grounded theory analysis, eventually interpreting the selective codes using a CCA framework. The interpretation of findings, therefore, is grounded within a culture, structure, agency reading narrowing the interpretations of the findings to the dialectics of power and subaltern agency. Future studies can interrogate how migrant health is constructed and shaped in mainstream discourse post-COVID-19 crisis, tracing the disruption of hegemonic discourses on migrant health and labor. It is also worthwhile to trace the policy changes in migrant worker treatment in the context of media advocacy about them post-COVID-19 outbreak.

A critical discussion on the role of the mainstream press in conveying migrant worker health during the pandemic includes the media's role in pushing for alternative discourses that sought to transform local attitudes and opinions toward migrant workers. What role does the media play in both disrupting societal norms, and intervening for structural change? Are there spaces for the mainstream press to push the claw of control from calibrated techniques of a neoliberal authoritarian ideology of labor management? The culture-centered project of coding and mapping absences creates opportunities to depict how the context of the margins can be better situated in media representations and the dialogic moments that rupture in creating openings by mainstream press for the margins. As of 11th May 2020, 90% of cases in Singapore were primarily made up of the migrant worker population (Ng and Ong, 2020), laying out the scale of outbreak inequality among migrant workers during COVID-19. Media discourses perform a central role in articulating these gross disparities with significant implications for structural change in the future. When mainstream sites create opportunities for voices in the margins to emerge, it not only seeks to debunk the assumptions of the status quo but allow for bottom-up rationalities to be centered in policymaking and intervention design.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: FACTIVA.

AUTHOR CONTRIBUTIONS

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

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

APPENDIX

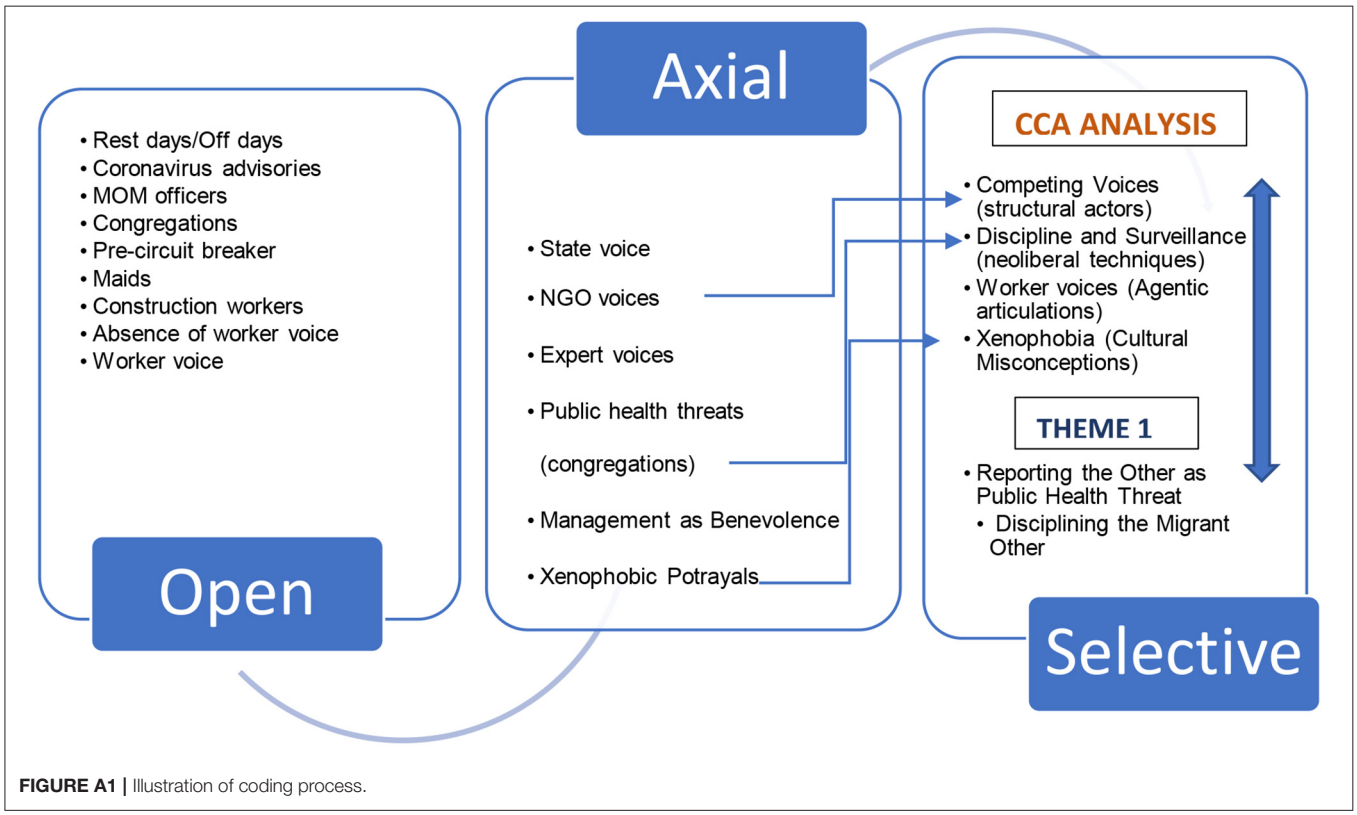

FIGURE A1 | Illustration of coding process.

TABLE A1 | Illustration of newspaper articles and timeline.

Date	Title	No. of words	Newspaper
09/02	Maids, employers get coronavirus advisory amid reports of foreign domestic workers denied rest days	501	The Straits Times
10/02	Advisory issued to maids, employers on rest-day arrangements	649	The Straits Times
11/02	Despite MOM's advisory, some employers don't intend to keep their domestic workers at home	628	Today
13/02	Covid-19: 8 more new cases in Singapore, 5 of these from Grace Assembly of God cluster	1,003	Today
16/02	Covid-19: SAF regular among 3 new victims as Grace Assembly of God cluster grows to 18 cases	1,465	Today
19/02	Coronavirus: migrant worker NGOs under strain from outbreak, but persisting	1,035	The Straits Times
20/02	Coronavirus outbreak; Coronavirus: migrant worker NGOs under strain but push on	1,023	The Straits Times
23/02	Gifts of masks, food and flowers	1,195	The Straits Times
25/02	Relief package of $10,000 to help infected migrant worker	340	The New Paper
26/02	Parliament: MOM to issue guidelines to employers on 5-day MC during coronavirus outbreak	783	The Straits Times

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Prognostic Factors for COVID-19 Pneumonia Progression to Severe Symptoms Based on Earlier Clinical Features: A Retrospective Analysis

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Approximately 15–20% of COVID-19 patients will develop severe pneumonia, and about 10% of these will die if not properly managed. Earlier discrimination of potentially severe patients basing on routine clinical and laboratory changes and commencement of prophylactical management will not only save lives but also mitigate the otherwise overwhelming healthcare burden. In this retrospective investigation, the clinical and laboratory features were collected from 125 COVID-19 patients who were classified into mild (93 cases) or severe (32 cases) groups according to their clinical outcomes after 3–7 days post-admission. The subsequent analysis with single-factor and multivariate logistic regression methods indicated that 17 factors on admission differed significantly between mild and severe groups but that only comorbidity with underlying diseases, increased respiratory rate (>24/min), elevated C-reactive protein (CRP >10 mg/L), and lactate dehydrogenase (LDH >250 U/L) were independently associated with the later disease development. Finally, we evaluated their prognostic values with receiver operating characteristic curve (ROC) analysis and found that the above four factors could not confidently predict the occurrence of severe pneumonia individually, though a combination of fast respiratory rate and elevated LDH significantly increased the predictive confidence (AUC = 0.944, sensitivity = 0.941, and specificity = 0.902). A combination consisting of three or four factors could further increase the prognostic value. Additionally, measurable serum viral RNA post-admission independently predicted the severe illness occurrence. In conclusion, a combination of general clinical characteristics and laboratory tests could provide a highly confident prognostic value for identifying potentially severe COVID-19 pneumonia patients.

Keywords: COVID-19, SARS-CoV-2, risk factor, clinical manifestation, prognostic factor

BACKGROUND

The novel coronavirus (SARS-CoV-2) has seemed to sweep across the globe ever since its first successful jump from bat to human being through a still unknown intermediate(s) in approximately late Nov 2019; it still shows a tendency toward significant surges in incidence worldwide (1–3). The SARS-CoV-2 virus seems more contagious than its sibling virus, severe acute respiratory syndrome (SARS) virus, which broke out in 2003; as of March 11, over 120,000 individuals have contracted

COVID-19 pneumonia within 3 months, which was about 15 times that of the total SARS cases (8,000 in 7 months) (4). The surging increase in COVID-19 patients within a short time window will severely impact the limited medical resources, including physicians, nurses, protective suits, masks, and goggles. Data from the Chinese mainland showed that the majority of total infected patients will recover under simple supervision management, such as quarantine in a compartment hospital isolation ward, but that the overall case fatality rate was 2.3% (5). For the clinical treatment of COVID-19 patients under shortage of enough medical supplies, the critical issues and priorities are to treat the severe COVID-19 patients [about 20% of the whole population (5)] and to save their lives with preventive and intensive medical care. However, the clinical presentation of COVID-19 patients differs substantially, and this can include asymptomatic infection, mild upper respiratory tract illness, and severe viral pneumonia (2, 6–8). The most crucial issue is therefore to identify these patients and prioritize their treatment strategy by applying prophylactic medical treatment and management before they progress to the severe stage.

As we know, respiratory function worsens in the severe stage. In clinical practice, saturated oxygen (<93% in rest state), reparatory rate (>30 times/min), and deteriorated chest radiology imaging (X-Ray and CT more high resolution) provide references to confirm their severity (5, 9, 10). Because of the hypoxia stress, most patients will experience an over-reactivated immune storm, including elevated expression levels of some specific immunological cytokines and changes in certain types of immune cell counts (6, 11). Biopsy analysis also showed that the lung bilateral diffuse alveolar damage with cellular fibromyxoid exudates (12). However, CT imaging and immunology detection is not only expensive but also largely unavailable an unable to cope with the significant rise in suspected cases, particularly in those hospitals that are not well-equipped. Can some routine clinical characteristics or/and laboratory measurements (or their combination) predict the occurrence of severe cases?

In this study, we retrospectively analyzed the clinical characteristics of those patients who progressed to severe pneumonia later and found that five simple clinical features and laboratory detection at an earlier time point could serve as prognostic factors facilitating discrimination of severe cases in advance.

METHODS

Patients

COVID-19 diagnosis was determined according to the criteria in the new Coronavirus pneumonia diagnosis and treatment plan (trial version 6) issued by the National Health and Health Commission (13). All 298 COVID-19 patients admitted to Guangzhou Eighth People's Hospital from January 20 to February 29, 2020, were included in this study. This study complied with the medical ethics of Guangzhou Eighth People's Hospital. We obtained written consent from the patients.

For this analysis, inclusion criteria were the following: (1) diagnosed as mild or ordinary on admission and (2) length of hospitalization >3 days and overall duration of the disease >7 days. Qualified patients were then classified into a mild symptom group and a severe symptom group based on the clinical manifestation. The severe symptom diagnosis was determined according to the following criteria: (1) respiratory distress, RR ≥ 30 times/min in the resting state; (2) oxygen saturation ≤93% in the resting state; and (3) arterial blood oxygen partial pressure (PaO_2)/oxygen concentration (FiO_2) ≤300 mmHg). The rest of the patients were in the mild group.

Data Collection

Patient general information, including gender, age, underlying diseases, epidemic history, etc., and their clinical data including symptoms, signs, clinical classification (course duration >7 days), laboratory test results, and SARS-CoV-2 viral test results were obtained with standardized data collection forms from electronic medical records.

Statistical Analysis

Quantitative data was firstly tested to be normality distribution with the Kolmogorov-Smirnov method. Then, for normalized distributed data, *t*-test and Tamhane T2 methods were used for variance of even and uneven data, respectively. For non-normal data, which was expressed as the median (quartile) [M (P25, P75)], the Mann-Whitney *U*-test was employed. The chi-square test (or Fisher exact probability method) was utilized for analyzing qualitative data. Logistic regression analysis and the receiver operating characteristic curve (ROC) analysis was employed to analyze the independent risk factors. The difference was statistically significant at $P < 0.05$. All analysis was performed using SPSS software (version 20.0).

RESULTS

Patient General Information

A total of 298 COVID-19 cases (about 85% of total cases in Guangzhou, China) were admitted to Guangzhou Eighth People's Hospital for treatment from January 20 to February 29, 2020 (**Figure 1**). According to the inclusion criteria, 173 cases were excluded because 23 cases were already in the severe symptom stage, 52 cases had a short hospitalization time of <7 days, and 98 patients had other defects, such as being short of a complete set of detection. Finally, 125 cases, including 63 males and 62 females, were qualified to be included for further investigation, and all their disease courses were over 7 days, with a maximum of 32 days. Based on the severity of disease at 3 days post-admission, 93 patients fell in the mild group (38 general cases and 55 mild cases) and 32 patients in the severe group (25 severe cases and seven critical cases).

All included patients were aged between 1.5 and 91 years (averaged 44.87 ± 18.55 years) (**Table 1**). Among them, 37 cases had at least one underlying disease, including 20 cases with hypertension, eight cases with diabetes, five cases with coronary heart disease, two cases with chronic obstructive pulmonary disease, two cases with chronic kidney disease, two cases with chronic liver disease, and two cases with sleep apnea syndrome. Five individuals with two or more basic disorders and 7 cases with obesity (BMI > 26). Epidemiologically, 88 cases had a history of

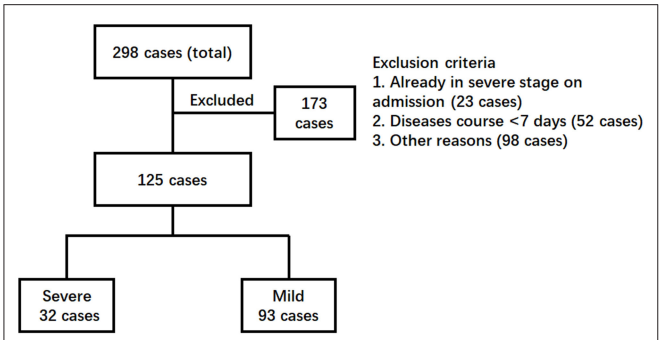

FIGURE 1 | Enrollment chart of COVID-19 patients. The clinical information of a total of 298 patients admitted to the hospital was reviewed. Patients were excluded according to the criteria (1) already in severe stages, (2) with a disease course <7 days, and (3) other reasons such as incomplete detection panel. Finally, 125 patients were further divided into two groups. The severe group included the patients who developed severe COVID-19 pneumonia later (>3 days post-admission). The patients remaining were kept in the mild group.

traveling to or living in the Hubei epidemic area before disease onset. Interestingly, we observed that seven patients developed serum SARS-CoV-2 viral RNA positive after admission but ahead of diagnosis to be a severe symptom.

Factors Differed Between the Mild Group and the Severe Group

The single-factor analysis was applied for each factor between the mild group and the severe group (**Table 1**). More patients in the severe group were old, obese (BMI > 26), and had underlying diseases, particularly hypertension and diabetes ($P < 0.05$), compared with the mild group. Among the general factors, no significant difference could be seen with regards to gender, history of traveling to or living in an epidemic region, coughing, sneezing, muscle joint pain, headache, fatigue, and gastrointestinal symptoms between these two groups ($P > 0.05$). However, more patients in the severe group exhibited high fever, chest tightness, and shortness of breath (fast respiratory rate) ($P < 0.05$). The serum concentration of C-reactive protein, procalcitonin, D-dimer, albumin, and lactate dehydrogenase (LDH) increased significantly in the severe group ($P < 0.05$).

Compared to the mild group, patients in the severe group had lower absolute lymphocyte counts, higher eosinophil counts ($P < 0.05$), and similar levels of other parameters, including white blood cells, neutrophils, platelets, hemoglobin, prothrombin time, activated partial thromboplastin time, blood lactic acid, blood creatinine, and creatine kinase. Interestingly, the levels of glutamate aminotransferase (ALT) and aspartate aminotransferase (AST) significantly increased for severe patients ($P < 0.05$). However, the median values of ALT and AST were still within the normal range, indicating that most of the severe COVID-19 patients had no significant liver damage.

Importantly, all seven patients with the presence of SARS-CoV-2 viral RNA in blood during the hospitalization, but before being in the severe stage, finally progressed to the severe stage; they included two severe cases and five critical cases ($P < 0.05$).

Binary Logistic Regression Analysis of COVID-19 Severe Risk Factors

Next, all categorical variables were converted into covariates, including age, presence of underlying diseases (Yes or No), hypertension (Yes or No), diabetes (Yes or No), obesity (Yes or No), Temperature (<37.4, 37.4–38.5, >38.5°C), fast respiratory rate (Yes or No), elevated C-reactive protein (>10 mg/L), decreased lymphocyte count (<1.1*10E9/L) and eosinophil count (<0.02*10E9/L), elevated procalcitonin (>0.05 ng/L), elevated D-dimer (>=2.25 μg/L), decreased albumin (<35 g/L), and elevated lactate dehydrogenase (LDH, >250 U/L), and were then subjected to single-factor logistic regression together with multiple independent variables. Those variables with statistical significance were chosen for subsequent binary logistic regression analysis to test the model coefficients, goodness-of-fit, and multicollinearity. Four factors identified to be significantly relevant to the severity of COVID-19 were underlying diseases (X1), fast respiratory rate (>24 times/min) (X2), elevated C-reactive protein level (CRP > 10 mg/L) (X3), and elevated lactate dehydrogenase level (LDH > 250 U/L) (X4) (**Table 2**). Finally, the multifactor logistic regression equation was obtained: $P = -6.488 + 2.752X1 + 4.056X2 + 2.424X3 + 5.392X4$. The β values and odds ratios (OR) for each factor are shown in **Table 2**. The result indicated that elevated LDH ranks as having the highest correlation to severe symptom development (OR = 219.608), followed by the fast respiratory rate (OR = 57.726), underlying diseases (OR = 15.67), and elevated CRP (OR = 11.289).

The Prognostic Capacity for Severe Symptom Development

To better evaluate the prediction capacity of each of the independent risk factors, we plotted their receiver operating characteristic curve (ROC) for the development of severe COVID-19 pneumonia and calculated the area under the ROC curve (AUC value), sensitivity, specificity, Cut-off value, Youden index, and *p*-value (**Table 3**) for all of them. According to the general standard that AUC values between 0.7 and 0.9 mean a medium level of diagnostic values and AUC values over 0.9 mean a high level of diagnostic values, we observed that all the factors (AUC < 0.9) failed to provide a high prognostic value when used alone. A two-factor combination test then showed that the combination of fast respiratory rate and elevated LDH could provide a highly confident prediction (AUC = 0.944, sensitivity = 0.941, and specificity = 0.902) (**Table 3**). The AUC values of elevated LDH plus underlying diseases or plus elevated CRP were both over 0.9, but their sensitivity or specificity was lower than 0.9. Then, triple factor combination significantly increased the prognostic efficacy, and all combinations had increased sensitivity and specificity (**Table 3**). Finally, we calculated the prognostic value of the combination of all four factors and found that the AUC value was significantly increased to 0.985 (95% CI 0.968–1.000), the sensitivity to 0.912, and the specificity to 0.957 (**Table 3**).

TABLE 1 | Characteristics of COVID-19 patients.

	All cases	Mild ($n = 93$)	Severe ($n = 32$)	p-value
Gender				0.96
Male	63	47	16	
Female	62	46	16	
Age (years)***	44.87 ± 18.55	40.49 ± 17.66	59.43 ± 13.47	<0.05
Underlying disease (cases)	37	17	20	<0.05
Hypertension (cases)	20	7	13	<0.05*
Diabetes (cases)	8	2	6	<0.05*
Obesity (BMI>30) (cases)	7	2	5	<0.05*
Travel to epidemic area (cases)	88	65	23	0.388
Temperature (cases)				<0.05
<37.4°C	57	48	9	
37.4–38.5°C	48	34	14	
>38.5°C	20	11	9	
Coughing (cases)	76	56	20	0.819
Running nose (cases)	21	14	7	0.533
Muscle joint pain (cases)	27	23	4	0.147
Headache (cases)	24	16	8	0.334
Fatigue (cases)	48	32	16	0.061
Digestive Symptoms (cases)	19	15	4	0.622
Fast respiratory rate (cases)	20	4	16	<0.05*
Serum viral RNA positive (cases)	7	0	7	<0.05*
White cell counts (10E9/L)***	5.57 ± 1.76	5.65 ± 1.73	5.33 ± 1.86	0.411
Absolute neutrophil counts (10E9/L)***	3.43 ± 1.43	3.26 ± 1.28	34.97 ± 1.77	0.053
Absolute leukocyte counts (10E9/L)&	1.32 (1.05–2.18)	1.43 (1.23–2.21)	0.82 (0.57–1.05)	<0.05**
Absolute eosinophil counts (10E9/L)&	0.02 (0–0.09)	0.04 (0.1–0.12)	0(0–0)	<0.05**
Platelets (10E9/L)***	200.56 ± 56.24	206.01 ± 55.61	182.46 ± 55.47	0.052
Hemoglobin (g/L)***	134.39 ± 18.02	135.31 ± 17.92	131.32 ± 18.32	0.306
Prothrombin time (sec)***	13.69 ± 1.13	13.66 ± 0.89	13.80 ± 1.70	0.668
Activated partial prothrombin time (sec)***	39.30 ± 4.74	38.93 ± 4.49	40.49 ± 5.37	0.13
C-reactive protein (CRP) (mg/L)&	6.32 (1.63–23.50)	4.00 (1.06–12.41)	46.345 (28.97–60.50)	<0.05**
D-dimer (μg/L)&	910 (700–1,400)	780 (560–1,050)	1,760(1297.5–3,265)	<0.05**
Procalcitonin (ng/ml)&	0.047 (0.03–0.076)	0.037 (0.027–0.063)	0.070 (0.051–0.145)	<0.05**
Lactic acid (mmol/L)***	1.78 ± 0.71	1.72 ± 0.76	1.93 ± 0.55	0.207
Alanine aminotransferase (ALT, U/L)&	18.90 (13.40–25.20)	16.70 (12.40–22.15)	27.5 (19.70–41.25)	<0.05**
Aspartate aminotransferase (AST, U/L)&	18.40 (14.20–27.15)	17.20 (13.75–21.00)	31.50 (23.25–37.75)	<0.05**
Albumin (g/L)***	38.30 ± 5.30	39.83 ± 4.49	33.49 ± 4.79	<0.05
Creatinine (umol/L)***	67.15 ± 28.21	64.02 ± 26.95	77.56 ± 42.19	0.271
Creatine kinase (CK, U/L)***	82.89 ± 48.39	77.93 ± 46.05	100.27 ± 59.73	0.08
Lactate dehydrogenase (LDH, U/L)&	175 (150–241.5)	161 (145–192)	322 (279.75–400)	<0.05**

**Fisher's Exact Test.*
***Mann-Whitney U-Test.*
****average ± standard deviation (STD).*
& average (95% confidence interval).

DISCUSSION

Our study showed that underlying disease, fast respiratory rate (>24 times/min), elevated serum C-reactive protein level (CRP, >10 mg/L), and elevated lactate dehydrogenase level (LDH, >250 U/L) were four independent risk factors for predicting the progression of some COVID-19 patients from mild to severe conditions. Firstly, elevated lactate dehydrogenase levels ranked as number 1 (OR = 219.332) and fast respiratory rate as number 2 (OR = 57.726) among the four factors (**Table 2**). Interestingly, an elevated lactate dehydrogenase level was associated with severe SARS infection (14), which broke out in 2003, but was absent in the severe MERS infection (15), which is still circulating. When used individually, all four factors have a moderate prediction value for their low specificity and sensitivity (AUC values < 0.9) (**Table 3**). Secondly, we found that the combination of two

TABLE 2 | Independent factors associated with severe symptom development in COVID-19 patients.

Variables	β	S.E.	chi-square	*P*-value	OR (95% confidence interval)	
X_1	Underlying disease	2.752	1.066	6.666	0.01	15.67 (1.94–126.55)
X_2	Fast respiratory rate (>24 times/min)	4.056	1.183	11.76	0.001	57.726 (5.685–586.191)
X_3	CPR (>10 mg/L)	2.424	1.004	5.823	0.016	11.289 (1.577–80.838)
X_4	LDH (>250 U/L)	5.392	1.24	18.911	<0.001	219.608 (19.332–2494.742)
Intercept		−6.488	1.499	18.738	<0.001	0.002

S.E., standard error;
OR (95% CI), Odd Ratio (95% confidence interval).

TABLE 3 | Prognostic values for severe COVID-19 pneumonia development.

	Factor	AUC (95% CI)	Sensitivity	Specificity	Cut-off value	Youden Index	*P*-value
Single factor	Underlying diseases (1)	0.722 (0.614–0.829)	0.618	0.826	0.367	0.444	<0.001
	Fast respiratory rate (2)	0.758 (0.648–0.867)	0.559	0.957	0.492	0.516	<0.001
	Elevated CRP (3)	0.774 (0.685–0.864)	0.853	0.696	0.298	0.549	<0.001
	Elevated LDH (4)	0.855 (0.766–0.944)	0.765	0.946	0.461	0.711	<0.001
Two factors	(1) + (2)	0.853 (0.767–0.939)	0.824	0.793	0.223	0.617	<0.001
	(1) + (3)	0.854 (0.779–0.928)	0.853	0.696	0.274	0.549	<0.001
	(1) + (4)	0.940 (0.894–0.987)	0.971	0.783	0.156	0.754	<0.001
	(2) + (3)	0.870 (0.795–0.944)	0.912	0.663	0.18	0.575	<0.001
	(2) + (4)	0.944 (0.892–0.996)	0.941	0.902	0.315	0.843	<0.001
	(3) + (4)	0.918 (0.856–0.981)	0.765	0.946	0.365	0.711	<0.001
Three factors	(1) + (2) + (3)	0.910 (0.850–0.969)	0.765	0.902	0.253	0.667	<0.001
	(1) + (2) + (4)	0.976 (0.955–0.998)	0.912	0.935	0.411	0.846	<0.001
	(1) + (3) + (4)	0.963 (0.933–0.993)	0.912	0.891	0.227	0.803	<0.001
	(2) + (3) + (4)	0.964 (0.919–1.000)	0.912	0.934	0.355	0.847	<0.001
Four factors	(1) + (2) + (3) + (4)	0.985 (0.968–1.000)	0.912	0.957	0.374	0.869	<0.001

AUC (95% CI), Area under receiver operating characteristic curve (95% confidence interval). The highlighted combinations are those with AUC > 0.900 plus sensitivity > 0.900 plus specificity > 0.900, which indicates significant and reliable prognostic values for clinical practice.

factors, fast respiratory rate plus elevated LDH, could provide a high prognostic value for severe symptom development (AUC = 0.944, sensitivity = 0.941, and specificity = 0.902). Combinations of triple factors could significantly increase the prognostic value (AUC > 0.9). Finally, a combination of all four factors, provide an excellent prognostic efficacy, achieving AUC = 0.985 (95% CI 0.968–1.000) with high sensitivity (0.953) and specificity (0.968).

Our hospital has treated over 80% of COVID-19 patients in Guangzhou city-−298 cases as of February 29, 2020—including 55 severe cases but only one death case. All the patients except two patients recovered as of March 15. A retrospective analysis of all the cases revealed that the extremely low fatality rate in our hospital, one of 298 cases (0.0336%)—significantly lower than the overall fatality rate (2.3%) in China (5), was largely attributed to the effect of an expert panel, consisting of physicians from multiple disciplines, including infectious diseases, respiratory diseases, and intensive care unit (ICU), and radiology. Patients newly admitted were reviewed by the panel, and patients who meet several of the following criteria were transferred immediately to the ICU isolation ward for close supervision, including, (1) the illness onset has entered 7–10 days; (2) over 50 years old; (3) obesity, pregnant women, children; (4) with underlying diseases, especially hypertension, diabetes, COPD; (5) fast respiratory rate; (6) obvious decline in spirit and appetite; (7) significant reduction and/or progressive decline of peripheral blood lymphocytes; (8) decrease in albumin; (9) elevated C-reactive protein; (10) elevated lactate dehydrogenase; and (11) quickly deteriorated or with two or more lesions in lungs revealed by chest imaging. Once they progressed to the severe stage, they received treatment immediately. The above four prognostic factors, as routine and affordable clinical characteristics, were included in these criteria, and their immediate and preventive therapies were facilitated retrospectively.

All seven patients who were detected to be serum viral RNA positive developed severe symptoms very soon, which further confirmed our previous observation that detectable 2019-nCoV viral RNA in blood is a reliable indicator for further clinical severity (16). However, as the viral RNA positive rate was low high (seven of 32 cases, 21.8%) in this study and other reports (17) and viral RNA detection is expensive, we do not recommend the continuous detection of viral RNA. In this regard, we suggest reserving the precious reagent for confirming virus infection.

In conclusion, our study indicated that underlying disease, a fast respiratory rate, elevated serum C-reactive protein level, and elevated lactate dehydrogenase level significantly correlated to the development of severe COVID-19 pneumonia;

additionally, elevated lactate dehydrogenase and a fast respiratory rate (possibly plus one or two more other factors) can serve as prognostic factors for the discriminating potential severe cases among the mild COVID-19 patients. Our study provided convenient, reliable, and affordable references for both patients and physicians to make a highly confident decision to commence management and treatment safely.

SUMMARY

With our successful experience of treating COVID-19 patients, we retrospectively found that routine clinical features could reliably predict severe pneumonia development and could thus provide quick and affordable references for physicians to save patients with otherwise fatal COVID-19 using their limited medical resource.

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

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.

AUTHOR CONTRIBUTIONS

HH, FL, and XD conceived the study and wrote the manuscript. HH and SC collected data and performed the data analysis. HH, SC, YuL, YoL, YF, and XD participated in the clinical treatment. LL, CL, and XT supervised the clinical treatment. FH analyzed the results. All authors read the manuscript and approved the final version. All authors contributed to the article and approved the submitted version.

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Designing a Network Proximity-Based Drug Repurposing Strategy for COVID-19

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The ongoing COVID-19 pandemic still requires fast and effective efforts from all fronts, including epidemiology, clinical practice, molecular medicine, and pharmacology. A comprehensive molecular framework of the disease is needed to better understand its pathological mechanisms, and to design successful treatments able to slow down and stop the impressive pace of the outbreak and harsh clinical symptomatology, possibly via the use of readily available, off-the-shelf drugs. This work engages in providing a wider picture of the human molecular landscape of the SARS-CoV-2 infection via a network medicine approach as the ground for a drug repurposing strategy. Grounding on prior knowledge such as experimentally validated host proteins known to be viral interactors, tissue-specific gene expression data, and using network analysis techniques such as network propagation and connectivity significance, the host molecular reaction network to the viral invasion is explored and exploited to infer and prioritize candidate target genes, and finally to propose drugs to be repurposed for the treatment of COVID-19. Ranks of potential target genes have been obtained for coherent groups of tissues/organs, potential and distinct sites of interaction between the virus and the organism. The normalization and the aggregation of the different scores allowed to define a preliminary, restricted list of genes candidates as pharmacological targets for drug repurposing, with the aim of contrasting different phases of the virus infection and viral replication cycle.

Keywords: COVID-19, network medicine, drug repurposing, network-based, pharmacologic (drug) therapy

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INTRODUCTION

The worldwide ongoing COVID-19 pandemic outnumbers 23.9M confirmed cases and a death toll above 819,000 (∼3.4% global case fatality rate), at the time of writing[1] (Dong et al., 2020). Worse, in several densely populated countries, especially those in the South of the world, it is still difficult to forecast when a significant slowing down of the pace of the new infections will occur, and if, when

[1]Source: COVID-19 Dashboard by the Center for Systems Science and Engineering (CSSE) at Johns Hopkins University (JHU), available at https://gisanddata.maps.arcgis.com/apps/opsdashboard/index.html#/bda7594740fd40299423467b48e9ecf6, retrieved August 26th, 2020.

and with what intensity a new global wave will arise. The ultimate goal in fighting a pandemic is to completely stop the spread, but slowing it down is also crucial, to mitigate otherwise devastating effects on health and socioeconomic systems on a local and global scale. Thus, it is necessary to interfere by every possible means with the natural, deadly flow of the outbreak, in order to reduce and flatten the epidemic curve and relieve the pressure on hospitals capacity (Qualls et al., 2017; Anderson et al., 2020).

In this perspective, aside all already implemented epidemiological, clinical and immunological measures and efforts, a deployment, via drug repurposing, of the vast, existing and potentially effective pharmacological arsenal is timely and needed, witnessed by the numerous ongoing clinical trials on several off-the shelf drugs (source: DrugBank)[2]. This work is committed to aid in the fight against the health consequences of the COVID-19 pandemic by providing a data-driven, viable drug repurposing approach.

In this study, we give account of the complexity of the molecular interactions and processes underlying the SARS-CoV-2 host response, and provide an integrated molecular picture to be exploited for a drug repurposing strategy. Such a picture includes the charting of the protein interaction map involving host genes that in the current state of knowledge have been observed to interact with SARS-CoV-2 viral proteins, and/or are considered critical in the host infection processes,

[2]Source: https://www.drugbank.ca/Covid-19, retrieved July 6th, 2020.

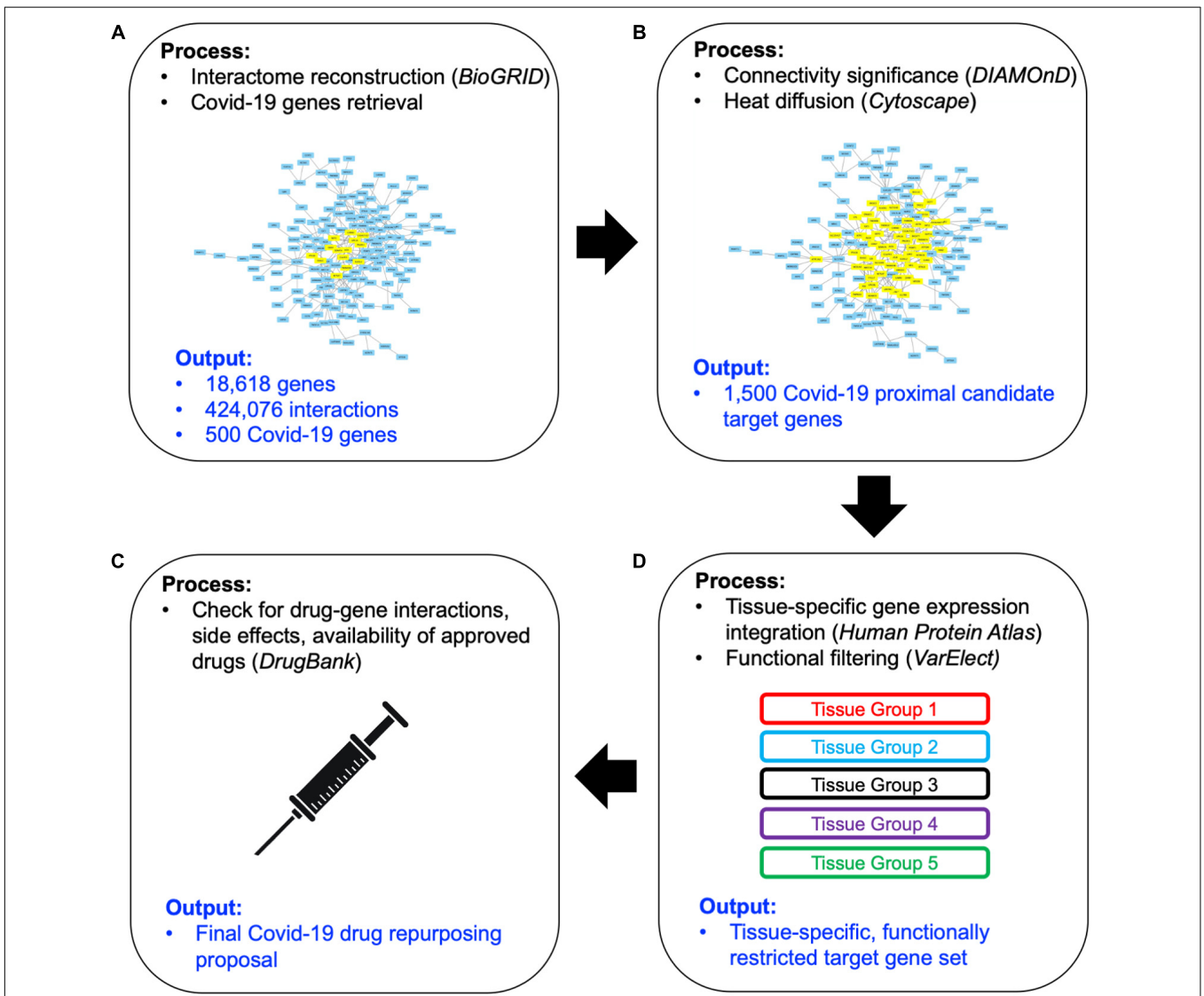

FIGURE 1 | Scheme of the workflow adopted: starting from available human interactome data and the set of COVID-19 experimentally associated genes **(A)**, a network proximity approach (based on connectivity significance and heat diffusion) has been carried out to select genes that are proximal to the initial set of COVID seed genes **(B)**. Filtering via gene expression in specific tissues and association to the most common COVID-19 symptoms and phenotypes **(C)** allowed the design of the proposed drug repurposing strategy **(D)**.

also considering previous knowledge related to other relevant Coronaviruses. In the wider context of network medicine (Bauer-Mehren et al., 2011; Silverman and Loscalzo, 2017), the protein-protein interaction (PPI) framework provides a widely assessed and effective heuristic approach for the identification of disease genes (Taylor et al., 2009; Gustafsson et al., 2014; Tieri et al., 2019; Silverman et al., 2020). The complexity of the organism's response to the viral invasion is mirrored by the wide variability of the clinical symptoms observed in patients, ranging from asymptomatic infections to extremely critical conditions, up to the death of the patient in around 3.4% of cases worldwide (see text footnote 1). With this study we therefore intended to expand the molecular landscape of the host proteins observed to directly interact with viral proteins (Gordon et al., 2020) to include actors who could be neglected when focusing only on the direct interactors set, and that could potentially prove to be important pharmacological targets to engage in order to propose an effective drug repurposing strategy aimed to improve the clinical outcome of the disease.

MATERIALS AND METHODS

The workflow of our approach has been sketched in **Figure 1**. Here we briefly describe each step of the method, providing detailed explanations in forthcoming subsections. We started by collecting updated human PPI data (**Figure 1A**) -from which a network of 18,618 human proteins and 424,076 binary interactions has been built- and SARS-CoV-2/Coronavirus/human PPI data, constituted by a set of 500 human genes potentially involved in the COVID-19 disease (see section "COVID-19 Associated Host Genes, Protein-Protein Interaction Data and Interactomes Reconstruction"). On such data, a network medicine approach has been applied by using connectivity significance and network diffusion algorithms in order to provide a COVID-19 "proximity" or "involvement" gene ranking (**Figure 1B**, details in section "Connectivity Significance" and "Network Diffusion").

The top 1,000 genes in the proximity ranking added to the original 500 Sars-CoV-2 related genes gives the final dataset of 1,500 mostly involved proteins in the COVID-19 disease. In order to further refine the selected list of genes, their gene expression levels in COVID-19-relevant tissues have been investigated (**Figure 1C**). The human tissues mostly involved in the COVID-19 infection have been identified and divided into five groups (see **Figure 2** and section "Gene Expression Data"). The genes that are not expressed in those tissues have been excluded. The remaining genes, for each tissue group, are ranked based on the most common COVID-19 symptoms. The rankings have been provided through VarElect functional filtering, whose details have been discussed in section "Functional Analysis," and they have been aggregated (see section "Rank Aggregation"), so that a restricted ranked list has been considered. Finally (**Figure 1D**), the proposed drug repositioning strategy was designed and implemented via dedicated drug-gene interaction information (see section "Design of Drug Repositioning Strategy via Drug-Gene Interaction Data").

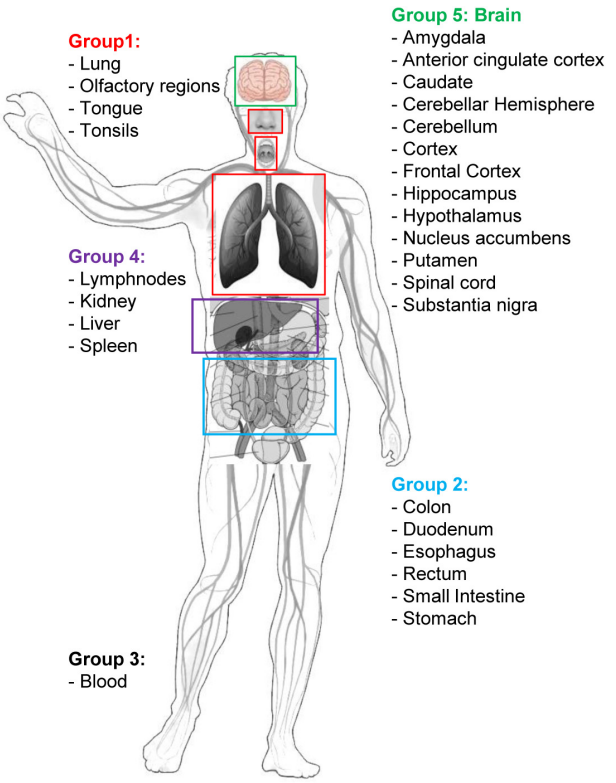

FIGURE 2 | Graphical sketch showing the selected five groups of organs/tissues representative of potential sites of interaction between SARS-CoV-2 and the organism. Group 1: respiratory tract tissues; Group 2: organs of the digestive system; Group 3: blood cells; Group 4: filtering organs; Group 5: brain areas. Group-specific gene expression data have been retrieved by the Human Protein Atlas web portal (www.proteinatlas.org).

COVID-19 Associated Host Genes, Protein-Protein Interaction Data and Interactomes Reconstruction

Protein-protein interaction data for interactome reconstruction have been retrieved from the BioGRID (Oughtred et al., 2019), one of the most comprehensive interaction repositories with freely provided data compiled through manual curation efforts, currently containing more than 1.7 million protein and genetic interactions from major model organism species, including Homo sapiens. The repository provides both the whole human-only interactome, as well as, in the effort to provide valuable data to fight the pandemic, the SARS-CoV-2/human protein interaction dataset, derived from several sources as described on the dedicated BioGRID webpage[3]. For this study, the latest version available at the time of the analysis of the human interactome, and of COVID-19-associated host genes, i.e., version 3.5.186 (.tab2 and .tab3 format types) have been used. The dataset includes 338 human proteins interacting with SARS-CoV-2 [i.e., the genes identified by the seminal work of Gordon and colleagues (Gordon et al., 2020)], 47

[3]https://wiki.thebiogrid.org/doku.php/covid

human proteins considered critical for the virus host entry and response, and further 115 proteins experimentally observed to interact with other, SARS-relevant Coronaviruses, finally totaling 500 involved human genes (**Supplementary Table S1**). The reason for including the last 115 genes is found in the fact that it is known that there is marked similarity and a close relationship between SARS-CoV-2 and SARS-CoVs or SARS-like bat CoVs (Wu A. et al., 2020), similarities that could play a relevant role when comparing the host tropism and transmission features of the SARS-CoV-2 and SARS-CoV and that are thus worthy of investigation. Besides these considerations, and despite the efforts in experimental PPI mapping, it is also known that the number of missing interactions greatly exceeds the number of experimentally detected interactions (Kovács et al., 2019). In this perspective, these further viral-human interactions related to other Coronaviruses provided by BioGRID in the same dataset represent a very significant information from the heuristic point of view, partly due to structural similarities.

The whole human interactome has been gathered from BioGRID data as well (BIOGRID-ORGANISM-3.5.186.tab3.zip), and the largest connected component (LCC) has been extracted to undergo network analysis, consisting of 18,618 genes and 424,076 unique pairwise interactions among them (**Supplementary File S1**).

Connectivity Significance

The concept of connectivity significance, originally proposed by Ghiassian et al. (2015), has been used to uncover genes associated with a particular path phenotype, via the observation that proteins associated to specific diseases show peculiar patterns of interaction among each other, patterns that in turn help in the identification of neighborhoods not previously associated to the disease. An efficient algorithm (a.k.a. DIAMOnD, DIseAse MOdule Detection) to compute this measure is publicly available (Ghiassian et al., 2015), and it has been used to rank the genes in the interactome showing the highest connectivity significance

TABLE 1 | Groups of tissue/organs matched with disease phenotypes **(A)** and number of target genes selected as potential candidates for drug repurposing by VarElect aggregate and single group ranks **(B)**.

(A) Groups of tissue/organs matched with disease phenotypes

Group # ID	Organ systems	Organs and tissues	Disease phenotypes (symptoms or disease manifestations)
Group 1	Respiratory tract	Lungs, tongue, tonsils, olfactory epithelium	"Fever" OR "cough" OR "pneumonia" OR "dyspnea" OR "pain" OR "hemoptysis " OR "sore throat" OR "chills" OR "inflammation"
Group 2	Digestive system	Stomach, esophagus, colon, duodenum, small intestine and rectum	"Fever" OR "diarrhea" OR "pain" OR "nausea" OR "vomiting" OR "inflammation"
Group 3	Blood cells	n/a	"Fever" OR "chills" OR "inflammation" OR "hemorrhagic"
Group 4	Filtering organs	Spleen, liver, lymph nodes, kidney	"Fever" OR "cough" OR "diarrhea" OR "pain" OR "nausea" OR "vomiting" OR "chills" OR "inflammation" OR "hemorrhagic"
Group 5a	Brain areas	Amygdala, Basal Ganglia, Cerebellum, Cerebral Cortex, Hippocampus, Hypothalamus, Midbrain, Olfactory region, Pons and Medulla, Thalamus	"Dizziness" OR "headache" OR "consciousness" OR "encephalopathy" OR "encephalitis" OR "seizures" OR "stroke" OR "delirium"
Group 5b	Brain areas	Amygdala, Basal Ganglia, Cerebellum, Cerebral Cortex, Hippocampus, Hypothalamus, Midbrain, Olfactory region, Pons and Medulla, Thalamus	"Ageusia" OR "dysgeusia" OR "hypogeusia" OR "anosmia" OR "hyposmia" OR "myalgia" OR "myelitis" OR "pain" OR "Guillain-Barre"

(B) Number of target genes selected as potential candidates for drug repurposing by VarElect aggregate and single group ranks

Group # ID	Selected gene targets (potential candidates for drug repurposing)	Selected genes	Selection of the first 15 genes
Group 1+2+4 (G124)	101	See **Supplementary Table S11**	See **Table 2**
Group 1+2+3+4+5 (G12345)	99	See **Supplementary Table S12**	See **Table 3**
Group 3 (G3)	20	See **Supplementary Table S13**	See **Table 4**
Group 5a (G5a)	20	See **Supplementary Table S14**	See **Table 5**
Group 5b (G5b)	20	See **Supplementary Table S15**	See **Table 6**

TABLE 2 | VarElect aggregated score obtained analyzing groups 1, 2, and 4 (G124; 15 top-ranking genes).

Aggregated score (normalized)	Gene	Gene description	Protein class	Biological process	Molecular function	Disease involvement	Subcellular Location
0.999501151	TNF	Tumor necrosis factor	Cancer-related genes, Candidate cardiovascular disease genes, Disease related genes, FDA approved drug targets, Plasma proteins, Predicted intracellular proteins, Predicted secreted proteins		Cytokine	Cancer-related genes, FDA approved drug targets	Secreted
0.624534796	TNFRSF1A	TNF receptor superfamily member 1A	Cancer-related genes, Candidate cardiovascular disease genes, CD markers, Disease related genes, FDA approved drug targets, Plasma proteins, Predicted membrane proteins, Predicted secreted proteins	Apoptosis, Host-virus interaction	Receptor	Amyloidosis, Cancer-related genes, Disease mutation, FDA approved drug targets	
0.529423274	TP53	Tumor protein p53	Cancer-related genes, Disease related genes, Plasma proteins, Potential drug targets, Predicted intracellular proteins, Transcription factors, Transporters	Apoptosis,Biological rhythms, Cell cycle, Host-virus interaction, Necrosis, Transcription, Transcription regulation	Activator, DNA-binding, Repressor	Cancer-related genes, Disease mutation, Li-Fraumeni syndrome, Tumor suppressor	Nucleoplasm
0.479435236	NLRP3	NLR family pyrin domain containing 3	Cancer-related genes, Disease related genes, Predicted intracellular proteins	Immunity, Inflammatory response, Innate immunity, Transcription, Transcription regulation	Activator	Amyloidosis, Cancer-related genes, Deafness, Disease mutation, Non-syndromic deafness	
0.406134194	TGFB1	Transforming growth factor beta 1	Cancer-related genes, Candidate cardiovascular disease genes, Disease related genes, Plasma proteins, Predicted intracellular proteins, Predicted secreted proteins		Growth factor, Mitogen	Cancer-related genes, Disease mutation	Golgi apparatus, secreted
0.404538712	EGFR	Epidermal growth factor receptor	Cancer-related genes, Disease related genes, Enzymes, FDA approved drug targets, Plasma proteins, Predicted intracellular proteins, Predicted membrane proteins, Predicted secreted proteins, RAS pathway related proteins	Host-virus interaction	Developmental protein, Host cell receptor for virus entry, Kinase, Receptor, Transferase, Tyrosine-protein kinase	Cancer-related genes, Disease mutation, FDA approved drug targets, Proto-oncogene	Plasma membrane
0.393038016	ICAM1	Intercellular adhesion molecule 1	Cancer-related genes, Candidate cardiovascular disease genes, CD markers, FDA approved drug targets, Plasma proteins, Predicted intracellular proteins, Predicted membrane proteins	Cell adhesion, Host-virus interaction	Host cell receptor for virus entry, Receptor	Cancer-related genes, FDA approved drug targets	Plasma membrane
0.37555677	FAS	Fas cell surface death receptor	Cancer-related genes, Candidate cardiovascular disease genes, CD markers, Disease related genes, Predicted membrane proteins, Predicted secreted proteins	Apoptosis	Calmodulin-binding, Receptor	Cancer-related genes, Disease mutation	Plasma membrane
0.352296	STAT3	Signal transducer and activator of transcription 3	Cancer-related genes, Disease related genes, Plasma proteins, Predicted intracellular proteins, Transcription factors	Host-virus interaction, Transcription, Transcription regulation	Activator, DNA-binding	Cancer-related genes, Diabetes mellitus, Disease mutation, Dwarfism	Nucleoplasm, cytosol

(Continued)

TABLE 2 | Continued

Aggregated score (normalized)	Gene	Gene description	Protein class	Biological process	Molecular function	Disease involvement	Subcellular Location
0.337175731	STAT1	Signal transducer and activator of transcription 1	Disease related genes, Plasma proteins, Predicted intracellular proteins, Transcription factors	Antiviral defense, Host-virus interaction, Transcription, Transcription regulation	Activator, DNA-binding	Disease mutation	Nucleoplasm, cytosol
0.336625898	KRAS	KRAS proto-oncogene, GTPase	Cancer-related genes, Disease related genes, Predicted intracellular proteins, RAS pathway related proteins			Cancer-related genes, Cardiomyopathy, Deafness, Disease mutation, Ectodermal dysplasia, Mental retardation, Proto-oncogene	Cytosol
0.324061896	CTNNB1	Catenin beta 1	Cancer-related genes, Disease related genes, Plasma proteins, Predicted intracellular proteins	Cell adhesion, Host-virus interaction, Neurogenesis, Transcription, Transcription regulation, Wnt signaling pathway	Activator	Cancer-related genes, Disease mutation, Mental retardation	Plasma membrane
0.315947057	AKT1	AKT serine/threonine kinase 1	Cancer-related genes, Disease related genes, Enzymes, Potential drug targets, Predicted intracellular proteins, RAS pathway related proteins	Apoptosis, Carbohydrate metabolism, Glucose metabolism, Glycogen biosynthesis, Glycogen metabolism, Neurogenesis, Sugar transport, Translation regulation, Transport	Developmental protein, Kinase, Serine/threonine-protein kinase, Transferase	Cancer-related genes, Disease mutation, Proto-oncogene	Nucleoplasm
0.314911716	CCND1	Cyclin D1	Cancer-related genes, Disease related genes, FDA approved drug targets, Predicted intracellular proteins	Cell cycle, Cell division, DNA damage, Transcription, Transcription regulation	Cyclin, Repressor	Cancer-related genes, FDA approved drug targets, Proto-oncogene	Nucleoplasm
0.312644606	ERBB2	Erb-b2 receptor tyrosine kinase 2	Cancer-related genes, CD markers, Disease related genes, Enzymes, FDA approved drug targets, Plasma proteins, Predicted intracellular proteins, Predicted membrane proteins	Transcription, Transcription regulation	Activator, Kinase, Receptor, Transferase, Tyrosine-protein kinase	Cancer-related genes, FDA approved drug targets	Plasma membrane

with respect to the 500 COVID-19-associated seed genes (in **Supplementary Table S2** were reported the first 200 ranked genes).

Network Diffusion

Network diffusion (or network propagation) is a methodology able to identify those genes which are proximal to a starting list of seed genes by using network topology (and optionally other features). In network medicine it can be used to identify genes and genetic modules that underlie human diseases (Mosca et al., 2014; Cowen et al., 2017; Sumathipala et al., 2019) or to identify causal paths linking mutations to expression regulators, or to discover significantly mutated subnetworks in cancer (Vandin et al., 2011; Paull et al., 2013). The methodology exploits the concept of heat diffusion, i.e., how the heat distribution spreads over time in a medium, here consisting of the PPI network, as it flows from nodes where it is higher toward nodes where it is lower according to the diffusion coefficient and their mutual connections. In practice, starting with an arbitrary subset of seed nodes (e.g., genes associated with a

TABLE 3 | VarElect aggregated score obtained analyzing groups 1, 2, 3, 4, and 5 (G12345; 15 top-ranking genes).

Aggregated score (normalized)	Gene	Gene description	Protein class	Biological process	Molecular function	Disease involvement	Subcellular Location
0.999992851	TNF	Tumor necrosis factor	Cancer-related genes, Candidate cardiovascular disease genes, Disease related genes, FDA approved drug targets, Plasma proteins, Predicted intracellular proteins, Predicted secreted proteins		Cytokine	Cancer-related genes, FDA approved drug targets	
0.646667342	TNFRSF1A	TNF receptor superfamily member 1A	Cancer-related genes, Candidate cardiovascular disease genes, CD markers, Disease related genes, FDA approved drug targets, Plasma proteins, Predicted membrane proteins, Predicted secreted proteins	Apoptosis, Host-virus interaction	Receptor	Amyloidosis, Cancer-related genes, Disease mutation, FDA approved drug targets	
0.469465581	TP53	Tumor protein p53	Cancer-related genes, Disease related genes, Plasma proteins, Potential drug targets, Predicted intracellular proteins, Transcription factors, Transporters	Apoptosis, Biological rhythms, Cell cycle, Host-virus interaction, Necrosis, Transcription, Transcription regulation	Activator, DNA-binding, Repressor	Cancer-related genes, Disease mutation, Li-Fraumeni syndrome, Tumor suppressor	Nucleoplasm
0.415888546	TGFB1	Transforming growth factor beta 1	Cancer-related genes, Candidate cardiovascular disease genes, Disease related genes, Plasma proteins, Predicted intracellular proteins, Predicted secreted proteins		Growth factor, Mitogen	Cancer-related genes, Disease mutation	Golgi apparatus, Cytosol
0.413794221	NLRP3	NLR family pyrin domain containing 3	Cancer-related genes, Disease related genes, Predicted intracellular proteins	Immunity, Inflammatory response, Innate immunity, Transcription, Transcription regulation	Activator	Amyloidosis, Cancer-related genes, Deafness, Disease mutation, Non-syndromic deafness	
0.374658935	FAS	Fas cell surface death receptor	Cancer-related genes, Candidate cardiovascular disease genes, CD markers, Disease related genes, Predicted membrane proteins, Predicted secreted proteins	Apoptosis	Calmodulin-binding, Receptor	Cancer-related genes, Disease mutation	Plasma membrane
0.332023563	STAT1	Signal transducer and activator of transcription 1	Disease related genes, Plasma proteins, Predicted intracellular proteins, Transcription factors	Antiviral defense, Host-virus interaction, Transcription, Transcription regulation	Activator, DNA-binding	Disease mutation	Nucleoplasm, Cytosol
0.330861395	ICAM1	Intercellular adhesion molecule 1	Cancer-related genes, Candidate cardiovascular disease genes, CD markers, FDA approved drug targets, Plasma proteins, Predicted intracellular proteins, Predicted membrane proteins	Cell adhesion, Host-virus interaction	Host cell receptor for virus entry, Receptor	Cancer-related genes, FDA approved drug targets	Plasma membrane, Cytosol
0.325734154	STAT3	Signal transducer and activator of transcription 2	Disease related genes, Predicted intracellular proteins, Transcription factors	Antiviral defense, Host-virus interaction, Transcription, Transcription regulation	Activator, DNA-binding		Plasma membrane, Cytosol

(Continued)

TABLE 3 | Continued

Aggregated score (normalized)	Gene	Gene description	Protein class	Biological process	Molecular function	Disease involvement	Subcellular Location
0.285387576	AKT1	Signal transducer and activator of transcription 3	Cancer-related genes, Disease related genes, Plasma proteins, Predicted intracellular proteins, Transcription factors	Host-virus interaction, Transcription, Transcription regulation	Activator, DNA-binding	Cancer-related genes, Diabetes mellitus, Disease mutation, Dwarfism	Nucleoplasm
0.280849434	CTNNB1	Catenin beta 1	Cancer-related genes, Disease related genes, Plasma proteins, Predicted intracellular proteins	Cell adhesion, Host-virus interaction, Neurogenesis, Transcription, Transcription regulation, Wnt signaling pathway	Activator	Cancer-related genes, Disease mutation, Mental retardation	Plasma membrane
0.267915871	SOD1	Superoxide dismutase 1	Cancer-related genes, Disease related genes, Enzymes, Plasma proteins, Potential drug targets, Predicted intracellular proteins		Antioxidant, Oxidoreductase	Amyotrophic lateral sclerosis, Cancer-related genes, Disease mutation, Neurodegeneration	Nucleoplasm, Plasma membrane
0.267304947	SPP1	Secreted phosphoprotein 1	Cancer-related genes, Plasma proteins, Predicted secreted proteins	Biomineralization, Cell adhesion	Cytokine	Cancer-related genes	Golgi apparatus
0.266717153	KRAS	KRAS proto-oncogene, GTPase	Cancer-related genes, Disease related genes, Predicted intracellular proteins, RAS pathway related proteins			Cancer-related genes, Cardiomyopathy, Deafness, Disease mutation, Ectodermal dysplasia, Mental retardation, Proto-oncogene	
0.25614674	PTEN	Phosphatase and tensin homolog	Cancer-related genes, Disease related genes, Enzymes, Potential drug targets, Predicted intracellular proteins, Predicted secreted proteins	Apoptosis, Lipid metabolism, Neurogenesis	Hydrolase, Protein phosphatase	Autism spectrum disorder, Cancer-related genes, Disease mutation, Tumor suppressor	Nucleoplasm, Cytosol

disease), a diffusion algorithm is applied to the initial values assigned to the seed nodes that propagate through the network according to its topology. Fixing a stopping time for the diffusion algorithm, the final distribution of the propagated values generates a proximity ranking that can be used to identify a subset of genes that are closely associated to the selected seed genes. The Cytoscape network analysis platform (Shannon et al., 2003), version 3.7, and the Cytoscape-embedded function "Diffuse," based on a heat diffusion algorithm, have been used for the analysis (Carlin et al., 2017). The diffusion algorithm has been run considering as seed genes the 500 COVID-19-associated human genes with initial heat $h_s(0) = 1$; non-seed genes have been set with initial heat $h_{ns}(0) = 0$. The heat diffusion has been observed at the following times t: 0.002, 0.005, 0.01, 0.02, 0.05 (arbitrary algorithm diffusion time units; **Supplementary Table S3**), and the quantities of heat in non-seed genes $h_{ns}(t)$ have been computed. The appropriate time has been identified by considering, for each time t, the intersection of the most significant genes obtained via the DIAMOnD algorithm and the most relevant genes in the diffusion process, i.e., the ones with highest $h_{ns}(t)$ values, and selecting the time showing the largest overlap, that turned out to be $t = 0.005$. More in detail, we considered the overlap of the top 200 genes obtained via the DIAMOnD algorithm with the top 1000 genes obtained via the heat diffusion algorithm at each stopping time. The combination of the two methods, the heat diffusion that favors genes well-connected to the seed genes or with high degrees, and the DIAMOnD that privileged those genes that are well-connected to the set of the seed genes, generates a proximity ranking of topologically well-connected genes to the COVID-19-associated genes. Moreover, since the overlap in the intersection is about 50%, the number of genes that is surely well-connected to the seed genes is very significant.

Rank Aggregation

Rank aggregation deals with the aggregation of several lists of preferences obtained from different methodologies. It is very

TABLE 4 | VarElect aggregated score obtained analyzing group 3 (G3; 15 top-ranking genes).

Normalized score	Gene	Gene description	Protein class	Biological process	Molecular function	Disease involvement	Subcellular Location
1	TBK1	TANK Binding Kinase 1	Disease related genes, Enzymes, Potential drug targets, Predicted intracellular proteins, RAS pathway related proteins	Antiviral defense, Host-virus interaction, Immunity, Innate immunity	Kinase, Serine/threonine-protein kinase, Transferase	Amyotrophic lateral sclerosis, Disease mutation, Glaucoma, Neurodegeneration	Nucleoplasm, Vesicles
0.855967078	TNFAIP3	TNF Alpha Induced Protein 3	Cancer-related genes, Disease related genes, Enzymes, Plasma proteins, Potential drug targets, Predicted intracellular proteins	Apoptosis, Inflammatory response, Ubl conjugation pathway	DNA-binding, Hydrolase, Multifunctional enzyme, Protease, Thiol protease, Transferase	Cancer-related genes, Disease mutation	Vesicles
0.695473251	RANBP2	RAN Binding Protein 2	Cancer-related genes, Disease related genes, Plasma proteins, Potential drug targets, Predicted intracellular proteins, Transporters	mRNA transport, Protein transport, Translocation, Transport, Ubl conjugation pathway	RNA-binding, Transferase	Cancer-related genes	Nuclear membrane, Vesicles
0.582167353	APP	Amyloid Beta Precursor Protein	Disease related genes, FDA approved drug targets, Plasma proteins, Predicted intracellular proteins, Predicted membrane proteins, Transporters	Apoptosis, Cell adhesion, Endocytosis, Notch signaling pathway	Heparin-binding, Protease inhibitor, Serine protease inhibitor	Alzheimer disease, Amyloidosis, Disease mutation, FDA approved drug targets, Neurodegeneration	Golgi apparatus; Vesicles
0.573662551	PPARG	Peroxisome Proliferator Activated Receptor Gamma	Cancer-related genes, Disease related genes, FDA approved drug targets, Nuclear receptors, Plasma proteins, Predicted intracellular proteins, Transcription factors	Biological rhythms, Transcription, Transcription regulation	Activator, DNA-binding, Receptor	Cancer-related genes, Diabetes mellitus, Disease mutation, FDA approved drug targets, Obesity	Nucleoplasm, Vesicles
0.560219479	GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	Enzymes, FDA approved drug targets, Plasma proteins, Predicted intracellular proteins	Apoptosis, Glycolysis, Translation regulation	Oxidoreductase, Transferase	FDA approved drug targets	Plasma membrane, Cytosol, Vesicles
0.465569273	NEU1	Neuraminidase 1	Disease related genes, Enzymes, Potential drug targets, Predicted intracellular proteins	Carbohydrate metabolism, Lipid degradation, Lipid metabolism	Glycosidase, Hydrolase	Disease mutation	Vesicles
0.456241427	NTRK1	Neurotrophic Receptor Tyrosine Kinase 1	Cancer-related genes, Disease related genes, Enzymes, FDA approved drug targets, Predicted membrane proteins, RAS pathway related proteins	Differentiation, Neurogenesis	Developmental protein, Kinase, Receptor, Transferase, Tyrosine-protein kinase	Cancer-related genes, Disease mutation, FDA approved drug targets, Proto-oncogene	Vesicles, Cytosol
0.438134431	MTOR	Mechanistic Target Of Rapamycin Kinase	Cancer-related genes, Disease related genes, Enzymes, FDA approved drug targets, Plasma proteins, Predicted intracellular proteins	Biological rhythms	Kinase, Serine/threonine-protein kinase, Transferase	Cancer-related genes, Disease mutation, Epilepsy, FDA approved drug targets, Mental retardation	Vesicles, Cytosol

(Continued)

TABLE 4 | Continued

Normalized score	Gene	Gene description	Protein class	Biological process	Molecular function	Disease involvement	Subcellular Location
0.427160494	LYN	LYN Proto-Oncogene, Src Family Tyrosine Kinase	Cancer-related genes, Disease related genes, Enzymes, Potential drug targets, Predicted intracellular proteins	Adaptive immunity, Host-virus interaction, Immunity, Inflammatory response, Innate immunity	Kinase, Transferase, Tyrosine-protein kinase	Cancer-related genes, Proto-oncogene	Golgi apparatus, Plasma membrane
0.421124829	CHUK	Component Of Inhibitor Of Nuclear Factor Kappa B Kinase Complex	Disease related genes, Enzymes, Potential drug targets, Predicted intracellular proteins, RAS pathway related proteins		Kinase, Serine/threonine-protein kinase, Transferase		Nucleoplasm, Vesicles, Cytosol
0.417283951	TPI1	Triosephosphate Isomerase 1	Cancer-related genes, Disease related genes, Enzymes, Plasma proteins, Potential drug targets, Predicted intracellular proteins	Gluconeogenesis, Glycolysis	Isomerase, Lyase	Cancer-related genes, Disease mutation, Hereditary hemolytic anemia	Nucleoplasm, Vesicles
0.376680384	ATM	ATM Serine/Threonine Kinase	Cancer-related genes, Disease related genes, Enzymes, Plasma proteins, Potential drug targets, Predicted intracellular proteins, Predicted membrane proteins	Cell cycle, DNA damage	DNA-binding, Kinase, Serine/threonine-protein kinase, Transferase	Cancer-related genes, Disease mutation, Neurodegeneration, Tumor suppressor	Nucleoplasm, Vesicles
0.372565158	RUNX1	RUNX Family Transcription Factor 1	Cancer-related genes, Disease related genes, Plasma proteins, Predicted intracellular proteins, Transcription factors	Transcription, Transcription regulation	Activator, DNA-binding, Repressor	Cancer-related genes, Disease mutation, Proto-oncogene	Nucleoplasm, Vesicles
0.368998628	BSG	Basigin (Ok Blood Group)	Blood group antigen proteins, CD markers, Predicted intracellular proteins, Predicted membrane proteins, Transporters		Blood group antigen		Vesicles

useful in all those situations in which preferences can be set according to several features, none of them prevailing on the others. This is actually our case with different lists of best genes associated with the different preferences, none being preferred over the others. Many methods have been proposed in literature to aggregate rank, they are mainly divided into three groups, namely heuristic algorithms, methods based on Markov chains and stochastic optimization methods, see Lin (2010) for a detailed overview. The most suitable method in this particular situation turned out to be a stochastic optimization method. Namely, a new ranking is obtained through an optimization problem whose objective is to minimize the distance between the new ranking and all the others. This approach usually considers two distances, the L1, also known in the rank aggregation literature as Spearman's distance, and the Kendall distance. The main difference between these two measures is that the first one considers the distances between the different scores of the genes in the different lists of preferences, while the second one takes into account the partial order of the ranking counting the number of pairwise discordance between two lists of preferences. The optimization has been carried out using the L1 distance over the list of preference obtained from the VarElect tool detailed in section "Functional Analysis."

Gene Expression Data

Human tissue-specific gene expression data have been retrieved by the Human Protein Atlas web portal[4] (Uhlén et al., 2015). The Tissue Atlas includes information about the expression profiles of human genes on mRNA and protein level. The protein data covers 15,313 genes (78%) for which there are antibodies available. The mRNA expression data are derived from

[4]www.proteinatlas.org

TABLE 5 | VarElect aggregated score obtained analyzing group 5 related to the central nervous system (G5a; 15 top-ranking genes).

Normalized score	Gene	Gene description	Protein class	Biological process	Molecular function	Disease involvement	Subcellular Location
1	TNF	Tumor Necrosis Factor	Cancer-related genes, Candidate cardiovascular disease genes, Disease related genes, FDA approved drug targets, Plasma proteins, Predicted intracellular proteins, Predicted secreted proteins		Cytokine	Cancer-related genes, FDA approved drug targets	
0.744887478	MAPT	Microtubule Associated Protein Tau	Candidate cardiovascular disease genes, Disease related genes, FDA approved drug targets, Plasma proteins, Predicted intracellular proteins			Alzheimer disease, Disease mutation, FDA approved drug targets, Neurodegeneration, Parkinsonism	Plasma membrane
0.64954711	APP	Amyloid Beta Precursor Protein	Disease related genes, FDA approved drug targets, Plasma proteins, Predicted intracellular proteins, Predicted membrane proteins, Transporters	Apoptosis, Cell adhesion, Endocytosis, Notch signaling pathway	Heparin-binding, Protease inhibitor, Serine protease inhibitor	Alzheimer disease, Amyloidosis, Disease mutation, FDA approved drug targets, Neurodegeneration	Golgi apparatus, vesicles
0.570314689	APOE	Apolipoprotein E	Cancer-related genes, Candidate cardiovascular disease genes, Disease related genes, Plasma proteins, Predicted secreted proteins	Cholesterol metabolism, Lipid metabolism, Lipid transport, Steroid metabolism, Sterol metabolism, Transport	Heparin-binding	Alzheimer disease, Amyloidosis, Cancer-related genes, Disease mutation, Hyperlipidemia, Neurodegeneration	Vesicles
0.534036791	TP53	Tumor Protein P53	Cancer-related genes, Disease related genes, Plasma proteins, Potential drug targets, Predicted intracellular proteins, Transcription factors, Transporters	Apoptosis, Biological rhythms, Cell cycle, Host-virus interaction, Necrosis, Transcription, Transcription regulation	Activator, DNA-binding, Repressor	Cancer-related genes, Disease mutation, Li-Fraumeni syndrome, Tumor suppressor	Nucleoplasm
0.530675133	IRF3	Interferon Regulatory Factor 3	Disease related genes, Predicted intracellular proteins, Transcription factors	Antiviral defense, Host-virus interaction, Immunity, Innate immunity, Transcription, Transcription regulation	Activator, DNA-binding	Disease mutation	Cytosol
0.530301615	PSEN1	Presenilin 1	Cancer-related genes, Disease related genes, Enzymes, Potential drug targets, Predicted intracellular proteins, Predicted membrane proteins, Transporters	Apoptosis, Cell adhesion, Notch signaling pathway	Hydrolase, Protease	Alzheimer disease, Amyloidosis, Cancer-related genes, Cardiomyopathy, Disease mutation, Neurodegeneration	Golgi apparatus, Nucleoplasm
0.519422915	SPTAN1	Spectrin Alpha, Non-Erythrocytic 1			Actin capping, Actin-binding, Calmodulin-binding	Disease mutation, Epilepsy, Mental retardation	Vesicles, Microtubules
0.503408348	SNCA	Alpha Synuclein	Disease related genes, Plasma proteins, Potential drug targets, Predicted intracellular proteins, Transporters			Alzheimer disease, Disease mutation, Neurodegeneration, Parkinson disease, Parkinsonism	

(Continued)

TABLE 5 | Continued

Normalized score	Gene	Gene description	Protein class	Biological process	Molecular function	Disease involvement	Subcellular Location
0.485059296	UNC93B1	Unc-93 Homolog B1, TLR Signaling Regulator		Adaptive immunity, Antiviral defense, Immunity, Innate immunity			Nucleoplasm
0.48440564	SOD1	Superoxide Dismutase 1	Cancer-related genes, Disease related genes, Enzymes, Plasma proteins, Potential drug targets, Predicted intracellular proteins		Antioxidant, Oxidoreductase	Amyotrophic lateral sclerosis, Cancer-related genes, Disease mutation, Neurodegeneration	Nucleoplasm, Plasma membrane
0.441777944	TBK1	TANK Binding Kinase 1	Disease related genes, Enzymes, Potential drug targets, Predicted intracellular proteins, RAS pathway related proteins	Antiviral defense, Host-virus interaction, Immunity, Innate immunity	Kinase, Serine/threonine-protein kinase, Transferase	Amyotrophic lateral sclerosis, Disease mutation, Glaucoma, Neurodegeneration	Nucleoplasm, vesicles
0.424596134	TGFB1	Transforming Growth Factor Beta 1	Cancer-related genes, Candidate cardiovascular disease genes, Disease related genes, Plasma proteins, Predicted intracellular proteins, Predicted secreted proteins		Growth factor, Mitogen	Cancer-related genes, Disease mutation	Golgi apparatus, Cytosol
0.420767579	ACADM	Acyl-CoA Dehydrogenase Medium Chain	Disease related genes, Enzymes, Potential drug targets, Predicted intracellular proteins	Fatty acid metabolism, Lipid metabolism	Oxidoreductase	Disease mutation	Mitochondria
0.41894668	TSC2	TSC Complex Subunit 2	Cancer-related genes, Disease related genes, Plasma proteins, Predicted intracellular proteins, Predicted membrane proteins	Host-virus interaction	GTPase activation	Cancer-related genes, Disease mutation, Epilepsy, Tumor suppressor	Cytosol

RNA-seq of 37 different healthy individuals. Genes expressed in 5 organs and tissue groups, representative of potential sites of interaction between SARS-CoV-2 and the organism, were first selected (**Table 1** and **Figure 2**), based on up-to-date information[5]. Indeed, it is actually recognized that, beside its impact on the respiratory system, SARS-CoV-2 induces multi-organ dysfunctions (Bal et al., 2020; Wu T. et al., 2020) indicating a potential virus-host interaction extended to several organs/systems. Respiratory tract tissues (lungs, tongue, tonsils, and olfactory epithelium) were included in group 1. In group 2, organs and tissues of the digestive system (stomach, esophagus, colon, duodenum, small intestine and rectum) were included. Groups 1 and 2 are therefore representative of the highest probability of virus-host interaction, affecting the epithelial cells (Cong and Ren, 2014). All blood cells were included in group 3. In group 4 the filtering organs and tissues (spleen, liver, lymph nodes, and kidney) were included. Finally, all brain areas for which RNA expression data were available in Protein Atlas (Amygdala, Basal Ganglia, Cerebellum, Cerebral Cortex, Hippocampus, Hypothalamus, Midbrain, Olfactory region, Pons and Medulla, and Thalamus) were included in group 5. The need to include tissues belonging to the nervous system in the analysis derives from the emerging evidence of a specific involvement of the latter in the development of symptoms currently named Neuro-COVID (Ahmad and Rathore, 2020; Helms et al., 2020; Mao et al., 2020). For each group, genes with an expression level <2 (for details about normalized RNA expression data see "Normalization of transcriptomics data" section in the Protein Atlas web portal)[6] in all tissues/organs belonging to each group were excluded from the analysis. In each of the groups, the 1,500 mostly involved proteins in the COVID-19 disease were selected according to their expression level and used for the functional analysis through the VarElect tool (Stelzer et al., 2016), see section "Functional Analysis" for details.

Functional Analysis

We took advantage from the VarElect tool, a comprehensive phenotype-dependent gene prioritizer, based on the widely used GeneCards, which helps in identifying causal gene-phenotype associations with extensive evidence (Stelzer et al., 2016). The sets of COVID-host interacting genes, selected for each group of tissue/organs, were matched with disease phenotypes

[5]Wadman M. et al., "How does coronavirus kill? Clinicians trace a ferocious rampage through the body, from brain to toes". doi: 10.1126/science.abc3208

[6]https://www.proteinatlas.org/about/assays+annotation#rna

TABLE 6 | VarElect aggregated score obtained analyzing group 5 related to the peripheral nervous system (G5b; 15 top-ranking genes).

Normalized score	Gene	Gene description	Protein class	Biological process	Molecular function	Disease involvement	Subcellular Location
1	NTRK1	Neurotrophic Receptor Tyrosine Kinase 1	Differentiation, Neurogenesis	Developmental protein, Kinase, Receptor, Transferase, Tyrosine-protein kinase	Cancer-related genes, Disease mutation, FDA approved drug targets, Proto-oncogene	Evidence at protein level	Vesicles, Cytosol
0.460741389	APOE	Apolipoprotein E	Cancer-related genes, Candidate cardiovascular disease genes, Disease related genes, Plasma proteins, Predicted secreted proteins	Cholesterol metabolism, Lipid metabolism, Lipid transport, Steroid metabolism, Sterol metabolism, Transport	Heparin-binding	Alzheimer disease, Amyloidosis, Cancer-related genes, Disease mutation, Hyperlipidemia, Neurodegeneration	Vesicles
0.457968476	MTOR	Mechanistic Target Of Rapamycin Kinase	Cancer-related genes, Disease related genes, Enzymes, FDA approved drug targets, Plasma proteins, Predicted intracellular proteins	Biological rhythms	Kinase, Serine/threonine-protein kinase, Transferase	Cancer-related genes, Disease mutation, Epilepsy, FDA approved drug targets, Mental retardation	Vesicles, Cytosol
0.378283713	COMT	Catechol-O-Methyltransferase	Disease related genes, Enzymes, FDA approved drug targets, Plasma proteins, Predicted intracellular proteins, Predicted membrane proteins	Catecholamine metabolism, Neurotransmitter degradation	Methyltransferase, Transferase	FDA approved drug targets, Schizophrenia	Vesicles
0.369819031	APP	Amyloid Beta Precursor Protein	Disease related genes, FDA approved drug targets, Plasma proteins, Predicted intracellular proteins, Predicted membrane proteins, Transporters	Apoptosis, Cell adhesion, Endocytosis, Notch signaling pathway	Heparin-binding, Protease inhibitor, Serine protease inhibitor	Alzheimer disease, Amyloidosis, Disease mutation, FDA approved drug targets, Neurodegeneration	Golgi apparatus, vesicles
0.319614711	SLC6A4	Solute Carrier Family 6 Member 4		Neurotransmitter transport, Symport, Transport		FDA approved drug targets	Golgi apparatus, Vesicles
0.299036778	PPARG	Peroxisome Proliferator Activated Receptor Gamma	Cancer-related genes, Disease related genes, FDA approved drug targets, Nuclear receptors, Plasma proteins, Predicted intracellular proteins, Transcription factors	Biological rhythms, Transcription, Transcription regulation	Activator, DNA-binding, Receptor	Cancer-related genes, Diabetes mellitus, Disease mutation, FDA approved drug targets, Obesity	Nucleoplasm, vesicles
0.288674839	LRRK2	Leucine Rich Repeat Kinase 2	Cancer-related genes, Disease related genes, Enzymes, Potential drug targets, Predicted intracellular proteins	Autophagy, Differentiation	GTPase activation, Hydrolase, Kinase, Serine/threonine-protein kinase, Transferase	Cancer-related genes, Disease mutation, Neurodegeneration, Parkinson disease, Parkinsonism	Nucleoplasm, Vesicles
0.26619965	TNFAIP3	TNF Alpha Induced Protein 3	Cancer-related genes, Disease related genes, Enzymes, Plasma proteins, Potential drug targets, Predicted intracellular proteins	Apoptosis, Inflammatory response, Ubl conjugation pathway	DNA-binding, Hydrolase, Multifunctional enzyme, Protease, Thiol protease, Transferase	Cancer-related genes, Disease mutation	Vesicles
0.260945709	SQSTM1	Sequestosome 1	Disease related genes, Predicted intracellular proteins	Apoptosis, Autophagy, Differentiation, Immunity		Amyotrophic lateral sclerosis, Disease mutation, Neurodegeneration	Vesicles, Cytosol
0.24270286	GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	Enzymes, FDA approved drug targets, Plasma proteins, Predicted intracellular proteins	Apoptosis, Glycolysis, Translation regulation	Oxidoreductase, Transferase	FDA approved drug targets	Plasma membrane, Cytosol

(Continued)

TABLE 6 | Continued

Normalized score	Gene	Gene description	Protein class	Biological process	Molecular function	Disease involvement	Subcellular Location
0.214681845	DNM1L	Dynamin 1 Like		Biological rhythms, Endocytosis, Necrosis	Hydrolase	Disease mutation	Cytosol, Vesicles
0.193812026	TOR1A	Torsin Family 1 Member A	Disease related genes, Predicted intracellular proteins		Chaperone, Hydrolase	Disease mutation, Dystonia	Nuclear membrane, Vesicles
0.150175131	CAVIN1	Caveolae Associated Protein 1		Transcription, Transcription regulation, Transcription termination	RNA-binding, rRNA-binding	Congenital generalized lipodystrophy, Diabetes mellitus	Plasma membrane, Vesicles
0.140542907	LDHA	Lactate Dehydrogenase A			Oxidoreductase	Cancer-related genes, Disease mutation, FDA approved drug targets, Glycogen storage disease	Cytosol, Vesicles

(symptoms or disease manifestations) that were considered peculiar to each group of organs/tissues (**Table 1**). Accordingly, for group 1 the phenotype query: "fever" OR "cough" OR "pneumonia" OR "dyspnea" OR "pain" OR "hemoptysis " OR "sore throat" OR "chills" OR "inflammation" was used (**Supplementary Table S5**). Group 2 was analyzed for the phenotype query: "fever" OR "diarrhea" OR "pain" OR "nausea" OR "vomiting" OR "inflammation" (**Supplementary Table S6**). Group 3 was analyzed for the phenotypes: "fever" OR "chills" OR "inflammation" OR "hemorrhagic" (**Supplementary Table S7**). The phenotype query for group 4 was: "fever" OR "cough" OR "diarrhea" OR "pain" OR "nausea" OR "vomiting" OR "chills" OR "inflammation" OR "hemorrhagic" (**Supplementary Table S8**). For group 5 (brain tissues) 2 sets of disease-related phenotypes were used in separate VarElect analyses, accounting for the reported neurological symptoms related to the central nervous system (group 5a, phenotype query: "dizziness" OR "headache" OR "consciousness" OR "encephalopathy" OR "encephalitis" OR "seizures" OR "stroke" OR "delirium," **Supplementary Table S9**) or the peripheral nervous system (group 5b, phenotype query: "ageusia" OR "dysgeusia" OR "hypogeusia" OR "anosmia" OR "hyposmia" OR "myalgia" OR "myelitis" OR "pain" OR "Guillain-Barre," **Supplementary Table S10**) (Ahmad and Rathore, 2020; Lahiri and Ardila, 2020; Tsivgoulis et al., 2020).

The VarElect scores related to each tissue group have been normalized so that they can be compared and used in a rank aggregation procedure. In particular, we considered two rank aggregation, the first by aggregating groups 1,2, and 4 (G124) and the second by aggregating groups 1,2,3,4, and 5 (G12345).

The VarElect analysis on single and aggregate groups allowed the selection of 260 (arbitrary cutoff, subject to extension in forthcoming analysis) gene targets potential candidates for drug repurposing (complete lists in **Supplementary Tables S11–S15**, selection of the first 15 genes for each aggregate or single group ranks in **Tables 2–6**). In particular, 101 genes were selected for the aggregate rank G124 (**Supplementary Table S11**), 99 genes were selected for the aggregate rank G12345 (**Supplementary Table S12**), and 20 genes were selected for each of the single analysis performed on group 3 (G3 blood cells, **Supplementary Table S13**), group 5a (G5a brain, VarElect analysis related to the central nervous system, **Supplementary Table S14**), and group 5b (G5b brain, VarElect analysis related to the peripheral nervous system, **Supplementary Table S15**).

Selected genes from aggregate ranks G124, G12345, and from single ranks G3, G5a, and G5b and subjected to the evaluation about the development of anti-COVID-19 pharmacology based on the repositioning of drugs already on the market (see section "Drug Repurposing Strategy").

Design of Drug Repositioning Strategy via Drug-Gene Interaction Data

The DrugBank repository[7] (Wishart et al., 2018) was manually queried for the selection of drugs on the basis of their possible interference with the direct or indirect virus-host interaction. The criteria applied for selecting a restricted list of gene targets and the corresponding drugs were: (a) the highest place occupied in the aggregate VarElect ranks G124 (**Table 2** and **Supplementary Table S11**) and G12345 (**Table 3** and **Supplementary Table S12**) and in the single ranks G3, G5a, and G5b (**Tables 4–6** and **Supplementary Tables S13–S15**); (b) the main cellular location of the target protein, selected on the basis of the possible virus-host interaction during cell entry (plasma membrane), RNA duplication (cytosol), RNA translation (endoplasmic reticulum), viral protein maturation and virus assembly (Golgi apparatus) and virus secretion

[7]https://www.drugbank.ca/

TABLE 7 | Summary of drugs potentially relevant for COVID-19 chosen via the data-driven drug repositioning strategy.

Drug(s)	DrugBank ID	Target gene	Possible compartment for Target-SARS-Cov-2 interaction	COVID-19-related phase	Drug description	Status	Approved conditions	Potential alternative targets	Reference/Note
Afatinib	DB08916	EGFR	Plasma membrane	Virus entry	Small Molecule Inhibitor	Approved	Metastatic Non-Small Cell Lung Cancer, Refractory, metastatic squamous cell Non-small cell lung cancer	ERBB2, ERBB4	
		ERBB2	Plasma membrane	Virus entry	Small Molecule, Inhibitor	Approved	Metastatic non-small cell lung cancer (NSCLC) with common epidermal growth factor receptor (EGFR) mutations	EGFR, ERBB4,	https://www.boehringer-ingelheim.us/press-release/fda-approves-new-indication-gilotrif-egfr-mutation-positive-nsclc
Artenimol	DB11638	FLNA	Plasma membrane	Virus entry	Small Molecule	Approved, investigational	Antimalarial agent	ANXA2, CAST, DPYSL2, DSP, HSPB1, IQGAP1, MAP4, RPL4, RPL10, RPL13, RPL14, RPL35, RPL17, RPL18, RPL19, RPL23A,	https://apps.who.int/iris/bitstream/handle/10665/162441/9789241549127_eng.pdf;jsessionid=F0093888/B29AE0/CE2AF698/13FFCFF6FD?sequence=1
Bosutinib	DB06616	LYN	Golgi apparatus, Plasma membrane	Virus entry, virus assembly	Small Molecule	Approved	Philadelphia chromosome-positive (Ph+) chronic myelogenous leukemia (CML)	BRC, ALB1, HCK, SRC, CDK2, MAP2K1, MAP2K2, MAP3K2, CAMK2G	https://pubmed.ncbi.nlm.nih.gov/23674887/
		SRC	Plasma membrane	Virus entry	Small Molecule	Approved	Inhibitor for the treatment of Philadelphia chromosome-positive (Ph+) chronic myelogenous leukemia (CML)	LYN, HCK, CDK2, MAP2K1, MAP2K2, MAP3K2, CAMK2G, ABL1, BCR,	https://pubmed.ncbi.nlm.nih.gov/23098112/
Calcium Phosphate	DB11348	CALR	Endoplasmic reticulum	Viral protein synthesis	Small Molecule	Approved	Calcium and phosphate supplement, antacid,	CASR, CIB1, SRI, CHP1, CANX, FBN2, S100B, CASQ2, RGN, PEF1, S100A6, TPT1, CIB2, CALM1, FBN3	
Calcium phosphate dihydrate	DB14481	CALR	Endoplasmic reticulum	Viral protein synthesis	Small Molecule	Approved	Counter calcium and phosphate supplement, antacid	CASR, PDCD6, SPARC, CANX, S100A6, TPT1, CIB2, FBN2, SRI, S100B, CASQ2, RGN, PEF1, CHP1,	

(Continued)

TABLE 7 | Continued

Drug(s)	DrugBank ID	Target gene	Possible compartment for Target-SARS-Cov-2 interaction	COVID-19-related phase	Drug description	Status	Approved conditions	Potential alternative targets	Reference/Note
Carboplatin	DB00958	SOD1	Nucleoplasm, Plasma membrane	Virus entry	Small Molecule	Approved	Antineoplastic activity	XDH, MPO, GSTT1, GSTM1, GSTP1, NQO1, MT1A, MT2A	https://patents.google.com/patent/US7259270?oq=07259270
Cetuximab	DB00002	EGFR	Plasma membrane	Virus entry	Monoclonal Antibody, Antagonist	Approved	Metastatic Colorectal Cancer	FCGR3B, C1QA, C1QB, C1QC, FCGR3A, FCGR1A, FCGR1B	http://www.ncbi.nlm.nih.gov/pubmed/11408594
Cisplatin	DB00515	SOD1	Nucleoplasm, Plasma membrane	Virus entry	Small Molecule	Approved	Sarcomas, small cell lung cancer, ovarian cancer, lymphomas and germ cell tumors	MPG, A2M, TF, ATOX1	
Docetaxel	DB01248	MAPT	Plasma membrane	Virus entry	Small Molecule	approved, investigational	Anti-mitotic chemotherapy for breast, ovarian, non-small cell lung, androgen independent metastatic prostate, gastric adenocarcinoma and head and neck cancer.	TUBB1, BCL2, MAP2, MAP4, NR1I2, CYP3A4,	https://pubmed.ncbi.nlm.nih.gov/18068131/
Entacapone	DB00494	COMT	Endoplasmic reticulum, vesicles	Viral protein synthesis, virus release	Small Molecule, Inhibitor	Approved	Parkinson's disease		http://www.ncbi.nlm.nih.gov/pubmed/11440283
Everolimus	DB01590	MTOR	Vesicles, Cytosol	Virus replication and release	Small Molecule	Approved	Immunosuppressant to prevent rejection of organ transplants		
Ferric derisomaltose	DB15617	TFRC	Plasma membrane	Virus entry	Small Molecule	Approved	Anemia, non-hemodialysis dependent chronic kidney disease	HBA1	https://www.accessdata.fda.gov/drugsatfda_docs/label/2020/208171s000lbl.pdf
Fostamatinib	DB12010	TBK1	Nucleoplasm, vesicles	Virus release	Small Molecule	Approved, investigational	Rheumatoid Arthritis and Immune Thrombocytopenic Purpura (ITP)	CTSL, ABL1, RPS6KA6, MET, TEK, TGFBR1, TGFBR2, SYK,	https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-fostamatinib-tablets-itp
Lansoprazole	DB00448	MAPT	Plasma membrane	Virus entry	Small Molecule	approved, investigational	Ulcerative, gastroesophageal reflux disease (GERD), and other pathologies caused by excessive acid secretion	ATP4A	https://pubmed.ncbi.nlm.nih.gov/19006606/
Natalizumab	DB00108	ICAM1	Plasma membrane	Virus entry	Monoclonal Antibody	Approved, Investigational	Multiple sclerosis	ITGA4, FCGR3B, FCGR1A	Natalizumab was voluntarily withdrawn from United States market because of risk of Progressive multifocal leukoence-phalopathy (PML). It was returned to market July, 2006

(Continued)

TABLE 7 | Continued

Drug(s)	DrugBank ID	Target gene	Possible compartment for Target-SARS-Cov-2 interaction	COVID-19-related phase	Drug description	Status	Approved conditions	Potential alternative targets	Reference/Note
		CD209	Plasma membrane	Virus entry	Monoclonal Antibody	Approved, Investigational	Multiple sclerosis	FCGR1A, ITGA4, ICAM1, FCGR3B	Natalizumab was voluntarily withdrawn from United States market because of risk of Progressive multifocal leukoencephalopathy (PML). It was returned to market July, 2006
Nintedanib	DB09079	LYN	Golgi apparatus, Plasma membrane	Virus entry, virus assembly	Small Molecule	Approved	Pulmonary fibrosis, systemic sclerosis-associated interstitial lung disease, and non-small cell lung cancer	KDR, LCK, SRC, PDGFRA, PDGFRB, FGFR1, FGFR2, FGFR3, FLT1, FLT3, FLT4,	https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/205832s010lbl.pdf
		SRC	Plasma membrane	Virus entry	Small Molecule	Approved	Pulmonary fibrosis, systemic sclerosis-associated interstitial lung disease, and non-small cell lung cancer (NSCLC)	FLT1, KDR, FLT4, PDGFRA, PDGFRB, FGFR1, FGFR2, FGFR3, FLT3, LCK, LYN,	https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/205832s010lbl.pdf
		FGFR1	Plasma membrane	Virus entry	Small Molecule	Approved	Pulmonary fibrosis, systemic sclerosis-associated interstitial lung disease, and non-small cell lung cancer (NSCLC)	FLT1, KDR, FLT4, PDGFRA, PDGFRB, FGFR1, FGFR2, FGFR3, FLT3, LCK, LYN,	https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/205832s010lbl.pdf
Osimertinib	DB09330	EGFR	Plasma membrane	Virus entry	Small Molecule Inhibitor	Approved	Metastatic Non-Small Cell Lung Cancer		http://www.ncbi.nlm.nih.gov/pubmed/26522274
Palifermin	DB00039	FGFR1	Plasma membrane	Virus entry	Small Molecule	Approved	Oral mucositis	FGFR2, NRP1, FGFR4, FGFR3, HSPG2,	
Pertuzumab	DB06366	ERBB2	Plasma membrane	Virus entry	Monoclonal antibody, Inhibitor	Approved	Metastatic HER2-positive breast cancer.		https://www.accessdata.fda.gov/drugsatfda_docs/label/2020/125409s124lbl.pdf
Regorafenib	DB08896	FGFR1	Plasma membrane	Virus entry	Small Molecule	Approved	Metastatic colorectal cancer and advanced gastrointestinal stromal tumors	FLT1, KDR, FLT4, KIT, PDGFRA, PDGFRB, FGFR2, DDR2, EPHA2, RAF1, BRAF, MAPK11, FRK, ABL1, RET, TEK, NTRK1	
Stiripentol	DB09118	LDHA	Cytosol, Vesicles	virus replication	Small Molecule	Approved	Anticonvulsant drug used in the treatment of epilepsy	LDHB, GABA(A) Receptor (Protein Group)	https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm?event=reportsSearch.process
Temsirolimus	DB06287	MTOR	Vesicles, Cytosol	Virus replication and release	Small Molecule	Approved	Renal cell carcinoma (RCC)		

(Continued)

TABLE 7 | Continued

Drug(s)	DrugBank ID	Target gene	Possible compartment for Target-SARS-Cov-2 interaction	COVID-19-related phase	Drug description	Status	Approved conditions	Potential alternative targets	Reference/Note
Tromethamine	DB03754	APP	Plasma membrane, Golgi apparatus, vesicles	Virus entry, virus assembly	Small molecule, Inhibitor	Approved	Prevention and correction of metabolic acidosis		http://www.ncbi.nlm.nih.gov/pubmed/8380642
Urea	DB03904	CTNNB1	Plasma membrane	Virus entry	Small Molecule	Approved, Investigational		ARG1, CA2, yedY, DHFR	
Vandetanib	DB05294	EGFR	Plasma membrane	Virus entry	Small Molecule Inhibitor	Approved	Non-resectable, locally advanced, or metastatic medullary thyroid cancer	VEGFA	

(secretory vesicles); (c) the existence of approved drugs as suitable candidates for repositioning.

RESULTS

Selection of Targets for Drug Repurposing

The application of the methodology detailed in section "Materials and Methods" leads to the selection of 260 target genes being potential candidates for drug repurposing. It turned out that out of these 260 genes, only 14 of them were ranked once (CDH1, CHEK2, TOP1, ADRB2, BIRC3, PRKAR1A, IKBKG, NEU1, CHUK, BSG, XPO1, WWOX, LDHA, and HSPA1A), while all of the others were repeated in two or more different ranks, with a total of 130 genes represented over 260 total entries in the pooled ranks. As for the main cellular locations, the majority of virus potential interactors were associated with cell nucleus (51), with less gene products located on plasma membrane and cytosol (15 each), Golgi/endoplasmic reticulum (12), vesicles (11), and mitochondria (7). The molecular function most represented was "enzyme" (46), while 16 activators/transcription factors, 9 membrane-bound receptors, 10 secreted proteins, 27 DNA-binding, 7 RNA-binding, 6 chaperones, 8 repressors were detected. Of note, 37 of the 130 unique gene targets were indicated in the Protein Atlas database as generic Virus-Host interactors, while 8 genes codify for proteins with antiviral activities. Finally, the analysis of "protein class" fields in **Supplementary Tables S11–S15**, revealed that 65 out of 130 genes were previously identified as non-COVID-19 specific potential drug targets, yet subjected to evaluation or approved by Regulatory Agencies (FDA and EMA).

Drug Repurposing Strategy

Following our extensive, multi-level analysis, we identified high ranking genes that may be potential pharmacological targets, fulfilling the requirements for a fast and safe drug repositioning strategy (**Table 7**). Most of them are enzymes (kinases, phosphatases, etc., i.e., AKT1, CDK4, LYN, MAPK14, TBK1, CHEK2, ATM, LRRK2, CHUK, SRC, MTOR, and MAPK1) belonging to various downstream signaling pathways, or involved in essential cell physiological processes, such as DNA replication, RNA synthesis and translation (i.e., RANBP2, SMARCA4, FUS, XPO1, DDX58, CAVIN1, and IFIH1), protein processing (i.e., PLAT, CASP8, PSEN1, APP, CASP3, XIAP, SERPING1, and TNFAIP3) energy consumption (i.e., GLA, NEU1) metabolic pathways (i.e., LDHA, WWOX, HMOX1, SDHB, SDHA, HADHA, ACADM, SOD1, GAPDH, PLOD1, and NOS2). Few proteins belongs to the class of secreted factors (i.e., PLAT, FBN1, TGFB1, SPP1, TNF, SERPING1, APOE, C4A, FN1, and TNFRSF1A). Some of the identified targets, based on their cellular compartmentalization, most probably may be activated/repressed in the process of virus entry and replication or viral proteins post-translational processing (i.e., HMOX1, APP, LYN, XIAP, SOD1, HIF1A, EGFR, ICAM1, FAS, CD209, CDH1, SRC, FLNA, DDX58, MAPT, CTNNB1, ERBB2, ADRB2, GAPDH, HSPB1, and CAVIN1), or may interact with virus proteins during the last phase of virus secretion through secretory vesicles (i.e., DNM1L, LDHA, NKX2-1, SLC6A4, CAVIN1, APOE, TNFAIP3, COMT, NEU1, BSG, SCARB1, MTOR, SQSTM1, NTRK1, and SPTAN1). A list of 18 potentially effective pharmacological targets with associated approved drugs, is presented in **Table 7**. Such genes have been selected prioritizing the existence of an already approved, safe and effective pharmacology. Then, gene candidates that were not considered as directly involved in virus-host protein interactions were discarded, i.e., those located in cell nucleus structures or those involved in essential, redundant and/or non-targetable cell metabolic/physiologic processes. Finally, all potential (and strong) candidates already under clinical investigation as potential drug targets for COVID-19 pandemics (i.e., TNF, highest in more than one aggregate rank in the VarElect analysis) were also excluded. The resulting list encompass plasma membrane receptors (i.e., EGFR, ERRB2, FGFR1, among others), proteins mainly localized in the Golgi and endoplasmic reticulum (CALR, APP, LYN, and COMT), Cytosol (LDHA, MTOR), vesicles (TBK1, COMT, APP, LDHA, and MTOR). Some of the proposed genes are potentially targeted by the same or similar

drugs (as evidenced in the "potential alternative targets" fields in **Table 6** drugs). Moreover, some of the proposed drugs are potentially effective on pharmacological targets already identified as potential drug targets or under investigation in ongoing clinical trials on COVID-19 patients (i.e., VEGFA, C1QA, C1QB, and C1QC)[8]. All of the selected genes were relatively high in their aggregated ranks (see **Tables 2–6**).

DISCUSSION

In this work, we identified and prioritized a number of target genes involved in different ways in the host SARS-CoV-2 invasion and response via a network proximity-based procedure. Subsets of such target genes were subsequently identified in different organs and systems of the human organism, with the aim of isolating and classifying, in functionally coherent tissue/organ groups (respiratory and digestive epithelia, blood, filter/excretory tissues, and nervous system), the mostly suited target genes for the development of a pharmacology based on the repositioning of drugs already on the market. For each group of tissues, relevant target classifications have been established, on the basis of the potentially associated pathological phenotypes, previously described as characterizing the COVID-19 disease (Adhikari et al., 2020). The highest target genes in the individual tissue ranking were then grouped to reach the selection of 130 unique targets, 90% of which were significant in two or more of the tissues considered. Finally, by analyzing each relevant target, a pharmacological proposal has been defined for 18 target genes and expected to interfere with the virus-host interaction in the various infectious phases and the viral replication cycle.

Computationally based approach has been already considered for drug repurposing: for example Zhou et al. (2020) prioritize sixteen potential repurposable drugs against SARS-CoV-2 using a network proximity analysis. In particular, the authors mapped the drug-target network into a selected COVID-19 host interactome to search for cellular target; (Cheng et al., 2020) proposed a combination of anti-inflammatory and antiviral therapeutics using a network based approach in which proximity measure quantifies the relationship between COVID-19 disease modules and drug targets in the Human PPIs network. Our computationally driven approach revealed that it is possible to hypothesize unequivocal and functional pharmacological interventions to counteract the development of symptoms affecting various organs and systems. This consideration arises from the evidence that some of the pharmacological targets identified (i.e., EGFR, ERBB2, APP, ICAM1, and FAS), may be important to prevent the interaction of the virus with the cell surface in different target organs. However, it is also necessary to conceive pharmacological strategies based on the combination of different drugs, able to counter, by targeting different players of the virus-host interaction, the various stages through which the infection develops at the cellular level (virus entry, replication, viral protein processing, and release of new virus). Finally, the association of therapies interfering with virus-host interaction with strategies aimed at bringing back under control the inflammatory phenomena, with which the body fights the infection and which have often proved fatal (Astuti and Ysrafil, 2020), is deserved.

Computational criteria and methods brought to the definition of COVID-19 proximal target genes. Then, biological criteria lead to select the relevant interactions, potential targets for drug repurposing, associated with different stages of viral infection and the development of the constellation of symptoms already described in COVID-19 patients (Adhikari et al., 2020; Ahmad and Rathore, 2020). Virus-host interactions may stand as physical interactions between viral and human proteins or as indirect interactions based on the triggering, after virus challenge, of the complex network of metabolic processes characterizing eukaryotic cells. In the analysis presented in this work, in addition to the classifications of relevant target genes, their cellular localization was also taken into consideration, with the aim of hypothesizing possible specific interactions for the individual compartments of the cell, in which the viral proteins could relate with human ones. Based on such rationale, plasma membrane-bound proteins have been considered as alternative interactors for virus entry. Cytoplasm-located proteins may conceivably interact with the virus during its replication phase, while endoplasmic reticulum and Golgi proteins could interact with the viral M protein and the viral proteins post-translational processing (Astuti and Ysrafil, 2020). Finally, vesicles-associated interactors have been hypothesized to play a role in the virus secretion.

It is known that the receptor-binding domains on the SARS-CoV-2 S protein bind with high affinity to human ACE2 (Wrapp et al., 2020), an interaction accounting for virus entry in the host cell and for its transmissibility. The analysis of COVID-19 extended interactome indicates several membrane bound gene/proteins (i.e., ICAM1, EGFR, ERBB2, APP, ADR2, FAS, CDH1, and MAPT), whose activity and/or expression could be affected by SARS-CoV-2 challenge. Evidence for alternative interaction of virus S protein with receptors other than ACE2 have been not only already suggested by computational analysis (Milanetti et al., 2020), but also demonstrated in vitro (Ulrich and Pillat, 2020). Furthermore, some of the selected proteins could also account for additional host interactions, not necessarily related with the transmission of disease. RNA-binding proteins present in the cytosol, part of the extended interactome and with a high position in the VarElect ranks (i.e., RANBP2, XPO1, and CDKN2A), could reasonably participate in the replication and translation phases of the viral RNA. Similarly, proteins associated with the endoplasmic reticulum and Golgi membranes (i.e., CALR, COMT, CAV1, and PTCH1) could be involved in the translation processes of the viral RNA and in the subsequent protein processing. Lastly, it is worth mentioning the interactions foreseen by computational analysis with secreted proteins. Among the most important are those with TNF, which plays a central role in the cytokine storm that characterizes the most severe phase of the disease, and which already constitutes a drug target challenged in intensive care units worldwide.

[8]https://www.drugbank.ca/covid-19#drug-targets

There are actually dozens of drug targets tested for COVID-19 in more than 1200 clinical trials worldwide, as reported in the DrugBank repository (see text footnote 8). Among these, only TNF has been identified by our analysis as being part of the COVID-19 host target genes. Recently, a list of more than 300 possible target genes has been experimentally observed to interact with Sars-CoV-2 proteins and thus considered for the development of anti-COVID, repositioning-based therapies (Gordon et al., 2020), of which only 11 (NEU1, SCARB1, TBK1, COMT, HMOX1, FBN1, GLA, ACADM, DNMT1, PLAT, and TOR1A) are shared with those predicted through the methodology applied in the present work after a data-driven prioritization. In addition, despite the apparent abundance of potential pharmacological targets proposed through data analysis, relatively few of these lend themselves to being used in drug repositioning strategies. The final data of the present work, summarized in the **Table 6**, indicate that among the potential first 130 targets identified, because at the top positions in the ranks of potential efficacy elaborated through our methodology, only 18 preliminary appear as suitable candidates for drug repositioning. The reasons lie in the lack, for most of the ranked genes, of pharmacologically active drugs already approved by the Regulatory Agencies, or in the impossibility of developing, for many of them participating in essential processes in cellular physiology, a pharmacological approach that modifies their activity, or, finally, in the difficulty of using drugs with a significant impact on physiology or with a high risk of inducing side effects in patients already deeply debilitated by SARS-CoV-2 infection.

CONCLUSION AND PERSPECTIVES

The pandemic caused by SARS-CoV-2 represents an open and unresolved challenge for the global health system. The need to identify drugs that demonstrate efficacy in countering both the mechanisms of interaction of SARS-CoV-2 with host cells and to control the devastating inflammatory phenomena that characterize the late stages of viral infection, requires increasingly urgent answers. The biomedical research approach based on the repurposing of already approved drugs seems to be one of the most viable strategies in this struggle. This work, via a data-driven network-based procedure, provides a viable and alternative drug repurposing strategy to be considered for clinical trial. The proposed approach has been conceived to support the comprehension of the molecular landscape of COVID-19 as well as the identification of genes that are not immediately associated to SARS-CoV-2 invasion, or not taken into consideration in respect to the host defense regulation and dynamics, and may thus suggest new directions for further studies and analyses. We leave open the possibility of extending our preliminary analysis by increasing the number of genes present in the currently proposed COVID-19 proximal target genes and/or by extending the selection of potential target genes identified through functional analysis to a greater number than the current one. Under the computational point of view further approaches could be considered, for instance several network topological measures and/or a combination of them could be considered to select COVID-19 proximal candidate target genes and to investigate whether/how changes in the drugs proposal occur.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

PT conceived the study. All authors collected the data, ran the analysis, wrote the manuscript, 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/fcell.2020.545089/full#supplementary-material

Supplementary File 1 | Cytoscape session file (.cys) with human interactome data.

Supplementary Table 1 | List of the 500 COVID-19 associated host genes derived from the BioGRID dataset (BIOGRID-CORONAVIRUS-3.5.186.tab3.zip).

Supplementary Table 2 | Output of the DIAMOnD algorithm: first 1000 genes ranked for connectivity significance starting from the 500 COVID-19 associated seed genes.

Supplementary Table 3 | Output of the network propagation/heat diffusion algorithm run with 5 different diffusion times starting from the 500 COVID-19 associated seed genes.

Supplementary Table 4 | Aggregated chart of the first 1500 genes ranked for COVID-19 proximity.

Supplementary Tables 5–10 | VarElect scores obtained for genes in the COVID-19 extended interactome expressed in different groups of tissues/organs (reported in each Table) and selected for their association with disease phenotypes (reported in each Table) specifics for each tissue/organ.

Supplementary Tables 11–15 | Aggregated and normalized VarElect scores for the first 100 (G124 and G12345) and 20 (G3, G5a, and G5b) genes in the VarElect analysis depicted in **Supplementary Tables 5–10**.

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Epidemiology of the First Wave of COVID-19 ICU Admissions in South Wales—The Interplay Between Ethnicity and Deprivation

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On the 9th March 2020, the first patient with COVID-19 was admitted to ICU in the Royal Gwent Hospital (RGH), Newport, Wales. We prospectively recorded the rate of ICU admissions of 52 patients with COVID-19 over 60 days, focusing on the epidemiology of ethnicity and deprivation because these factors have emerged as significant risk factors. Patients were 65% (34 of 52) male and had a median (IQR) age of 55 (48–62) years. Prevalent comorbidities included obesity (52%); diabetes (33%), and asthma (23%). COVID-19 hospital and ICU inpatient numbers peaked on days 23 and 39, respectively—a lag of 16 days. The ICU mortality rate was 33% (17 of 52). People of black, Asian, and minority ethnic descent (BAME group) represented 35% of ICU COVID-19 admissions (18 of 52) and 35% of deaths (6 of 17). Amongst the BAME group, 72% (13 of 18) of patients were found to reside in geographical areas representing the 20% most deprived in Wales, vs. 27% of patients in the Caucasian group (9 of 33). Less than 5% of the population within the area covered by RGH are of BAME descent, yet this group had a disproportionately high ICU admission and mortality rate from COVID-19. The interplay between ethnicity and deprivation, which is complex, may be a factor in our findings. This in turn could be related to an increased prevalence of co-morbidities; higher community exposure; larger proportion of lower band key worker roles; or genetic polymorphisms.

Keywords: COVID-19, ethnicity, BAME, deprivation, mortality

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INTRODUCTION

Severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) infection, originating from Wuhan, China is the underlying cause of coronavirus disease-2019 (COVID-19) (1). The pandemic reached Italy in February and caused significant strain on the Lombardy critical care units (2). The first known patient with SARS-CoV-2 infection was discovered in Wales on the 2nd March 2020. The first COVID-19 patient admitted to an ICU in Wales was admitted to the Royal Gwent Hospital (RGH) in Newport on the 9th March. Heeding the warning from Italy, the RGH moved early to cancel elective activity and prepare for a significant increase in admissions (3).

The RGH in Aneurin Bevan University Health Board (ABUHB) is the major specialist center, which provides secondary services to a population of ∼400,000 people. The overall geographical

region from which RGH's acute admissions are received, known as the catchment area, includes a higher number of deprived neighborhoods than any other hospital in Wales according to the 2011 Census data from the UK Office of National Statistics (ONS).

We have previously reported that social deprivation is an independent risk factor of long-term outcome following critical care discharge (4). Social deprivation is often linked to reduced access to healthcare and this has been a particular problem amongst the Black, Asian and Minority Ethnic (BAME) population group. The acronym "BAME" is used for research purposes in the UK and includes people not of Caucasian ethnicity. BAME is an umbrella term, which the UK census reports as four broad subgroups: black/black British, Asian/Asian British, mixed-race, and other non-Caucasian minority ethnic people.

During the first wave of the COVID-19 pandemic, data emerged indicating that people of BAME descent in the UK are at higher risk of intensive care unit (ICU) admission and death with COVID-19 (5). In this case-series analysis we report the epidemiology of the first wave of COVID-19 patients in South-East Wales admitted into ICU, and describe the connection between ethnicity and social deprivation using data from the first 60 days of the COVID-19 pandemic in the RGH.

METHODS

Conceptualization and Approval

Our prospective service evaluation on the ICU was developed before the first patient with COVID-19 was admitted. The ABUHB Risk Review Committee approved the project and waived the need for written informed consent. All data collected was fully anonymised and summarized as an absolute number (with percentage), or as a median value (with interquartile range), as appropriate.

Setting

RGH is a medium-size district general hospital with 800 inpatient beds in Newport, South Wales. The critical care unit is normally a 16-bedded combined ICU (maximum of 12 beds for invasive ventilation with 1:1 nurse:patient ratios) and high-dependency unit (for patients needing other organ support, including non-invasive ventilation with 1:2 nurse:patient ratios) located in two areas on the same floor. We have previously described the case-mix and our approach to flexibly use the critical care resources (6).

During the first-wave of COVID-19 admissions, the critical care capacity has been upscaled to a 40 bedded ICU spanning three separate areas with increased nurse-to-patient ratios (3). All patients with suspected or confirmed COVID-19 disease were enrolled in a multicenter clinical trial as appropriate (7, 8). We have not used any novel disease modifying agents or therapies outside these clinical trials.

Clinical Data Sources

Data on COVID-19 patients admitted to the ICU was collected prospectively from 9th March 2020 (day 1) to the 7th May 2020 (day 60). COVID-19 was defined as confirmed (respiratory failure and radiological changes with SARS-CoV-2 RNA detected on PCR testing), or suspected (respiratory failure and radiological changes consistent with COVID-19 but without a confirmed PCR test result). We recorded patient age; sex; BMI; need for any assistance with daily activities; APACHE II score; time from hospital admission to ICU, and ICU mortality.

Epidemiological Data Sources

To put the ICU admissions into context and to aid in national comparisons, we utilized the daily situation report data from the Welsh Government Acute Secondary Care COVID-19 Group, available from the 23rd March 2020 (day 16) onwards. This administrative data summarized the daily patient admissions and discharges, including the COVID-19 related deaths reported in each Welsh hospital.

We recorded ethnicity for each patient in the study according their Caucasian or BAME subgroup status, while background data on ethnicity within RGH catchment area was obtained from the ONS 2011 census data. We used patients' postcodes in order to place them into their respective census neighborhood localities - known as "lower super output areas" (LSOAs). In census terms, LSOAs are geospatial statistical units possessing a similar population size, and homogeneity regarding tenure of households and dwelling type.

Deprivation within each LSOA was ranked using the Welsh Index of Multiple Deprivation (WIMD) quintiles from the ONS. Patients' postcodes (assumed to be a surrogate marker of deprivation) and ethnicity were plotted in their corresponding LSOAs using the interactive online WIMD map tool at https://wimd.gov.wales/.

RESULTS

Overview

Between 9th March 2020 (day 1) and 7th May (day 60), 52 patients (46 confirmed and 6 suspected) with COVID-19 disease were admitted to the ICU. All patients were mechanically ventilated on admission. **Table 1** describes the demographics of the 52 patients admitted to the ICU. One out of 52 patients needed assistance with daily activities of living before admission. **Figure 1** shows the daily ICU admission figures from 9th March and their relation to the total number of patients in the hospital with COVID-19 disease from 23rd March onwards (day 15).

Exceeding Maximum Capacity

The RGH critical care unit went exceeded normal maximum capacity on the 28th March, 20 days after the first admission with COVID-19 and remained above maximum capacity for 40 days (**Figure 2**). Ward inpatient numbers peaked on the 31st March (day 23) while ICU numbers peaked, after a 16 day lag, on the 16th April (day 39), when calculated using a seven-day moving mean across the 60 day study. **Figure 3** demonstrates how the first patient was discharged alive on the 25th March, 17 days following the first admission with COVID-19, while the first death occurred on the 31st March (day 23).

TABLE 1 | Demographics of COVID-19 ICU patients.

	Patients ($n = 52$)
Age; years	55 (48–62)
Gender	
Female	18 (34.6%)
Male	34 (65.4%)
APACHE II score on admission	12 (10–15)
Hospital admission to ICU time; days	1.32 (0.36–3.66)
Ethnicity	
White	34 (65.4%)
Mixed	0 (0.0%)
Asian/Asian British	12 (23.1%)
Black/Black British	3 (5.8%)
Other	3 (5.8%)
Wales index of multiple deprivation	
Quintile 1—least	6 (11.5%)
Quintile 2	7 (13.5%)
Quintile 3	11 (21.2%)
Quintile 4	5 (9.6%)
Quintile 5—most	22 (42.3%)
Common comorbidities	
Essential hypertension	18 (34.6%)
Diabetes (type 1 and 2)	17 (32.7%)
Asthma (all severities)	12 (23.1)
Ischaemic heart disease	4 (7.7%)
Chronic kidney disease	2 (3.8%)
BMI	
18.5–<25	6 (11.5%)
25–<30	17 (32.7%)
30–<40	24 (46.2%)
40+	3 (5.8%)
Unknown	2 (3.8%)

Values are median (IQR) or number (proportion).

Ethnicity, Admissions, and Mortality

We contrasted the admission and death rates of ICU of patients in the Caucasian and BAME subgroups to their local representation in the 2011 census of the RGH catchment area background population (**Table 2**). Asians/British Asians had the highest ICU admission rate (116.4 per 100,000) and ICU death rate (48.5 per 100,000) of any ethnic group, compared to Caucasians at 8.6 per 100,000 ICU admissions and 2.8 per 100,000 ICU deaths.

Ethnicity and Deprivation

In terms of deprivation, nine out of 33 patients (27%) in the Caucasian group compared to 13 of 18 patients (72%) in the BAME group, lived in areas in the bottom quintile of the WIMD (most deprived). The Asian/British Asian subgroup had the highest proportion, 11 of 13 (85%), within the bottom quintile.

Out of patients in the BAME group, 12 of 18 patients (66%) lived in a multi-generational household; three of them were unemployed, and eight of them were employed in a lower band key-worker role. In contrast, none of the Caucasian patients lived in a multi-generational household; one was a pensioner; one was unemployed, and none worked in key-worker roles.

The absolute number of deaths was highest in the bottom quintile for both Caucasian and BAME patients. More patients died in the BAME group within the bottom quintile: 4 of 6 deaths vs. 3 of 11 deaths in the Caucasian group. **Figure 4** shows the interplay between socioeconomic deprivation, ethnicity and outcome.

DISCUSSION

During the first wave of the COVID-19 pandemic, we found that patients from the BAME group are at significantly higher risk of ICU admission and death from COVID-19. Although the hospital's catchment area covers an overwhelmingly white ethnic population, over a third of the admissions to the critical care unit were from the BAME group. Notably, we observed a significant interplay between deprivation and ethnicity.

The lag between the peak of hospital admissions and the peak of ICU admissions provides an important buffer and could be used in future modeling of ICU capacity for anticipated further waves of COVID-19 outbreaks. We have further observed a sharp decline in hospital admissions, but a much slower return to normal number of patients cared for on the ICU after the stricter lockdown measures were implemented in Wales. This is in part explained by our long ICU length of stay for both survivors and non-survivors.

As our patient population was relatively young, with fewer comorbidities and frailty than the Welsh average, we have opted for keeping active treatment as long as feasible in a bid to improve outcomes (3, 9). Compared to some large European cohorts, our patients were significantly younger, however this was in line with the national experience in the UK and Wales and similar to the United States (10–13). The relatively low APAPCHE II scores on admission were also in line with the data from the whole of UK and likely represent the fact that our patients were primarily admitted with single organ lung failure, as opposed to the more traditional multi-organ involvement seen in other community acquired illnesses (13). Our cohort was closely matched in terms of age, sex, comorbidities and need for assistance for daily living to the ventilated cohort of 7,425 patients in the ICNARC database (13).

Wales has a significantly lower number of critical care beds compared to England or the rest of Western Europe, a shortage which has been known for over 20 years (3, 14). Despite increasing ICU surge capacity to 9.0/100,000 population from a baseline of 4.2/100,000, and thereby reaching the pre-surge level in Lombardy, initial estimations of excess death from COVID-19 predicted that the Welsh ICU capacity could be a limiting factor in the response (2, 15).

Fortunately, the lockdown measures reduced the pressures on critical care capacity and allowed critical care units to manage beyond their normal maximal capacity, albeit with increased nurse-to-patient ratios. Importantly, we did not see any correlation between peak ICU occupancy and mortality. Despite initial concerns that critical care units may be overwhelmed

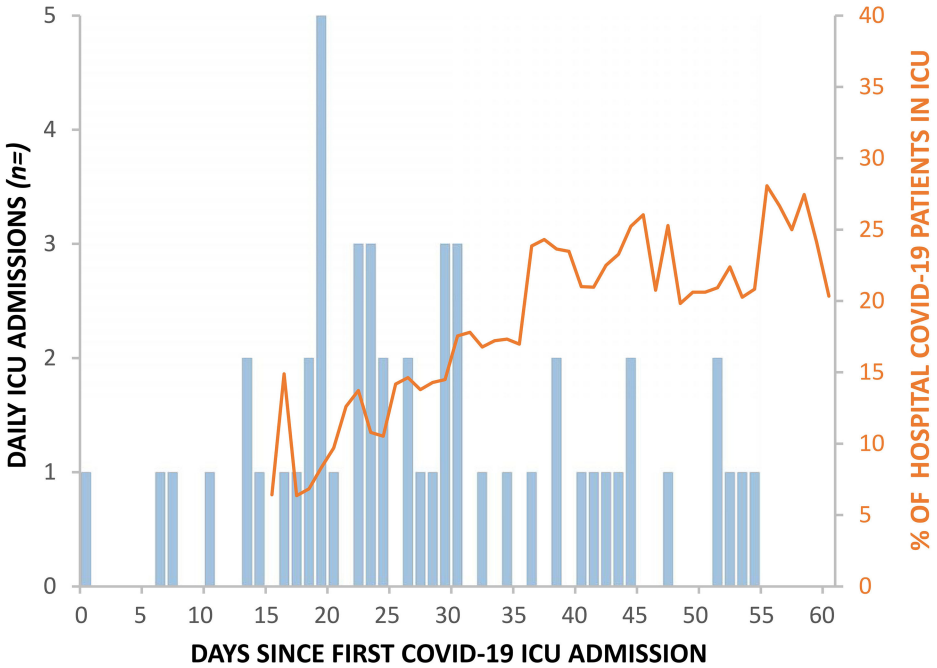

FIGURE 1 | Daily ICU COVID-19 admissions (blue bars) alongside corresponding percentage of total hospital COVID-19 inpatients in ICU (orange line).

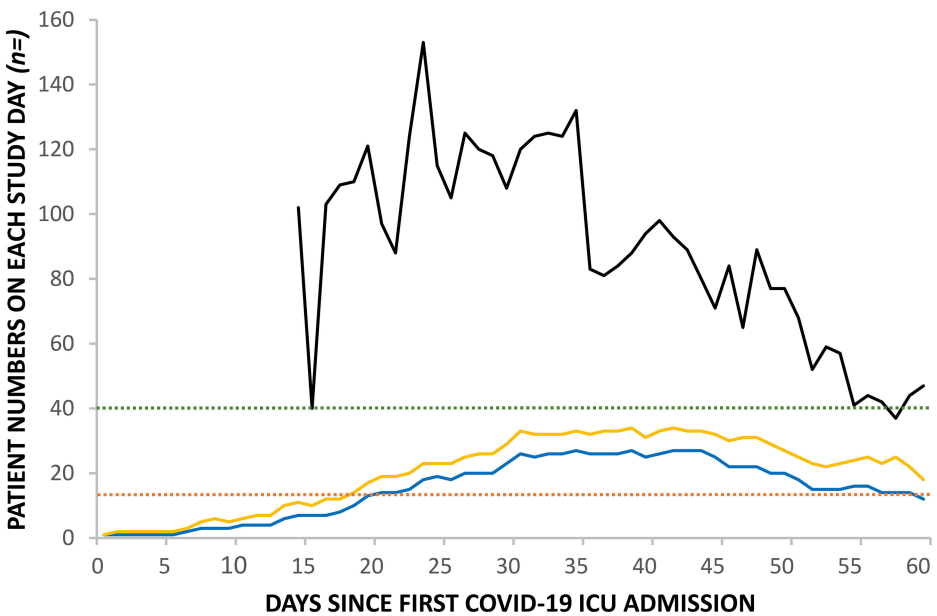

FIGURE 2 | Change in daily inpatient numbers in RGH: COVID-19 ward numbers (black line), COVID-19 ICU numbers (blue line), and total ICU numbers (yellow line). Pre-pandemic ICU maximum capacity for invasively ventilated patients (red dashed line); upscaled pandemic maximum capacity for invasively ventilated patients (green dashed line).

with admissions, there were no ethical dilemmas of having to triage patients out of the ICU required (16). Despite the operational difficulties, RGH mortality figures compare favorably with others reported in the international literature and in the UK (10, 11, 13, 17).

At the beginning of the pandemic wave, critical care patients represented ∼10–15% of the patients admitted to hospital, however after day 35, when the lockdown started to curb the number of hospital admissions, this had doubled. The reducing number of patients on the general wards enabled

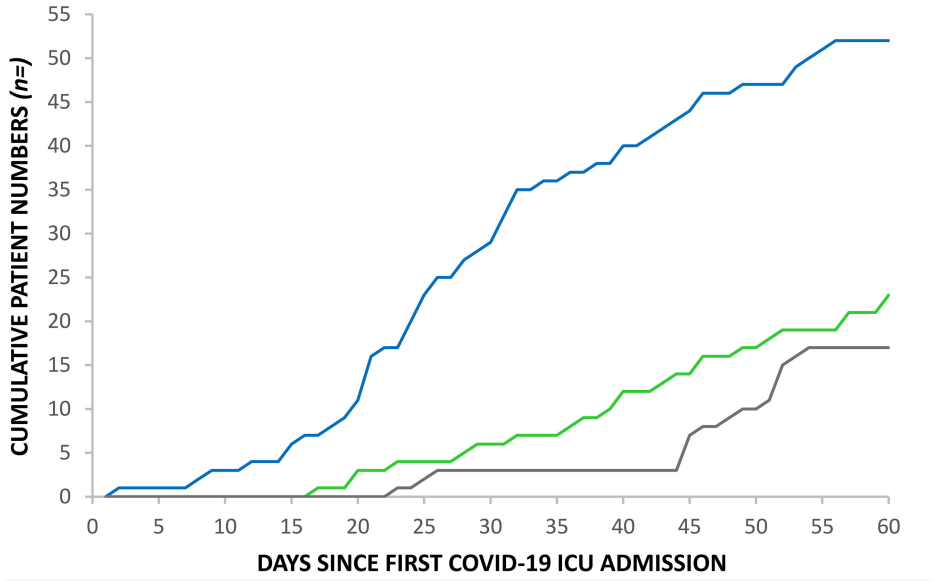

FIGURE 3 | Cumulative admissions, discharges and deaths to RGH ICU: COVID-19 ICU admissions (blue line), COVID-19 ICU discharges (green line) and COVID-19 ICU deaths (gray line).

TABLE 2 | COVID-19 ICU admissions and mortality by ethnicity—Causasian group and BAME subgroups.

	RGH catchment area—Background population (n = 415,617)	RGH ICU—Covid-19 patients	
		Admissions (n = 52)	Mortality (n = 17)
Caucasian	396,101 (95.3%)	34 (65.4%)	11 (64.7%)
BAME:	19,516 (4.7%)	18 (34.6%)	6 (35.3%)
Mixed	4,529 (1.1%)	0 (0.0%)	0 (0.0%)
Asian/Asian British	10,309 (2.5%)	12 (23.1%)	5 (29.4%)
Black/Black British	2,964 (0.7%)	3 (5.8%)	1 (5.9%)
Other	1,732 (0.4%)	3 (5.8%)	0 (0.0%)

Values are numbers (proportion).

the redeployment of staff to the much higher work-intensity areas, including the ICU. This flexibility helped to maintain operational capabilities even when the RGH reached over 250% of conventional ICU occupancy levels (18).

The higher risk of death in the BAME group and in patients living in the most deprived areas of the country has been highlighted recently in the UK (4). The weekly analysis of the data supplied to the Intensive Care National Audit and Research Centre (ICNARC) showed that Asian/Asian British ethnic origin and living in an area at the bottom quintile of the deprivation index scale are both independently associated with mortality (19). Our results are in agreement with the findings of these reports.

Furthermore, we postulate that BAME status and social deprivation may have a synergistic effect in our population. It is known that in the UK, the BAME population is more likely to have comorbidities associated with higher susceptibility for severe COVID-19 disease such as diabetes and hypertension. One or more of these attributes were present in every patient of BAME descent in our study. Interestingly, the APACHE II scores did not differ between the Caucasian and BAME population (data not shown), further emphasizing the potential deleterious effects of the combination of chronic comorbidities and other deprivation not detected by the APACHE II score.

The risk of adverse health events, ICU admission and death are thought to be higher in people living in more deprived areas and this has been indicated in England and more recently in our study in Wales (3, 20). Interestingly international comparisons do not support this, notably in France multiple studies have shown no adverse association with low socioeconomic status and initial severity of illness on ICU admission or long-term outcome (21, 22). Currently, there is no definitive data on this subject in relation to COVID-19 disease.

Based on previously published data from England and evaluating mortality figures up to the end of April 2020, Razaq et al. proposed a risk matrix to evaluate the risk of increased vulnerability due to SARS-CoV-2 exposure in the BAME group (19). Interestingly, most of our patients admitted to the ICU were in the low risk group: aged between 30 and 69 and living in a multigenerational household.

Our data indicates that low socio-economic status in the BAME group disproportionately increases the risk of ICU admission and death from COVID-19. The exact mechanisms for this increased risk and vulnerability from COVID-19 are not clear. There has been speculation that this may be due to the higher prevalence of particular health conditions, such as cardiovascular disease or diabetes, or predisposing factors such as low vitamin D levels, among the BAME population. The findings of the OpenSAFELY collaborative, based on 17 million adult patients in England with more detailed primary care data, show

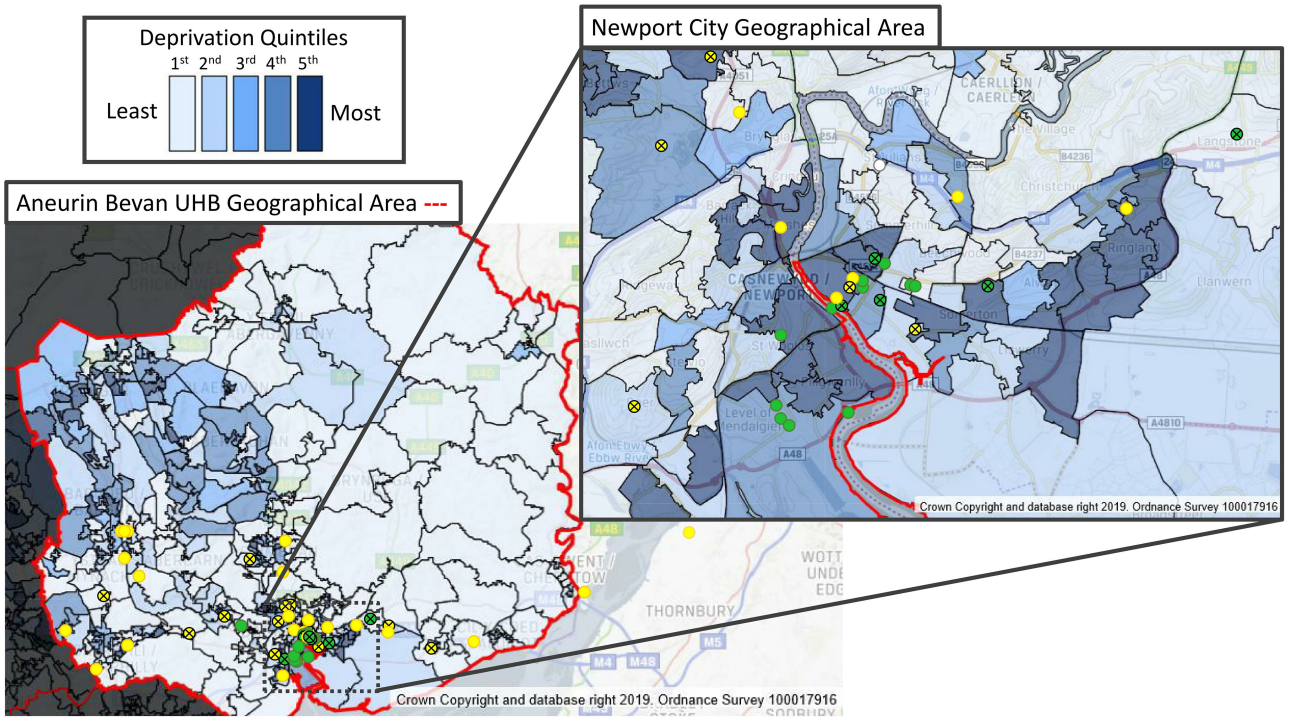

FIGURE 4 | Ethnicity, deprivation and COVID-19 ICU admissions from the RGH ICU catchment area postcodes of COVID-19 ICU individual patients, plotted on a map of "Welsh Index of Multiple Deprivation" (WIMD) quintiles, indicating ethnicity and ICU outcome. Caucasian (yellow circles); BAME (green circles); survivor (open circles); deceased (crossed circles).

that this is only a small part of the excess risk (23). Increased susceptibility for SARS-CoV-2 infection in BAME people due to genetic polymorphisms of the ACE-2 gene has also been postulated, however, this will need to be confirmed in large multi-center studies such as the one run by the ISARIC 4C Collaborators (24, 25).

Our results suggest that poorer outcomes in BAME people in COVID-19 disease may be due to exposure risks in the community, as our patients were over-represented in lower socio-economic groups. This may include higher household density with increased risk of transmission due to the lockdown, or disproportionate employment in lower band key worker roles, who either work in high exposure care environments or are unable to implement safe social distancing due to their roles, as was the case for the vast majority of our BAME patients. It is also possible that BAME status combined with low socioeconomic status delayed the presentation of patients to healthcare facilities, leading to more severe disease on admission. However, we haven't observed significant differences in the acute physiology of our patients, further pointing toward the combined chronic deleterious effect of ethnicity and deprivation.

There are significant limitations to our study: the sample size is limited and our findings might be unique to the significantly over-represented low socio-economic areas within the RGH catchment area. The small sample size also precludes more sophisticated analysis beyond simple descriptive statistics. There also might be selection bias, due to the admission policies of the ICU, however, criteria for admission into ICU have not been based on race or age. It is still possible that change in referral threshold from the other primary and secondary care providers have changed the case-mix of our population. However, comparison with the national ICNARC report suggests our case-mix is similar to elsewhere in the UK, when looking at patient factors other than socio-economic deprivation, and our findings could be extrapolated to other areas (13).

We could not compare ethnicity between hospitalized patients and those who were admitted to the critical care unit as this information has been generally poorly recorded in the hospital information system. To date there is no reliable information in the UK to corroborate our data presented here.

In conclusion we report that the first 52 patients with suspected and confirmed COVID-19 admitted to the Royal Gwent Hospital ICU has significantly stretched the critical care capacity, and it appears that the effect of the lockdown prevented the health service from being overwhelmed beyond additional capacity created in preparation for the COVID-19 pandemic. We observed a disproportionately higher admission rate and mortality rate amongst the BAME group, and it appears this may be closely linked to socio-economic deprivation which is highly prevalent in the area. Improving access to healthcare, including increasing critical care bed numbers might prevent excess mortality in this population.

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

TS: conceptualization, methodology, writing—original draft preparation, supervision, and project administration. EP and TB: investigation and data curation. TB, AD, and TS: writing—review and editing. EP and TS: visualization. All authors contributed to the article and approved the submitted version.

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Identification of a Novel Pathogen Using Family-Wide PCR: Initial Confirmation of COVID-19 in Thailand

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In resource-limited countries, early detection of novel pathogens is often challenging, due to financial and technical constraints. This study reports the efficacy of family-wide polymerase chain reaction (PCR) in screening, detecting, and identifying initial cases of the novel SARS-CoV-2 in Thailand. Respiratory secretions were collected from suspected individuals traveling from Wuhan, China to Thailand at the beginning of January 2020. Family-wide PCR assays yielded positive results for coronavirus in one traveler within 12 h on January 8, 2020. Nucleotide sequences (290 bp) showed 100% similarity to SARS-CoV-2. The whole genome sequence was further characterized by Next Generation Sequencing (NGS) for confirmation. Combining family-wide PCR, as a rapid screening tool, with NGS, for full genome characterization, could facilitate early detection and confirmation of a novel pathogen and enable early containment of a disease outbreak.

Keywords: SARS-CoV-2, coronavirus, COVID-19, family-wide PCR, novel pathogen, NGS, Thailand

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BACKGROUND

The accurate and timely identification of novel pathogens presents an obvious challenge in resource-limited settings, requiring expensive laboratory infrastructure and equipment along with the associated consumable supplies and reagents, and highly-trained technical staff. Timely diagnosis helps contain or prevent potential outbreaks, as in the case of the first imported Middle East Respiratory Syndrome (MERS) case in Thailand (1).

Bangkok was among the highest risk locations for the pneumonia-causing Corona Virus Disease 2019, given the large number of Chinese visitors annually. From beginning of 2020 until January 28, 2020 alone, Immigration Division 2, Thailand indicated that Thailand received 930,965 Chinese travelers, of whom 20,271 were from Wuhan (2). Total 41,000 people traveled from Wuhan to Thailand between December 1, 2019 and January 30, 2020 (2). According to the official Immigration Bureau website, Thailand received 1,054,891, 170,840, and 65,617 travelers from China in January, February, and March 2020, respectively (3).

A cluster of pneumonia of unknown origin in Wuhan, China in December 2019, presented a challenge for laboratories in resource-limited countries. Currently, the most straightforward approach to identifying unknown pathogens in humans and animals is metagenomics next generation sequencing (NGS) technology, which however has the disadvantage of being costly and time-consuming (4–6).

Family-wide polymerase chain reaction (PCR) assays have previously been used to detect various pathogens such as paramyxoviruses and MERS-CoV (7–10). Our laboratory modified and applied an existing family-wide PCR assay (11–14) to detect the novel pathogen from individuals suspected of being part of the Wuhan outbreak. We detected and successfully identified the virus on January 8, 2020. On January 10, 2020, the novel Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) was publicly identified as the cause of the outbreak (15). In the weeks following the viral genome publication, numerous diagnostic PCR assays with specific primers and probes became available both publicly and commercially (16–18). In the interim, however, diagnostically applicable data were unavailable, and family-wide PCR was used to screen and confirm 14 COVID-19 patients in Thailand. Here, we report the process of rapidly detecting the novel virus in an imported case of COVID-19 from Wuhan to Bangkok, Thailand.

METHODS

Beginning on January 3, 2020, Thailand implemented measures for screening patients arriving at four international airports from Wuhan and stepped up surveillance at public and private hospitals. Public health nurses evaluated all arriving passengers for signs of fever (>37.5°C) and respiratory symptoms. Between January 4 and 8, 2020, specimens were collected from five individuals exhibiting fever or signs of respiratory symptoms [patients under investigation (PUIs)] identified by the Department of Disease Control (DDC), Ministry of Public Health Thailand, and sent to laboratories for diagnostic testing. Two specimen types were collected from each PUI, sputum (SP), and nasopharyngeal and throat swabs (NT) placed in viral transport media. All PUIs were transported to the Bamrasnaradura Infectious Disease Institute (BIDI) and quarantined.

All specimens collected were sent to three separate laboratories to test for 33 common respiratory pathogens using a commercial diagnostic multiplex PCR assay (Fast Track Diagnostics, Luxembourg) at BIDI, PCR analysis for SARS- and SARS-related coronaviruses (CoVs) at the Department of Medical Sciences laboratory, Ministry of Public Health (DMSc), and to the Thai Red Cross Emerging Infectious Diseases Health Science Centre (TRC-EID) laboratory, where family-wide PCR testing was performed for influenzas and coronaviruses using established protocols (11–14) (**Table 1**).

The family-wide approach uses three pan-CoV nested Reverse Transcription PCR (RT-PCR) assays labeled W, Q, and C to amplify different regions of the RNA-dependent RNA polymerase gene (RdRp) (8–10). All three PCR assays were run separately using the same reagents for all patient samples. The 5 μL RNA template was added to 45 μL One Step RT-PCR master mix reagents (Qiagen) containing 10 μL of 5× buffer, 2 μL of dNTP (10 mM), 2 μL of Enzyme mix, and 1.5 μL of each primer (10 μL). Thermal cycling was performed according to each published protocol (12–14).

All PCR positive amplicons were further characterized by direct sequencing for confirmation. Additionally, whole genome sequencing (WGS) using NGS technology was performed on respiratory specimens from patients whose family-wide CoV PCR was positive for SARS-CoV-2. WGS was performed using TruSeq RNA library preparation and an Illumina MiSeq 3000 sequencer, according to the manufacturer's instructions with subsequent *de novo* assembly and reference mapping analysis. WGS was also performed at DMSc (19).

Once specific primer was available, real-time PCR (qPCR) assay targeting the receptor-binding domain of the spike gene using SYBR-Green One Step RT-PCR was later performed according to the published protocol (17) on respiratory tract specimens from patients whose SARS-CoV-2 testing was previously positive by family-wide PCR.

RESULTS

Detailed results of the 5 PUIs are shown in **Table 1**, where Patients #1–4 were negative for SARS-CoV-2, and positive for various different pathogens. Patient #5, whose specimen was collected on January 8, 2020, was found positive for novel coronavirus, which was later confirmed as SARS-CoV-2. The patient's NT and SP specimens were positive for 2 pan-CoV PCR protocols (Q and C), and negative for protocol W and all other viruses. Upon further investigation, sequence mismatch (up to 7 in 20 nucleotides) between the primers in protocol W and SARS-CoV-2 (GenBank accession no. NC_045512.2) was observed, which seemed to reduce the efficacy of the assay for SARS-CoV-2.

Direct sequencing was conducted from these PCR amplicons and the nucleotide sequences from both Q and C protocols [290 and 182 base pairs (bp), respectively] which were both positive for SARS-like-CoV, albeit different regions of the RdRp gene. A GenBank BLAST search performed on January 9, 2020 showed the sequences were best-matched (83.1% and 93.4%, respectively) to bat SARS-like-CoV isolate 4231 (GenBank accession no. KY417146.1). Repeat analysis on January 11, 2020, 1 day following the online publication of the novel 2019 CoV (i.e., SARS-CoV-2, GenBank accession no. MN908947.3) genome (15), showed a complete match (100% concordance) for both fragments (the 290 nucleotide sequence from sputum is published in GenBank; Accession no. MN970003).

WGS of this patient's NT specimen using NGS, (GenBank Accession no. MT447155), yielded a 29,805 nucleotide sequence identified as SARS-CoV-2, based on a 100% (29,805/29,805 bp) match to the virus identified in patients at the beginning of the outbreak in Wuhan (GISAID accession no. PI_ISL_402124) (17).

Upper and lower respiratory tract specimens from the index patient were retested over 7 subsequent days, with positive results in most specimens (**Table 2**). Testing was discontinued after

TABLE 1 | PCR results of 33 known respiratory pathogen and family-wide PCR detection for 5 PUIs identified.

Specimen Type	Known pathogen detected	3 CoV family-wide PCRs (Sequencing result)	Pan-Influenza A PCR (Sequencing result)
PATIENT #1			
Throat Swab	Influenza A virus	Negative	Positive (Influenza A virus)
	Moraxella catarrhalis		
	Haemophilus influenzae		
Suction	Influenza A virus	Negative	Positive (Influenza A virus)
	Moraxella catarrhalis		
	Haemophilus influenzae		
PATIENT #2			
Nasopharyngeal and throat swabs	Respiratory Syncytial Virus A/B	Negative	Negative
	Staphylococcus aureus		
	Klebsiella pneumoniae		
Sputum	Respiratory Syncytial Virus A/B Influenza C virus	Negative	Negative
	Staphylococcus aureus		
	Klebsiella pneumonia		
	Haemophilus influenzae		
PATIENT #3			
Nasopharyngeal and throat swabs	*Haemophilus influenzae*	Negative	Negative
Sputum	*Haemophilus influenzae*	Negative	Negative
PATIENT #4			
Nasopharyngeal and throat swabs	Coronavirus OC43	Positive—Protocol W	Negative
	Haemophilus influenzae	Positive—Protocol Q	
		Positive—Protocol C (Coronavirus OC43)	
PATIENT #5			
Nasopharyngeal and throat swabs	*Staphylococcus aureus*	Positive—Protocol Q	Negative
	Haemophilus influenzae	Positive—Protocol C (SARS-CoV-2)	
Sputum	*Staphylococcus aureus*	Positive—Protocol Q	Negative
	Haemophilus influenzae	Positive—Protocol C (SARS-CoV-2)	

yielding negative results on 2 consecutive days and the patient returned to China.

To determine the sensitivity and specificity of family-wide PCR, the specimens from the index patient were re-tested using qPCR (17). The qPCR analysis gave concordant results to family-wide PCRs (both Q and C protocols) when the qPCR threshold cycle (ct) was lower than 29.6 (**Table 2**). The qPCR provided better sensitivity than family-wide PCR in specimens with lower viral load. Specimens from Patients 1 to 4 were all negative when re-tested with qPCR using specific primer for SARS-CoV-2.

DISCUSSION

The magnitude of an outbreak can substantially be reduced by rapid and early identification of cases (20). This report shows the value of family-wide PCR when applied to detect unknown viruses, which led to the rapid identification of SARS-CoV-2 in Thailand. This report focuses on diagnoses of the initial patients and is not a comprehensive examination of the clinical, epidemic, and laboratory review of early COVID-19 cases in Thailand. SARS-CoV-2 was detected and identified in Thailand 1 day before China confirmed the cause of the Wuhan epidemic (15). The suspected importation of the novel coronavirus was unofficially communicated to the World Health Organization in-country office. All positive PCR amplicons were further sequenced for confirmation, to identify the viral species and verify that it was not a false positive from non-specific binding or contamination. The efficient detection of this pathogen is the result of coordinated public health efforts with collaborations between several Thai laboratories, allowing rapid results and proficient outbreak containment measures. Despite being the first country to confirm a case of COVID-19 outside of China at the beginning of January, <100 confirmed COVID-19 cases were registered in Thailand for more than 2 months, until the marked increase in new cases in mid-March (21).

The short PCR amplicon from family-wide PCR generates less sequence information when compared to the sequence information from NGS. This study showed that the two

TABLE 2 | PCR results of the specimens from Patient #5 collected during January 8 to January 18, 2020.

Collection date	Specimen #	Specimen Type	CoV PCR Protocol Q	CoV PCR Protocol C	qPCR (threshold cycle)
8 January 2020	200040-NT	NT	100% identity to SARS-CoV-2	100% identity to SARS-CoV-2	20.166
	200040-SP	Sputum	100% identity to SARS-CoV-2	100% identity to SARS-CoV-2	NA*
9 January 2020	200049-NT	NT	Not Detected	Not Detected	30.847
	200049-SP	Sputum	Not Detected	Not Detected	29.628
12 January 2020	200107-NT	NT	100% identity to SARS-CoV-2	100% identity to SARS-CoV-2	22.949
	200107-SP	Sputum	100% identity to SARS-CoV-2	100% identity to SARS-CoV-2	NA*
14 January 2020	200129-NT1	NT	Not Detected	Not Detected	32.032
	200129-SP1	Sputum	100% identity to SARS-CoV-2	100% identity to SARS-CoV-2	21.929
	200129-NT2	NT	Not Detected	Not Detected	32.032
	200129-SP2	Sputum	100% identity to SARS-CoV-2	100% identity to SARS-CoV-2	23.516
15 January 2020	200136-NT	NT	Not Detected	Not Detected	31.47
	200136-SP	Sputum	100% identity to SARS-CoV-2	100% identity to SARS-CoV-2	25.66
16 January 2020	200154-SP	Sputum	100% identity to SARS-CoV-2	100% identity to SARS-CoV-2	29.2
17 January 2020	200182-NT	NT	Not detected	Not detected	Not Detected
18 January 2020	200209-NT	NT	Not Detected	Not Detected	Not Detected

**NA, specimen was not available for testing. NT, Nasopharyngeal and throat swabs.*

family-wide PCR protocols (protocol Q and C) exhibited similar sensitivities despite having different amplicon fragment lengths (328 and 228 bp, respectively), which could potentially affect diagnostic accuracy, as sequence length correlates with specificity. Our data from Patient #5 (**Table 2**) suggests that the sensitivity of family-wide PCR is lower than qPCR, which may result in false negative results if the viral load in the specimen is low. The qPCR (commercially available or published in-house protocols) has been used at our laboratory for routine diagnosis of COVID-19 once specific primers and probes were available, which provides more rapid results. A shortcoming to family-wide PCR is the need for guesswork involved in choosing which viral family to amplify. CoV and influenza viral families were chosen for first tier testing during this investigation as these are common respiratory pathogens causing pneumonia.

Despite the shortcomings, family-wide PCR is convenient and sensitive as a screening assay for an unknown pathogen. Moreover, the method has several advantages, notably being more cost effective (about 100× less costly), more efficient (2–4× faster than NGS), and simpler, as no special training is required for laboratory personnel beyond routine PCR. Previously, this laboratory approach was successfully used to identify the first MERS case in Saudi Arabia from viral culture specimens (8). Family-wide PCR has also been used for pathogen detection in wildlife under USAID's PREDICT project, Emerging Pandemic Threats (EPT) program since 2010 (9, 10). It is conventionally recommended as a supplemental method for the detection of novel viruses rather than the main detection assay, as it is less sensitive than qPCR (11).

CONCLUSION

Our results demonstrate the practical applications of family-wide PCR as the initial detection assay for novel viruses of extreme pathogenicity in limited-resource setting when specific primers are not available, generating results within 12 h. The short turnaround time is much preferred to NGS which can be both time-consuming and costly. The efficient containment of an outbreak of any novel pathogen requires rapid identification of the novel pathogen, and family-wide PCR was well suited to this purpose.

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 here: https://www.ncbi.nlm.nih.gov/nuccore/MN970003 [GenBank Accession No. MN970003].

ETHICS STATEMENT

Ethical review and approval and written informed consent for participation was not required for clinical specimens collected for outbreak investigation purposes in accordance with the national legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

SW, RB, SI, WC, SS, PO, NM, OK, SU, AV, TPl, and TH involved in the study design. TPo, CR, CS, PK, and SY conducted the diagnostic analyses. SG drafted the manuscript. SW and SG critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Blood Test Results of Pregnant COVID-19 Patients: An Updated Case-Control Study

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Background: Coronavirus disease (COVID-19) is a current global public health emergency. However, current research on the blood test results of pregnant women with COVID-19 is insufficient.

Methods: A case-control study was carried out based on clinical blood test results. Pregnant COVID-19 patients, pregnant COVID-19 patients with diabetes, and pregnant COVID-19 patients with hypertension, were assessed in this study. Also, 120 controls were matched by age, parity, fetus number, and presence of chronic disease. *T*-tests, Chi-square tests, Wilcoxon signed-rank tests, and Kruskal-Wallis tests were used to compare data from the blood tests and liver function indices among the selected groups.

Results: Between January 24 and March 14, 2020, 60 pregnant COVID-19 patients delivered at the Maternal and Child Health Hospital of Hubei Province. The average maternal age of pregnant COVID-19 patients was 30.97 years and the mean gestational period was 37.87 weeks. 71.67% (43/60) of pregnant COVID-19 patients gave birth by cesarean delivery. In total, 21.67% (13/60) were diagnosed with diabetes and 18.33% (11/60) were diagnosed with hypertension during pregnancy. Compared to controls, pregnant COVID-19 patients showed significantly lower numbers of blood lymphocytes and higher numbers of neutrophils, as well as higher levels of C-reactive protein and total bilirubin. Among the three groups, pregnant COVID-19 patients with diabetes had significantly higher levels of neutrophils and lower levels of total protein. Aspartate transaminase levels were higher in pregnant COVID-19 patients with hypertension than in pregnant COVID-19 patients with no comorbidities and controls with hypertension.

Interpretations: Blood and liver function indices indicate that chronic complications, including hypertension and diabetes, could increase the risk of inflammation and liver injury in pregnant COVID-19 patients.

Keywords: COVID-19, blood lab test, case-control, pregnancy with hypertension, pregnancy with diabetes

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INTRODUCTION

SARS-CoV-2, which causes coronavirus disease (COVID-19), is a new coronavirus discovered in 2019. It is similar to previously studied zoonotic viruses, including the severe acute respiratory syndrome coronavirus (SARS-CoV-1) and the Middle East respiratory syndrome coronavirus (MERS-CoV) (Liu Y. et al., 2020). Since its first report in Wuhan, China in December 2019,

over 3.8 million people have been infected with SARS-CoV-2, and it has caused over 260,000 deaths worldwide (Rothan and Byrareddy, 2020; World Health Organization, 2020). The World Health Organization (WHO) declared the SARS-CoV-2 outbreak to be a public health emergency of international concern on January 30, 2020, indicating a high global risk (Sohrabi et al., 2020).

To provide practical evidence for the prevention and control of COVID-19, many studies have been conducted on the general population. However, pregnant women are more vulnerable to COVID-19 because they are in an immunocompromised state (Liang and Acharya, 2020). Pneumonia arising from any infectious etiology is an important cause of morbidity and mortality among pregnant women (Schwartz and Graham, 2020). In addition, both hypertension and diabetes are common complications during pregnancy, which have long-term negative health impacts on both mothers and their offspring (Davenport et al., 2018). Thus, pregnant women, especially those with chronic complications, should be evaluated as a high-risk group during the current COVID-19 pandemic (Liu D. et al., 2020).

To date, most studies (Chen H. et al., 2020; Chen N. et al., 2020; Schwartz, 2020) on pregnant women with COVID-19 have described their clinical characteristics including symptoms, pregnancy outcomes, CT manifestation, and mother-child vertical transmission. However, the indication for the adoption of cesarean delivery was controversial at the beginning of COVID-19 epidemic because parturient with COVID-19 might be associated with increased risk of miscarriage, preterm birth, and neonatal death (Boelig et al., 2020; Chen Z. et al., 2020; Wu et al., 2020). And there are few studies assessing pregnant women with COVID-19 who have comorbidities including hypertension and diabetes. The purpose of this study was to compare blood test results and liver function indices among pregnant COVID-19 patients and control groups of ordinary pregnant women.

METHODS

Participants

Patients included in the study delivered at the Maternity and Child Health Hospital in Wuhan, Hubei province. In 2018, 22.55% of newborns in Wuhan were delivered at this hospital, making it the largest maternal hospital in Hubei. From January 24 to March 14, 2020, 3,730 pregnant women delivered in this hospital, and 60 of them (1.61%) were confirmed to have COVID-19 by laboratory and clinical diagnosis (Juan et al., 2020). Moreover, we retrospectively selected 120 controls matched by age (within 3 years), parity, fetus number, and chronic disease (including diabetes, hypertension, hypothyroidism, and hepatic disease). Control patients were selected 1 year before the COVID-19 epidemic. Sociodemographic information and blood samples were collected upon admission and results were obtained from medical health records. All research subjects were recruited in the third trimester.

Abbreviations: CRP, C-reactive protein; TBil, total bilirubin; TP, total protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; WBC, white blood cell; ALB, albumin; GLB, globulin.

Data Analysis

Maternal and neonatal characteristics of continuous variables are presented as mean ± SD. *T*-tests, Chi-square tests, Wilcoxon signed-rank tests, and Kruskal-Wallis tests were used to compare the characteristics between the pregnant COVID-19 patients and control groups. Data analyses were conducted using the Statistical Analysis System (SAS 9.4). Two-sided $p < 0.05$ were considered statistically significant.

RESULTS

Sociodemographic characteristics of pregnant COVID-19 patients and control groups are shown in **Table 1**. The average age of the pregnant COVID-19 patients was 30.97 years and

TABLE 1 | Sociodemographic characteristics of pregnant COVID-19 patients and control group.

	COVID-19 patients	Control patients	*P*
	***N* (%)/*N* ± SD**	***N* (%)/*N* ± SD**	
Age	30.97 ± 4.13	29.97 ± 3.43	0.0871
Parity			
1	40 (66.67)	80 (66.67)	–
2	19 (31.67)	38 (31.67)	
3	1 (1.67)	2 (1.67)	
Fetus number			
1	59 (98.33)	118 (98.33)	–
2	1 (1.67)	2 (1.67)	
Chronic disease			
None	36 (60.00)	72 (60.00)	–
Diabetes	10 (16.67)	20 (16.67)	
Hypertension	8 (13.33)	16 (13.34)	
Diabetes and hypertension	3 (5.00)	6 (5.00)	
Hypothyroidism	2 (3.33)	4 (3.33)	
Hepatic disease	1 (1.67)	2 (1.67)	
Gestational week of delivery	37.87 ± 2.36	38.81 ± 1.81	0.0078
Gestational week of delivery			
<37	10 (16.67)	9 (7.50)	0.0599
≥37	50 (83.33)	111 (92.50)	
Delivery			
Vaginal delivery	17 (28.33)	69 (57.50)	0.0002
Cesarean delivery	43 (71.67)	51 (42.50)	
First symptoms			
None	26 (28.33)	0	–
Fever before or during delivery	11 (18.33)	0	
Fever after delivery	23 (38.33)	0	
Respiratory symptoms	9 (15.00)	0	
Neonatal birthweight (g)	3220 ± 622	3251 ± 520	0.7247
1-min Apgar scores			
10	38 (63.33)	14 (58.33)	0.5152
<10	22 (36.67)	10 (41.67)	
5-min Apgar scores			
10	57 (95.00)	23 (95.83)	0.8105
<10	3 (5.00)	1 (4.17)	

the mean gestational period was 37.87 weeks. These patients presented with mild COVID-19 infection. A high percentage of diabetes and hypertension was detected among the pregnant COVID-19 patients (21.67 and 18.34%, respectively). The majority of pregnant patients gave birth by cesarean delivery (71.67%), significantly higher than that of control groups (42.50%). Most pregnant COVID-19 patients showed no fever or respiratory symptoms, and only 15% reported respiratory symptoms during hospitalization. There were 61 live-birth newborns without COVID-19 infection. Moreover, **Table S1** presents the characteristics of maternal COVID-19 patients with chronic disease. The difference of clinical symptoms showed no statistical significance between pregnant COVID-19 patients with chronic disease and COVID-19 patients without chronic disease. Cesarean delivery in COVID-19 patients with chronic was significantly higher than in COVID-19 patients without chronic disease (91.67 vs. 58.33%, $p = 0.0054$).

Blood test results of the study groups are presented in **Table 2**. There was a statistically significant difference in the number of blood lymphocytes, neutrophils, and C-reactive protein (CRP) between pregnant COVID-19 patients and control women. Moreover, blood total bilirubin (TBil) levels in pregnant COVID-19 patients were significantly higher than those in controls (8.25 vs. 6.57, $p < 0.05$). The results of Chi-square analysis further confirmed that the proportion of lymphocytopenia among COVID-19 patients was significantly higher than control groups (43.33 vs. 15.83%, $p = 0.0003$) (**Table S2**). The proportion of COVID-19 patients with elevated CRP was higher than the control groups (60.00 vs. 38.33%, $p = 0.0061$). The blood test results of liver function showed that the proportion of COVID-19 patients with elevated aspartate aminotransferase (AST) was higher than the ordinary patients (8.33 vs. 0.83%, $p = 0.0084$). Moreover, the proportion of COVID-19 patients with decreased blood TBil level was lower than the control groups (11.67 vs. 29.17%, $p = 0.0091$).

Blood test results of pregnant COVID-19 patients and controls with diabetes are presented in **Table 3**. The white blood cell (WBC) count of pregnant COVID-19 patients without diabetes was significantly higher than that in the pregnant controls with diabetes (10.20 vs. 8.49, $p < 0.05$). The number of neutrophils

TABLE 2 | Blood test results of pregnant COVID-19 patients and control group.

	COVID-19 patients		Control patients		*P*
	Mean	**SD**	**Mean**	**SD**	
WBC, 10^9/L	10.68	3.87	9.60	3.00	0.0771
Lymphocytes, 10^9/L	**1.25**	**0.53**	**1.66**	**1.18**	**0.0001**
Neutrophils, 10^9/L	**80.83**	**8.88**	**77.17**	**6.10**	**0.0051**
CRP, mg/L	**20.16**	**41.96**	**8.87**	**20.41**	**0.0015**
ALT, U/L	15.07	25.21	10.03	4.81	0.7200
AST, U/L	22.33	26.81	16.59	5.00	0.1796
TBil, umol/L	**8.25**	**3.24**	**6.57**	**2.81**	**0.0010**
TP, g/L	66.79	4.70	66.91	4.78	0.8843
ALB, g/L	35.86	3.03	35.78	3.40	0.9853
GLB, g/L	30.93	3.63	31.43	4.15	0.5720

WBC, white blood cell; CRP, C-reactive protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBil, total bilirubin; TP, total protein; ALB, albumin; GLB, globulin. Bold represented statistical significance.

TABLE 3 | Blood test results of pregnant COVID-19 patients and control pregnant patients with diabetes.

	COVID-19 patients with diabetes		COVID-19 patients without diabetes		Control patients with diabetes	
WBC, 10^9/L	9.95	3.08	**10.20**	**3.38**	**8.49**	**2.16**
Lymphocytes, 10^9/L	1.22	0.43	1.57	1.16	1.49	0.36
Neutrophils, 10^9/L	**80.98**	**7.73**	**78.67**	**7.56**	**75.61**	**4.75**
CRP, mg/L	10.61	22.96	13.65	28.64	3.60	1.77
ALT, U/L	14.45	10.08	11.69	17.16	9.87	4.42
AST, U/L	19.79	10.77	18.67	18.20	16.16	3.41
TBil, umol/L	7.76	3.74	**7.23**	**3.11**	**5.93**	**1.83**
TP, g/L	**64.64**	**3.74**	66.69	4.80	**68.52**	**4.51**
ALB, g/L	35.48	2.76	35.61	3.40	36.92	2.83
GLB, g/L	29.17	3.28	31.35	4.18	31.60	3.38

WBC, white blood cell; CRP, C-reactive protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBil, total bilirubin; TP, total protein; ALB, albumin; GLB, globulin. Bold represented statistical significance.

in pregnant COVID-19 patients with diabetes was higher than that in both pregnant COVID-19 patients without chronic disease and controls with diabetes (80.98 vs. 78.67 and 75.61, respectively, $p < 0.05$). The TBil level in COVID-19 patients without diabetes was higher than that in pregnant controls with diabetes (7.23 vs. 5.93, $p < 0.05$). Finally, total protein (TP) levels in the pregnant COVID-19 patients with diabetes were significantly lower than that in the controls with diabetes (64.64 vs. 68.52, $p < 0.05$). **Table S3** shows that the proportion of participants with decreased albumin (ALB) was higher than the ordinary patients with diabetes (46.15 vs. 19.23%, $p = 0.042$).

Blood test results of pregnant COVID-19 patients and controls with hypertension are presented in **Table 4**. WBC, lymphocytes, neutrophils, and CRP were unchanged across the different groups. In contrast, AST levels in pregnant COVID-19 patients with hypertension were significantly higher than that in pregnant COVID-19 patients without chronic disease and controls with hypertension (38.97 vs. 16.61 and 19.09, respectively, $p < 0.05$). Additionally, ALB levels in pregnant COVID-19 patients with hypertension were significantly lower than that in pregnant COVID-19 patients without hypertension (33.67 vs. 36.08, $p < 0.05$). **Table S4** shows that the proportion of lymphocytopenia of COVID-19 patients with hypertension was significantly higher than that of control patients with hypertension (18.18% vs. 9.09, $p = 0.049$).

DISCUSSION

This is an updated case-control study comparing the blood test results of pregnant women with COVID-19. Among the pregnant COVID-19 patients, there were 43 of 60 cases (71.67%) underwent cesarean section. This result is in line with previous studies which reported a range of 56–93% of cesarean section (Chen L. et al., 2020; Huntley et al., 2020; Walker et al., 2020; Yang et al., 2020). The high adoption of cesarean delivery might be partly explained by the COVID-19 infection combined with the presents of chronic disease and the concern about the effects of COVID-19 on the pregnancy. We found that the most prevalent symptom of pregnant COVID-19 patients was fever, and a majority had no respiratory symptoms. These findings are consistent with other studies (Chen L. et al., 2020; Guan et al., 2020). Zaigham and Andersson (2020) also reported that most COVID-19 patients did not have any symptoms upon admission. Therefore, it is necessary to separate COVID-19 infected patients from ordinary patients by laboratory indicators upon hospital admission. Moreover, maternal symptoms of COVID-19 during the first or second trimester of pregnancy require further research.

This study showed a high percentage of diabetes and hypertension among pregnant COVID-19 patients. A previous study based on 30 pregnant COVID-19 patients confirmed that the most common comorbidities were hypertension (16.7%) and diabetes (6.6%) (Wang et al., 2020). The associations between COVID-19 infection and the development of diabetes and hypertension among pregnant women were still unclear. The potential mechanism might be explained by the expression of Angiotensin-converting-enzyme 2 (ACE2), the receptor for the COVID-19 spike protein, on pancreatic β cells can have a direct effect on β cell function, which might result in the development of diabetes among pregnant COVID-19 patients (Bornstein et al., 2020). Moreover, the hyper-inflammatory state in COVID-19 may be associated with a higher risk of developing pre-eclampsia (Abbas et al., 2020).

As far as we know, this is the first study to explore the blood test results of pregnant women with chronic disease who are diagnosed with COVID-19. Compared to the pregnant women without COVID-19, the blood indices of pregnant COVID-19 patients showed a significantly lower lymphocyte count than the controls. Other indices including neutrophil count and CRP levels of the pregnant COVID-19 patients were significantly higher than those of the control pregnant women. These differences in inflammatory indices are in accordance with typical clinical characteristics of pneumonia. Chen L. et al. (2020) reported that lymphopenia was present in 51 of 116 pregnant

TABLE 4 | Blood test results of pregnant COVID-19 patients and control pregnant patients with hypertension.

	COVID-19 patients with hypertension		COVID-19 patients without hypertension		Control patients with hypertension	
WBC, 10^9/L	8.95	2.39	9.87	3.11	10.72	4.29
Lymphocytes, 10^9/L	1.39	0.36	1.54	1.14	1.55	0.46
Neutrophils, 10^9/L	77.10	8.91	78.59	7.17	77.68	7.57
CRP, mg/L	26.01	59.39	10.16	20.77	15.66	29.94
ALT, U/L	28.56	51.17	10.62	8.70	9.39	3.87
AST, U/L	**38.97**	**56.56**	**16.61**	**7.30**	**19.09**	**5.28**
TBil, umol/L	7.25	2.18	7.19	3.18	6.22	2.29
TP, g/L	65.10	4.68	67.03	4.47	66.35	6.28
ALB, g/L	**33.67**	**2.89**	**36.08**	**2.94**	35.09	4.92
GLB, g/L	31.44	2.68	30.95	3.23	32.85	7.53

WBC, white blood cell; CRP, C-reactive protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBil, total bilirubin; TP, total protein; ALB, albumin; GLB, globulin. Bold represented statistical significance.

women diagnosed with COVID-19, while. Liu D. et al. (2020) found that among 15 pregnant COVID-19 patients, 12 had a decreased lymphocyte count and 10 had increased CRP values. It is recommended that the blood indices of pregnant COVID-19 patients should be carefully monitored and the changes in these inflammatory indices were correlated with the prognosis of patients (Chen L. et al., 2020). The long-term effects of the COVID-19 infection and treatment on blood indices of pregnant COVID-19 patients need further study.

Additionally, pregnant women with COVID-19 showed significantly higher TBil values. Elevated TBil levels are considered biomarkers for liver disease (Weaver et al., 2018). Thus, COVID-19 could affect the liver function of pregnant women. However, there was no difference in ALT and AST levels in pregnant women with COVID-19. A cohort study (Guan et al., 2020) containing 1,099 COVID-19 cases showed that 10.5% of patients presented with abnormal bilirubin, 21.3% with ALT elevation, and 22.2% with AST elevation. It has been reported that 2–11% of patients with COVID-19 have liver comorbidities and 14–53% have abnormal levels of AST and ALT during disease progression (Zhang et al., 2020). The limited number of pregnant COVID-19 patients in this study may limit the power to detect changes in ALT and AST. Moreover, the majority of the COVID-19 patients in this study had a mild infection and liver injury was not obvious. Therefore, liver damage caused by COVID-19 during pregnancy is still unclear and needs further research.

In our study, pregnant COVID-19 patients with chronic diseases, including diabetes or hypertension, showed a stronger inflammation response and markers of liver injury compared to pregnant COVID-19 patients without chronic diseases and control pregnant women with chronic diseases. Compared to the other two groups, pregnant COVID-19 patients with diabetes had significantly higher neutrophil counts and lower TP values. The main feature of diabetes is hyperglycemia, which can increase the oxidative stress response and enhance the inflammatory response. Diabetes could also compromise the ability of the liver to regenerate (Mendes-Braz and Martins, 2018). Insulin resistance in diabetes influences liver cell apoptosis and causes liver dysfunction (Schattenberg and Schuchmann, 2009). Hajam and Rai (2019) found that diabetic rats had a significant increase in lipid peroxidation (LPO) in the liver, while ALT, AST, and ALP levels were significantly decreased in the antioxidative enzymatic system. Thus, under the combined effects of COVID-19 and diabetes, suboptimal inflammatory processes and liver function biomarkers might indicate a worse prognosis in pregnant women.

Moreover, pregnant COVID-19 patients with hypertension showed a significantly higher level of blood AST, which is an important indicator of liver function (Sookoian and Pirola, 2015). Arima et al. (2014) reported that hypertension exacerbated liver injury and hepatic fibrosis in spontaneously hypertensive rats. Han et al. (2015) revealed that trophoblastic mitochondrial damage may result in liver injury in preeclampsia-like mouse models. Therefore, it was hypothesized that pregnant COVID-19 patients with hypertension are more likely to suffer from liver injury than the other groups. However, few studies have focused on liver injury caused by hypertension and this hypothesis requires further verification.

Our study has several limitations. First, pregnant women with COVID-19 were only enrolled during their third trimester. The effects of COVID-19 on pregnant women in their first or second trimester are not clear. Second, this study only assessed pregnant COVID-19 patients during hospitalization, but long-term effects on the liver and other organs as well as newborns require further research. Third, we only compared the blood indices of pregnant COVID-19 patients with hypertension and diabetes. The mechanisms underlying the effects of COVID-19 on these parameters also need further exploration.

CONCLUSION

The results of this study indicate that chronic diseases may strengthen the inflammatory response to COVID-19 and mediate liver damage in pregnant women. Therefore, during the treatment of pregnant women with COVID-19, focusing on inflammation and liver injury is necessary, especially for those with chronic diseases.

DATA AVAILABILITY STATEMENT

The datasets generated for this article are not readily available because The application of the raw data should be approved by the Ethics Committee of Maternal and Child Health Hospital of Hubei Province. Requests to access the datasets should be directed to Yao Cheng, chengyao2014@sina.com.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of Maternal and Child Health Care Hospital of Hubei Province. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

GS, YC, and QL: wrote the manuscript. GS, YC, and YZ: conceived and designed the experiments. YC: analyzed the data and performed the experiments. All of the authors: gave final approval of the version to be submitted and agree to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

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Triage for Potential Percutaneous Coronary Intervention During the Coronavirus Disease 2019 (COVID-19) Pandemic

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INTRODUCTION

Since the outbreak of coronavirus disease 2019 (COVID-19), this public health emergency has caused 5,701,337 infections and 357,688 deaths (1). As one of the first few affected countries, China has gradually gained control of the emergency. The West China Hospital of Sichuan University is a regional public health center located in Chengdu, Sichuan province, which mainly provides medical services for southwest China. Our center is also a certified COVID-19 tertiary care hospital and designated hub center within the Sichuan province. The daily volume of our center is ∼80,000 visits. For better management of patients with chest pain and those who need percutaneous coronary intervention (PCI), our Department of Cardiology and Chest Pain Center issued a workflow for PCI at the beginning of COVID-19 pandemic. From January 1, 2020, to May 31, 2020, over 4,300 PCIs were performed. However, there were no hospital-acquired COVID-19 cases.

PCI WORKFLOW AND PATIENT TRIAGE

In our medical center, the Chest Pain Center is attached to the Department of Emergency for better management of patients with chest pain, especially those who need emergency medical intervention. Once patients who present with chest pain come to our Chest Pain Center, their vital signs and health status will be evaluated within 10 min. If cardiac emergencies, including acute coronary syndrome, bradyarrhythmia, severe myocarditis, or other situations needing endovascular intervention, are considered, patients will be enrolled in our PCI workflow and patient triage process. The workflow consists of three steps and is presented in **Figure 1**.

Step 1. Tele-Communication

The resident, chief resident, and attending-on-shift of the Cardiac Catheterization Laboratory (Cath Lab) are required to tele-communicate with residents of the Department of Emergency and get the exact information of the enrolled patients. The information includes temperature, epidemiological history of COVID-19, and COVID-19-related symptoms (i.e., fever, cough, fatigue, etc.). Based on the information, patients are classified into two categories, non-suspected COVID-19 patients and suspected COVID-19 patients, according to the Novel Coronavirus-Pneumonia diagnostic criteria and treatment regimens defined by the National Health Commission of the People's Republic of China (2).

Step 2. Consultation

The medical consultation is carried out by the resident of Cath Lab. The consultation route from Cath Lab to Chest Pain Center is set and managed by our center.

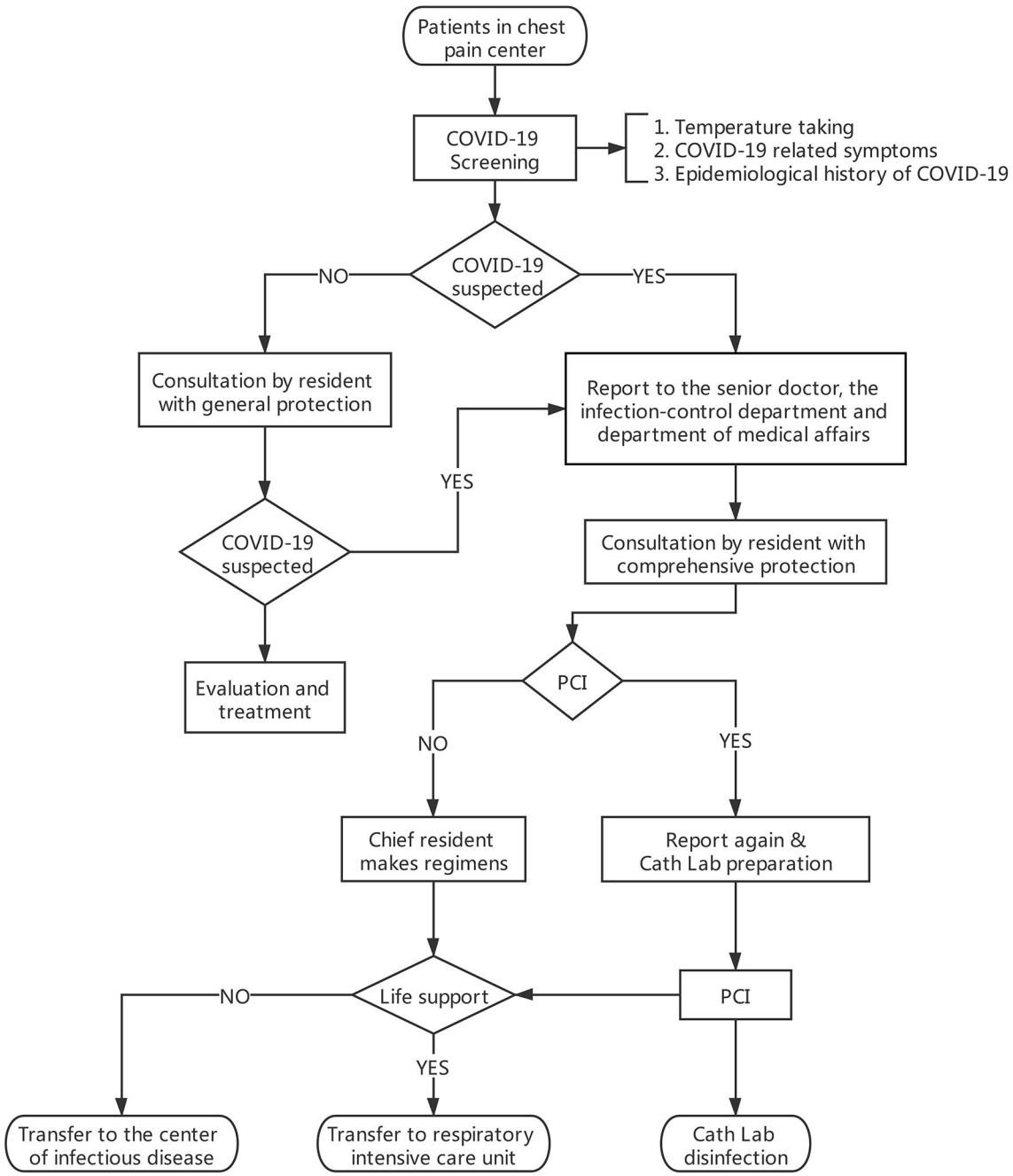

FIGURE 1 | PCI workflow and patient triage process. General protection: medical uniforms, surgical mask, medical cap, and rubber gloves. Comprehensive protection: gown, medical N95 mask, goggles, medical cap, and rubber gloves.

For non-suspected COVID-19 patients, consultation doctors are required to wear medical uniforms, surgical mask, medical caps, and rubber gloves. The COVID-19-related information will be checked again, and the temperature will be re-taken. PCI will be carried out in non-suspected COVID-19 patients if endovascular intervention is deemed necessary.

For suspected COVID-19 patients, a reminder will be generated on the consultation system. Consultation doctors ares required to fetch gowns, medical N95 masks, goggles, medical caps, and rubber gloves, and put on the personal protective equipment before the consultation. If PCI is not necessary, a medical regimen and transfer plan to the Center of Infectious Disease or Respiratory Intensive Care Unit (RICU) should be made. If emergent thrombolysis is required, the chief resident should instruct the thrombolysis in the negative-pressure ward of the Chest Pain Center. A further transfer plan to the Center of Infectious Disease or RICU should then be made. When PCI is deemed necessary, consultation doctors should report to the attending-on-shift and the director. Additionally, a report to the Infection-Control Department and Department of Medical

Affairs is required so that the hospital can react immediately if any unexpected situation takes place. Then the suspected COVID-19 patient can be sent to the Cath Lab.

Step 3. Transfer and Procedure

Before the transfer of suspected COVID-19 patients, all medical workers are required to wear a full set of protective equipment including apron, N95 mask, goggles, and rubber gloves,. The protective equipment is available at the Cath Lab. Only after the staff are equipped with protection is the transfer administrated. The transferring route (including the elevator), Cath Lab, and the entrance are previously set. After the procedure, patients who do not need further life support will be transferred to the Center of Infectious Disease, while patients who need further life support are transferred to the RICU. Additionally, the patients' families and companions will be evaluated by the Infection-Control Department and appropriate isolated measures will be taken. Comprehensive disinfection is carried out after the procedure.

DISCUSSION

Since the outbreak of COVID-19, public health systems worldwide have been confronted with great challenges on medical supply and hospital management. Certain adaptations must be made in the face of COVID-19 (3). During the pandemic, some reforms and suggestions have been made (4). Some centers have also designed crisis management plans (5). An optimized and effective management of PCI workflow will also help control the spread of COVID-19.

Our center is located in Chengdu, where medical resources are not overwhelmed. With the implementation of the proposed workflow, no hospital-acquired COVID-19 has been reported in our Chest Pain Center or Cath Lab. Centers that share similar situations with us may find it useful to implement our workflow. A better management of patients with chest pain will help in this world pandemic.

AUTHOR CONTRIBUTIONS

YH and MC had the idea for this article. QL, SH, and T-YX contributed to the writing of the manuscript. YP, J-FW, CL, YZ, LZ, MW, HW, M-XZ, YB, and Y-LW participated in the workflow design and update. All authors contributed to the article and approved the submitted version.

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Timely Sharing of Data on Infection and Death of Medical Workers

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Keywords: COVID-19, medical workers, infection, death, overwork

INTRODUCTION

Since December 2019, an outbreak of a new type of coronavirus pneumonia (COVID-19) has attracted worldwide attention. COVID-19 has rapidly evolved into a global pandemic that has required urgent action from the international community (1). The epidemic has now spread all over the world, with the number of confirmed cases and deaths still rising. As of 2:00 a.m. CEST, August 1, 2020, there have been 17,406,428 confirmed cases worldwide, of which 669,740 have died (2).

BASELINE CHARACTERISTICS OF DEATH MEDICAL WORKERS IN CHINA, THE UNITED STATES AND ITALY

According to incomplete statistics from official and public reports (3, 4), as of August 1, 2020, at least 64 medical workers in China have given their lives whilst working to prevent and control COVID-19, compared to at least 851 in the United States (of which 156 have complete clinical data), and these numbers continue to increase (**Supplementary Table 1**). The virus has resulted in the death of a higher proportion of male medical workers in both China (79.7%) and the United States (55.8%). In China, the most deceased medical workers were between 50 and 59 years old (**Supplementary Figures 1A,B**), accounting for ∼54%, with a median age of 50 years (48.4 ± 12.8 years). Among both male and female workers in the United States, the age group with the highest number of deaths among medical workers was 60–69 years old (**Supplementary Figures 1A,B**), about 35.3%, with a median age of 57 years (55.2 ± 12.8 years). In addition, nearly half of Chinese medical workers died of other diseases due to overwork, 24 (38.7%) died from COVID-19 infection, and eight died from accidents (**Supplementary Table 1**).

According to statistics, the peak months for medical workers' deaths were February and April in China and the United States, respectively (**Supplementary Figures 1C,D**). In China, most of the medical worker deaths were doctors (37, 57.8%), while in the United States, nurses accounted for the majority of deaths among medical workers (75, 48.1%). In both China and the United States, medical worker deaths were more concentrated in the hardest-hit areas (New York and New Jersey in the United States and Hubei Province in China).

In China, the peak of medical worker deaths occurred ∼1 week after the highest number of daily new cases, whereas in the United States and other countries such as Italy, the number of medical worker deaths has increased since the COVID-19 pandemic (**Supplementary Figures 1C,D**). In the United States, it appears that the number of medical worker deaths ceased to increase in June, but the above data do not include the deaths of medical workers due to incomplete clinical data, overwork, and other accidental factors.

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DISCUSSION

Compared with the current basic stability of the epidemic in China, the situation abroad is still relatively serious. In the United States, Brazil and India, more than 1.5 million cases have been confirmed, especially in the United States, where the number of confirmed cases has exceeded 4 million. Moreover, the number of new confirmed cases in India is increasing exponentially. In some states of the United States, medical personnel account for as many as 20% of known coronavirus cases (4). With the increasing severity of the epidemic, the number of confirmed cases has skyrocketed, and the workload, working hours, and burden of medical workers have also increased (5, 6). In addition, infections among medical workers are also a worrying problem (7). With the increasing number of COVID-19 patients, there is a severe shortage of personal protective equipment for medical workers, including masks, goggles, protective clothing, and so on. Due to the severe shortage of protective equipment, medical workers are forced to reuse disposable masks and homemade protective clothing, which is still facing a huge risk of infection.

There are also serious issues concerning the infection of medical workers in Spain. According to the Spanish Ministry of Health, as of April 6, 2020, ∼19,400 medical workers were confirmed to be infected with COVID-19, accounting for about 14% of the confirmed cases. Of these, 13 medical workers have died, including 11 doctors, 1 nurse and 1 health worker.

In the United States, as of August 1, 2020, there were 11 emergency medical technicians among deceased medical workers. These first-responders work in ambulance and emergency medical services and were often the first medical professionals to have contact with patients with COVID-19. Infections of these personnel may therefore be due to a failure to take adequate preventive measures and insufficient protection in the epidemic (8). First-line emergency personnel need clearer measures and to be given adequate protective equipment.

With the intensification of the pandemic, medical health care systems across the world continue to overloaded. The sacrifice of medical workers reminds governments that they need to invest in more medical resources, strengthen the protection of medical workers, give priority to ensuring the supply of resources to front-line medical workers, and take good care of the physical and mental health of these workers by reducing stress. This may involve more rational arrangements for the rotation of shifts and transfer of staff to allow for rest and to enhance medical confidence and resistance to the epidemic (9). The timely sharing of data on the infection and related deaths of medical workers by governments could enable us to better understand and assess the risks faced by medical workers, enabling us to provide real-time guidance, early intervention and better responses to the COVID-19 pandemic.

AUTHOR CONTRIBUTIONS

WM and MC drafted the manuscript, studied the concept, and design. WM collected 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.2020.552409/full#supplementary-material

Supplementary Figure 1 | Distribution of deceased medical workers in China, the United States and Italy. **(A)** pie chart of age distribution; **(B)** histogram of age distribution of different genders; **(C)** Daily new cases; **(D)** Daily deaths of medical workers.

Supplementary Table 1 | Baseline characteristics of death medical workers in China, the United States and Italy[a].

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Taiwan Government-Guided Strategies Contributed to Combating and Controlling COVID-19 Pandemic

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Coronavirus disease 2019 (COVID-19) is highly contagious, and thus has become an emerging health crisis worldwide. The optimal strategies to prevent the spread of this disease are inconclusive, and therefore, the adopted measurements to combat COVID-19 varies in different countries. In mid-March and late-August 2020, we performed internet searches to collect relevant information, from sources such as the website of the World Health Organization. The epidemiological data of COVID-19 from several countries were collected and we found that Taiwan had a comparably successful story for combating the pandemic. As of mid-March, Taiwan had high rates of diagnostic testing (688.5 tests per million citizens) with a lower infection rate (49 cases, 2.1 cases per million people). As of late-August, there were 488 cases (20 cases per million people). Furthermore, Taiwanese government-guided strategies and hospital data were also reviewed. We summarized some important strategies to combat COVID-19, which include: (1) border control; (2) official media channel and press conferences; (3) name-based rationing system for medical masks; (4) TOCC-based rapid triage, outdoor clinics, and protective sampling devices; and (5) social distancing, delaying the start of new semesters, and religious assembly restriction. In conclusion, Taiwan had lower rates of COVID-19 compared with other countries, and Taiwan government-guided strategies contributed to the control of the disease's spread.

Keywords: novel coronavirus, COVID-19, SARS-CoV-2, quarantine, 2019-nCoV, pandemic, government strategy

INTRODUCTION

In December 2019, novel coronavirus disease (COVID-19), caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), was detected in central China and then spread throughout the country and to the rest of the world rapidly (1–4). The number of infected people increased exponentially, and more than 150,000 confirmed cases were reported by mid-March 2020; the phenomenon was officially recognized as a pandemic by the World Health Organization (WHO) on 11 March 2020 (1, 5).

The basic reproductive value of the virus was estimated at 2-3, and the case fatality rate was ∼3% (1, 6–8). There are no effective antiviral drugs or vaccines available. The clinical manifestations are protean, and it is estimated that 80% of infected people are asymptomatic or experience mild cases; therefore, it is difficult to detect potentially infected patients at an early stage and disrupt the transmission chains (9–11). International cooperation is especially crucial to combat this disease.

Due to the recent emergence of SARS-CoV-2 in humans, researchers are developing best practices in real-time to combat this new virus. Several strategies were adopted very early to block transmission, including the unprecedented lockdown of Hubei and other provinces, and travel bans within China and many other countries globally (1, 6, 12). But geographic characteristics, medical resources, population densities, and social norms vary widely across countries and the adopted strategies facing COVID-19 differ widely too. Based on the observed high degree of contagiousness of SARS-CoV-2, blocking the spread of this disease poses steep challenges. However, by focusing upon slowing down the speed of transmission and flattening the curve of coronavirus cases, governments have been able to prevent the collapse of their medical systems, reduce mortality, and buy time for the development of antiviral drugs and vaccinations (13). Furthermore, technological advances may be beneficial in combating the pandemic. Big data analytics, new technology, and proactive testing have been applied in Taiwan to combat COVID-19 (14, 15). These strategies may be collectively beneficial in reducing virus transmission.

Taiwan is a small and populous island which is geographically very close to China. Social interaction between China and Taiwan is frequent, thus leading to a high risk of virus transmission. Central Epidemic Command Center (CECC) was assembled to combat the COVID-19 pandemic in Taiwan on 20 January 2020. The commander of the CECC was the Taiwanese Ministry of Health and Welfare minister, and the organization's members included experts from various fields. The CECC executed several strategies to reduce disease transmission and its government-guided strategies may have contributed to mitigating the disease's spread. Taiwan's first confirmed case was detected on 21 January 2020, and by mid-March there were 49 cases (16). As of late August, there were more than 25 million confirmed cases worldwide; but Taiwan has a lower incidence of infection with only 488 cases reported in total. We conducted this retrospective study to investigate the incidence of COVID-19 in some countries and the varying effects of government-guided strategies. Reviewing these measurements and experiences may be helpful for policy makers and healthcare providers.

STUDY DESIGN AND DATA COLLECTION

Study Design and Incidences of COVID-19

Our study was approved by the ethical committee of MacKay Memorial Hospital, Taipei, Taiwan (registry number: 20MMHIS140e). As of mid-March 2020, we prospectively searched the websites of WHO, Taiwan Centers for Disease Control (CDC)—which is a key department under the Ministry of Health and Welfare and is responsible for disease prevention and control—and other websites to extract data regarding patient numbers and diagnostic tests of COVID-19 in some countries based on the epidemic conditions and completeness of publicly available data (1, 16–18). The total population of each country was also obtained to calculate the case numbers per million citizens. The trend chart was also plotted.

Government-Guided Strategies and Hospital-Adopted Measurements

Taiwan's CECC delivered information to the populace via public broadcasting; this included television, newspaper, and the internet (16). Additionally, official documents were provided to each hospital or healthcare clinic. In accordance with the government-guided strategies, individual healthcare units adapted the optimal measurements according to their own equipment, patient characteristics, human resources, and clinical settings. We retrospectively reviewed the government-guided strategies and our hospital measurements. Some photos are provided to demonstrate practical situations.

RESULTS OF EPIDEMIOLOGICAL DATA

Incidences of COVID-19 and Diagnostic Tests Performed

As of 13 March 2020, 49 cases were confirmed as COVID-19 in Taiwan with an overall low incidence rate (2.1 cases per million citizens, **Table 1**). Compared with other countries, aggressive tests for COVID-19 were also observed in Taiwan and 16,089 people had received diagnostic testing (688.5 tests per million citizens). The positivity rate was relatively low (49/16 089, 0.3%). Additionally, Taiwan kept a relatively low rate of increase for disease transmission (**Figure 1A**). The number of infected people increased exponentially and there were more than 25 million infected people worldwide as of late August 2020 (**Figure 1B**). There were 488 cases in Taiwan, and Taiwan retained a low positive rate (20 cases per million citizens, **Table 1**).

MAJOR MEASURES OF GOVERNMENT-GUIDED STRATEGIES AND HOSPITAL-ADOPTED MEASUREMENTS

Taiwan's CECC has executed many strategies to combat COVID-19. We have summarized five important strategies and hospital-adopted measurements.

Border Control

Many Taiwanese people either work or have business in China, and it is impossible to cut off all transportation between the two regions. With gradually decreased flights, the number of incoming people from China declined from more than 100,000 per month in 2019 to 5,000 in February 2020. At the same time, the CECC cooperated with other government departments to perform immediate border inspections. For people traveling from endemic areas, home quarantine for 14 days was required and enforced by chiefs of villages (16). If people went out without permission, they were searched by police and fined

TABLE 1 | Tests for COVID-19 and positive patients in selected countries.

	Tests performed	Population (millions)	Tests per million citizens	Positive cases	Positive test rate (%)	Positive cases per million citizens
As of 11 March 2020						
South Korea	210,144	51.3	4,099	7,755	3.69	151.3
Italy	60,761	60.5	1,005	10,149	16.70	167.9
UK	26,261	67.9	387	456	1.73	6.7
USA	8,554	331	26	696	8.08	2.1
Japan	9,600	126.5	76	568	5.92	4.5
Taiwan	16,089	23.4	688.5	49	0.31	2.1
As of late August 2020						
South Korea	1,909,329	51.3	37,236	19,400	1.02	378
Italy	8,410,510	60.4	139,138	265,409	3.16	4,391
UK	16,273,209	67.9	239,511	331,644	2.04	4,881
USA	80,305,101	331	242,383	6,097,763	7.59	18,405
Japan	1,420,589	126.4	11,238	65,573	4.62	519
Taiwan	86,983	23.4	3,651	488	0.55	20

up to 300,000 New Taiwan Dollars (roughly 10,000 USD). The definition of endemic areas changed over time according to the risk assessment of the CDC.

Official Media Channel and Press Conference

Whenever there was a new confirmed case or important policy, the CECC gave a press conference to announce the current information. As the pandemic's events changed rapidly, the frequency of press conferences was almost daily. Fear comes from ignorance and misinformation. Fake news is not uncommon in the internet era, especially during a pandemic (19, 20). Fake news causes panic, violence, unnecessary stockpiles, discrimination, and an increase in the spread of disease (21). Therefore, in Taiwan, people who release fake news are fined. An authorized and timely official channel was helpful to reduce fake news and public anxiety. Line® is one of the most popular instant messaging applications in Taiwan, thus, the CECC has built an official Line® channel to deliver correct information (**Supplementary Figure 1**). The application of new technology is beneficial in providing timely and correct information (14, 15).

Name-Based Rationing System of Mask Plan

The CECC recruited all mask factories and governed the allocation of masks. For the general public, a "name-based rationing system of mask plan" was performed. Everyone could buy two masks per week at corner pharmacies. As the production capacity increased, everyone could purchase nine masks over a 2-week period beginning in April. In April, masks were donated to other countries and, starting in June, masks could be sent to people abroad. New technology was applied to the rationing plan and the purchase process was certificated by each citizen's national health insurance card. A real-time "mask map" website was also created to provide a clear instruction of storage and people could also make mask reservations using a mobile app (16) (**Supplementary Figure 2**).

TOCC-Based Rapid Triage, Outdoor Clinics, and Protective Sampling Device

TOCC refers to travel, occupation, contact, and cluster, which has been promoted in Taiwan for years. Patients who look for medical aid will always be asked their TOCC history. Hospitals can potentially be places for disease to spread, hence, outdoor tent clinics were set up to provide medical care for patients with travel or contact history (**Figure 2**). Patients with travel or contact history of COVID-19 were guided to specific outdoor clinics to prevent the virus' spread within hospitals. Inside the special outdoor clinics, healthcare providers wore medical masks, gloves, and isolation gowns to protect themselves. If COVID-19 was suspected, a nasopharyngeal swab was performed using a homemade sampling shield—or a protective cover transformed from an old incubator to offer the clinician protection against exposure (**Figure 3**) (22). Physicians and patients were also separated by polyvinyl chloride clear sheets. This creates a physical barrier to block the aerosols, droplets, or vomitus during sampling. Telemedicine was also used to assist in medical care and to reduce exposure. For people under quarantine having mild medical illness, such as itching skin rashes or superficial burn injuries, a history-taking and inspection was arranged via telemedicine (**Supplementary Figure 3**).

Social Distancing, Delay of New Semesters, Restriction of Religious Assembly

Education and religion are essential, but crowds of any kind pose a high risk for virus transmission. In order to

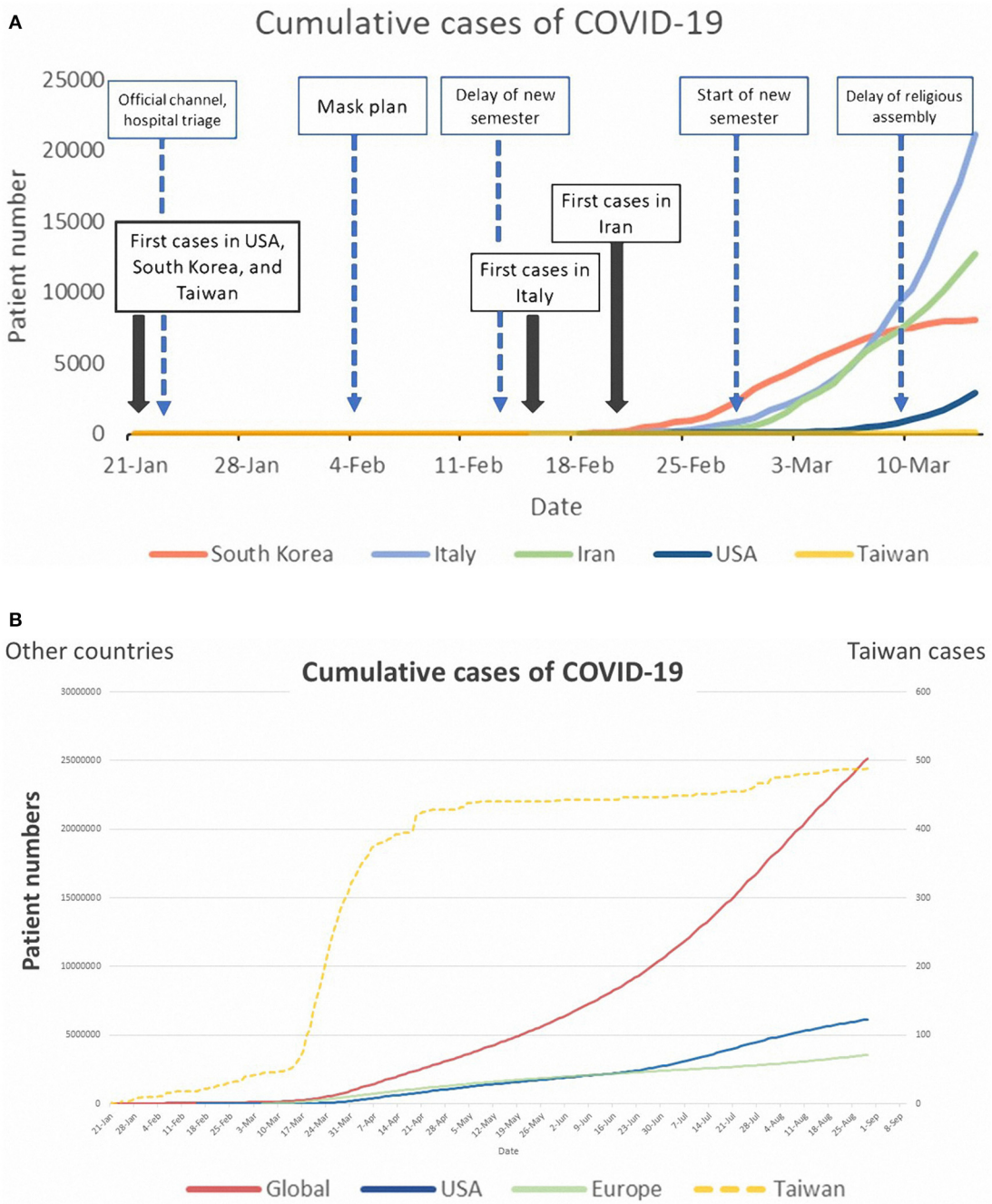

FIGURE 1 | (A) Trend chart of cumulative cases of COVID-19 in some countries and government-guided strategies in Taiwan (as of 11 March 2020). **(B)** Trend chart of cumulative cases of COVID-19 in some countries (as of late August 2020).

reduce transmission of COVID-19 in schools, the CECC cooperated with the Ministry of Education to postpone the second semester of all schools to late February. Moreover, religion is an important cultural component, but several outbreaks of COVID-19 tied to religious assembly have been reported (1). The Dajia Matsu pilgrimage is an important religious assembly in Taiwan which is listed in the UNESCO Intangible Cultural Heritage. It is usually held in late March and spans 9 days and 8 nights; millions of people participate. The religious assemblies were also postponed for months (no local cases for ∼2 months) to mitigate viral transmission.

FIGURE 2 | Tents as "outdoor clinics".

FIGURE 3 | Homemade "sampling shield" for nasal swab testing.

MINOR MEASURES, SOME DETAILS OF IMPLEMENTATION, AND DISCUSSION

The coronavirus pandemic is a severe crisis worldwide and the optimal strategies to combat COVID-19 in each country remain unclear. Our study found Taiwan had aggressive diagnostic tests (high number of tests performed per million citizens) and low infection rates compared with other countries; in fact, it had one of the lowest incidences in the world. Government-guided strategies contributed to controlling the disease's spread and may be beneficial for reference by other countries' health policy makers and healthcare providers.

Timely quarantine and identification of infectious sources are essential to reduce virus transmission (23, 24). However, people may be afraid of quarantine and may even conceal their travel history. Taiwan's broadly covered health insurance system also shows an advantage. By rapidly connecting its medical and immigration systems, the government was able to generate a notice of travel-abroad history within 30 days to remind healthcare providers when the health insurance card was connected (16). Prompt disposition and management could be arranged. Additionally, isolation and quarantine may create huge psychological stress (25), and in order to provide humane support, a quarantine bag was delivered to people under quarantine, which included instant noodles, food, rice, facemasks, hand sanitizer, and coupons for in-home movies and music, such as Netflix and Line TV (**Supplementary Figure 4**). If people under quarantine felt sick, they could use a hotline (1922) to be scheduled at a designated hospital for further assistance. These measures and strategies cumulatively contributed to reduced exposure and breaking transmission chains in local transmission.

Public education with correct information about virus transmission and disease prevention is crucial during a pandemic. The CECC invited famous internet celebrities and YouTubers to make videos in various languages and to share correct information. For health personnel, teleconferences were held to share knowledge and standard procedures of medical care. All these measurements reduce unnecessary fear and panic.

The innovative "name-based rationing system for masks" was believed to have contributed to disease control during the COVID-19 pandemic (26). The major route of transmission of COVID-19 is thought to be through respiratory droplets and wearing mask is a simple and effective step to prevent infection (1, 16, 27–29). Stockpiling masks and hand sanitizer is not reasonable and is hazardous for disease control as it may cause some individuals to lack necessities (30). Taiwan's government-guided strategy to recruit mask factories and re-allocate masks ensured that healthcare providers had enough personal protective equipment (PPE) to care for highly contagious patients. PPE played a vital role in the battle against COVID-19 and shortages of PPE was an important issue. In a large-scale study in China, 3.8% of infected individuals were healthcare personnel, and the risk of healthcare-associated infection was emphasized (6). Allocation of PPE by the government and reducing stockpiling behaviors were beneficial for front-line healthcare providers. For the general public, the name-based rationing system for masks ensured everyone could have an adequate supply of masks. This strategy avoided mask stockpiling and public panic also decreased. Community transmission might be reduced when all residents wear a mask.

Unnecessary hospital visits were prohibited. Patients who look for medical aid in Taiwan are always asked for TOCC history. At the hospital entrance, a short version of COVID-19 symptoms was posted to provide a simple, graphic, and clear reminder of the disease (**Supplementary Figure 5**). People were asked about the purpose of their hospital visits and TOCC history was confirmed. If entry was allowed, the visitor then went through infrared thermal imagers, which decreases contact during body temperature reading (**Supplementary Figure 6**), and they must then apply hand sanitizer to clean their hands. These measures contributed to a decreased risk of exposure.

Our study had some limitations. First, we recognize that there is no single best strategy and that the true impact of each strategy remains unclear. The optimal strategy will differ by geographic region, culture, population density, and healthcare resources and norms. Second, due to the lack of relative quantification of the impact for each one of the components that are mentioned as part of the public health strategy, it's difficult to investigate the independent impact of each measurement.

CONCLUSION

In conclusion, the emerging COVID-19 pandemic is an important health crisis worldwide. The number of infected people increased exponentially in many areas, while Taiwan experienced a relatively controllable situation. These government-guided strategies may contribute to the reduction of disease transmission.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

C-CC, C-YT, W-MC, C-MC, and C-YL were responsible for conception. C-CC, C-YT, Y-CL, C-YH, P-HL, and Y-LT were involved in study methodology and data collection. W-MC, T-HS, and S-LW supervised study. C-YT, Y-CL, and C-MC performed data analysis. C-YL wrote the first draft. C-CC, C-YT, and W-MC contributed to this work equally. All authors approved 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.2020.547423/full#supplementary-material

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SYSTEMATIC REVIEW
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Current Understanding of COVID-19 Clinical Course and Investigational Treatments

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Importance: Currently, there is no unified framework linking disease progression to established viral levels, clinical tests, inflammatory markers, and investigational treatment options.

Objective: It may take many weeks or months to establish a standard treatment approach. Given the growing morbidity and mortality with respect to COVID-19, this systemic review presents a treatment approach based on a thorough review of scholarly articles and clinical reports. Our focus is on staged progression, clinical algorithms, and individualized treatment.

Evidence Review: We followed the protocol for a quality review article proposed by Heyn et al. (1). A literature search was conducted to find all relevant studies related to COVID-19. The search was conducted between April 1, 2020, and April 13, 2020, using the following electronic databases: PubMed (1809 to present); Google Scholar (1900 to present); MEDLINE (1946 to present), CINAHL (1937 to present); and Embase (1980 to present). The keywords used included *COVID-19, 2019-nCov, SARS-CoV-2, SARS-CoV,* and *MERS-CoV*, with terms such as *efficacy, seroconversion, microbiology, pathophysiology, viral levels, inflammation, survivability,* and *treatment and pharmacology*. No language restriction was placed on the search. Reference lists were manually scanned for additional studies.

Findings: Of the articles found in the literature search, 70 were selected for inclusion in this study (67 cited in the body of the manuscript and 3 additional unique references in the Figures). The articles represent work from China, Japan, Taiwan, Vietnam, Rwanda, Israel, France, the United Kingdom, the Netherlands, Canada, and the United States. Most of the articles were cohort or case studies, but we also drew upon other information, including guidelines from hospitals and clinics instructing their staff on procedures to follow. In addition, we based some decisions on data collected by organizations such as the CDC, FDA, IHME, IDSA, and Worldometer. None of the case studies or cohort studies used a large number of participants. The largest group of participants numbered <500 and some case studies had fewer than 30 patients. However, the review of the literature

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revealed the need for individualized treatment protocols due to the variability of patient clinical presentation and survivability. A number of factors appear to influence mortality: the stage at which the patient first presented for care, pre-existing health conditions, age, and the viral load the patient carried.

Conclusion and Relevance: COVID-19 can be divided into three distinct stages, beginning at the time of infection (Stage I), sometimes progressing to pulmonary involvement (Stage II, with or without hypoxemia), and less frequently to systemic inflammation (Stage III). In addition to modeling the stages of disease progression along with diagnostic testing, we have also created a treatment algorithm that considers age, comorbidities, clinical presentation, and disease progression to suggest drug classes or treatment modalities. This paper presents the first evidence-based recommendations for individualized treatment for COVID-19.

Keywords: COVID 19, infectious disease, disease management, directed treatment, cover-19 testing, clinical course

HIGHLIGHTS

- **Question:** What are the most effective treatment recommendations for COVID-19?
- **Findings:** COVID-19 can be divided into three distinct Stages, beginning at the time of infection (Stage I), sometimes progressing to pulmonary involvement (Stage II, with or without hypoxemia) and less frequently to systemic inflammation (Stage III). In addition to modeling the stages of disease progression, we also created a treatment algorithm which considers age, comorbidities, clinical presentation, and disease progression to suggest drug classes or treatment modalities.
- **Meaning:** This paper presents the first evidence-based recommendations for individualized treatment for COVID-19.

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic has spread throughout the globe. According to the Centers for Disease Control and Prevention (CDC), in the United States alone there were 5,460,429 cases along with 171,012 deaths, as of August 19, 2020 (2). A mathematical model created by The Institute for Health Metrics and Evaluation (IHME) predicts that in the United States the number of deaths may climb to over 295,000 by December 1, 2020 (3). This creates a critical and immediate need for medical treatment and resources.

Preliminary data in the US suggests that COVID-19 may be more infectious and lethal than Influenza H1N1. To place this in context, **Figure 1** provides a comparison of the reproduction rate and case-fatality rates for major respiratory virus pandemics (4–6). In the general population, case-fatality rates for COVID-19 are about 1.4% (7). Data strongly emphasizes early intervention to reduce case-fatality and inhibit reproductive rates.

To date, a number of articles have been published on the clinical course and treatment of the disease (8–10). The majority of patients present with more than one symptom on admission,

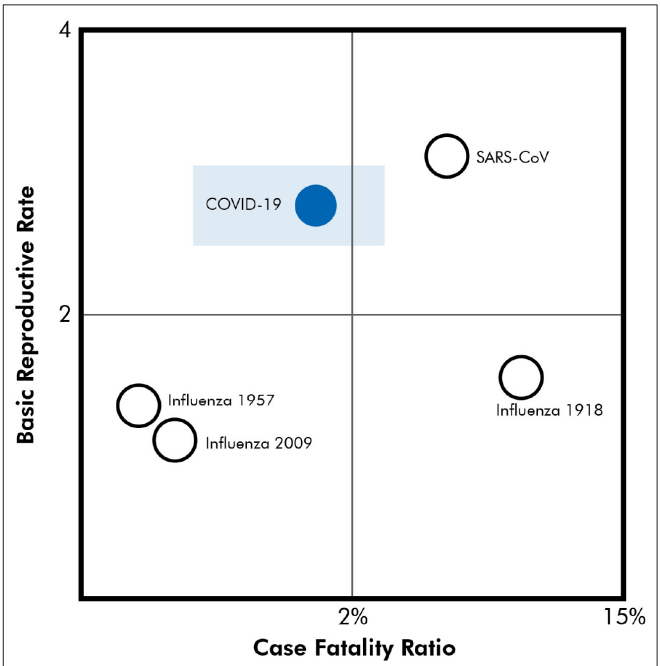

FIGURE 1 | Reproduction rate and case-fatality rates for major respiratory virus pandemics (4, 5). Rectangle donates case fatality range from multiple publications.

although the combination of fever, cough, and shortness of breath is rare. Siddiqi and Mehra proposed a staged progression model based on observed clinical courses in published studies (11). In Stage 1, or the mild phase, the virus multiplies and establishes residence in the host, predominantly in the respiratory tract. In Stage 2, there is viral multiplication and localized inflammation in the lungs. Stage 3 is marked by extra-pulmonary systemic hyperinflammation syndrome. The prognosis and recovery from Stage 3 is generally poor. Rapid recognition of which stage the

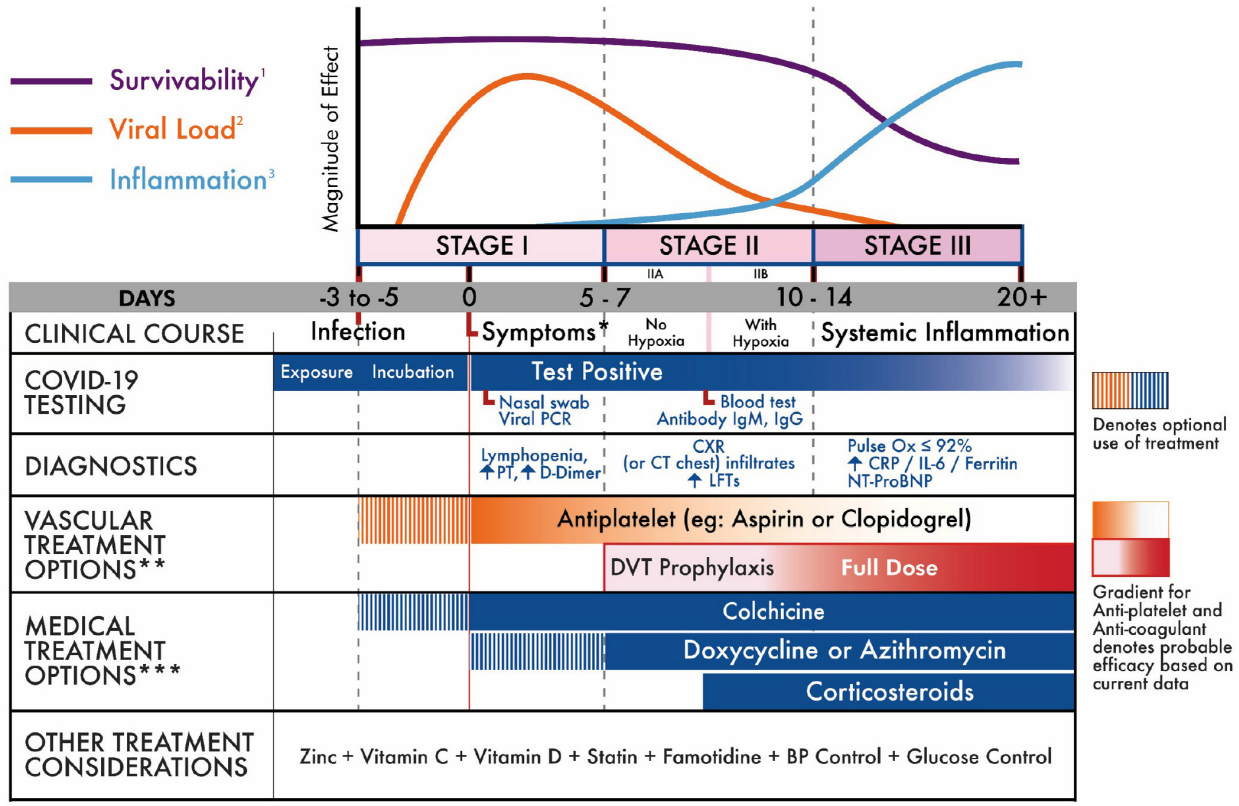

FIGURE 2 | COVID-19 clinical stages and management strategy (15–17). *Initially mild in stage I (fever, cough, myalgia, other non-specific). May progress in stage II-III to severe dyspnea and respiratory distress (16, 18–20). **As with all treatment options, risks, and benefits should be carefully reviewed with the patient. ***No treatments are currently FDA approved for COVID-19 treatment. The FDA has approved remdesivir and convalescent plasma for inpatient use.

patient is in and the deployment of appropriate therapy may have the greatest yield.

Common correlating factors that tend to lead to poorer outcomes include age, hypertension, diabetes, coronary artery disease, chronic lung disease, and malignancies (12). Research also finds variations in outcomes due to a dysregulated and exuberant immune response. Patients requiring intensive care have significantly higher levels of IL-6, CRP, ferritin, and D-Dimer. An important therapeutic modality may be to downregulate the cytokine storm, particularly in severe illness (13). The literature also suggests that disease progression can be predicted. During the severe acute respiratory syndrome (SARS) pandemic, a retrospective analysis revealed that 2-week cumulative case data could help estimate the total case numbers with accuracy—well before the date of the last reported case (14).

As we have found, there is no unified framework linking disease progression to established viral levels, clinical tests, inflammatory markers, and investigational treatment options. Given that it may take many weeks or months to establish a standard treatment approach and that rates of morbidity and mortality are increasing, we present an initial treatment approach based on a thorough review of currently available scholarly articles and clinical reports. Our focus is on staged progression, clinical algorithms, and individualized treatment.

METHODS

We followed the protocol for a quality review article proposed by Heyn et al. (1). A literature search was conducted to find all relevant studies related to COVID-19. The search was conducted between April 1, 2020, and April 13, 2020, using the following electronic databases: PubMed (1809 to present); Google Scholar (1900 to present); MEDLINE (1946 to present), CINAHL (1937 to present); and Embase (1980 to present). The keywords used in this search included *COVID-19, 2019-nCoV, SARS-CoV-2, SARS-CoV,* and *MERS-CoV*, with terms such as *efficacy, seroconversion, microbiology, pathophysiology, viral levels, inflammation, survivability,* and *treatment and pharmacology*. No language restriction was placed on the search. Reference lists were manually scanned for additional studies. From this systematic review, a model was created that incorporated clinical course, diagnostics, disease management, and treatment.

Our results focus on recommendations for individualized treatment, by selecting the most appropriate drug or modality for the patient, carefully weighing risks and benefits. Clinicians and patients should understand the staged progression of COVID-19 (**Figure 2**). As such, we present a treatment algorithm that recommends no treatment for some and specific treatment

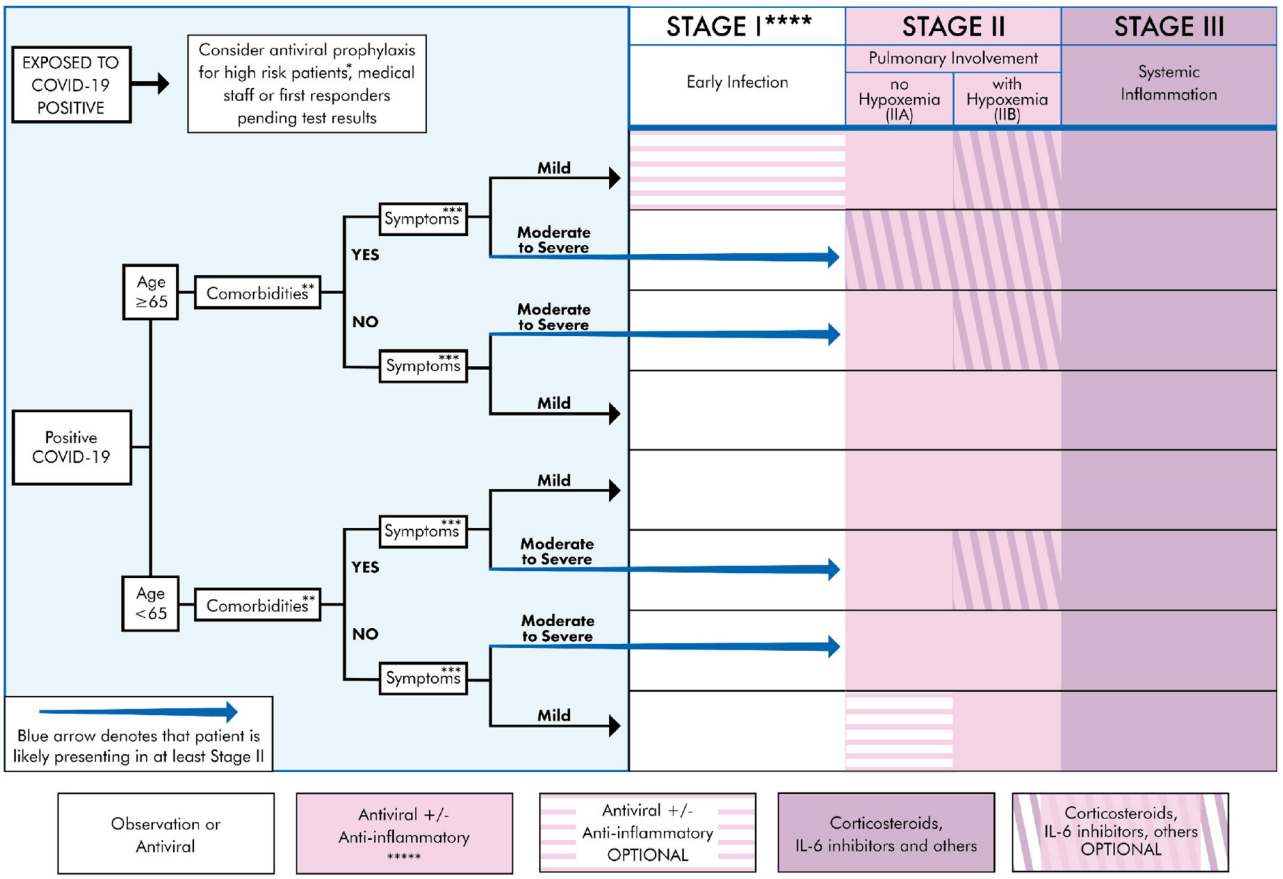

FIGURE 3 | Treatment algorithm for COVID-19+ patients based on clinical presentation and therapeutic staging. *High risk patient: Anyone that is ≥65 y/o or meets comorbidities criteria as defined below. **Comorbidities: Defined as any two of the following: HTN, DM, CVD, CKD, Pre-existing lung disease, CHF, diabetes >7.6%, use of biologicals, HIV+, history of transplant, morbid obesity (BMI ≥ 40) (21, 22). ***Symptoms Mild: Fever, cough, fatigue, myalgia, headache, anosmia. Rarely, patients may also present with diarrhea, nausea, and vomiting (8, 21, 23). Moderate: Symptomatic viral pneumonia with possible hypoxemia (PaO2/FiO2 < 300). Confirmed by chest imaging (CXR or CT) which demonstrate bilateral infiltrates or ground glass opacities (21). Severe symptoms: Systemic (extra-pulmonary) hyperinflammation with one of the following: respiratory rate > 30 or SpO_2 < 92% on room air (11, 17). Will also include abnormal chest imaging (CXR, CT scan, or lung ultrasound) characterized by bilateral opacities that are not primarily due to volume overload or lung collapse (partial or full). Echocardiogram can be used rule out of primary cardiac causes (24, 25). ****See **Table 1** for appropriate Rx for stage. Treatment must be individualized to the patient by considering risks, benefits, and contraindications of the particular Rx. Note: there may be a potential for combining multiple agents if no drug interaction exists, as there are pleural mechanisms of actions. *****Convalescent plasma can be used during any stage, though likely more beneficial earlier in the disease course (63).

for others, depending on age, comorbidities, and symptom severity (**Figure 3**).

RESULTS

Based on our thorough review of the literature, we correlated the disease course to COVID-19 testing, diagnostic options, and treatment strategies (see **Figure 2**). COVID-19 can be divided into three distinct stages, beginning at the time of infection (Stage I), sometimes progressing to pulmonary involvement (Stage II, with or without hypoxemia), and less frequently to systemic inflammation (Stage III). We also created a treatment algorithm that considers age, comorbidities, clinical presentation, and disease progression to suggest drug classes or treatment modalities (see **Figure 3**). The specific treatments are summarized in **Table 1** (15, 21–62, 64).

Comorbidity

Data exists for early identification of cases at high risk of progression to severe COVID-19. One promising model created in China found that patients who developed severe COVID-19 possessed one of the following diseases: hypertension, diabetes, coronary heart disease, chronic respiratory disease, or tuberculosis. The same model cited age and various serological indicators [such as C-reactive protein (CRP), lactate dehydrogenase (LDH), bilirubin, and others] as factors associated with worse outcomes (65). Additional research confirmed, in a case-control study, that subjects with high Sequential Organ Failure Assessment (SOFA) scores, with age >65, with hypertension, diabetes, and/or coronary heart disease were at greatest risk (66). Lastly, research focusing on viral load and survival found that higher initial viral load is independently associated with worse prognosis (2).

TABLE 1 | Summary of investigational treatments by COVD-19 effect.

Agent	Effect	Dosing	Stage	Mechanism	Commentary
Remdesivir	AV	• 200 mg IV ×1, followed by 100 mg qd for 5–10 days	I–III	• RNA polymerase inhibitor (26)	• Adverse effects include elevated ALT and AST, phlebitis, constipation, headache, nausea. • Theoretical risk of renal injury. • Should not be used in pregnancy due to lack of data. • Has limited drug-drug interactions (no significant CYP effect) • Clinical trials underway in the US, UK, and China • Showed efficacy in COVID-19 treatment (27)
Lopinavir/Ritonavir	AV	• 200 mg/50 mg/capsule, 2 capsules PO bid for no more than 10–14 days	I–III	• Protease inhibitor (28)	• Nearly 14% of patients cannot complete a course due to GI side effects (29). • Ritonavir is a potent CYP3A4 inhibitor (interacting with Rx such as apixaban, tacrolimus, and amiodarone). • In rare cases, Lopinavir/Ritonavir can cause liver injury, pancreatitis, and cardiac toxicity. • Treatment in mostly Stage II–III patients was not found to be superior to standard of care. Subgroup analysis suggestive that earlier treatment (Stage I) might be beneficial (28)
Favipiravir	AV	• 1,600 mg PO bid x1d, then 600 mg PO bid for up to 14 days	I–III	• Broad spectrum inhibitor of RNA-dependent RNA polymerase (30, 31)	• Increases liver function parameters (AST, ALT, and total bilirubin) • Testis toxicity and has a risk for teratogenicity and embryotoxicity • Was found to be superior to Lopinavir/Ritonavir in a small controlled study (32)
Umifenovir	AV	• 200 mg q8h for up to 14 days	I–III	• S protein/ACE-2 membrane fusion inhibitor (33)	• Metabolism by CYP3A4. Caution with strong inhibitors or inducers. • Hypersensitivity risk increases in children under 2 years of age • Limited clinical evidence shows promise in COVID-19 (34)
Hydroxychloroquine	AV A-IN	• Stage I–II −400 mg PO bid for first day followed by 200 mg bid daily for 5 days (35, 36) • Stage III—May consider extending treatment (200 mg bid) for up to 14 days (35)	I–III	• AV: replication-neutralization of the pH cellular organelles for gene replication • A-IN: inhibition of macrophage activation and reducing release of tissue TNF-a, IL-1, IL-6 (37–39) • A-IN: interfere with lysosomal activity and autophagy, disrupt membrane stability, alter signaling, and transcriptional activity, which can then inhibit immune activation and cytokine production (38)	• Adverse effects include: rash, nausea, and diarrhea. GI symptoms can be mitigated by taking with water; use with caution in diabetic patients may cause hypoglycemia (40) • Increased risk of retinopathy with a recommended maximal daily dose of 5.0 mg/kg. Avoid if history of retinal disease, macular degeneration, or previous treatment with tamoxifen (41) • Caution in patient at risk for QT prolongation. EKG at baseline and following initiation is generally advised, particularly in critically ill patients. • Contraindicated with Epilepsy, Porphyria, G6PD, and Myasthenia Gravis • Not proven effective for Pre-Exposure/Post-Exposure prophylaxis. IDS recommends for patients hospitalized with Pneumonia, as part of a clinical trial (29) • No definitive evidence from randomized controlled trials that it is effective (29)

(Continued)

TABLE 1 | Continued

Agent	Effect	Dosing	Stage	Mechanism	Commentary
Chloroquine	AV A-IN	• Stage I–II −500 mg bid for 5 days • Stage III—may consider extending treatment for up to 10 days	I–III	• A-IN: decrease secretion and/or receptor expression of cytokines such as TNF-a (39, 42) • Interfere with lysosomal activity and autophagy, similar to Hydroxychrloroquine (38)	• Has greater adverse event profile than Hydroxychloroquine and possibly less efficacy. Most common symptoms include abdominal cramps, nausea, anorexia. • Can increase QTc and result in hematologic effects (including hemolysis with G6PD deficiency). Can cause retinal toxicity and hypoglycemia. • As with Hydroxychloroquine, additional clinical trial data is necessary to determine efficacy and safety in COVID-19 patients (43). • No definitive evidence from randomized controlled trials that it is effective
Ivermectin	AV	• 45–64 kg: 9 mg orally single dose • 65–84 kg: 12 mg single dose • 85 kg or more: 0.15 mg/kg orally single dose	I–II	• Broad-spectrum antiviral activity *in vitro* through inhibition of nuclear import of host and RNA viral proteins (44)	• May consider prophylactic use of Ivermectin in patients on corticosteroids who have high risk of Strongyloides hyperinfection • Possible considerations as adjunct therapy or replacement when other agents contraindicated • Contraindicated in Pregnancy
Ribavirin	AV	• IV 500 mg each time, bid or tid, no more than 10 days	II–III	• Inhibits viral RNA dependent RNA polymerase	• Can cause birth defects or death in an unborn baby (45) • Hematologic toxicity is observed in dose-dependent fashion. • Caution when used with azathioprine or HIV/AIDS medicines • Ribavirin is likely not effective when used alone and must be used in combination with IFN-α or lopinavir/ritonavir
Convalescent plasma donor containing SARS-CoV-2–specific antibody (IgG)	AV A-IN	• 200–250 mL of ABO-compatible convalescent plasma × 2 (achieving 400 mL in total) on the same day it was obtained from the donor	I–III	• Neutralizing activity against SARS-CoV-2	• Allergic transfusion reactions • Likely most beneficial in early disease course (e.g., Stage I or Stage II), as its mechanism of action is to neutralize viral particles (46)
Azithromycin	A-IN	• 500 mg qd × 1, then 250 mg bid for 4 days	II–III	• Inhibits RNA-dependent protein synthesis • Multiple immunomodulatory effects (47)	• Previous studies have shown some efficacy against viruses such as Influenza, Ebola, RSV, and Rhinovirus (48) • May confer benefit when added to Hydroxychloroquine (36, 49) • Should be used if superimposed bacterial Pneumonia. • Does increase QT interval, especially when added to Hydroxychloroquine. An EKG is recommended prior to start (and EKG or telemetry monitoring while on Tx is recommended) (50)
Doxycycline and other Tetracyclines	A-IN	• 200 mg qd × 1, then 100 mg qd for 4 days. May consider extending treatment for up to 14 days	II–III	• Downregulation of NFkB pathway as well as TNFa, IL-1B and IL-6 • Possible inhibition of RNA replication (51)	• May confer benefit when added to Hydroxychloroquine. • Should be used if superimposed bacterial Pneumonia
Prednisone Methylprednisolone Dexamethasone Hydrocortisone	CS	• 40–60 mg prednisone PO or 30–60 mg methylprednisolone IV, or 5–10 mg dexamethasone IV qd for up to 7 days • 50 mg hydrocortisone IV q6H until improvement in shock	II–III	• Multiple immunomodulatory effects, including suppression of PMN migration and reversal of increased capillary permeability (52)	• Should not be used in Stage I (unless another indication) as it may increase viral load (53) • Indicated for asthma or COPD exacerbation or any shock with a history of chronic steroid use in excess of 10 mg prednisone daily. Also used for multipressor (>2 pressor) shock. • Use in patients with hypoxemia may confer a mortality benefit. If ARDS, higher doses may be required (54, 55)

(Continued)

TABLE 1 | Continued

Agent	Effect	Dosing	Stage	Mechanism	Commentary
Tocilizumab and other IL-6 inhibitors	A-IN	• 4–8 mg/kg IV (usually 400 mg) × 1 dose. If inadequate response, may repeat one time after 12 h (56)	IIb–III	• Inhibits inflammatory cytokine storm • Inhibits IL-6 and signal transduction of RNA viruses but not of DNA viruses (57)	• Side effects include upper respiratory tract infections, mild stomach cramps. • Black box warning for a risk of serious infections, including tuberculosis and other opportunistic infections. Patients treated with this medication should be tested for latent tuberculosis prior to discharge from the hospital • Caution in neutropenia or thrombocytopenia • May interact with cholesterol-lowering medications, seizure medications, heart rhythm medications • May be beneficial for use in Cytokine Activation Syndrome (58)
IFN-α and other Type 1 Interferons	AV A-IN	• 5 million U or equivalent dose each time, 2 times/day for Vapor inhalation	IIb–III	• Interfere with viral replication • Slowdown of cell metabolism and secretion of cytokines	• Inhalation pharmacodynamics and pharmacokinetics have never been assessed. • IV and SC modes of administration are well-described and proven safe in several clinical trials (under expert use), with similar pharmacodynamics and pharmacokinetics (59)
Prazosin and other alpha-1 adrenergic receptor (AR) antagonists	A-IN	• 1 mg bid or tid, titrating up as tolerated	I–III	• Reduces catecholamine and cytokine response through alpha-1 AR antagonism	• Contraindicated if hypotension. • No current evidence for starting the Rx if patient is not already on it. A recent retrospective review found that patients previously treated with alpha-1AR antagonists had improved end points (60)
Atorvastatin and other Statins	A-IN	• Atorvastatin 40 mg qhs	I–III	Pleiotropic effects, anti-inflammatory	• If there is an indication for a statin, the statin should be started or continued (15)
Baricitinib	A-IN AV?	• Eli Lilly and National Institute for Allergies and Infectious Diseases (NIAID) announced that the drug will begin its first large randomized trial in COVID-19 patients, in late April in the U.S., and additional sites in Asia/Europe (61)	IIb–III	• JAK1/JAK2 inhibitor • Theoretic (but proven) antiviral properties	• FDA approved for treatment of rheumatoid arthritis. • Side effects include upper respiratory tract infection and reactive of herpes simplex and herpes zoster. • Black box warning for serious infections including TB. Patients must be tested for TB prior to starting treatment. • Increase risk of malignancy (including Lymphoma), and thromboembolism (61)
Colchicine	A-IN	• 1.5 mg loading dose + 0.5 mg after 60 min, and then 0.5 mg bid for up to 3 weeks (62)	I–II	• Anti-inflammatory, through a variety of mechanisms including inhibition of neutrophil chemotaxis and IL-1 activation	• FDA approved for the treatment of gout and familial Mediterranean fever • Most common adverse effects are abdominal pain and diarrhea. • Adequate cardiovascular safety profile
Heparin, Enoxaparin, and other Anticoagulants	AC	• DVT Prophylaxis Dosing (e.g., Enoxaparin 40 mg SC qd, or Heparin 5000 SC tid) • Full anticoagulation—individualize to patient	II–III	Tissue factor pathway inhibition (54)	• Indicated as DVT prophylaxis for all hospitalized patients (Stage II) without contraindication for anticoagulation. • Full anticoagulation may be beneficial in Stage III, as it has shown benefit for those suffering from sepsis associated coagulopathy, ARDS, or D-Dimer levels >6-fold the upper limit of normal (54)

AV, Antiviral; A-IN, Anti-inflammatory; CS, Corticosteroid; AC, Anti-coagulant.

**None of these Rx are considered standard of care for treatment of COVID-19, and ideally should be used as part of a clinical trial. Moreover, this table is not meant to be a comprehensive review of adverse effects and drug-drug interactions. Treatment must be individualized to the patient, considering the patient's age, comorbidities, clinical course, drug interactions, and hypersensitivities. Lastly, this table is meant to be updated as new evidence (and perhaps new agents or classes of agents) is presented.*

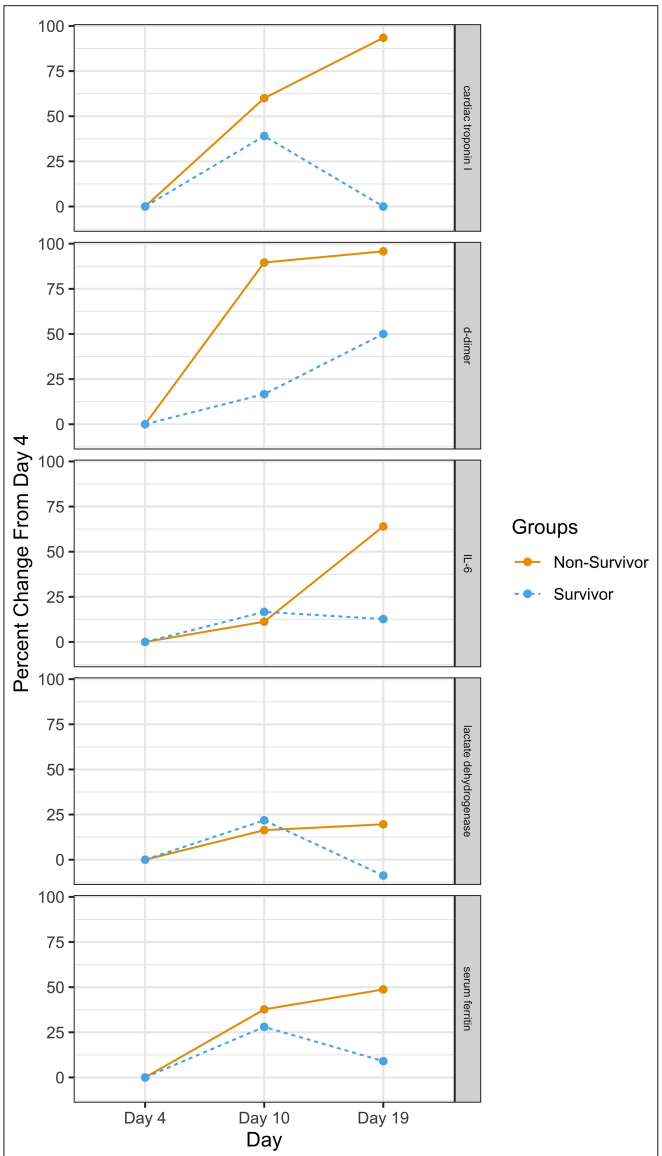

FIGURE 4 | Percent change in clinical measures between survivors and non-survivors. Source: (21).

Disease Progression

The most common presenting symptoms are fever and cough, followed by myalgia and fatigue. Less commonly, patients may present with sputum production, headache, or abdominal symptoms like diarrhea (21). In terms of disease progression, a case study of the first five patients diagnosed with COVID-19 in Europe points the way to two different clinical evolutions of the disease: 1. Presenting few symptoms, but showing high viral load from the respiratory tract; 2. A two-step disease process, with worsening of symptoms around 10 days of symptom onset despite decreased viral load in respiratory samples. In our model, we plot the disease progression as a function of infection, survivability, and inflammation (**Figure 2**).

We identify the inflection point where survival decreases as inflammation increases—approximately day 10 from symptom onset. Support for this is found in research by Chen et al. published in *The Journal of Infection* (37). Their research found that sepsis and ARDS in hospitalized patients start at around days 10 and 11, respectively. They also found temporal changes in inflammatory laboratory markers beginning at day 4 of illness onset. These included temporal changes in D-dimer, IL-6, serum ferritin, high-sensitivity cardiac troponin I, and lactate dehydrogenase. The differences were statistically significant between survivors and non-survivors for all time points. **Figure 4** provides the percent change between survivors and non-survivors from day 4. In addition, Yang et al. found that the patients admitted to the ICU with severe hypoxemia had a 50% probability of survival at day 7 of ICU admission (corresponding to Day ∼17 in **Figure 2**) (16).

Stage I

The incubation period is on average 5 days. In most patients, initial presenting symptoms are mild (though a small number of patients can be asymptomatic throughout the disease). Stage I symptoms include fever, cough, fatigue, and body aches. In a minority of cases, symptoms may also include headache, abdominal symptoms, anosmia, as well as others. The duration of initial symptoms is 5–7 days, correlating with a peak in viral load (21). During this time, the appropriate diagnostic test is a nasopharyngeal PCR. Laboratory studies may include an elevated D-Dimer and prothrombin time, as well as lymphopenia (see **Figure 2**). Given that symptoms in this stage are mild, and correlated with viremia, the appropriate treatment modality is supportive care or antiviral medication. Nevertheless, treatment must be individualized, based on a patient's age, comorbidities, presenting symptoms, and drug interactions (see **Figure 3** and **Table 1**).

Stage II

Some patients progress into Stage II, which is characterized by a decrease in viral levels and an increase in inflammation that initially localizes to the lungs. Infiltrates are typically seen on chest x-ray (CXR) or computed tomography (CT). Similar to symptom duration in Stage I, the typical symptom course in Stage II is also 5–7 days. Treatment with antivirals is still indicated, but given an average decrease in viral levels during this stage, that treatment is theoretically less effective than in Stage I. Moreover, Stage II is divided into two sub-stages (IIA and IIB), depending on whether a patient is hypoxemic or not. This distinction is important for management (see **Figure 2**). In Stage IIB, the patient is significantly dyspneic and may benefit, depending on age and comorbidities, from the use of corticosteroids or other anti-inflammatory treatments (see **Figure 3**).

Stage III

Although only a minority of patients (estimated at 10–15%) progress to Stage III, mortality within this stage is considerable (estimated at 20–30%). The morbidity and mortality are generally

due to uncontrolled inflammation, which at this point is systemic. The most important symptom is respiratory distress (correlating in a typical patient to a Pulse Ox $\leq$ 92%). Laboratory markers include significantly increased CRP and IL-6 levels (16, 67). As in Stage II, treatment may include antivirals (if the patient is still viremic), but agents to counteract inflammation and its effects (such as microthrombi) must be considered (see **Figure 2**). A summary of investigational therapies can be found in **Table 1**. It should be noted that many ongoing clinical trials will more clearly define COVID-19 specific treatment risks and benefits.

Pre-exposure and Post-exposure Prophylaxis

A number of clinical trials are exploring pre-exposure and post-exposure prophylaxis. There is no definitive evidence that any particular treatment modality is effective but antivirals, anti-parasitics, and convalescent plasma have been proposed. Antivirals, like Remdesivir, may prove beneficial at any stage of disease (26, 27). Convalescent plasma provides the antibody support needed to envelop and destroy the virus while preventing the exuberant immune response or cytokine release that leads to significant pathology, particularly in Stages IIb and III (46).

LIMITATIONS

This review has several limitations. First, the incredible volume and speed at which data is published about the treatment of COVID-19 indicates that research findings and recommendations may change. Second, the research used to create this review came from small studies, often-times with very few controls. Third, the articles were limited to English-language publications or translations, so relevant international data could be lacking.

CONCLUSION

This paper presents the first evidence-based recommendations for individualized treatment for COVID-19. Based upon the observed transmission and mortality rates, health professionals urgently need to align patient baseline risk to disease stage and investigational treatment options. The COVID-19 pandemic represents the greatest public health crisis in three generations: the need for comprehensive management cannot be overstated.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MH and RA revised the project, the main conceptual ideas and proof outline. BM, DS, JP, JPM, JD, YS, AC, and LA worked out almost all of the technical details with MH and RA assistance. PH, KH performed the numerical calculations and verified the numerical results. All authors contributed to writing and editing the manuscript.

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Positive Selection of ORF1ab, ORF3a, and ORF8 Genes Drives the Early Evolutionary Trends of SARS-CoV-2 During the 2020 COVID-19 Pandemic

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In this study, we analyzed full-length SARS-CoV-2 genomes from multiple countries to determine early trends in the evolutionary dynamics of the novel COVID-19 pandemic. Results indicated SARS-CoV-2 evolved early into at least three phylogenetic groups, characterized by positive selection at specific residues of the accessory proteins ORF3a and ORF8. Also, we are reporting potential relevant sites under positive selection at specific sites of non-structural proteins nsp6 and helicase. Our analysis of co-evolution showed evidence of epistatic interactions among sites in the genome that may be important in the generation of variants adapted to humans. These observations might impact not only public health but also suggest that more studies are needed to understand the genetic mechanisms that may affect the development of therapeutic and preventive tools, like antivirals and vaccines. Collectively, our results highlight the identification of ongoing selection even in a scenario of conserved sequences collected over the first 3 months of this pandemic.

Keywords: evolution, epistasis, positive selection, COVID-19, SARS-CoV2

INTRODUCTION

The first case of pneumonia confirmed to be caused by the novel virus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was a patient admitted in a hospital of Wuhan, Hubei province, China on December 12, 2019 (Wu et al., 2020). As of April 9, 2020, the World Health Organization (WHO) has confirmed 1,439,516 cases, 85,711 deaths, and the presence of COVID-19 in 209 countries, areas, or territories. Of the confirmed cases, 71% are from seven countries: United States of America (395,030), Spain (146, 690), Italy (139, 422), Germany (108,202), China (83,249), France (81,095), and Iran (66,220). As of the writing of this report, the number of COVID-19 cases continue to increase worldwide, with multiple epicenters. Remarkably, by the time of the revision of this manuscript (September 21, 2020), the number of

both confirmed cases and deaths has dramatically increased to 30,905,162 and 958,703, respectively, becoming the Americas the center of the pandemic with 15,580,622, and 530,373 confirmed cases and deaths, respectively, and corroborating the huge impact of this pandemic for the public health around the world.[1]

The International Committee on Taxonomy of Viruses (ICTV) initially named this pathogen 2019-nCoV (also referred to as COVID-19 by WHO) and included it within the *Coronaviridae* viral family (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020). Later, based on the close phylogenetic relationship of COVID-19 with other human and bat SARS-CoVs, ICTV renamed the virus as SARS-CoV-2 (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020).

The *Coronaviridae* family encompasses a group of single-stranded, positive-sense RNA viruses with a genome length varying between 27 and 32 kb. These are zoonotic viruses with the potential to infect humans and animals. Coronaviruses may cause acute and chronic respiratory, enteric, and central nervous system infections (Weiss and Navas-Martin, 2005; Phan et al., 2018). In the case of SARS-CoV-2, a meta-analysis of 50,466 patients indicates that fever and cough are the most common symptoms (95% CI: 81.8–94.5% and 65.7–78.2%, respectively; Sun et al., 2020). The disease may worsen, and the percentages of severe cases and fatality rate vary between 12.7 and 24.3% and 2.7 and 6.1% (95% CI), respectively (Sun et al., 2020). Interestingly, new clinical evidence obtained by the time of this revision shows the ability of SARS-CoV-2 to produce arrhythmia, septic shock, coagulation dysfunction, and multiple organ functional failure (Wang et al., 2020).

The genome organization of SARS-CoV-2 is similar to viruses from the genus *Betacoronavirus*, one of the four genera included in the *Coronaviridae* subfamily Orthocoronavirinae. The ~29,903 nucleotide (nt) genome is organized as follows, 5' to 3': replicase ORF1ab, S (encoding the structural spike glycoprotein), ORF3a (ORF3a protein), E (structural envelope protein), M (structural membrane glycoprotein), ORF6 (ORF6 protein), ORF7a (ORF7a protein), ORF7b (ORF7b protein), ORF8 (ORF8 protein), N (structural nucleocapsid phosphoprotein), and ORF10 (ORF10 protein). ORF1ab (~21,291 nt) encodes 16 non-structural proteins: leader protein, nsp2, nsp3, nsp4, 3C-like proteinase, nsp6, nsp7, nsp8, nsp9, nsp10, RNA-dependent RNA polymerase, helicase, 3' to 5' exonuclease, endoRNAse, 2'-O-ribose methyltransferase, and nsp11 (Wu et al., 2020).

Much speculation regarding the origin of SARS-CoV-2 emanates from unfounded theories, such as a man-made laboratory origin; however, a recent study supports the hypothesis that SARS-CoV-2 was the result of cross-species transmission followed by natural selection in the novel human host (Andersen et al., 2020). This hypothesis is strongly supported by studies examining amino acid differences between SARS-CoV-2 and some phylogenetically related betacoronaviruses (e.g., Bat-RatG13 isolate and the human SARS-CoV isolate Urbani) at the receptor-binding domain (RBD) of the spike protein, where such differences seem to increase the ability of SARS-CoV-2 to bind to the human receptor angiotensin-converting enzyme 2 (ACE2; Andersen et al., 2020). This increased affinity for binding ACE2 might help to explain the infectiousness of SARS-CoV-2 in human populations (Wan et al., 2020).

Considering the extraordinary plasticity shown by other human viral RNA pathogens, for example, HIV-1, Influenza viruses, SARS-CoV, and hepatitis C virus, to undergo adaptative changes to evade innate and adaptive immune responses, develop drug resistance, or establish an infection in a new host (Frost et al., 2018), multiple questions arise regarding the adaptative changes that SARS-CoV-2 has undergone during the pandemic. SARS-CoV-2 has spread throughout many countries resulting in the infection of people with diverse immunological backgrounds and demographics (age, sex, environmental conditions, etc.) that potentially impose significant selective pressures on SARS-CoV-2.

Here, we evaluate the phylogenetic and evolutionary dynamics of SARS-CoV-2 during the early phase of the COVID-19 pandemic. Using different analyses based on a codon-based phylogenetic framework, we identified critical sites in the genome undergoing positive selection, which might favor viral divergence and emergence of multiple viral variants. Our findings are discussed in terms of the potential effects that the early evolution of SARS-CoV-2 might have on the outcome of this pandemic.

MATERIALS AND METHODS

Data Collection

Eighty-six full-length SARS-CoV-2 genomes representing early viral isolates from patients living in diverse geographic regions were used for this study. Viral sequences available to be downloaded from the NCBI SARS-CoV-2 data hub as of March 10, 2020 represent the total number of full-length viral genomes at the time that the analysis was conducted (**Figure 1**).

Phylogenetic Analysis

A phylogenetic tree of SARS-CoV-2 was reconstructed using a Bayesian approach on the program MrBayes 3.2.7 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). For this propose, Hasegawa-Kishino-Yano 85 (HKY85) was used as nucleotide substitution model (Hasegawa et al., 1985). This model was chosen based on the Bayesian information criterion (BIC) score (84484.082), and in its availability on MrBayes. HKY85 represented the third best option among 24 substitution models (**Supplementary Figure 1**). This analysis was conducted on the software Mega 7 (Kumar et al., 2016)

Settings on MrBayes included: number of substitutions types (Nst) = 2 (allowing transitions and transversions have potentially different rates), rates = Gamma, and Markov Chain Monte Carlo (MCMC) = 100,000,000. The run was diagnosed using Tracer 1.7.1 (Rambaut et al., 2018) to ensure an ESS larger than 200. The tree was built using a 15% burnin proportion

[1]https://www.who.int/emergencies/diseases/novel-coronavirus-2019

Genbank accession number	Genome bases	isolation source	Host	Country	State/City/Province	Collection Date
LC528232.1	29902	throat swab	Homo sapiens	Japan	Kanagawa	10-Feb-20
LC528233.1	29902	throat swab	Homo sapiens	Japan	Kanagawa	10-Feb-20
LR757995.1	29872	N/A	Homo sapiens	China	Wuhan	26-Dec-19
LR757996.1	29868	N/A	Homo sapiens	China	Wuhan	1-Jan-20
LR757998.1	29866	N/A	Homo sapiens	China	Wuhan	26-Dec-19
MN908947.3	29903	N/A	Homo sapiens	China	Wuhan	Dec-19
MN938384.1	29838	nasopharyngeal swab	Homo sapiens	China	Shenzhen	10-Jan-20
MN975262.1	29891	sputum	Homo sapiens	China	Shenzhen	11-Jan-20
MN985325.1	29882	oropharyngeal swab	Homo sapiens	USA	Washington	19-Jan-20
MN988668.1	29881	bronchoalveolar lavage fluid	Homo sapiens	China	Wuhan	2-Jan-20
MN988669.1	29881	bronchoalveolar lavage fluid	Homo sapiens	China	Wuhan	2-Jan-20
MN988713.1	29882	sputum	Homo sapiens	USA	Illinois	21-Jan-20
MN994467.1	29882	nasopharyngeal swab	Homo sapiens	USA	California	23-Jan-20
MN994468.1	29883	nasopharyngeal swab	Homo sapiens	USA	California	22-Jan-20
MN996527.1	29825	bronchoalveolar lavage fluid	Homo sapiens	China	Wuhan	30-Dec-19
MN996528.1	29891	bronchoalveolar lavage fluid	Homo sapiens	China	Wuhan	30-Dec-19
MN996529.1	29852	bronchoalveolar lavage fluid	Homo sapiens	China	Wuhan	30-Dec-19
MN996530.1	29854	bronchoalveolar lavage fluid	Homo sapiens	China	Wuhan	30-Dec-19
MN996531.1	29857	bronchoalveolar lavage fluid	Homo sapiens	China	Wuhan	30-Dec-19
MN997409.1	29882	buccal swab	Homo sapiens	USA	Arizona	22-Jan-20
MT007544.1	29893	N/A	Homo sapiens	Australia	Victoria	25-Jan-20
MT012098.1	29854	throat swab	Homo sapiens	India	Kerala	27-Jan-20
MT019529.1	29899	bronchoalveolar lavage fluid	Homo sapiens; male; age 65	China	Wuhan	23-Dec-19
MT019530.1	29889	bronchoalveolar lavage fluid	Homo sapiens; female; age 49	China	Wuhan	30-Dec-19
MT019531.1	29899	bronchoalveolar lavage fluid	Homo sapiens; male; age 41	China	Wuhan	30-Dec-19
MT019532.1	29890	bronchoalveolar lavage fluid	Homo sapiens; female; age 52	China	Wuhan	30-Dec-19
MT019533.1	29883	bronchoalveolar lavage fluid	Homo sapiens; male; age 61	China	Wuhan	1-Jan-20
MT020781.2	29806	N/A	Homo sapiens	Finland	N/A	29-Jan-20
MT020880.1	29882	nasopharyngeal swab	Homo sapiens	USA	Washington	25-Jan-20
MT020881.1	29882	oropharyngeal swab	Homo sapiens	USA	Washington	25-Jan-20
MT027062.1	29882	nasopharyngeal swab	Homo sapiens	USA	California	29-Jan-20
MT027063.1	29882	oropharyngeal swab	Homo sapiens	USA	California	29-Jan-20
MT027064.1	29882	oropharyngeal swab	Homo sapiens	USA	California	29-Jan-20
MT039873.1	29833	sputum	Homo sapiens; male	China	Hangzhou	20-Jan-20
MT039887.1	29879	nasopharyngeal swab	Homo sapiens	USA	Wisconsin	31-Jan-20
MT039888.1	29882	oropharyngeal swab	Homo sapiens	USA	Massachusetts	29-Jan-20
MT039890.1	29903	N/A	Homo sapiens	South Korea	N/A	Jan-20
MT044257.1	29882	sputum	Homo sapiens	USA	Illinois	28-Jan-20
MT044258.1	29858	respiratory swab	Homo sapiens	USA	California	27-Jan-20
MT049951.1	29903	sputum	Homo sapiens	China	Yunnan	17-Jan-20
MT050493.1	29851	throat swab	Homo sapiens	India	Kerala	31-Jan-20
MT066156.1	29867	sputum	Homo sapiens	Italy	N/A	30-Jan-20
MT066175.1	29870	N/A	Homo sapiens	Taiwan	N/A	31-Jan-20
MT066176.1	29870	N/A	Homo sapiens	Taiwan	N/A	5-Feb-20
MT072688.1	29811	oropharyngeal swab	Homo sapiens	Nepal	N/A	13-Jan-20
MT093571.1	29886	N/A	Homo sapiens	Sweden	N/A	7-Feb-20
MT093631.2	29860	throat swab	Homo sapiens	China	Wuhan	8-Jan-20
MT106052.1	29882	nasopharyngeal swab	Homo sapiens	USA	California	6-Feb-20
MT106053.1	29882	nasopharyngeal swab	Homo sapiens	USA	California	10-Feb-20
MT106054.1	29882	sputum	Homo sapiens	USA	Texas	11-Feb-20
MT118835.1	29882	bronchoalveolar lavage	Homo sapiens	USA	California	23-Feb-20
MT121215.1	29945	throat swab	Homo sapiens	China	Shanghai	2-Feb-20
MT123290.1	29891	oropharyngeal swab	Homo sapiens	China	Guangzhou	5-Feb-20
MT123291.2	29982	bronchoalveolar lavage fluid	Homo sapiens	China	Guangzhou	29-Jan-20
MT123292.2	29923	sputum	Homo sapiens	China	Guangzhou	27-Jan-20
MT123293.2	29871	stool	Homo sapiens	China	Guangzhou	29-Jan-20
MT126808.1	29876	nasopharyngeal swab	Homo sapiens	Brazil	N/A	28-Feb-20
MT135041.1	29903	N/A	Homo sapiens	China	Beijing	26-Jan-20
MT135042.1	29903	N/A	Homo sapiens	China	Beijing	28-Jan-20
MT135043.1	29903	N/A	Homo sapiens	China	Beijing	28-Jan-20
MT135044.1	29903	N/A	Homo sapiens	China	Beijing	28-Jan-20
MT152824.1	29878	mid-nasal swab	Homo sapiens	USA	Washington	24-Feb-20
MT159705.1	29882	nasopharyngeal swab	Homo sapiens	USA	N/A	17-Feb-20
MT159706.1	29882	nasopharyngeal swab	Homo sapiens	USA	N/A	17-Feb-20
MT159707.1	29882	nasopharyngeal swab	Homo sapiens	USA	N/A	17-Feb-20
MT159708.1	29882	nasopharyngeal swab	Homo sapiens	USA	N/A	17-Feb-20
MT159709.1	29882	oropharyngeal swab	Homo sapiens	USA	N/A	20-Feb-20
MT159710.1	29882	nasopharyngeal swab	Homo sapiens	USA	N/A	17-Feb-20
MT159711.1	29882	nasopharyngeal swab	Homo sapiens	USA	N/A	20-Feb-20
MT159712.1	29882	oropharyngeal swab	Homo sapiens	USA	N/A	25-Feb-20
MT159713.1	29882	nasopharyngeal swab	Homo sapiens	USA	N/A	18-Feb-20
MT159714.1	29882	nasopharyngeal swab	Homo sapiens	USA	USA	18-Feb-20
MT159715.1	29882	nasopharyngeal swab	Homo sapiens	USA	N/A	24-Feb-20
MT159716.1	29687	nasopharyngeal swab	Homo sapiens	USA	N/A	24-Feb-20
MT159717.1	29882	nasopharyngeal swab	Homo sapiens	USA	N/A	17-Feb-20
MT159718.1	29882	nasopharyngeal swab	Homo sapiens	USA	N/A	18-Feb-20
MT159719.1	29882	nasopharyngeal swab	Homo sapiens	USA	N/A	18-Feb-20
MT159720.1	29882	nasopharyngeal swab	Homo sapiens	USA	N/A	21-Feb-20
MT159721.1	29882	oropharyngeal swab	Homo sapiens	USA	N/A	21-Feb-20
MT159722.1	29882	nasopharyngeal swab	Homo sapiens	USA	N/A	21-Feb-20
MT163716.1	29903	N/A	Homo sapiens	USA	Washington	27-Feb-20
MT163717.1	29897	N/A	Homo sapiens	USA	Washington	28-Feb-20
MT163718.1	29903	N/A	Homo sapiens	USA	Washington	29-Feb-20
MT163719.1	29903	N/A	Homo sapiens	USA	Washington	1-Mar-20
MT163720.1	29732	N/A	Homo sapiens	USA	Washington	1-Mar-20
NC_045512.2	29903	N/A	Homo sapiens	China	Wuhan	Dec-19

FIGURE 1 | Sample summary. Description of the 86 SARS-Cov-2 full-length genome sequences included in this study. All sequences were obtained form I from the NCBI severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2) data hub, accession number, genome length, isolate name, source, host, and country of origin are provided. N/A indicates information not available.

and the half compatible rule in order to collapse all the nodes with a posterior probability lower than 0.5. The final tree was visualized using Figtree 1.4.3.[2]

Rates of Evolutionary Change

Rates of evolutionary change of SARS-CoV-2, expressed as substitutions/site/year, were calculated using the programs BEAST2.4.3, BEAUti, and Tracerr, introducing the sampling date as a trait (Drummond et al., 2012). MCMC was run for 100 million generations, using the HKY85 substitution model and a gamma distribution with four categories as the site heterogeneity model. The resulting file was analyzed with Tracer 1.7 to check for convergence and to determine the evolutionary rate.

Population Structure Analysis

The extent of genetic differentiation (population structure) between different phylogenetic groups of SARS-CoV-2 was evaluated by the fixation index (F_{ST}; Hudson et al., 1992). This test was developed by Sewall Wright and determines the overall genetic divergence among subpopulations by evaluating the difference between mean pairwise intra-subpopulation diversity with mean pairwise inter-subpopulation diversity in order to establish population structure. F_{ST} values range between 0 and 1, reflecting undifferentiated to fully differentiated populations, respectively. Overall, a value <0.33 for viral populations suggests lack of genetic divergence between subpopulations (Wei et al., 2009; Zu et al., 2019). Analysis was conducted on the software HyPhy (Pond et al., 2005), and a randomization test with 1,000 replicas was carried out to determine statistical significance ($p < 0.001$).

Pairwise Distance Calculations

Nucleotide and amino acid pairwise distance calculations among SARS-CoV-2 sequences were conducted using the SSE 1.3 Sequence Distances program (Simmonds, 2012), as previously described for the genome characterization of hepatitis C virus genotype 7 (Salmona et al., 2016). However, based on the high level of identity found in the set of SARS-CoV-2 sequences evaluated in our research, we decided to use a sliding window of 50 nt, instead of 300 nt as reported by Salmona et al. (2016), with a shift of 25 nt. Additionally, *p*-distances in nucleotide and amino acid sequences between phylogenetic groups were calculated using MEGA 7 (Kumar et al., 2016).

Evolutionary Rate per Site Analysis

Mean (relative) evolutionary rates for each site in the alignment were estimated under the General Time Reversible model, including all three codon positions. These rates were scaled, considering the average evolutionary rate across all sites is 1. This means that sites showing a rate < 1 are evolving slower than average, and those with a rate > 1 are evolving faster than average. This analysis was conducted using MEGA 7 (Kumar et al., 2016).

Inference of Selective Pressures

Since natural selection can be manifested as different modes (diversifying, directional, or purifying), we used a combination of different evolutionary analyses to enhance the detection of relevant sites in the genome of SARS-CoV-2 experiencing diversifying (positive) and purifying (negative) selection: single likelihood ancestor counting (SLAC; Kosakovsky Pond and Frost, 2005), fixed effects likelihood (FEL; Kosakovsky Pond and Frost, 2005), mixed effects model of evolution (MEME; Murrell et al., 2012), and fast unbiased Bayesian approximation (FUBAR; Murrell et al., 2013). These methods use a maximum likelihood or Bayesian approach (FUBAR) to infer nonsynonymous (dN) and synonymous (dS) substitution rates on a per site basis for given coding alignment and corresponding phylogeny (Weaver et al., 2018). SLAC, FEL, and FUBAR were methods used to identify sites experiencing pervasive diversifying or purifying selection, while MEME was used to detect sites experiencing both pervasive and episodic diversifying selection (Spielman et al., 2019).

The presence of recombination in the sequence dataset potentially affecting the detection of positive selection was assessed using the algorithm genetic algorithm for recombination detection (GARD; Kosakovsky Pond et al., 2006). All methods were performed on the adaptive evolution server Datamonkey 2.0 (Weaver et al., 2018).

Evidence of directional selection was assessed on amino acid sequences using the directional evolution of protein sequences (DEPS) method, implemented on the Datamonkey webserver (classic; Delport et al., 2010). This method is a model-based phylogenetic maximum likelihood test that looks for evidence of preferential substitution toward a given residue at individual positions of a protein alignment (Kosakovsky Pond et al., 2008). DEPS has the ability to overcome diverse evolutionary scenarios that confound most existing evolutionary tests (Kosakovsky Pond et al., 2008). For additional details about the evolutionary methods used in this research, see **Supplementary Figure 2**.

Coevolution Analysis

Evidence of coevolution among different sites in the SARS-CoV-2 genome was evaluated using the method Bayesian Graphical Models for co-evolving sites (BGM; Poon et al., 2007). This method detects coevolutionary interactions between amino acids in a protein, where amino acid substitutions are mapped to branches in the phylogenetic tree.

Blosum 62 Substitution Matrix

Blosum 62 substitution matrix (BSM62) was used to infer the nature of amino acid replacements found during the evolutionary analysis of SARS-CoV-2, where positive values reflect that the substitution is most likely a product of random substitution, while negative values may be indicative of selection (Henikoff and Henikoff, 1992).

[2]http://tree.bio.ed.ac.uk/software/figtree

RESULTS

Phylogenetic Dynamics of SARS-CoV-2

To evaluate potential divergence events of SARS-CoV-2, indicating the rise of new variants early during the pandemic, we reconstructed the evolution of SARS-CoV-2 using full-length genome sequences of viruses collected between late December of 2019 and early March of 2020 from patients infected in different countries around the world. The results of the phylogenetic analysis demonstrate the rapid divergence of SARS-CoV-2 into three distinct phylogenetic groups (A, B, and C). The divergence of these groups was strongly supported by high values of posterior probability that range from 0.82 to 1 (**Figure 2A**). Interestingly, F_{ST} analysis also supported the early divergence of these three groups, showing statistical significant values ($p < 0.001$), and F_{ST} values >0.33 between all group comparisons (~0.51 s 0.4; **Figure 2B**).

Group A includes one of the first viral sequences generated during the outbreak in Wuhan, China, collected on December of 2019 (NC_045512.2), as well as multiple viral isolates from different Chinese provinces. The position of these sequences among multiple branches within the Group A cluster suggests the emergence of multiple viral variants in China, especially from Wuhan before the start of the global pandemic. Furthermore, the basal branch position of some of these variants indicates that they were the ancestors of viral isolates obtained from patients in the United States, Japan, Finland, Taiwan, Nepal, and India between January and February of 2020.

Similarly, in the Group B cluster, we found viral isolates from multiple Chinese provinces between December of 2019 and January of 2020. These isolates are likely ancestors of viral isolates recovered from patients in the United States, India, and Taiwan between January and March of 2020. Interestingly, one isolate from Wuhan (LR757995.1) is part of the Group B cluster, supporting the hypothesis that multiple viral variants emerged in China before the start of the pandemic.

The Group C cluster was the only cluster that did not contain sequences from China. This cluster includes viral isolates collected from the United States, Italy, Australia, Sweden, Brazil, and South Korea between January and February of 2020. The absence of viral isolates from China and the increased genetic distance from Group A suggests that the emergence of these variants might have come from a second wave of transmission outside of China after the start of the pandemic.

Importantly, by the time of the revision of this manuscript (September 20, 2020), different classifications have been published regarding the clade and lineage nomenclature of SARS-CoV-2. In this sense, the groups arbitrarily named and reported in our study as A, B, and C are now classified as follows: A as B, L, or 19A, B as A, S, 19B, and group C as B.2, V, or 19A (Alm et al., 2020).

Evolutionary Divergence in the Genome of SARS-CoV-2

Once we reconstructed the phylodynamic of SARS-CoV-2 isolates obtained early during the pandemic event, we attempted to determine which nucleotide positions in the SARS-CoV-2 genome were related to the early divergence of this virus. Overall, the evolutionary rate of SARS-CoV-2 is 1.15×10^{-3} substitutions/site/year (95% HPD 7.41×10^{-4}–1.57×10^{-3}), while pairwise analysis at nucleotide and amino acid levels revealed an average identity of 99.93–99.98% and 99.86–99.97%, respectively. Given the short divergence time, a high level of identity is to be expected; however, a few synonymous and non-synonymous substitutions were observed in the ORF1ab, S, OFR3a M, ORF8, N, and OFR10 genes (**Figures 3A,B**). When pairwise distances were calculated based on gene length, the highest levels of divergence were observed within genes ORF10 and ORF8 when considering synonymous and non-synonymous substitutions, respectively (**Figures 3C,D**).

Also, the estimated per site evolutionary rate in the coding regions revealed that 98.85% of the sites in the genome are evolving at expected rates of evolution, while 1.15% of the sites are evolving faster than expected (**Figure 3E**). In this context, and consistent with the length of the OFR1ab gene, most of these synonymous and non-synonymous substitutions (82 sites) were distributed among different protein-encoding segments of this gene; the segment encoding nsp3 had the highest number of polymorphic sites (**Figure 3E**).

Detection of Purifying and Diversifying Selection

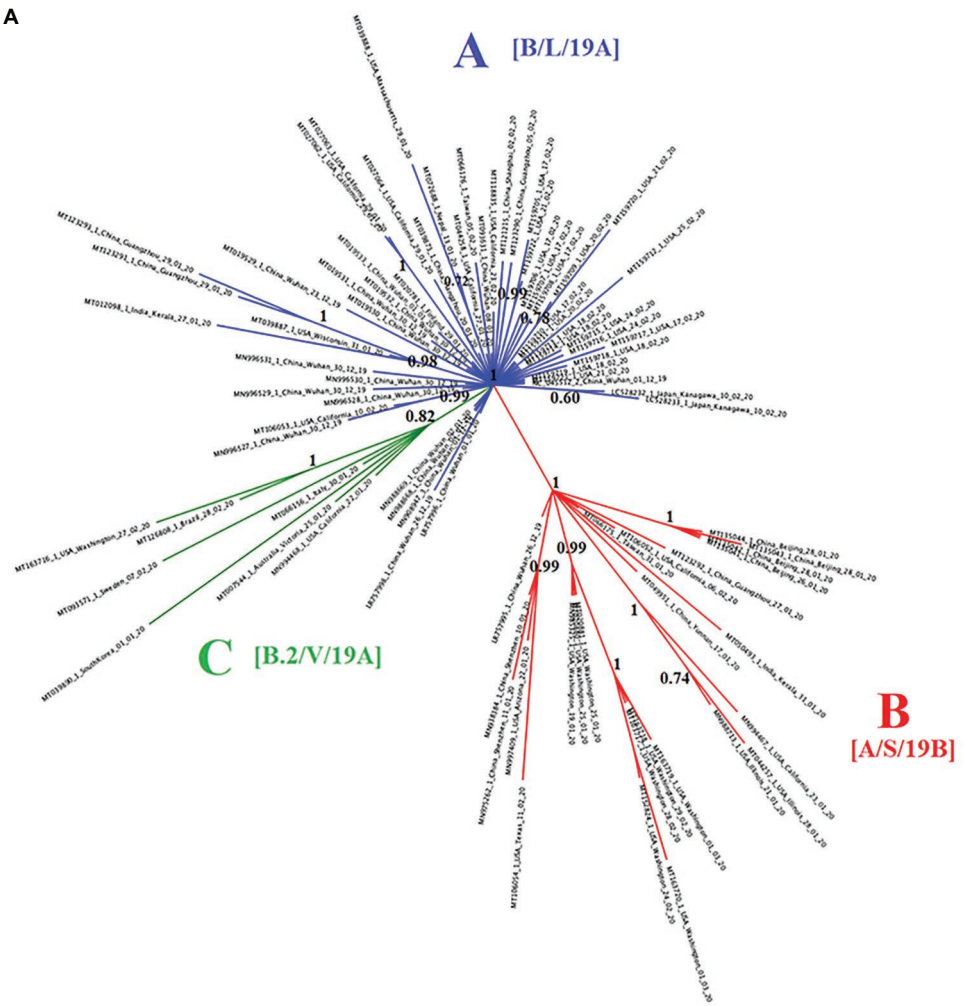
Once we identified fast-evolving positions within different genes of SARS-CoV-2, we used a combination of different algorithms centered on a codon-based phylogenetic framework to detect specific codons evolving under natural selection. Overall, no recombination events potentially affecting the results of these analyses were detected using the GARD algorithm.

Using SLAC, we obtained a broad picture of the extent of natural selection acting upon the SARS-CoV-2 genome. We found an overall dN/dS ratio of 0.937 along the genome. In particular, 75 codons located within five genes (ORF1ab > S > N > ORF8 > ORF3a) showed evidence of increased fixation of non-synonymous mutations (dN/dS >1). Conversely, a small number of codons (35 codons) located within five genes (ORF1ab > N > S > M = ORF10) were accumulating a higher number of synonymous mutations (dN/dS < 1). Interestingly, evaluation of dN/dS at the level of individual genes showed higher ratios for the ORF3a and ORF8 genes (**Figure 4A**).

Significant purifying (negative) selection was observed in 12 out of the 35 codons evolving at dN/dS < 1 using the FEL (12 sites), SLAC (1 site), and FUBAR (1 site) methods; the codons were located in the ORF1ab, S, and N genes (**Figure 4B**). At these codons, increased fixation of synonymous substitutions seems to be favoring the phenotypic preservation of SARS-CoV-2 at specific residues of the proteins encoded by these genes. Interestingly, negative selection of codon position 84 was the only codon supported by statistical significant values of all three methods, highlighting the relevance of this result.

Furthermore, by tracking these mutations within different isolates, we observed that these changes could explain the divergence of different viruses within different phylogenetic groups. In some

A



B

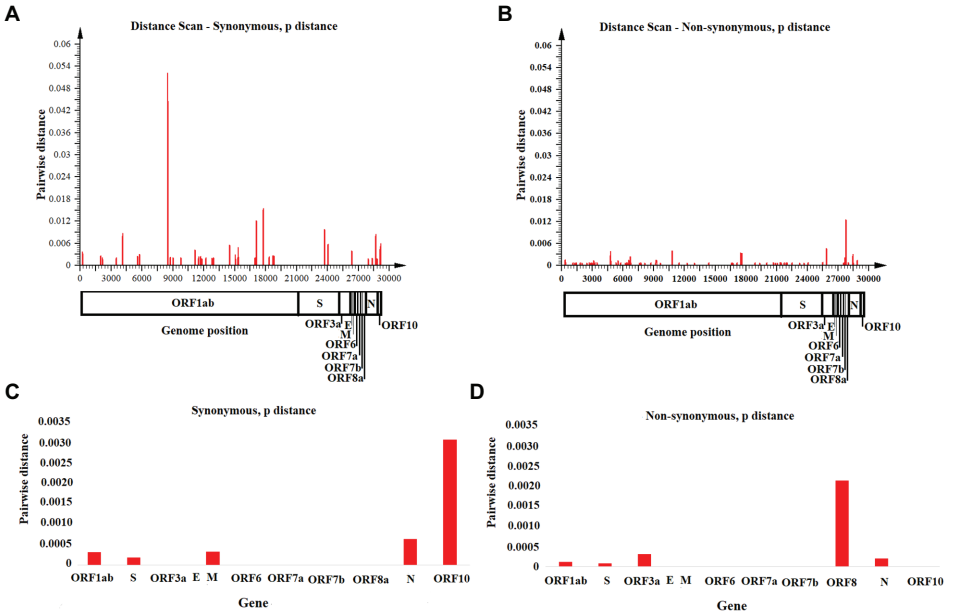
Comparison groups	Metapopulation diversity	Mean subpopulation diversity	Mean interpopulation diversity	F_{ST}	p
Group A vs Group B	0.000144	0.0001	0.000201	0.501	<0.001
Group A vs Group C	0.000112	0.000089	0.000199	0.553	<0.001
Group C vs Group B	0.000216	0.000166	0.000313	0.468	<0.001

FIGURE 2 | Phylogeny and population structure analysis of SARS-Cov-2. **(A)** Bayesian tree reconstructed using 86 SARS-Cov-2 full-length genomes collected from patients naturally infected at different countries, showing the existence of three phylogenetic groups: A (blue), B (red), and C (green). Numbers over the nodes represent their posterior probability. Information in the brackets corresponds with the current nomenclature proposed to describe different lineages reported in our study (https://www.gisaid.org/references/statements-clarifications/clade-and-lineage-nomenclature-aids-in-genomic-epidemiology-of-active-hcov-19-viruses/). **(B)** Intra- and inter-subpopulation diversity among phylogenetic groups was compared to determine the extent of population structure. F_{ST} values >0.33 ($p < 0.001$) were consider significant.

cases, mutations were associated with multiple isolates, supporting the relevance of these findings.

On the other hand, evidence of diversifying positive selection on non-synonymous sites was detected in just 4 of the 75 codons evolving at dN/dS > 1 in genes ORF1ab and ORF3a, with the FUBAR and FEL methods providing the highest power of detection (**Figure 4C**). Based on this analysis, these four sites appear to be evolving under pervasive diversifying selection.

Interestingly, all sites detected under positive selection were found in at least two isolates, and in case of codon 3606



E

Gene	Total of evolving sites >1	# of synonymous sites	Gene nucleotide position	# of non-synonymous sites	Gene nucleotide position
ORF1ab	82	28	(349)[1], (2004, 2181)[2], (3513, 4137, 5766)[3], (8517, 8769, 9009, 9771)[4], (11145)[5],(11691)[6], (11850,12208)[7],(12807, 12960)[8], (14541, 15060, 15333, 15343)[9], (16983, 17109, 17112)[10], (17796, 18339,18711,18801)[11]	54	(225,389)[1], (1120, 1283, 1426, 1826, 2012, 2452)[2], (2706,2834, 2912, 2994, 3141, 3146, 3253, 3473, 4797, 4819, 5307, 5519, 5580, 5761, 6236, 6371, 6430, 6554, 6703, 6731, 6751, 7601, 7736, 8123)[3], (8722, 9226, 9269, 9659)[4], (10818, 11485, 11499)[5], (12269)[7], (12961)[8], (14393)[9], (16613, 16736, 17159, 17483, 17594)[10], (18911, 19346)[11], (20017)[12], (20672, 20878, 21052)[13]
S	11	3	471, 2472, 2763	8	82, 145, 662, 741, 1223, 1963, 2390, 2789
ORF3a	2	0		2	383, 752
M	1	1	207	0	
ORF8	3	0		3	32, 184, 251
N	10	5	105, 519, 822, 957	5	136, 581, 605, 1028, 1030
ORF10	1	1	78	0	

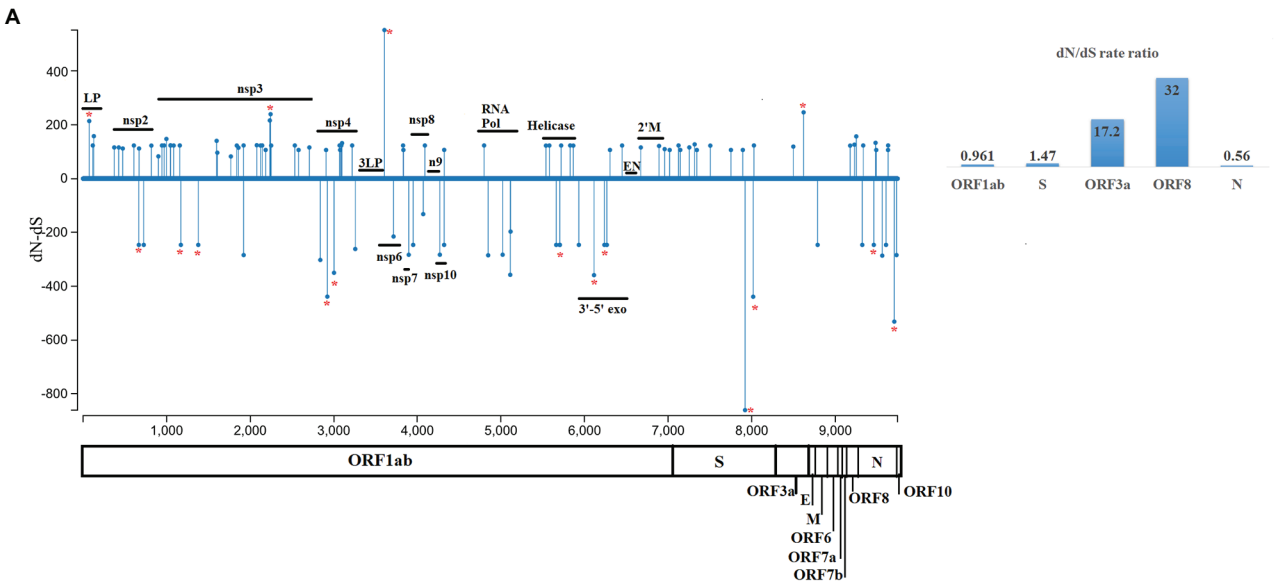
FIGURE 3 | Pairwise distance analysis. Pairwise distance analysis at **(A)** synonymous and **(B)** non-synonymous nucleotide sites was conducted using the program Sequence Distances (software SSE). Red bars represent pairwise distance comparisons using a sliding window of 50 nucleotides. Average nucleotide pairwise distance for different genes is shown at **(C)** synonymous and **(D)** non-synonymous sites. **(E)** Fast-evolving synonymous and non-synonymous sites at each coding region are shown. For these sites, evolutionary rates oscillated between 4.97 and 4.95. Red numbers represent nucleotides at: (1) leader protein, (2) nsp2, (3) nsp3, (4) nsp4, (5) nsp6, (6) nsp7, (7) nsp8, (8) nsp10, (9) RNA independent polymerase, (10) helicase, (11) 3' to 5' exonuclease, (12) endoRNAse, and (13) 2'-O-ribose methyltransferase.

(nsp6), positive selection was significantly supported by three different tests. Also, the selection of this site was observed in isolates from all three phylogenetic groups, thus supporting the reliability of these findings. As a way to assess the nature of different amino acid substitutions, we used the Blosum score. In two cases at ORF1ab codons 75 (D–E = 2) and 3,606 (L–F = 1) replacements were made between amino acids with similar biological characteristics. Conversely, at codons 251 of the ORF3a (G–V; BSM62 = −3) and ORF1ab codon 2,244 (I–T; BSM62 = −1) replacements were made between amino acids of different biological proprieties. Interestingly, change at codon position 251 is highly conserved within isolates of group C, suggesting that this change might have promoted the divergence of this group.

Detection of Directional Selection

To maximize the inference of potential sites experiencing positive selection, amino acid alignments of SARS-CoV-2 were analyzed using the DEPS algorithm. Overall, DEPS identified a total of four amino acid residues that are experiencing directional selection. Of these four residues, isoleucine (I) has the strongest bias, affecting 16 out of 19 sites evolving *via* directional selection (**Figure 5A**).

The majority of selected sites were located in nonstructural proteins (nsp) encoded by the ORF1ab gene, with nsp3 accounting for the highest proportion (**Figure 5B**). Overall, just a low proportion of the total number of predicted sites resulted in a conservative amino acid substitution (residues at positions 902, 1,769, 2,235, and 2,908). Remarkable, among those residues experiencing replacements between amino acids with different biological properties, residue 84 of protein ORF8 appeared to be synapomorphic in all Group B sequences. Also, similar to previous algorithms, DEPS identified positive selection of residue 251 of ORF3a, supporting the potential significance of this site in the early evolution of SARS-CoV-2.



B

Gene	Gene codon position	SLAC dN-dS	SLAC P [dN/dS < 1]	FEL dN/dS	FEL p-value	FUBAR dN-dS	FUBAR Prob[dS>dN]	Inferred substitution	Phylogenetic group
ORF1ab/ nsp2	668	-246.55	0.33	0	0.05	-18.84	0.88	$_{(V)}GCA_{85\ (V)}GCU_1$	Group C (MT066156.1)
ORF1ab/ nsp3	1171	-246.55	0.33	0	0.06	-18.81	0.88	$_{(T)}ACA_{85\ (T)}AGG_1$	Group A (MT019529.1)
ORF1ab/ nsp3	1379	-246.55	0.33	0	0.10	-18.15	0.88	$_{(L)}CUU_{82\ (L)}CUC_4$	Group B (MT135041.1,MT135042.1, MT13043.1, MT135044.1)
ORF1ab/nsp4	2923	-439.02	0.21	0	0.05	-19.03	0.88	$_{(K)}AAA_{85\ (K)}AAG_1$	Group A (MT066176.1)
ORF1ab/nsp4	3003	-350.33	0.26	0	0.05	-18.99	0.88	$_{(R)}AGA_{85\ (R)}AGG_1$	Group C (MT093571.1)
ORF1ab/helicase	5704	-246.55	0.33	0	0.06	-18.80	0.88	$_{(A)}GCC_{82\ (A)}GCU_4$	Group A (MT012098.1, MT039887.1, MT123291.1) Group B (MT123292.1)
ORF1ab/3'-to-5' exonuclease	6113	-359.07	0.23	0	0.09	-18.48	0.88	$_{(H)}CAU_{85\ (H)}CAC_1$	Group B(MT106054.1)
ORF1ab/3'-to-5' exonuclease	6267	-246.55	0.33	0	0.09	-18.18	0.88	$_{(P)}CCU_{85\ (P)}CCC_1$	Group C (MT007544.1)
S	824	-861.17	0.02	0	0.07	-39.51	0.98	$_{(N)}AAC_{81\ (N)}AAU_3$	Group A (MT039888.1, MT072688.1) Group B (MN988713.1, MN994467.1, MT044257.1)
S	921	-439.36	0.21	0	0.05	-19.03	0.88	$_{(K)}AAA_{84\ (K)}AAG_2$	Group A (MT106053.1, MN996527.1)
N	173	-246.55	0.33	0	0.05	-18.85	0.88	$_{(A)}GCA_{85\ (A)}GCU_1$	Group B (MN994467.1)
N	418	-531.95	0.22	0	0.10	-18.69	0.88	$_{(Q)}CAG_{84\ (Q)}CAA_2$	Group A (MT123291.1) Group B (MT123292.1)

C

Gene	Gene codon position	SLAC dN-dS	SLAC P [dN/dS < 1]	FEL dN/dS	FEL p-value	MEME dN/neutral evolution component	MEME p-value	FUBAR dN-dS	FUBAR Prob[dS<dN]	Inferred substitution	Phylogenetic group
ORF1ab/leader protein	75	214.12	0.60	Infinity	0.25	293.63	0.26	31.52	0.94	$_{(D)}GAU_{84\ (E)}GAA_2$	Group B (MN988713.1, MT04427.1)
ORF1ab/nsp3	2244	239.71	0.47	Infinity	0.10	480.91	0.12	31.52	0.95	$_{(I)}AUC_{84\ (T)}ACC_2$	Group A (MT019531.1, MT123293.1)
ORF1ab/nsp6	3606	552.28	0.14	Infinity	0.08	3129.69	0.10	41.36	0.97	$_{(L)}UUG_{80\ (F)}UUU_{5\ (F)}UUC_1$*	Group A (LC528232.1, LC5282233.1) Group B (MN997409 MT049951.1*) Group C (MT163716.1, MT126808)
ORF3a	251	246.55	0.44	Infinity	0.19	517.70	0.21	31.38	0.95	$_{(G)}GGU_{79\ (V)}GUU_7$	Group C (all 7 taxon)

FIGURE 4 | Diversifying and purifying selection on SARS-CoV-2. **(A)** General overview obtained by SLAC analysis, showing the evolutionary rate (dN-dS or dN/dS) along the genome and at individual genes of SARS-CoV-2. Statistically significant codons were inferred by multiple evolutionary tests used in this study. Red asterisks represent codons with significant evidence for selection. Codons evolving at **(B)** purifying (negative) or **(C)** diversifying (positive) selection are shown numbers in red represent evolutionary tests with significant values according to the analysis: SLAC, FEL, MEME (p = 0.1), and FUBAR (posterior probability = 0.9). The criteria for considering a site positively or negatively selected was based on their identification by at least one of the tests. The phylogenetic group column (assigned according with **Figure 2A**) shows also the isolates carrying the substitutions. LP, leader protein; 3LP, 3C-like proteinase; n9, nsp9; 3'-5' exo, 3' to 5' exonuclease; EN, endoRNAse; and 2'M, 2'-O-ribose methyltransferase.

Evidence of Coevolution Among Sites

Finally, we attempted to find coevolutionary correlations between different codons within the genome that result in the positive selection of sites. Analysis by BMG produced evidence of 14 coevolving codon pairs; these interactions took place mostly within codons located within the ORFB1ab gene (**Figure 6**). Although most of the interactions were detected between nonsynonymous codons, coevolution between codons 4,090–4,269 and 818–4,320 was detected by a synonymous substitution at one of the codons. Also, based on the nature of the amino acid replacement, just 6 of the 14 interactions resulted in replacements between amino acids with different biological properties. Interestingly, 8 of the 14 interactions appeared associated with sites evolving under some type of positive selection, suggesting that the selection of these sites might be the result of epistatic events.

A

Residue	p-value	Bias term	Proportion of affected sites	Directionally evolving sites
C	0.0028	10.89	50.00%	1
I	0	716.43	0.69%	16
S	0	47.28	1.71%	1
V	0	24.36	3.80%	1

B

Protein	Site	Composition in the alignment	Reconstructed most common ancestor at site	Inferred Substitutions	DEPS EBF	Blosum 62 Matrix Score	Phylogenetic group
ORF1ab/nsp2	609	$T_{85}I_1$	T	$I_1 \leftrightarrow_0 T$	I: 375.3	-1	Group A (MT027064.1)
ORF1ab/nsp3	902	$M_{85}I_1$	M	$I_1 \leftrightarrow_0 M$	I: 224.4	1	Group C (MT039890.1)
ORF1ab/nsp3	945	$T_{85}I_1$	T	$I_1 \leftrightarrow_0 T$	I: 374.2	-1	Group A (MT159717.1)
ORF1ab/nsp3	1769	$M_{85}I_1$	M	$I_1 \leftrightarrow_0 M$	I: 223.3	1	Group C (MT163716.1)
ORF1ab/nsp3	1840	$T_{84}I_2$	T	$I_2 \leftrightarrow_0 T$	I:>10^5	-1	Group B (MT152824.1, MT163720.1)
ORF1ab/nsp3	2124	$T_{85}I_1$	T	$I_1 \leftrightarrow_0 T$	I: 375.3	-1	Group A (MT159712.1)
ORF1ab/nsp3	2185	$S_{84}I_2$	S	$I_2 \leftrightarrow_0 S$	I:>10^5	-2	Group A (MT123291.1, MT123293.1)
ORF1ab/nsp3	2235	$L_{85}I_1$	L	$I_1 \leftrightarrow_0 L$	I: 367.5	2	Group A (MT019529.1)
ORF1ab/nsp4	2908	$F_{85}I_1$	F	$F_0 \leftrightarrow_1 I$	I: 558.4	1	Group A (MN996531.1)
ORF1ab/nsp4	3090	$T_{85}I_1$	T	$I_1 \leftrightarrow_0 T$	I: 375.3	-1	Group A (LR757998.1)
ORF1ab/nsp8	4090	$T_{85}I_1$	T	$I_1 \leftrightarrow_0 T$	I: 375.3	-1	Group A (MT123292.1)
ORF1ab/helicase	5538	$T_{85}I_1$	T	$I_1 \leftrightarrow_0 T$	I: 378.5	-1	Group B (MT050493.1)
ORF1ab/helicase	5579	$T_{85}I_1$	T	$I_1 \leftrightarrow_0 T$	I: 375.3	-1	Group C (MN994468.1)
ORF1ab/helicase	5865	$Y_{81}C_5$	Y	$C_5 \leftrightarrow_0 Y$	C: 695.1	-2	Group B (MT163717.1, MT163718.1, MT163719.1, MT163720.1, MT152824.1)
ORF1ab/ 3'-5' exonuclease	6449	$T_{85}I_1$	T	$I_1 \leftrightarrow_0 T$	I: 375.5	-1	Group A (MT123291.1)
S	408	$R_{85}I_1$	R	$I_1 \leftrightarrow_0 R$	I: 652.7	-3	Group A MT012098.1
ORF3a	251	$G_{79}V_7$	G	$G_0 \leftrightarrow_7 V$	V:>10^5	-3	Group C (all 7 taxon)
ORF8	11	$T_{85}I_1$	T	$I_1 \leftrightarrow_0 T$	I: 376.3	-1	Group B (MT106054.1)
ORF8	84	$L_{61}S_{25}$	L	$L_0 \leftrightarrow_{25} S$	S:>10^5	-2	Group B (all 25 taxon)

FIGURE 5 | Directional selection analysis on SARS-CoV-2. **(A)** An amino acid alignment was evaluated by DEPS and four different residues producing 19 directionally evolving sites in the proteome of SARS-CoV-2 are reported. Values of *p* show the statistical significance of each residue considering a model test of selection vs. not selection. Bias term: alignment-wide relative rate of substitution toward target residue. Proportion of affected sites: percentage of sites evolving under a directional model vs. a standard model with no directionality. Directionally evolving sites: number of sites that show evidence of directional selection for focal residue. **(B)** Description of 19 directionally evolving sites. Sites were detected by Empirical Bayesian Factor (EBF) considering a cut-off of 100 or more. Numbers in red represent replacements between amino acids with different properties. The phylogenetic group column (assigned according with **Figure 2A**) shows also the isolates carrying the substitutions.

Gene site 1	Codon position	Gene site 2	Codon position	Probability that sites 1 and 2 are not conditionally independent	Inferred substitution site 1	Blosum 62 matrix score	Inferred substitution site 2	Blosum 62 matrix score	Phylogenetic group
ORF1ab/leader protein	75 +	ORF8	62	0.5	$_{(D)}GAU_{84\,(E)}GAA_2$	2	$_{(V)}GUG_{83\,(L)}CUG_3$	1	Group B (MT044257.1, MN988713.1) MN994467.1*[1]
ORF1ab/leader protein	117	ORF1ab/nsp3	1607	0.76	$_{(A)}GCU_{84\,(T)}ACU_2$	0	$_{(I)}AUA_{84\,(V)}GUA_2$	3	Group A (MT027062.1, MT027063.1)
ORF1ab/leader protein	130	ORF1ab/nsp3	2244 +	0.56	$_{(G)}GGA_{85\,(E)}GAA_1$	-2	$_{(I)}AUC_{83\,(T)}ACC_2$	-1	Group A (MT123293.1) MT019353.1*[1]
ORF1ab/nsp2	609 +	S	49	0.74	$_{(T)}ACU_{85\,(I)}AUU_1$	-1	$_{(H)}CUA_{85\,(Y)}UAU_1$	2	Group A (MT027064.1)
ORF1ab/nsp2	818	ORF1ab/nsp10	4320	0.76	$_{(G)}GGU_{85\,(S)}AGU_1$	0	$_{(S)}UCC_{85\,(S)}UCG_1$	N/A	Group C (MT093571.1)
ORF1ab/nsp3	1047	S	655	0.73	$_{(E)}GAA_{85\,(D)}GAC_1$	2	$_{(H)}CAU_{85\,(Y)}UAU_1$	2	Group B (MT163720.1)
ORF1ab/nsp3	1049	ORF1ab/nsp3	1769 +	0.76	$_{(A)}GCU_{85\,(V)}GUU_1$	0	$_{(M)}AUG_{85\,(I)}AUU_1$	1	Group C (MT163716.1)
ORF1ab/nsp3	2124 +	ORF1ab/nsp6	3829	0.75	$_{(T)}ACU_{85\,(I)}AUU_1$	-1	$_{(L)}CUC_{85\,(I)}UUC_1$	2	Group A (MT159712.1)
ORF1ab/nsp3	2235 +	ORF1ab/nsp6	3833	0.74	$_{(L)}CUA_{85\,(I)}AUA_1$	2	$_{(N)}AAU_{85\,(K)}AAA_1$	0	Group B (LR757998.1)
ORF1ab/nsp3	2251	ORF1ab/2'-O-ribose methyltransferase	6958	0.74	$_{(G)}GGU_{85\,(S)}AGU_1$	0	$_{(K)}AAG_{85\,(R)}AGG_1$	2	Group A (MN99629.1)
ORF1ab/nsp3	2579	ORF1ab/nsp4	3090	0.72	$_{(D)}GAU_{85\,(A)}GCU_1$	-2	$_{(T)}ACU_{85\,(I)}AUU_1$	-1	Group A (MN996531.1)
ORF1ab/nsp3	2708	ORF1ab/nsp4	2908	0.73	$_{(N)}AAC_{85\,(S)}AGC_1$	1	$_{(F)}UUU_{85\,(I)}AUU_1$	0	Group A (MT019529.1)
ORF1ab/nsp8	4090 +	ORF1ab/nsp10	4269	0.73	$_{(T)}ACU_{85\,(I)}AUU_1$	-1	$_{(F)}UUC_{85\,(F)}UUU_1$	N/A	Group B (MT123292.1)
ORF1ab/3'-to-5' exonuclease	6304	ORF8	11 +	0.73	$_{(D)}GAU_{85\,(A)}GCU_1$	-2	$_{(T)}ACA_{85\,(I)}AUA_1$	-1	Group B (MT106054.1)

FIGURE 6 | Coevolution between codon pairs in the genome of SARS-CoV-2. BMG analysis was conducted to detect coevolving codon pairs. Evidence of 14 coevolving codon pairs was detected and the specific locations of those in the genome of SARS-CoV-2 are presented. Posterior probability of pair associations was supported by Markov Chain Monte Carlo Analysis at cut-off of 50 or more. Numbers in red represent replacements between amino acids with different properties. The phylogenetic group column (assigned according with **Figure 2A**) shows also the isolates carrying the substitutions. *[1]Represents viral isolated where the changes were not detected. Red + represents codons under positive selection, in which coevolution with other codon might represent and epistatic event.

DISCUSSION

Herein, we evaluated the phylogenetic and evolutionary dynamics of SARS-CoV-2 during the first month of the pandemic event in 2020. Our phylogenetic analysis revealed the complex dynamic of the spread of infection throughout the world, suggesting that multiple viral variants might have emerged in China before the start of the pandemic event. The evolutionary rate calculated for SARS-CoV-2 in this study was consistent with previous reports for SARS-CoV (Salemi et al., 2004; Zhao et al., 2004), explaining the high levels of identity at nucleotide and amino acid levels calculated for SARS-CoV-2 in our study. In this context, the high conservation observed in the genome of SARS-CoV-2 early during the

pandemic might also be attributed to the unique RNA correction machinery of coronaviruses (Ferron et al., 2018).

However, and despite the relative genome stability observed in SARS-CoV-2 at this stage of the pandemic, we were able to describe the existence of at least three phylogenetic groups. Interestingly, these findings are consistent with the results of a previous research published just 2 days before the submission of our manuscript (Forster et al., 2020). Nevertheless, both studies have significant methodological differences, which increases the reliability of the results obtained. First, different datasets were analyzed in both studies: sequences reported by Forster et al. (2020), were obtained from The Global Initiative on Sharing Avian Influenza and Coronavirus public-private partnership database (GISAID).[3] Second, although similar cut-offs were considered for the analysis of viral sequences (spanning for December 2019 to March 4 and 10, 2020 for Forster et al., 2020 and ours, respectively), our analysis was conducted with about half the number of sequences used by Forster et al. (2020). Since both studies revealed similar clustering patterns, it may indicate that the sequences sampled in the present study accurately represent the existent diversity. Lastly, the methodologies employed used to infer the early evolutionary events of SARS-CoV-2 were different. In the present study, a Bayesian phylogenetic analysis was carried out (Nascimento et al., 2017). Forster et al. (2020) utilized a phylogenetic network analysis (Bandelt et al., 1999), an alternative methodology used to visually represent the evolutionary relationships between different taxa, when the levels of data incongruence are large (Schliep et al., 2017). Although phylogenetic tree based methods appear as the most common analytical choice, the use of one of the two methodologies can be justified based on the evolutionary complexity of the data (Schliep et al., 2017). Also, the similarity between the our results and the ones obtained by Forster et al. (2020) can help to clarify some concerns regarding the usefulness of the phylogenetic networks to infer the evolution of SARS-CoV-2 (Sanchez-Pacheco et al., 2020).

In this context, we consider that the limited number of variable sites in the genome of SARS-CoV-2 at the early phase of the pandemic might represent a real challenge for different phylogenetic reconstruction methods. As a part of our research, multiple attempts to infer early evolutionary trends in the evolution of SARS-CoV-2 were conducted using neighbor joining and maximum likelihood approaches. Still, in both cases, these methods failed to give optimal clade resolution and significant statistical support to the evolutionary inferences (data not shown). Conversely, we found that Bayesian phylogenetic analysis offers a good alternative to reconstruct the evolutionary trends of SARS-CoV-2.

Furthermore, we supported the results of our phylogenetic analysis by conducted a F_{ST} analysis. This analysis has been applied to infer population structure in other RNA viruses like deformed wing virus, Israel acute paralysis virus (Cornman et al., 2013), black-streaked dwarf virus (Zu et al., 2019), and rice stripe virus (Wei et al., 2009). Since the interpretation of statistically significant F_{ST} values can vary among different species, being, for example, values between 0.05 and 0.2 considered significant for mammal populations (vonHoldt et al., 2016), we decided to use a conservative value for viral populations (>0.33; Wei et al., 2009; Zu et al., 2019). The average F_{ST} values of 0.51 depicted between the pair comparisons among all three different SARS-CoV-2 phylogenetic groups indicates that 50% of the genetic variation in the SARS-CoV-2 population analyzed in our study might be attributed to genetic differentiation rather than genetic flow.

Given the presumed origin of SARS-CoV-2 (Andersen et al., 2020), where the infectious cycle in nature of these viruses is mostly maintained between bats or rats and domestic or wild animals (Ye et al., 2020), it may be expected the existence of early events of divergence in SARS-CoV-2 as a result of adaptation to human populations. In this context, similar results were seen during the evolution of the epidemic of SARS-CoV, where phylogenetic analysis shows the existence of early evolutionary events during this epidemic is possible to see that strains originated form early and middle phases of the epidemic event showed higher diversity (appearing in distinct phylogenetic clusters) than strains originated late during the epidemic, thus supporting the hypothesis that multiple strains originated the epidemic event of SARS-CoV (Yip et al., 2009).

Interestingly, our evolutionary analysis supported the hypothesis regarding that early divergence events produced during the pandemic of SARS-CoV-2 might be associated with positive selection of specific sites at ORF3a and ORF8. By using a combination of different evolutionary algorithms, we attempted to maximize the detection of codon sites that may be promoting the divergence of SARS-CoV-2 by diversifying or directional selection. In this sense, two primary considerations must be addressed regarding the biological relevance of multiple sites detected in this study. First, a considerable number of polymorphisms were detected in just one viral isolate, which might be a consequence of the small number of viral isolates available at the time we started this study. Another possibility is that some of the polymorphisms might be due to sequencing errors. Second, it is important to consider that some of the codons detected as a positive selected sites might have been product of a false positive results, since all these algorithms are not exempt of this issue (Murrell et al., 2013).

In this context, MEME might be expected to be the most sensitive test since it can detect both pervasive and episodic selection (Spielman et al., 2019). However, in our analysis, we observed a superior performance of FUBAR over the other three codon-based tests. This fact is consistent with a previous research showing that FUBAR is expected to have a better performance over SLAC and FEL over most circumstances (Murrell et al., 2013), being also methodological differences between different algorithms another factor to explain the differences observed in our study (Spielman et al., 2019). On the other hand, despite of the discrepancies among different algorithms, in all cases, positive codons detected by FUBAR showed large dN-dS values with borderline p-values in the other tests. Hence, we may assume that these sites are likely to be under diversifying selection. Also, as previously reported (Kosakovsky Pond et al., 2008), we observed that the addition of

[3]https://www.gisaid.org/

DEPS as a part of our methodology increased the detection of potential important residues of SARS-CoV-2 by detecting sites evolving under directional selection, suggesting that the combination of both FUBAR and DEPS may be used to support future evolutionary analysis of SARS-CoV-2.

Additionally, we used the Blosum 62 matrix score to evaluate if physicochemical properties of different amino acid replacements were preserved or modified by the evolutionary process. Interestingly, we observed larger negative scores indicating changes in the physicochemical properties on selected residues, promoting the divergence among three different phylogenetic groups (ORF3a V251G, score 3, and ORF8 L84S, score 2), indicating that these changes may produce potential effects in the function of these proteins. Conversely, positive selection of residue 3,606 [ORF1ab was associated with a conservative replacement (L–F, score 1)]. However, and despite the nature of this replacement, a previous amino acid stability analysis in SARS-CoV-2 indicated that this change might confer lower stability to the nsp6 structure, thus potentially affecting viral autophagy (Benvenuto et al., 2020). In all cases, experimental evidence is needed to define the relevance of these findings in SARS-CoV-2.

Based on the results of different evolutionary algorithms, and supported by the number of sequences affected by these polymorphisms, we are reporting four potentially relevant residues that may be driving the early evolution of SARS-CoV-2 in human populations. Firstly, the positive selection of residue 3606 (nsp6) supported by three different tests, indicating that this residue is under strong pervasive diversifying selection, affecting isolates from three different phylogenetic groups. As explained above, this change might be relevant for the virulence of SARS-CoV-2 since the function of nsp6 in different coronavirus is implicated in limiting autophagosome expansion, potentially favoring viral infection by limiting the delivery of viral proteins for degradation (Cottam et al., 2014).

Secondly, residue 251 of the ORF3a protein appears to be positive selected by FUBAR and DEPS tests, suggesting, as seen in influenza virus, that diversifying and directional selection processes are not mutually exclusive (Kosakovsky Pond et al., 2008). The selection of this site in SARS-CoV-2 seems to be relevant since it might be related to the emergence of viruses in phylogenetic Group C. The early selection of this site might have a biological relevance since the ORF3a protein has been associated with virulence of human coronaviruses by controlling not only the expression of cytokines and chemokines but also inducing necrotic cell death (Shi et al., 2019). In fact, a recent publication comparing the ability of ORF3a proteins between SARS-CoV and SARS-CoV-2 to induce apoptosis indicates that ORF3a of SARS-CoV-2 decreases levels of apoptosis in infected cells, potentially allowing the virus to spread more widely during the infection (Ren et al., 2020).

Thirdly, a residue located at position 84 of the ORF8 protein was found to be evolving under directional selection and might be related to the emergence of Group B. Mutations at ORF8 might be highly relevant since this protein has been implicated in viral pathogenesis by regulating the initial innate response in SARS-CoV (McBride and Fielding, 2012; Shi et al., 2019). In this context, a potential mechanism in which ORF8 can regulate the virulence of SARS-CoV-2 might be associated with its ability to interact MCH-1 molecules to downregulate their surface expression in different cell types, disrupting antigen presentation and viral clearance by cytotoxic cells (Park, 2020; Zhang et al., 2020). Furthermore, in terms of evolution, and based on the notably increased dN-dS values, our results indicate that early during the pandemic, the ORF8 gene was under intense evolutionary pressure. These results are consistent with SARS-CoV evolutionary signatures, suggesting that ORF8 might facilitate cornaviruses-host shifts (Forni et al., 2017). Conversely, relaxed purifying selection rather than positive selection must be considered an alternative to explaining the high dN-dS values in ORF8 observed in our study. This evolutionary signature was already described in the evolution of ORF8 gene during the epidemic of SARS-CoV, suggesting that this gene might not have had an important adaptative role during this epidemic (Forni et al., 2017). Further studies are needed to confirm these findings in SARS-CoV-2.

Additionally, we found that residue 5,865 (ORF1ab/helicase) is evolving under directional selection and might be related to the divergence of five isolates from Washington, United States, forming a sub-cluster in Group B. The relevance of this residue in SARS-CoV2 helicase's ability to inhibit interferon production in infected cells (Yuen et al., 2020) remains to be established.

Finally, our analysis of coevolution revealed some potential epistatic interactions that might be driving the evolution of SARS-CoV-2. This mechanism has been proposed to explain the emergence of an Ebola virus variant in 2014 (Ibeh et al., 2016), and its relevance in the evolution of coronaviruses should be explored in future studies. Also, it is interesting to mention that most of the co-evolving sites were located in nsp3; given the role of this protein in the virulence of coronaviruses (Fehr et al., 2015), this observation may be key in understanding the evolution of SARS-CoV-2. Furthermore, since two of the interactions detected by BGM were associated with synonymous mutations, the relevance of this type of substitution to viral fitness should not be underestimated, since selection of synonymous substitutions has been reported in other RNA viruses like VSV (Novella et al., 2004; Velazquez-Salinas et al., 2018).

Collectively, our results describe the early evolutionary events of SARS-CoV-2 during the current pandemic and the findings may support the hypothesis that different variants of SARS-CoV-2 might be circulating in the world. However, in the absence of experimental work showing phenotypic differences among different isolates of SARS-CoV-2, we cannot rule out the alternative hypothesis claiming that early events of divergence of SARS-CoV-2 might have been the product of founder effects (Chookajorn, 2020; Mavian et al., 2020). In this context, the results reported in our research must be taken with caution.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found free available in the NCBI database (https://www.ncbi.nlm.

nih.gov/nucleotide/). For accession numbers see information in **Figure 1**.

AUTHOR CONTRIBUTIONS

LV-S and SZ conceived and designed the experiments. LV-S, SZ, and SE performed the experiments. LV-S, SZ, SE, DG, IN, and MB analyzed the data and wrote 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/fmicb.2020.550674/full#supplementary-material

Supplementary Figure 1 | Substitution model calculations for SARS-CoV-2.

Supplementary Figure 2 | Overview about the evolutionary selection algorithms used in this study.

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REVIEW
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A 21st Century Evil: Immunopathology and New Therapies of COVID-19

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Coronavirus Disease 2019 (COVID-19) has been classified as a global threat, affecting millions of people and killing thousands. It is caused by the SARS-CoV-2 virus, which emerged at the end of 2019 in Wuhan, China, quickly spreading worldwide. COVID-19 is a disease with symptoms that range from fever and breathing difficulty to acute respiratory distress and death, critically affecting older patients and people with previous comorbidities. SARS-CoV-2 uses the angiotensin-converting enzyme 2 (ACE2) receptor and mainly spreads through the respiratory tract, which it then uses to reach several organs. The immune system of infected patients has been demonstrated to suffer important alterations, such as lymphopenia, exhausted lymphocytes, excessive amounts of inflammatory monocytes and macrophages, especially in the lungs, and cytokine storms, which may contribute to its severity and difficulty of establishing an effective treatment. Even though no specific treatment is currently available, several studies have been investigating potential therapeutic strategies, including the use of previously approved drugs and immunotherapy. In this context, this review addresses the interaction between SARS-CoV-2 and the patient's host immune system during infection, in addition to discussing the main immunopathological mechanisms involved in the development of the disease and potential new therapeutic approaches.

Keywords: Severe Acute Respiratory Syndrome Coronavirus 2, Coronavirus Disease 2019, immunopathology, cytokine storm, cellular exhaustion, treatment, coronavirus

INTRODUCTION

The ongoing outbreak of Coronavirus Disease 2019 (COVID-19) has been classified as a threat of international concern and a public health emergency, having affected almost 30 million people and killed more than 900,000 around the world so far, according to the World Health Organization (WHO) (1). The etiologic agent of this pandemic is Severe Acute Respiratory Syndrome

Coronavirus 2 (SARS-CoV-2), the third coronavirus to have emerged as a public health issue and to cause an outbreak in the human population over the past two decades (2). This is the nomenclature referred to in this paper (3), which is derived from its similarity to the SARS-CoV virus that caused the outbreak in 2003, which is now known as "SARS-CoV-1".

Coronaviruses belong to a group of single-stranded RNA viruses and are regarded as one of the main types of viruses that affect the human respiratory system. SARS-CoV-2 is the seventh coronavirus known to have infected humans; SARS-CoV-1, MERS-CoV, and SARS-CoV-2 can cause serious illness, whereas HKU1, NL63, OC43, and 229E are associated with mild symptoms (4).

SARS-CoV-2 emerged at the end of 2019 in Wuhan, China, with reports of infection in humans and quickly spread around the world. The virus causes COVID-19, which consists of a spectrum of clinical syndromes, ranging from fever and breathing difficulty to acute respiratory distress and death, critically affecting older patients and people with comorbidities, including heart disease, diabetes, and other health conditions (5).

SARS-CoV-2 infection can be subdivided into the following three general phases: the spread of the virus in the body – known as viremia –; the acute phase with the appearance of clinical signs; and the stage of convalescence, which progresses either to recovery or death (6).

The pathological mechanism, so far unraveled, has proposed the role of the host's angiotensin-converting enzyme 2 (ACE2) and its affinity with viral receptors, especially the glycoprotein spike. The high affinity between these molecules facilitates viral dissemination in the body and allows the infectious condition to be established (7, 8). The immune systems of infected patients have demonstrated important changes, such as lower effector T cells, loss of the antiviral capacity of $CD8^+$ T lymphocytes and natural killer cells (NK), and the excessive release of inflammatory mediators, which may contribute to the disease severity and difficulty in establishing an effective treatment (9, 10).

This is a highly transmissible virus, whose contagion usually occurs through droplets released by infected individuals when they cough, sneeze, or talk, directly contaminating other people by reaching mucous membranes on the face or contaminating the environment, later acting as a transmission source. Until now, we have relied on quarantine, social isolation, and infection-control measures to prevent disease spread, as well as supportive care for infected individuals. A specific antiviral agent to treat the infected individuals and decrease viral transmission (11, 12) is yet to be found. Several research groups around the world have been working on possible therapeutic strategies against SARS-CoV-2 by applying commercially available drugs, hoping to accelerate the discovery of an effective treatment (13, 14).

Since many studies are made available online on a daily basis, both in journals and in preprint servers, for this review we used only studies already published and peer-reviewed in order to avoid biased information. In this scenario, understanding the virus dynamics and host response is essential to formulate strategies for antiviral treatment, vaccination, and epidemiological control of COVID-19. Thus, our goal is to review SARS-CoV-2's interaction with the patient's host immune system during infection and discuss the main immunopathological mechanisms involved in COVID-19, as well as potential new therapeutical approaches.

CORONAVIRUS: AN OVERVIEW

Coronaviruses (CoVs) consist of a group of enveloped, non-segmented, positive-sense single-stranded RNA viruses from the order Nidovirales, family Coronaviridae, and subfamily Orthocoronavirinae. Coronaviruses have the largest genome of all RNA viruses, encoding viral proteins involved in transcribing viral RNA, replication, structure, and accessory proteins (15). The virus has four main proteins – spike, envelope, membrane, and nucleocapsid (S, E, M, and N, respectively) – important for the virus to enter and replicate in the host cell (16), also representing the main molecules used for diagnosis, antiviral treatment, and potential vaccines.

According to antigenic and genetic criteria, CoVs are classified into three groups: α-CoVs, β-CoVs and γ-CoVs (17). Coronaviruses of human infection (hCoVs) are detected in both α-CoVs (hCoV-229E and NL63) and β-CoVs (MERS-CoV, hCoV-OC43, hCoV-HKU1, SARS-CoV-1, and SARS-CoV-2) (18). In addition to infecting humans, α-CoVs and β-CoVs can infect several species of mammals, including bats and pigs, while γ-CoVs infects birds, wild cats, pigs, and some species of marine mammals (19–22). CoVs have a high potential of jumping between species and their genome is characterized by high-frequency recombination and a high mutation rate (23).

hCoVs are responsible for the common cold and other respiratory pathologies with different degrees of severity, especially in babies, the elderly, and immunocompromised patients, characterized by human-to-human transmission (24). Coronavirus severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) (25–27) are caused by human β-CoVs and represent a serious illness with a case-fatality ratio of 9 - 10% and 35%, respectively (8, 28).

In contrast, according to data provided by the WHO, COVID-19 caused by SARS-CoV-2 shows an estimated lethality of ~5% of reported cases (data reported until July 2020) (29), reaching rates of up to 15% among elderly patients and patients with comorbidities. Despite the lower case-fatality rate, the high viral transmissibility of SARS-CoV-2 generates an overall number of cases that far outweighs SARS or MERS for spreading more easily among people (5, 28).

The first report of a COVID-19 case in Wuhan, China, occurred in December 2019, and in February the WHO declared the matter a public health emergency of international concern. Until now (September 2020), reports of COVID-19 account for almost 30 million cases and more than 900 thousand deaths in more than 220 countries, territories, or areas (1, 29). Imperial College, UK (30) proposed a mathematical model whose prospects indicate 7 billion infections and 40 million deaths in 2020 in the absence of mitigation measures.

Both SARS-CoV and MERS-CoV were initially believed to have resulted from a zoonotic spread from a bat population (31).

α-CoVs and β-CoVs are believed to have evolved over thousands of years, restricted to bats and intermediate mammalian hosts (civet cats for SARS-CoV-1 and dromedary camels for MERS-CoV), which probably contributed to the zoonotic transmission of the new coronavirus to humans (32).

Regarding SARS-CoV-2 transmission, several works have demonstrated that coronaviruses found in pangolins (*Manis javanica*) and SARS-CoV-2 share a genomic similarity of approximately 91%. The presence of the virus in samples of pulmonary fibrosis in pangolins found around the COVID-19 outbreak suggests that these animals were the hosts responsible for spreading the virus among humans (4, 21, 33). In contrast, some researchers claim that SARS-CoV-2 did not come directly from pangolins since, despite their similarity, the viruses found in these animals do not have the essential tools needed to infect human cells (4, 34). Thus, the possibility of other animals, such as ferrets and snakes, acting as intermediate hosts for SARS-CoV-2 and being responsible for zoonotic transmission is still under consideration (35).

Since SARS-CoV-2 genomes' information is still scarce and genomes of other coronaviruses closely related to this virus have limited availability (36), the evolutionary origin of SARS-CoV-2 is yet to be fully understood. So far, it is known that, compared with other β-CoVs, SARS-CoV-2 shows 50, 79, and 88 - 96% of genome similarity with MERS-CoV, SARS-CoV-1, and the bat SARS-like virus, respectively (37, 38).

The genomic changes of SARS-CoV-2 appear in both non-structural and structural proteins – notably in proteins S, M, and N – affecting viral multiplication, encapsulation, tropism, and transmission (39). Two important characteristics were described in the genome of SARS-CoV-2 that lead to alterations in the S protein: (i) receptor-binding domain (RBD), which is the most variable part of the viral genome, appears to be optimized for binding to the human ACE2 receptor, and (ii) presence of a polybasic (furin) cleavage site at the S1 and S2 boundary, *via* the insertion of twelve nucleotides, which allows effective cleavage by furin and other proteases and has a role in determining viral tropism, infectivity, and host range (4).

Such genomic changes also affected the recognition of these viruses by immune cells. Baruah and Bose (40) demonstrated that Sars-CoV-2 has specific regions for B cell and cytotoxic T cell glycoproteins recognition, which does not coincide with those found in bat-derived CoV, SARS-CoV-1, or MERS-CoV (**Figure 1**). Such distinguished interaction of cells and viruses can promote unusual immunomodulation or immune responses that contribute to the severity of the disease. All aspects of immunomodulation and immune evasion will be discussed in the subsequent topics.

PATHOGENESIS OF SARS-COV-2

Viral Entry and Replication

A virus starts its infection by binding viral particles to the host's surface cellular receptors. The recognition of cellular receptors is the first step towards viral entry into host cells, in addition to determining their tropism. The ability to engage receptors and

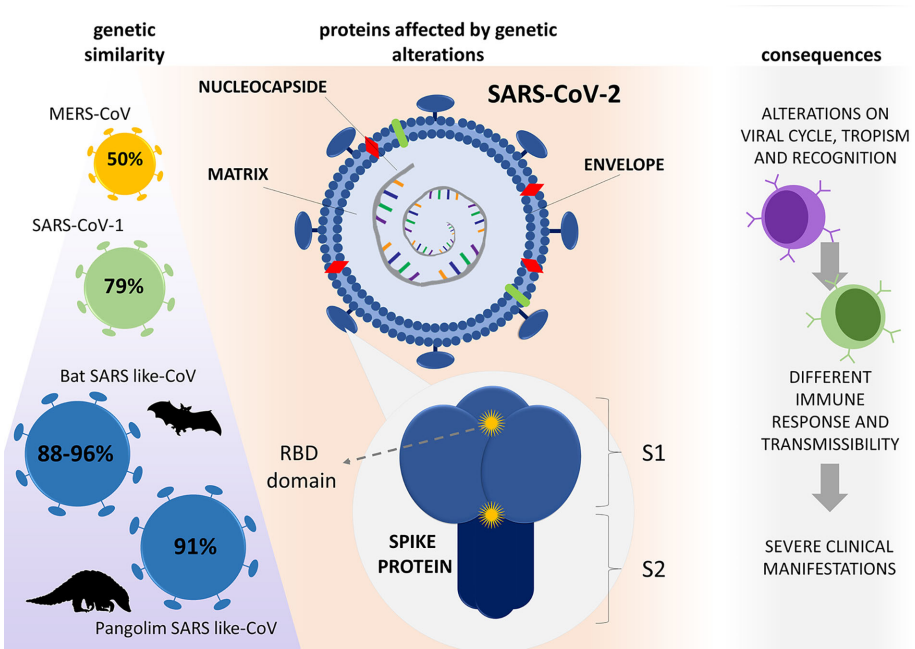

FIGURE 1 | Genetic evolution of SARS-CoV-2 and its consequences. Compared with other β-CoVs, SARS-CoV-2 has similarities of 50, 79, and 88 - 96% to MERS-CoV, SARS-CoV-1, and bat SARS-like-CoV genome, respectively, with 91% similarity with SARS-like CoV found in pangolins. The virus resulted from mutations that caused changes in important proteins for its virulence; notably, the spike, matrix, envelope, and nucleocapsid proteins caused alterations in host cell interactions, which culminated in a new aggressive disease (COVID-19). RBD (receptor binding domain), S1 (subunit 1) S2 (subunit 2).

the affinity of binding can define the efficiency of a virus when infecting an organism, while the amount of these receptors present in cells can indicate the intensity of infection. Viruses that have a high capacity to bind to more conserved receptors are more likely to migrate between different species, which may also reflect the susceptibility of hosts and increase viral pathogenicity (40, 41).

As well as the other β-CoVs, the SARS-CoV-2 genome has a long open reading frame (ORF) 1ab region, followed by regions that encode S, E, M, and N proteins (42). Homotrimers of S proteins are present on the viral surface and are responsible for attaching to host receptors (43). The E protein plays a role in the assembly and release of the virus, in addition to being involved in viral pathogenesis (44). The M protein has three transmembrane domains and shapes the virions, promotes membrane curvature, and binds to the nucleocapsid (45, 46). Lastly, the N protein contains two domains that can bind to the RNA virus and is also an antagonist of interferon (IFN) and a virally-encoded repressor of RNA interference, which appears to benefit viral replication (47, 48).

The S protein of SARS-CoV-2 plays an important role in determining tropism for being able to activate receptors in host cells and induce the invasion process. This protein is cleaved by proteases into the S1 and S2 subunits, which are responsible for receptor recognition and membrane fusion, respectively (39). Several articles have experimentally demonstrated that the RDB in the S protein, especially in the S1 region, binds to the peptidase domain (PD) of the ACE2 receptor, which is part of the renin-angiotensin-aldosterone system, an enzyme present in the plasma membrane mainly of pulmonary, endothelial, cardiac, renal, and intestinal cells (7, 22, 38, 49, 50). The S2 subunit is known to contain the fusion peptide, in which it is inserted into the host cell membrane to trigger the fusogenic reaction (7, 51, 52). The interaction of the S glycoprotein with the CD26 receptor and CD209L (39, 53, 54) is also suggested, however, its role remains unclear.

The binding of the virus to the ACE2 receptor causes stabilization of the RBD in the standing-up state and triggers conformational changes in the S complex, resulting in the release of the S1 subunit and activation of S2 fusogenic activity (55). The S2 subunit contains an N-terminal fusion peptide (FP), heptad repeat 1 (HR1), heptad repeat 2 (HR2), a transmembrane region (TM), and a cytoplasmic tail (CT). During the fusion process, the FP portion is exposed and inserts into the membrane of the target cell, leading to a modification in S2, then the HR1 and HR2 come together to form a six-helical bundle (6-HR) structure, which allows the fusion between the membranes (55–57).

Therefore, CoVs need to elicit exogenous proteases to perform modifications of their binding receptors necessary for the connection to occur. SARS-CoV-2 has its own furin-like proteases, which play a role in these changes, providing it with an evolutionary advantage in relation to other coronaviruses and improving the process of cell infection and viral dissemination. Concerning exogenous proteins, SARS-CoV-2 can also use host proteins to prepare its S glycoprotein for receptor binding (49). Hoffman et al. (7) demonstrated *in vitro* that strains of the virus isolated from COVID-19 patients can use both the host protease transmembrane serine protease 2 (TMPRSS2) and cathepsins B/L to prime the S protein.

The entry mechanism of CoVs in host cells depends on the strain and species considered, as well as tissue and cell-type specificities (receptor/protease availability and local microenvironment) (58). After binding to a target host cell *via* interactions with cellular receptors, viral entry of CoVs can occur in two manners: (i) the endosomal pathway and (ii) the non-endosomal pathway (59, 60). The endosomal pathway is facilitated by low pH and pH-dependent endosomal cysteine protease cathepsins, helping to overcome the energetically unfavorable membrane fusion reaction and facilitating endosomal cell entry of CoVs (61, 62). The non-endosomal pathway is dependent on TMPRSS2, which allows the activation of the S protein for viral entry (63).

Once the viral genome is inside the host cell cytoplasm, translation of viral RNA produces RNA-dependent RNA polymerase (RdRp), which uses viral RNA as a template to generate virus-specific mRNAs (subgenomic mRNAs) from subgenomic negative-strand intermediates (64–66). Translation of subgenomic mRNAs leads to the production of structural and nonstructural viral proteins. Thus, after their formation, structural proteins are inserted into the membrane of the endoplasmic reticulum or Golgi, and viral particles germinate into the endoplasmic reticulum-Golgi intermediate compartment. Finally, the vesicles containing the virus particles fuse with the plasma membrane to release the virus (65, 67, 68).

Another possible mechanism for CoV entry may occur through antibodies. During the binding of the virus-antibody complex, simultaneous binding of viral proteins to antigen-binding fragment (Fab) regions of immunoglobulin G (IgG) and of the fragment crystallizable (Fc) portion of the antibody to Fc gamma receptors (FcγRs) that are expressed by immune cells occurs, promoting viral entry without the use of the ACE2 receptor (69, 70). However, the presence of viral RNA in the endosomes signals *via* the Toll-like 3 (TLR3), TLR7, or TLR8 receptor, activating the host cell to release pro-inflammatory cytokines that lead to exacerbated tissue damage, a phenomenon called antibody-dependent enhancement (ADE) (71).

Such a mechanism for SARS-CoV-2 is not yet fully understood, but previous coronavirus infections or SARS-CoV-2 convalescent patients with different SARS-CoV-2 strains could promote ADE, as experimentally shown for antibodies against the MERS-CoV or SARS-CoV-1 spike S protein (72). Several studies have shown that sera administration induced increased SARS-CoV-1 viral entry into cells that express the Fc receptor, and serum-dependent SARS-CoV-1 entry does not pass through the endosome pathway (73, 74).

This mechanism was characterized by Yip et al. (75) and Wang et al. (76), who revealed that the anti-Spike protein antibodies were in fact responsible for the infection of immune cells, and the enhancement of the infection can be improved by increasing the dilutions of antibodies. In relation to MERS-CoV, a similar mechanism has been demonstrated, since neutralizing monoclonal antibodies (nAb) are able to bind to the spike-S

surface protein, allowing conformational changes and being subject to proteolytic activation. Meanwhile, nAb binds to the cell surface IgG Fc receptor, guiding viral entry through canonical pathways dependent on the viral receptor (77). Recent studies with COVID-19 patients reported that there was a strong IgG antibody response against the nucleocapsid protein and a delay in eliminating the virus, leading to an increase in the severity of the infection and contributing to the hypothesis of ADE of SARS-CoV-2 (78).

In view of this, the geographic discrepancy in pathogenesis can be explained, since individuals who have experienced previous exposure to coronaviruses are experiencing the effects of ADE due to the heterogeneity of the antigenic epitope (79). In addition, the potential of human antibodies for vaccination will depend on whether antibodies play a role in disease progression or in protecting against viral infection (70).

As an evasion mechanism, CoVs use a glycan conformational shield to prevent the recognition of the virus by the immune system, and, for this reason, S glycoproteins are found in trimers form and require structural alterations to engage with cellular receptors. In most of the hCoVs described, these S trimers are found in a naturally closed conformation, however, this mechanism also causes a delay in the process of cell infection due to the need for major changes in the glycoprotein conformation. It was described that, in SARS-CoV-2, the S trimers seem to exist in a partially open state, which prevents recognition by the immune system, but accelerates the initiation of conformational changes in the receptor and the processes of binding and fusion (49).

Pathogenic Mechanisms

Considering the similarity between SARS-CoV-1 and SARS-CoV-2, it is likely that their biochemical interactions and pathogenesis are also similar (80, 81). Once SARS-CoV-2 was reported to use ACE2 to enter host cells, it is suggested that the virus may target a cell spectrum similar to SARS-CoV-1 (38, 82, 83). SARS-CoV-1 is known to mainly infect macrophages and pneumocytes in the lungs, as well as other extrapulmonary tissues that express ACE2, which can also be expected for SARS-CoV-2 (82–84). However, the affinity of SARS-CoV-2 to ACE2 is 10–20-fold higher than that of SARS-CoV-1, which could explain its higher transmissibility and demonstrate that it can bind more efficiently to host cells, having a robust infection in $ACE2^{+}$ cells in the upper respiratory tract (7).

ACE2 is an enzyme belonging to the renin-angiotensin system, located on the cell surface of type II alveolar epithelial cells in the lungs and cells of other tissues, and plays a crucial role in controlling vasoactive effects in the body. Despite their similarities, ACE and ACE2 have different substrate specificities with distinct functionalities that perform opposite actions in the body. In brief, ACE cleaves angiotensin I to generate angiotensin II, the peptide that binds and activates angiotensin type 1 receptor (AT1R) to constrict blood vessels, thereby raising blood pressure. In contrast, ACE2 inactivates angiotensin II (Ang-II) while generating angiotensin 1-7 (Ang-1-7), a potent heptapeptide that acts in vasodilation and attenuation of inflammation (85).

Therefore, considering that SARS-CoV-2 uses ACE2 to enter cells, the main hypothesis of pulmonary pathology is that the increased activity of ACE (Ang-II) over ACE2 (Ang-1-7) may cause acute lung injury since the binding of the S protein to ACE2 leads to its blockage. Thus, the suppression of ACE2 occurs due to the increased internalization and release of ACE2 from the cell surface, which leads to a decrease in tissue ACE2 and the generation of Ang-1-7, and consequently higher Ang-II levels. Because of this, as shown in an experimental SARS-CoV-1 model, this process can drive an Ang II-AT1R-mediated inflammatory response in the lungs and potentially induce direct parenchymal injury (67, 80, 86, 87).

Another hypothesis states that SARS-CoV-2 infection blocks ACE2 function when binding to host cells, inhibiting its role of cleaving bradykinin and, as a consequence, bradykinin accumulates in the lung, promoting pulmonary edemas due to vasodilator activity and consequent respiratory failure. The increased bradykinin activation in the pulmonary endothelium can also induce neutrophil migration, enhancing tissue damage caused by the respiratory burst of these cells (88).

ACE2 is also highly expressed and co-expressed with TMPRSS2 in nasal epithelial cells, chalices, and hair cells (89). This finding is in accordance with the high detection of viral RNA in the upper airways present in nasal swabs and throats of both symptomatic and asymptomatic patients, demonstrating that the nasal epithelium is an important site for the infection to initiate and can represent an essential reservoir for viral dissemination and transmission (38).

Although the virus mainly affects the lungs, there are reports that SARS-CoV-2 also has organotropism, accompanied by dysfunction, in multiple organs, including the kidneys, liver, heart, and brain, which can influence the course of the disease and possibly worsen pre-existing conditions. It has been reported that ACE2, TMPRSS2, and cathepsin L can be expressed on glial cells and neurons, cardiomyocytes, liver cells, bile duct cells, and renal tubular cells (90, 91).

Evidence indicates that SARS-CoV-2 "neuroinvasion" can establish a direct entry along the olfactory nerve, mainly through the nasal olfactory epithelium, which expresses ACE2 and TMPRSS2, allowing access to the central nervous system (CNS). The spread of the virus through the hematogenous or transsynaptic pathway has also been widely discussed, however, it is known that the different levels of neurotropism and neurovirulence in patients with COVID-19 can be explained by a combination of viral factors and their interaction with the host (41, 92, 93).

Regarding the evolution of infected individuals, aging, comorbidities, and weakening of the immune system are factors that generally cause the infection to intensify at the acute phase, leading to the manifestation of more severe conditions (6). Thus, according to epidemiological studies, it is known that patients with chronic conditions, such as hypertension, diabetes, and chronic obstructive pulmonary disease (COPD), are more likely to develop a critical form of the disease (94–96).

The risk of applying medication commonly used in hypertension treatments to COVID-19 patients (97, 98) has

raised different hypotheses over the issue of invoking a higher expression of ACE2 (99–101). A systematic review assessing the clinical outcomes for SARS-CoV-2-infected individuals regarding treatment using angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin receptor blockers (ARBs) concluded that these types of drugs have no deleterious effects and should continue to be used in COVID-19 patients (102), reinforcing the recommendations of several medical societies, including the American Heart Association (103) and European Society of Cardiology (104).

Respiratory diseases, such as COPD and asthma, cause a reduced lung function and greater susceptibility to lung inflammation, and are expected to show a potentially critical course of COVID-19. COPD patients are already considered more susceptible to the development of pneumonia based on the clinical characteristics exhibited, such as lung structural damage, alterations in local/systemic inflammatory response, impaired host immunity, microbiome imbalance, persistent mucus production, and the presence of potentially pathogenic bacteria in the airways (105). Additionally, in the scenario of COVID-19, smokers and individuals with COPD have shown to have increased airway expressions of ACE-2 (106). It is still worth mentioning that patients who have this type of disorder often use corticosteroid immune-suppressing drugs, whose effect of reducing the immunity to respiratory infections may represent another contributing factor to a higher risk of infection (107).

Clinical and Radiological Changes

Most COVID-19 patients exhibit mild to moderate symptoms, but approximately 15% progress to critical pneumonia and 5% eventually develop acute respiratory distress syndrome (ARDS), septic shock, multiple organ failure, and death (26, 108). Once the infection is installed, the spectrum of clinical presentations has been reported to range from asymptomatic infection to critical respiratory failure.

According to the severity of symptoms, patients can be classified as mild, severe, and critical. In general, the most commonly reported symptoms are fever, cough, myalgia, fatigue, pneumonia, dyspnea, as well as the loss of smell and taste, whereas less common reported symptoms include headache, diarrhea, hemoptysis, and a runny nose (108, 109). Most critically ill patients present progressive respiratory failure due to alveolar damage caused by hyper inflammation, which can result in lethal pneumonia (26).

A retrospective study conducted by Liu et al. (110) demonstrated that older patients with SARS-CoV-2 showed higher pneumonia severity index scores and had a higher chance of multiple lobe involvement compared with young patients. Elderly adults are more susceptible to SARS-CoV-2 and have a high risk of morbidity and mortality (111). This can be explained by factors such as physiological changes and multiple age-related comorbidities, in addition to associated polymedication (112).

Regarding the potential involvement of COVID-19 in the CNS, studies have investigated the neurological changes developed throughout the course of the disease. Nonspecific symptoms (dizziness, headache, and seizure) and specific symptoms (loss of smell or taste and stroke) were described (91, 113–115). Epidemiological studies have reported that some patients infected with SARS-CoV-2 did report headaches (8%), nausea, or vomiting (1%). A more recent study investigating 214 COVID-19 patients found that about 88% of critically ill patients displayed neurologic manifestations, including acute cerebrovascular diseases and impaired consciousness (26, 116).

Among patients diagnosed with SARS-CoV-2, it has been reported that renal dysfunction is characterized by high levels of blood urea nitrogen, creatinine, uric acid, and D-dimer, associated with proteinuria and hematuria (90, 117–119). Recent studies have reported an incidence between 3-9% of acute kidney injury in COVID-19 patients, demonstrating renal abnormalities (94, 96, 111, 120). Cardiovascular complications are also associated with COVID-19 infection, including myocardial injury, myocarditis, acute myocardial infarction, heart failure, dysrhythmias, and venous thromboembolic events, being significant contributors to the mortality associated with this disease (121, 122).

Several studies found that CoVs can also affect other body regions, such as the gastrointestinal tract and ocular tissues (123, 124); some of them specifically investigated changes in the gastrointestinal tract and identified the presence of SARS-CoV-2 RNA in samples of anal/rectal swabs and feces of infected patients, establishing that the virus could be transmitted orally or fecally as well. Additionally, symptoms such as diarrhea, vomiting, and intestinal pain (125) have also been reported for SARS-CoV-2-positive patients, which can be associated with the expression of ACE2 in gastrointestinal epithelial cells, present especially in the small and large intestines, contributing to viral infection and replication in these cells (126).

Regarding ocular tissues, some studies have also identified the manifestation of conjunctivitis in patients with COVID-19 (<1%) (96), however, it is an underestimated number (127). Currently, it is still unclear how SARS-CoV-2 can cause conjunctivitis, but theories include: (i) conjunctiva can be a direct inoculation site for the virus, (ii) the virus can reach the upper respiratory tract through the nasolacrimal duct, or (iii) infection can occur *via* hematogenous through the lacrimal gland (123).

Histologically, biopsy samples of lungs reveal evident desquamation and hyaline membrane formation of pneumocytes, in addition to bilateral diffused alveolar damages along with cellular fibromyxoid exudate, indicating ARDS. In addition, the cytopathic effects found include multinucleated syncytial cells, increased atypical pneumocytes, and the presence of inflammatory infiltrates of mononuclear cells (26, 108).

More recently, reports on COVID-19 have included the occurrence of coagulation abnormalities in most critically ill patients (128–131). Tang et al. (132) reported the occurrence of disseminated intravascular coagulation in 71.4% of non-surviving COVID-19 patients and in only 0.6% of surviving patients, suggesting a high frequency in severe COVID-19 patients. Autopsies performed on patients with COVID-19 also demonstrated small fibrinous thrombi in pulmonary arterioles with endothelial tumefaction, the presence of megakaryocytes, and indications of coagulation cascade activation (133).

Although it is important to consider the direct procoagulant properties of SARS-CoV-2, the combination of immobility, systemic inflammation, platelet activation, endothelial dysfunction, and stasis of blood flow can lead to thrombotic complications that mimic systemic coagulopathies associated with severe infections, such as sepsis-induced coagulopathy (SIC), disseminated intravascular coagulation (DIC), and thrombotic microangiopathy (130). However, COVID-19 has some distinct features that may establish a new category of coagulopathy, denominated COVID-19 associated coagulopathy (CAC), whose main markers are higher D-dimer concentration and fibrinogen levels, a relatively lower platelet count, and longer prothrombin time (129). In COVID-19 patients, CAC has been associated with higher mortality (131).

Chest computed tomography (CT) in patients with COVID-19 has commonly demonstrated multifocal "*ground-glass*" opacity (GGO) in the lungs, which can occur concurrently with consolidation in posterior and peripheral areas, suggesting a pneumonia pattern in the organization of lung injury and indicating disease progression (134–136). Another important manifestation found through chest CTs is reticular pattern formation with interlobular septal thickening, which might be associated with interstitial lymphocyte infiltration and determine the disease course (108, 137, 138).

CT has highlighted many other alterations, including the "*crazy-paving*" pattern, which may result from the alveolar edema and interstitial inflammation in acute lung injury, and air bronchogram with a pattern of air-filled (low-attenuation) bronchi, but with gelatinous mucus and several airway changes, such as bronchiectasis and bronchial wall thickening resulting from the destruction of bronchial wall structure, proliferation of fibrous tissue, and fibrosis (137–140).

IMMUNE RESPONSE AGAINST SARS-COV-2

Cytokine Storm

Antiviral immune response is usually coordinated by IFN-type cytokines that activate cells and intensify the response against these invading agents, triggered by the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), such as toll-like receptors (TLR), fundamental for pathogen recognition and activation of innate immunity. Type 7 of TLR (TLR7) – expressed on the surface of endosomes predominantly in the lungs, placenta, and spleen – might play a central role in COVID-19. This receptor has been reported to quickly recognize single-stranded SARS-CoV-1 RNA, inducing the production of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-12 in plasmacytoid dendritic cells (141–143).

The recognition of SARS-CoV-2 RNA by TLR7 can mediate the release of cytokines in response to the virus, a context in which IL-6 may play an important role. It has been well described that IL-6 is a pleiotropic cytokine with distinct functions in different contexts in the immune system, being fundamental for the formation of follicular T helper lymphocytes and the generation of long-lived plasma cells. However, this cytokine can also inhibit the activity of $CD8^+$ cytotoxic lymphocytes by inducing the expression of PD1 in these cells, in addition to inhibiting suppressors of cytokine signaling 3 (SOCS3), an important protein responsible for controlling cytokine production, leading to an excessive release of inflammatory mediators (144).

The pathophysiology of COVID-19 is yet to be fully elucidated and several gaps still need to be filled, however, several studies have shown an increase in cytokines, notably pro-inflammatory, in the serum of infected patients, which has been associated with hyper inflammation and the lung injury particular to the disease. The main cytokines described include TNF-α, IFN-γ, IL-1β, IL-1Ra, IL-2R, IL-6, IL-7, IL-8, IL-9, IL-10, basic FGF, G-CSF, GM-CSF, IP-10, MCP-1, MIP-1a, PD6F, and VEGF, in addition to an increase in other inflammation biomarkers, such as C-reactive protein, ferritin, and procalcitonin. However, mediators related to the complement system, such as C3 and C4, did not present any difference in healthy individuals. Furthermore, even higher levels of these mediators were found in patients of critical COVID-19 cases, suggesting that the severity of the disease may be associated with this huge amount of inflammatory mediators, called cytokine storm, which overloads the immune system with information, preventing the establishment of an effective immune response (26). For example, a study published by Valle et al. showed that COVID-19 patients have higher levels of IL-6, IL-8, and TNF-alpha than healthy individuals on hospital admission; moreover, when they stratified the population by low versus high cytokine levels and applied a risk competition model, it was found that each cytokine is an independent predictive factor of the patients' overall survival and is significantly associated with worse clinical outcomes (145).

On the other hand, in theory, a type I IFN-mediated response activates the JAK-STAT signaling pathway that should be able to suppress viral replication and prevent the virus from spreading early in the infection. This is probably what occurs in asymptomatic individuals who can establish an effective response against SARS-CoV-2 (9, 141). However, in several viruses, viral proteins can modulate the production of this type of IFN, impairing the generation of an effective antiviral response (141, 143, 146, 147). Li et al. (148) conducted an *in vitro* experiment that revealed a strong capacity of ORF6, ORF8, and nucleocapsid proteins of SARS-CoV-2 to inhibit IFN-β and NF-κB activity, in addition to genes containing interferon-stimulated response elements (ISREs), suggesting that the virus has an important IFN antagonist activity.

By monitoring the production of type I IFN in SARS-CoV-2-positive patients, Trouillet-Assant et al. (149) found a peak in IFN-α2 production between 8 and 10 days after the onset of symptoms, in general, which reduces overtime. However, as many as one critically ill patient in five was unable to produce any amount of type I IFN and had a higher viral load, respiratory failure, and worse clinical outcome. Nonetheless, Zhou et al. (150) conducted a study that demonstrated that SARS-CoV-2 infection induced a markedly elevated expression of IFN-related inflammatory genes, which appears to decrease over time in mild cases, but not in severe ones.

Additionally, Major et al. (151) described the role of types I and III of IFN in lung repair during viral infection. The production of IFN-α/β and IFN-λ in C57BL/6 mice was detected immediately at the early stage of influenza virus infection and decreased over time, having reached undetectable levels at the onset of epithelial recovery. Interestingly, the treatment with IFN-α, β, or λ during the recovery phase reduced the proliferation type II alveolar epithelial cells by activation of IFN-induced p53, aggravating lung injury, disease severity, and susceptibility to coinfections. Therefore, time and duration of IFN are critical factors for viral infection response and should be thoroughly considered as a COVID-19 therapeutic strategy.

Similarly, an experimental study conducted with MERS patients indicated that type I IFN has protective activity against this infection and the blockade of its signaling resulted in delayed virus clearance, enhanced neutrophil infiltration, and impaired MERS-CoV–specific T cell responses. Additionally, early treatment using this type of IFN prevented fatal infections in mice. However, the late treatment did not cure the animals and failed to effectively inhibit virus replication, increased infiltration, and activation of monocytes, macrophages, and neutrophils in the lungs, in addition to having enhanced proinflammatory cytokine expression, which led to fatal pneumonia, indicating that type I IFN plays a central role at the very beginning of the infection (152).

Therefore, using IFN in SARS-CoV-2 treatment seems to be beneficial at the early infection stage, especially for patients unable to produce this type of response. Furthermore, as the disease progresses, the use of inflammatory cytokine blockers for patients who fail at regulating their production over time could represent a better strategy.

COVID-19 patients also have high levels of production of anti-inflammatory cytokines, such as IL-10, perhaps as a way of compensating for the exacerbated inflammatory response, which can lead to a picture of immune dissonance and anergy towards the infection (26, 153–155). It is fundamental to perform further studies that elucidate the mechanisms of the immune response and the balance between pro-inflammatory and anti-inflammatory response patterns to understand the immunopathogenesis of COVID-19.

IL-7 is a pleiotropic cytokine that plays an essential role in the differentiation and clonal expansion of lymphocytes. Chi et al. (156) described the production of IL-7 in COVID-19 patients; when compared to healthy controls, both asymptomatic and symptomatic individuals in the acute phase show an increase in the levels of this cytokine, however convalescent individuals return to the basal state equal to that observed in healthy individuals. When symptomatic individuals were stratified according to the severity of the disease, those with moderate to severe conditions had higher levels of IL-7. In addition, SARS-CoV-2-specific T cells from the peripheral blood of convalescent individuals of COVID-19 show high expressions of CD127, a receptor necessary for homeostatic cell proliferation triggered by IL-7, which may be related to the recovery observed (157). Patients with a severe COVID-19 condition, on the other hand, have an increased IL-7 production, but contradictorily they also have severe lymphopenia. Thus, we speculate that the deficiency in the expression of CD127 might occur in severely ill patients, which culminates in the deficiency of cell proliferation induced by IL-7 and consequent lymphopenia. However, studies that seek to evaluate the expression profile of IL-7 and CD127 in COVID-19 patients need to be carried out. In addition, the use of IL-7 as a treatment for COVID-19 patients has been evaluated and will be discussed further.

Several pro-inflammatory cytokines have been described in COVID-19 patients and are associated with the disease's immunopathogenesis. Among them, IL-1β and TNF-α stand out for playing a central role in this context (26, 156). The respiratory failure characteristic of SARS-CoV-2 infection, especially in individuals who develop the most severe forms of the disease, occurs independently of infection or viral replication in the epithelial bronchial cells and probably occurs due to exacerbated inflammatory dysregulation, resulting from activation of the NLRP3 inflammasome pathway and consequent release of IL-1β (158). However, although several articles have shown an increase in IL-1β production in COVID-19 patients and early treatment with IL-1 receptor blockers has helped prevent respiratory failure (159), its exact role in the immunopathogenesis of the disease has not yet been fully described.

Cytokine storms may have great relevance in the pathogenesis of COVID-19. The induction of inflammatory mediators can induce cell damage, especially in lung tissues, causing respiratory failure. In addition, several of these mediators have potent vasodilator activity, which at the local level can cause pulmonary edemas, while at the systemic level leads to septic shock, worsening the clinical condition of these individuals. Similarly, several studies have shown that viral infections can induce cytokine storms, or take advantage of it, to establish infection and escape from the immune system, intensifying pathological phenomena such as those observed in sepsis, in addition to increasing the mortality rate of this population (160, 161).

Despite the absence of direct evidence of the role of cytokines and chemokines in lung injury, initial studies have shown that the increase in these pro-inflammatory mediators is associated with lung injury in patients with COVID-19 and has a central role in the pathogenesis of the disease (153). The balance of the innate immune response is essential at the beginning of the infection, while its imbalance can culminate in excessive inflammation, which hinders the establishment of an effective immune response against the virus.

Therefore, using hemoperfusion can be an important tool to treat severe COVID-19 patients who developed cytokine storms, as well as other treatments focusing on controlling and reducing hyper inflammation using specific blockers or monoclonal antibodies directed against the mediator or to antagonize its receptor (144).

Innate Immune Response

The innate immune system is the first line of defense against pathogens through the activation of PRR in macrophages,

neutrophils, and dendritic cells by the interaction with PAMPs. An effective innate immune response against viruses like SARS-CoV-2 is essential not only to initiate the response but also to structure the basis for the production of a robust and more specific adaptive response (162). Changes in this process, commonly observed in viral infections, can cause an immune imbalance and susceptibility of the host (163).

Patients who develop severe COVID-19 exhibited a marked increase in neutrophil and reduced lymphocytes counts compared with patients with mild signs of the disease (10). A general increase in the number of circulating neutrophils and the reduction of lymphocytes enhance the neutrophil/lymphocyte ratio, which has been used as a predictor of the infection severity and development of pneumonia. In addition to being a predictor of a worse prognosis, an increase in this ratio also indicates a serious immune imbalance in these patients (153).

In addition to having high levels of cell-free DNA, myeloperoxidase-DNA, and citrullinated histone H3 – important markers of neutrophil extracellular traps (NETs) –, the serum of COVID-19-positive patients was able to strongly trigger NETosis in healthy neutrophils *in vitro* (164). Despite representing important strategies to eliminate pathogens by neutrophils, NETs damage healthy tissue and induce inflammation (165), in addition to featuring a variety of oxidizing agents and being involved in several vascular diseases, as well as pathogen-induced acute lung injury. The release of NETs by neutrophils can be triggered by several factors, such as virus-damaged epithelial cells, activated platelets, activated endothelial cells, and inflammatory cytokines, such as IL-1β, IL-8, and G-CSF, among others (95, 166–169). In this context, it is fundamental to conduct studies assessing the role of neutrophils and NETs to better understand COVID-19 pathogenesis.

Concerning monocytes, COVID-19 patients have shown an abundant circulation of $CD14^+$ $CD16^+$ cells, with a sharper increase in patients who developed severe respiratory syndrome. This subtype of monocytes can over-secrete TNF-α, IL-1β, and IL-6 and expand quickly in systemic infections, implying that they must play an important role in the rapid defense against pathogens. Controversially, these cells are the main producers of IL-10, which makes their exact function in immune responses elusive (170, 171). Additionally, Dutertre et al. (172) demonstrated that $CD14^+$ $CD16^+$ monocytes are responsible for TNF overproduction in HIV infections and might be considered the major actor in immune hyperactivation in disease (172).

A study assessing bronchoalveolar lavage of SARS-CoV-2-positive individuals found an abundance of monocytes-derived inflammatory macrophages in critically ill patients. In addition, the authors observed through single-cell analysis that these macrophages can contribute to local inflammation by recruiting inflammatory monocytes and neutrophils through CCR1 and CXCR2 chemokine receptors. However, in patients who presented a moderate form of the disease, macrophages produced chemo-attractants for the recruitment of T cells, such as CXCR3 and CXCR6. Such a difference in response might be the key to understanding the pathogenesis of respiratory failure in COVID-19 (173).

Furthermore, critically ill patients have also manifested rapid proliferation of another subpopulation of monocytes characterized by $GM\text{-}CSF^+$ $IL\text{-}6^+$, which may be related to inflammatory risk and impairment of the lungs when migrating in large quantities (170). GM-CSF has been described as an active part of the pathogenesis of autoimmune and inflammatory diseases, mainly in the involvement of myeloid cells, such as monocytes, which can initiate tissue damage in a dependent manner on this marker (174, 175). In addition, high levels of mediators, such as IL-6, TNF-, and IL-10, found in these patients are likely to have been produced by these monocytes and to be highly involved in cytokine storm and pathogenesis of SARS-CoV-2, since as the disease progresses these mediators reduce, which is correlated to the restoration of the immune function of $CD4^+$ and $CD8^+$ T lymphocytes, which is further discussed later (154).

Critical COVID-19 patients have shown excessive activation of circulating $HLA\text{-}DR^-$ monocytes, which has been associated with the onset of respiratory failure, suggesting its role as a predictive factor. The lack of expression of HLA-DR in monocytes may indicate a modulatory capacity of the virus, which prevents the antigen presentation and hampers the formation of an adaptive immune response (176, 177).

During an *in vitro* experiment, Yang et al. (178) found that, despite being permissive to infection by SARS-CoV-2, human monocyte-derived macrophages and dendritic cells are not able to effectively produce viral replicates. Despite their central role in pathogenesis, this may indicate that these cells are not important reservoirs for viruses. In addition, neither of the cell types developed a response based on type II IFN, but macrophages had lower production of type I and III IFN than the control, indicating that the virus can inhibit a response mediated by these types of IFNs. Additionally, macrophages were able to trigger an exacerbated inflammatory response with higher TNF-α, IL-8, IP10, MIP1α, and IL-1β. Dendritic cells had not been reported to show such inflammatory phenomenon, which is due to the ability of SARS-CoV-2 to inhibit STAT1 phosphorylation. Such important attenuation of dendritic cell response caused by the virus may have important implications for humans to develop effective immunity, therefore, further studies should seek to better elucidate such a relevant relationship.

Similarly, in the presence of IFN-α and GM-CSF, circulating monocytes should quickly differentiate into monocyte-derived dendritic cells (mDC), which are important antigen-presenting cells capable of phagocyting viruses and initiating the adaptive immune response process, as well as activating $CD4^+$ T cells, generating immune memory in the process, and refining the body's defense against infections (179, 180).

The number of mDCs has not increased in patients infected with SARS-CoV-2 compared with healthy controls, even in the most severe cases of the disease. Interestingly, the levels of GM-CSF in the serum of these patients are highly elevated, which should lead these cells to increase, demonstrating that the virus may have a mechanism to control the production of IFN-α and

consequent differentiation of mDCs (9, 26). In the same way, individuals infected and not infected with SARS-CoV-2 have similar levels of IL-12, an important cytokine produced by mDC that is involved in the differentiation of naïve T cells (9, 181).

Thus, we hypothesize that the lack of mDC generation and consequent inability of the infected individual to produce IL-12 may be among the main factors of innate immunity-related pathogenesis of COVID-19. As further discussed, the increase in naïve T cells, reduced cell functionality of $CD4^+$, $CD8^+$, and natural killers (NK), and delay in the appearance of humoral response found in these patients indicates a failure in the generation and function of mDC. Therefore, it is urgent to carry out studies aimed at analyzing the effect of SARS-CoV-2 on dendritic cells.

T Helper Cells

Establishing and maintaining immune response and memory generation against viruses depends on the activity of T cells. These lymphocytes originate from bone marrow progenitor cells and migrate to the thymus for maturation, selection, and peripheral export. Peripheral T cells are subdivided into groups that include naïve T cells, which are capable of responding to new antigens, memory T cells derived from previous antigen activations and maintain long-term immunity, and regulatory T cells that coordinate the immune response (163).

The immune response begins when naïve T cells encounter antigens and co-stimulatory molecules presented by antigen-presenting cells, such as dendritic cells that phagocytize the virus, resulting in the production of IL-2, proliferation, and differentiation of effector T cells, which migrate to various sites to promote the elimination of pathogens (163, 182).

Inflammatory factors induced by viruses can trigger a storm of mediators that cause changes in the differentiation and activation of T cells, disturbing the homeostasis of the immune system. In patients with COVID-19, the overall percentage of T lymphocytes is generally reduced, especially $CD4^+$ $CD3^+$ T lymphocytes, which have an activation phenotype, a reduction much more pronounced in severely ill patients. Furthermore, a higher percentage of $CD4^+$ $CD45RA^+$ naïve cells and lower $CD4^+$ $CD3^+$ $CD45RO^+$ memory T cells were also found in COVID-19 patients (10, 153, 183, 184).

Polyfunctional $CD4^+$ T cells are characterized by the expression of activation markers as well as their capacity to produce IFN-γ, IL-2, and TNF-α. These cells have been linked to an excellent response against viral infections and during the development of immunity by vaccination (185, 186). Even though COVID-19 patients have shown an increase in the expression of molecules related to T $CD4^+$ activation, such as CD69, CD38, and CD44, molecules related to their function, such as intracellular IFN-γ, IL-2, and TNF-α, are reduced, especially in individuals with a more severe stage of the disease (9, 170), indicating an impairment of polyfunctional T cells.

Li et al. (187) demonstrated that patients infected with SARS-CoV-1 had elevated levels of polyfunctional $CD4^+$ T cells, especially those in a severe condition but who progressed to clinical improvement. In contrast, critically ill patients with SARS-CoV-2 demonstrated a drastic reduction of this cell subtype, which may indicate that this virus has developed its own mechanisms to control cellular responses, thus differing from other coronaviruses (9).

Similarly, Chen et al. (154) demonstrated that $CD4^+$ T lymphocytes from COVID-19 patients showed increased expressions of T cell immunoglobulin-3 (Tim-3), a type I transmembrane protein that acts as a negative regulator of Th1 pattern. $CD4^+$ cells showed low expression of this marker at the early phase of infection, having progressively increased over time, indicating that the exhaustion of these cells occurs as the disease progresses.

Many studies with SARS-CoV-2 positive patients have described the generation of these exhausted pathological lymphocytes that exacerbate the inflammatory response at the early stage of infection, initiating a cytokine storm, followed by cell exhaustion and loss of functionality, a phenomenon that has appeared mainly in more severe cases of the disease (9, 10, 170, 188).

The vast majority of studies to date show impairment of proliferation, maturation, and response of T cells, especially in sicker patients. This may indicate that SARS-CoV-2, similarly to other viral infections, can interfere with the function of $CD4^+$ cells at the very beginning of the infection, causing excessive release of inflammatory mediators and exhaustion of the response capacity of these cells over time, reducing the host's antiviral immunity (189). What seems to happen in COVID-19 is that the total lymphocyte count is reduced in these patients and, among the remaining T cells, the highest percentage is from naïve $CD4^+$ T lymphocytes, while the activated subpopulations, although few, present a phenotype with excess and reduced markers related to activation and function, respectively, indicating that, despite overactivation, these cells fail to exercise effective immune activity.

Another important point is the reduction of regulatory T cells $CD4^+$ $CD25^+$ $Foxp3^+$ verified in these patients. These cells have a fundamental role in the negative regulation of inflammation, control of cell proliferation, and the effector function of several cells, which probably has contributed to the excessive inflammation observed in critically ill patients (153, 190). It has been described that regulatory T cells play a central role in mitigating the immune response in several viral infections (191); reducing the number of these cells in patients with COVID-19 can lead to loss of regulatory functions and consequent cytokine storm.

It has been described in several studies that COVID-19 patients have a reduced number of circulating Treg cells, which may be due to the increase in soluble IL-2 receptors (IL-2R or CD25) that potentially scavenges IL-2, reducing their bioavailability for binding to CD25 on the cell surface, thus preventing the induction of the clonal expansion signal of Treg cells (153, 192, 193).

Cytotoxic Cells

T lymphocytes $CD8^+$ and NK are essential to control viral infections due to their cytotoxic effect. These cells become activated after recognizing antigens attached to molecules of

MHC-I presented by infected cells, which usually leads to the death of the infected cell by effector mechanisms (163).

Kamiya et al. (194) demonstrated that SARS-CoV-2 infection in humans dramatically reduces the total $CD8^+$ and NK cell count, especially in patients who have developed more severe disease. The inhibition of these cells was characterized by an increase in the expression of NK inhibitory receptor CD94/NK group 2 member A (NKG2A), a type C lectin receptor of cytotoxic cells that acts as a potent suppressor when binding to minimally polymorphic MHC-I that present peptide sequences of other MHC-I molecules, inducing an inhibitory signal through two receptors with tyrosine-based inhibition motifs that suppress cytokine secretion and cytotoxic activity.

In patients who recovered from COVID-19, $CD8^+$ and NK cell counts and the reduction in NKG2A expression were restored, suggesting that the inhibition of these cells is a result of SARS-CoV-2-mediated immunomodulation (10). Corroborating these data, it has been reported that other viral infections also manage to increase the expression of NKG2A in NK cells as a way to escape from the immune system (195).

SARS-CoV-2 studies involving $CD8^+$ T cells have shown exhaustion of the effector capacity of these cells over time by the reduction of granzyme B, perforins, and lysosome-associated membrane protein 1, also known as LAMP-1 or CD107a, described as a marker of cytotoxic cells' degranulation and an important parameter to assess the activity of these cells. $CD8^+$ T cells from COVID-19 patients have a very marked activation phenotype with an increase in CD69, CD38, CD137, and CD44, especially in critically ill patients. However, despite presenting an increase in these activation molecules, these cells also have enhanced cell exhaustion proteins, such as PD1, Tim3, CTLA-4, and TIGIT, especially in more critically ill patients (9, 154, 170).

TIGIT receptor, present in T and NK cells, can bind to dendritic cell CD155 receptors and induce an increased expression of IL-10 and reduce IL-12, in addition to inhibiting T cell activation and blocking cytotoxicity of NK cells (196). The use of specific blockers for these receptors, such as anti-PD1 and anti-TIGIT, has helped in the recovery of the function of these cells. Therefore, it is logical to assume that specific NKG2A blockers could be an important tool to assist in the treatment of SARS-CoV-2 infections, restoring the functionality of cytotoxic cells (10, 195).

Together, these data described the increase in activation markers and cellular exhaustion, in addition to the reduction in functionality markers indicating that, like $CD4^+$ T lymphocytes, these cells were probably hyperactivated right at the beginning of the infection, collaborating with the generation of a cytokine storm, until they became exhausted and lost their functional capacity, causing reduction of antigen-specific response and loss of its antiviral effects (**Figure 2**).

Humoral Response

Detecting a humoral response against SARS-CoV-2 has been the focus of attention to developing faster and more accurate diagnostic tools. A study assessing the presence of IgA, IgM, and IgG against SARS-CoV-2 in infected patients found that IgA levels begin to rise in the first seven days after the onset of symptoms and continue increasing until it stabilizes near the 14^{th} day after the onset of symptoms. Additionally, IgM production appears as early as IgA, the antibody titration in the first seven days after the onset of symptoms was very low, starting to increase only from the eighth day, reaching a plateau after the 14^{th} day. The average time of appearance of specific IgG against SARS-CoV-2 starts 14 days after the appearance of symptoms and grows exponentially until the 21^{st} day (197).

These data corroborate the disease stages proposed by Lin et al. (6) (presented in the introduction), and the changes in the immune response present in the disease. The early appearance of IgA results from the first contact of the virus with the individual's mucosa at the moment of contagion and continues to increase until the acute phase. Despite viremia and symptom onset, IgM levels only begin to rise from the eighth day after symptom onset, indicating anergy in the immune response during this period, perhaps caused by dysfunction in antigen-presenting cells, such as dendritic cells, and also by reducing the amount of activated T helper cells, which play a central role in triggering the immunity acquired by the activation and clonal expansion of B lymphocytes, in addition to the formation of germinal centers and generation of plasma cells that produce high affinity and avidity antibodies (198, 199).

The appearance of IgG near the third stage of the disease may be related to the clinical evolution of patients and those who fail to establish an efficient immune response might be at risk of death. Interestingly, Guo et al. (197) found that approximately 22% of COVID-19 patients confirmed by RTq-PCR did not present detectable levels of IgM. Most of these individuals were tested in the first seven days after the onset of symptoms, therefore the lack of IgM can be justified by the delay in generating a humoral response against SARS-CoV-2. However, some critically ill patients followed for a longer period remained negative for IgM even 22 days after the onset of symptoms. As for IgG levels, some patients took 30 to 40 days after the appearance of symptoms to show some detectable level of IgG, suggesting a possible failure in the production of antibodies that may have contributed to the severity of the disease. It is possible that the generation of antibodies in more advanced stages of COVID-19 does not benefit the recovery process since most pathological mechanisms at this stage might be more related to the excess of inflammatory mediators than the presence of the virus itself.

In recovered patients, the magnitude of the production of neutralizing antibodies (nAb) against SARS-CoV-2 is positively correlated with the severity of the disease; while asymptomatic individuals have little or no capacity to produce nAb, individuals who recovered from severe cases of COVID-19 had robust nAb production. Also, these severe recovered patients showed an increase in B cell receptor (BCR) rearrangement, which may demonstrate that the effective production of nAb requires enhanced and prolonged BCR stimulation. Asymptomatic or mild symptomatic patients may possibly mount robust SARS-CoV-2 specific CD8 + T cell responses, which can provide protection by directly eliminating the target cells infected by the virus. However, due to the lack of immunity provided by

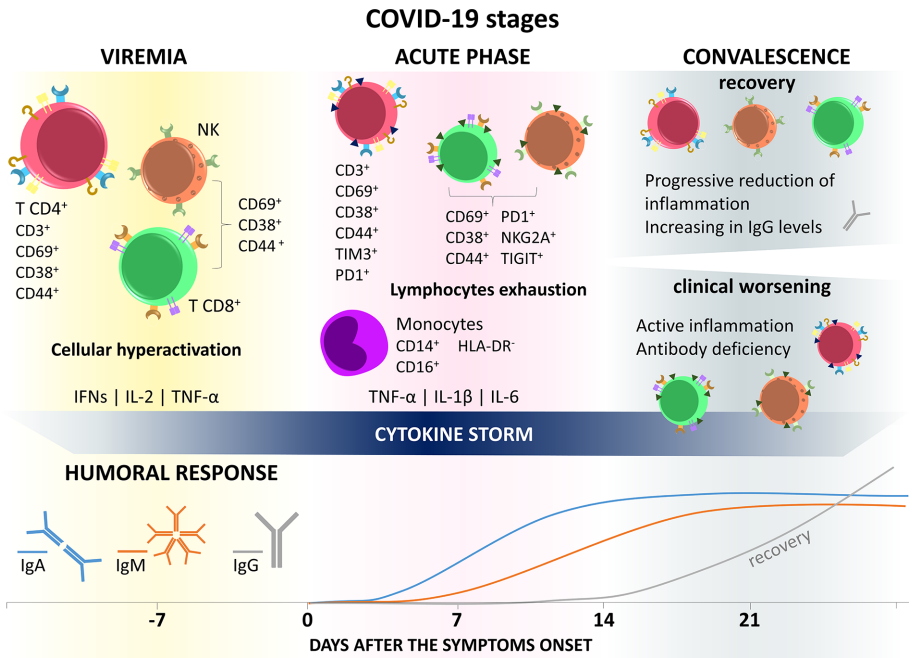

FIGURE 2 | Immune response in COVID-19 stages. SARS-CoV-2 infection is divided into three general phases. In the first one, called viremia, the virus spreads through the body and there is excessive activation of immune cells with exacerbated production of inflammatory mediators, such as IFN-γ, IL-2, and TNF-α, triggering cytokine storms and immune impairment. The second (acute) phase, characterized by the appearance of COVID-19 symptoms, presents a profile of immune cells still hyperactivated, but with the presence of cell exhaustion markers, such as Tim3, PD1, TIGIT, and NKG2A, in addition to losing the functional capacity of producign IFN, IL-2, and TNF-α. In this period there is still the appearance of $CD14^+$ $CD16^+$ hyperinflammatory monocytes, with a high production capacity of TNF-α, IL-1β, and IL-6, which will migrate to the lungs, contributing to the pathogenesis of respiratory failure and maintaining the cytokine storm. The lethargic state of the immune system in the early stages of infection may be related to the delay in the generation of a humoral response. In the third, or convalescence, phase, the individual can evolve in two opposite directions, recovery or clinical worsening/death. In recovery, cells of lymphoid origin recover their effector function and lose markers of exhaustion, while IgG levels improve. On the other hand, in patients with clinical worsening, this status of immune anergy continues.

nAb, these individuals might suffer from SARS-CoV-2 reinfection (200, 201).

In the same way, Zhang et al. (202) also demonstrated that patients who recovered from severe COVID-19 have high levels of BCR clonal expansion and B cell activation, indicating a more robust humoral response than patients with mild disease, thus asymptomatic individuals or those with mild COVID-19 probably have different cell and humoral responses than individuals who developed the severe form of the disease.

In an article published by Chen et al, the serum of 26 patients who recovered from COVID-19 were analyzed for the production of IgG anti-SARS-CoV-2 S1 protein antibodies. It was found that, despite the majority of patients presenting high IgG titers, only three individuals had antibodies that effectively neutralized the binding of the viral glycoprotein to the human ACE2 receptor. In addition, the authors successfully managed to clone two different neutralizing antibodies from these patients with the ability to inhibit virus-cell binding, opening up the potential for using them as a possible source of treatment for COVID-19 (203).

In theory, the production of specific neutralizing antibodies against SARS-CoV-2 should be able to combat the virus and reduce viral load. The production of immune memory verified in the blood of recovered patients has also been used to treat COVID-19 patients, as we will discuss further (204).

COVID-19 TREATMENT

To date, no effective vaccines or therapeutic antiviral agents have been approved for the treatment of COVID-19 or any other human CoV infection. The main approach to disease management focuses on supportive care. To contain the viral transmission and disease, rapid public health interventions using immune cell-based therapies, antibodies, antivirals, new drugs, or vaccine strategies have focused on reducing mortality, virus spread, and mitigating potential future outbreaks. In this context, we conducted a survey of the main SARS-CoV-2 drugs/treatments following three criteria: peer-reviewed published scientific literature, with clinical trials that are underway, and that display a broad spectrum of action in the face of various viral and parasitic disease. The researched data (until September 2020) for ongoing and completed trials were searched in "clinicaltrials.gov".

Enhancing Immunity

As exposed in the previous topics, currently there are no proven treatments for SARS-CoV-2 infections, thus, much has been discussed about the maintenance of a healthy immune system. In this sense, the use of vitamins and other essential components for the proper functioning of the immune response can be an

important approach in times of risk like this (205). Several studies have shown that the use of supplements helps in enhancing the immune response and recovery from viral infections, as is the case with the use of vitamin A and D or selenium to improve the humoral immunity of individuals vaccinated against influenza virus (206, 207), or the use of zinc to improve the immune response of individuals infected with torquetenovirus (208).

Among vitamin supplements, vitamin D stands out for having an immunomodulatory effect on both adaptive and innate immune responses, helping in the development of B, T, and NK cells. In addition, it has the ability to stimulate the production of antioxidant responses and microbicidal molecules such as defensins and cathelicidins (209). The use of vitamin D has also been associated with the prevention of respiratory diseases associated with viral infections (210), and epidemiological data suggest that vitamin D deficiency increases the susceptibility to acute viral respiratory infections (211). However, a study in the United Kingdom that evaluated plasmatic concentrations of vitamin D in samples from COVID-19 patients found no association between circulating vitamin D levels and the risk for disease severity (212).

The use of supplementation with other types of vitamins has also been described in viral infections; the use of vitamin C, for example, a potent antioxidant and an important enzyme cofactor, contributes to the development of the immune response, helping in the production of type I IFN. However, a systematic review with meta-analysis found no evidence that the use of vitamin C has any effect in preventing common cold infections (213). As for vitamin E, it has been shown that its deficiency can impair cellular and humoral immune responses (214). However, the use of vitamin E has been associated with an increased risk of pneumonia and has shown no significant effect in preventing lower respiratory tract infections (215, 216).

In view of the controversial results, more than 50 ongoing clinical trials are seeking to clarify the role of vitamins, minerals, and other dietary supplementation in the prophylaxis and treatment of COVID-19, analyzing parameters such as the risk of infection, risk of hospitalization, and clinical outcome.

Immunotherapy

Antibody-Based therapy

Considered an efficient method for the clinical treatment of different infectious diseases, including MERS-CoV and SARS-CoV-1 (217), antibody-based immunotherapy has been studied as a potentially applicable tool to treat COVID-19. The mechanisms involved with its effects against SARS-CoV-2 are related to preventing the virus from entering the host cells, blocking its replication.

The virus entry block was studied for acting both in the cell receptor ACE2 and directly on the virus (neutralizing antibodies [nAbs]), specifically in the S1 subunit of the S protein (218–220). Regarding the blocking of ACE2 receptors, the application of some mechanisms stand out: the soluble version of ACE2 fused to an immunoglobulin Fc domain (ACE2-Fc), RDB domain attached to Fc (RDB-Fc), and receptor-targeted monoclonal antibodies (mAb) (221).

Viral neutralization by nAbs is also an immunotherapeutic approach and directly recognizes epitopic regions of SARS-CoV-2. This effect can be achieved either directly through mAbs manufactured in laboratories or by using polyclonal antibodies (pAbs) (218). nAbs act directly on the virus, preventing its infectivity by activating several pathways, such as the complement system, cell cytotoxicity, and phagocytic clearance (222–224).

The therapeutic use of mAbs has shown good outcomes, mainly due to its high specificity. Recently, several mAbs against viruses have been developed, including SARS-CoV-1 and MERS-CoV, in which the S protein is the major target described both *in vitro* and *in vivo*. According to some studies, the specific nAbs against SARS-CoV-1 RBD in the S protein could effectively block SARS-CoV-2 entry (218, 225). However, Wrapp et al. (226) tested several published SARS-CoV-1 RBD-specific nAbs and found that they do not have substantial binding to the S protein of SARS-CoV-2, suggesting that the cross-reactivity may be limited. Thus, the combination of nAbs with different viral targets and sources could improve treatment efficacy. In addition to experimental studies, to date, more than 10 clinical trials have aimed at testing human mAbs against SARS-Cov-2 (227–235), which could also represent an alternative, effective treatment.

Furthermore, some immunomodulatory mAbs have been tested in the context of COVID-19. It is remarkable that until now most of the data published regarding the use of immunomodulatory mAbs derive from studies using either anti-IL-6 or anti-IL-6R, probably because using IL-6 blockers seems promising at controlling the cytokine storm associated with the development of ARDS in more aggressive patterns of SARS-CoV-2 infection. However, clinical observations remain controversial.

Although some studies found considerable clinical improvements resulting from treatment with IL-6 blockers (236–239), others do not report any significant difference between the clinical features of groups treated with anti-IL6/IL-6R mAbs and their respective controls (without anti-IL-6/IL-6R) (240–243). These controversial results can be explained by the pleiotropic function of IL-6, which also play an important anti-inflammatory role, questioning the use of IL-6 blockade to suppress inflammation-induced tissue damage (244). Additionally, severe side effects have been associated with the use of IL-6 blockers, including enhanced hepatic enzymes, thrombocytopenia, severe bacterial and fungal infections, and sepsis (241, 245). In general, data from analyses on the use of this type of mAbs remain inconclusive (243, 246, 247).

Recent findings are optimistic, but data validation by robust scientific evidence has been hampered by the small sample size in most case reports and studies on the use of mAbs blocking other immune mediators, such as IL-1β, GM-CSF, and complement protein C5 (238, 248–250). However, seeking to verify the effectiveness of using mAbs blocking inflammatory mediators, dozens of clinical trials are currently underway.

Aiming at reducing the hyper inflammation found in the lungs of SARS-CoV-2-infected patients, different clinical studies are currently investigating the activities of mAbs anti-JAK, anti-GM-CSF, anti-GM-CSF receptor, anti-M-CSF receptor, anti-CD14,

anti-IFNγ, anti-VEGF, anti-BKT, anti-CCR5, anti-IL-6, anti-IL-6 receptor, anti-TNFα, anti-IL1β, anti-IL1β receptor, and complement C5 inhibitor (220, 251, 252). Similarly, ongoing clinical trials have sought to reverse the hyper-thrombotic state found in critically ill patients by using anti-P-selectin, anti-CTGF, and factor XIIa antagonist mAbs (253, 254). Furthermore, to restore the exhausted T lymphocytes' and NK cells' immunity, other clinical studies applied anti-PD1 mAbs under the hypothesis of a stimulus of anti-viral response and prevention of ARDS (255–257).

More recently, the passive administration of pAbs has also been tested in COVID-19 patients (222–224, 258–267), also known as convalescent plasma (CP) or immune plasma, which is already used effectively and safely in the treatment of other severe acute respiratory syndrome infections of viral etiology, such as SARS, MERS, and H1N1, and offers only a short-term but rapid immunity to the susceptible individuals (268).

A strict criterion to select the CP donor states that the individual must show clinical recovery and test negative for the virus presence. Thus, after being confirmed, a high titer of neutralizing antibodies against SARS-CoV-2 must be stored in blood banks (269, 270).

Some reviews related to patients who received transfusion with CP showed a reduction in viral load, improvement in clinical symptoms, better radiological findings, and improved survival (260, 261, 271–273). In addition, after having received CP containing nAbs, COVID-19 patients had significant improvements from the beginning of treatment (until 22 days), presenting lower fever, decreased viral load, and higher nAbs levels. Further, 60% of the patients were discharged one month after the treatment (271). Better outcomes were found in early administration of CP (before SARS-CoV-2 seroconversion), preferably on day 5, for obtaining maximum efficacy (268).

More recently, Li et al. found no statistically significant clinical improvement or mortality reduction in a randomized clinical trial with CP-treated COVID-19 patients (274). However, the authors reported that CP treatment is potentially beneficial to critically ill patients by suggesting a possible antiviral efficiency of high titer of nAbs. Notably, there are clinical controversies, ethical issues, and potential risks associated with convalescent plasma therapy (275), such as the possibility of ADE development, exacerbating the disease severity, and causing a significant illness in future exposure to coronaviruses infection (268, 276, 277) (REF). Divergences between studies may be caused by variations in the composition of CP, which is highly variable and includes a variety of blood-derived components, timing of CP administration, titer of the specific antibody in administered plasma, and presence of blood borne pathogens (268). Nonetheless, understanding the efficacy and safety of CP therapy relies on the completion of the ongoing clinical trials.

Another therapeutic strategy using antibodies is intravenous immunoglobulin (IVIg) that contains polyclonal IgG isolated from healthy donors, which can be further enhanced by using IgG antibodies collected from recovered COVID-19 patients in the same geographical region as the patient. Results have been mostly positive, although many of these therapies have not been formally evaluated through a randomized, double-blind, placebo-controlled clinical trial (278). According to recent studies, IVIg can be used effectively in early stages of SARS-CoV-2 infection (before the initiation of systemic damage), reducing the use of mechanical ventilation, preventing the progression of pulmonary lesions, and promoting early recovery (268). Also, cross-neutralization activity was shown against SARS-CoV-2 in commercial IVIg manufactured prior to the COVID-19 pandemic and are currently under evaluation as potential therapies for COVID-19 (279). Thus, intravenous use of immunoglobulins can prove helpful in therapy against SARS-CoV-2, however, adjustments in the therapeutic regimen are necessary for all IVIg possibilities, as well as a complete understanding of the possible adverse effects, such as the risk of ADE (278, 279), that are being studied in more than 10 ongoing clinical trials.

Some works have shown that therapies focusing on the interaction between SARS-CoV-1 and the ACE2 receptor may be extended for use in SARS-CoV-2 patients as an immunotherapy tool (218). However, other authors refute this idea based on the fact that recent studies showed limited cross-neutralization between SARS-CoV-1 antibodies and SARS-CoV-2 (280, 281). Furthermore, it was shown that SARS-CoV-2 S protein binds ACE2 with a higher affinity than SARS-CoV-1, suggesting that such interaction differs between the two viruses (266).

Immune Cell-Based Therapy

In addition to antibody-based therapies, scientists have been studying immune cell-based therapies as a tool to combat COVID-19, focusing especially on NK and T cells. The importance of NK cells as the first antiviral responders can be seen in patients with NK cell deficiency and immunocompromised individuals who have increased susceptibility to viral infections (282). In this sense, Market et al. (282) gathered the main reports so far addressing potential therapies focusing on mediating NK cell activity to mitigate the immunopathological consequences of COVID-19, and consequently lighten the load on our health systems.

Some ongoing clinical trials have been studying the use of NK cell therapy through different approaches. A randomized phase I/II trial studied the infusions of CYNK-001 cells, an allogeneic off-the-shelf cell therapy enriched for $CD56^+/CD3^-$ NK cells expanded from human placental $CD34^+$ cells in 86 hospitalized patients with moderate COVID-19 disease (283). Another randomized phase I/II study explored the use of NKG2D-ACE2 CAR-NK cells with each common, severe, and critical type COVID-19. The authors hypothesize that these cells target the S protein of SARS-CoV-2 and NKG2DL on the surface of infected cells with ACE2 and NKG2D, respectively, seeking out the elimination of SARS-CoV-2 virus particles and their infected cells (284).

The unregulated profile of the immune response in critically ill COVID-19 patients may be due to the reduction of Treg cells, which culminates in excessive release of inflammatory mediators and cytokine storms (153, 191–193). Thus, the use of adoptive

transfer of these cells as a measure of inflammatory control in critically ill patients is a promising therapeutic approach. The infusion of autologous polyclonal Treg has already been used to treat inflammatory diseases, such as type 1 diabetes (285), however the use of autologous cells takes a long time, due to the period necessary for differentiation and clonal expansion, making this an unviable and costly method for infectious diseases, as is the case with COVID-19 (286).

A viable alternative is the use of allogeneic human leukocyte antigen-matched umbilical cord-derived Tregs (UBC-Treg) which can be widely expanded and used on a larger scale. A recent case study used $1x10^8$ administration of UBC-Treg in two patients with COVID-19 who had severe respiratory failure, and both demonstrated significant clinical improvement and reduced inflammatory markers four days after starting treatment (287).

There are currently two clinical trials underway that aim to infuse Treg cells in patients with severe COVID-19 and ARDS. The first one is a multi-center, prospective, double-blinded, placebo-controlled phase 1 randomized clinical trial, which has 45 patients who will receive cryopreserved UBC-Treg (288). The second one is a randomized, double-blind, placebo-controlled phase 2 study with 88 participants who will receive off-the-shelf allogeneic hybrid Treg/Th2 cells (RAPA-501-ALLO). RAPA-501-ALLO cells will be generated from healthy donors, cryopreserved, banked, and made available for off-the-shelf therapy. The cells are manipulated *ex vivo* to differentiate into two anti-inflammatory phenotypes simultaneously, generating hybrid Treg/Th2 cells, with the potential to reduce inflammation and mediate a protective effect on tissues (289).

In addition to therapeutic approaches using Treg cell infusion, another three clinical trials are underway with the aim of evaluating treatment using specific SARS-CoV-2 T cells isolated from individuals who recovered from COVID-19 (290–292). The use of virus-specific T cells for off-the-shelf treatment has been used in several viral infections, such as cytomegalovirus, HHV6, adenoviruses, Ebola virus, and BK virus (293–296). Although vaccination provides T cells-based virus-specific immunity, the path to its development is long, so the use of adoptive cell transfer techniques from healthy individuals who recovered from COVID-19 and developed an effective cell response is probably the fastest way to treat critically ill individuals (297). Besides that, as mentioned before, asymptomatic or mild symptomatic patients may possibly mount robust SARS-CoV-2 specific CD8+ T cell responses (200, 201), therefore, the use of these individuals' cells to treat critically ill patients with COVID-19 can be a promising tool.

The clinical use of IL-7 has been implemented in the treatment of cancer patients and infectious diseases, mainly with the objective of improving the immune response by stimulating the generation of lymphocytes (298, 299). In addition, IL-7 administration has been reported to increase CD4 + and CD8 + T lymphocyte counts without inducing the production of pro-inflammatory mediators, making it a promising method of recovering immune function in patients with disorders related to cytokine storms, such as sepsis and COVID-19 (300).

In a case study conducted by Monneret et al. (301), compassionate administration of IL-7 to a patient with severe COVID-19 significantly improved total lymphocyte count and HLA-DR expression in circulating monocytes four days after administration of the first dose. The patient also showed a significant improvement in lung involvement and negative viral load. Another study conducted by Laterre et al. (302), who administered IL-7 to COVID-19 patients found that there was a significant improvement in the lymphocyte count after starting treatment, in addition, the patients did not show any change in TNF-α levels, IL-1β, and IL-12p70, which may indicate that IL-7 therapy may be safe for patients with severe inflammatory changes. Thus, the use of IL-7-based immunotherapy can be an important tool to be used in future clinical trials in patients with severe lymphopenia.

Therefore, the data available to date do not ensure the success of immunotherapy applied in patients with COVID-19, thus, further studies specifically targeting SARS-CoV-2 should be performed to provide more specific data. However, immunotherapy is effective and of immediate use, being of short duration. This approach also presented limitations, such as the possibility of abnormal reactions and other serious risks, such as induction of severe acute lung injury or ADE (225). Although we are living through a unique moment in science, with some mismatched information and novel, important discoveries being made every day, immunotherapy seems to be a possibly effective option to help patients until an effective, safe vaccine or treatment is developed.

Drug Options Against SARS-CoV-2

Although some drugs appear to be effective against SARS-CoV-2 and are able to improve COVID-19 symptoms, there is no specific antiviral compound for this virus. In the face of such a global health emergency, several clinically used drugs are being reviewed and redirected to be tested in patients who have critical complications of COVID-19 in an attempt to eliminate the virus and modulate the patient's immune response.

Antivirals

Due to the large amount of experimental and clinical studies assessing the effectiveness of antiviral therapy against SARS-CoV-2, we have seen the importance of this class of drugs in reducing the viral load peak at the beginning of the infection. Evidence from laboratory, animal, and clinical studies demonstrate that the use of associated or isolated antivirals can delay the progression of lung lesions and decrease the possibility of respiratory transmission of SARS-CoV-2. In this study, we selected the following most promising treatment options: lopinavir/ritonavir, arbidol, ribavirin, remdesivir, favipiravir, and type I IFN.

In the context of discovering new drugs, it is efficient to test the efficacy of existing antiviral drugs regarding the treatment of related viral infections. After the emergence of SARS in 2003, the screening of approved drugs identified an effective SARS-CoV-2 antiviral-drug candidate: the combination of the human immunodeficiency virus (HIV) protease inhibitors lopinavir and ritonavir. However,

lopinavir has insufficient oral bioavailability for significant therapeutic activity due to rapid catabolism by the cytochrome P450 enzyme system. Thus, ritonavir is a cytochrome P450 and glycoproteins inhibitor, which increases the lopinavir plasma half-life, enhancing the pharmacokinetic and pharmacodynamic activities against the viral HIV-protease (303).

Chu et al. (304) described the possible mechanism of action of these drugs on SARS-CoV-1, suggesting that they act by inhibiting intracellular viral multiplication, preventing the action of the protease enzyme, which leads to the formation of an immature and less infectious virus with no ability to replicate.

Choy et al. (305) were successful at demonstrating the antiviral effect of lopinavir against SARS-CoV-2, but this was not the case for ritonavir. In turn, Kang et al. (306) found a lower viral load in infected SARS-CoV-2 Vero cells treated with lopinavir/ritonavir in relation to the untreated infected control. Although no consensus has been reached on its efficacy, dosage, or administration period, the literature includes some case reports, case series, and observational studies reporting a protective effect of the lopinavir/ritonavir combination in COVID-19 patients (110, 307–312).

Conversely, Cao et al. (313) conducted a controlled open-label study with 199 hospitalized severe COVID-19 patients randomly divided into two groups: a standard care group and a lopinavir/ritonavir treatment group (400 mg/100 mg). No benefit was observed in the lopinavir/ritonavir treatment group, showing no significant results for faster clinical improvement, lower mortality, or decreased viral RNA detectability. Although there are 85 clinical trials in progress testing lopinavir/ritonavir associated with other drugs on SARS-CoV-2 and/or COVID-19, WHO stopped the study of lopinavir/ritonavir in the Solidarity Trial.

Deng et al. (312) have studied the association of lopinavir/ritonavir with arbidol treatment and demonstrated a significant improvement in COVID-19 patients compared with a group treated only with lopinavir/ritonavir. Arbidol (umifenovir) is a broad-spectrum antiviral and immunomodulatory compound used to treat influenza and many other viruses (314). Analyses of molecular dynamics and structure-guided drug-binding have suggested an efficiency of arbidol at blocking or hampering the trimerization of the SARS-CoV-2 spike glycoprotein, in addition to inhibiting virus-cell interactions, which supports the potential use of arbidol to treat COVID-19 (315).

Chen et al. (316) demonstrated that arbidol therapy was able to shorten the course of the disease and promote clinical improvement, resulting in low fever and improvements in dry cough without side effects faster than the control group. Zhu et al. (317) also demonstrated the effects of arbidol by retrospectively analyzing the clinical data from 50 COVID-19 patients. The study demonstrated that the use of arbidol monotherapy, without association with other drugs, was more effective than the treatment with lopinavir/ritonavir, showing clinical improvement of the disease, presenting a total elimination of viral load over a shorter duration; in addition, no fever or ARDS were reported compared with those in the lopinavir/ritonavir group.

Ribavirin is another antiviral drug used in association with lopinavir/ritonavir to treat SARS-CoV-1 and was able to reduce viral load, risk of adverse clinical outcomes, ARDS, or death in SARS patients (304, 318). Ribavirin has a broad antiviral spectrum as it is a nucleotide analog that competes for the active site of RdRp, a crucial enzyme in the life cycle of RNA viruses, inhibiting viral replication and transcription (221, 319, 320).

Elfiky (320) conducted an *in silico* study demonstrating that ribavirin and other antivirals such as sofosbuvir can strongly bind to coronavirus RdRp, preventing the transcription of new copies of viral RNA. Only a few clinical studies have investigated the effect of ribavirin on COVID-19 patients, with the studies available generally focusing on the association of ribavirin and other therapeutic schemes (321–323). Nevertheless, China's government (324) has recommended the use of ribavirin in COVID-19 patients.

Remdesivir (RDV) is also among the several potential drugs tested for SARS-CoV-2 treatment. Originally developed to treat Ebola virus infection, RDV is active against RNA viruses from different families, including Coronaviridae (e.g., SARS-CoV-1 and MERS-CoV) (325). RDV showed an *in vitro* effective antiviral activity against SARS-CoV-2 (326). Grein et al. (327) conducted a cohort study with 53 COVID-19 patients treated with RDV and found that 68% of them had improved oxygen-support class, whereas 57% of the patients receiving mechanical ventilation were extubated. Overall mortality reached 13% over a median follow-up of 18 days, however, viral load data were not collected to confirm the antiviral effects of RDV. The biggest issue with this study is that the authors did not include a group without RDV, which hampers the performance of comparative statistical analyses to prove whether the data found resulted from the treatment with RDV.

Another double-blind, randomized, placebo-controlled trial of intravenous RDV conducted in adults hospitalized with COVID-19 with evidence of lower respiratory tract involvement was performed in different parts of the world (328). The study of Beigel and colleagues (328) enrolled 1,063 COVID19 pneumonia patients, 538 of whom were assigned to the treatment with RDV and 521 to a placebo, showed the effectiveness of RDV in treating COVID-19 patients. The drug was superior to the placebo in reducing the recovery time in hospitalized COVID-19 patients and decreased the mortality rate in the RDV group, however, this result did not reach statistical significance.

Antionori et al. (329), analyzing patients with severe COVID-19 pneumonia in an intensive care unit (ICU) who were treated for 10 days with RDV, found that on the 28th day, 38.9% showed improvement, 16.7% were still on mechanical ventilation, and 44.4% died. The data suggest that this treatment can benefit hospitalized patients who are not in the ICU, where the clinical result was better and adverse events are observed less frequently.

Alternatively, the randomized, double-blind, placebo-controlled, multicenter trial with 273 ill individuals performed by Wang et al. revealed that RDV intravenous administration was well-tolerated in COVID-19 patients. However, the authors

did not find any clinical improvement or significant antiviral effect. Goldman et al. (330), in another phase 3 clinical trial on 397 patients with severe COVID-19 without mechanical ventilation support, also did not find differences between 5-day and 10-day courses of RDV therapy. The RDV data currently available are still controversial, however, dozens of clinical studies are currently using this drug as an alternative treatment for COVID-19, possibly further elucidating its effects.

The efficiency of favipiravir, another anti-influenza RdRp inhibitor, has also been clinically assessed and was approved for COVID-19 treatment in China, March 2020 (331, 332). An experimental study carried out with the VERO cell line showed that the drug has *in vitro* activity against SARS-CoV-2 (326). Aiming at comparing the effects of favipiravir and lopinavir/ritonavir, Cai et al. (333) conducted an open, non-randomized, before-after controlled study with 80 patients and found that favipiravir favored viral clearance and improved chest CT, having caused fewer adverse effects than the lopinavir/ritonavir group. Currently, 31 clinical trials using this medication are in progress.

Regarding antivirals, type I IFN is a group of cytokines comprising the α and β subtypes, among others, with an important role in antiviral immunity that interferes with viral replication, as discussed above. Many studies have shown the protective effect of type I IFN associated with antiviral therapies for patients with SARS and MERS [reviewed by Sallard et al. (334)], which arouses the interest of the scientific community in type I IFN as a potential treatment against SARS-CoV-2 (334–337). Despite their efficacy against SARS-CoV-2 (338, 339), the results of *in vitro* studies using IFN-α and -β to treat COVID-19 patients remain inconclusive.

Such uncertain nature of the results is associated with biases present in these studies, which include limited-size sample, heterogeneous experimental designs/clinical status, and the type of IFN isoform tested. In addition, since COVID-19 treatments rarely involve monotherapy, it is difficult to assess whether the results derived from the tested IFN or the drugs used in combination (322, 323, 340–343). It is also worth mentioning that, as discussed above, type I IFN appears to exacerbate inflammation in the progression to severe COVID-19; the timing of administration and subgroups targeted for treatment with type I IFN need to be considered with caution.

A recent retrospective multicenter cohort study of 446 Chinese patients with COVID-19 reported that among severe to critical COVID-19 patients, early administration (≤5 days after admission) of IFN-α2b decreased mortality in comparison with no admission of IFN-α2b, whereas no significant benefit was associated with IFN-α2b use in moderately ill patients. However, late use of IFN-α2b increased mortality and delayed recovery of severe to critical COVID-19 patients (344).

Zhou et al. (341), investigated the isolated effect of IFN-α in a cohort study comparing 77 patients with moderate COVID-19 treated with nebulized IFN-α2b (5 mU b.i.d.), oral arbidol (200 mg t.i.d.), or a combination of both. Although the study did not include a control group, the treatment with IFN-α2b, either containing arbidol or not, significantly reduced the duration of detectable virus in the upper respiratory tract and the circulating of inflammatory markers (IL-6 and C-reactive protein levels).

Still, in a retrospective multicenter cohort study with 141 mild COVID-19 patients by Xu et al. (342), the arbidol/IFN-α2b combination proved more effective in accelerating pneumonia recovery than IFN-α2b monotherapy, but this was not the case for viral clearance or reducing the length of hospital stay than IFN-α2b monotherapy.

Hung et al. (322) assessed the effect of IFN-β on COVID-19 patients and found that the triple combination of IFN-β1b, lopinavir/ritonavir, and ribavirin was safer and more effective than lopinavir/ritonavir alone at alleviating symptoms, shortening the duration of viral shedding and hospital stay in patients with mild to moderate COVID-19. Similarly, an open randomized clinical trial was carried out by Danoudi-Monfared et al. (345), analyzing treatment with IFN-β-1a. The IFN group of COVID-19 patients (n=42) received IFN β-1a in addition to the protocol medications (hydroxychloroquine plus lopinavir-ritonavir or atazanavir-ritonavir) while the control group (n=39) received only the protocol medications. The IFN-β-1a-treated patients showed a significantly increased discharge rate on day 14 and decreased mortality within 28 days. A better survival rate was also observed when patients received IFN- β-1a in the early stage of the disease.

The COVID-19 treatment guidelines of many countries already recommend the use of IFNs α/β (335). Currently, all over the world, more than 20 clinical trials are using IFN-α and/or β alone or in association with other drugs.

Chloroquine and Hydroxychloroquine

Chloroquine and hydroxychloroquine have been used worldwide for more than 70 years, and they are part of the WHO model list of essential medicines (346). They were synthesized specifically for the treatment and chemoprevention of malaria, but their immunomodulatory activity led these drugs to be used against autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and other inflammatory rheumatic diseases; they also show broad-spectrum antiviral effects (347–349).

Regarding the chemical structure, hydroxychloroquine differs from chloroquine in the presence of a hydroxyl group at the end of the side chain: the N-ethyl substituent is β-hydroxylated. Both drugs have similar pharmacokinetics, with rapid gastrointestinal absorption and renal elimination, but different clinical indications and toxic doses, in which hydroxychloroquine is less toxic and more clinically used in the malaria model (348, 350).

The action mechanism of these drugs has direct molecular effects on lysosomal activity, autophagy, and signaling pathways (347). As antivirals, chloroquine is known to block SARS-CoV-1-infection by increasing endosomal pH required for virus entry, as well as interfering with the glycosylation of cellular receptors (351, 352). The possible mechanism against SARS-CoV-2 is the inhibition of virus entry by altering the glycosylation of ACE2, reducing the binding efficiency between ACE2 in host cells and the S protein on the surface of the SARS-CoV-2, thus preventing the virus from binding to target cells (348, 351, 353). In addition

to a potent antiviral inhibition, the immunomodulatory activity of these drugs is well established in the literature. Proposed effects of chloroquine on the immune system include increasing the export of soluble antigens into the cytosol of dendritic cells, the blocking of TLR7 and TLR9 signaling, thus reconstructing $CD8^+$ cytotoxic viral response, and inhibiting and/or reducing the production of inflammatory cytokines like IL-1, IL-6, TNF, and IFN-α (347, 354–359), which has an important role in the immunopathogenesis of COVID-19, as previously reported in item 4.1.

In vitro studies on SARS-CoV-2 have demonstrated the low-dose action of these drugs, having found the lowest half-maximal effective concentrations (EC50s). In addition, their association with azithromycin significantly inhibited viral replication (326, 360–362). In humans, a study by Gao, Tian, and Yang (363) showed that patients treated with chloroquine phosphate had inhibited exacerbation of pneumonia, improving lung imaging findings, promoting a virus-negative conversion, and shortening the COVID-19 course.

The association of hydroxychloroquine with other drugs is also suggested, with emphasis on studies using azithromycin, a broad-spectrum macrolide antibiotic primarily used to treat respiratory, enteric, and genitourinary bacterial infections. Despite not yet being approved for antiviral therapy, it has been studied *in vitro* and in clinical trials for activity against several viruses (364).

Gautret et al. (365) demonstrated the effectiveness of the hydroxychloroquine-azithromycin combination in a non-randomized clinical trial with 36 COVID-19 patients. A 57.1% rate of cure was attributed to the patients treated with hydroxychloroquine, however, when combined with azithromycin, 100% of the patients were cured. The authors suggested a synergistic effect of the drug combination since both were reported to have antiviral and immunomodulatory activity in the literature.

Gautret et al. (366) conducted another analysis to provide evidence of a beneficial effect of co-administration of hydroxychloroquine with azithromycin in a non-comparative and uncontrolled observational study with 80 mildly infected SARS-CoV-2 patients. The hydroxychloroquine/azithromycin treatment showed that 81.3% of the patients had a favorable result with a rapid decrease in nasopharyngeal viral load at day 8 (93%), reducing the mean length of stay in the hospital.

Arshad et al. (367) performed a multicenter observational study, which included 2541 COVID-19 patients. Patients were separated into four groups: untreated (n = 409), treated with hydroxychloroquine (n = 1202), the association of hydroxychloroquine and azithromycin, and azithromycin only (n = 147). The authors suggested that the treatment with hydroxychloroquine alone and in combination with azithromycin was associated with a reduction in the hazard ratio for death when compared with receipt of neither medication.

However, a lot of controversy has been raised about these data, and many important limitations of this study were considered by several authors (368–373), threatening the validity of the reported findings. Among these, there is the potential for immortal time bias and selection bias, the administration of corticosteroids in most patients treated with hydroxychloroquine than in other groups, and a disproportionately high share of patients with cardiovascular comorbidity in the untreated group.

Seeking to analyze the efficacy of early treatment using hydroxychloroquine and azithromycin, Million et al. (374) carried out a retrospective study with 1061 SARS-CoV-2 infected patients. In the study, 91.7% of the patients reached good clinical results and virological cure within 10 days, while 4.3% had a poor outcome associated with advanced age. However, it is worth mentioning that the study did not include a control group to establish a comparison.

To assess the use of hydroxychloroquine as a prophylactic measure, Boulware et al. (375) performed a randomized, double-blind trial in adults who had been exposed to individuals diagnosed with COVID-19, either in the home or work environment. The authors found that postexposure prophylaxis did not prevent the development of the disease.

An important question that may be considered about chloroquine and its derivate is the numerous adverse effects reported, such as nausea, pruritus, headache, hypoglycemia, neuropsychiatric effects, and idiosyncratic hypersensitivity reactions. In long-term treatments, effects such as retinopathy, vacuolar myopathy, neuropathy, restrictive cardiomyopathy, and cardiac conduction disorders are also reported. Furthermore, its concomitant use with azithromycin may predispose patients to arrhythmias (213), which represents a major negative implication.

Huang et al. (376) conducted a randomized clinical trial with 22 patients in China to compare the effects of chloroquine and lopinavir/ritonavir. Even though chloroquine led to some clinical improvement, half of the patients experienced adverse effects such as vomiting, abdominal pain, nausea, diarrhea, skin rashes, cough, and shortness of breath.

Satlin et al. (377), Magagnoli et al. (378), Rosenberg et al. (379), and Ip et al. (380) reported that treatment with hydroxychloroquine, azithromycin, or both were not associated with a survival benefit among patients and there were no significant differences in mortality for patients receiving hydroxychloroquine during hospitalization. Similarly, Mahévas et al. (381) analyzed the efficacy of hydroxychloroquine in patients hospitalized with coronavirus pneumonia who needed oxygen but not intensive care, through a comparative observational study. 181 patients were analyzed, 84 of whom received hydroxychloroquine. Data showed there was no effect on reducing admissions to intensive care or deaths on day 21 after hospital admission and the hydroxychloroquine treatment did not have any effect on survival without acute respiratory distress syndrome on day 21 after hospital admission.

Tang et al. (382) carried out a multicenter, open, randomized, and controlled clinical trial evaluating 150 patients admitted with confirmed mild to severe COVID-19; of these, 75 were treated with hydroxychloroquine. The authors demonstrated that treatment does not contribute to the elimination of the virus.

Borba et al. (383) conducted a phase IIb, double-blind, randomized clinical trial comparing the effects of high doses (600 mg/twice daily for 10 days) and low doses (450 mg twice

daily at day 1 and once daily for 4 days) of chloroquine in 81 and 40 patients, respectively. The results did not evidence lower viral load in respiratory secretions, not even in combination with azithromycin. The mortality rate for the high-dose group was over twice as high as the low-dose group (39.0% vs. 16.0%). Additionally, some patients, mainly in the high-dose group, showed adverse effects, such as increased creatine phosphokinase (CK) and CK-MB, while the high-dosage group exhibited more corrected QT (QTc) interval prolongation. Neither of the dosages was able to influence lethality. The authors concluded that critically ill patients should not receive chloroquine at high doses.

In the meantime, a cohort study with 201 patients showed that the use of chloroquine or hydroxychloroquine combined with azithromycin generated a higher increase in QT prolongation than chloroquine or hydroxychloroquine monotherapy (384). More recently, another large observational study involving 1376 cases of COVID-19 from New York found no significant association between the use of hydroxychloroquine and intubation or death (385).

Currently, chloroquine and hydroxychloroquine are the most largely studied compounds in the context of COVID-19 treatment, encompassing at least 320 ongoing clinical trials. However, considering that more recent studies failed to prove any favorable effect of their use in COVID-19 patients, the WHO discontinued the study of hydroxychloroquine in the Solidarity Trial (13).

Antihelminthics

Amid the COVID-19 pandemic, the search for active molecules against the coronavirus should use advanced tools of computational biology and artificial intelligence for the recognition of drugs already approved and commercialized with potential effects on the replication of SARS-CoV-2 (386).

In this context, over the past few years, research has shown the antiviral potential *in vitro*, especially against RNA viruses, of Ivermectin, the best known and most widely used antiparasitic drug in human and veterinary medicine, with promising results against SARS-CoV-2 (387). The model of Vero/hSLAM cells infected with a SARS-CoV-2 isolate showed the ivermectin antiviral effect in which 24h-ivermectin treatment reduced 93% of RNA viral load in the cell supernatant and 99.8% of the intracellular viral RNA. The authors hypothesized that its probable mechanism of action occurs through the inhibition of nuclear import of importin-α/β1–mediated the IMPα/β1 heterodimer of viral proteins, as shown for other RNA viruses (387, 388). Corroborating, Lehrer and Rheinstein (389) identified the ivermectin docking site between the region of leucine 91 of viral spike and the histidine 378 of the ACE2 receptor, which may interfere with the attachment of the spike to the human cell membrane.

Although the *in vitro* proliferation inhibition effect of Ivermectin against SARS-CoV-2 has been shown, there is no evidence that the IC50 of ~ 2 μM determined by Caly and colleagues can be achieved in the clinic where pharmacokinetics studies showed that even the maximum tested dosage of 1700 μg/kg presented only 0.28μM of plasma concentration (390).

According to Navarro et al. (391), no adverse effects of high doses of ivermectin have so far been demonstrated in clinical studies with patients, with only a few transient ocular events in those who experienced high doses (up to 400 μg/kg). However, Duthaler et al. (392) demonstrated that the adverse effects of ivermectin in the body can vary according to the patient's nutritional status, and the effects of high doses can be harmful, especially in patients with malnutrition levels. The general consensus of the authors is that further studies are needed to evaluate the efficacy and safety of ivermectin administered in high doses against SARS-CoV-2.

Xu et al. (386) published a review article regarding niclosamide, an old anthelmintic used to treat tapeworm infections, showing promising antiviral activity against various viral infections, such as SARS-CoV-1 and MERS-CoV. This drug has shown to act *in vitro* by enhancing autophagy and efficiently reducing MERS-CoV replication.

Originally developed as an antiprotozoal agent, nitazoxanide is another broad-spectrum antiviral agent that has been currently developed to treat influenza and other viral respiratory infections. Nitazoxanide exhibited *in vitro* activity against MERS-CoV by inhibiting the expression of viral N protein, in addition to reducing the production of IL-6 in an *in vivo* model (393, 394).

Despite the lack of studies in the literature showing the effect of these anthelmintics on the COVID-19 model, clinical trials have currently included this type of antiviral agent in many countries; there are 37 clinical trials using ivermectin alone or associated with hydroxychloroquine, and 19 with nitazoxanide. These studies are yet to be published and preliminary results are expected in the second half of 2020.

Anticoagulants

A high mortality risk in severe COVID-19 patients has been described, especially due to the development of disseminated intravascular coagulation and coagulopathy (395). Patients with sepsis and disseminated intravascular coagulation may develop thromboembolic complications or microvascular clot deposition, contributing to multiple organ failure. In patients with severe pneumonia, the activation of vascular endothelium, platelets, and leukocytes results in the unregulated generation of thrombin, both locally, in the lungs, and systemically, leading to fibrin deposition and subsequent tissue damage and microangiopathy (396). In COVID-19 patients, severe pulmonary inflammation is believed to be associated with the regulation of pro-inflammatory cytokines, which can cause the dysfunction of endothelial cells and consequently higher thrombin production. Therefore, the use of anticoagulant therapy could be beneficial for COVID-19 patients (397).

In a retrospective study with 449 patients with severe COVID-19, Tang et al. (395) observed a lower mortality rate in individuals treated with prophylactic heparin associated with coagulopathy compared with those who had not been treated with an anticoagulant. The study associated the use of thrombosis prophylaxis with lower 28-day mortality in COVID-19 patients, but only for those presenting a high value

of either sepsis-induced coagulopathy score (≥4) or D-dimer (≥3.0 mg/L).

Paranjpe et al. (398) carried out a large cohort analysis with 2773 COVID-19 patients in the United States, among which 28% received anticoagulant therapy, and also found an association of anticoagulant-based treatment with lower mortality risk. The mortality rate in patients who required mechanical ventilation and received anticoagulant therapy was lower than those who had not been treated with an anticoagulant.

It is important to highlight that heparin has an anti-inflammatory effect that can bind to inflammatory cytokines, chemokines, and proinflammatory proteins, inhibiting neutrophil chemotaxis and leukocyte migration (399–401). In the current COVID-19 context, there are over 60 ongoing clinical trials covering the use of thromboprophylaxis, which will certainly clarify the potential role of anticoagulants in patients with COVID-19.

Dexamethasone

Recent studies have demonstrated great interest in the role of corticosteroids to attenuate the pulmonary and systemic damage in COVID-19 patients because of their potent anti-inflammatory and antifibrotic properties, especially dexamethasone, a synthetic corticosteroid which is on the list of essential medicines of the World Health Organization and is readily available worldwide at low cost. This drug acts as a broad-spectrum immunosuppressor and has greater activity in inflammatory and autoimmune conditions (402, 403).

Recently, the randomized RECOVERY study, conducted by the University of Oxford, declared dexamethasone as the world's first treatment proven effective in reducing the risk of death among severely ill COVID-19 patients. The trial accompanied a total of 2104 patients treated with dexamethasone and 4321 who received conventional care. The dexamethasone group showed reduced 28-day mortality in COVID-19 patients receiving invasive mechanical ventilation or oxygen therapy without invasive mechanical ventilation, but not in patients who were not receiving any respiratory support (404).

Similar results were published by Tomazini et al. (405) in a Brazilian multicenter, randomized, open-label, clinical trial involving 299 adults with moderate or severe ARDS due to COVID-19. The study showed that 144 patients who received dexamethasone treatment plus the standard treatment showed a significant increase in the number of days without mechanical ventilation during the first 28 days. In the same way, Villar et al. (406) also published a multicenter randomized clinical trial and showed that early administration of dexamethasone in COVID-19 patients who had moderate and severe ARDS presented an increased average number of days without mechanical ventilation, as well as reduced mortality compared to the control group. There are currently 29 clinical trials evaluating the therapeutic efficacy of dexamethasone in COVID-19 patients.

In the face of the huge amount of studies involving clinical trials to test drugs for SARS-CoV-2 and COVID-19 treatment, in addition to the different research methodologies and criteria addressed, on March 22, 2020 the WHO and partners launched the "SOLIDARITY", an international clinical trial. The purpose is to help find an effective treatment for COVID-19, seeking to establish consistent endpoints, control arms, and inclusion-exclusion criteria for this umbrella trial (13).

The SOLIDARITY trial includes hospitalized patients with COVID-19 from more than 90 countries around the world to compare treatment options with standard care and assess their relative effectiveness against SARS-CoV-2. By enrolling patients from multiple countries, the SOLIDARITY trial aims at rapidly discovering if any of the drugs mitigate disease progression or improve survival. According to the WHO director-general, the study will dramatically cut the time needed to generate robust evidence on how the drugs work. Thus, the two most promising treatment options selected were Remdesivir or Lopinavir/Ritonavir with IFN-β. Other drugs can be added based on emerging evidence (13).

CONCLUSION

In conclusion, this collection of works suggests that the genomic changes of SARS-CoV-2 are responsible for its higher transmissibility rate and severity in relation to other hCoVs. Furthermore, the process of tropism and invasion of the virus is favored by its capacity of high-affinity bonding to the human ACE2 receptor. Cytokines have a direct role in the immunopathogenesis of COVID-19 by inducing the hyper inflammation and lung injury peculiar to the disease. Benefits of IFN-mediated response seem to occur only during early infection, and the failed control of its production over time might be related to the worsening of the disease. Monocytes and macrophages have an important role in respiratory failure during COVID-19; several studies have reported that these cells migrate to the lungs, producing pro-inflammatory cytokines, like IL-6, and inducing epithelial damage. Controversially, at later stages, COVID-19 patients present an impaired immune response due to exhausted phenotype and lower effector T cells, $CD8^+$ T lymphocytes, and NK cells, culminating in antiviral immunity loss. Theoretically, the production of specific antibodies against SARS-CoV-2 by the immune system should be able to combat the virus and reduce viral load, but in critically ill patients, it does not seem to occur effectively, contributing to the severity of the disease. Unfortunately, no effective vaccines or therapeutic antiviral agents have been approved for the treatment of COVID-19 so far, but immunotherapy and some repositioned drugs originally used to treat inflammatory and coagulation disorders and viral and parasitic infections are ongoing clinical trials. This is a unique moment in science and humanity, with some mismatched information, as well as novel, important discoveries being made every day, therefore, all information must be interpreted carefully. Our review encompassed the most relevant articles in the area seeking to disseminate good-quality information.

AUTHOR CONTRIBUTIONS

TS, FT-P, RS: study design, data collection, and manuscript writing. MG, BB, MD, AR, AC, VC, and ES: data collection and manuscript writing. IC, WP, and IC-C: text correction and organization. MM-S: study design, text correction, and organization. All authors contributed to the article and approved the submitted version. The corresponding authors attest that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted.

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SYSTEMATIC REVIEW
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Trends in MERS-CoV, SARS-CoV, and SARS-CoV-2 (COVID-19) Diagnosis Strategies: A Patent Review

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The emergence of a new coronavirus (SARS-CoV-2) outbreak represents a challenge for the diagnostic laboratories responsible for developing test kits to identify those infected with SARS-CoV-2. Methods with rapid and accurate detection are essential to control the sources of infection, to prevent the spread of the disease and to assist decision-making by public health managers. Currently, there is a wide variety of tests available with different detection methodologies, levels of specificity and sensitivity, detection time, and with an extensive range of prices. This review therefore aimed to conduct a patent search in relation to tests for the detection of SARS-CoV, MERS-CoV, and SARS-CoV-2. The greatest number of patents identified in the search were registered between 2003 and 2011, being mainly deposited by China, the Republic of Korea, and the United States. Most of the patents used the existing RT-PCR, ELISA, and isothermal amplification methods to develop simple, sensitive, precise, easy to use, low-cost tests that reduced false-negative or false-positive results. The findings of this patent search show that an increasing number of materials and diagnostic tests for the coronavirus are being produced to identify infected individuals and combat the growth of the current pandemic; however, there is still a question in relation to the reliability of the results of these tests.

Keywords: coronavirus (2019-nCoV), COVID-19 (condition), MERS (middle east respiratory syndrome), SARS, ELISA (enzyme linked immuno sorbent assay), isothermal amplification, RT-PCR—polymerase chain reaction with reverse transcription

INTRODUCTION

Coronaviruses (CoVs) are enveloped positive-sense RNA viruses that belong to the Coronaviridae family, phylogenetically subdivided into the α, β, γ, and δ genera (1). β-coronaviruses include SARS-CoV, MERS-CoV, and SARS-CoV-2 (2), with these viruses being identified as the causative agents of zoonotic infections (3). The first one, Severe Acute Respiratory Syndrome (SARS), emerged in Southern China in 2003 (1). Middle East Respiratory Syndrome (MERS-CoV) appeared in Saudi Arabia, almost a decade after the SARS-CoV outbreak (1, 3). The last one, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), started in the Chinese province of Guangdong in November 2019 (4).

Commercially available CoV tests currently fall into two major categories: (A) Molecular assays for detection of viral RNA using RT-PCR-based techniques or nucleic acid hybridization-related strategies and (B) Serological and immunological assays that largely rely on detecting antibodies produced by individuals as a result of exposure to the virus or on through the detection of antigenic proteins in infected individuals (5).

CoVs may cause hepatic, neuronal, and gastrointestinal diseases (6) and various symptoms, such as lower respiratory tract disease, which can lead to progressive and potentially lethal atypical pneumonia with clinical symptoms that include fever, malaise, lymphopenia, breathing difficulty, and in some cases also diarrhea (6, 7). There are many ways to transmit the virus, including close person-to-person contact, aerosol transmission, and touch transmission. "Hidden" transmission can occur through asymptomatic infected individuals transmitting the virus (8).

The rapid and accurate detection of CoVs has been shown to be useful in preventing the spread of the disease, and also in the decision-making of public health managers (9). The first step in identifying the possible presence of the virus is through taking individuals' temperatures, and physical examination may help to identify patients with a more severe condition (6). Furthermore, samples, such as saliva, nasal swabs, trachea and nasopharynx extracts, lung tissue, sputum, feces, and blood should be isolated and used for testing (10, 11). Virus isolation and viral nucleic acid detection are the principal ways of identifying the pathogen (11). Real-time reverse-transcriptase polymerase chain reaction (rRT-PCR) has been the main CoV diagnosis method, and is characterized by rapid detection, good sensitivity, and specificity (9, 12). Although PCR is the "gold standard" for virus detection, other methods have also been developed for the detection of CoVs RNA, these include several molecular, non-PCR-based methods, such as isothermal nucleic acid amplification (Loop-mediated isothermal amplification—LAMP), and nucleic acid sequence-based amplification (9).

In addition, virus detection using methods such as immunofluorescence assay, direct fluorescent antibody assay, protein microarray, semiconductor quantum dots, MAb-based rapid nucleocapsid protein detection, and microneutralization assays, that can be used to rapidly investigate the presence of viruses, have also been proposed (6, 11). Immunoassays are particularly advantageous as they can use monoclonal antibodies to detect viral antigens in <30 min without the need for expensive instruments (6).

Detection kits can accelerate accurate diagnosis, but they have different levels of test sensitivity and specificity (8, 13). The sensitivity of a test is characterized by its ability to detect a true positive, that is, to correctly identify individuals who have the disease. On the other hand, specificity identifies true negative, correctly identifying individuals who do not have the disease (14). However, achieving the specificity and sensitivity values claimed by the tests can be affected by how the tests are applied and by how the samples are treated. Parameters that can be modified include the process for the collection of the sample, its transportation and storage, and the preparation and testing of the sample (15). The tests need to be fast and reliable to identify virus outbreak sites and enable health authorities to promote appropriate measures (16).

Thus, this review aims to assess patents that address trends in strategies for the diagnosis of those infected with SARS-CoV, MERS-CoV, and SARS-CoV-2. Through providing these data, we aim to contribute to efforts to combat the current pandemic.

METHODS

In the present patent review, the European Patent Office (EPO) and World Intellectual Property Organization (WIPO) databases were searched for titles and abstracts that contained the descriptors "coronavirus and MERS," "coronavirus and COVID," "coronavirus and 2019-nCoV," and "coronavirus and SARS." A total of 402 patents were identified for preliminary assessment from the databases, of which 224 were excluded due to being duplicates. After a careful check of the titles and abstracts, 120 patents were excluded for being outside the focus (diagnosis of the disease) of our review. A further 18 were excluded because the full-texts were not available. After reading the full patents 13 more patents were excluded for being outside the scope of the review. This selection process resulted in 27 patents being selected for our critical analysis according to the study objective. **Figure 1** illustrates the systematic search and screening strategy used in this review, which was based on the PRISMA statement.

PATENT SEARCH AND SCREENING

This review covered patents published between 2003 and 2020, a period that encompasses the emergence of SARS-CoV epidemics in Asia, MERS-CoV in Saudi Arabia, and the current SARS-CoV-2 pandemic. The largest number of patents on diagnostic methods targeting these pathogens were registered between 2003 and 2011 (19 patents) (**Figure 2A**).

China (CN) produced the most patents, with nine, followed by the Republic of Korea (KR) with six patents, and the United States (US) with four patents (**Figure 2B**). These countries are usually well-represented in the field of patent filing due to their advanced technological and scientific sectors and, in this area, their strong records of innovation in the production of disease diagnosis methods. Moreover, a large number of healthcare and biotechnology companies (including startups) are based in these countries which are world leaders in the sector of diagnostics and molecular biology (17, 18).

A patent can be applied for by several different scientific entities, including industrial laboratories, universities, and/or independent researchers. As expected, the industrial laboratory sector applied for the largest number of patents (19 out of a total of 27 patent applications) (**Figure 2C**). In the industrial sector, companies applying for patents included Gen-Probe Incorporated, Biomerieux B.V., Beijing Applied Biological Tech Co. Ltd., Adaltis Inc., the Korea Research Institute of Bioscience and Biotechnology, Shanghai Institute Biological Sciences, Mogam Biotechnology Institute, and Samsung Electronics Co. Ltd. Universities filed eight patents, sometimes in partnership with companies, and sometimes

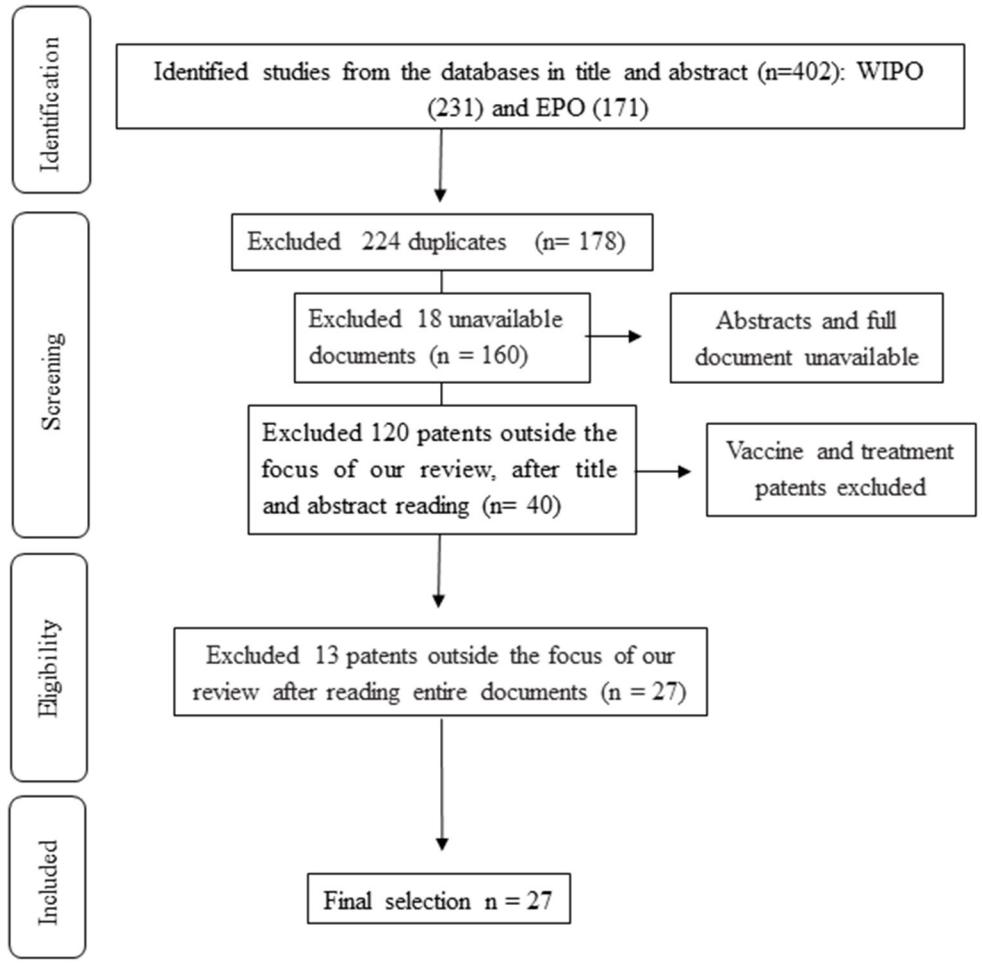

FIGURE 1 | Flowchart of patent search and screening.

independently. Of the patents identified in the review, seven were filed by independendent researchers. The cooperative process between universities and industry for the development of innovative health products and in other areas is a global trend that yields benefits for both universities and industry, and, ultimately, for society (19). Every patent filed has an International Patent Classification (IPC), which classifies the invention based on the technological area to which it belongs. In the present review, the code C12Q, which refers to *"Measuring or testing processes involving enzymes, nucleic acids or microorganisms; compositions or test papers therefore, processes of preparing such compositions; condition-responsive control in microbiological or enzymological processes"* was presented in 15 patents (**Figure 2D**), followed by C07K (Peptides) and G01N (Investigating or analyzing materials by determining their chemical or physical properties) with four patents each. The patents identified in the review used two main techniques target amplification and enzyme linked immunosorbent assay (ELISA).

TECHNIQUES AND METHODS USED IN THE PATENTS

Target Amplification Techniques

Amplification techniques seek to use different methods to repeatedly amplify certain regions of a genetic material present in the sample to detectable levels of diagnostic. Different methods improve both the sensitivity and specificity of technique, whether by adding oligonucleotides and enzymes or by controlling specific reaction conditions. Most methods are automated and provide quantitative and accurate results in a short period of time. They also eliminate the need for specialized training and reduce the risk of contamination and human error (20). These techniques can produce cost-effective, reproducible tests with high sensitivity and specificity that provide reliable diagnoses (21). There are two main amplification techniques: polymerase chain reaction (PCR), and isothermal amplification technologies (IAT), each with a number of methods that are described below.

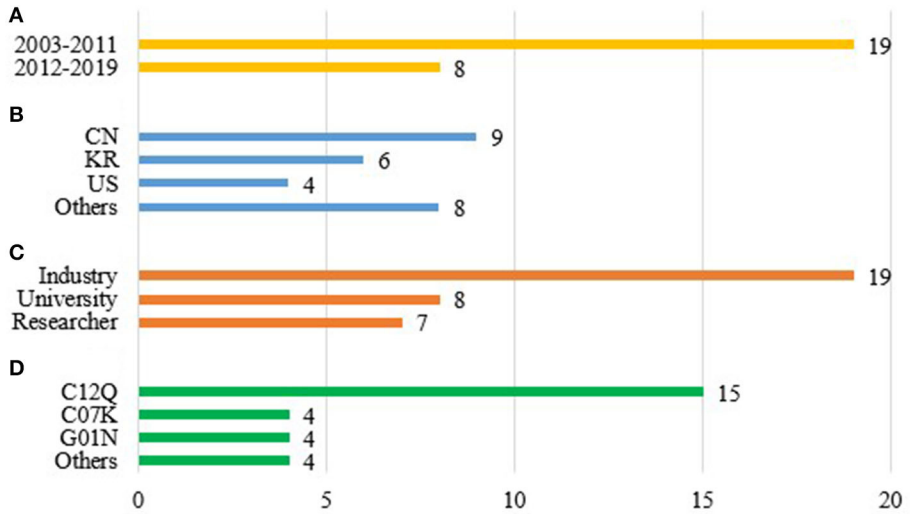

FIGURE 2 | Final selection of patents. **(A)** Publication date. **(B)** Country of patent publication. CN, China; KR, Republic of Korea; US, United States of America. Others: CA, Canada; DE, Germany; JP, Japan; NL, Netherlands; SG, Singapore; WO, World Intellectual Property Organization. **(C)** Patent applicant. **(D)** International Patent Class (IPC). Others: A61K, Preparations for medical, dental, or toilet purposes; C07H, Sugars; derivatives thereof; nucleosides; nucleotides; C12N, Microorganisms or enzymes; compositions thereof; propagating, preserving, or maintaining microorganisms; mutation or genetic engineering; culture media; nucleic acids.

Polymerase Chain Reaction (PCR)

PCR is an enzymatic method that separates the two strands of DNA to produce numerous copies of a gene, using a primer to mark the location and a DNA polymerase to continuously assemble a copy in each segment (9). Real time reverse-transcriptase PCR (rRT-PCR) is a recently developed PCR-based detection method used to detect and quantify multiple species from a sample (22). Viral antigens, viral RNA, DNA, and biomarkers can be detected using rRT-PCR blood/serum and tissue samples (23). rRT-PCR is a popular method as it has multiple advantages including its speed in providing a simple and sensitive quantitative assay (9, 24). However, there have been situations in which rRT-PCR has produced false positives, thereby limiting its clinical use in detection (25). Another recognized disadvantage of rRT-PCR is its relative high costs related to equipment acquisition, maintenance and the required reagents when compared to other methodologies (26). It is noteworthy that a lack of the reagents required for rRT-PCR during the SARS-CoV-2 outbreak has been a serious limitation on the use of this methodology, especially in developing countries (27).

The amplified product and probe melting during rRT-PCR were recognized by continuous fluorescence (25), and is measured after each cycle, with its intensity reflecting the amount of DNA in the sample at a time-specific (28). Several kinds of rRT-PCR have been developed such as multiplex rRT-PCR, which produces results based on the amplicon size of the pathogen in gel electrophoresis. However, this method exhibits some disadvantages in comparison to conventional PCR, such as the inability to monitor the amplicon size without opening the system and its incompatibility with some other platforms (29). Quantitative rRT-PCR (RT-qPCR) uses the same methodology, but the technique is more efficient than multiplex rRT-PCR, and also avoids contamination (30).

RT-PCR test kits suffer from some limitations, such as their complicated operation; long turnaround times, taking on average over 2–3 h to generate the results; their inability to function with a low viral load or with samples that have not been very carefully collected; variation in the diagnosis rate; and the requirement of expensive equipment and trained technicians to use them (31, 32). The experimental method leads to the efficiency of the technique. For example, in RT-PCR tests the number of thermal cycles can reflect in the concentration of viral RNA, through the increase of the cycles leading to higher uncertainty in the test accuracy, due to the small viral load (33). For this reason, high false-negative rates of COVID-19 infection have been reported (34).

Patents identified in the review using PCR

Table 1 presents a summary of published patents of SARS and MERS coronavirus tests. Mei et al. (39), in 2004, patented a multi-channel combined micro-fluidic chip suitable for SARS virus detection. The invention uses the PCR method to realize RT-PCR intermodal detection to provide better accuracy, sensitivity, stability, ease of operation, and fast detection. The test takes 25 min and is low-cost because the primer does not need a fluorescent label. In general, nested double PCR is used to detect pathogens; however, its operation is considered tedious and accuracy is only 40–60%. However, SARS virus can be detected at very low concentrations, with the detection limit reaching 10^{-2} copies/100 μl. The type of sample used is the patient's saliva. The microfluidic chip is based on a pipeline, a liquid containing tank, and a reaction tank arranged on a sample feeding pipeline between the liquid containing tank.

The materials that compose the PCR micro-fluidic chip such as polycarbonate (PC) plastic, quartz, and glass are resistant to light permeability. The structure of the chip comprises a plurality of liquid reservoirs, microchannels, and millimeter-sized reaction tubes or reaction wells (pools) (62, 63).

Artus Ges Fuer Molekular Biolog (40) in 2004 described a quantitative real-time PCR method for SARS detection. The kits use oligonucleotides to detect the SARS virus using a biological sample, such as body fluid (in particular sputum), feces or blood. The inventors state the invention provides an efficient, sensitive, and reliable qRT-PCR method for virus detection, and is able to detect all known variants of the virus and excluded all other near-related Coronaviridae. The method brings as an advantage, the quantitative detection of SARS-associated virus with a theoretical yield point of 10 genome, corresponding to 120 RNA copies per ml of the biological sample. Therefore, the RT-PCR method is correct in at least 95% of the cases examined.

Briese et al. (36) in 2004, developed a PCR and RT-PCR assay for SARS-CoV detection. The invention uses biological samples, such as body fluids, including cerebrospinal fluid, pericardial fluid, peritoneal fluid, saliva, serum, feces, and urine, and allows a rapid, sensitive and specific molecular diagnosis of the virus. In addition, the invention provides a synthetic nucleic acid sequence comprising 10–30 consecutive nucleotides, including the N region of SARS. Moreover, the synthesized DNA strands can subsequently serve as additional templates for the same primer sequences, and the PCR can therefore be used to detect the existence of a defined sequence in a DNA sample. The sensitivity of the method showed 500 copies in ∼100 ng of total RNA extracted post-mortem from a SARS victim.

Wu and Gao (43) in 2004 patented a target sequence to identify SARS-related CoV using a gene diagnosis technology with an RT-PCR primer, a short-handle circular ring probe, and a kit. The invention uses blood to determine the presence of the virus, in a rapidly, timely, specific and sensitive way. The detection can be done in 2 h, for 1–10 numbers of copies, which greatly improves the sensitivity of hybridization detection. Moreover, the target point selection was performed with a stable point without a mutated region having a length in the range of 120–180 bp. The invention kit includes the RNA extract, the PCR solution, a positive control, and a self-contained reagent.

The invention by Inoue and Hong (44), in 2005, provided a simple, sensitive, and specific diagnostic test when compared to three commercial tests available. A one-step PCR method rather than the usual two was used to detect SARS-CoV. This test is based on a qualitative nucleic acid amplification assay for the detection of SARS-CoV in patient samples, such as plasma, throat swab, serum, saliva, sputum, and uses specific primer pairs designed from the SARS-CoV non-structural protein 1 (NSPl), a putative proteinase. The invention provides a gel-based RT-PCR detection kit, which includes one or more primers and/or probes, and may contain a positive control nucleic acid or at least a portion, thereof comprising the NSPl region, as either RNA or DNA.

The primers used in the invention should be between 16 and 20 nucleotides in length, and the amplification product can be detected by determining the amplification product' length. The preferred part of the NSP1 region for amplification is between 4,609 and 7,003 nucleotides. Three other tests (Eiken, Artus, and Roche SARS diagnostics) were used to compare the efficiency of the invention, which provided the most sensitive detection. The invention is sufficiently sensitive to detect a few molecules of RNA in each RT-PCR reaction, with the results being acquired in hours.

Kostrikis (45) in 2005, described a molecular-beacon-based multi-allelic qRT-PCR assay for the detection and discrimination between SARS-associated and other CoV isolates in clinical samples, such as nasopharyngeal aspirate, stool or whole blood. The method comprises mismatch-tolerant molecular beacons, four sets of PCR primers for four different viral genes, and four different molecular beacons, an exogenous RNA standard that is added to the sample that can be reverse-transcribed and amplified by one of the primer sets, and a fifth molecular beacon that is labeled with a different fluorophore, specific for the exogenous RNA standard.

The multiple targets sequences of the invention are the S, E, M, and N genes in the SARS-CoV. The samples tested using the four genes showed 100% specificity. Therefore, the detection of the four target alleles in the same tube minimizes the likelihood of missing the presence of the virus in the sample, and increases the sensitivity and specificity of the method. The kit contains reagents for performing amplification reactions including PCR and also for sample pretreatment including the reagent required for CoV release and/or purification (45).

In 2005, Lim et al. (46) developed a PCR method and kit for detecting SARS-CoV. This invention provides a set of primer-CoV specific primers, and the targets were the ORF1ab, S, E, M, N genes of the virus. The primer set could specifically detect a virus without cross-reactivity with other CoV, and reducing the possibility of detecting false or false positives. PCR can be carried out in a variety of materials, such as polypropylene tubes, a 96-well plate, or in a silicon-based micro PCR chip. However, when used in a silicon-based micro PCR chip, PCR also can be carried out by thermal cycling, shortening the reaction to 30 min.

Park et al. (50) in 2006, patented a kit to detect SARS using oligonucleotides including a primer and/or a probe, designed to be more sensitive and specific than conventional tests. The invention could detect the early stage of infection using RT-PCR and biological samples such as feces to isolate and purify viral RNA. The method can mix the enzymes comprising the DNA polymerase and/or reverse transcriptase with a reaction mixture comprising the oligonucleotides, and can add an RNA specimen to the prepared mixture, and the amplifying reaction solution comprising the specimen RNA prepared using an RT-PCR process.

In 2008, Park (51) described a method using nucleocapsid or a spike protein antigen to diagnose SARS-CoV. The invention includes a solution containing SARS ATP-ceramide-N_monoclonal antibody, a chromogenic substance. The antibody comprises a monoclonal antibody, and the chromophore comprises an enzyme or gold. The method uses blood as a sample type, and IgG or N and S genes as a target to detect the virus. The patient can only be diagnosed if two different samples test positive or the same sample tests positive twice.

TABLE 1 | Published patents of SARS and MERS coronavirus tests.

References	Year	Country	Product	Test type	Target	Sample type and/or result time	Benefits
Wang (35)	2003	CN	Reagent kit diagnosis of SARS-CoV antibody	IBT	IgG	Serum sample	Comprehensive antigens, high sensitivity and strong specificity
Briese et al. (36)	2004	US	Synthetic nucleic acid sequence to detect SARS-CoV	RT-PCR	N gene	Feces and blood.	High sensitivity and specificity
Che et al. (37)	2004	CN	Monoclonal antibodies that bind to the SARS-CoV N protein	ELISA	IgG1 or IgG2b	Serum and lung tissue	High specificity, good repeatability, easy operation, low cost
Houde and Lacroix (38)	2004	CA	Diagnostic peptides for SARS-CoV	ELISA, Immunochromatography; Antigen filter test	IgG	Serum or biological fluid	High sensitivity and specificity
Mei et al. (39)	2004	CN	Multi-channel combined microfluidic chip to detect SARS-CoV	rRT-PCR	NS	Saliva. 25 min.	High sensitivity, precise, stable and easy to operate, specificity, fast detection speed, low cost
Artus Ges Fuer Molekular Biolog (40)	2004	DE	SARS-CoV detection kit	rrRT-PCR	NS	Sputum, feces, or blood.	Efficient, sensitive and reliable
Sillekens and Biomerieux (41)	2004	NL	Nucleic acid sequences as primers for detection of SARS-CoV	NASBA	N gene	Nasopharyngeal aspiration, feces, or blood	NS
Vijaysri et al. (42)	2004	CA	Oligonucleotide for detecting SARS-CoV	Amplification test	Rep gene	Hours.	Sensitivity
Wu and Gao (43)	2004	CN	Short-handled circular probe system	rRT-PCR	NS	Blood. 2 h.	High specificity and sensitivity
Inoue and Hong (44)	2005	SG	Test to detect SARS-CoV	rRT-PCR	NSP1	Plasma, throat swab, sérum, saliva, sputum. Hours.	Fast, sensitive and specific compared to available commercial tests
Kostrikis (45)	2005	US	Multi-allelic molecular detection of SARS-CoV	rRT-PCR	S, E, M and N genes	Nasopharyngeal aspiration, feces, or whole blood	High sensitivity and specificity
Lim et al. (46)	2005	KR	Primer set for detecting SARS-CoV silicone-based micro PCR chip	rRT-PCR	ORF1ab, S, E, M, N genes	30 min.	Reduces the possibility of determining false or false positives; detect SARS virus without cross-reactivity
Ma and Jie (47)	2005	CN	Antibody against a SARS-CoV NC protein	Test strip	N protein	Serum, plasma, urine, semen, saliva, sweat, tears. 10 min.	Sensitivity 10 pg/ml recombinant protein N
Minekawa et al. (48)	2005	JP	SARS-CoV detection method	RT-LAMP	ORF1ab, R2, R3	Any sample derived from human living body; 20–35 min.	High sensitivity and speed, does not require temperature control
Qin et al. (49)	2005	CN	Antigenic determinant of SARS-CoV NC protein epitope	Enzyme immunoassay	IgG and IgM	Sputum or serum	High affinity for SARS anti-virus antibodies, simple, sensitive method and high precision
Park et al. (50)	2006	KR	Oligonucleotides to detect SARS	rRT-PCR	NS	Feces	Detects virus at the initial stage. Good specificity and sensitivity
Park (51)	2008	KR	Detection of SARS by NC antigen or S protein	ELISA or PCR	IgG or N and S gene	Blood	Fast and safe
Lou et al. (52)	2009	US	Oligonucleotide for detecting SARS-CoV	All types of amplification reactions	Rep gene	Any sample that contains SARS nucleic acid. Hours.	Analytical sensitivity and specificity
Kacian (53)	2010	US	Detection probe for SARS-CoV	TMA	ORF1ab genes	Nasopharyngeal swab	Selective and sensitive detection
Jeong et al. (54)	2012	KR	Aptamer specific to SARS-CoV	rRT-PCR or ELISA	N protein or IgG	NS	NS

(Continued)

TABLE 1 | Continued

References	Year	Country	Product	Test type	Target	Sample type and/or result time	Benefits
Kaiyuan et al. (55)	2012	CN	Multiplex fluorescent PCR in tube for 5 types of CoV—OC43, 229E, NL63, HKU1 and SARS	rRT-PCR	NS	Nasopharyngeal swab	Detection of 5 types of CoV in one tube. Sensitive, fast, accurate, saves materials, and reagents
Yana et al. (56)	2017	JP	Antibodies against MERS-NP	ELISA, Immunochromatography; Antigen filter test	IgG	Serum, plasma, urine, semen, saliva, sweat, tears.	Specifically detects only MERS-CoV. Precision, speed and simplicity.
Ahn et al. (57)	2018	KR	Primer set for detection MERS-CoV	RT-LAMP	ORF1b and N gene	Sputum, lung tissue	High specificity, does not need temperature control, or expensive equipment
Wang et al. (58)	2018	CN	Primer probe set and kit for detecting SARS-CoV and MERS-CoV	RPA	NS	25 min.	Short time, good specificity, minimum detection limit, lower cost, prevention of false negatives
Zhou et al. (59)	2018	CN	Fluorescent primer MERS-CoV	rRT-RAA	NS	Throat Swab; 20 min.	Closed reaction, does not depend on PCR, tested at normal temperature 37–39°C. High specificity and sensitivity
Han et al. (60)	2019	KR	Fusion protein based on MERS-CoV NC and mAbs	ELISA	IgG	Blood, body fluid, saliva, and sputum.	Standard positive/negative control; High sensitivity and specificity.
Jeong et al. (61)	2019	WO/KR	Antibody to detect MERS-CoV binding to the fusion protein of the N-terminal and C-terminal domain fragment of the NC protein	ELISA	IgG	10–15 min	High specificity

ITB, Immunoblotting; mAbs, Monoclonal antibodies; NASBA, Nucleic acid sequence-based amplification; NS, Not Specified; NSP1, Non-structural protein 1; RPA, Recombinase Polymerase Amplification; RT-LAMP, Reverse Transcription Loop-Mediated Isothermal Amplification Method; rRT-PCR, Real time Reverse Transcription Polymerase Chain Reaction; RT-PCR, Reverse Transcription Polymerase Chain Reaction; rRT-RAA, Real Time Reverse Transcription Recombinase Aid Amplification; RT-RAA, Reverse Transcription Recombinase Aid Amplification; TMA, Transcription-Mediated Amplification; WO, World Intellectual Property Organization.
Countries: CN, China; JP, Japan; KR, Republic of Korea; TW, Taiwan; US, United States of America.

In 2012, Jeong et al. (54) formulated an oligonucleotide of a specific sequence and pharmaceutical composition as a physiologically acceptable carrier that can be used for detecting SARS-CoV. The invention provides a method of treatment and diagnosis using an oligonucleotide aptamer that has a special affinity with the nucleocapsid, and has more effect than an antibody, being smaller in size. Furthermore, the nucleotide molecule is not sensitive to temperature changes, and regenerates within a short time. The target used in this invention can be an N protein or IgG. The aptamers used in the invention include single-stranded DNA ligands because they have a high affinity and complex structure that binds to the target protein and can be identified using the systemic evolution of ligands by exponential enrichment (SELEX). The SELEX method separates high-affinity DNA and RNA ligands into the target molecules, including proteins and organic small molecules. The pharmaceutical composition may be prepared by mixing and using substances, such as lubricants, disintegrants, solubilizers, dispersants, stabilizers, suspending agents, pigments, and others. However, its use is limited as samples analyzed by this method need to be processed in certified laboratories, causing a delay in the results (64).

In 2012, Kaiyuan et al. (55) described a tube multiplex fluorescent PCR detection method for five types of human coronavirus, OC43, 229E, NL63, HKU1, and SARS, which can be used as a detection reagent for scientific research and clinical uses. The sample used in this technique is a nasopharyngeal swab, and the probes can hybridize with the nucleic acid sequence amplified by the primers.

Isothermal Amplification of the Target

Unlike PCR, isothermal amplification methods require only one temperature, thus eliminating the need for thermal cyclers (65). The method is fast, sensitive, does not require strong energy sources, and is easy to implement in service locations or in situations with limited resources. However, the method has some disadvantages, such as the challenge of designing compatible primer pairs, the potential to generate non-specific amplified products (20, 66). Target isothermal amplification technologies include nucleic acid sequence-based amplification (NASBA), transcription-mediated amplification (TMA), loop-mediated isothermal amplification (LAMP), recombinases aided amplification (RAA), and recombinase polymerase amplification (RPA), which are discussed below (67).

Introduced in 1991 by Compton, NASBA is a technique commonly used for selective amplification of RNA fragments (68). It is a transcription-sensitive system, ideal for specific replication of nucleic acids *in vitro* (69), which uses two specific oligonucleotide primers and three avian myeloblastosis virus (AMV) reverse transcriptase enzymes (70). RNAase H, RNA polymerase, and T7, together with the primers, amplify the RNA targets at 41°C (71), producing at the end of the reaction the terminal product, ssRNA, detected by methods, such as electrochemiluminescence, gel electrophoresis, sphere-based enzymatic detection, and enzyme-linked gel assay (20).

Similar to NASBA, TMA also uses isothermal amplification with the use of reverse transcriptase to produce cDNA from the target RNA. RNA polymerase then generates complementary RNA derived from cDNA, thus amplifying the original target RNA of interest. It presents fast kinetics, producing up to 1,000 copies of target RNA per reaction. The obtained amplicons can be detected by gel electrophoresis or oligonucleotide probes (20, 72). Like NASBA, it needs the reaction temperature needs to be carefully controlled to denature the secondary structures. The results from commercially available TMA assays for Human Immunodeficiency Virus (HIV), Hepatitis C Virus (HCV), and Hepatitis B Virus (HBV) are similar to those for commercial RT-PCR (73).

Over the past 10 years, LAMP has become a frequently used technique due to its effectiveness, sensitivity, and specificity for diagnosis. The method is based on the use of four specific external and internal primers responsible for amplifying nucleic acid (65). It presents fast detection (around an hour), ease-of-use, and only a single temperature for incubation (74). Several LAMP assays have been applied to detect a variety of pathogens, such as parasites, bacteria, and viruses, including influenza, Ebola, Zika, yellow fever, MERS-CoV, and SARS-CoV-2 (75). Moreover, it can be performed with a variety of samples, such as blood, urine, saliva, and semen (76).

In a study by Wang et al. (77), the LAMP assay demonstrated 100% sensitivity and specificity, with the reaction being completed in 60 min, while a RT-PCR assay required 82 min. The results obtained were visual and easy to observe. The method appeared to be a powerful tool to monitor suspected patients and risk groups through the identification of SARS-CoV-2. A study by Park et al. (78) used a non-purified sample directly with the LAMP technique because its high amplification efficiency made it possible to detect the results through colorimetric methods. Studies have already demonstrated that the RT-LAMP assay can be used for MERS-CoV detection, using primers directed to the viral N protein sequence.

RPA is characterized by a minimum need for sample preparation, a low operating temperature (37°C), the use of freeze-dried reagents, simplicity, sensitivity, selectivity, and rapid amplification (about 104 times in 10 min). This technique uses two primers and one probe, and the unwinding of the DNA and annealing of the primers uses recombinase enzymes (79, 80). It can use several samples, such as blood, serum, plasma, feces, urine. Also, as it is reagents have been freeze dried, the RPA kit can be kept at room temperature for several months (81).

RAA makes use of two primers, three specific enzymes, and three proteins to amplify DNA at 39°C in about 30 min. The enzymes include a UvsX recombinase extracted from *E. coli* to anneal the model DNA primers, single-stranded DNA binding protein (SSB), to form a D-loop structure to maintain a single-stranded state of model DNA, with the help of DNA polymerase for amplification and extension (82). In this methods, it is possible to use reverse transcriptase with or without a fluorescent probe for real-time detection of RNA amplicons (83, 84) has high specificity and sensitivity, is easy to use, and can produce a clinical diagnosis in minutes (85).

Patents using isothermal amplification of the target

In the patents identified in this review, Silleke and Biomerieux B.V. (41) used nucleic acid sequences as primers for SARS-CoV detection through NASBA. The target regions chosen for amplification correspond to the gene that encodes the SARS-CoV nucleocapsid protein. The invention also addresses the use of the methodology and the proposed primers to quantify the virus before and after therapy, through sample collection from nasopharyngeal aspiration, feces, or blood. When the analytical sensitivity of the primers was evaluated, they showed 2.5 copies of RNA *in vitro* in the amplification (41).

The 2005 invention by Mineka et al. (48) also uses a method to detect SARS-CoV through RT-LAMP by detecting ORF1ab, R2, and R3 genes in 20 to 35 min, with the presence of 2.5–10 copies. An oligonucleotide primer was first prepared that selectively hybridizes to a nucleotide sequence specific to the SARS-CoV, and then uses the LAMP method to identify the virus.

The 2010 patent by Kacian and Gen-Probe Inc. (53), describes a detection method for SARS-CoV through TMA. The tests obtained a sensitivity of 100–1,000 copies, having 100% reactivity, with an endpoint of detection of 80 copies/mL. The tests were performed using a nasopharyngeal swab as a sample. In assessing specificity and sensitivity, the detection probe did not cross-react with HIV, HBV, parvovirus, and HCoV-229E (viral nucleic acid from the human coronavirus strain).

The 2018 patent by Ahn et al. (57) used primers targeting the ORF1b and N genes in sputum and lung tissue samples to identify MERS-CoV. The method has high specificity and does not require temperature control so the equipment was relatively inexpensive. To verify the specificity of the primers in relation to MERS-CoV six different viruses were used: influenza-A (H1), influenza-A (H3), influenza-B1, influenza-B2, human metapneumovirus (MPV), and 229E were submitted to RT-LAMP. The primers amplified only the sample containing MERS-CoV, thus proving its specificity. Finally, the efficiency of LAMP was compared with that of RT-PCR assays available in the market, and was shown to have better sensitivity and a shorter reaction time.

The 2018 patent by Wang et al. (58) describes a kit to detect SARS-CoV and MERS-CoV using RPA. The specificity of the probe and primer were evaluated and no cross-reactions with other types of viruses tested were observed. The minimum detection limit was 10 copies in the SARS-CoV model and 100 copies in the MERS-CoV model. The tests were shown to have a

useful life 1 year, being one of the main advantages of this test, in addition to its low-cost and prevention of false negatives.

The patent by Zhou et al. (59) published in 2018 describes a reverse transcription RAA (RT-RAA) method for detecting MERS-CoV using a primer and a fluorescent probe using. The tests take ∼20 min and use a nasopharyngeal swab sample. It presented a detection limit of 10 copies/mL and specificity was proven by submitting common pathogens, such as influenza-A H1N1 virus, influenza-N, respiratory syncytial virus and rhinovirus to the method.

Enzyme Linked Immunosorbent Assay (ELISA)

Finally, quantitative analytical methods that perform antigen-antibody reactions through colorimetric change with the aid of an enzyme conjugate and substrate are useful for quantitative and qualitative results in respect of molecules in biological fluids. One of the most popular of these methods is the enzyme linked immunosorbent assay (ELISA) because it can be used to quantify substances at very-low concentrations (86).

This method consists of an analytical biochemical assay with high sensitivity and specificity for the detection and qualitative or quantitative analysis of an analyte without using expensive and sophisticated devices. Any substance, whether it is a specific protein or a mixture of it, can be used as an analyte. Its methodology comprises the production of monoclonal or polyclonal antibodies using antigens. Radioimmunoassay techniques, with the use of radioisotopes or fluorescence markers, are often selected to detect proteins. In the latter method, protein quantification occurs indirectly, with the absorbance of the color generated by the chemical bond due to the the presence of the dye being proportional to the amount of protein. These techniques demonstrate good sensitivity and detection limits, and ability to quantify below the nanoscale (87). A study developed by Xiang et al. (88) reported the use of ELISA in tests for IgM and IgG antibodies directed at the diagnosis of COVID-19, obtaining strong sensitivity and specificity in relation to their detection.

However, false-negative results can occur in tests based on antibody detection. The IgM antibodies are produced as part of the early immune response during the initial stage of the infection, while the IgG antibodies indicate that the disease has entered a recovery period, or may be present if there has been prior infection (89, 90). The antibody tests are used in cases where RT-PCR is negative and there is an epidemiological bond to SARS-CoV-2 infection and during the period when symptons are first presented and the viral load is high (91). The false-negative cases, in this type of test, can occur in situations where the antibodies are close to the germline, being able to bind to the SARS- CoV-2 antigens (92). Another issue that the immunological assay presents is the high incidence of false-positive cases in seronegative patients. This is probably related to inappropriate sample collection time in relation to the stage of the infection, as well as to naive IgM antibodies, which can produce an incorrect result due to the antibodies low action. However, the search for class-switched isotypes, in this case IgG, might help to decrease the risk of errors. In addition, a target antigen is also essential when the virus being tested is capable of mutating because the same viral antigens will be present in its structure (93).

Patents Using ELISA

In 2004, Che et al. (37) developed and patented a group of monoclonal antibodies belonging to IgG1 or IgG2b with specific binding capacity to the SARS-CoV N protein through a hybridoma, in addition to providing the reagent for SARS-CoV antigen deletion. In a clinical application study, the double antibody sandwich ELISA kit detected the SARS-CoV antigen in the patients' serum, obtaining high specificity and sensitivity. There was no cross-reactivity with cell cultures that were not infected with SARS-CoV.

Houde and Lacroix (38) in 2004, created an *in vitro* diagnostic method for detecting the presence or absence of antibodies indicative of SARS-CoV by binding them to a peptide, or analog of it, to form an immune complex. In the ELISA assay the peptide was adsorbed or covalently coupled in wells of a microtiter plate treated with the serum, or the biological fluid to be tested. After washing with anti-human IgG or anti-human IgM, IgA was labeled with peroxidase and added to the wells and for peroxidase determination with a corresponding substrate. Clinical samples can range from cultured cells, cell supernatants, cell lysates, serum, plasma, biological fluid to tissue samples.

In 2005, Qin et al. (49) developed the SARS-CoV nucleocapsid protein epitope. This polypeptide has a high affinity for SARS anti-virus antibodies and the antibody developed in the invention has a high affinity for SARS-CoV. The detection method was reported to be simple, sensitive and with high precision. It uses IgG and IgM and serum or sputum samples from patients to detect the virus. Park (51) formulated a method to diagnose SARS-CoV using a nucleocapsid protein antigen (SARS-CoV-N) or spike protein (SARS-CoV-S). The method uses HRP-conjugated human anti-IgG antibody or the SARS-CoV-N monoclonal antibody mixed with a sample containing SARS-CoV to adsorb the SARS-CoV-N antibody. Finally, in 2019, Jeong et al. (61) and Han et al. (60) developed an antigen for MERS-CoV diagnosis using the NC fusion protein which included a fragment of the N-terminal domain and a fragment of the C-terminal domain of the CoV N protein. They used IgG and compared their innovations with a kit already available in the market from Euroimmun®. The invention by Han et al. showed positive results for human serum diluted 128 times, while the commercial kit showed positive results only in serum diluted 16 times. This patent used blocking ELISA, and as samples blood, body fluid, saliva, and sputum. Although both patents revealed high specificity, the latter one exhibited higher sensitivity (60).

DIAGNOSTIC TESTS FOR COVID-19

A similar search of Google Patents was performed using the keywords "SARS-CoV-2 and diag*" with the IPC C12Q for patents published from January 2020 for tests to identify the new coronavirus. This allows current trends in this area to be highlighted, and similarities and differences in the patented tests for SARS-CoV-1 and MERS-CoV to be compared with those

TABLE 2 | Published patents of SARS-CoV-2 (COVID-19) tests.

References	Year	Country	Product	Test type	Target	Sample type and/or result time	Benefits
Xu et al. (94)	2020	CN	Novel nucleic acid kit for rapidly detecting SARS-CoV-2	Real-time fluorescent PCR	NS	Throat swab, nasopharynx extract, sputum. 2 h	Simple, economical, reduction of cross contamination.
Gu et al. (95)	2020	CN	Primer pair with mutation resistance	Real-time fluorescent quantitative PCR	N gene	Throat swab, alveolar lavage, saliva, blood, urine, and feces	Avoids phenomena of sensitivity reduction and false negatives
Yan et al. (96)	2020	CN	COVID-19 nucleic acid detection kit	Multiple fluorescence PCR	ORF1ab gene	Pharyngeal swab, sputum, and alveolar lavage fluid. 70 min	Good sensitivity and specificity
Wang et al. (97)	2020	CN	Novel micro-drop digital PCR kit	Digital PCR	ORF1ab and N genes	NS	Stability, repeatability, detection of low viral load, reduction of false negatives
Song and Baek (98)	2020	KR	Primer sets for detecting SARS-CoV-2	Isothermal amplification	N gene	90 min	NS
Wan et al. (99)	2020	CN	Rapid detection kit for SARS-CoV-2 dry powder LAMP	LAMP	ORF1ab and N genes	One or 2 h	Detection reagent storage at room temperature
Cui et al. (100)	2020	CN	Novel rapid detection kit for SARS-CoV-2	LAMP	ORF1ab gene	30 min	Good specificity, sensitivity, and visual identification of the result

related to SARS-COV-2. **Table 2** shows the main patents found and the characteristics of each invention.

Some of the patents identified also used PCR methods similarly to the previous patents. In one of the inventions, Xu et al. (94) published a SARS-CoV-2 rapid detection kit was developed using the fluorescent RT-PCR method and a hydrolysis probe. The kit consists of the probe, primers and a positive and negative control for detection. Three fragments of reverse transcription of segments of the virus and complementary human DNA were used as the positive and negative controls, respectively. Among the advantages of the kit described by the inventors are simplicity of use, savings on reagents and a reduction in cross contamination. The detection time was around 2 h, demonstrating good specificity by using three regions of the virus for amplification and detection. Samples obtained by pharyngeal swab, nasopharynx extract and sputum can be used in the kit [A].

Gu et al. (95) described a primer pair invention for detecting viral RNA of the new coronavirus by quantitative fluorescence PCR. The nucleocapsid gene is the target of amplification in this invention, as it allows high detection due to its low molecular weight and the generation of a high number of amplified copies. In addition, the developed primer is resistant to mutation of the virus, thereby avoiding reduced sensitivity and the generation of false negatives. The method allows the use of several samples, such as pharyngeal swab, alveolar lavage, saliva, blood, urine and feces.

Using the same PCR methodology of multiple fluorescence, Yan et al. (96) developed another kit was to simultaneously detect several SARS-CoV-2 genes. It has a 70-min detection process and can be used with pharyngeal swabs, sputum, and alveolar lavage fluids. The test showed a minimum detection limit of 2 pg/mL. Another patent by Wang et al. (97) used a digital PCR micro-drop kit to detect SARS-CoV-2 by amplifying the ORF1ab and N genes. The advantages of this test are described as being the production of results with a more direct interpretation, and greater detection sensitivity than quantitative PCR, as well as high stability, repeatability, low viral load detection capacity and a reduction in false negatives.

Other patents related to COVID-19 used isothermal amplification methods, kits and components for the detection of SARS-CoV-2 were also patented. Song Min-Seok and Baek Yoon-hee (98) developed a set of primers to allow the detection of the virus by any method of isothermal amplification within 90 min from the nucleocapsid gene. In the same vein, another invention by Wan et al. (99) presented a kit for rapid detection using the LAMP method, having as target genes ORF1ab and N. In this invention, the detection reagent was obtained by freeze drying, enabling its transport at room temperature rather than in the severe storage conditions at −20°C that these reagents often need to be kept in. The kit has a detection time of around 1–2 h and can be read by the naked eye. The LAMP method was also used in another invention by Cui et al. (100), using the ORF1ab target gene. The applied methodology makes it possible to identify the presence of the virus by a change in the color of the sample. After a detection time of 30 min, a bright green color indicates a positive result, while yellow/orange a negative result.

Alternative Test Methodologies

Peptide-based magnetic chemiluminescence enzyme immunoassay (101, 102) is another method with good sensitivity but was not found in the search for patents. It can also be used in combination with rRT-PCR, having a rate positivity for IgG and IgM of 71.4 and 57.2%, respectively. Therefore, combining

this immunoassay with real-time RT-PCR may enhance the diagnostic accuracy of COVID-19 (103–106).

Regarding the ELISA method, it is usually well-used, due to its practicality, low-cost and easy execution, ideally with each country developing with its own technologies, purifying its local antigens for good test performance (107–109).

Other methodologies not found in our survey, but with promising features are CRISPR-based methodologies (110–113), lateral flow immunoassay (5), viscoelastic testing (114), and biosensors for COVID-19 (115), being conceived as alternatives to the usual methods. In the patents identified, isothermal amplification proved to be a faster, simpler, and more sensitive method for detecting SARS-CoV-2 (116–119).

Possible Limitations of Patented Tests

We know that antibody testing is necessary, but reagent IgG results may not guarantee new positive results for rRT-PCR, and further studies are needed to demonstrate protection against COVID-19 in reagent IgG patients (109, 120). There is a huge range of tests, but they are being used with literature-based criteria, because only mass testing can guarantee criteria for opening and closing cities (47, 121–125). The specificity and sensitivity of the tests described in this review may change due to environmental factors. According to Younes et al. (126), in ELISA assays, false-positive results may occur because protein N is the most conserved viral protein among human beta-coronaviruses. Thus, the antigens used in the kits can produce inaccurate results. Among the other possible reasons for inaccurate results are cross-linking with other coronaviruses (MERS-CoV, SARS-CoV-1) or because the antigens used have the ability to react with viruses responsible for the common cold (HKU1, 229E, OC43, NL63) in the winter when they circulate in large quantities (126). To circumvent this problem, diagnostic methods have been improved with the use of the spike protein, which detects two domains of protein S (S1 and S2) (127).

In a study by Yang et al. (128), LAMP demonstrated similar sensitivity to PCR, and specificity of 99% in the 208 clinical samples tested. This was due to the use of six to eight initiators to identify different regions. Despite being considered the gold standard, PCR is susceptible to environmental factors that can cause changes in the parameters discussed. Issues related to viral load, and slow or no antibody response can interfere in the results. Its use is recommended from the third day of symptom onset, when there is a high viral load (129, 130). Interestingly there is also a noticeable lack of technology-based products coming from developing countries and even from countries in Europe that normally play an important role in the development of diagnostic products. Thus, there is a need for greater investment to develop practical, fast and reliable technologies that can be used to provide the mass testing required to win this battle against COVID-19. Moreover, the current public patent knowledge can be a limiter in the development of new pharmaceutical product or processes, and at a particular time like a COVID-19 outbreak, so these barriers should be on the ground. Thus, these gaps in knowledge only create more doubts that build a foundation (131).

STRENGTHS AND LIMITATIONS

Among the strengths of this study is the focus on patents in the review, which provides an overview of the situation and growth trends in a particular area of knowledge or product of interest. In addition, patents often have technological information that is not found in its entirety in articles, as companies are careful to protect their inventions, and this can provide a better overall understanding of the tests. Regarding the limitations of this review, some innovative diagnostic methods are not patented immediately, with authors preferring to have their data published quickly by means of scientific articles. Thus, most of the patents found for the diagnosis of COVID-19 are based on known methods such as PCR, isothermal amplification and ELISA. The potential bias in the identification and inclusion patent can occur because to the 18-month confidentiality period that patent offices grant to inventors. However, the search was carried out on the relevant patent databases and using comprehensive search terms and a careful selection process, in order to avoid this risk.

CONCLUSION

It is known that making a fast and reliable diagnosis of a disease is of paramount importance to take fundamental measures for the control and treatment of the disease. This is particularly so in the case of viruses, especially CoVs, which affect humans in a number of different ways and can require rapid and special care in case of infection. We describe the patents that contain diagnostic methods focused on CoV, essential for the detection of SARS-CoV, MERS-CoV, and SARS-CoV-2. We also presented in this review some data from studies of trials already carried out by researchers as well as from patents aimed at other infections caused by CoV. Molecular methods, such as RT-PCR, ELISA, and isothermal amplification technologies positively contribute to simple, fast, sensitive, specific, and low-cost tests. The knowledge obtained with other types of CoV can contribute to the fight against COVID-19 and the current pandemic.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

JN, AS, and AO analyzed and interpreted the data and performed the draft. AG, LQ-J, and LB did the critical review of intellectual content. MS realized the conception and design of the manuscript. HC and NM contributed to the final draft of the manuscript and with resources for the

research. All authors contributed to the article and approved the submitted version.

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REVIEW
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Systems Biology Approaches for Therapeutics Development Against COVID-19

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Understanding the systems biology approaches for promoting the development of new therapeutic drugs is attaining importance nowadays. The threat of COVID-19 outbreak needs to be vanished for global welfare, and every section of research is focusing on it. There is an opportunity for finding new, quick, and accurate tools for developing treatment options, including the vaccine against COVID-19. The review at this moment covers various aspects of pathogenesis and host factors for exploring the virus target and developing suitable therapeutic solutions through systems biology tools. Furthermore, this review also covers the extensive details of multiomics tools *i.e.*, transcriptomics, proteomics, genomics, lipidomics, immunomics, and *in silico* computational modeling aiming towards the study of host–virus interactions in search of therapeutic targets against the COVID-19.

Keywords: systems biology, multiomics, *in silico*, database (DB), COVID-19, coronavirus, pathogenicity

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INTRODUCTION

COVID-19 (coronavirus disease 2019) outbreak is caused by an animal virus belonging to the family Coronaviridae (Ahmed et al., 2020; Cascella et al., 2020). This animal virus is transmitted to humans and causes severe respiratory syndromes (Mohd et al., 2016; Fung et al., 2020). The associated syndromes are Middle East respiratory syndrome (MERS) (Wernery et al., 2017), severe acute respiratory syndrome (SARS) (Lai et al., 2020), acute respiratory distress syndrome (ARDS) (Wu et al., 2020b), and most recently coronavirus disease (COVID-19). With the outbreaks related to the above mentioned coronavirus related syndromes, it is evident that human pathogenic coronavirus related mutants and strains occur and emerge from infected animal livestock during the past decade (Deng and Peng, 2020). In the present situation, COVID-19 has been spread globally (Khachfe et al., 2020). This crisis started in China in December 2019. The Wuhan market (China) was associated with animals and their meat products for domestic cooking purposes. The consumption of coronavirus infected meat products by nearby local people is the starting point of the pandemic (Schwartz and Graham, 2020). Within one month approximately, 9,066 positive coronavirus infected cases were found, and 213 patients died till January 2020 (Riou and Althaus, 2020). Moreover, the cases increased continuously at multiple rates around the world, leading to a global health emergency. The coronavirus outbreak has proven a threat to

humanity. The coronavirus associated with COVID-19 shows 75–80% similarity with the severe acute respiratory syndrome coronavirus (SARS-CoV) and is more directly connected to numerous bat coronavirus. Unlike other coronaviruses, COVID-19 grows better in epithelial cells of human airway rather than in the cultured cells in the laboratory. It uses human angiotensin-converting enzyme 2 as its cellular receptor, so the infection is transmitted only after the infection of the lower respiratory tract (Perlman, 2020). The novel coronavirus causes severe respiratory disease, COVID-19. The patients suffer from pneumonia. They develop a cold, dry cough and a sudden rise in body temperature (Zhu et al., 2020). Human to human transmission of COVID-19 occurs through respiratory droplets of the sneezed particles or from the close contact of an infected person (Chen, 2020). The WHO (World Health Organization) is currently engaged in managing the pandemic situation with nations around the world by releasing guidelines for health workers. For the novel coronavirus 2019, the unavailability of vaccine tenders the importance of antiviral drugs and therapeutics for pandemic control programs and preventive measures in pandemic reoccurrence (Sohrabi et al., 2020). The current outbreak can be controlled by maintaining social distance and reducing the person to person transmission. The immediate step required to control disease (Archana et al., 2015) outbreak includes isolation, early diagnosis, and other supportive treatment (Czernin et al., 2020). It can also be preventive by maintaining personal hygiene, avoiding crowded places, wearing of fitted masks, and ventilation. Special measures should be taken for the children, old age, and immuno-compromised people as they are more prone to COVID-19 (Marchand-Senécal et al., 2020). The therapeutic drugs available to clinical workers for the treatment of coronavirus infections are only as a temporary option. This new demand gives the opportunity for researchers to save humankind from this menace. The SARS viruses are difficult and quite dangerous to handle *in vivo*, but the information of their genes, proteins, or the RNA acquired by sequencing is simple and easy to handle through artificial intelligence. The expectation from systems biology for therapeutic agent development is mentioned. Furthermore, the role of different components of multiomics is discussed for virulence assessment of coronavirus. Along with the importance of artificial intelligence in generating data for drug development and the requisite of data mining from the database, the *in silico* appeal for host–virus interaction study (*viz*. protein–protein interaction, computational modeling) and vaccine development are also described. Molecular docking studies have been used for the detection of medications to inhibit SARS-CoV-2 spike protein and protease enzyme in the past. Thus, molecular docking can pave the way for computational drug designing, which can further be utilized for the treatment of COVID-19 (Hall and Ji, 2020).

PATHOGENESIS AND VIRULENCE STRATEGY

Coronavirus is an enveloped and single-stranded RNA virus. It is classified into four categories: α-coronavirus, β-coronavirus, δ-coronavirus, and γ-coronavirus (Yang and Wang, 2020). Earlier, there were six coronaviruses that infect humans and cause diseases. Despite the SARS-CoV and MERS-CoV, COVID-19 is caused by the seventh member of the coronavirus family to infect human, the novel SARS coronavirus and SARS-CoV share almost 79% genome similarity (Dawood, 2020). Like SARS-CoV and MERS-CoV, COVID-19 is considered in the family of β-coronavirus (Guo et al., 2020). These two (SARS-CoV and SARS-CoV-2) have identical domains for receptor binding and use angiotensin-converting enzyme 2 (ACE2) as the receptor. Significantly, S protein present on the surface is responsible for the identification of the receptors in the target host, facilitating the entry into the host cell (Hofmann and Pöhlmann, 2004). The binding efficiency of SARS-CoV-2 is ten times higher than the SARS-CoV. This shows that ACE2 could be a possible candidate for treatment (Wang X. et al., 2020). The fact that there is less information about the pathogenesis of SARS-CoV-2 and also that systems biology and omics technology cannot cover every specific cellular or physiological process for hindering the virulence strategy of virus are the main limitations. Patients suffering from COVID-19 have symptoms similar to SARS-CoV and MERS-CoV like fever, fatigue, non-productive coughs, myalgia, pneumonia, and decrease leukocyte count (Daga et al., 2019). Metabolic acidosis showing dysfunction of microcirculation was also observed. Additionally, kidney and liver functions were also affected in some patients. The blood and lower respiratory tract specimen cultures turned out to be negative for bacteria and fungus in 76% sepsis patients in a COVID-19 cohort. Therefore, viral sepsis would be more accurate to describe the clinical manifestations of severe or critically ill COVID-19 patients. Understanding the mechanism of viral sepsis in COVID-19 is warranted for exploring better clinical care for these patients (Zhou et al., 2020).

Therefore the pathogenesis mechanism of SAR-CoV and MERS-CoV will help in understanding the pathogenesis of SARS-CoV-2. Significantly spike proteins are determined to facilitate the entry of the virus into the host body (Xia et al., 2020). They bind to the receptor of the cells like ACE2 and CD209L. Initially, it was reported that the virus enters the cell by the fusion of the virus with the plasma membrane. The proteolytic cleavage of spike protein at position S2′ is essential for membrane fusion following viral infection (Belouzard et al., 2009; Walls et al., 2020). While in MERS-CoV, the membrane fusion was initiated by furin activation. Besides, SARS-CoV-2 uses clathrin-dependent and independent endocytosis methods to enter into the cell (Wang et al., 2008). The virus releases RNA genome into the cell to begin the process of replication. The glycoproteins form to facilitate the formation of the nucleocapsid. The germination of the virus particle takes place in an endoplasmic reticulum golgi intermediate compartment (ERGIC) (Risco et al., 2002). Afterward, the virus particle fused to the plasma membrane to release.

After entering into the host cell, the antigen peptides presented by major histocompatibility complex (MHC) and virus-specific

cytotoxic T lymphocytes (CTLs) help in the identification. Therefore, the knowledge of antigen presentation of SARS-CoV-2 will significantly assist in interpreting the pathogenesis of COVID-19 (Prompetchara et al., 2020). As there is less information about antigen presentation for COVID-19, so the information behind SARS-CoV and MERS-CoV will significantly help the researchers in planning the methodologies (Yuen et al., 2020). Mainly SARS-CoV involves MHC I molecules for antigen presentation and also susceptible to different HLA (Human Leukocyte Antigen) polymorphisms. Most of the alleles like HLA-A∗0201, HLA-DR0301, and HLA-Cw1502 help in the protection from SARS disease. The alleles, like HLA-DRB1∗11:01 and HLA-DQB1∗02:01 are more sensitive to MERS-CoV disease (Risco et al., 2002). Nguyen et al., 2020 studied the binding efficiency of HLA and mentioned that HLA-B*15:03 is responsible for maximum binding with conserved peptide of SARS CoV2. Additionally Wang W. et al. (2020) applied next generation sequencing method and found that HLA-C*07:29 *and* B*15:27 are highly significant in COVID-19 infected patients.

The mannose-binding lectin (MBL) is an important molecule in innate immunity and starts its function before the response of a specific antibody (Ip et al., 2005). People infected with COVID-19 have low level of MBL in their serum as compared to healthy ones. It is observed that MBL is associated with antigen presentation and is also linked with the infection of SARS (Mason and Tarr, 2015). Furthermore, this evidence will be a helping hand in understanding the mechanism of SARS-CoV-2 infection. In comparison with the humoral response, cellular immunity is more significant in the case of coronavirus (Wang F. et al., 2020).

Immune dysfunction such as severe respiratory failure is observed in COVID-19 patients in a case study by Giamarellos-Bourboulis et al. (2020). The macrophage activation syndrome, less human HLA-DR expression along with the reduced number of CD4 lymphocytes, natural killer cells, and CD19 lymphocytes were shown in severe respiratory failure along with sustained production of TNF-α and IL-6 (Magro et al., 2020). The inhibition of HLA-DR expression was performed by plasma of COVID-19 patients, and it could be partially restored *via* IL-6 blocker. Herein, IL-6 based HLA-DR expression is a characteristic feature and deals with hyper inflammation and cytokine production. Generally SARS-CoV-2 causes hyper inflammation by impairing the host immune response and subsequently dearranging the renin–angiotensin–aldosterone system (Henry et al., 2020a). Acute lung injury and coagulopathy were caused by an imbalance in RAAS (renin–angiotensin–aldosterone system) and hyper inflammation. RAAS is an essential hormone system that performs the function of blood pressure regulation and is also helpful in balancing the fluid within the body. Moreover, it would result into fibrinolysis, immunothrombosis, and multiple organ damage (Henry et al., 2020a). The patients in later stage have deteriorated conditions and die within a short period of time because of organ failure and acute respiratory distress syndrome. All these happen due to cytokine storm and it is significant in increasing the symptoms. Cytokine storm was also validated by clinical studies studying critical patients. Hence, the suppression of cytokine storm is another way to treat COVID-19 infected patients (Ye et al., 2020).

Host Factors

During the 2002–2003 SARS epidemics, the human population got infected from the cross-transmission of civet, raccoon, and Chinese ferret-badger. Initially, the animal handlers got infected from the wet market (Perlman and Netland, 2009). Although they do not have any symptoms of SARS-CoV during detection, their serum gave a high positive result. The infection rate increases when a physician gets infected by treating them, and consequently, the epidemic started. The genetic analysis of the isolated virus reveals its fast rate of adaption in the host cell. It was found in the live animal market and isolated from *Rhinolophus* spp. Hence the virus is transmitted from bats to mammals and then to humans (He et al., 2014). In civets and humans, the virus gets entry through the ACE2 receptor, which was not observed in the case of the bat. Apart from its wild ruminants, canine and feline were also susceptible to contamination with the same virus (Malik et al., 2020).

The study by sequence data analysis and molecular biology reveals that approximately 60 novel bat coronaviruses were found in Africa, North America, Europe, and China (Hu et al., 2015). This strain probably originated from the same source and got diverted based on adaption in a different host. The coronavirus isolated from *Delphinapterus leucas* was also categorized in sub-group, infecting mammals. The spike protein of SARS-CoV and new SARS-CoV-2 shares around 76.5% amino acid identity (Zhang et al., 2020). The coronavirus can quickly enter into the host cells *via* spike protein. The spike protein undergoes cleavage before entering into the target cell. Mostly, SARS-S involves angiotensin-converting enzyme 2 (ACE2) for receptor and also incorporate TMPRSS2, a cellular serine protease that is clinically proved to block the entry of the virus into the cell (Hoffmann et al., 2020). The study about SARS-S/ACE2 elucidated that it contributes an essential role in virus transmission, pathogenesis, and target as a therapeutic agent. SARS-CoV mainly infects pneumocytes and macrophages present in the lungs (Liu J. et al., 2020). Apart from lungs, ACE2 was also expressed in the extra-pulmonary surface. SARS-CoV-2 enters the cell by membrane diffusion and slows down the regulation of ACE2 receptor (Qian et al., 2013; Silhol et al., 2020). TMPRSS2 based entry of SARS-CoV-2 has been observed when cleavage of the S1/S2 site is mediated by furin in the virus-infected cell (Coutard et al., 2020). Thus, it can be deciphered that TMPRSS2 is a vital host factor responsible for COVID-19 spread like coronavirus and influenza A viruses. Iwata-Yoshikawa et al. (2019) reported that TMPRSS2 is a drug target as it contributes to the development of homeostasis. The Camostat mesylate is a serine protease inhibitor that can be sufficient to block the function of TMPRSS2, and Japan approved it for humans (D'Amico et al., 2020). Therefore, the above-discussed compounds possess antiviral activity and were suggested for the treatment of COVID-19. In SARS-CoV, lysosomotropic agents make the infection more severe and sensitive by disturbing endosomal pH (Docea et al., 2020). In

SARS-CoV infection, mainly protease treatment nullifies the effect of blocking mediated by lysosomotropic-agent. Cathepsin L, which is an endosomal protease, can also block the SARS-CoV-2 infection (Liu T. et al., 2020) and is also significant in triggering the membrane fusion and is one of the extraordinary phenomena in the pathogenesis of SARS-CoV (Smieszek et al., 2020). The age is also an important factor in COVID-19 infection. In a clinical research, Lighter et al. (2020) reported that people more than sixty years are at higher risk of the infection. However, Jin et al., in a study found that men are more prone to the infection and their surviving potency is lower than females (Jin et al., 2020). Conditions of the patient become more severe if they are suffering from other diseases like hypertension, respiratory disorder, and cardiovascular diseases. Involvement of these diseases may enhance the mortality in this case (Yang Y. et al., 2020).

Drugs Available and Treatment

Presently, no specific drug and vaccine are available to combat COVID-19 infection. Although, various drug compounds are in the experimental and trial pipelines till now. EIDD-2801 is one of the potential clinical candidates for seasonal and pandemic influenza (Hampton, 2020). Hence, it can be suggested as a potential drug to treat COVID-19 only after clinical trials. Besides the implementation of neuraminidase inhibitors, RNA synthesis inhibitors, Lopinavir/Ritonavir, and peptide (EK1) can be used for its treatment (Rothan and Byrareddy, 2020). However, they are not sufficient to combat SARS-CoV-2 infection. According to recent reports, the antiviral remdesivir and chloroquine have safe records and can be efficiently implemented to treat COVID-19 infection (Zhang and Liu, 2020). Initially, it was suggested by the Washington Department of Health to use remdesivir intravenously to protect against COVID-19. Remdesivir is sufficient to block RNA synthesis by targeting RNA-dependent RNA polymerase and is being potentially used as an antiviral drug for the various RNA viruses (Patankar, 2020). Subsequently, remdesivir and chloroquine were implanted to treat COVID-19 infection. Favipiravir, ribavirin, and galidesivir are the nucleoside analog to be potentially used. Non-structural proteins *i.e.*, chymotrypsin and papain-like protease, are required for virus replication and host immune response inhibition (Chen Y. W. et al., 2020). Inhibitors against them like cinanserin, flavonoids, and PLP inhibitors can be alternatively used for the treatment of the disease. More novel therapeutic agents are urgently required globally to fight against it. Furthermore, a list of non-specific drugs available to cure COVID-19 infection is mentioned in **Table 1**. Alternatively, some antiviral, *i.e.*, nucleoside analogs and HIV-protease inhibitors, can be used to attenuate coronavirus viral infection. The treatment course included various drugs such as oseltamivir, lopinavir, and ritonavir. Along with the intravenous administration of ganciclovir, the patients are advised to take them twice a day for 3 to 14 days (Tobaiqy et al., 2020). However, in first-line treatment, paracetamol is used to treat fever, and expectorants (guaifenesin) should be given for non-productive cough. Oxygen therapy is required in critical conditions like severe acute respiratory infection and hypoxemia. The oxygen supply rate is 5 L/min in most of the children and non-pregnant women. However, in pregnant women, the supply rate is more than 92–95% (Huang et al., 2020). Patients suffering from AKI (acute kidney injury) should be subjected to renal replacement therapy. Antibiotic therapy starts within one hour after the confirmation of the symptoms. The bacterial and fungal infections can occur in the patients in the late and middle stages of diseases. So, it should be advisable to follow conventional and rational antibiotics followed as precision medicine applicable to a patient's condition under critical care units (**Figure 1**). The implementation of IFN-α and lopinavir/ritonavir is recommended by the National Health Commission of the People's Republic of China (Wang and Zhu, 2020). The implementation of the above medicines reduces the mortality rate in SARS infected patients (Chu et al., 2004). Methylprednisolone may also consider the children for a maximum of five days (Mouton et al., 2020). The patients suffering from the severe immune response are advised to take glucocorticoids. Different vaccine types such as subunit vaccines, attenuated viruses, and viral vector-based vaccines, inactivated

TABLE 1 | List of proposed therapeutic agents for the treatment of COVID-19.

S.No.	Proposed Drugs	Action of mechanism	References
1.	Ribavirin	Inhibit RNA synthesis	Khalili et al., 2020
2.	Ritonavir	Inhibit HIV viral proteinase enzyme	Cheng et al., 2020
3.	Methylprednisolone	Activation of specific nuclear receptors, alter gene expression and inhibit cytokine production	Yang Y. et al., 2020
4.	Hydrocortisone	Inhibitor of neutrophil apoptosis, phospholipase A2, NF-Kappa B	Russell et al., 2020
5.	Mycophenolate mofetil	Inosine monophosphate dehydrogenase inhibitor	Seminari et al., 2020
6.	Hexamethyleneamiloride	Inhibitor of HCoV-229E and inhibit replication of parent coronaviruses	Farag et al., 2020
7.	Chloroquine	Increase endosomal pH for virus/cell fusion, and interfere with glycosylation of cellular receptors of SARS-CoV	Wang M. et al., 2020
8.	Chlorpromazine	Inhibit clathrin-mediated endocytosis	Zumla et al., 2020
9.	Amodiaquinedihydrochloride	Heme polymerase activity inhibition	Lee et al., 2020
10.	Lycorine	Cell division inhibition, antineoplastic and antiviral	Liu T. et al., 2020
11.	Emetine	RNA, DNA, and protein synthesis inhibition, antiviral	Bleasel and Peterson, 2020
12.	Mycophenolic acid	Inhibitor of inosine-5′-monophosphate dehydrogenase.	Weißbarth et al., 2020
13.	Pyrviniumpamoate	Mitochondrial respiration complex 1 inhibition and suppression of unfolded protein response	Jeon et al., 2020
14.	Remedisivir	Nucleic acid inhibition	Choy et al., 2020

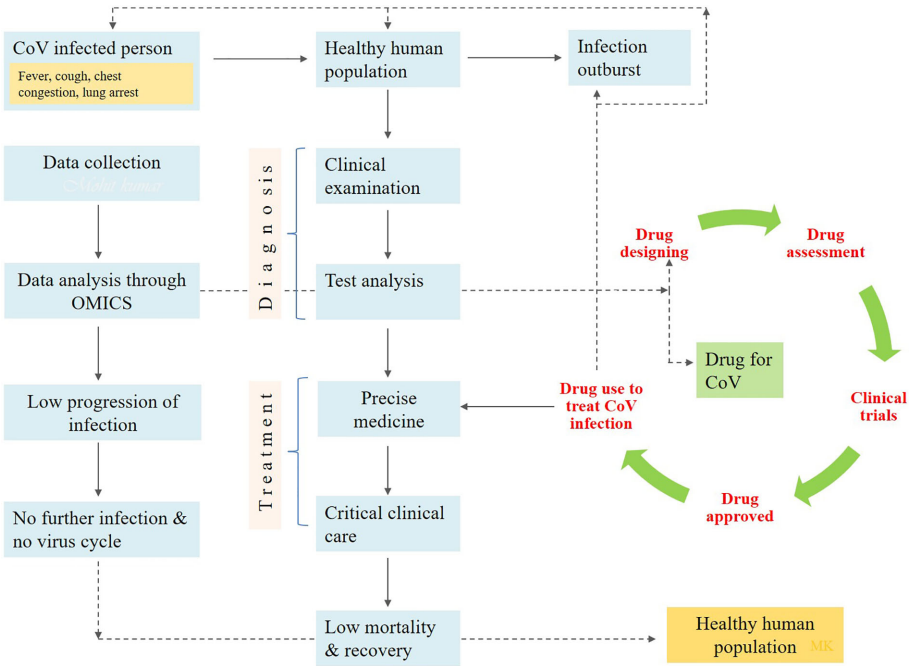

FIGURE 1 | The diagrammatic flow chart showing the implementation of drug to prevent the human health from COVID-19 infection.

viruses, DNA vaccines, and recombinant proteins can probably be used to cure COVID-19 infection (Saif, 2020). Trials on animal models are conducted to study the biological behavior of COVID-19. Presently, researchers worldwide are working for the development of a non-human primate model to know the mechanism of its interaction with the host (Chen et al., 2019).

Development of Novel Biomarkers

The COVID-19 patients are generally diagnosed based on the clinical data. This takes time as the symptoms are generated after the infection sets deeper in the lungs. In such a situation, it becomes difficult for healthcare workers to treat the patients speedily. Biomarker identification provides an advantage over clinical diagnosis. Ulhaq and Soraya identified interleukin-6 (IL-6) as a potential biomarker for COVID-19 infection. As COVID-19 is linked with swift replication and a propensity to infect the lower respiratory tract, so it results in an increased response of IL-6-promoted severe respiratory distress. Thus, the levels of IL-6 can be linked to the disease progression in patients which can prove helpful in further treatment (Ulhaq and Soraya, 2020). C-reactive protein (CRP) is produced by the liver and induced by a range of inflammatory intermediaries such as IL-6. Regardless of its non-specificity, this acute phase reactant is used clinically as a biomarker for different inflammatory situations; an augmentation in CRP intensity is related with an increase in severity of disease. Lactate dehydrogenase is related to severity of pneumonia. Significant rise in LDH levels was observed among refractory COVID-19 patients. The COVID-19 infection leads to thrombocytopenia so platelet count is also a reliable marker for diagnosis of disease severity (Kermali et al., 2020). The Hematologic biomarkers include increase in leukocyte count as a distinguishing factor among infected and non-infected people. In a meta-analysis (Yang J . et al., 2020) carried out by Henry and colleagues in 2020, they found that among 2,984 COVID-19 patients there was significant difference in leukocyte count among severe and non-severe patients (Henry et al., 2020b). According to Bernheim et al., 2020, chest tomography (CT)/X-ray imaging is also a vital component to diagnose COVID-19 suspects when the number is large for diagnosis. In a study they found that CT scan of 56% infected individuals of COVID 19 came normal. Thus it has limited sensitivity and negative predictive value in the early stage of infection (Bernheim et al., 2020).

THE EXPECTATION FROM SYSTEMS BIOLOGY FOR THERAPEUTIC AGENTS

With the advent of next-generation technologies, the systems biology (Hu et al., 2020; Tang et al., 2020) is applied for the assessment of microbial virulence and associated pathogens (Pezeshki et al., 2019; Çakır et al., 2020; Eckhardt et al., 2020). Information and risk assessment of novel pathogens that emerged with time due to mutations, recombinants, horizontal/vertical gene transfer and reoccurrence of outbreaks need to be presented into the databases (McCormick, 2003; Pybus et al., 2007; Escobedo-Bonilla, 2013; Hartzell and Blaylock, 2014; Kaiser et al., 2015; Deneke et al., 2017; Liu et al., 2019; Olanya et al., 2019).

This update to the existing databases of newly emerged or novel pathogens create a challenge and an opportunity for multiomics experts to collect information/data, reunite and organize new standard datasets/databases or update the already existing databases (Kong et al., 2006; Winnenburg et al., 2007; Schumacher et al., 2014; Xie et al., 2017; Bloch and Bailin, 2019; Dong et al., 2019; Duncan et al., 2019; Yan et al., 2019; Chan et al., 2020). The new information will help the clinical and medical researchers to plan research methodologies for therapeutic and drug development. For instance, the novel coronavirus is a newly emerged pathogen which is creating nuisance to humankind. It is estimated that, globally, approximately 0.2 million patients out of thirteen million infected cases died with coronavirus infection, and the rates are still increasing (Baud et al., 2020; Chaurasiya et al., 2020; Kobayashi et al., 2020). The risk, hazards, and exposure characterization and assessment (Njage et al., 2019; Perera et al., 2019; Pavelić et al., 2020) are essential for building the background knowledge to evaluate the possibilities of novel therapeutics and drugs discovery against coronavirus. The systems biology majorly consists of multiomics, databases, and *in silico* studies (Arora and Singh, 2018). *In silico* approach is mainly dependent on computational designing (Koutsoukas et al., 2011; Lavecchia and Cerchia, 2016) and analyzing the interaction of proteins (Bultinck et al., 2012; Oany et al., 2014). The database mining also contributes to the *in silico* approach (Loging et al., 2007; Rao et al., 2014). As prerequisite to the database (Boissel et al., 2004; Nagata and Pastan, 2009), researching the information is required for the practical planning of methodologies (Wu et al., 2011). The study of heritable phenotypic changes, called as epigenomics, is supposed to play an important role in understanding the mortality rate among black and white individuals (Holmes et al., 2020). These changes may be inherited by the cell system as the memory (Holliday, 1987). The covalent modifications on lysine acetylation, lysine methylation, arginine methylation, serine, and threonine phosphorylation, lysine ubiquitination are the major epigenetic mechanisms which can determine the many genetic and phenotypic modulations (Holliday, 1987; Zhang et al., 2019). The heterogeneity in treatment success is also believed to be influenced by these epigenomic changes (Holmes et al., 2020). As the corona virus targets on the lung cells (Conti et al., 2020), the epigenetic control of ACE2 in the lungs cannot be denied. As Woo and Alenghat highlighted the regulation of transcription during host–microbe interaction under the epigenetic modifications, it may be possible that the rate of transcription of virus may be negatively influenced under genetic environmental pressure *e.g.* strong immune cells. Hence, the more epigenetic exploration is needed to prevent the corona virus infection (Woo and Alenghat, 2017). DNA methylation may be one of the major factors in providing fewer sites for attack of the viral genetic material. The components related to biological entities like DNA, RNA, proteins, metabolites (Jacob et al., 2019; MacMullan et al., 2019) correspond to genomics, proteomics, and metabolomics, respectively (Yan et al., 2019; Jenkins and Orsburn, 2020). Various other systems biology approaches which can be utilized for the development of a potent drug against COVID-19 include immunomics, host lipid omics, public health omics and quantitative dynamic omics. This multiomics approach (**Figure 2**) is also taking attention to therapeutic development (Donovan et al., 2019; Lee and Ruppin, 2019).

Host Virus Interaction Study by Computational Tools—*In Silico* Approach

The interaction of any protein with its receptors always depends on the receptor-binding domains on the proteins (Lan et al., 2020a). These receptor binding domain recognizes their interactive sequences on the receptors and binds them with mostly non-covalent bonds in the human system. However, the SARS-Cov-2 viruses mainly attack the mucus membranes of the human system as their first site of attachment, but they are then bound to their receptors, *i.e.*, angiotensin-converting enzyme 2 (ACE2), and finally helps the viruses to come inside the host cells (Lan et al., 2020b). Moreover, after entering the cell, they start their replication with the help of replication proteins and followed by multiplication steps, as shown in schematic **Figure 3**.

During the multiplication and their amplification steps, SARS-CoV-2 are responsible for the release of some proteases, which leads to the generation of reactive oxygen species (ROS) in the host cell (Chen Y. et al., 2020; Nasi et al., 2020). These ROS are toxic to the cell system and its environment. On the other hand, host defense mechanism activates in the form of their immune system, and the free-flowing neutrophils of the blood reached the target site, the lungs (mainly in the alveoli, where the severe acute respiratory virus reached after attaching the nasal, oral or mucus surfaces). In addition to neutrophils, monocytes differentiate into specific tissue macrophages, in response to the infection. However, neutrophils, basophils, monocyte *etc.* are myeloid progenitor cells and are parts of the innate immunity. These cells, upregulated in any foreign particle, enter into the human body, specifically the virus (van de Laar et al., 2016). The release of cytokines is also supposed to be initiated through adaptive and innate immunity cell signaling. In response to toxicity and foreign particles, the metabolism of the macrophages fluctuates, and they tend to release the inflammatory cytokines which finally affects the molecular signaling of responsive target cells and their neighbor cells. When the normal cellular signaling fluctuates either by ROS stress or by inflammatory cytokines resulting from the SARS-CoV-2 entering the host system, they lead to mucus accumulation in the lungs, abnormal and painful breathing, abnormal BP, *etc.* Here, a small study through computational biology and *in silico* tools for a better understanding of host–virus interaction is designed.

The *Homo sapiens* angiotensin I-converting enzyme 2 (ACE2), transcript variant 1, mRNA of Human origin, with a length of 3,339 bp, was extracted from the NCBI with the accession number NM_001371415.1. The mRNA was converted to the protein sequence by ExPASy; its 5–3 and 3–5 predicted sequences were found. Further, through protein blast,

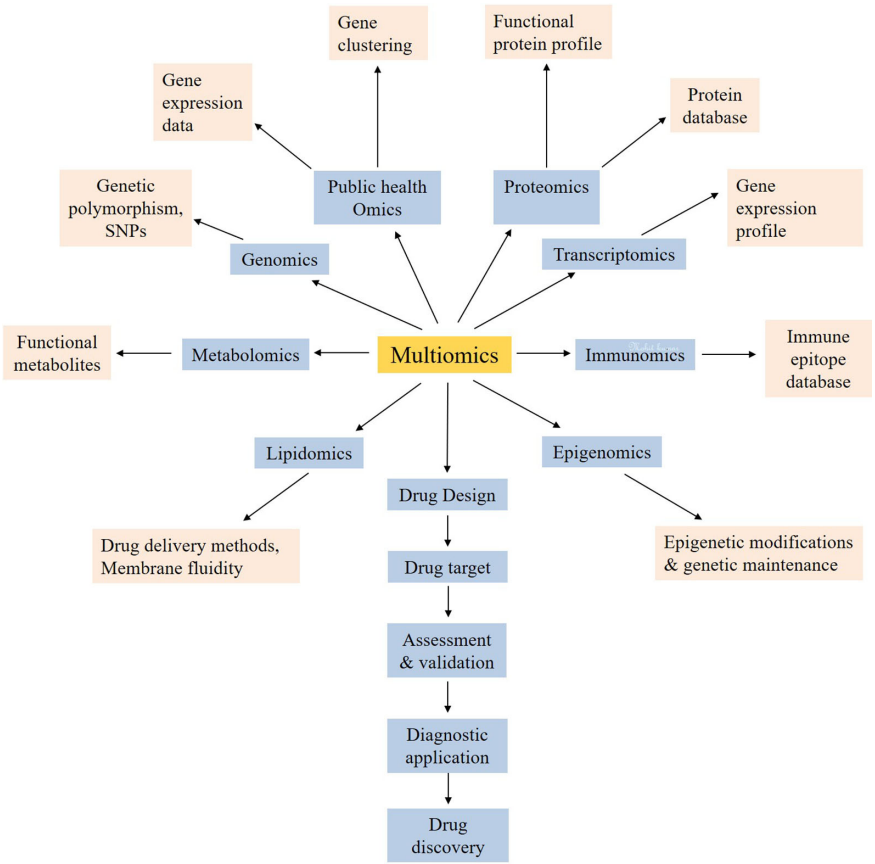

FIGURE 2 | The multiomics approach for the systemic use of technologies for the development of new drug targets for coronavirus.

angiotensin-converting enzyme 2 precursor was finalized for the study based on its 100% identity with our query sequence. The accession number of the angiotensin-converting enzyme 2 precursor was NP_001358344.1, and this was the only protein showing 100% identity with our ExPASy query sequence.

The 3D modeling of the protein was done using SWISS-MODEL. The obtained protein model was a monomer containing ligands, as N-acetyl-D-glucosamine and Zinc. The template used by the SWISS-MODEL (**Table 2**) for model prediction was 6m17 PDB, and it showed the 100% sequence identity coverage with the predicted model, and that is angiotensin-converting enzyme 2. Now, interestingly, this ACE2 showed to be responsible for acting as a receptor for the coronavirus entry to the cells. The QMEAN value for the predicted protein model was −0.99 and is supposed to be of good quality to be used for the study. **Figure 4** depicts the overall protein 3D model with side and top views, along with its QMEAN value to examine its quality. The Quality of the predicted model was also validated through the ProSA web server, based on NMR and X-ray data. For further validation of the protein model, the ProQ webserver was used, and it predicts the model of extreme quality was good based on the LG score. The Ramachandran plot assessment also suggests its 98% amino acids in the favored region of the plot.

Similarly, the spike S1 protein of the coronavirus (source organism; Wuhan seafood market, *Pneumonia virus*) was downloaded from the RCSB protein data bank with the PDB ID, 6M17 (Yan et al., 2020). It was present as a 2019-nCoV RBD/ACE-B0AT1 complex in its PDB format, but to check the protein–protein interaction, the PDB file was modified except for one of the SARS-CoV-2 receptor binding domain. The 3D structure of the protein is shown in **Figure 5**.

The tentative protein–protein interactions are crucial to understand further mechanisms, and a preview of protein–protein docked complex is presented in **Table 3** (van de Laar et al., 2016). Among all the docked complexes, the best structure of cluster 5 complex was depicted in **Figure 5** for its molecular interactions. The cluster 5 has the best HADDOCK score (883.4 +/– 9.7), the lowest Van der Waals energy (–171.7 +/– 9.8), and the Z-score (–2.2). This gives a good understanding towards future tools for understanding such interactions.

Role in Vaccine Development

Vaccine development is in demand due to the increasing rate of mortality and morbidity of COVID-19 infection. Vaccines play an important role in the reduction of toxicity and the elimination of diseases (Dubey et al., 2018; Amanat and Krammer, 2020).

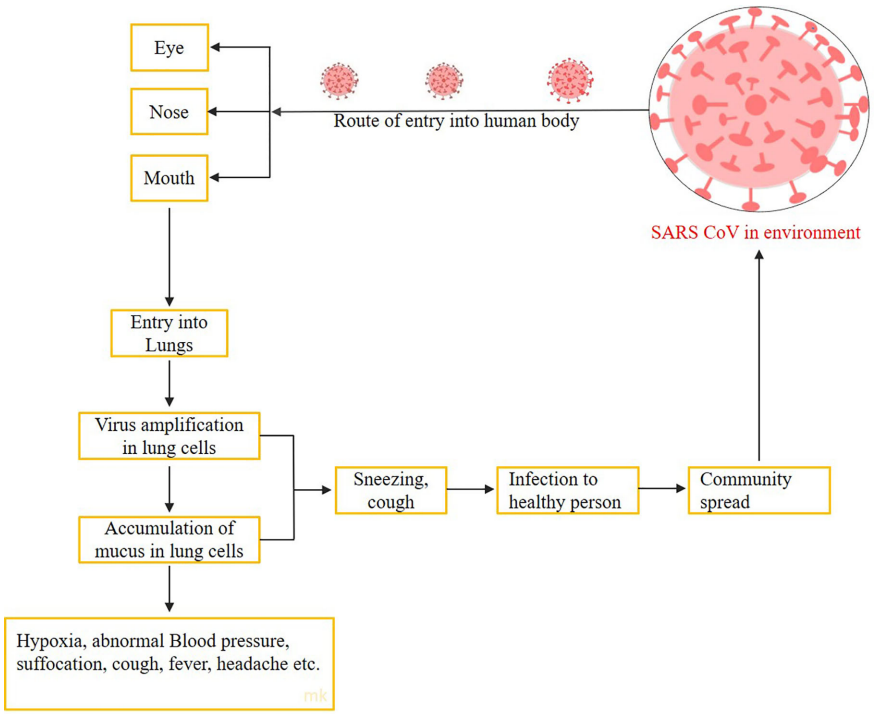

FIGURE 3 | The schematic diagram showing the entry routes and spread of COVID-19.

TABLE 2 | *In silico* tools used in present study (Protein-protein/Protein-ligand study for drug target and protein target inhibition).

S. No.	*In silico* tool	Function	References
1.	Chemdraw	Ligand structure analysis	Cousins, 2011
2.	SWISS-MODEL	Protein structure homology modeling	Waterhouse et al., 2018
3.	ERRAT	Protein structure validation	Colovos and Yeates, 1993
4.	ProSA	Protein structure validation	Wiederstein and Sippl, 2007
5.	ProQ	Protein structure validation	Wallner, 2005
6.	RAMPAGE	Protein structure validation	Lovell et al., 2003
7.	UCSF Chimera	Protein analysis	Dromey, 1996
8.	PyMOL	Protein analysis	DeLano, 2002
9.	AutoDock	Molecular docking	Norgan et al., 2011
10.	Schrödinger	Molecular docking	Schrodinger, 2011

The conventional technologies of vaccine designed have many limitations, including time consumption, laborious, costly, and many more. Perhaps somehow, the involvement of *in silico* tools is sufficient to overcome the mentioned limitations (Rauch et al., 2018). Immunoinformatics approach like reverse vaccinology, epitope prediction, rational vaccinology, and structural vaccinology is advantageous to be used for the designing of vaccines (Kazi et al., 2018). Moreover, side chain and backbone modeling are significant in designing the antibody structure to act as vaccine target. SCWRL and SCAP are the *in silico* tools used to identify the mutation in the proteins (He et al., 2015). RAMBLE and RAPER are the additional software applied for side chain prediction analysis. Multivant scaffolding is another tool applied for the designing of potential epitope (He et al., 2015). However, an epitope can be grafted by using multigraft interface technology. ORF-FINDER, GS FINDER, and GLIMMER are some of the *in silico* tools that performed the screening of ORFs and selected the most immunogenic peptide that alternatively help in vaccine designing (Davies and Darren, 2007).

Structural biology is an important area of immunoinformatics and the basis for the structure of proteins. On the basis of structure, rational vaccinology analyzes the structure of novel protein antigens that will be targeted as potential vaccine candidates (Hegde et al., 2018). Rational technology of vaccine design was significantly used against viral pathogens like influenza, HIV (Van Regenmortel, 2019) and Hepatitis C (He and Zhu, 2015). Furthermore, approaching systems biology enhances the understanding of host–pathogen interaction and also develops adjuvant that provides long-lasting immunity.

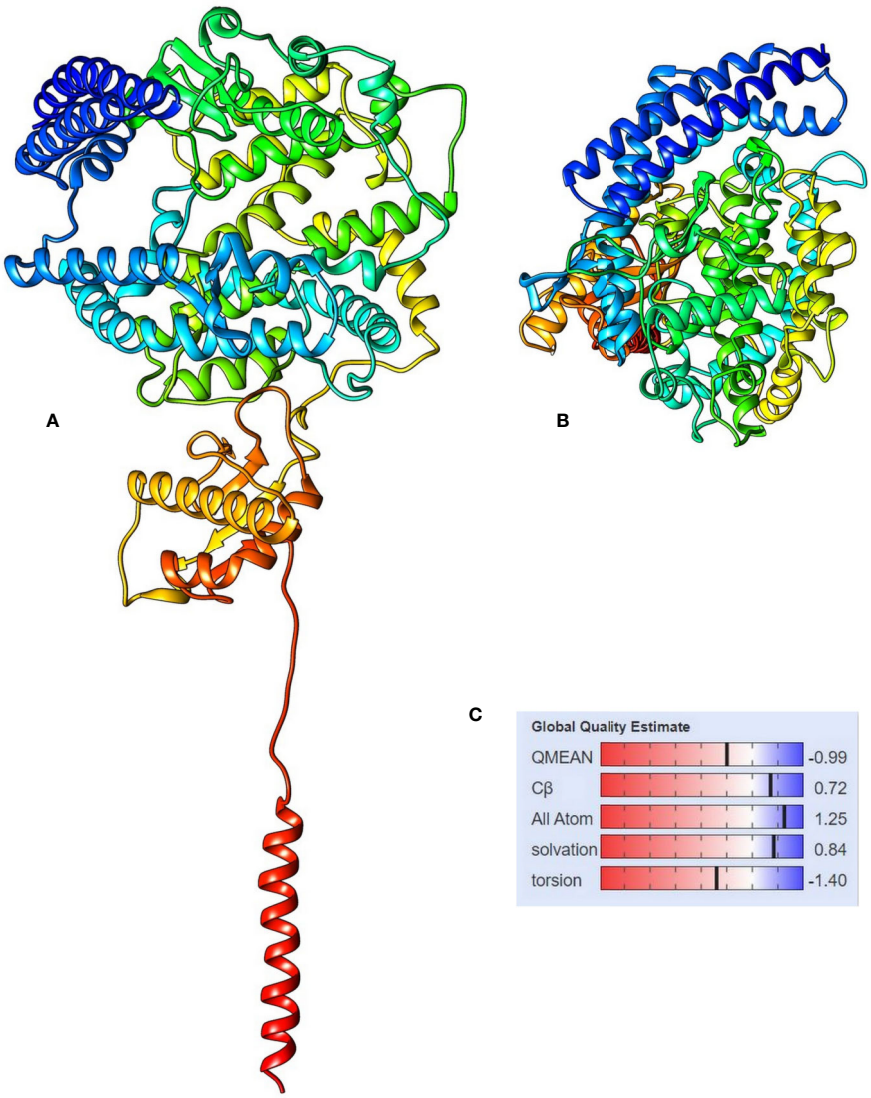

FIGURE 4 | The 3D structure of angiotensin-converting enzyme 2 isoform X1, **(A)** side view and **(B)** top view, and **(C)** the QMEAN value for the protein model showing the statistical representation of the protein model quality.

Vaxijen is another important computation tool that contributes to vaccine development. It is an online software that uses the alignment-free approach to predict the antigenic nature of the proteins (Sinha and Shukla, 2019; Bappy et al., 2020). Majorly the critical process in vaccine development is the identification of epitopes that can be targeted as a vaccine candidate. A number of epitope prediction tools are available that can be significantly used for epitope prediction (Naz et al., 2020). The B-cell and T-cell epitopes of novel SARS-CoV-2 were analyzed by Ahmed et al. by approaching IEDB and other computational tools (Grifoni et al., 2020). Ong et al. (2020) reported the reverse vaccinology and machine learning approach were used to identify the potential vaccine candidate against COVID-19 infection (Ong et al., 2020). As reported by Chen and Wu (2020), ABCpred and BepiPred and IEDB are the epitope prediction tools used for the identification of epitopes in the novel SARS-CoV-2. Besides, the multigraft, multivalent scaffolding, codon optimization, and antibodyomics tools are also helpful in the recognition and construction of potential vaccine candidates (Sunita et al., 2020). However, at present computational tools are the first to be used for vaccine designing of emerging diseases. Later on, these will be validated by experimental studies. Collectively, the actual implementation of these disciplines accelerates the process of vaccine development (Chin et al., 2019).

The Chemdraw software is used to draw the molecular structure of a molecule or compound in the computer and is easy to handle in an offline mode. This gives an immediate and clear sharp image of the structure and can be saved in many file types, *eg*; mol file. The file needs not to be redrawn if any

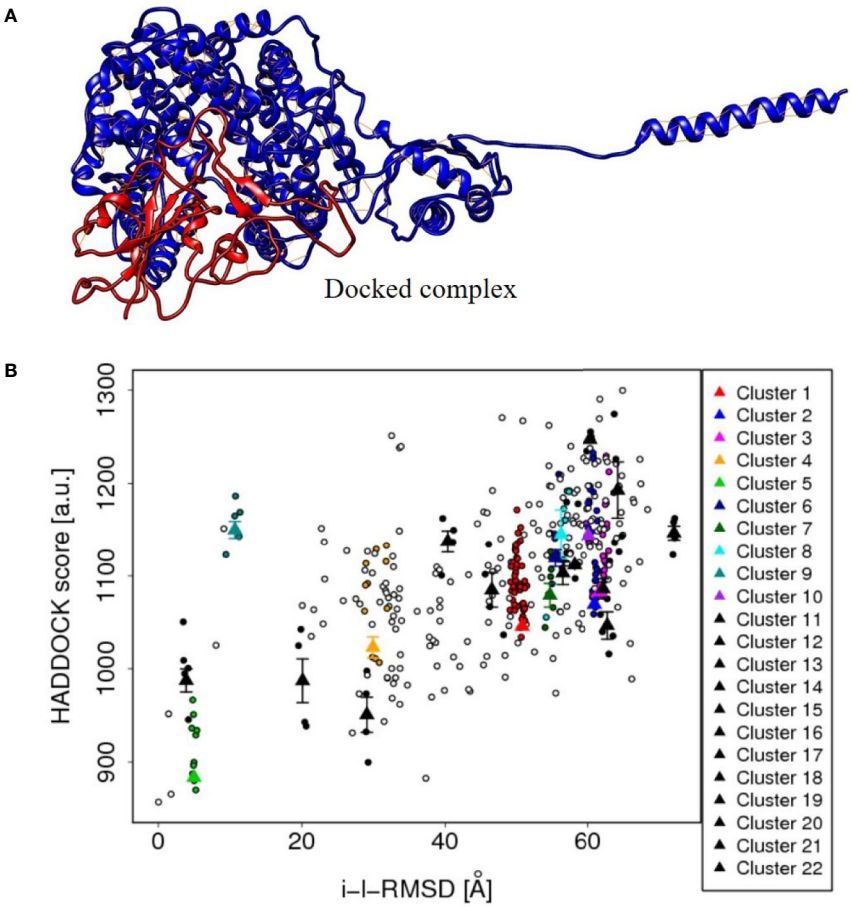

FIGURE 5 | **(A)** The protein-protein docked complex (cluster 5) of ACE2 isoform X1of Human cell (red color), and spike S1 protein (blue color) of corona virus, showing the close interaction. **(B)** The plot between HADDOCK score and RMSD values of the protein–protein docked clusters, showing the best suitability of cluster 5 in the plot and hence to be used to depict the interaction results of ACE2 and spike protein.

TABLE 3 | The docking approaches between ACE2 isoform X1 and spike S1 protein of the corona virus by HADDOCK.

S.No.	S.No.	Cluster	Haddock score	Vander Waals energy	Z-score
1.		5	883.4 +/– 9.7	–171.7 +/– 9.8	–2.2
2.		22	950.7 +/– 37.6	–141.7 +/– 13.4	–1.1
3.		21	987.2 +/– 46.9	–114.0 +/– 11.9	–0.5
4.		14	987.5 +/– 24.8	–139.6 +/– 11.2	–0.5
5.		4	1,023.0 +/– 22.6	–115.2 +/– 5.7	–0.1

correction has to be added. Hence this provides an immediate output of the structures to be used in any docking study. The software, SWISS-MODEL is designed for the homology modeling of the proteins. The software works easily in windows system and gives the output in a simple manner so as the beginners in the field of systems biology can easily benefit. The protein model quality is also validated on the basis of Q mean score, which is the collective information of many parameters *e.g.* X-ray and NMR. After protein homology modeling, there is a need of the formed structure validation, and this task can be done by many online software applicatioms *e.g.*; ERRAT, ProSA, ProQ, RAMPAGE *etc*. Further, the ProQ also gives its own score called as LG score, for the highest ranked models in protein validation. This model validation can be analyzed based on their scores (software gives different scores more or less similar based on their inbuilt programming).

The Ramachandran plot provides the quantitative data for favored and unfavored regions, which basically used the protein backbone dihedral angles and is necessary for protein validation. The Schrödinger and AutoDock software is used for the molecular docking of protein–ligand or protein–protein. However, both the software programs can be used for the docking, but an expert should be needed to deal with these software programs, and it needs some prior knowledge to work with them. The software programs, UCSF Chimera and PyMOL are used for the molecular structure analysis, and it need some knowledge to handle the software programs correctly. But with basic knowledge of molecules and bioinformatics, the online tools can be handled easily and can give the best results in the drug discovery and exploration of proteins. The HADDOCK

online server provides the quantitative description for the protein–protein molecular interaction. It provides many clusters of protein–protein interactions based on the possibility of the bond formations and the analysis involved many parameters *eg*; Haddock score, Z-score. These parameters are necessary for choosing the best cluster for further analysis of the molecular interactions using systems biology approach. Van der Waals interaction is the distance dependent interaction between atoms, and Haddock provides us the series of interactions (strongest to weaker). However, so software can easily give results based on their input algorithm, we have to analyze the parameters for the perfect study.

Role of Omics Techniques for Virulence Assessment

The MRA (Microbial Risk Assessment) (Brul et al., 2012; Haddad et al., 2018) is a clinical evaluation of virulence associated with pathogen, mainly foodborne pathogens *i.e.*, animal meat and their products (Wassenaar et al., 2007; Franz et al., 2016). Nowadays, the utilization of multiomics datasets for improving and redesigning the role model of microbial risk assessment is being practiced by researchers (Buchanan et al., 2000; denBesten et al., 2018). The dose–response models are designed by clinical researchers for studies, especially for diseases associated with RNA viruses (Gale et al., 2014). This probabilistic approach firstly consists of the evaluation of viral infections *via* oral path, *i.e.*, the host and virus interaction *via* cellular receptors with and without conquering the host defense system and replication in the host cell (Voysey and Brown, 2000; Huang and Haas, 2009), followed by designing the models to study disease progress immediately after infection. The virulence markers are the viral gene sequences representing the viral disease-related traits (Haddad et al., 2018; Liang J. et al., 2020). Therefore, it is observed that the above-discussed appeal must be undertaken for coronavirus probabilistic virulence assessment (Benvenuto et al., 2020). Since dose–response models are particular for RNA viruses thus, the possibility and chances of multiomics application (Zhang et al., 2018) to search the antiviral targets rise for the development of therapeutics and drugs against coronavirus (Fritsch et al., 2018; Jeon et al., 2020).

Genomics

Genomics offers the task to reveal the characteristics of drugable genomes (Hopkins and Groom, 2002), which consist of sequences and alignments signifying the virulence trait (Losada et al., 2016; Lee et al., 2017). Data mining from databases can make it possible for the researcher to seek out queries related to coronavirus (Hatcher et al., 2017). For the purposive research regarding the newly emerged virus, the National Institute of Health (NIH) of United States created a resource (http://www.niaid.nih.gov), an integrated surveillance data for supporting researchers in collaboration with various research institutes working on systems biology (Squires et al., 2012; Uyeki et al., 2016). The main aim is to make the comparative genomic research for correlative analysis of coronaviridae family-related genus and strains for the predicted data and annotated genome sequences (Zhu et al., 2016; Liang J. et al., 2020; Randhawa et al., 2020). In this process, the MSA (Multiple Sequence Alignment) signifies the closely related RefSeq constructing the virus ortholog groups with associated protein playing role in virulence (Fumagalli et al., 2010; Chapman et al., 2011; Claytor et al., 2017). Till now, there is very little scientific data and literature available related to coronavirus. The genomics approach is leading and generating the manually curated research data (Brister et al., 2015; Manzoni et al., 2018) about the clinical coronavirus strains aiding the scientific literature country-wise in the above-discussed manner (Huang et al., 2007).

Transcriptomics and Metabolomics

Transcriptomics is mainly concerned with gene expression profile (Irigoyen et al., 2016) *i.e.*, by ribosome profiling (Irigoyen et al., 2016), RNA sequencing (Depledge et al., 2019), and high throughput DNA microarrays (Wang et al., 2009). The dose–response models are developed to study the factors affecting gene expression (Hashem et al., 2019) at different concentrations of virulence proteins (Haas, 2020). They also check the mRNA abundance at various levels of infection progression (Albariño et al., 2018). The metabolic enzymes run cellular metabolism (Ahmed Idris et al., 2020). The cellular metabolism is revealed through the intense study of genomics, transcriptomics, and proteomics (Fanos et al., 2020), which are directly and indirectly linked to pathways involved in metabolomics (Haas et al., 2016). The primary significance of metabolomics comes for diagnostic assessment (To et al., 2016). The concentrations of metabolites (Sinha et al., 2019) are detected by NMR (Nuclear Magnetic Resonance), HPLC/MS (High-performance Liquid Chromatography/Mass Spectrometry) (Peng and Liu, 2017). Metabolic profile analysis would reveal the binding and inactivation of metabolites by drugs (Eisfeld et al., 2017) that would arrest further disease progression (Zhao and Lin, 2014). In this manner, transcriptomics and metabolomics increase the possibility for developing therapeutics and drugs against the coronavirus.

Proteomics

Proteomics study reveals the functional role of proteins associated with host and pathogen (Zheng et al., 2018). With available resources of database and drug targets obtained by previous studies on influenza virus (Sadewasser et al., 2017), hepatitis C virus (Lupberger et al., 2019), poxvirus (Grossegesse et al., 2017), Nipah virus (Vera-Velasco et al., 2018), *etc.*, it is feasible to develop novel drugs. The studies on GPCR (G-protein coupled receptors) (Sriram and Insel, 2018), ion channels (Lin, 2019; Duncan et al., 2020), and enzymes (Ding et al., 2017) provide the platform for researchers to study drug–target interactions. Zheng and Perlman, in 2018 discussed the importance of proteomics in the host immune system and respiratory virus interaction response. They insighted the landscape proteomics analysis formed by prediction of clinical data and the role of immune response gathered *via* host lipid omics, immunomics, phosphoproteomics, and public health omics (Zheng and Perlman, 2018). The recent study done by Kang et al., 2020, the western blotting, protein categorization, gel

digestion, SDS-PAGE analysis, SILAC labeling for protein analysis, protein identification, separation and quantification methodologies for identification of structural proteins of coronavirus, mainly bronchitis virus particles were done. The above-discussed method is likely to be the strategy for finding the novel antiviral against coronavirus.

Immunomics

Immunomics is based on the efficiency of the host to eliminate pathogens that enter the human body. The immune system of organisms contains many cellular, molecular, and physical components that provide defense against invasive microorganisms. Dysregulation of immune cells such as inflammatory monocyte–macrophage and type I interferon (IFN) led to the occurrence of lethal pneumonia in mice infected with SARS-CoV (Channappanavar et al., 2016). This indicates that immune cells play a vital role in combating pathogens. The immune memory cells are able to protect the host from the early invasion of respiratory pathogens. Bioinformatics tools and sequence homology can be used to find potential immune targets and designing of a vaccine against COVID-19. Grifoni et al. used the Immune Epitope Database (IEDB) and Analysis Resource for prediction of COVID-19. They used SARS-CoV to predict epitope responses as it shows higher similarity to SARS-CoV-2. They found conserved regions in SARS-CoV and SARS-CoV-2 caused COVID-19. Vaccination approach proposed to target the immune response toward these conserved epitope regions could generate immunity. This will not only protect from *Betacoronaviruses* but also against moderately challenging virus that will emerge in future (Grifoni et al., 2020). Carbohydrates present on the host and viral proteins are potential targets for modulating the immune response. The use of computational tools and integrated omics approaches can lead to the development of vaccines and drugs for such targets of viral infection and receptors (Zheng and Perlman, 2018).

Host Lipid Omics

Lipids play a vital role in the interaction of the virus with the host cells. Lipids can act straightly as the receptors or co-factors of entry for viruses at the surface of cell or endosomes. Viral replication complex highly depends on them, and lipids also provide energy for replication of the virus. Lipids can help to order the suitable cellular allocation of viral protein and also the trafficking, assemblage, and liberation of viral particles. Thus, host lipid studies can play indispensable role in understanding virus propagation (Diamond et al., 2010; Das, 2020). Coronaviruses seize intracellular membranes of the host cells to produce fresh partitions called double-membrane vesicles (DMVs). These partitions help in the viral genome amplification. A current study showed that a primary lipid processing enzyme, cytosolic phospholipase A2α enzyme (cPLA2α), was directly linked with DMVs' development and replication of coronaviruses (Müller et al., 2018). Coronaviruses require a specific composition of lipids for their replication. If this lipid homeostasis is broken, then the viral replication is affected. Yan and his co-workers found in a study that glycerophospholipids and fatty acids (FAs) were considerably increased in the HCoV-229E-infected cells, and the linoleic acid (LA) to arachidonic acid (AA) metabolism was strikingly disturbed upon infection of HCoV-229E. Exogenous supplementation of LA and AA decreased the replication of coronavirus. They came to the conclusion that there was an upregulation of lipids that were responsible for replication and membrane synthesis. The virus maintains homeostasis for its better replication, but when this homeostasis is broken by supplying lipids from outside the cell, the replication of the virus is disturbed. Thus, lipidomics can provide better treatment strategies if integrated with immunological data (Yan et al., 2019).

Public Health Omics

Public health omics takes into consideration the entire kinetic response of the host. In public health omics, the expressions of genes and transcriptome are studied. The interaction and regulation of different transcriptome datasets are studied. It takes into consideration the upregulation and down-regulation of different genes during infection. It takes account of molecular as well as clinical conditions of the host and pathogen. The pathway interaction and response of host are analyzed after the infection by utilizing this methodology. This renders the whole set of the idea in host–pathogen interaction with respect to the time of infection. Such systems biology methods draw attention to the significance of time-related factors in the study of multifactorial diseases such as influenza and coronavirus. A study by Dimitrakopoulou and his group revealed the temporal effect of the influenza virus by studying the interactome and signaling pathways. Their findings cooperatively update the budding area of public health omics and potential clinical trials intended to interpret dynamic host reactions to pathogens (Dimitrakopoulou et al., 2014). This is perhaps the unnoticed field of omics technology, but its application can give better results for understanding the spread of COVID-19. This technique will help in the development of time dependent drug development in case of infection (Pawelek et al., 2012).

ARTIFICIAL INTELLIGENCE FOR DATA GENERATION AND DRUG DEVELOPMENT

Artificial intelligence plays a vital role in this global scenario to fight against COVID-19. Artificial intelligence and machine learning techniques have helped to group data of genomic taxonomic classification, detection assay based on CRISPR (Dangi et al., 2018; Vashistha et al., 2018), endurance calculation of patients, and identifying probable drug candidates for COVID-19. Metsky et al. screened SARS-CoV-2 by machine learning designs employing a CRISPR-based virus detection system with high speed and sensitivity (Metsky et al., 2020). Similarly, artificial intelligence can be used for the management of critical patients of COVID-19. Rahmatizadeh et al. applied a three-stage model based on input, process, and output. They took into consideration paraclinical, clinical, epidemiologic data, personalized medicine, diagnosis, risk stratification, treatment, prognosis, and management.

The AI (Artificial Intelligence) approach is helpful in stratifying patients and their timely cure (Rahmatizadeh et al., 2020).

Thus, computational tools not only help in virus detection but also in drug development. Wu and his co-workers analyzed the proteins coded by the SARS-CoV-2 virus and modeled them for target prediction. They predicted potential targets and probable drugs against them. They screened 3-chymotrypsin-like protease (3CLpro), spike, RNA-dependent RNA polymerase (RdRp), and papain like protease (PLpro) thoroughly. 78 generally used antiviral drugs and compounds from ZINC database were used for positioning and structural analysis. Thus, *in silico* studies provide drug repositioning to treat COVID-19 (Wu et al., 2020a). A deep-learning based analysis structure of thoracic CT images was built for computerized recognition and observation of COVID-19 patients over time. Swift development of computerized diagnostic systems based on artificial intelligence and machine learning cannot only give improved diagnostic accurately and rapidly, but will also defend healthcare workers by diminishing their contacts with COVID-19 patients (Alimadadi et al., 2020).

CONCLUSION AND FUTURE PERSPECTIVE

The present review gives an insight into the applicability of systems biology tools for developing drugs against COVID-19 infection. The ultimate aim is to find the possible viral targets by exploring the pathogenicity and virulence strategy of coronavirus. The promising *in silico* application of molecular interaction and simulation study is done purposely for the understanding of host (human) and virus interaction to plan the future strategies for managing the situations of virus pandemics. It is worthy to mention that omics data and systems biology algorithms can combine data from cytokines, blood cell populations, proteomics, transcriptomics, clinical parameters, and epidemiological data to develop personalized medicine strategies and patient stratification based on omics. Although it is difficult to make the strategies or policies by the non-medical expertise of the administration, with the help of systems biology, the possibilities increase. The molecular docking and simulations study are presented to make it simple and approachable to non-target audience also, to ensure the seriousness of the COVID-19 as a global pandemic. In conclusion, it can be stated that the systems biology can lead based on the obtained sequencing data, to ensure the understanding of molecular and physiological mechanisms/phenomena and definitely help in breaking the coronavirus like epidemic outbreaks in future by potential antiviral drugs acting on target for preventing the associated disease progression and increasing the infected patient's treatment effectiveness.

AUTHOR CONTRIBUTIONS

SJ wrote the first draft of the manuscript with contributions from MK, Mandeep, and Sunita. The final draft was read and edited by YS and PS. All authors contributed to the article and approved the submitted version.

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An Overview of Epidemiology of COVID-19 in Macau S.A.R

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The Greater Bay Area of southern China has a population of over 71 million people. The area is well-connected with Hubei province, the epicenter of the COVID-19 outbreak. Macau, as the most densely populated city in the world, is very vulnerable to infectious disease outbreaks. Since its return to the sovereignty of China 20 years ago, the city has experienced outbreaks such as severe acute respiratory syndrome (SARS), Swine flu, and COVID-19. At the time of writing, 10 confirmed imported/local transmission cases were recorded. The government undertook measures to attain and then maintain 40 days without new cases. In this article, we report on the 10 confirmed cases and discuss the measures that the Macau Special Administrative Region (S.A.R.) government undertook during the COVID-19 pandemic.

Keywords: COVID-19, Macau, Greater Bay Area, China, coronavirus (2019-nCoV) outbreak

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INTRODUCTION

Since the beginning of the COVID-19 pandemic (formerly known as the "novel coronavirus"), the government of the Macau S.A.R has undertaken serious actions to prevent its spread in the community. The Macau S.A.R is located in the megalopolis of the Greater Bay Area, together with the Hong Kong S.A.R. and cities in the Pearl River Delta in Guangdong province. The Greater Bay Area (GBA) has a population of 71 million, the fourth largest bay area in the world after the New York metropolitan area, the San Francisco Bay area, and the Greater Tokyo area. Rapid infrastructure development has meant that the 71 million residents can commute from one city to another within an hour in the area. This region is only 5 h away from Wuhan by high-speed train. There are four international airports in the GBA: Hong Kong, Guangzhou, Shenzhen, and Macau, the region is well-connected. When the severe acute respiratory syndrome (SARS) outbreak hit the region in 2003, the Macau S.A.R. recorded one imported case, while the rest of the region was badly affected by the outbreak. At that time, traffic and economy from mainland China and Hong Kong were restricted by policies and limited infrastructure. The Macau S.A.R. is the most densely populated region in the world [a population of ∼670,000 and a population density of 21,717 people per square km (1)] with up to 38 million tourists per year (2), so ineffective infectious disease control can be catastrophic. Despite the potential risk factors for local outbreaks, Macau managed to keep the number of confirmed patients with COVID-19 at 10 for 40 consecutive days (since the last confirmed case on February 4, 2020). Despite its good profile on infectious control, very limited documentation has been published regarding the outbreak response in Macau. This article reviews official publications available in the public domain regarding the COVID-19 pandemic in Macau, with focus on clinical data of the initial 10 confirmed cases and on the strategies that the Macau government adopted to minimize the impact caused by this worldwide pandemic.

EARLY RESPONSE TO THE LOCALIZED WUHAN OUTBREAK

On January 3, 2020, the government of the Macau S.A.R. announced its concern of the cluster of cases of unknown pneumonia in Wuhan, Hubei province, China. At that time, there were 44 cases of pneumonia of unknown etiology reported from Wuhan, 11 (25%) of the patients were severely ill (3). In response to this, the government has kept in close contact with the National Health Commission of People's Republic of China from the beginning and has also embraced immediate preventative measures. These measures included measuring the body temperature of arriving air passengers from Wuhan, and also requiring that they complete a health declaration form. The implementation of a health declaration form was also legislated during the 2003 SARS outbreak, arriving visitors were required to declare their personal contact details, travel history in the past 14 days, and health conditions, later the declaration was applied to everyone that entered the Macau S.A.R.

After a cross-departmental meeting, the Health Bureau of Macau issued a Level III Alert on January 5 in response to the outbreak in Wuhan. This system was revised since the 2003 SARS outbreak, the alert meant that there was a moderate risk to public health and activated the following: (1) the Macau Customs Service implemented temperature monitoring at all ports, (2) the Municipal Affairs Bureau executed strict animal import regulations, (3) the Tourism Office kept in close contact with the tourism sector and assisted the Health Bureau in providing infectious control training to the hospitality sector, (4) casinos were obligated to have appropriate equipment to monitor the body temperature of employees and visitors, (5) the Government Information Bureau issued the latest updates on the viral outbreak and information on preventative measures for the public, and (6) paramedics and relevant healthcare-related staff guaranteed the supply of personal protective equipment (PPE) when managing suspicious cases. Visitors and staff to any governmental offices were advised to wear surgical masks and to seek early medical assistance if they experienced fever or respiratory symptoms. Furthermore, government representatives (including the Director of the Health Bureau Center for Disease Control and Prevention) visited Wuhan to inspect the local situation and to reference their approach on diagnosis, treatment, and prevention of the virus. On January 21 2020, the Macau Government established the "Novel Coronavirus Response and Coordination Center." This 24 h operating center was responsible for coordinating all cross-departmental strategies and polices regarding the prevention, and if necessary, the control of the outbreak (at the time of writing, there were no new confirmed cases of COVID-19 in Macau).

REINFORCEMENT ON PREVENTATIVE MEASURES

The first confirmed case of COVID-19 infection in Macau was confirmed on January 22 2020. Thereafter, the government embraced further action to prevent a community outbreak by implementing several public measures. For instance, trained medical personnel were allocated in addition to the existing body monitoring facilities across all the ports and operation hours in these ports were also reduced. The government also urged those who were still in China to return to Macau as soon as possible, preferably within 14 days, to self isolate in case they were asymptomatic. Tour groups from Wuhan were suspended and flights and ferry services to Hong Kong and China were also suspended from early February.

At the same time, the authorities estimated that there were 1,113 visitors from Hubei province entering Macau between the December 1 2019 and January 26 2020. The authority advised these visitors to return to China, whilst those still opting to stay in Macau were quarantined in designated government premises. By January 27, the majority of Hubei visitors had left, leaving behind 371 people (200 of which were from Wuhan) in Macau (4). Thereafter, any Hubei citizens that entered Macau were required to submit a medical certificate stating that they were free from COVID-19 infection.

As the outbreak of COVID-19 continued to progress worldwide, the authorities closely monitored the situation in its neighboring countries. Immigration policies were constantly updated. Since February 24, a mandatory 14 day quarantine was required for visitors who had traveled from South Korea. Subsequently, the same policy applied accordingly to travelers with recent travel history from other high-risk countries (including Italy and Iran) in the past 14 days.

In terms of preventing community transmissions, the Health Bureau and Police Force Department conducted contact tracing of the confirmed cases. These close contacts, once identified, were screened for the COVID-19 virus and quarantined in specific assigned premises for 14 days if initial viral tests were negative. The use of surgical face masks in public were further encouraged and the importance of strict hand-hygiene was widely advertised. The Municipal Affairs Bureau upgraded its routine sanitizing work to a daily basis to ensure that all markets and hawkers' areas were clean. To apply social distancing practices, in early January all public large-scale events were canceled and public libraries, museums, and leisure activity venues were closed down. Citizens were advised to avoid social gatherings and public transport services were reduced. Kindergartens, primary and secondary schools, and all higher education institutions were closed to ensure students were kept safe at home. From January 30 to February 10, 2020, most government non-emergency services were closed down and related civil servants were exempted from work. Later, in February, the Chief Executive of Macau announced for the first time in history that there would be a suspension on the gambling industry, including casinos and associated entertainment facilities, for a period of 15 days.

As part of the early response, the Macau government began actively sourcing medical equipment, including mechanical ventilators, as early as the beginning of January. Moreover, the Health Bureau stressed that their use was to primarily protect all healthcare-related staff from the contagious virus. They ensured that at least a 3 month supply of protective equipment, including PPEs and protective visors were available. To minimize the risk of cross-infection, "Dirty Team" practices were adopted among

TABLE 1 | Demographics and clinical course of the first 10 confirmed cases in Macau.

Case	Date of diagnosis	Age	Region of residence	Background	Recent travel history	*Transmission classification	Clinical complications	Clinical outcome	Duration of hospitalization
1	2020/1/22	45–60	Wuhan	Tourist	Wuhan (China), Zhuhai (China), Macau	Imported	None	Cured	15 days
2	2020/1/21	60+	Wuhan	Tourist	Wuhan (China), Zhuhai (China), Macau	Imported	None	Cured	28 days
3	2020/1/26	45–60	Wuhan	Tourist	Wuhan (China), Zhuhai (China), Macau	Imported	Hypoxemia, received steroid therapy	Cured	19 days
4	2020/1/26	30–45	Wuhan	Tourist, mother of case 6	Wuhan (China), Hong Kong, Macau	Imported	None	Cured	21 days
5	2020/1/26	15–30	Wuhan	Tourist	Wuhan (China), Zhuhai (China), Macau	Imported	None	Cured	17 days
6	2020/1/27	15–30	Wuhan	Tourist, son of case 4	Wuhan (China), Hong Kong, Macau	Imported	None	Cured	22 days
7	2020/1/27	60+	Wuhan	Tourist	Wuhan (China), Guangzhou (china), Zhuhai (China), Macau	Imported	None	Cured	28 days
8	2020/2/2	60+	Macau	Resident	Zhuhai (China), ZhongShan (China), Macau	Imported	Pneumonia at presentation, hypoxemia, received steroid therapy	Cured	34 days
9	2020/2/4	15–30	Macau	Resident, history of contact with case 8	Macau	Imported/local	None	Cured	29 days
10	2020/2/4	45–60	Macau	Resident	Guangzhou (China), Macau	Imported/local	None	Cured	24 days

**Transmission classification is based on WHO analysis of available official data and may include: (a) community transmission—where it is unable to relate the confirmed cases through chains of transmission for a large number of cases, (b) local transmission—which indicates locations where the source of infection is within the reporting location, and (c) imported cases—which indicates cases that have been acquired outside the location of reporting. A publicly accessible dataset was analyzed in this study. This can be found here: https://www.ssm.gov.mo/apps1/PreventCOVID-19/en.aspx#clg17046.*

frontline staff in the government hospitals, for paramedics, and fire brigades.

FACE MASKS

To ease public anxiety, after communication with major pharmacies and suppliers in mid-January, the Macau government reassured the public that there would be a sufficient supply of surgical face masks. Since the first imported case of COVID-19, legislation was passed to demand that all staff at casinos and hotels wear surgical face masks. On January 22 2020, the Health Bureau announced a campaign to guarantee that all Macau residents would have an adequate supply of surgical face masks. The government had successfully purchased 20 million face masks which were then distributed to district health centers, these included more than 50 pharmacies and later some non-government organization units. These face masks would be available for purchase at the original cost by Macau residents and non-resident workers, in a batch of 10 face masks at a price of MOP$0.80 each (approximates to USD$0.10). Each person would be able to purchase 10 face masks every 10 days. The government put enormous effort into searching for suppliers of face masks worldwide and ensuring that the campaign would continue during the pandemic period, including chartering flights to transport these masks ensuring there was a sufficient supply for the public. On February 3, the Macau Transport Bureau (DSAT) announced that all public transportation drivers and passengers would be required to wear face masks when embarking public transport vehicles (5). Furthermore, people were strongly advised to wear face masks in public.

THE 10 CASES OF COVID-19 IN MACAU

Macau had its first confirmed case of COVID-19 2 weeks after the level III alert was announced. A confirmed case is defined as

TABLE 2 | Comparison of epidemiology and demographics of cases of COVID-19 in Macau with neighboring regions during the initial phases of the pandemic.

	Macau S.A.R (up to March 14 2020)	Hong Kong S.A.R (8, 9) (up to April 19 2020)	Taiwan (10) (up to March 18 2020)	Singapore (11) (up to March 31 2020)
Population (12)	0.6 million	7.5 million	23.8 million	5.9 million
Population density (per km^2)	21,717	7,140	673	8,358
Percentage of urbanity	100%	100%	78.9%	100%
Incidence of COVID-19	10	1025	100	926
Mode of transmission:				
Import	10 (100%)	604 (59%)	71 (72%)	525 (57%)
Non-import	0	421 (41%)	29 (28%)	401 (43%)
Number of mortality	0	2	3	3

Numbers in () represent percentage of cases of COVID-19 classified by mode of transmission.

a person with laboratory confirmation of COVID-19 infection, irrespective of clinical signs and symptoms (6). On February 2, there were another nine new confirmed cases. **Table 1** illustrates the demographics and clinical course of these 10 confirmed cases in Macau. Out of the 10 cases, seven were tourists from Wuhan and three were Macau residents. The first eight cases were classified as imported cases whilst the last two were either imported or local transmission cases since these two patients had both recent travel history to China and contact with high risk groups. Transmission classification is based on the WHO analysis of available official data and may include: (a) community transmission—where it is unable to relate the confirmed cases through chains of transmission for a large number of cases, (b) local transmission—which indicates locations where the source of infection is within the reporting location, and (c) imported cases—which indicates cases that have been acquired outside the location of reporting.

During the clinical course of these 10 confirmed cases, all patients were treated in an isolation ward in the hospital. According to information provided by the Novel Coronavirus Response and Coordination Center, all 10 patients received anti-viral treatment. A total of (20%) experienced hypoxemia during hospitalization and required the used of steroid therapy. None of them needed intubation and mechanical ventilation, mortality rate was zero. All (100%) patients were cured and discharged. Discharge criteria was in accordance with the "Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia" published by the National Health Commission, China, that includes the absence of fever, the clinical resolution of symptoms, improvement in imaging tests of the chest, and repeatedly negative nucleic acid results in nasopharyngeal swabs. All seven patients from Wuhan left for China on the same day of discharge. Meanwhile the three local Macau residents were discharged to a designated venue for a 14 day quarantine during the recovery period, they were to be tested again for the virus at regular time intervals. This extended quarantine standard is twice as long when compared to the standards advised by the National Health Commission in China. The extended quarantine period was to ensure that the patients' virology tests would not turn positive again before they returned to the community.

COVID-19 IN NEIGHBORING REGIONS

The pandemic progression of COVID-19 in places neighboring Macau appeared to develop in a similar pattern and magnitude. Nearby regions that share comparable age and gender distribution, ethnic diversity, cultural background, and economic status include the Hong Kong S.A.R., Taiwan, and Singapore. **Table 2** shows a summary of the epidemiology of COVID-19 in these regions in the time frame of January to March/April 2020. As illustrated, these regions all had a relatively low incidence and mortality rate. Similar to Macau, given the high population density and close geographical connections to mainland China, these regions were highly vulnerable to a community-wide outbreak. In other overseas countries, the first wave of COVID-19 case importation was usually followed by widespread community transmission. However, these did not appear to be the case here. These regions (and Macau) all demonstrated a higher proportion of imported cases. This can be explained by the execution of early public interventions in each of these regions—namely city-wide lockdown, social distancing, and voluntary facemask-wearing in the community. These interventions, in fact, have later been identified to have had a positive impact on public health by several studies (13, 14).

DISCUSSION

Under the one-country two-system framework, the S.A.R. enjoys a high degree of autonomy including its own immigration customs border and policies. In the 2003 SARS outbreak, Macau had only one imported case. Entry to the Macau S.A.R. at the time was restricted to only a small number of tour groups or transiting tourists from the mainland. Connections to Hong Kong were limited to helicopters and ferries, air traffic was also relatively low. The government at the time implemented the following policies to control the outbreak: they established an inter-departmental coordination workgroup, health declaration, body temperature measures at entry ports, specialized fever areas at emergency sections in hospitals, a hand wash and household disinfection campaign, and an

infectious disease reporting system between Hong Kong and Guangdong province.

Some of the policies still remain active today, the monitoring of body temperature at major ports and communication within the region are still in place. After the SARS outbreak, China opened a free individual traveler scheme for their citizens to visit the two S.A.R.s as an economy stimulus package. The policy attracts millions of tourists every year, and the measures were deployed again in the successful control of the swine flu outbreak. Since then, Macau has gradually integrated into the region. Over the past 17 years, the area has rapidly developed into a world class megalopolis and infrastructure, Macau has received 21 times more tourists than in 2003, the original measures were inadequate for the increasing capacity.

The government upgraded some of the existing measures, and introduced new measures to contain the new virus. This included digitizing the health declaration form and integrating with the national immigration network to verify the travel history of visitors. Public health promotion was also adopted on social media. Information on places and transport routes used by confirmed individuals could be released and circulated in a short time frame. The introduction of the mask guarantee scheme reassured the public and maintained public confidence in the government, the masks were distributed with the help of a centrally registered network to provide a real-time track record on the stock. The government also operated a closed management system for those who had returned from high risk regions, individuals were quarantined in designated venues. In addition to this, the government also provided social care support to those who were quarantined. These measures heightened the confidence of the public in the government with the added benefit of keeping the public informed and ensuring that they learnt the correct response.

CONCLUSION

At the time of writing, no new cases of COVID-19 infections have been reported in Macau for the past 40 days (from February 4 to March 14, 2020). Import transmission was minimized by an early response by the government and the isolation of potential infectious sources by identifying visitors from high incidence areas. Many of the policies abided by the WHO's guidance on protective measures—including promoting hand hygiene and social distancing. Moreover, being a densely populated region, the government understood that social distancing is not always possible. Therefore, the government advised the use of face masks, and this seems to have been effective in limiting community transmission.

Up until this paper was submitted, there had been 34 new cases of confirmed COVID-19 infections in Macau (7), all of which were imported cases. These imported cases were mainly overseas students and residents returning to Macau from Europe or North America, and this corresponded to the increasing incidence of COVID-19 in those parts of the world. Nonetheless, the Macau government continued to pursue effective policies to combat this pandemic—which can be summed up in the government's message to the public: "Let's all persist: avoid crowd gathering, wash hands frequently, wear a mask properly, declare health conditions, reduce leaving Macau." The region has a lot of logistic, medical, and economic developments planned. In the future, there should be more resources given to a disease control system within the region, these development should be synchronized along with the economic development in the region.

DATA AVAILABILITY STATEMENT

Publicly accessible dataset were analyzed in this study. These can be found here: https://www.ssm.gov.mo/apps1/PreventCOVID-19/en.aspx#clg17046.

AUTHOR CONTRIBUTIONS

SI contributed to the current policy and confirm cases review and the writing of the manuscript. IC contributed background and previous experience review. All authors contributed to the article and approved the submitted version.

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OPINION
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COVID-19: Dogma Over Potential for Prolonged Droplet Dispersal in Air

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INTRODUCTION

A typical cough or sneeze produces thousands of different droplets, some of which are relatively large, say 0.5–3 mm in diameter and these typically travel less than a few meters but have been reported to go up to 6 m (1). Many more droplets produced are much smaller (ranging from <0.3 to 2.5 μm) (2) and as a result, just like some wind-dispersed spores and pollen, can remain suspended in air for many seconds, hours, and even days in certain conditions aided by turbulence, updrafts and thermals. Although the smallest droplets are much more numerous (97% reported to be <1 μm^3), the volume of larger droplets exceeds that of the combined volume of smaller droplets and it is not known how many virus particles are contained in small or large droplets. There is a great deal of variation in reported droplet production between different studies [e.g., Duguid (3) reported most droplets produced from sneezes, coughs, and from talking loudly were between 25 and 75 μm, with some droplets up to 2,000 μm in diameter, while more recent studies focussing on coughing primarily report much smaller droplets to be more numerous, mostly 0.5–12 μm in diameter (4–6) and one recent study suggest that no droplets are produced over 16 μm in diameter (7)]. Some studies use the term droplet nuclei for the smallest droplets (<5 μm diameter) and "droplet" for those over 5 μm diameter (8) "that fall rapidly to the ground under gravity, and therefore are transmitted only over a limited distance (e.g., ≤1 m)" (8). We will examine this point later in this article by referring to all size categories as droplets and discussing the fall speeds of small droplets in still air and studies on plant pathogens in droplets produced from rain-splashes, which have shown dispersal of airborne droplets <1 mm in diameter exceeding several meters in moderately windy conditions (9). These intermediate-sized droplets ranging from 10 to 1,000 μm may therefore pose a risk in terms of remaining airborne for significant periods (e.g., 0.3–10 min) and could contain a relatively large source of inoculum.

In conditions of low relative humidity, evaporation of water molecules from the surface of droplets makes them ever smaller and lighter and for droplets containing virus or other constituents, such as mucus, salts and proteins, evaporation while the droplet is suspended in air, leaves the virus particles and other materials originally in the droplet, suspended in air as a smaller dry particle made up of the different constituents clumped together as one. In some cases, such desiccation and concentration of salts may denature a virus but it is plausible that some virus particles can remain active for at least short periods when dry and longer if humid conditions prevent evaporation so that the virus remains protected in a droplet. Both small and large droplets can be responsible for contaminating nearby surfaces that they fall onto, or the hands of the affected person, leading to spread of the disease by later contact with an uninfected person. This is thought to be the main route of transmission following studies on influenza (9). For this reason, advice to avoid SARS-CoV-2, the virus that causes COVID-19 disease, and more generally to avoid other infections is to wash hands frequently and avoid touching the face or close contact with another person. In addition, common advice given as a response to the COVID-19 epidemic is to keep a distance of at least 2 m from another person (USA and UK government advice). This is because coarse droplets could be projected by coughs, sneezes, and even speech to contaminate another

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person's face, hands, or clothes, while microscopic droplets (<10 μm in diameter) can remain in the air as an aerosol and in addition to falling onto clothes and surfaces, they could be inhaled directly by an uninfected person to infect their upper or lower respiratory tract. Clearly, the further away from a potentially infected person we stand, the more we reduce the risk of catching the infection. But how far away is a safe distance? Here, we review what factors affect the risk of infection by dispersal of droplets in air and how that can guide advice to avoid COVID-19 infection with reference to an under-reported size-range of fine spray droplets that could carry the virus.

It is often difficult to study dispersal of human pathogens in real conditions although a lot can be inferred from epidemiological studies and mathematical modeling. However, there is a wealth of literature on the dispersal of plant pathogen spores that we can use as a proxy to estimate droplet dispersal of human viruses in this case. We are not considering dispersal of dry spores or plant pollen that are adapted for long-distance dispersal, only the dispersal of water droplets that may contain fungal or oomycete spores, bacteria or virus particles. In the case of plant pathogens, these can be splashed from infected leaves, fruit or plant debris. The behavior of a water droplet once in the air is the same, whether it holds a biological particle or not and once the droplet's momentum from the initial release event has subsided and the droplet is suspended as an aerosol.

DROPLET DISPERSAL IN STILL AND MOVING AIR

A wealth of studies has shown splash dispersal of plant pathogens in controlled conditions by dropping simulated raindrops down a shaft or tower onto an infected plant or by sampling in field conditions (10–14). Spore-containing droplets splashed from the infected plant or other media may be collected on water-sensitive paper, microscope slides or by other passive air-sampling devices arranged at different distances downwind. Just as with coughs and sneezes, different sizes of droplet are produced. They comprise relatively large droplets (1–5.5 mm diameter) that are dispersed ballistically up to a few meters [i.e., following a trajectory based on their speed of release and size, which affects their momentum; aerosolised microscopic droplets (<10 μm), which remain airborne for considerable periods (e.g., over a hour) even in still air; and intermediate-sized spray droplets (>10 μm <1 mm diameter) that form a fine spray that fall in still air but remain airborne for much longer than ballistic droplets and are able to be blown in the wind for distances up to tens of meters]. Perryman et al. (3), using a wind-tunnel in combination with a rain-tower to produce splash droplets from the surface of orange fruits infected with a fungus, found that as wind speed increased, an increasing number of fine droplets (>10 μm <1 mm diameter) were blown downwind. At the maximum wind speed investigated (7 m s^{-1}), these fine droplets were detected 8 m downwind of the source and their maximum vertical position with distance downwind showed that some were still moving upwards, meaning that they were behaving as an aerosol affected by eddy diffusion (15). Evaporation of these droplets was considered to be negligible during a flight covering 8 m in <1.15 s and because the ambient conditions were relatively cool and humid (around 15°C and 70–80% relative humidity).

The fall-speed (cm per second) of a spherical object with the density of water in still air at 20°C is approximated to 0.00308 multiplied by the square of the particle diameter (in μm) (16). So, a roughly spherical droplet of 2 μm diameter would fall at 0.012 cm/s, a droplet of 20 μm diameter, would fall at around 1.2 cm/s and a particle of about 200 μm diameter (0.2 mm) would fall at 123 cm/s. In contrast to the plant pathology studies, where the kinetic force producing the splash droplets came from the falling rain drop, Lindsley et al. (2) found that most aerosol droplets produced by a person coughing were under 2.5 μm diameter, so these would fall at negligible speeds. This means that in still conditions, a fine aerosol produced from coughing could remain airborne for hours, while larger droplets (larger than the aerosol fraction) generated by a cough or sneeze from a person would fall onto nearby surfaces. Intermediate-sized droplets (>10 μm <1 mm diameter) seem to be relatively rarely produced by coughing (1, 2) but if these moved due to the turbulence caused by the force of a cough or sneeze to say 2 m height, they would then take about 30 s for a 20 μm droplet to fall back 40 cm, to where somebody 1.6 m in height might breath it in.

SARS-CoV-2 SURVIVAL IN DROPLETS

Factors affecting the survival and dispersal of the virus include the material the droplet is formed of (i.e., a solution of mucus, salts, and cell contents in water, and the conditions the droplet is exposed to during dispersal). The loss of water by evaporation from the surface of droplets during their flight may affect the activity (activity is used here because the virus particle itself is not a viable organism but when active can infect a living cell) or loss of activity of the infectious agent being transported because the water may partially protect the enclosed virus from desiccation and variations of temperature, and exposure to oxygen, ozone, and other chemicals. Estimates made for SARS-CoV-2 persistence of activity while suspended in air range from up to 30 min to a half-life in air of several hours (17–19). However, it is unclear whether different droplet sizes, ambient conditions (temperature and relative humidity) and droplet compositions (concentration of virus and cell contents) have been studied. A virus in a microscopic water droplet may become a free virus particle suspended in air if the water droplet around it evaporates. Such a dry and microscopic virus particle is more likely to lose activity but could remain airborne and in a dry state, may not be completely prevented from being inhaled even by a specialist facemask such as the N95 or FFP2 mask (1). The "infective dose" for SARS-CoV-2 is not known but the idea of an infective dose is really down to a combination of probabilities of infection occurring based on the percentage of virus particles that are actually active, evade immune responses and whether they end up at a potential infection site. Although the chances of infection decline as the number of active virus particles a person is exposed to reduces, in theory, just one active virus

particle falling onto exactly the right receptor could still lead to infection, while a high exposure to virus, increases this risk of infection considerably.

DISCUSSION

Following the study of Perryman et al. (10), it is clear that effects of turbulence in moving air can cause intermediate-sized droplets (10 μm to 1 mm) to remain airborne and even disperse upwards as they travel down wind. This size-range of droplets is larger than those normally considered to form an aerosol and may have been neglected in previous medical studies but many other biological particles in this size range are known to disperse in air (e.g., uredospores of cereal rust fungi are 22 μm in aerodynamic diameter (15) and have been reported to disperse in air over continents and oceans (20), moss spores up to 40 μm diameter have been collected in the arctic having dispersed from warmer production sites (15) and grass pollen ranging from 20 to 50 μm diameter (15) is known to be primarily produced in the countryside in great amounts but is dispersed to affect people prone to hay-fever in city centers, often several Km from the nearest large sources of flowering grass). Although these and any aerosolised microscopic droplets would dilute as they disperse over distance and time, just like a plume of smoke, it is possible they could be breathed in by an unsuspecting person seconds, minutes, and even hours later. To sample very small droplets or small dry particles, it is important to use the correct air sampling devices such as wet cyclones or liquid impingers, which are designed to collect particles as small as below 1 μm (aerodynamic diameter) with good collection efficiency (21). One COVID-19 study (22) concluded that the virus wasn't airborne but had used an air sampler that directs the airflow via a perforated plate to impact onto solid culture media, and this may not collect small particles efficiently. Data for similar devices show EC_{50} (the cut-off for collection of 50% of particles) values to be over 4 μm, meaning that droplets smaller than this are increasingly less likely to be collected (21).

The persistence of activity of SARS-CoV-2 particles in airborne droplets appears to be sufficient to pose a threat. Droplets may not even require coughing because production of fine droplets containing influenza virus particles has been demonstrated simply from normal breathing (23). If subsequent dispersal of aerosolised droplets shown in plant pathogen studies pertain to aerosolised droplets containing SARS-CoV-2 virus particles, and assuming estimates of up to 1–7-h half-life (duration of activity) (18, 19) of the SARS-CoV-2 virus in typical ambient conditions are correct, logic suggests there must be potential for an aerosol-based infection route for this disease, which could occur at significant time after droplet production and also at distances downwind of an infected person in outdoor conditions. Huang (1) suggests that larger droplets may also pose a risk based on the inner surfaces of the nose acting as a potential infection site. If that is the case, the fact that many thousands of microscopic droplets are produced by a cough and with the possibility that intermediate-sized droplets can also remain in air for many seconds, traveling many meters in outdoor moving air, greater importance should be placed on using facemasks to prevent these droplets being inhaled, in addition to the current advice to wash hands regularly.

AUTHOR CONTRIBUTIONS

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REVIEW
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Implementing COVID-19 (SARS-CoV-2) Rapid Diagnostic Tests in Sub-Saharan Africa: A Review

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Introduction: For the COVID-19 (SARS-CoV-2) response, COVID-19 antigen (Ag), and antibody (Ab) rapid diagnostic tests (RDTs) are expected to complement central molecular testing particularly in low-resource settings. The present review assesses requirements for implementation of COVID-19 RDTs in sub-Saharan Africa.

Methods: Review of PubMed-published articles assessing COVID-19 RDTs complemented with Instructions for Use (IFU) of products.

Results: In total 47 articles on two COVID-19 Ag RDTs and 54 COVID-19 Ab RDTs and IFUs of 20 COVID-19 Ab RDTs were retrieved. Only five COVID-19 Ab RDTs (9.3%) were assessed with capillary blood sampling at the point-of-care; none of the studies were conducted in sub-Saharan Africa. *Sampling:* Challenges for COVID-19 Ag RDTs include nasopharyngeal sampling (technique, biosafety) and sample stability; for COVID-19 Ab RDTs equivalence of whole blood vs. plasma/serum needs further validation (assessed for only eight (14.8%) products). *Sensitivity—Specificity*: sensitivity of COVID-19 Ag and Ab RDTs depend on viral load (antigen) and timeframe (antibody), respectively; COVID-19 Ab tests have lower sensitivity compared to laboratory test platforms and the kinetics of IgM and IgG are very similar. Reported specificity was high but has not yet been assessed against tropical pathogens. *Kit configuration:* For COVID-19 Ag RDTs, flocked swabs should be added to the kit; for COVID-19 Ab RDTs, finger prick sampling materials, transfer devices, and controls should be added (currently only supplied in 15, 5, and 1/20 products). *Usability and Robustness:* some COVID-19 Ab RDTs showed high proportions of faint lines (>40%) or invalid results (>20%). Shortcomings were reported for buffer vials (spills, air bubbles) and their instructions for use. *Stability:* storage temperature was ≤30°C for all but one RDT, in-use and result stability were maximal at 1 h and 30 min, respectively. *Integration in the healthcare setting* requires a target product profile, landscape overview of technologies, certified manufacturing capacity, a sustainable market, and a stringent but timely regulation. In-country deployment depends on integration in the national laboratory network.

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Discussion/Conclusion: Despite these limitations, successful implementation models in triage, contact tracing, and surveillance have been proposed, in particular for COVID-19 Ab RDTs. Valuable experience is available from implementation of other disease-specific RDTs in sub-Saharan Africa.

Keywords: COVID-19, diagnostics, low-resource settings, sub-Saharan Africa, rapid diagnostic tests (RDT), SARS—CoV-2

THE COVID-19 PANDEMIC AND SUB-SAHARAN AFRICA: THE NEED FOR POINT-OF-CARE DIAGNOSTICS

On January 30 2020, the World Health Organization (WHO) declared the coronavirus disease COVID-19 (caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), in this paper referred to as COVID-19) outbreak as a Public Health Emergency of International Concern, and shortly thereafter called for research on point-of-care (POC) *in-vitro* diagnostics (IVDs) for use at the community level (1). In response, numerous POC IVDs are in development or have entered the market, many of which are so-called rapid diagnostic tests (RDTs) (2).

WHO recommends nucleic acid amplification tests (NAAT) for identification of COVID-19 infection in triage and the tracing of contacts (3, 4). However, in low-income countries, sub-Saharan Africa (sSA) in particular, molecular testing is frequently only available in central reference laboratories. Moreover, testing capacity is limited, leading to long turnaround times which preclude the use for patients and infection control management (5–8).

RDTs are equipment-free, generate a result in a short time (mostly within 30 min), can be operated at the POC level, and by minimally trained healthcare workers outside central laboratory test facilities (9). As of August 18 2020, the Foundation for Innovative New Diagnostics (FIND) (2) lists 18 SARS-CoV-2 antigen-detection RDTs and 163 SARS-CoV-2 antibody detection RDTs that are currently marketed or in development, of which, respectively, 17 and 155 have regulatory approval by the European Community [Conformité Européenne (CE) mark] and five antibody detection RDTs have approval from the United States Federal Drug Agency (US FDA, Emergency Use List). Countries in sSA have successfully deployed RDTs for HIV and malaria diagnosis (10), adding to the expectation for the implementation and successful roll-out of RDTs for the detection of the COVID-19 infection. However, published evidence of performance of these RDTs so far is limited (see below). Most studies focused on diagnostic accuracy and were carried out in reference settings in high- and middle-income countries early affected by the COVID-19 pandemic (11). By contrast, few studies have assessed POC use and RDT user-friendliness and, to the best of our knowledge, so far none have assessed their integration in the healthcare setting in sSA.

THE SCOPE OF THIS PAPER, TERMS USED

The present article aims to pinpoint product- and healthcare-related requirements for the implementation of RDTs in detecting the SARS-CoV-2 infection in the context of sSA. The term "low-resource settings" (LRS) refers to low-income countries (of which 29 out of 33 are located in sSA) (12) as well as to remote and under-served areas in middle-income countries.

The ASSURED criteria [affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to those who need it (13)] were used to interpret the WHO request for "POC diagnostics for use in the community" (14) and to define the COVID-19 RDT products. Rather than aggregating and comparing diagnostic accuracy of the COVID-19 RDTs, the present review reviews their design (format, package, and configuration), specimen and sampling, usability, robustness, and stability, all in view of the end-user and large-scale implementation in sSA. Where relevant, comparisons are made with the deployment of RDTs in sSA targeting malaria, HIV, and other infectious diseases. Among those listed by the WHO, the testing scenarios considered for the COVID-19 response are (i) case management of suspects (detect active infection, triage), (ii) contact tracing (detect asymptomatic and symptomatic acute infection), and (iii) surveillance (detect acute or past exposure or infection). The scenarios of monitoring response/recovery, tool for prognosis, vaccine response, and environmental monitoring are not addressed (15).

From a communication perspective (i.e., avoiding confusion with the SARS virus epidemic from 2002), the WHO decided to name the disease which was caused by the Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) not after the virus; instead the WHO proposed the name "COVID-19 disease" (16). The name COVID-19 has been widely adopted by the scientific community as well as by health authorities and the lay press. For convenience and easy reading, the present text, IVDs and RDTs for COVID-19 disease are therefore further referred to as COVID-19 IVDs and COVID-19 RDTs, respectively, with antigen-detection and antibody-detection RDTs written as COVID-19 Ag RDTs and COVID-19 Ab RDTs. When referring to the virus or IVD brand names, the term SARS-CoV-2 is used.

SEARCH STRATEGY

We have reflected on COVID-19 RDTs that are currently being developed and marketed. Guidelines and policy briefs from international organizations [WHO, Africa and Europe Centers

for Disease Control and Prevention (Africa CDC, ECDC)], US FDA, International Medical Device Regulators Forum) and published literature (English and French language) were searched for the implementation of RDTs in response to COVID-19 and the control of other infectious diseases in sSA (malaria, HIV, cholera, respiratory tract viruses). Selected items were further explored by the snowball strategy using PubMed and gray literature, complemented with our own field observations.

To assess the published evidence about COVID-19 RDTs, a literature search was performed on PubMed using the strings "(COVID-19) AND diagnostic" "(COVID-19) AND antigen," and "(COVID-19) AND antibody." Based on successive screening of the title, abstract, and full text, original research articles that reported the evaluation of an IVD for COVID-19 were included. Articles assessing COVID-19 IVDs that met the ASSURED criteria were analyzed in detail, excluding non-commercialized products and those which did not evaluate clinical specimens or did not include controls. For each RDT, the following data were extracted and imported into a Microsoft Excel worksheet (**Supplementary Table 1**): PMID, title, assay type and target (antigen/antibody), brand, authors, citation, product code, and lot number. Technical specifications and product performance characteristics were extracted. Pre-publication papers were not included. The search was last updated on August 10 2020.

For a subset of one COVID-19 Ag RDT product and of 20 COVID-19 Ab RDTs retrieved by the literature search, the instructions for use (IFU) were obtained from the manufacturer's website or by correspondence with the manufacturer. Complementary information about format, configuration, package, eligible specimens, and stability were retrieved from the IFU and added to the worksheet.

To discuss the utility of COVID-19 Ab RDTs in the different testing scenarios, we used published accuracy data from two recent meta-analysis studies addressing COVID-19 Ab RDTs, as one of the studies provided a comparison between RDTs and laboratory-confined antibody testing by ELISA and chemiluminescence assays (CLIA) (11, 17). Data about the review was primarily presented with the number of RDT products (rather than the number of studies) as the denominator.

COVID-19 AG RDTS AND COVID-19 AB RDTS: PRODUCTS AND STUDIES RETRIEVED

A total of 47 articles on COVID-19 RDTs were retrieved, 42 (89.4%) of them assessed COVID-19 Ab RDTs comprising a total of 54 RDT products. Another 5 (10.6%) studies assessed COVID-19 Ag RDTs, all assessing the two products, i.e., the COVID-19 Ag Respi-Strip (CORIS BioConcept®, Gembloux, Belgium), further shortly referred to as CORIS COVID-19 Ag Respi-Strip and the BIOCREDIT COVID-19 Ag test (RapiGEN Inc. Gyeonggi-do, Republic of Korea) further shortly referred to a BIOCREDIT COVID-19 Ag test. To compare the latter products, the single other POC IVD for COVID-19 Ag detection retrieved in the literature search was used, i.e., the 2019-Novel Coronavirus (2019-nCoV) Antigen Rapid Test Kit (BIOEASY Biotechnology Co., Shenzhen, China), further referred to as BIOEASY 2019-nCoV Ag Rapid Test Kit. The BIOEASY 2019-nCoV Ag Rapid Test Kit is based on immunofluorescence and needs a reader, so does not fit the ASSUR*E*D criteria (*E* = equipment-free).

In addition to the COVID-19 RDTs, a number of low-complexity cartridge-based NAAT platforms (comprising sample preparation, amplification, and signal visualization in a closed format) were identified during the literature search, as well as simplified (e.g., isothermal) COVID-19 based IVDs which are in development (18–20). Although some are promising for POC testing, they are not equipment-free and are thus not discussed here.

All 56 COVID-19 RDTs were based on the lateral flow immunochromatographic test platform comprising a nitrocellulose strip embedded in a cassette or applied in a tube format and with test results presenting as colored lines read by the naked eye.

As to regulation, according to the FIND SARS-CoV-2 Diagnostic Pipeline (2), the CORIS COVID-19 Ag Respi-Strip and the BIOCREDIT COVID-19 Ag RDTs as well as the BIOEASY 2019-nCoV Ag Rapid Test Kit were CE marked. Of the 54 COVID-19 Ab RDTs, 40 (74.1%) were also listed on the FIND SARS-CoV-2 Diagnostic Pipeline; 34 (85%) of them were CE-marked (63.0% of all COVID-19 Ab RDTs), two products had FDA-Emergency Use Approval.

Both the CORIS COVID-19 Ag Respi-Strip and the BIOEASY 2019-nCoV Ag Rapid Test Kit detected the nucleocapsid protein of the SARS-CoV-2 virus. This choice was based on the 2003 SARS-CoV epidemic, which identified the nucleocapsid protein as the best target for antigen detection, with high sensitivity in an ELISA and RDTs (21–24). The nucleocapsid protein is relatively conserved, immunogenic, and abundantly expressed during infection (22, 25). The antigen detected by the BIOCREDIT COVID-19 Ag test was not indicated in the article evaluating the product, the IFU of this product could not be retrieved.

The product specifications of the COVID-19 Ab detecting RDTs retrieved from the published papers and the IFUs are listed in **Tables 1**, **2**, respectively. Over 90% of products detected both IgG and IgM; three of these products had separate strips for both antigens. Products used either recombinant spike or nucleocapsid protein or both as the detection antigen (**Table 1**). The spike protein is of interest as it is highly conserved and specific and its receptor-binding domain protein (RBD-S) is expected to be neutralizing (25, 26).

For two-thirds (36/54, 66.0%) of the products, the identity of the recombinant detection antigen was not mentioned in the article and neither was it mentioned in 70% of product IFUs (**Table 2**). This proportion is in line with the observation of Pallett et al. (27). They reported that the majority of the 284 COVID-19 Ab immunodiagnostics assessed (of which many had regulatory approval) either made a non-specific reference to the SARS-CoV-2 antigen and antibody targeted (59.2%) or listed no information whatsoever (17.3%) about the nature of the antigen or antibody targeted—these proportions were higher compared to the ELISA platform (27). For the International Medical Device Regulators Forum (28), proprietary information

TABLE 1 | Selected product specifications and study design for 54 COVID-19 antibody detection rapid diagnostic tests (RDTs) retrieved from 45 peer reviewed original research articles.

Product Specifications Study Design	RDT products	
	Nr	%
ANTIBODIES DETECTED		
○ IgG	1	1.9
○ IgM	1	1.9
○ IgG & IgM	50	92.6
○ Total antibodies	2	3.7
DETECTING ANTIGEN (BINDS ANTIBODIES)		
○ Spike protein	8	14.8
○ Nucleocapsid protein	4	7.4
○ Spike protein and Nucleocapsid protein	6	11.1
○ Could not be retrieved by investigator	4	7.4
○ Not mentioned	32	59.3
SPECIMEN ASSESSED		
○ Serum or plasma only	29	53.7
○ Venous whole blood (with/without other specimens)	19	35.2
○ Capillary whole blood (with/without other specimens)	5	9.3
○ Not mentioned	1	1.9
○ Equivalence of claimed specimen types	6	11.1
○ Equivalence of claimed anticoagulants	0	0
ORIGIN OF SAMPLES FROM INDEX PATIENTS:		
○ Hospitalized patients	23	42.6
○ Outpatients	7	13.0
○ Not specified if in- or out-patients	26	48.1
○ Disease severity mentioned	7	13.0
ORIGIN OF SAMPLES FORM CONTROL PATIENTS		
○ Hospitalized patients	18	33.3
○ Outpatients	9	16.7
○ Not specified if in- or out-patients	26	48.1
○ Disease severity mentioned	1	1.9
GEOGRAPHIC ORIGIN OF PATIENTS ASSESSED		
○ Asia	12	22.2
○ North America	8	14.8
○ South America	4	7.4
○ Europe	42	77.8
○ Australia	5	9.3

All RDTs are lateral-flow immunochromatography assays. Numbers refer to the number of COVID-19 RDT products.

does not need to be disclosed in the IFU. Information about the nature of the antigen and antibody targeted, however, cannot be labeled as proprietary information and is essential for the comparison and monitoring of the diagnostic accuracy but also for the interpretation of seroprevalence studies and presumed immunities (27). In addition, the WHO recommends that the IFU of RDTs should contain enough and detailed information about the test principles including identification of the antibody and antigen and the chemical principles of detection (29).

The origin of patient and control samples were not specified for nearly half of the COVID-19 Ab RDTs assessed and disease

TABLE 2 | Selected specifications and test characteristics of a subset of 20 COVID-19 antibody detection rapid diagnostic tests (RDTs) retrieved from 20 products' instructions for users (IFU).

Product specifications	Nr	%
RECOMBINANT DETECTION ANTIGEN		
○ Spike protein	3	15.0%
○ Nucleocapsid protein	0	0.0%
○ Spike and Nucleocapsid protein	3	15.0%
○ Not mentioned	14	70.0%
FORMAT, CONFIGURATION, PACKAGE		
○ Strip-in-cassette	20	100.0%
○ Strip-in-tube	0	0.0%
○ Sampling material in kit	4	20.0%
○ Transfer device in kit	15	75.0%
○ Self-contained kit (containing both sampling materials and transfer device)	20	100.0%
○ Controls included in the kit	1	5.0%
CLAIMED SPECIMENS		
○ Plasma / Serum	2	10.0%
○ Serum/ Plasma/ Whole blood	8	40.0%
○ Serum/ Plasma/ Whole blood including capillary finger prick blood	8	40.0%
○ Serum/Plasma/Whole blood but not recommended for finger prick blood	2	10.0%
REPORTING OF SENSITIVITY		
○ Sensitivity expressed in function of time since symptom onset	5	25%
STORAGE TEMPERATURE		
○ 2/4°C up to 30 °C	20	100.0%
OPERATING CONDITIONS AND IN-USE STABILITY (STABILITY AFTER OPENING THE DEVICE POUCH)		
○ Operating conditions mentioned	0	0%
○ In-use stability 30 min	1	5.0%
○ In-use stability 1 h	3	15.0%
○ No in-use stability mentioned, "process immediately"	16	80.0%
SHELF-LIFE		
○ 2 months	1	5.0%
○ 6 months	1	5.0%
○ 12 months	3	15.0%
○ 18 months	2	10.0%
○ Not mentioned in IFU	13	65.0%
RESULT STABILITY		
○ 15 min	5	25.0%
○ 20 min	11	55.0%
○ 30 min	1	5.0%
○ Not mentioned in IFU	3	15.0%
○ **SAMPLE STABILITY**		
○ Capillary blood finger prick: "perform immediately" (all 8 products)		
○ Venous whole blood: 2–7 days at 4–8°C (median 3)		
○ Serum/Plasma: 2–7 days at 4–8°C (median 3)		
○ Not mentioned for 9 (45%) products		

All RDTs are lateral-flow immunochromatography assays. Numbers refer to the number of COVID-19 RDT products.

severity was only reported for a minority of product evaluations [seven products assessed in four studies (**Table 1**)]. The origin of patients (hospitalized vs. non-hospitalized) was not reported for nearly half of the products, and details of disease severity were provided for only a few products. Of note, viral load is expected to be higher in hospitalized vs. non-hospitalized patients (30), and sensitivity and test line intensities are lower in mild COVID-19 disease (31, 32). Providing relevant patient information is part of the Standards for the Reporting of Diagnostic Accuracy Studies (STARD) checklists (33) and essential to understand a product's performance in different settings (11). Further, <10% (5/56) of COVID-19 RDTs assessed in our literature review were evaluated in the POC setting and no study was conducted in sSA (**Table 1**). Among 17 studies evaluating COVID-19 Ab RDTs reviewed in a recent meta-analysis, only two were conducted at the point of care, representing only 2% of the total tests assessed (11).

IMPLEMENTING COVID-19 RDTS FOR SUB-SAHARAN AFRICA: THE RDT PRODUCT

Specimen and Sampling

COVID-19 Ag RDTs

So far nasopharyngeal secretions are the preferred specimen for COVID-19 Ag RDTs as well as for NAAT reference testing (34). Specimen equivalence of the CORIS COVID-19 Ag Respi-Strip has been evaluated in one study, demonstrating equivalence of nasopharyngeal aspirates, and nasopharyngeal swabs (21). In addition, the product's IFU mentions nasopharyngeal washes as an eligible specimen—however, this information was not supported by published evidence. The BIOCREDIT COVID-19 Ag test was evaluated on saliva, nasopharyngeal swabs, nasopharyngeal aspirates, throat swab, throat swabs, and sputum (35). The BIOEASY 2019-nCoV Antigen Rapid Test Kit has published an evaluation of nasal/nasopharyngeal swabs and oropharyngeal swabs as eligible specimens (36); the product's IFU in addition mentions sputum as a specimen with no published data referred. Given patients' reluctance for diagnostic sampling in LRS (37), alternative specimens (such as saliva) would be more acceptable than a nasopharyngeal swab or aspirate (38, 39). COVID-19 has been detected in self-collected saliva samples using NAAT methods but this needs further study (40, 41).

For COVID-19 antigen detection, sample stability is a concern: in the studies published on the CORIS COVID-19 Ag Respi-Strip, the BIOEASY 2019-nCoV Antigen Rapid Test Kit, and the BIOCREDIT COVID-19 Ag test, samples were kept at 4°C or −70°C when testing could not be done immediately, which indicates the need for a cold chain (21, 35, 36). The IFU of the CORIS COVID-19 Ag Respi-Strip indeed confirms the need for freezing at −20°C if immediate testing of the sample is not possible and mentions a loss of signal intensity when samples are stored at 4°C. By consequence, sample stability of the COVID-19 Ag RDTs is a concern. As a comparison, the WHO draft specifications for COVID-19 POC IVDs deployable at triage list as a minimum ("acceptable") requirement a pre-testing sample stability of 30 min at 10–35°C, 2–4 h at 2 to 8°C and 8 h in a generic preservative at 2–8°C (39).

To facilitate logistics and prevent patients being lost to follow-up, the sample or sample-buffer mixture for the COVID-19 antigen testing should be appropriate for downstream NAAT-testing (sufficient volume, RDT buffer compatible with the NAAT assay, preserved stability, and contained in a leak-free tube). In the publications on both the aforementioned COVID-19 Ag RDTs the same sample was used for NAAT and Ag detection, indicating the possibility of downstream NAAT (21, 35, 36).

COVID-19 Ab RDTs

For COVID-19 Ab RDTs, finger prick capillary blood specimens stand out as the preferred specimen (6, 11, 42), as finger pricks are minimally invasive and safe and easy to perform. In addition, in sSA, healthcare workers and patients are familiar with finger prick sampling, particularly in malaria-endemic areas. Probably explained by the use of stored (left-over) samples, published evaluations of the COVID-19 Ab RDTs were done on only serum or plasma for half (29/54 products, 53.7%) of the COVID-19 Ab products; 19 (35.2%) were also evaluated on venous blood and five (9.3%) on capillary whole blood, all of them in a POC setting (**Table 2**). Only eight COVID-19 Ab RDTs (14.8%) in four studies have published evidence about equivalence of venous whole blood with serum or plasma (31, 42–45). In these studies, plasma was obtained by centrifugation of EDTA whole blood and over 97% agreement was found between both specimen types. Only one article (assessing a single product) studied specimen equivalence between plasma, venous whole blood, and finger prick blood and found no difference in the 10 paired samples (seven COVID-19 patients and three healthy controls) assessed (29). Although so far venous whole blood and serum appears to be equivalent, further study is needed to validate the specimen equivalence, as serum and plasma are expected to have higher antibody titers compared to whole blood (11). None of the studies retrieved had assessed the equivalence of different anticoagulants (**Table 2**).

Specimen type may affect diagnostic performance of RDTs: as an example, for HIV 1/2 RDTs, higher numbers of false positives in whole blood as compared to plasma specimens were shown (46). Further, the concentration of antibodies is higher in serum and plasma than in whole blood, which may lead to differences in sensitivity and specificity if the same volume is used (47). In their IFUs, all 20 COVID-19 Ab RDTs mentioned both serum and plasma. Two products had only plasma and serum mentioned as eligible specimens in their IFU, and two products indicated the use of serum, plasma, and whole blood but specified that finger prick blood was not recommended.

ASSURED: Sensitivity and Specificity of the COVID-19 RDTs, Utility in Testing Scenarios

COVID-19 Ag RDTs

At reference testing, the specificity of the COVID-19 Ag RDTs was 100% in all studies for both products (21, 36, 48–50) but diagnostic sensitivity was low for the CORIS COVID-19 Ag

Respi-Strip: sensitivity was 82–100% for samples with high viral load but overall sensitivity ranged from 24 to 58% (21, 48–50). The BIOEASY 2019-nCoV Antigen Rapid Test Kit showed a higher overall sensitivity (95%), which however declined to 72% in patients with low viral loads (36). The higher sensitivity may be explained by the fact that the fluorescent signal was detected by equipment as compared to a colorimetric reading by the naked eye in the case of the CORIS COVID-19 Respi-Strip.

The pattern of moderate sensitivity/high specificity of the CORIS COVID-19 Ag Respi-Strip is comparable with those of influenza RDTs (51) and its consequences are twofold. Firstly—provided confirmation of the high specificity in large prospective series—a positive test result can be confidently accepted as a diagnosis of acute COVID-19 infection. Secondly, given the low sensitivity, negative test results imply referral of the patient (or sample) for subsequent NAAT testing (21). In a similar scenario in Kenya, influenza Ag RDTs have been proposed for surveillance and even clinical management in remote settings where capacity is limited (52). Although COVID-19 Ag RDTs would be of benefit in a triage scenario [short time-to-result, cost-saving, alleviating central testing (21)], the sensitivity of the CORIS COVID-19 Ag Respi-Strip is below the required sensitivity for a decentralized stand-alone POC triage (≥70% acceptable, ≥80% desirable) (39). Furthermore, an even higher sensitivity (≥95% acceptable, ≥98% desirable) is needed in the scenario of COVID-19 contact tracing and diagnosis of cases with subacute infection, as both viral load and pre-test probability (prevalence) are lower compared to the triage setting of acute symptomatic patients (15).

By consequence, sensitivity needs to be improved while maintaining a high specificity, as has been achieved for influenza Ag RDTs and potentially the BIOEASY 2019-nCoV Antigen Rapid Test Kit (53) by optimization of test chemistry and signal detection through digital reading equipment.

In order to use COVID-19 Ag RDTs as a tool to demonstrate viral clearance after recovery (e.g., for reasons of infection control or safely resuming work), further data about the SARS-CoV-2 antigen and viable virus dynamics during the COVID-19 infection are needed. The WHO interim guidelines for COVID-19 laboratory testing (March 2020) (54) do not mention COVID-19 Ag tests for any testing scenario. In a scientific brief, the WHO did not recommend COVID-19 Ag RDTs for patient care (55). FIND mentions the possibility of using COVID-19 Ag tests for case management in high prevalence and active outbreak settings, i.e., (i) at triage (with confirmatory molecular testing of negative samples), (ii) to monitor active infections as well as (iii) in contact tracing (56).

COVID-19 Ab RDTs

Data about diagnostic performance of COVID-19 Ab RDTs were recently aggregated in two independent meta-analyses, both including data from peer-reviewed as well as pre-printed articles (11, 17). In addition, a Cochrane review concluded at the end of April 2020 assessed COVID-19 antibody detecting immunoassays but without stratifying for COVID-19 RDTs (30). An overview of COVID-19 antibody kinetics can be found in references (13, 26, 57–59). Briefly, IgM antibodies appear 5–10 days after the first day of symptoms, closely followed but sometimes overlapped by IgG antibodies. IgG and IgM antibodies increase during week 2 and peak in week 3, mean times for seroconversion (60) are 9–11 days after symptom onset for total antibody, 10–12 days for IgM and 12–14 days for IgG. Levels of IgM decline from week 5 onwards and are almost non-detectable by week 7 (26).

For the detection of IgG and/or IgM, both RDT meta-analysis studies computed for COVID-19 Ab RDTs in similar pooled sensitivities of 64.8 and 66.0%, which were much lower than the corresponding sensitivities of 97.8 and 84.3% for the laboratory-confined CLIAs and ELISAs, respectively (11). Pooled sensitivities of IgG (65%) and IgM (62%) were almost identical—precluding their differential use in diagnostic algorithms—and increased in parallel during the course of infection: in week 1 post symptom onset, aggregated sensitivity for IgM and IgG was 25.3 and 13.4% respectively, increasing to 51.8 and 50.1% in week 2 and exceeding 70% only from week 3 onwards (69.9 and 79.8%, respectively), with high variations between different products (11). Pooled specificity for COVID-19 Ab in the study of Bastos et al. was high (96.6%) but lower compared to ELISAs (99.7%) (11); Ricco et al. computed a specificity of 98.0%, respectively (17). However, as mentioned by the authors, specificities might be biased by the case-control design used in most studies (11, 17) as well as by reporting bias—i.e., exclusion from publication of products with low specificity (17).

The sensitivity and specificity findings of the studies retrieved in our literature review (assessed for 28 articles published until June 2020), are in line with the above: combined IgG/IgM sensitivity ranged from 42.3 to 100.0% and specificity from 89.2 to 100.0%. In addition, in these articles, we looked in detail at the control panels used for assessing specificity: they included other coronaviruses (SARS-CoV, NL63, HKU1, 229E, OC43), cytomegalovirus, Epstein-Barr virus, severe fever with thrombocytopenia syndrome virus, dengue virus, human hepatitis B virus, *Mycoplasma pneumoniae*, parvovirus infection, *Bartonella henselae*, *Brucella spp.*, and autoimmune pathologies. Apart from dengue, no other tropical disease was evaluated for potential cross-reactions and only few products were challenged with HIV 1/2 positive samples. Of note, tropical diseases such as malaria, dengue, schistosomiasis, and sleeping sickness have been associated with false positives in antibody detection RDTs for HIV1(/2) and malaria (61–65) but have, to the best of our knowledge, not yet been assessed for cross-reactions with COVID-19 Ab RDTs. Assessing COVID-19 Ab RDTs for contextual pathogens in sSA is urgently required (6) and the WHO requirements for Emergency Use Listing (see below) of COVID-19 Ab RDTs lists HIV and malaria among the list of organisms to be tested for cross-reactions (66).

Recommendations about the use of COVID-19 Ab IVDs in general (i.e., all diagnostic platform comments) are as follows: WHO interim guidelines for COVID-19 clinical management state that COVID-19 Ab IVDs have no place in the diagnosis of current COVID-19 infection (triage scenario) (4) except for patients presenting late who may have negative NAAT results. In these cases and provided there is a strong epidemiological

link to COVID-19 infection, paired serum samples (in the acute and convalescent phase) can support diagnosis through the demonstration of seroconversion (5, 67). Further, COVID-19 Ab IVDs can be used in the case of sero-epidemiological studies (which define levels and geographic extent of population exposure) (68). COVID-19 Ab tests should not be used as criteria to discharge patients from hospitals (as the presence of antibodies does not mean "non-infectivity") nor as criteria for (health care) workers to return to work [as the presence of antibodies does not mean "protection" (6, 69)]. Further, population screening in low prevalence settings is not recommended, as it will probably result in more false-positive than true positive results (60)—this will be particularly the case of RDTs given their lower specificity.

As for COVID-19 Ab RDTs, in a scientific brief from April 2020, the WHO recommended them only for research settings but not for patient care (55). Given the poor performance (in particular sensitivity of COVID-Ab RDTs compared to ELISA and CLIA platforms, both aforementioned meta-analysis studies share this conclusion and question the utility of using (or continuing to use) COVID-19 Ab RDTs for medical decision making (11, 17). For seroprevalence studies, the WHO mentions the option of COVID-19 RDTs, provided confirmatory testing by ELISA and with serum as the preferred specimen (68). FIND mentions the use of COVID-19 Ab RDTs for screening of contacts ≥10 days post exposure (56). The interim guidance on COVID-19 Ab RDTs from the African Union, Africa CDC, and WHO Africa (June 2020) also mentions three indications, particularly for areas with limited or no access to NAAT (6): COVID-19 Ab RDTs can be used as an initial screening at POC triage (with sampling patients who tested negative for molecular testing), screening for contacts (also with molecular testing of negative contacts), and surveillance (sero-epidemiological studies). In a viewpoint paper, the authors refer to the latter scenarios for the successful deployment of COVID-19 Ab RDTs in triage (Peru) and contact tracing (Singapore) (60).

Test Format, Configuration, and Package

COVID-19 Ag RDTs

The strip-in-tube format of the CORIS COVID-19 Ag Respi-Strip is less suitable to POC testing compared to the strip-in-cassette format which is preferred by healthcare workers performing malaria diagnosis (70) (**Figure 1**). Compared to the cassette, the tube format is more difficult to manipulate and writing the patient's identification is challenging (not enough space on the strip, a felt pen is needed to write on the tube). Moreover, there are biosafety issues: in similar strip formats, we demonstrated viable bacteria on processed cholera RDT strips (71). The BIOEASY 2019-nCoV Antigen Rapid Test Kit uses a cassette format, but sample preparation is at a similar level of complexity as the "strip-in-tube" format: the swab has to be inserted and mixed into a dropper bottle and next the mixture is applied from the dropper bottle to the cassette.

Sampling material is not included in either of the COVID-19 Ag RDTs we analyzed. However, despite adding to the cost, a "single pack" format (containing everything for a single sample test) could be more convenient for decentralized testing and in addition ensure the use of the correct buffer and buffer volume (72). Humidity in tropical countries accelerates RDT deterioration (70) and therefore a humidity-indicating desiccant should be added to the RDT strip package (73). Given their impact on sampling, the recommended flocked swabs for COVID-19 Ag RDTs (providing a higher volume uptake than conventional swabs) with an aluminum or plastic shaft (74, 75) should be included in the package and categorized as a kit component (i.e., essential to the RDT) rather than as an accessory (i.e., a replaceable item) (76).

COVID-19 Ab RDTs

All COVID-19 Ab RDTs we analyzed were based on the strip-in-cassette format (**Table 2**). While Africa CDC states as one of the advantages of COVID-19 Ab RDTs that they often include all the materials needed to perform the test including sampling materials and sample transfer devices (capillary tube or pipette) (6), finger prick material (lancets and alcohol swabs) were included in only 4/20 COVID-19 Ab RDT products for which the IFU was assessed. In the case of the FaStep COVID-19 IgG/IgM Rapid Test Device (Assure Tech. Hangzhou Co., Ltd, Hangzhou, China) the supply of finger prick material was especially confusing as only serum and plasma were listed as eligible specimens in its IFU. Five COVID-19 Ab RDTs did not include a sample transfer device in the test kit, requiring a micropipette to be present on site. The use of the sample transfer device provided in the kit, which is calibrated for a certain volume, can be problematic when transfer volumes for plasma/serum and whole blood are different, as was the case for Zheijang Orient Gene Biotech (Huzhou, China), where 5 μl of plasma/serum but 10 μl of whole blood should be applied and only a single transfer device was included.

Controls—Waste Management

In lateral immunochromatographic RDTs (such as COVID-19 RDTs), the integrated control line only confirms migration of the sample-buffer-conjugate along the nitrocellulose strip and does not include a check for the antibody-antigen interactions. For some (2/22, 9.1%) of the COVID-19 RDT products, lyophilized negative and positive controls were available but should be procured separately. In LRS, the inclusion of positive and negative controls within the RDT test kit itself is however an asset and is also listed as "desirable" in the WHO draft specifications for COVID-19 POC IVDs (39). Among the IFUs assessed, only a single product provided positive controls in the test kit: the StrongStep SARS-CoV-2 IgG/IgM Antibody Rapid Test (Liming Bio-Products, Jiangsu, China).

Finally, as is the case for other RDTs, materials of components, package, and accessories should be compatible with local waste management capacities such as field incinerators; compostable plastics are an asset for minimal environmental impact (39, 77).

ASSURED: Usability, Robustness, and Environmental Stability

Usability and Robustness

In addition to complying with STARD guidelines (33), studies should actively observe and assess the product's usability.

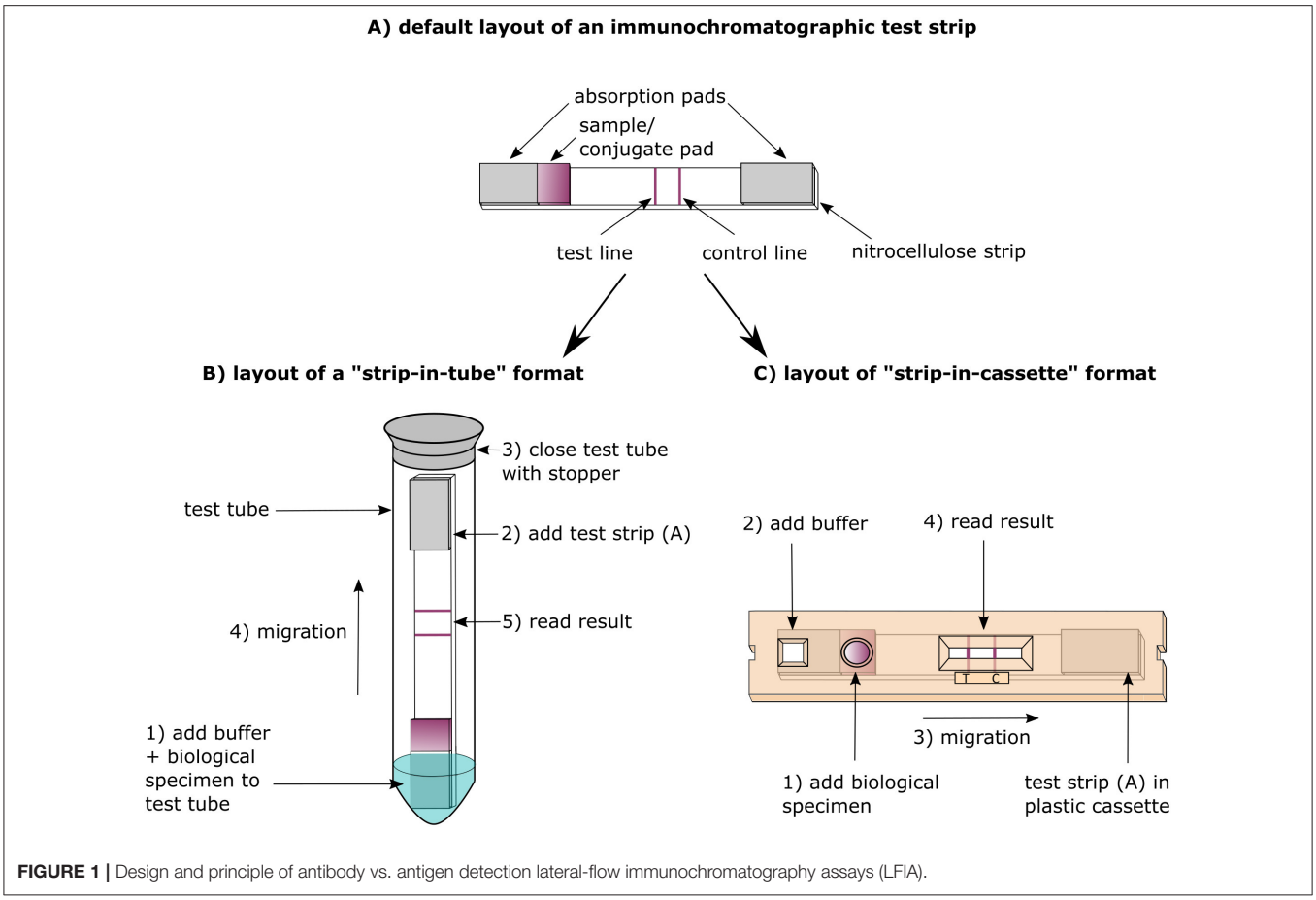

FIGURE 1 | Design and principle of antibody vs. antigen detection lateral-flow immunochromatography assays (LFIA).

Usability studies—also referred to as ease of use or user-friendliness studies assess the product design and IFU as to be understood and manipulated by the intended user. Usability studies are an essential part of IVD development (78); originally most encouraged for RDTs used for HIV-self testing, the WHO also recommends them for other RDTs such as syphilis, hepatitis B, and hepatitis C (9).

For usability studies of RDTs in LRS, the WHO recommends the inclusion of label comprehension studies, result interpretation studies, and trained user observations (9). Usability studies should address the intended user (representative for level of education, literacy, auxiliary skills, and language) in the usual setting and with the RDT product as marketed, i.e., with the components, accessories, and IFU as supplied with the RDT product (29). Depending on the target or disease program, intended users of RDTs in LRS may be clinical healthcare workers and trained lay providers (such as in the case of malaria and cholera) (29). Alternatively, some RDTs—although conceived for POC testing outside the laboratory—are mainly used within (basic) laboratories and with laboratory technicians as the user—an example RDTs used for influenza diagnosis (79). Given the surveillance component of the COVID-19 response, this may be the case for part of the COVID-19 RDTs, too.

Robustness (sturdiness) of the RDT is measured in so-called flex studies which study the RDT performance while mimicking procedural (user) errors (such as adding too few or too much sample volume) and harsh environmental conditions for storage (humidity, light, temperature). Usability and flex studies identify and mitigate potential user-related hazards, orient training and supervision needs, improve workflow and ergonomics, and promote integration of the IVD in the healthcare system (79).

Table 3 lists topics of product- and user-related factors that may influence user-friendliness of RDTs and may be assessed in robustness studies. Both usability and flex studies cross-reference with product specifications and analytical performance studies: as an example, inter-operator agreement of test and control line readings (precision testing) is related to product characteristics such as the presence of crisp and clear test lines. Labeling and IFU including accessible "bench-aids" or quick reference guides and should anticipate user- and product-related failures that cannot be mitigated by design. To be effective, IFUs should be adapted to the literacy and performance level of the user working in stressful conditions (84, 85).

The CORIS COVID-19 Respi-Strip has been assessed for user-friendliness with a European context method [Scandinavian Evaluation of Laboratory Equipment for Point of Care testing (SKUP, https://skup.org/)], based on satisfactory interviews and ratings (21) expressed by laboratory technicians (21). In addition, proportions of weak test line intensities were recorded (33.0 and 12.3% in two studies, respectively) (21, 49) as well as

TABLE 3 | Rapid diagnostic tests applied in low-resource settings: examples of factors related to ease of use (user friendliness, usability) or robustness.

Product specifications/characteristics	Human (user) factors—comments
FORMAT, PACKAGE, AND CONFIGURATION	
Format: ○ Strip-in-cassette vs. strip-in-tube Configuration: ○ Kit with individual tests ($n = 25$), transfer devices, and 1 buffer vial ○ Self-contained kit which also contains sampling materials • Alcohol swabs, finger prick lancets (Ab tests) • Flocked swabs, transport medium (Ag tests) ○ Positive and negative controls available in the test kit Package: ○ Single pack: individual tests packed with small dedicated buffer vial	○ Cassette more familiar and preferred to tube (similarity with malaria and HIV RDTs) but in the case of COVID-19 Ag RDTs, the use of cassette may increase the number of steps including sample transfer ○ Self-contained kit easier for field testing (procedure, logistics) ○ Single pack assures correct use of buffer vial and volume
SPECIMEN TYPE, SAMPLE COLLECTION, AND SAMPLE STABILITY	
○ Biological specimens (validation of specimen types/anticoagulants) • COVID-19 Ag RDTs: upper respiratory tract specimen - nasopharyngeal swab, oropharyngeal swab, saliva • COVID-19 Ab RDTs: - serum, plasma, whole blood: venous vs. capillary ○ Biosafety aspects of sample collection ○ Sample stability • COVID-19 Ag RDTs: samples need to be frozen if immediate testing is not possible ○ Shelf-life	○ COVID-19 Ag RDTs: • Upper respiratory tract sampling is challenging for laboratory technicians [influenza, (79)] • Suboptimal sampling is associated with false-negative results (80) • Self-collected saliva is associated with false-negative results (81) ○ COVID-19 Ab RDTs: • Capillary blood is the most common specimen for malaria and HIV testing • Most RDTs have been validated on serum/plasma only • Test kit must be adapted to match serum/plasma vs. whole blood (e.g., samples transfer device)
DEVICE COMPONENTS AND ACCESSORIES	
○ Device (cassette, tube) easily writable, large read-window ○ Transfer device: easy to handle, self-regulating, stable volume mark ○ Lancets: auto-retractable, painless ○ Alcohol swabs: large, with enough content ○ Desiccant with humidity indicator	
OPERATING CONDITIONS	
○ Environmental temperature ○ Relative humidity ○ Light	○ Particularly relative humidity is harmful as it affects the nitrocellulose strip and the applied antigens/antibodies ○ Poor light conditions (evening and night shifts) hamper visual detection of faint test lines, particularly in staff with presbyopia
PROCEDURE	
○ Time to let the RDT adjust to room temperature ○ Numbers of steps (particularly timed steps) • Collecting/Preparing specimen: ≤1 operator step preferable • Assay performance RDT: ≤2 timed steps preferable ○ Transfer of sample volume: • not too small (tendency to apply too much) • nor too high (sampling, transfer) • easy use of transfer device (large enough, visible volume mark) ○ Process time (time-to-result) ○ Hands-on time	○ RDTs with storage temperature <30°C are frequently stored in the refrigerator ○ Error-prone procedures (in particular when self-testing) include • Transferring sample to cassette or tube • Adding volumes of sample and/or buffer: tendency to add too high volumes • Mixing buffers vials from different production lots (or products) ○ Too-long process time incites too early reading
STABILITY	
○ Storage temperature ○ Open pouch stability, in-use stability (stability once the package has been opened)	○ Stability up to 30°C only implies the need for a "cool chain" In-use stability is important (relative humidity)
READING AND INTERPRETATION	
○ Swift migration with excellent background clearance of the strip ○ Crisp, high intensity test lines (no faint or blurred lines) ○ No ghost lines (application site of control/test antibody lines) ○ Low frequency of invalid results (i.e., absence of control line or control line obscured by blood-buffer mixture) ○ Clear instructions for interpretation ○ Extended stable read time (e.g., 60 min duration of valid result) ○ Low proportion of invalid test lines ○ Low proportion of faint/weak line intensities	○ User errors: • disregarding faint test lines as negative and ghost lines as positive • not recognizing invalid tests and anomalies • reading too early (false-negatives) or too late (backflow, false-positives) • interpret test line intensity as indicative for antigen concentration (and clinical severity) ○ Product errors: anomalies (70) • poor background clearance blurring test/control lines • incomplete migration • high numbers of absent control lines

(Continued)

TABLE 3 | Continued

Product specifications/characteristics	Human (user) factors—comments
LABELING AND INSTRUCTIONS FOR USE	
○ Visibility (lay-out and presentation) ○ Readability (grade of education needed for comprehension) ○ Clear and easy-to-understand instructions and labels ○ Real-life pictorial instructions is an asset (29)	○ Instructions for use must anticipate users' errors ○ Translations and understanding of international symbols need to be validated. (82) Complementary support documents: bench aids, flyers, videos…

According to references (9, 29, 39, 61, 73, 77, 79–81, 83).

the proportion of invalid test results (1.5%) and inter-observer agreement of result readings (98.3%) (21).

Table 4 lists findings of usability retrieved for the 54 COVID-19 Ab RDTs retrieved in the literature review. Of note, only a few of these studies (e.g., a study evaluating RDT products for self-testing) (92) were designed specifically for usability testing, whereas other studies reported product-related ease-of-use anecdotally observed alongside diagnostic accuracy evaluations. Despite the scarce and fragmented data and despite the fact that none of these studies addressed the LRS user, some observations are relevant for implementation. Firstly, usability differed between the selected products with most performing well (by trained laboratory staff) but some performing poorly, showing high proportions of invalid test lines (>40%) or invalid and inconclusive test results (>20%). Secondly, migration of the blood-buffer mixture was a substantial problem in certain products and affected test line reading which lead to a high proportion of invalid results. Thirdly, sampling and sampling transfer were confirmed as difficult procedure steps. In addition, incidental shortcomings were observed for instance in the buffer vial (spills, buffer) and IFU.

Stability

More than in high-resource settings, stability is an issue in tropical LRS. As to storage stability, unlike for instance malaria RDTs [of which many are stable up to 40°C (70)], all but one of the COVID-19 RDTs mentioned 30°C as the maximum storage temperature (the BIOCREDIT COVID-19 Ag test claims stability up to 40°C): this "cool storage" (39) is easily surpassed in tropical climate zones. For COVID-19 POC testing at triage, the WHO draft Target Product Profile lists as an acceptable and desirable target a shelf life of 12 months when stored at 30°C and of 18–24 months when stored at 40°C, respectively, at a relative humidity of 75 ± 5%. Required acceptable and desirable operating conditions (i.e. at the POC when performing the test) are 15–35°C at 25–80% humidity and 10–40°C at 25–90% humidity respectively (39)—none of the IFUs however mentioned operating conditions (**Table 2**).

Shelf-life was only retrieved from the IFUs for seven COVID-19 Ab RDTs, with three and two products reaching 12 and 18 months, respectively. In a comment, WHO mentions that COVID-19 IVDs "crosscuts cultures, climates, and economies" and acknowledges that the proposed stability and shelf-life requirements do not meet the conditions from tropical countries but encourages manufacturers to develop IVDs resistant to the environmental conditions in tropical countries (39). Further, it should be noted that, in view of the recent accelerated development and production of COVID-IVDs, few stability studies have been conducted. Storage conditions and shelf-life are inferred on extrapolations of small-scale accelerated stability testing design.

In-use stability (i.e., stability of the device (cassette) once the package is opened) was mentioned in the IFUs for only four COVID-19 Ab RDTs and was, respectively, 1 h for three of them and 30 min for the remaining product, much lower than the 1 and 4 h set as acceptable and desirable by the WHO draft Target Product Profile for COVID-19 IVDs (39). A similar observation was made for the result stability (i.e., the stable and readable presence of test and control lines beyond the read time): result stability mentioned for 17/20 COVID-19 Ab IVDs was consistently ≤30 min (**Table 2**), compared to ≥60 min listed as the desired specification for a frontline RDT differentiating bacterial and non-bacterial infections in LRS (77).

Implementation Monitoring

Unlike laboratory-based immunoassays such as ELISA assays, RDTs have no wash or dilution steps making them vulnerable to non-specific reactions (false-positives) and prozone effects (false-negatives) (94, 95). As noted above, tropical diseases and immunological conditions with low prevalence may cause false positive results (61, 62). Further, incidental product anomalies or malfunctioning may occur. To capture such rare events, some of which may be product related, consistent implementation monitoring is needed. The same goes for user errors and poor practices which can be traced only by regular exchanges inside a laboratory network and through supervision visits and vigilance. Here, the role of national reference laboratories (NRL) and the tiered national laboratory network is pivotal: NRLs should take the lead in selection, distribution, quality control, training, supervision, communication, and post-market surveillance (see below) (96, 97).

COVID-19 ANTIGEN-DETECTION RDTS FOR SUB-SAHARAN AFRICA: INTEGRATION INTO HEALTHCARE

Target Product Profile—A First Step

The COVID-19 RDTs have been developed for decentralized use in high-income countries. To fit the context of sub-Saharan Africa, a Target Product Profile (TPP) should be defined. TPPs include intended use, target population, diagnostic performance, operational characteristics, throughput, need for

TABLE 4 | Usability (ease-of-use, user-friendliness observations as assessed for 54 COVID-19 antibody detection rapid diagnostic tests (RDTs) retrieved from 45 peer reviewed original research articles.

Product specifications study design	Nr (%) of products assessed	Main findings—comments
○ Line intensities	24 (44.4%)	○ Presence of weakly colored test lines was reported in nine articles for 24 products • No further details about line intensity provided in three papers (86–88) • Proportions of very weak or weak test lines as 40.1 and 76.9% (27, 31, 89) • Correlation of line intensity with the time since onset of symptoms (two products) (90) • No difference in the number of weak test lines in whole blood vs. plasma (45) • Whole blood compared to plasma/serum: IgM band fainter, IgG line slightly stronger intensity (31) • More faint test lines observed for IgM compared to IgG (31) • Lower line intensities in patients with mild disease compared to severe disease (31) • More weak test line results in critical than mild-moderate cases (89)
○ Inter-operator agreement result reading	8 (14.8%)	○ Inter-operator agreement was evaluated in three articles for 8 products. • Agreement of 100% among laboratory scientists, four products (45) • Agreement between trained evaluators 95.3% (91) • Agreement between lay volunteers (home-testing) and health professionals (92) • - Product 1: 62.8% for positive tests, 100.0% for negative, and 98.5% for invalid tests • - Product 2: 93.9% for positive tests, 97.0% for negative, and 98.4% for invalid tests
○ Anomalies assessed	1 (1.9%)	○ A pink background was reported in 1 paper for 3/11 products assessed (32)
○ Ease-of-use of components and accessories	13 (24.1%)	○ Ease-of-use components and accessories was assessed in 2 studies for 13 products (32, 92). ○ Tollanes assessed 11 products in a reference setting (Norway) (32) • User: biomedical laboratory scientists, Method: no data provided • 2/11 products were less user-friendly at test performance and result reading/interpretation, • both products had also higher proportions of invalid/inconclusive results (16.0% and 23.0%) • 2/11 products were less user-friendly at result reading/interpretation • One of them had a high proportion of invalid/inconclusive results (21.0%) • Observations: • -Colored/strong pink background obscuring weak line intensities (three products) • -Blood drawn up to the IgM test lines (two products) • -Buffer vial spills easily, need for pre-analysis mixing of blood and buffer (two products) • -Air bubbles in buffer vial (1 product) ○ Atchison 2020 (92) (UK) assessed usability and acceptability of two products for home-testing (self-testing) • Significant usability issues with lancet and transfer pipet (transfer of blood into the sample well) • (Insufficient volume applied) • Minor problems with buffer vial (design) • Problems in migration across the reading window • Instructions for use (interpretation of results) not clear • Blurred photographs made by the participant • Not completing the test (2.5%): putting blood/buffer in the wrong well, spilling buffer, damaging the test)
○ Proportion of invalid test results	20 (37.0%)	○ Proportions of invalid test results were assessed in 6 studies for 20 products ○ For four products—all assessed on plasma and serum, proportions of invalid tests were 0–0.1% (27, 86, 90, 91). ○ For 11 products assessed on EDTA-anticoagulated venous blood (32), invalid or inconclusive results were absent or very low (<1%) for eight products, but 16/0, 21.0, and 23.0% the remaining three products, mostly caused by insufficient background clearing (see above) ○ For two products assessed for self-testing [see above (92)], invalid results were of 4.8 and 7.4%
○ Other investigations	1 (1.9%)	○ One study demonstrated a prozone effect for 1 product (93)
STABILITY TESTING:		
○ In-use stability	0	○ Result stability was assessed in one study assessing two products (27) • Result was visible and stable up to 2 h after processing • At 24 h post-test reading, changes were noted in both products (8.8 and 9.8% of tests done), mostly from negative to positive, some tests became unreadable, and few changed from positive to negative. ELISA results were concordant with initial readings at 15 min
○ Sample stability	0	
○ Result stability	2 (3.7%)	
FLEX/ROBUSTNESS TESTING		
○ Flex/robustness study done	1 (1.9%)	○ A flex study was conducted for one product (31) • Dilutions of samples were used to assess semi-quantification of RDT • Whole blood was intentionally applied without adding buffer (to mimic POC user error)
○ Label comprehension study	0	
○ Results interpretation study	1 (1.9%)	○ An observed untrained user study was performed on two products for home (self-testing) (92) • Online discussions, questionnaires, observations, and interviews of people who tried the test at home • Nationally representative survey of adults in England using the two products at home: the survey showed limitations with the usability of kits. Most people reported completing the test; however, they identified difficulties with practical aspects of the kit, particularly the lancet and pipette, a need for clearer instructions and more guidance on the interpretation of results (see above)
○ Observed untrained user study	0	
○ Other usability study performed	2 (3.7%)	

batching and turnaround time, as well as training needs, shelf-life, environmental stability, price, and after-sale support (98, 99). The involvement of multiple stakeholders is needed: laboratory staff and frontline healthcare workers, but also manufacturers, health policy makers, and regulators. Examples of TPP for IVDs in LRS have been published recently (77, 100) and the WHO recently published a drafted a TTP document for COVID-19 IVDs RDTs in different testing scenarios (see 5.2) (39). TPPs are living documents and flexibility must be built in to exploit upcoming data about virus dynamics, clinical presentation, and changes in the epidemic which may affect prevalence and pre-test probability (39). A TPP also offers the advantages of product harmonization.

Technology Landscape, Market Landscape, and Independent Product Evaluations

FIND collates a publicly available tracker list of COVID-19 IVDs and has started an independent product evaluation (2). The WHO-initiated independent evaluation "rounds" of malaria RDTs have shown that publication of head-to-head testing results is a valuable guide to procurement but also stimulates improvements in product performance and compliance (101). In our analysis we found that 24 studies (50.0%) compared multiple COVID-19 Ab products and four (8.3%) were conducted in different test centers. In addition, given the fast-moving research in COVID-19, "technology & market landscape," review documents are welcome: such documents merge research and market needs and opportunities. Examples are those published by UNITAID for priority diseases in LRS, such as the "fever diagnostics technology landscape" (102).

Manufacturing Capacity and Quality

Depending on the scale, persistence and potential resurgence of the COVID-19 epidemic, sufficient production volumes of RDTs must be foreseen (19). Leading manufacturers of HIV and malaria RDTs have spare production capacity (103) and manufacturing COVID-19 RDT cassette platforms will only require minor modifications to the existing production lines. In the case of COVID-19 Ag RDTs, the inclusion of sampling components (e.g., flocked swabs) and accessories (personal protective equipment) could be a (temporary) bottleneck. The manufacturer should provide evidence for compliance with a stringent quality management system such as ISO 13485.

Lot-to-lot variation is a well-known challenge for immunoassays and may affect performance (104) and has been well-documented for malaria RDTs (70, 96). The WHO has installed a system of pre-market lot-testing which can detect major product failures (96, 105) but such a system is underpowered to detect small changes between lots (106). Control for minor changes between lots will depend on pro-active implementation monitoring (see above), communication between laboratories and manufacturers, and post-market field effectiveness studies (104, 106). Of note, none of the studies evaluating COVID-19 RDTs retrieved in our search compared different lot numbers.

Market Intelligence and Interventions

A sustainable market is key for a stable supply. Past experience with malaria RDTs showed that a fast scale-up of production combined with downward pricing negatively impacted manufacturing quality (98). Tenders offering multi-year contracts, fixed volumes, and delivery allow manufacturers to plan productions while investing in quality management and innovation (107). Likewise, experiences in HIV RDTs have shown that the selection of multiple RDT products (all meeting the quality standards) increases competition and leads to a more diverse supply base; in addition it supports product development and innovation (103). In line with an economy of scale, the price of the COVID-19 RDTs will decrease at high-volume production. For malaria RDTs, the price in 2016 was <0.30 USD per test (98) but the price in particular for the COVID-19 Ag RDTs will probably be much higher given their more complicated format, components, and packaging. In addition, the development of COVID-19 Ag RDTs is more expensive compared to COVID-19 Ab RDTs (use of monoclonal antibodies vs. recombinant antigen and simpler sampling materials) (19). Further, indirect costs (transport, training, and quality control) of COVID-19 RDTs will be higher as in-country deployment cannot benefit from the logistics of a national vertical disease-control program as is the case for instance for malaria and HIV.

Stringent but Timely Regulation

IVD regulation safeguards their safety, quality, and performance but many countries in sSA are low-regulated and may rely on regulatory approvals from highly regulated countries. COVID-19 RDTs are currently CE-certified according to the IVD Directive 1998/79. However, this directive is not stringent, e.g., the CE mark is granted upon manufacturer's self-declaration and only minimum performance data are required (108). The new Directive EU 2017/746 (109) (effective from 2022 onwards) is more stringent than the expiring one, but neither covers the specific environmental and human conditions in sSA. On top of that, regulatory processes take time. The WHO Prequalification therefore established the Emergency Use Listing (EUL) procedure for COVID-19 IVDs, allowing fast-track evaluation of performance, quality, and safety (110). So far, the WHO EUL list of approved SARS-CoV-2 *in vitro* diagnostic products (111) (July 10 2020) only comprises NAAT tests, but 13 COVID-19 Ab RDTs are in the process of application according to the WHO EUL weekly update of ongoing applications dated August 11 (112). The Pan African Harmonization Working Party on Medical Devices and Diagnostics strives to harmonize regulation among the sub-Saharan—this will facilitate market entry of IVDs and avoid duplication of field evaluation studies (58).

In-Country Deployment of COVID-19 RDTs: Integration Into the Epidemic Response

As is the case for other RDTs, in-country approved policies for COVID-19 RDT use needs to be operationalized by strategic plans and by integration in national laboratory networks coordinated by NRL, in order to ensure quality, training, logistics, and monitoring (113). Training needs for a POC test for triage

as projected by the WHO draft TPP are 0.5 days (acceptable) to 2 h (desirable) (39). Further, connectivity with the COVID-19 epidemic response is essential for the timely communication of results but also for stock management and field assistance. Likewise, a swift sample to result flow for reference NAAT testing at the central level is needed. The opportunity of automated reading and transmission of RDT results should be explored: apart from enabling real-time reporting and spatial monitoring, it will ease the workload and reduce transcription errors (113, 114).

Post-market Surveillance

As described above, implementation monitoring—coordinated by NRL—can detect product or supply shortcomings and result in quality improvement (113). End user awareness, low-threshold monitoring by NRLs, and communication with manufacturers are essential. Major shortcomings should be assessed with the National Regulatory Authorities. The WHO has issued guideline documents about the regulatory framework for IVDs and post-market surveillance directed to WHO prequalified IVDs is currently being updated but the guiding principles can also be applied to COVID-19 IVDs (113).

Communication: Concern About Commercial Promotion of COVID-19 Antibody Tests

Communication with stakeholders is essential (115). Questions should be tackled early on. In particular, the diagnostic algorithm and limitations of the RDTs should be well-communicated (74) and misconceptions should be clarified. It may be difficult to discuss or explain concepts of test characteristics let alone predictive values or serial (orthogonal) testing: On-line "calculators" [such as provided by US FDA (116) and US CDC (117)] are useful to visualize concepts of test utility such as the relation between prevalence, specificity, and positive predictive value.

Another example of correct communication concerns information about RDTs in commercial promotion. COVID-19 Ab RDTs are intensely promoted but frequently their intended use is mentioned only vaguely and diagnostic sensitivity is presented in relation to NAAT reference testing without mentioning the day of sampling since the onset of symptoms or NAAT testing, as was the case for 75.0% of the IFUs that were presently retrieved (**Table 4**). Such practices may entail a high risk of wrongful use of RDTs—i.e., use to detect infection rather than exposure—and thereby missing ongoing disease (74). In addition, the ECDC reported several COVID-19 RDT devices with fraudulent documentation and unsubstantiated claims (118). Antibody tests with insufficient clinical performance data have been compiled by the FDA in a "removed" list (119) and the WHO and FDA recently warned against falsified COVID-19 IVDs and reagents (75, 120). It can be expected that in low-regulated countries such practices will become more frequent and NRLs should inform healthcare workers and the community.

DISCUSSION

Limitations

The present viewpoint review has inherent limitations—firstly, we only searched the PubMed database and retrieved English-language literatures and did not attempt to assess the gray literature nor the pre-print literature (e.g., medrxiv.org). In this way, relevant information may have been missed. Further, the present literature review was narrative and a systematic review and meta-analysis of performance data were not done. Conversely, the iterative approach allowed us to work fast and provide a "snapshot" of the recent situation (August 10, 2020) in a fast-evolving domain.

As to the panel of COVID-19 RDT products retrieved in the literature, it is clear that the actual discussed products represent a minority of the plethora of products marketed or in the pipeline of development. Moreover, in view of potential selection for evaluation and publication bias (17) they may represent the better end of the products. Likewise, the observations published about usability were made based on small sample sizes and in most cases not explored in detail. Finally, as mentioned by two recent meta-analysis studies, heterogeneity among COVID-19 IVDs is high (11, 17), and aggregated data as listed here probably obscure the better performing products (30).

With regard to implementation in LRS, we did not discuss the role and contribution of certain stakeholders specific to the implementation of IVDs in LRS, such as funders, implementers, technical experts—for a framework analysis and landscape analysis, see reference (121), neither did we discuss funding for diagnostics research and implantation.

The Way Forward

The multiple shortcomings of the COVID-19 RDTs listed above add to the unsatisfactory low sensitivity of the COVID-19 Ag and Ab RDTs which on its own already refrained experts and policy makers from recommending their use beyond research (4, 11, 17, 55, 67). Considering the WHO COVID-19 Research Roadmap (in which rapid POC IVDs figure at the first priority), leading international experts made a well-motivated plea to use COVID-19 Ab RDTs in areas where NAAT testing is not scalable or affordable, for the scenarios of triage, contact tracing, and surveillance (60). As for COVID-19 Ag tests, the gaps toward utility and implementation in LRS are probably wider, given their low sensitivity (60) but also because of their sample requirements (nasopharyngeal aspirate, biosafety, frozen storage), more complex design, and higher cost. Of note, despite acknowledging these limitations, the CORIS COVID-19 Respi-Strip has been satisfactorily applied in a field setting in the Democratic Republic of Congo as an initial test at triage, with subsequent NAAT testing of patients testing negative (8).

Which would be today's choice for a COVID-19 Ab RDT to be deployed in LRS? The information revealed by the present and prior reviews and publications are too scarce and fragmentary to enable the selection of a "best buy" product. Minimum hints distilled from the above are as follows: look for a product for which the antigen is described and information about specimen equivalence of whole blood is given. Retrieve

published evidence about accuracy and have a kit hands-on tested with emphasis on usability (**Table 3**) and precision, in particular a good inter-reader agreement. A self-contained kit with sampling materials included (provided ergonomic) is an asset. Stick to a single product (to ensure consistency of measurements), build-up good communication with the manufacturer. Monitor lot-to-lot variation and keep records of observations and incidents. If affordable and manageable, validate the RDT product with an ELISA or CLIA as the comparator (68) and build-in repeat (follow-up) sampling at contact tracing and surveillance to anticipate seroconversion delays (60).

Evaluation studies—so far under-powered (11) should be STARD-compliant (11, 33) and address challenges specific to COVID-19 RDTs such as specimen stability (COVID-19 Ag RDTs) and equivalence, product stability, robustness, and usability. Much of the validation work can be done in the reference setting and in head-to-head comparative study designs allowing researchers to preselect best scoring products for field testing. Prospective and well-documented biobanking at the country or regions of implementation is essential (122). Independent analytical and diagnostic performance data of COVID-19 IVDs that are publicly available [such as conducted by US FDA (123) and FIND (124)] must be encouraged (125). FIND invites researchers to submit validation data to a centralized repository accessible throughout the diagnostic community. Of note, practices such as withholding products names in studies—such as in the case of the RDT evaluation by the UK National COVID Testing Scientific Advisory Panel (126) are undesirable. To curb the risk of selective publication [e.g., COVID-19 specificity (11, 17)], the Cochrane systematic review on COVID-19 Ab IVDs makes a plea for the registration of diagnostic accuracy studies in publicly available registers (30). Regulation should find a compromise between the compelling need for POC testing and the scrutiny of product evaluation, documentation, and promotion (25).

CONCLUSION

Large scale implementation of COVID-19 RDTs in LRS faces numerous challenges. However, one should not overlook the extremely short period from concept to marketing of the present COVID-19 RDTs (a process which usually takes >5 years) (127) and the high potential for improvements in the short term, as many of the above discussed product shortcomings are easily remediable. Field evaluation studies in LRS should address usability and utility, i.e., integration of best performing COVID-19 RDTs in diagnostic algorithms. As to the integration in healthcare settings, the valuable expertise of the national disease control programs and laboratory networks can be capitalized.

AUTHOR CONTRIBUTIONS

JJ and OV had the rationale for this work. JJ did the literature review, writing of the initial draft, and revisions. LH and VK contributed to literature review, revisions, provided critical review, and did the figure design. OL, DA, and OV provided critical review and commentaries. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.557797/full#supplementary-material

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OPINION
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More Caution Needed for Patients Recovered From COVID-19

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INTRODUCTION

Since December 2019, the COVID-19 (Coronavirus Disease, 2019) pandemic has caused ∼35 million confirmed cases including over 1,000,000 deaths worldwide, and currently remains out of control in most countries (1). China is trying to bring COVID-19 under control by multiple drastic social and economic restrictions in combination with effective healthcare provision. As of Oct 7, 2020, among the 91,188 confirmed cases in China, only 386 cases remained hospitalized while there were 86,056 cases discharged after full recovery (1). The positive trend in China demonstrated a promising combat against the COVID-19 outbreak; however extreme caution is still needed given that the SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) remains largely unknown to us. For instance, a recent study revealed that four recovered patients from COVID-19 showed positive quantitative RT-PCR (reverse-transcriptase–polymerase-chain-reaction) test results again after discharged from hospital (2). Likewise, data from Guangdong Province, China indicated that ∼14% of the discharged patients would be tested positive for SARS-CoV-2 again (3). On April 10, the Korea Centers for Disease Control and Prevention also reported that more than 90 recovered patients showed recurrent positive test results for SARS-CoV-2 (4). These findings from recovered patients with re-detectable SARS-CoV-2 therefore raise a significant public health concern about whether they could spread SARS-CoV-2 to others again. To explore this public health concern, we systematically searched PubMed (up to Sep 9, 2020) to summarize the available evidence from studies that documented the recovered patients with re-detectable SARS-CoV-2, using the search terms ("novel coronavirus" OR "SARS-CoV-2" OR "COVID-19") AND ("recovered" OR "discharged") AND ("positive" OR "re-detectable") with no language or time restrictions. Of 383 potentially relevant records, 36 eligible studies, independently reviewed by two investigators (JZ and GuoL), were included for analyses. For the purpose of prompt and easy intake, we also summarized the progress, symptoms, infectivity, potential reasons, and treatments of re-detectable positive patients in **Table 1.**

POTENTIAL REASONS

Several possible reasons including virology, specimen detection and patients' condition, may help explain why the recovered patients with COVID-19 became retest positive for SARS-CoV-2.

TABLE 1 | Summary of the progress, symptoms, infectivity, potential reasons, and treatments of re-detectable positive patients.

Progress	Initial	In February 2020, four recovered patients from COVID-19 showed positive quantitative RT-PCR test result again.
	Developing	On April 10 2020, recurrent positive test results for SARS-CoV-2 were found in the Korea. Data showed nearly 14% of the discharged patients would be tested positive against.
Potential reasons	Virology	Viral residue, intermittent viral release, and periodic changes of virus replication.
	Detection of specimens	Low quality of throat swabs, different RT-PCR tests kits with imperfect accuracy, inadequate sampling and laboratory practices.
	Patients' condition	Older than 50 years and above, had comorbidities, received glucocorticoid therapy, longer hospital stay, lymphopenia, severe conditions, and lower immune
Symptoms	No symptoms or only mild symptoms.	
Infectivity	No reported and no conclusive evidence.	
Treatments	1. A combination of stool and different respiratory samples. 2. Employing the test of IgM-IgG antibodies. 3. Continual physical distancing or quarantines, use of respirator in public, close monitoring, and multiple laboratory tests for long-term follow-up after discharge.	

Virology

Currently nucleic acid detection represents the most widely used test to confirm SARS-CoV-2 infection. Following Guidelines for the Diagnosis and Treatment of Novel Coronavirus (COVID-19) Infection by the National Health Commission (Trial Version 7), 2 consecutive negative RT-PCR test results of respiratory specimens, mostly from throat swabs, are one key criterion for discharging patients with COVID-19 (5). However, the duration of SARS-CoV-2 RNA shedding has not been well-characterized (6). Viral RNA was detectable in different specimens, including throat swabs, stool, and urine, in patients for average 20 days (range, 8–37 days) after disease onset (7). Furthermore, recently studies have showed that higher viral loads are found in the nose and lower respiratory specimen than in the throat (8, 9); and the clearance of viral RNA in patients' stool is delayed compared to respiratory tract (10). A recent study with 98 patients showed that over half of stool samples of patients remained positive for SARS-CoV-2 for a mean of 11.2 days after respiratory tract samples became negative (11). The residuals and distribution of virus could thus be another possible reason for recovered patients' recurrence of positive viral test results.

Detection of Specimens

Several aspects including decreased viral loads in patients due to their improving conditions, low quality of throat swabs, different RT-PCR tests kits with imperfect accuracy, inadequate sampling, and laboratory practices may yield variation of duration of viral shedding and false negative results and therefore inaccurate diagnoses of recovery. While widespread use of bronchoalveolar lavage fluid specimen test to detect SARS-CoV-2 is infeasible and impractical to help determine whether patients can be discharged, a combination of stool and different respiratory samples (especially from lower respiratory tract) may serve as a better tool to reduce the false negatives and recovered patients' recurrence.

Patients' Condition

Once infected with SARS-CoV-2, the conditions of patients who were older than 50 years and above, had comorbidities or received glucocorticoid therapy might be more severe (7). SARS-CoV-2 in patients with severe COVID-19 had a longer duration of viral shedding and even could be detected until death (2, 7). Ultimately, longer hospital stay and lymphopenia due to severe conditions and lower immune function would more likely to result in retest positive of recovered patients (12).

THE SYMPTOMS AND IMMUNITY OF RECOVERED PATIENTS

No obvious symptoms were reported in the recovered patients regardless of their RT-PCR test results after discharged from hospital. Similar to SARS-CoV-1 infection (13), virus-specific immunoglobulin M (IgM), immunoglobulin G (IgG), and neutralizing IgG antibodies were detected in most recovered patients between 7 and 14 days after the onset of symptoms, and antibody titers persisted for weeks following virus clearance (6). A recent study of SARS-CoV-2 infection in rhesus macaques indicated neutralizing antibodies against SARS-CoV-2 might offer protection to reinfection during early recovery days (28 days) (14). However, some study revealed that in a high proportion of recovered patients, IgG levels, and neutralizing antibodies started to decrease within 2–3 months after infection (15, 16). If patients' RT-PCR test was positive after recovery or immune systems become weak or declined, there remain potential risks of a relapse of symptomatic COVID-19 (17, 18). The IgM and IgG antibodies that were significantly and positively related to disease severity, would be produced gradually in the infected cases with COVID-19, which could help evaluate the stage of infection in the body (19). Therefore, employing the test of IgM-IgG antibodies as an add-on may be used to lower the risk of false negatives before a decision of hospital discharge was made. The test of IgM-IgG antibodies may also aid in the evaluation of whether recovered patients would require further close clinical attention after the recurrent positive RT-PCR test results.

INFECTIVITY AND TREATMENT

Nevertheless, based on the literature there is no clear evidence showing that the SARS-CoV-2 found in patients recovered from COVID-19 is transmissible. Notably, detection of viral RNA does not necessarily indicate that infectious virus is present in specimens (16). A virological analysis of nine infected cases indicated no isolates of live virus after day 8 of symptoms onset, regardless of their ongoing high viral loads (20). Furthermore, one study found that only a low level of fragment genome could

be detected in the recovered patients, implying that they would hardly spread the coronavirus again (21).

However, more caution is required because some other infective viruses have been known to persist for longer periods of time. For instance, infective Ebola virus could persists in semen for several months after two consecutive negative tests in blood samples (22). If human-to-human transmissions of SARS-CoV-2 were confirmed in patients recovered from COVID-19, the prevention and control would become significantly challenging and COVID-19 "immunity passports" would be challenged. While more high-quality evidence is urgently needed to determine the potential propagation in recovered patients with positive viral test results, continual physical distancing or quarantines, use of respirator in public, close monitoring, and multiple laboratory tests for long-term follow-up after discharge would be important for the current combat of COVID-19.

CONCLUDING REMARKS

Although the phenomenon of discharged patients testing positive again for SARS-CoV-2 RNA was reported in some counties (e.g., Korea, Italy, and Russia), the studies were mostly documented from China. It is possible that these results from the strict and vigilant post-discharge monitoring policies in place in China (5). According to current reports, recovered patients with re-detectable SARS-CoV-2 account for a certain proportion of recovered patients. The underlying mechanism and infectivity of this population remains elusive and the evidence is only now starting to emerge. Considering the significance of this ongoing global public health emergency, we should take more caution needed for patients recovered from COVID-19 and perform more and urgent investigations of recovery cases to contain the epidemic.

AUTHOR CONTRIBUTIONS

JZ, JT, and GuoL: conception and design. JZ, HQ, CL, and GuoL: acquisition of data. JZ, ZL, JT, and GuoL: drafting the article. JZ, HQ, CL, ZL, GuaL, JT, and GuoL: revising it for intellectual content and final approval of the completed article. All authors: contributed to the article and approved the submitted version.

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A Risk Prediction Model for Evaluating the Disease Progression of COVID-19 Pneumonia

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Background and Objective: The epidemic of coronavirus disease 2019 (COVID-19) pneumonia caused by infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) has expanded from China throughout the world. This study aims to estimate the risk of disease progression of patients who have been confirmed with COVID-19.

Methods: Meta-analysis was performed in existing literatures to identify risk factors associated with COVID-19 pneumonia progression. Patients with COVID-19 pneumonia were admitted to hospitals in Wuhan or Hangzhou were retrospectively enrolled. The risk prediction model and nomogram were developed from Wuhan cohort through logistic regression algorithm, and then validated in Hangzhou and Yinchuan cohorts.

Results: A total of 270 patients admitted to hospital between Dec 30, 2019, and Mar 30, 2020, were retrospectively enrolled (**Table 1**). The development cohort (Wuhan cohort) included 87 (43%) men and 115 (57%) women, and the median age was 53 years old. Hangzhou validation cohort included 20 (48%) men and 22 (52%) women, and the median age was 59 years old. Yinchuan validation cohort included 12 (46%) men and 14 (54%) women, and the median age was 44 years old. The meta-analysis along with univariate logistic analysis in development cohort have shown that age, fever, diabetes, hypertension, CREA, BUN, CK, LDH, and neutrophil count were significantly associated with disease progression of COVID-19 pneumonia. The model and nomogram derived from development cohort show good performance in both development and validation cohorts.

Conclusion: The severe COVID-19 pneumonia is associated with various types of risk factors including age, fever, comorbidities, and some laboratory examination indexes. The model integrated with these factors can help to evaluate the disease progression of COVID-19 pneumonia.

Keywords: sSARS—CoV-2, meta-analysis, COVID 19, risk prediction model, disease progression

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INTRODUCTION

Since December, 2019, China reported a cluster of cases of pneumonia with unknown cause in Wuhan, Hubei (1). On Jan 7, 2020, Chinese health authorities have confirmed these cases were associated with a novel coronavirus, severe acute respiratory syndrome corona virus 2 (SARS-CoV2; previously called 2019-nCoV) via next generation sequencing analysis of patients' respiratory tract samples (2, 3). SARS-CoV2 is the seventh member of Coronaviridae, which has been shown to infect human cells through interacting with the angiotensin-converting enzyme 2 (ACE2) receptor on the cell surface (4). ACE2 receptor is wildly distributed in various types of human cells including type II alveolar cells, renal tubular cells, Leydig cells and so on (5). Thus, SARSCoV2 possesses a strong ability to infect humans.

Most of the original cases of coronavirus disease 2019 (COVID-19) pneumonia were reported to have been exposed to the Huanan seafood market in Wuhan (6). However, the medical and nursing staffs, patients without exposure to the market but with a history of travel to Wuhan have been found to be infected by SARS-CoV2, suggesting that human-to-human transmission is occurring (7, 8). The number of diagnosed cases has been increasing rapidly: by March 27 2020, more than 500,000 cases of COVID-19 pneumonia had been reported in China and other countries worldwide (including Japan, South Korea, Spain, Italy, the UK, and the USA), and over 23,000 patients had died, equivalent to a case fatality rate of around 4% (9). Epidemic prevention is becoming increasingly severe.

Similar as respiratory diseases caused by other beta-coronaviruses such as severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) (10, 11). Patients with both mild and severe COVID-19 pneumonia showed fever, dry cough, and dyspnea symptoms (6). Furthermore, patients with severe COVID-19 pneumonia were more likely to progress to acute respiratory distress syndrome (ARDS), which had relatively higher case fatality rate (12). However, little studies were reported about evaluating the risk of disease progression of COVID-19 pneumonia.

In this study, we employed a meta-analysis of 6,061 cases of COVID-19 from 32 studies. The results showed that severe COVID-19 pneumonia was obviously correlated with severe complications, including ARDS, shock, acute kidney injury and acute cardiac injury. And the comorbidities significantly increased the risk of progressive COVID-19. In addition, a total of 270 COVID-19 pneumonia patients were collected from Wuhan, Hangzhou, and Yinchuan. A predictive model and nomogram were then established based on previously identified risk factors including age, fever, comorbidities, CK, LDH, CREA, BUN, and neutrophil count to predict the risk of disease progression in Wuhan cohort. A nomogram is a statistical instrument that accounts for numerous variables to predict an outcome of interest (13). The nomogram showed great performance in predicting the probability of severe COVID-19 pneumonia, which was further validated in two independent cohorts.

TABLE 1 | Demographic and clinical characteristics.

Parameters	Development cohort ($n = 202$)	Hangzhou validation cohort ($n = 42$)	Yinchuan validation cohort ($n = 26$)
CLINICAL CHARACTERISTICS			
Age (median, range, y)	53 (20–88)	59 (20–87)	44 (15–70)
Gender			
Male	87 (43.1%)	20 (47.6%)	12 (46.2%)
Female	115 (56.9%)	22 (52.4%)	14 (53.8%)
Status			
Mild	102 (55.1%)	26 (61.9%)	20 (76.9%)
Severe	83 (44.9%)	16 (38.1%)	6 (23.1%)
Fever			
Yes	145 (72.1%)	30 (71.4%)	19 (73.1%)
No	56 (27.9%)	12 (28.6%)	7 (26.9%)
COMORBIDITIES			
Hypertension			
Yes	31 (15.9%)	7 (16.7%)	4 (15.4%)
No	164 (84.1%)	35 (83.3%)	22 (84.6%)
Diabetes			
Yes	17 (8.7%)	5 (11.9%)	0 (0%)
No	178 (91.3%)	37 (88.1%)	26 (100%)
LABORATORY INDICTORS			
BUN (mmol/L)	4.7 ± 2.3	5.7 ± 4.4	3.9 ± 1.1
CREA (μmol/L)	71.5 ± 26.2	80.6 ± 31.0	65.8 ± 18.2
CK (U/L)	171.1 ± 441.3	97.6 ± 86.0	56.9 ± 26.8
LDH (U/L)	284.2 ± 207.4	252.4 ± 181.5	241.0 ± 55.3
Neutrophil (10^9/L)	3.9 ± 3.7	5.0 ± 5.7	3.2 ± 1.5

MATERIALS AND METHODS

Meta-Analyses

Thirty-two eligible studies were analyzed by performing meta-analysis. OR, RR, SMD, 95% CI as well as forest plots were calculated via stata 12.0. Specific analysis procedures including search strategy, inclusive and exclusive criteria, data extraction, quality evolution and statistics were provided in **Supplementary Texts 1**, **2**.

Study Design and Participants

This was a retrospective study done at two centers in Wuhan and Hangzhou. Patients with confirmed COVID-19 pneumonia were admitted to Zhongnan Hospital of Wuhan University or the First People's Hospital of Xiaoshan District or The Fourth People's Hospital of Ningxia Hui Autonomous Region were retrospectively enrolled (**Table 1**).

Next-generation sequencing or real-time PCR of throat swab specimens were used to confirm the SARS-CoV2 infection of each patient according to a previously published protocol (14). The following primers or probe targeted to the envelope gene of SARS-CoV2 were used: Forward primer: 5′-GACCCCAAAATCAGCGAAAT-3′; Reverse primer: 5′-TCTGGTTACTGCCAGTTGAATCTG-3′; Probe: 5′-FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1-3′.

Patients with respiratory rate over 30 per min, or SpO_2 <93%, or PaO_2/FiO_2 <300 mmHg were considered as severe cases (15). The clinical characteristics, laboratory findings, and comorbidities of the patients were recorded at the time of admission to hospital. The study is approved by the Ethics Committee of the Zhongnan Hospital of Wuhan University, the First hospital of Xiaoshan District and The Fourth People's Hospital of Ningxia Hui Autonomous Region under the accession 2020035.

Statistical Analysis

Normality of the data was evaluated using the Kolmogorov-Smirnov test. Data with normal distributions were presented as mean ± SD, data with non-normally distribution were presented as median (IQR), and categorical variables as frequency (%). Differences between two groups were analyzed by Fisher's exact test (for categorical variables) or Mann-Whitney *U*-test (for continuous variables). The hazard ratios (HRs) and corresponding 95% CIs were calculated using univariate or multivariate logistic regression algorithm.

Model Development and Valudations

For the development of the nomogram, we tested the significance of potential risk factors by univariate logistic regression algorithm and further filtered by meta-analysis. As a result, eight important predictors of risk of severe COVID-19 pneumonia were to create the model via multivariate logistic regression. The models produce a linear predictor, which is represented as a patient's predicted log hazard. We tested the accuracy of the model as well as nomogram derived from the development cohort in the validation cohort by discrimination, ROC (receiver operating characteristic curve) analysis and calibration curve. The nomogram converts the risk of each variable into a points system, which can be added to produce an overall risk estimate.

The discrimination is evaluated by the c-statistic, which measures the capability of the model to predict a high risk for a patient who is considered as high risk. The closer that c-statistic is to one, the better the discrimination, and a value of 0.5 indicates that the model is not better than chance. The calibration curve of the model measures the relationship between the outcomes predicted by the model and the actual outcomes in indicated cohort. A 45° line indicates perfect calibration, which the predicted outcome of the model perfectly matches the patient's observed outcome. Any deviation above or below the 45° line indicates underprediction or overprediction, respectively. The ROC analysis is evaluated by AUC (area under the curve), which measures the sensitivity and specificity of the model. The closer that AUC is to one, the more sensitive and specific the model. All the statistics were performed using R project (version 3.4.4).

RESULTS

According to our search strategy, a total of 142 studies were collected from the online database. Filtered by the criteria of inclusion and exclusion, we finally retained 32 (**Supplementary Figure 1**) studies for further meta-analysis, and no publication bias exists (**Supplementary Figure 2**). Meta-analysis results revealed that acute cardiac injury [odds ratio [OR] = 37.93, 95% confidence interval [CI]: 17.92–80.28; $P < 0.001$], acute kidney injury [OR = 24.82, 95% CI: 11.40–54.02; $P < 0.001$), ARDS (OR = 49.03, 95% CI: 20.14–119.35; $P < 0.001$), shock (OR = 45.48, 95% CI: 19.85–104.18; $P < 0.001$) occurred frequently in patients with severe COVID-19 pneumonia compared with the ordinary cases (**Figure 1**). The death event [risk ratio [RR] = 30.09, 95% CI: 11.46–79.01; $P < 0.001$ was more common in disease progression in COVID-19 pneumonia. The detailed meta-analysis results were summarized in **Supplementary Table 1**.

To investigate the variables associated with severe COVID-19 pneumonia, we analyzed the relationship between patients' clinical characteristics and severe COVID-19 pneumonia risk through meta-analysis. **Supplementary Table 2** showed that age [standardized mean difference [SMD] = 2.04, 95% CI: 1.45–2.63, $P < 0.001$] and gender [OR = 1.57, 95% CI: 1.36–1.80, $P < 0.001$] held different distribution in severe and mild COVID-19 pneumonia groups. Additionally, patients with comorbidities, including hypertension [OR = 2.53, 95% CI: 1.89–3.37, $P < 0.001$), diabetes (OR = 2.43, 95% CI: 1.98–2.97, $P < 0.001$), cancer (OR = 1.73, 95% CI: 1.07–2.78, $P = 0.025$), cerebrovascular (OR = 4.02, 95% CI: 2.41–6.60, $P < 0.001$), cardiac disease (OR = 4.11, 95% CI: 3.15–5.35, $P < 0.001$), renal disease (OR = 5.44, 95% CI: 2.81–10.54, P < 0.001), and pulmonary disease (OR = 4.17, 95% CI: 2.86–6.08, $P < 0.001$) may be closely related with the risk of patients infected with SARS-CoV2 progressed to severe COVID-19 pneumonia (**Figure 2**). In addition, different clinical symptoms including cough, fatigue, fever, muscular soreness, and CT performance were also correlated with severe COVID-19 pneumonia. The above symptoms may frequently occur in patients with progressive COVID-19 pneumonia. Next, we analyzed the relationship between patients' laboratory findings and severe COVID-19 pneumonia risk. The results showed that hepatic function indexes albumin, ALT, AST, renal function indexes BUN, CREA, cardiac function indexes CK, CK-MB, LDH, cTnI, MYO, coagulation function indexes D-dimer, PLT, PT, blood routine indexes Hb, lymphocyte, neutrophil, CD4 lymphocyte, CD8 lymphocyte, inflammation indexes WBC, CRP, PCT, ESR, ferritin, and electrolyte indexes Na^+ were obviously changed in patients with severe COVID-19 pneumonia compared with mild COVID-19 pneumonia patients. Moreover, the **Supplementary Table 3** showed that blood routine indexes Hb, lymphocyte (SMD = −2.35, 95% CI: −2.85–1.86; **Supplementary Figure 3A**), WBC (SMD = 1.35, 95% CI: 0.49–2.22; **Supplementary Figure 3B**), neutrophil (SMD = 2.21, 95% CI: 1.70–2.73; **Supplementary Figure 3C**), CD4 lymphocyte, CD8 lymphocyte, inflammation indexes CRP (SMD = 4.28, 95% CI: 3.23–5.33; **Supplementary Figure 3D**), PCT, ESR, and ferritin were obviously changed, which indicated that the severe patients may suffer from inflammatory factor storm. At the same time hepatic function indexes ALT (SMD = 1.29, 95% CI: 0.84–1.74; **Supplementary Figure 4A**) and AST (SMD = 2.01, 95% CI: 1.20–2.81; **Supplementary Figure 4B**),

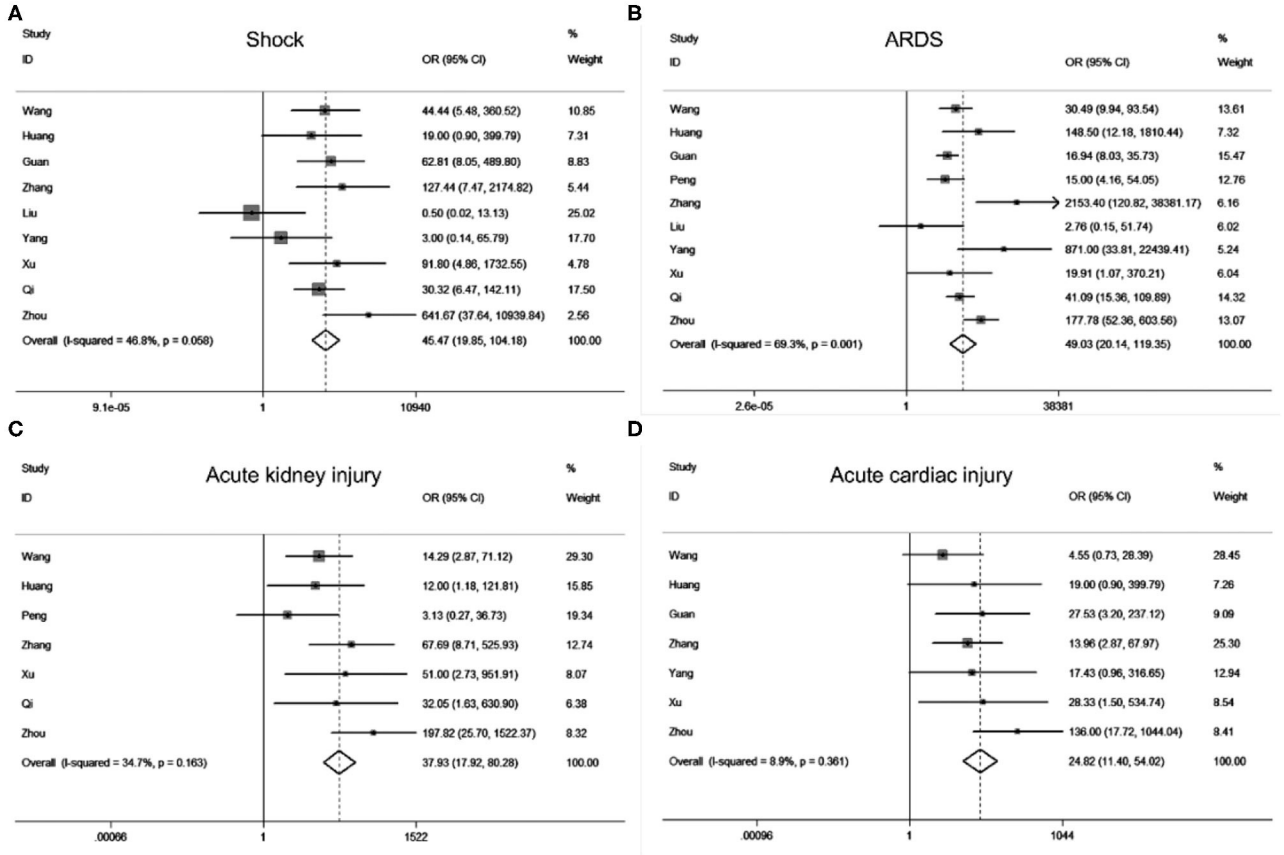

FIGURE 1 | Forest plots of odds ratios (OR) showed the associations between complications including shock **(A)**, ARDS **(B)**, acute kidney injury **(C)**, as well as acute cardiac injury **(D)** and disease progression of COVID-19 pneumonia.

renal function indexes CREA (SMD = 1.20, 95% CI: 0.72–1.68; **Supplementary Figure 4C**) and BUN (SMD = 2.44, 95% CI: 1.52–3.36; **Supplementary Figure 4D**), cardiac function indexes CK (SMD = 4.04, 95% CI: 2.73–5.36; **Supplementary Figure 4E**), LDH (SMD = 1.64, 95% CI: 0.71–2.56; **Supplementary Figure 4F**), coagulation function indexes D-dimer, PLT, PT, and electrolyte indexes Na^+ were increased in patients with severe COVID-19 pneumonia compared with mild COVID-19 pneumonia patients, which indicated patients with severe COVID-19 pneumonia may be suffering from multi-organ dysfunctions.

Together, the meta-analysis results revealed that age (HR = 1.31, 95% CI: 1.17–1.47; $P < 0.001$), diabetes (HR = 1.71, 95% CI: 1.16–2.54; $P = 0.007$), heart diseases (HR = 5.02, 95% CI: 1.47–17.10; $P = 0.01$), hypertension (HR = 2.11, 95% CI: 1.60–2.79; $P < 0.001$), pulmonary diseases (HR = 3.60, 95% CI: 1.91–6.78; $P < 0.001$), blood oxygen saturation (HR = 0.94, 95% CI: 0.89–0.99; $P = 0.025$), CREA (HR = 1.08, 95% CI: 1.02–1.15; $P = 0.007$), BUN (HR = 1.54, 95% CI: 1.28–1.87; $P < 0.001$), neutrophil (HR = 1.17, 95% CI: 1.00–1.38; $P = 0.050$), PCT (HR = 4.07, 95% CI: 1.05–15.73; $P = 0.042$), serum ferritin (HR = 4.59, 95% CI: 2.00–10.55; $P < 0.001$), CK (HR = 2.13, 95% CI: 1.44–3.15; $P < 0.001$), LDH (HR = 2.15, 95% CI: 1.32–3.51; $P = 0.002$) and cTnI (HR = 4.87, 95% CI: 1.48–16.01; $P = 0.009$) were potential risk factors for disease progression of COVID-19 pneumonia (**Supplementary Table 4**).

To further validate the potential risk factors for COVID-19 pneumonia progression, we collected 202 COVID-19 pneumonia cases from Zhongnan Hospital of Wuhan University (termed as Wuhan cohort). The median age of Wuhan cohort is 53 years [Interquartile Range [IQR], 20–88]. About 20% of all patients have comorbidities such as hypertension (15.3%) and diabetes (8.4%) (**Table 1**). Similarly, patients with severe COVID-19 pneumonia are elder and with different comorbidities including hypertension and diabetes (**Table 2**). We further analyzed the relationship between potential risk factors identified by meta-analysis using the univariate logistic regression (**Supplementary Figure 5**).

The results showed that age (HR = 1.07, 95% CI: 1.05–1.09; $P < 0.001$), fever (HR = 2.25, 95% CI: 1.28–4.04; $P < 0.001$), diabetes (HR = 7.45, 95% CI: 2.76–25.26; $P = 0.002$), hypertension (HR = 6.26, 95% CI: 3.02–14.07; $P < 0.001$), BUN (HR = 1.39, 95% CI: 1.21–1.63; $P < 0.001$), CREA (HR = 1.02, 95% CI: 1.01–1.04; $P = 0.002$), CK (HR = 1.00, 95% CI: 1.00–1.01; $P = 0.03$), LDH (HR = 1.01, 95% CI: 1.00–1.02; $P < 0.001$), and neutrophil (HR = 1.43, 95% CI: 1.25–1.68;

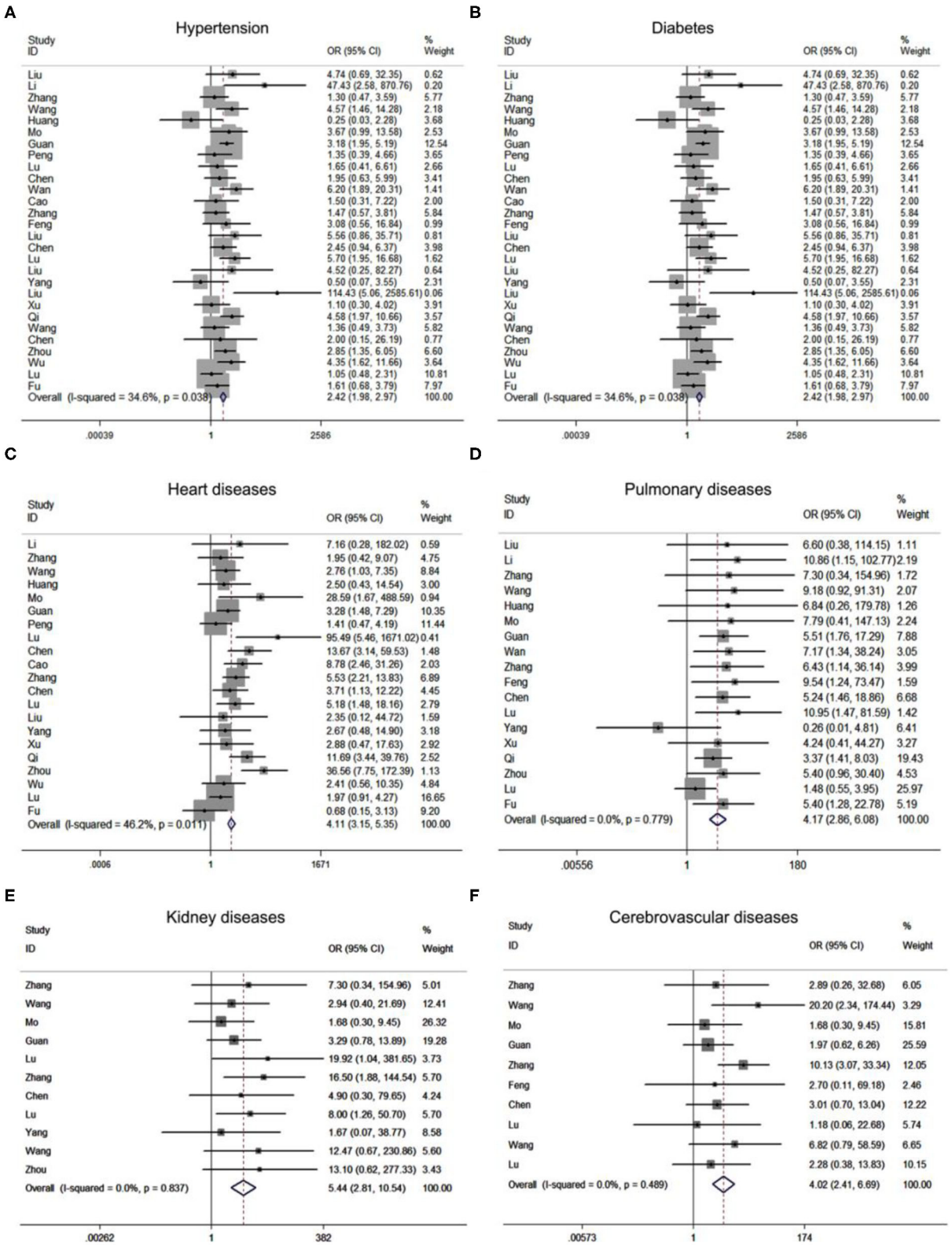

FIGURE 2 | Forest plots of odds ratios (OR) showed the associations between comorbidities including hypertension **(A)**, diabetes **(B)**, cardiac diseases **(C)**, pulmonary diseases **(D)**, renal diseases **(E)**, as well as cerebrovascular diseases **(F)**, and disease progression of COVID-19 pneumonia.

TABLE 2 | Clinical characteristic of 202 COVID−19 pneumonia patients from Zhongnan Hospital of Wuhan University.

Parameters	Total cases (*N* = 202)	Mild cases (*N* = 102)	Severe cases (*N* = 83)	*P*-value	Normal range
CLINICAL CHARACTERISTICS					
Age (median, range, y)	53 (20–88)	46 (20–83)	62 (23–88)	<0.001	
Gender				0.038	
Male	87 (43.1%)	36 (35.3%)	42 (50.6%)		
Female	115 (56.9%)	66 (64.7%)	41 (49.4%)		
Fever				0.021	
Yes	145 (72.1%)	66 (64.7%)	66 (80.5%)		
No	56 (27.9%)	36 (35.3%)	16 (19.5%)		
Cough				0.076	
Yes	106 (52.7%)	47 (46.1%)	49 (59.8%)		
No	95 (47.3%)	55 (53.9%)	33 (40.2%)		
COMORBIDITIES					
Hypertension				<0.001	
Yes	31 (15.9%)	7 (6.9%)	24 (31.6%)		
No	164 (84.1%)	95 (93.1%)	52 (68.4%)		
Diabetes				0.002	
Yes	17 (8.7%)	3 (2.9%)	14 (18.4%)		
No	178 (91.3%)	99 (97.1%)	62 (81.6%)		
LABORATORY INDICTORS					
Hepatic Function					
ALT (U/L)	35.6 ± 37.0	24.9 ± 23.0	46.8 ± 44.1	<0.001	7–45
AST (U/L)	42.6 ± 53.5	26.0 ± 12.7	60.0 ± 73.5	<0.001	13–35
Total bilirubin (μmol/L)	12.7 ± 10.5	11.1 ± 4.8	13.7 ± 7.8	0.029	5–21
Albumin(g/L)	37.7 ± 5.0	40.0 ± 3.5	35.0 ± 5.2	<0.001	40–55
Renal Function					
BUN (mmol/L)	4.7 ± 2.3	4.1 ± 1.6	5.6 ± 2.9	<0.001	2.8–7.6
CREA (μmol/L)	71.5 ± 26.2	65.3 ± 21.5	78.1 ± 29.2	<0.001	49–90
Cardiac Function					
CK (U/L)	171.1 ± 441.3	92.8 ± 95.5	238.8 ± 546.2	<0.001	<145
CK–MB (U/L)	18.0 ± 23.9	14.2 ± 22.6	19.1 ± 18.2	<0.001	0–25
LDH (U/L)	284.2 ± 207.4	192.8 ± 93.3	392.6 ± 254.2	<0.001	125–243
Myoglobin (U/L)	215.0 ± 371.6	31.0 ± 22.4	248.7 ± 413.0	0.040	<140.1
NT–proBNP (pg/mL)	542.7 ± 877.1	200.2 ± 326.3	671.5 ± 1000.5	0.313	0–900
Coagulation Function					
PT (s)	13.0 ± 1.6	12.6 ± 1.1	13.4 ± 2.0	0.008	9.4–12.5
APTT (s)	31.2 ± 3.2	31.3 ± 2.9	31.0 ± 3.7	0.163	25.1–36.5
D–dimer (ng/L)	1416.2 ± 5396.9	292.2 ± 453.9	3126.9 ± 8432.6	<0.001	0–500
Fibrinogen (mg/L)	410.0 ± 111.7	398.9 ± 105.1	418.3 ± 117.4	0.443	238–498
PLT (10^{12}/L)	185.5 ± 68.0	191.6 ± 61.9	178.4 ± 74.6	0.059	125–350
Inflammatory Index					
WBC (10^9/L)	5.3 ± 3.0	4.4 ± 1.8	6.4 ± 3.9	0.002	3.5–9.5
Monocyte (10^9/L)	0.5 ± 0.9	0.4 ± 0.2	0.6 ± 1.4	0.968	0.1–0.6
Neutrophil (10^9/L)	3.9 ± 3.7	2.8 ± 1.6	5.3 ± 5.1	<0.001	1.8–6.3
Eosinophil (10^9/L)	0.04 ± 0.17	0.07 ± 0.23	0.02 ± 0.05	0.008	0.02–0.52
CRP (mg/L)	39.8 ± 61.3	17.5 ± 21.7	77.9 ± 84.7	<0.001	0–10
Electrolyte					
K^+ (mmol/L)	4.03 ± 0.65	4.05 ± 0.67	3.99 ± 0.66	0.566	3.5–5.3
Na^+ (mmol/L)	138.4 ± 5.9	140.1 ± 6.2	136.9 ± 4.0	<0.001	137–147
Ca^+ (mmol/L)	2.16 ± 0.17	2.22 ± 0.12	2.07 ± 0.21	<0.001	2.11–2.52
Survival Status				0.001	
Alive	190 (94.1%)	95 (99.0%)	71 (86.6%)		
Death	12 (5.9%)	1 (1.0%)	11 (13.4%)		

TABLE 3 | Univariate logistic results of different variables in development cohort.

Parameters	HR	95%CI	Z-value	*P*-value
Age	1.07	1.05–1.09	5.56	<0.001
Gender (Male vs. Female)	1.88	1.15–3.10	2.09	0.04
Fever (Yes vs. No)	2.25	1.28–4.04	2.34	0.02
Cough (Yes vs. No)	1.85	1.13–3.06	2.03	0.04
Hypertension (Yes vs. No)	6.26	3.02–14.07	3.96	<0.001
Diabetes (Yes vs. No)	7.45	2.76–25.26	3.06	0.002
ALT	1.03	1.02–1.05	3.53	<0.001
AST	1.06	1.04–1.08	4.87	<0.001
Total bilirubin	1.07	1.03–1.12	2.54	0.01
Albumin	0.75	0.69–0.81	−5.79	<0.001
BUN	1.39	1.21–1.63	3.71	<0.001
CREA	1.02	1.01–1.04	3.08	0.002
CK	1.00	1.00–1.01	2.14	0.03
CK–MB	1.01	1.00–1.03	1.37	0.17
LDH	1.01	1.01–1.02	5.06	<0.001
Myoglobin	1.09	1.02–1.22	1.73	0.08
NT–proBNP	1.00	1.00–1.01	0.64	0.52
PT	1.42	1.17–1.77	2.80	0.01
APTT	0.97	0.89–1.06	−0.53	0.59
D–dimer	1.00	1.00–1.00	2.77	0.01
Fibrinogen	1.00	1.00–1.00	1.02	0.31
PLT	1.00	0.99–1.00	−1.26	0.21
WBC	1.30	1.16–1.47	3.69	<0.001
Monocyte	1.48	0.95–3.91	0.79	0.42
Neutrophil	1.43	1.25–1.68	4.02	<0.001
Eosinophil	0.02	0.00–0.65	−1.38	0.17
CRP	1.03	1.02–1.04	3.84	<0.001
K^+	0.86	0.54–1.32	−0.55	0.58
Na^+	0.81	0.74–0.89	−3.75	<0.001
Ca^+	0.0004	0.000012–0.0038	−4.76	<0.001

$P < 0.001$) remained significantly associated with the disease progression of COVID-19 pneumonia in both logistic and meta analyses (**Table 3**).

To evaluate the probability of the patient with severe COVID-19 pneumonia, we sought to develop a model for predicting the risk of disease progression using multivariate logistic regression algorithm with the above variables in Wuhan cohort. The final model contained nine investigated variables (including age, fever, hypertension, diabetes, BUN, CREA, CK, LDH, and neutrophil count) and one interaction term. The model for predicting the risk of severe pneumonia had a c-statistic of 0.86, with good calibration curve (**Figure 3**). The ROC analysis in Wuhan cohort revealed that the model had high sensitivity and specificity (**Figure 3**). Moreover, we collected another two independent cohorts of patients from Hangzhou and Yinchuan as validation cohorts to evaluate the performance of the model. The Hangzhou validation cohort consisted of 42 patients. 16 (38%) patients were diagnosed as severe COVID-19 pneumonia, and 26 patients were diagnosed as mild COVID-19 pneumonia (**Table 1**). The model for predicting the risk of severe pneumonia had a c-statistic of 0.93, with great calibration curve in validation cohort (**Figure 3**). The model had an AUC of 0.928, which indicated it also had good performance in validation cohort (**Figure 3**). The Yinchuan validation cohort consisted of 26 patients. 6 (23%) patients were diagnosed as severe COVID-19 pneumonia (**Table 1**). The model for predicting the risk of severe pneumonia had a c-statistic of 0.90, with great calibration curve in validation cohort (**Figure 3**). The model had an AUC of 0.908, which indicated it also had good performance in validation cohort (**Figure 3**).

To prevent the overfitting of our model, we performed correlation analysis between the biological test parameters used in our model, the results showed that the CREA level was significantly correlated with the BUN level in COVID-19 patients ($R = 0.77$, $P < 0.001$; **Supplementary Figure 6**). We sought to interact these two parameters in our model, through the AUC of model increased to 0.874 in the development cohort (**Supplementary Figure 6**), the AUC dropped to 0.837 and 0.900 in Hangzhou and Yinchuan validation cohort, respectively (**Supplementary Figure 6**). Therefore, we use all the variables to generate the model without interactions.

We created the nomogram to predict the probability of severe COVID-19 pneumonia (**Figure 4**). The line labeled Points was used to calculate the points referred to each of the nine risk factors. The subsequent lines (lines 2–10 in **Figure 4**) are the risk factors involved in this model. The value for each variable is located on these lines and a vertical line is drawn up to the points line to find the points associated with each value. All of the points are summed and the total located on the Total points line. According to the total points, we could get the COVID-19 pneumonia of severe COVID-19 pneumonia for each patient.

DISCUSSION

The Coronaviridae is a family of enveloped, non-segmented, positive-stranded RNA viruses, which are widely distributed in humans and other mammals such as bats, masked palm civets, and pangolins (16, 17). Six of these coronaviruses are found to infect humans previously, and four of them only mild respiratory symptoms similar to the common cold. However, MERS-CoV and SARS-CoV, two kinds of beta-coronaviruses, infected more than 1,000 and 8,000 patients with high case fatality rates (37% for MERS-CoV and 10% for SARS-CoV), respectively (18, 19). The novel coronavirus −19, which is also considered as SARS-CoV2, is the seventh member of the Coronaviridae family found to infect human beings (20). Though the case fatality rate of SARS-CoV2 so far is lower than that of SARS-CoV or MERS-Cov diseases, SARS-CoV2 is contagious in humans and is the cause of the ongoing pandemic that has been designated a Public Health Emergency of International Concern by the World Health Organization (WHO) (21).

In our study, we employed a meta-analysis to identify relevant variables for promoting COVID-19 pneumonia progression. Severe COVID-19 pneumonia was obviously correlated with severe complications including ARDS (OR = 49.03), shock (OR = 45.48), acute kidney injury (OR = 24.82), acute cardiac injury (OR = 37.93), progressive COVID-19 also enhanced the death

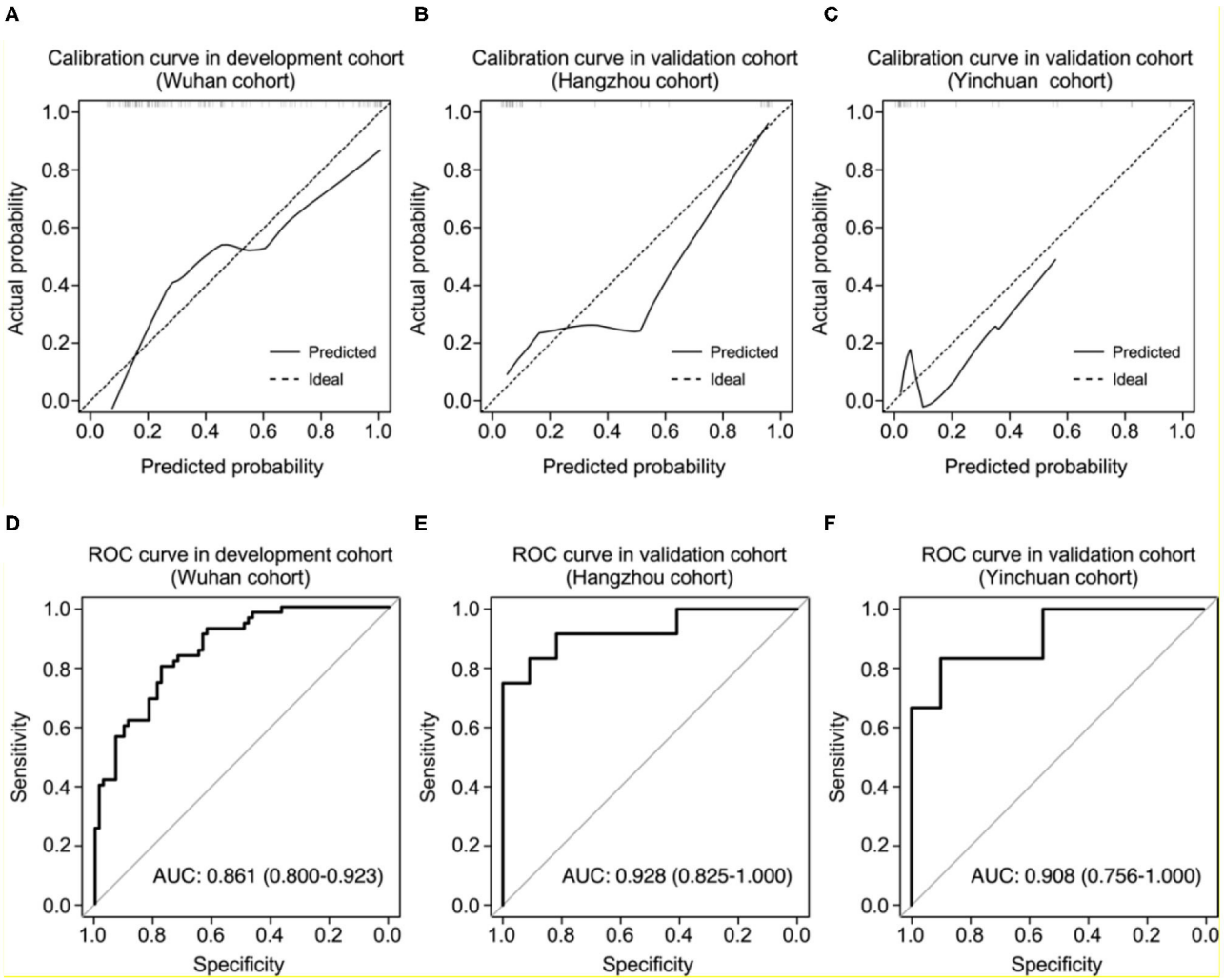

FIGURE 3 | Performance of the nomogram in development and validation cohorts. **(A–C)** Calibration curves of the nomogram for predicting of the probability of severe COVID-19 pneumonia in development **(A)**, Hangzhou validation **(B)**, and Yinchuan validation cohorts **(C)**. The histogram at the top of the plot shows the distribution of the predicted probabilities. **(D–F)** Receiver operating characteristic curves of sensitivity and specificity of the nomogram for predicting severe COVID-19 pneumonia in development **(D)**, Hangzhou validation **(E)**, and Yinchuan validation cohorts **(F)**.

risk (RR = 30.09). Previous studies have implied the SARS-CoV2 infection may cause multi-organ injuries, our results further supported their conclusion by making evidence-based medicine research (22, 23). Moreover, Age and comorbidities such as hypertension, diabetes, cancers, heart diseases, pulmonary diseases, kidney diseases as well as cerebrovascular diseases can significantly increase the risk of COVID-19 progression. Meanwhile, we also found that clinical symptoms might not contribute to the disease progression of COVID-19 pneumonia. Laboratory findings especially the renal function index (CREA, BUN), myocardial enzymonram (CK, LDH), and neutrophil counts in severe COVID-19 patients were sustained higher than that of the ordinary patients.

Age and comorbidities were important potential risk factors in progressive COVID-19 pneumonia (24). Elder patients could progress to COVID-19 pneumonia due to the hypo-immunity and comorbidities. Laboratory examinations could reflect the different statuses of organs. We observed that a range of indicators representing functions of different organs had changed in COVID-19 patients, especially in severe COVID-19 patients via meta-analysis, suggesting the existence of multi-organ dysfunctions in patients with severe COVID-19 pneumonia. Organ injuries and progressive COVID-19 pneumonia may be reciprocal causation, progressive COVID-19 caused organ injuries, and indexes of organ injuries could in turn help us to monitor the risk of COVID-19 pneumonia progression. The decision to evaluate disease progression COVID-19 patients based on solely index might be misleading. However, there are no explicit standards or risk models to assist diagnosis. Thus, we collected a total of 270 COVID-19 patients to establish a model for evaluating the risk of COVID-19 pneumonia progression.

In our development cohort, concentrations of C-reactive protein were increased in most patients, similar as observed in previous beta-coronavirus infections (10, 11). In the subgroup

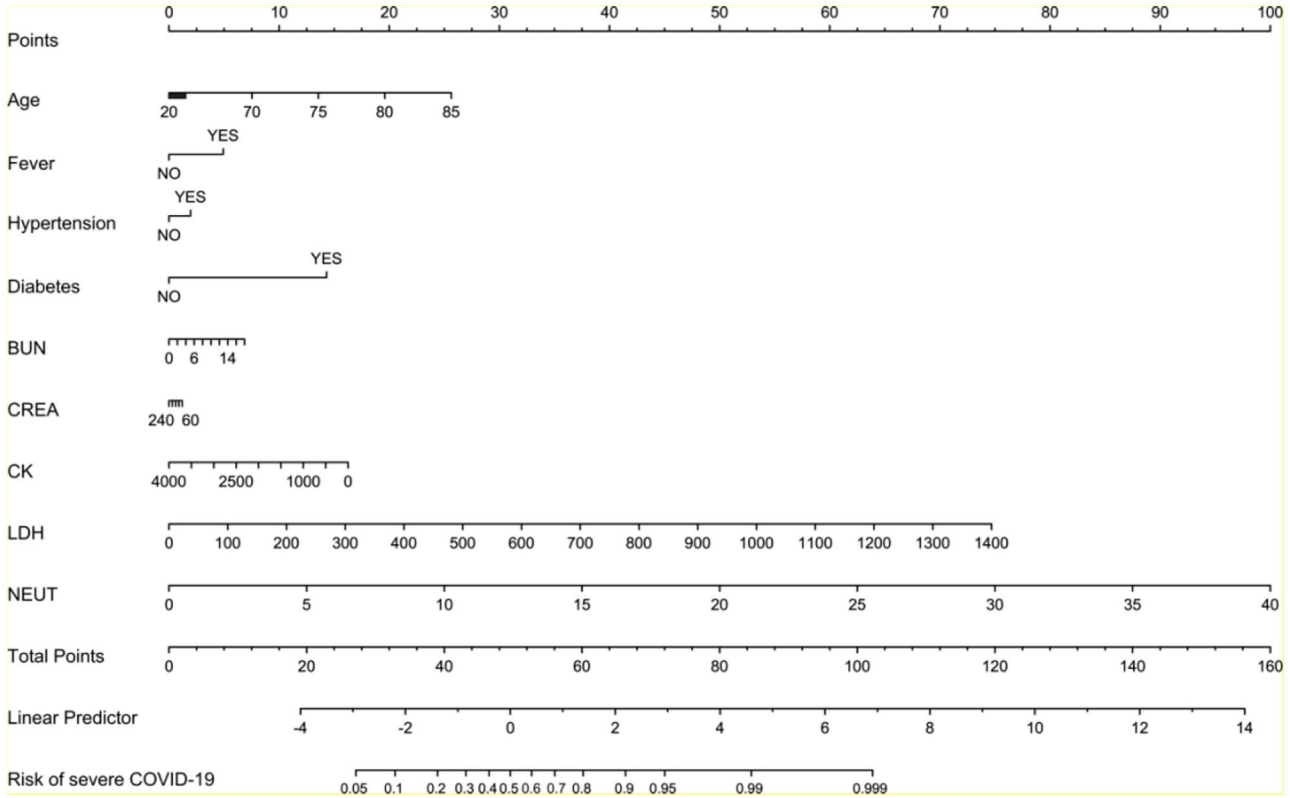

FIGURE 4 | Nomogram to predict chance of being severe COVID-19 pneumonia.

of patients with severe COVID-19 pneumonia, concentrations of C-reactive protein (74.3 mg/L) were higher than those in mild patients. The concentration of albumin was significantly reduced in severe group patients, which might lead to albuminemia. This finding is similar to those of a previous study of patients with H1N1 infection (25). Furthermore, renal function index (CREA and BUN), myocardial enzymonram (CK and LDH), and liver function index (ALT and AST) were elevated in patients with severe COVID-19 pneumonia. Previous studies also showed that the severe COVID-19 pneumonia could cause kidney dysfunctions based on biopsy samples (26). Thus, our findings indicate that patients with severe COVID-19 pneumonia may be suffered with multiple organ dysfunctions.

Several variables have been documented to be associated with severe COVID-19 pneumonia, such as cancer, hypertension, heart diseases and so on (24). However, no study integrates these risk factors to predict the probability of severe COVID-19 pneumonia. First, we screened the predictive variables for severe COVID-19 pneumonia using the 202 patients from Wuhan cohort. Eight risk factors were found to be significantly correlated with severe COVID-19 pneumonia, and we subsequently created a model and a nomogram based on these risk factors by multivariate logistic regression algorithm. To further validate the validity of the model and nomogram, we collected another two validation cohorts of COVID-19 pneumonia patients from Hangzhou and Yinchuan, the calibration, discrimination and ROC analysis together reveal that the model we built has good performance in two independent validation cohorts (**Figure 3**).

Our prediction model and nomogram can help doctors to evaluate the risk of disease progression of COVID-19 pneumonia patients on admission to hospital. The nomogram summed points showed the risk of each patient, sensitively reflecting the dysfunctions of multi-organs and the risk score might be related to the outcome of the patient. Accurate risk evaluation may also assist therapeutic decision-making for different patients. According to previous report, steroid drugs may bring no benefits for patients with mild COVID-19 pneumonia, indicating that patients with low risk may not accept steroid drugs treatment (27, 28). In addition, the decision to make disease managements for COVID-19 pneumonia is complex, which depends on various factors, including the patient's baseline disease burden and overall clinical picture, not solely on the risk of severe COVID-19 pneumonia. Thus, our model and nomogram can make assistance for doctors by providing an objective and quantifiable estimate of the probability progressed to severe COVID-19 pneumonia.

Our study still has several limitations. First, our nomogram does not include other important predictive variables including heart diseases, chronic obstructive pulmonary disease (COPD), and CT images because of relatively small cohort population. Second, multi-organ dysfunctions of patients with

severe COVID-19 pneumonia were evaluated by laboratory examinations of indicated indexes, which needs further pathological validations by biopsy specimens. Third, follow-up data were not available for majority of patients, we could not monitor the risk change of patients during the whole illness onset. Finally, although we have validated our nomogram in an independent cohort, large-scale cohort validation and long-term follow-up are still needed to confirm our findings. Therefore, our nomogram may be improved as additional predictive variables are incorporated, and it still needs follow-up data to accurately monitor the disease progression of COVID-19 pneumonia.

CONCLUSION

In conclusion, our study summarized all the existing literatures (published or preprinted), which studied the factors of disease progression of COVID-19 pneumonia. Severe COVID-19 pneumonia is significantly correlated with severe complications such as ARDS, shock, acute kidney injury and acute cardiac injury, and the comorbidities significantly increased the risk of progressive COVID-19 pneumonia. At the same time, a model followed by a nomogram based on previously identified risk factors including age, fever, comorbidities, CK, LDH, CREA, BUN and neutrophil count for predicting the risk of severe COVID-19 pneumonia was established in a development cohort, and further validated in an independent cohort.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The study is approved by the Ethics Committee of the Zhongnan Hospital of Wuhan University and the First hospital of Xiaoshan District under the accession 2020035. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GC, PL, and YC contributed equally as the co-author. QS and MG had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. GC, MG, RZ, and QS concept and design. GC, PL, KF, YC, SW, BC, XF, and QS acquisition, analysis, or interpretation of data. GC, PL, and QS drafting of the manuscript. MX and MG critical revision of the manuscript for important intellectual content. GC and QS statistical analysis. RZ and YC administrative, technical, or material support. MX, RZ, MG, and QS supervision. MG obtain funding. 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.2020.556886/full#supplementary-material

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Lifestyle Acquired Immunity, Decentralized Intelligent Infrastructures, and Revised Healthcare Expenditures May Limit Pandemic Catastrophe: A Lesson From COVID-19

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Throughout history, the human race has often faced pandemics with substantial numbers of fatalities. As the COVID-19 pandemic has now affected the whole planet, even countries with moderate to strong healthcare support and expenditure have struggled to contain disease transmission and casualties. Countries affected by COVID-19 have different demographics, socioeconomic, and lifestyle health indicators. In this context, it is important to find out to what extent these parametric variations are modulating disease outcomes. To answer this, this study selected demographic, socioeconomic, and health indicators e.g., population density, percentage of the urban population, median age, health expenditure per capita, obesity, diabetes prevalence, alcohol intake, tobacco use, case fatality of non-communicable diseases (NCDs) as independent variables. Countries were grouped according to these variables and influence on dependent variables e.g., COVID-19 positive tests, case fatality, and case recovery rates were statistically analyzed. The results suggested that countries with variable median age had a significantly different outcome on positive test rate ($P < 0.01$). Both the median age ($P = 0.0397$) and health expenditure per capita ($P = 0.0041$) showed a positive relation with case recovery. An increasing number of tests per 100 K of the population showed a positive and negative relationship with the number of positives per 100 K population ($P = 0.0001$) and the percentage of positive tests ($P < 0.0001$), respectively. Alcohol intake per capita in liter ($P = 0.0046$), diabetes prevalence ($P = 0.0389$), and NCDs mortalities ($P = 0.0477$) also showed a statistical relation to the case fatality rate. Further analysis revealed that countries with high healthcare expenditure along with high median age and increased urban population showed more case fatality but also had a better recovery rate. Investment in the health sector alone is insufficient in controlling the severity of the pandemic. Intelligent and sustainable healthcare both in urban and rural settings and healthy lifestyle acquired immunity may reduce disease transmission and comorbidity induced fatalities, respectively.

Keywords: COVID-19, socioeconomic, demographic, health indicator, urban population, median age

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INTRODUCTION

There have been rapid demographic changes in most regions and countries of the world since the middle of the last century. Increased population density, urban population, and life expectancy are noticeable examples of such changes (1, 2). The key objective of these transitions is connected to improving the socioeconomic level of a country's population. Socioeconomic development influences population health status through the regulation of the environment, lifestyle, and healthcare systems (3). Socioeconomic variables including population density, gross national income (GNI) per capita, and health expenditure per capita play an important role in achieving sustainable development (4, 5). The health policies of governments are important and perhaps the most critical aspect of these in ensuring adequate facilities and management in the population (6).

Over the last few decades, many governments have increased spending in health sectors to improve healthcare systems, treatments, research, and the development of new drugs and vaccines, and technologies for preventing and controlling diseases (7–12). The majority of this funding is being invested to prevent and treat diseases of a communicable and non-communicable nature. The amount spent on health is mostly dependent on GNI at purchasing power parity (PPP) (5, 13, 14) and the outcome is visible as reduced child mortality, increase in median age, and life expectancy at birth (15–18). Therefore, life expectancy, median age, and the percentage of the urban population have become the key indicators in human development indexes (19–21). The top-listed economies by GNI (PPP) are conventionally ahead in technology, research, and training (22).

Chronic respiratory diseases are responsible for almost four million premature deaths globally (23). Respiratory infections are usually worsened through population density, human behavior, insufficient public health safety, the genomic mutations of microbes, extraneous usage, and developing resistance to antibiotics. The lack of global coordination to prevent infectious disease outbreak and pandemic is partly due to weak policies, management, and expenditures in autocratic regimes, putting global health security at risk (24). In addition, developed countries have created facilities, readiness, and prevention from several life-threatening diseases without any divergences in rural and urban populations (24). However, these sustainably developed countries are facing devastating disasters during the Corona Virus Disease-19 (COVID-19) pandemic (25). COVID-19 emerged in December 2019 in Wuhan, China, with growing morbidity and mortality worldwide (26). As of May 22, 2020, there were more than 5.1 million positive cases and more than 0.3 million deaths (27).

The COVID-19 pandemic is caused by a positive-sense single stranded RNA (+ssRNA) virus named SARS-CoV-2 which belongs to the corona virus family. This family of viruses is capable of introducing human sickness (26) with an incubation period ranging from 2 to 14 days to develop symptoms (28). SARS-CoV-2 is mostly transmitted between persons via respiratory droplets, coughs, sneezes, and fomites (2). COVID-19 patients could be asymptomatic or develop flu-like symptoms with fever, dry cough, tiredness, and shortness of breath. Intensive care with ventilation and symptom-based therapies are needed for critical patients (2). The World Health Organization (WHO) declared the COVID-19 outbreak as a Public Health Emergency of International Concern (PHEIC) on January 30, a and pandemic on March 11, 2020 (29).

Clinical reports have confirmed that non-communicable disease (NCDs) including diabetes, heart disease, hypertension, respiratory disease (COPD/bronchial asthma), cancer, predominantly amongst the aged individuals, upsurge the susceptibility to COVID-19 (30). Surprisingly, countries with developed facilities to manage NCDs are struggling in the COVID-19 pandemic. Healthcare personnel are also being infected in all countries regardless of the country's economic status and demographic characters (24, 31). Several disease susceptibility patterns and predictions are also made to understand trends of infections, death, and recovery patterns (6, 32) and factors that influence transmission and fatalities (6). The associations of different environmental factors have also been investigated (6, 33, 34). COVID-19 requires sufficient public attention and needs to reprioritize financial involvement in appropriate segments of the health sector to confirm inclusive responses. Highly affected countries have used numerous tactics of financial distribution, depending on their capabilities, structures, and regulatory systems (35). In-depth investigations need to be conducted on the association of infection pattern, fatalities, and recovery in combination with population density, median age, percentage of the urban population, GDP per capita, and health expenditure per capita along with lifestyle and health indicators.

This study used publicly available demographic, socioeconomic, and health indicator data from COVID-19 affected countries to analyze and extrapolate the influence of categorical independent variables on disease outcome. The main research question asked to what degree these variables have a significant impact on the dependent variables including positive test rates, case fatality rates, and case recovery rates. These findings along with other studies of a similar nature might help to strengthen our preparedness to face any yet-to-come contagious pandemics in the near future. The findings of this study also highlight the importance of implementing an intelligent healthcare system in both urban and rural areas, coupled with a healthy lifestyle that boosts population immunity. Refocusing healthcare investment in poorly addressed sectors should be prioritized to minimize loss of life and related economic losses on a global scale.

METHODS

Data Characteristics

The study was designed to use available secondary data for all variables. We obtained data on COVID-19 data of total positive cases, total death cases, total recovered cases, and total tests from Worldometers.info (27). The socioeconomic and demographic

data, including total population, population density, median age, urban population percentages, male to female ratio, and financial information together with gross domestic product (GDP) in USD, gross national income (GNI) per capita (purchasing power parity, PPP) in USD, health expenditure (% of GDP) in USD were attained from the databank of the World Bank (36). However, the health expenditure per capita data of Hong Kong was obtained from the website of the Department of Health of the Government of the Hong Kong Special Administrative Region (37). In addition, GNI per capita (PPP) of Djibouti was obtained from the database of the International Monetary Fund (38). Lifestyle and health indicator data including the prevalence of obesity (39), the prevalence of insufficient physical activity (40), the prevalence of diabetes (41), NCD mortality rate per 100 K population (42), alcohol consumption per capita per liter per year (43), and tobacco use percentages of males (44) were obtained from the WHO and World Bank.

Inclusion and Exclusion Criteria

In total, 91 countries were selected, whose total infection cases were over 1,000 on May 9, 2020 (**Supplementary Data Sheet 1**). For some particular parameters, the data relating to some countries were unavailable and they were excluded from the relevant analysis. However, other data from these countries were used in the respective analysis.

Variables

The current study was conducted with several variables. Variables related to COVID-19, viz. total positive cases, total death cases, total recovered cases, the total number of tests were used. The percentage of positive tests were calculated against total number of tests performed. Case fatality and case recovered rates were calculated against total positive cases. The percentage of positive tests, case fatality, and case recovered rates were used as dependent variables instead of total positive cases, death cases, and recovery cases. This was because the socioeconomic conditions in a number of countries did not go through strategies of mass diagnosis, These countries instead conducted diagnosis when COVID-19 related symptoms first appeared. Population density, median age, percentage of the urban population, GNI per capita (PPP) in USD, and health expenditure per capita in USD were used as socioeconomic and demographic independent variables. Lifestyle and health related independent variables viz. prevalence of obesity, insufficient physical activity, diabetes, and any kind of tobacco use by men and women were obtained as percentages. Alcohol consumption per capita per liter was used as a variable, and the average number of liters consumed per year was considered. In addition, NCDs and mortality rates per 100 K of the population were also taken as another independent variable. Test numbers of countries were not uniform and depend on several factors including socioeconomic status and government initiatives. We calculated the number of tests per 100 K populations as well as positive cases per 100 K populations. Tests per 100 K of the population were used as independent variables and analyzed against positive test rates, case fatalities, and case recovered rate along with positive cases per 100 k as dependent variables. Linear regression analysis was performed with ungrouped independent variables with dependent variables as one to one analysis fashion. Later, the group wise distribution of independent variables were analyzed with dependent variables to find associations.

Independent Variables Processing and Grouping

Independent variables except for GNI per capita (PPP) were grouped for trends and scenarios concerning dependent variables (**Table 1**). The GNI per capita (PPP) in USD were grouped into categories according to the World Bank (45). Grouped independent variables were analyzed against dependent variables as mean ± SEM. Cross analysis of each independent variable was also performed to get in-depth information, as outlined in the discussion section of this study. Cross analysis derived inter relational table can be found in **Supplementary Table 2**.

Statistical Analysis

We performed one to one regression analysis of ungrouped data in SPSS version 26. GraphPad Prism version 6 was used to generate graphs. One- or two- way ANOVA were performed as required along with the individual group to group statistical variation analysis. All statistical significance was measured at a significant value < 0.05. All the final graphs generated in GraphPad Prism were combined using Inkscape version 0.92 graphics software.

RESULTS

The COVID-19 pandemic shows uneven epidemiological and clinical trends as it spreads to countries with climatic, socio-economic, lifestyle and demographic variations around the globe. In addition to mutation induced genomic variations in the virus, these factors might have a substantial influence on key outcomes like rate of infection, case fatality, and case recovery. This study was conducted to find possible links between the rate of COVID-19 infections, fatalities, and recovery with socioeconomic, demographic, lifestyles, and health indicators.

The association of dependent variables with non-grouped independent variables were measured with linear regression and results were shown in **Table 2**. One to one regression analysis showed that median age ($P = 0.005$), and any kind of tobacco use by men ($P = 0.004$) and women ($P = 0.02$) were significantly linked with positive test rate. Case fatality rates were associated and a significantly predicted by male/female ratio of the population ($P = 0.033$), median age ($P = 0.005$), health expenditure per capita in USD ($P = 0.008$), NCD mortalities per 100 K ($P = 0.003$), alcohol consumptions per capita in liter ($P = 0.001$), and tobacco use by women ($P = 0.007$). Moreover, case recovered rates were also significantly related to median age ($P = 0.004$), health expenditure per capita in USD ($P = 0.018$), and NCD mortalities per 100 K ($P = 0.038$).

The three dependent variables of the present study, covering 91 countries have mean percentages of positive cases per test, case fatality, and case recovery of 9.94 ± 1.25%, 4.26 ± 0.38%, and 44.98 ± 2.80%, respectively. The goal was to find any significant differences among these variables when countries were grouped according to different socio-economic, demographic, lifestyle, and health determinants.

TABLE 1 | Characteristics of grouped independent variables.

Variables	Groups description	Numbers of country	Unavailable data (no of country)
Population density Km^2	Up to 100	51 (56.04%)	0
	100+ to 250	26 (28.57%)	
	250+ to 500	8 (8.79%)	
	500+	6 (6.59%)	
Per capita GNI (PPP) in USD*	Up to 1026	0	0
	1,026+ to 3,995	4 (3.40%)	
	3,995+ to 12,375	19 (20.88%)	
	12,375+	68 (74.73%)	
Urban population, %	Up to 50%	12 (13.19%)	0
	50+ to 75%	38 (41.76%)	
	75%+	41 (45.05%)	
Median age, years	Up to 20	6 (6.59%)	0
	20+ to 30	21 (23.08%)	
	30+ to 40	30 (32.97%)	
	40+	34 (37.36%)	
Per capita health expenditure in USD	Up to 100	12 (13.19%)	0
	100+ to 500	28 (30.77%)	
	500+ to 2,000	26 (28.57%)	
	2,000+	25 (27.47%)	
Prevalence of obesity, %	Up to 15%	19 (20.88%)	3 (3.30%)
	15+ to 20%	41 (45.05%)	
	20%+	28 (30.77%)	
Prevalence of insufficient physical activities, %	Below 20%	9 (9.89%)	
	20 to 30%	22 (24.18%)	14 (15.38%)
	30+ to 40%	35 (38.46%)	
	40%+	11 (12.09%)	
NCD mortality per 100 K	Up to 300	7 (7.69%)	5 (5.49%)
	300+ to 400	30 (32.97%)	
	400+ to 500	22 (24.18%)	
	500+ to 600	14 (15.38%)	
	600+ to 700	11 (12.09%)	
	700+	12 (13.19%)	
Prevalence of diabetes, %	Up to 5%	16 (17.58%)	1 (1.10%)
	5+ to 10%	56 (61.54%)	
	10 to 15%	8 (8.79%)	
	15%+	8 (8.79%)	
Alcohol consumptions per capita in liters per year	Up to 5 L	32 (35.16%)	2 (2.20%)
	5+ to 10 L	35 (38.46%)	
	10+	16 (17.58%)	
Any kind of tobacco used by male, % of population	Up to 25%	24 (26.37%)	2 (2.20%)
	25 to 40%	29 (31.87%)	
	40%+	19 (20.88%)	

*Groups were formed according to the distribution. *Grouping of Per capita GNI (PPP) was done according to the World Bank classification by income level.*

Effect of Socioeconomic and Demographic Factors on the Percentage of Positive Tests

The population density, percentage of the urban population, median age, and health expenditure per capita were used as independent variables. These variables were grouped as mentioned in **Table 1**. Data on the total tests performed were not available for four countries (Cameroon, China, Guinea, and Sudan), therefore 87 countries were included in this section. Results showed that countries with low population density (below 100 people per km^2) had a high percentage of positive tests (**Figure 1A**, left panel). However, countries in other population density groups had lower positive test rates with minimum variability among them. The percentage of positive tests were slightly lower in countries with high urban density (75% above) compared to other groups (**Figure 1B**, left panel). Countries with increasing median age showed a statistically significant decrease in the percentage of positive tests (**Figure 1C**, left panel). The relation between the percentage of positive tests and health expenditure per capita showed no significance regardless of whether country spending had a higher or lower outcome (**Figure 1D**, left panel).

Effect of Socioeconomic and Demographic Factors on the Case Fatality Rate

The percentage of case fatalities and its association with population density was uneven and not statistically significant (**Figure 1A**, right panel), however, countries with a higher urban population showed higher case fatalities (**Figure 1B**, right panel). Although the percentage of positive tests had a significant association with median age, case fatalities did not show a trend (**Figure 1C**, right panel). But, countries with the highest number of older populations (median age over 40 years) had a higher fatality rate compared to the other three groups. Similarly, case fatality was found to be high in the countries where the government disburses more in the health sector per capita (**Figure 1D**, right panel).

Effect of Socioeconomic and Demographic Factors on Case Recovery Rate

The association of socioeconomic and demographic determinants with the case recovery rate is shown in **Figure 2**. Population density did not show any connection with the case recovery rate (**Figure 2A**). Similar to the case fatalities trend, countries with a high urban population showed a higher recovery rate than the countries with a lower dense city population (**Figure 2B**). Similar to the results for case fatalities, countries with increasing median age showed an increased recovery rate (**Figure 2C**) and variances of mean values were statistically significant by one-way ANOVA ($^*P = 0.0397$). **Figure 2D** indicates that countries that spend more on healthcare systems per capita, had more recovery rates from COVID-19 ($^{**}P = 0.0041$) (**Figure 2D**). Due to the unavailability of total recovery case information for the Netherlands and the United Kingdom, they were not included in this segment of analysis.

Effect of Number of Tests on the Rate of Positive Tests, Fatalities, and Recovery

All countries included in the study varied in COVID-19 diagnosis capacity for social, economic, and political reasons. Taking this

TABLE 2 | One to one linear regression analysis using ungrouped independent variables.

	Positive test rate				Case fatality rate				Case recovered rate			
	R^2	*F*	Regression coefficients (β)	*p*	R^2	*F*	Regression coefficients (β)	*p*	R^2	*F*	Regression coefficients (β)	*p*
Male/Female ration	0.001	0.059	−0.027	0.808	0.051	4.694	−0.225	*0.033*	0.035	3.109	−0.186	0.081
Population density/km^2	0.003	0.29	−0.058	0.592	0.029	2.667	−0.171	0.106	0	0.026	0.017	0.873
Urban population, %	0.001	0.067	−0.028	0.797	0.011	0.994	0.105	0.321	0.035	3.137	0.187	0.08
Median age, years	0.091	8.478	−0.301	*0.005*	0.086	8.393	0.294	*0.005*	0.092	8.787	0.303	*0.004*
PC GNI (PPP) in USD	0.035	3.063	−0.186	0.084	0.003	0.254	0.053	0.616	0.024	2.133	0.155	0.148
PC health expenditure in USD	0.013	1.123	−0.114	0.292	0.076	7.325	0.276	*0.008*	0.062	5.775	0.249	*0.018*
Obesity prevalence, %	0	0.032	0.020	0.859	0.009	0.793	0.096	0.376	0.006	0.501	0.077	0.481
Insufficient physical activity prevalence, %	0	0.008	−0.011	0.929	0.003	0.234	0.056	0.63	0.018	1.341	−0.133	0.251
NCD mortality per 100 K	0	0.005	0.008	0.945	0.099	9.236	−0.315	*0.003*	0.051	4.453	−0.226	*0.038*
Diabetes prevalence, %	0.005	0.425	0.071	0.516	0.008	0.666	−0.087	0.417	0.013	1.162	−0.115	0.284
Alcohol consumptions per capita per L	0.042	3.659	−0.205	0.059	0.11	10.767	0.332	*0.001*	0.04	3.554	0.199	0.063
Tobacco used by male %	0.097	8.95	−0.312	*0.004*	0	0.042	−0.022	0.838	0	0.001	−0.004	0.073
Tobacco used by female %	0.064	5.645	−0.252	*0.02*	0.081	7.678	0.285	*0.007*	0.017	1.522	0.132	0.221

Linear regression analysis was conducted as one to one analysis with dependent and independent variable. Significant value was calculated as $P \leq 0.05$ and mentioned in italic in the table.

into account, we aimed to further examine whether the number of tests performed per 100 K population had any significant effect on mean positive cases, percentage of case positives, case fatality, and case recovery. To do this analysis, we calculated the country specific number of positive cases per 100 K of the population, and tests performed per 100 K population from data on the number of total tests (27), total positive cases (27), and the total population of the country (36).

The association of COVID-19 test numbers per 100 K population is presented in **Figure 3**. Positive cases per 100 K of the population were significantly (****$P < 0.001$) boosted with an increased number of tests performed per 100 K population (**Figure 3A**), in contrast, the percentage of positive tests were high in the lowest COVID-19 tests per 100 K populations (****$P < 0.0001$) (**Figure 3B**). Case fatality did not change significantly among countries grouped into increasing test numbers (**Figure 3C**). Higher case recovery rates were observed in the groups of countries where a higher number of tests were performed (**Figure 3D**). Cameroon, China, Guinea, and Sudan were out of this analysis due to the unavailability of test data from these countries.

Lifestyle and Health Indicators

Individual, community, and/or social lifestyle can influence the transmission of viruses as well as death and recovery rates. Another study has shown that health indicators like the prevalence of diabetes and other NCDs also influence the number of death and recovery (46). Therefore, we considered the prevalence of obesity, insufficient physical activities, diabetes, NCDs mortality per 100 K population, consumption of alcohol per capita per liter per year as well as the percentage of general tobacco uses in male populations as independent variables.

Lifestyle and Health Indicators on Case Fatalities of COVID-19

In our investigation, countries with high obesity prevalence were witnessing more COVID-19 related deaths (**Figure 4A**) and correspondingly, countries where people were less physically active also had increased death rates (**Figure 4B**). Countries with high alcohol consumption were significantly ($P = 0.0046$) prone to death (**Figure 4C**). However, interestingly, tobacco use patterns showed different scenarios compared to alcohol consumption. Higher percentages of any kind of tobacco used in males showed fewer case fatalities compared to other groups of countries (**Figure 4D**). Surprisingly, countries with low diabetes prevalence showed a significantly ($P = 0.0389$) higher death rate (**Figure 4E**). NCD mortality per 100 K population showed a negative relation with the COVID-19 case fatality rate ($P = 0.0477$), and countries with high NCD mortality experienced less COVID-19 case fatality (**Figure 4F**).

Lifestyle and Health Indicators on Case Recovered Rate of COVID-19

Our analysis (**Figure 5A**) showed that countries with an increasing prevalence of obesity were related to the COVID-19 case recovery rate. Insufficient physical activity did not show any meaningful association with the recovery rate (**Figure 5B**). However, high alcohol consumption displayed a relationship

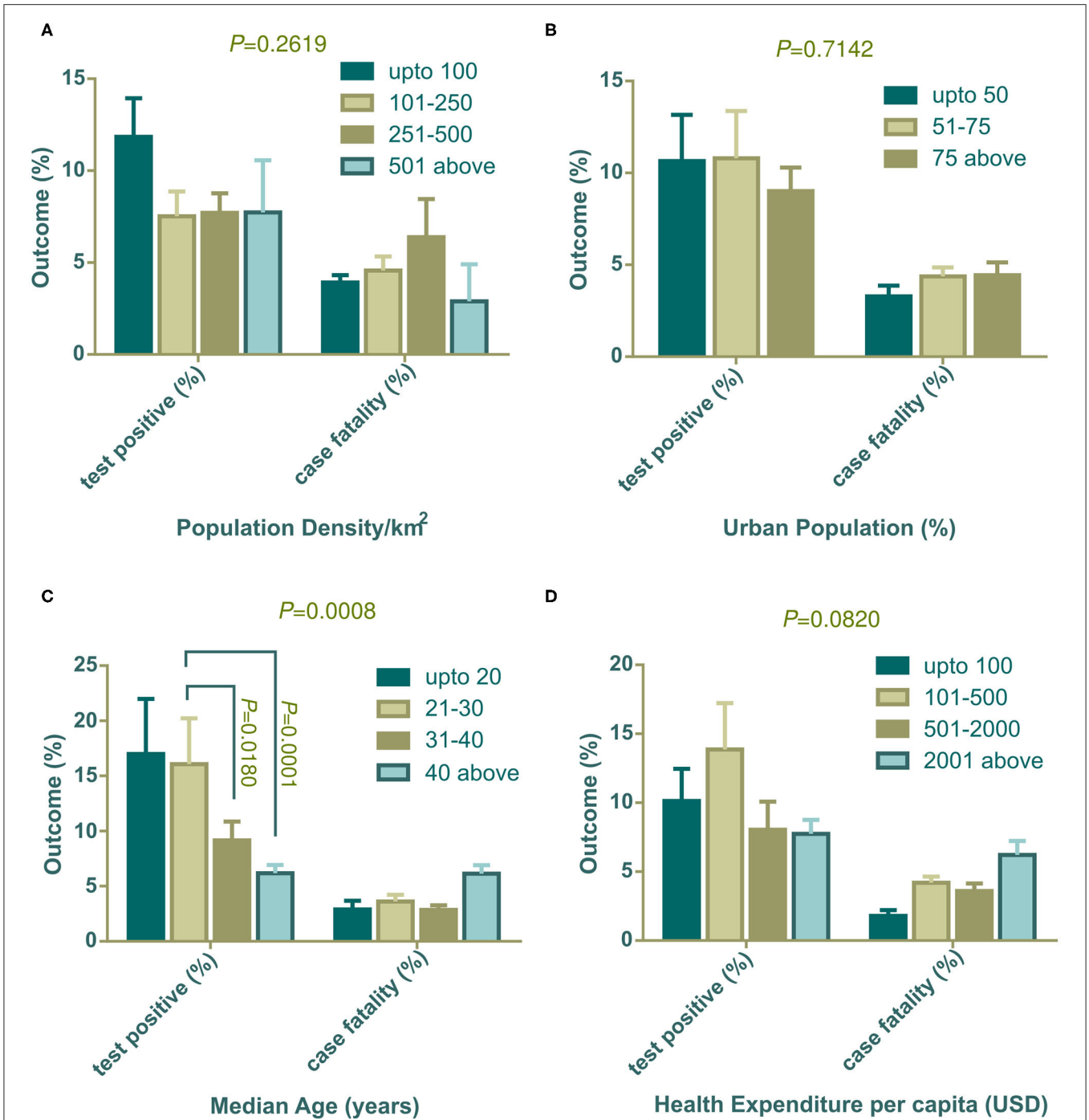

FIGURE 1 | Positive tests (%) and case fatality (%) distribution among countries categorized with population density **(A)**, urban population **(B)**, median age **(C)**, and health expenditure per capita **(D)**. All outcomes (%) are presented as mean ± SEM. In **(C)**, mean positive test (%) of countries with 21–30 years of median age is statistically significant against 31–40 years of median age ($P = 0.0180$) and median age over 40 years ($P = 0.0001$) by Tukey's multiple comparison test. Two-way ANOVA interaction *P*-value are shown on top of each graph. Statistically significant interaction *P*-value indicates that change in independent variable interacts differently on two dependent outcomes.

with high recovery, though it was not statistically significant (**Figure 5C**). The tobacco use pattern did not show any trends (**Figure 5D**). The highest prevalence of diabetes exhibited a low case recovery rate (**Figure 5E**) compare to other groups of countries. Low NCD mortalities per 100 K groups had a relatively high case recovery rate (**Figure 5F**).

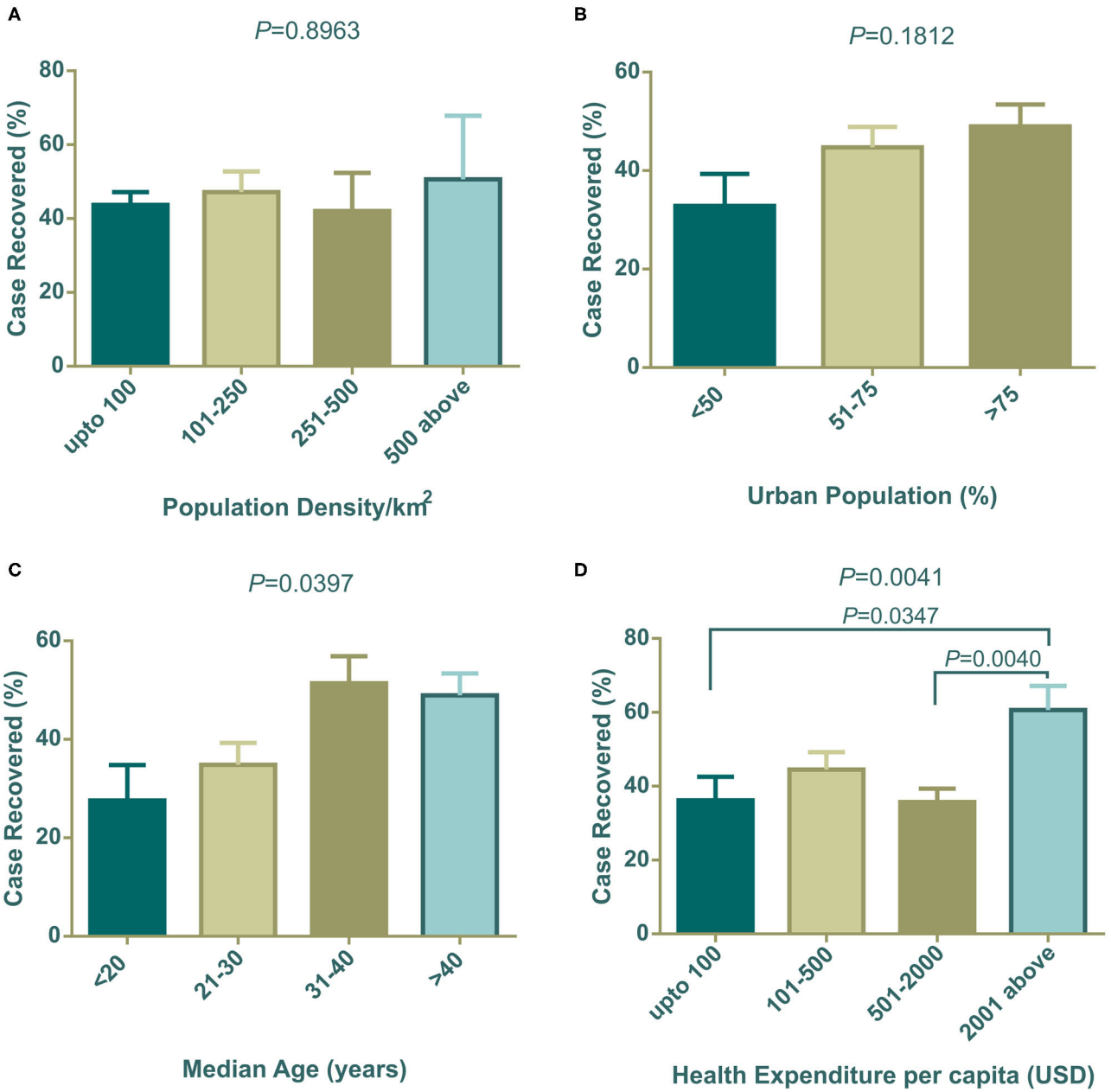

FIGURE 2 | Case recovery (%) distribution among countries categorized with population density **(A)**, urban population **(B)**, median age **(C)**, and health expenditure per capita **(D)**. One way ANOVA statistical differences among column means are shown as *P*-values on top of each graph. In **(C,D)**, differences among means are statistically significant. Data presented are mean ± SEM.

Parametric Distribution of Countries With Top Case Fatality Rates

The association of different factors with dependent variables showed a mixed pattern. In this context, we wanted to further explore countries with high fatality rates so that parametric trends can be observed. To do that, we selected 30 countries with more than 5% case fatalities from among 91 countries (**Table 3**). The mean ± SEM values of all parametric distributions are also shown in **Table 3**. Individual parameter distributions are shown as pie charts in **Figures 6**, **7**.

Socioeconomic and Demographic Distribution Among Countries With Top Case Fatality Rates

Socioeconomic and demographic distribution among the top 30 countries whose case fatality rates are more than 5% were illustrated in **Figure 6**. According to data, more than half of the

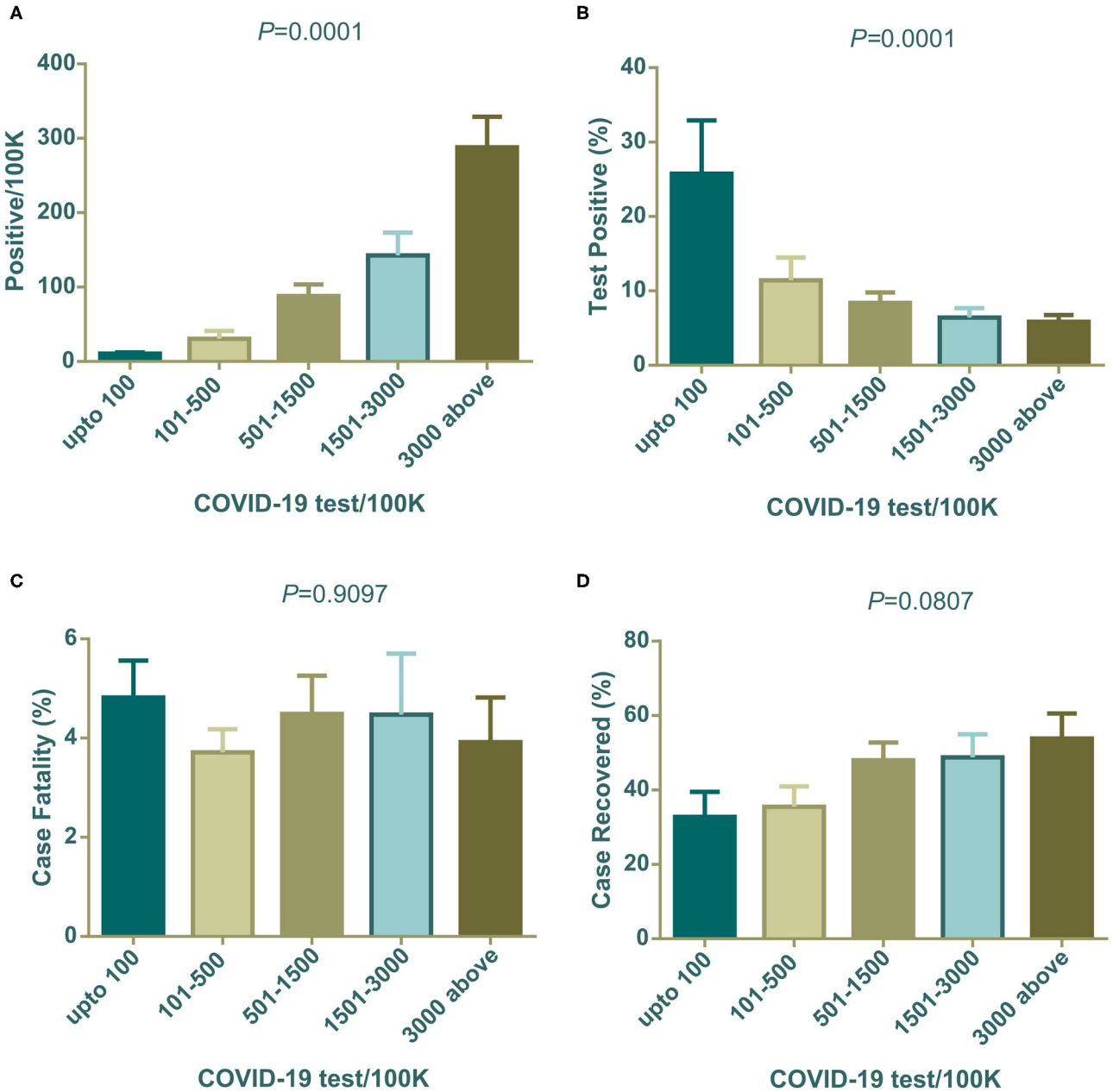

FIGURE 3 | Positives/100 K, positive test (%), case fatality (%), and case recovery (%) data distribution among countries grouped with COVID-19 test/100 K population. All are mean ± SEM. In one-way ANOVA variance among column means are statistically significant for **(A,B)**. Case fatality (%) and case recovered (%) were not statistically significant **(C,D)**. One way ANOVA differences among column means are shown as P-values on top of each graph.

top 30 countries (n = 17, 56.67%) had the lowest population density per km^2. The percentage of the urban population was high (above 50%) in 90% of countries (n = 27). Half of these countries had an older population (median age over 40 years). About 40% of countries (n = 12) spent more money in health sectors per capita and 93.33% (n = 28) of countries belonged to the high income group.

Lifestyle and Health Parameter Distribution Among Countries With Top Case Fatality Rates

The prevalence of obesity was observed in 28 countries. Among them, 50% of countries belong to a moderate percentage of the obese population (**Figure 7**). Physical activity data were available for 27 countries and among them, 44.44% (n = 12) of countries indicated that 30–40% of people were

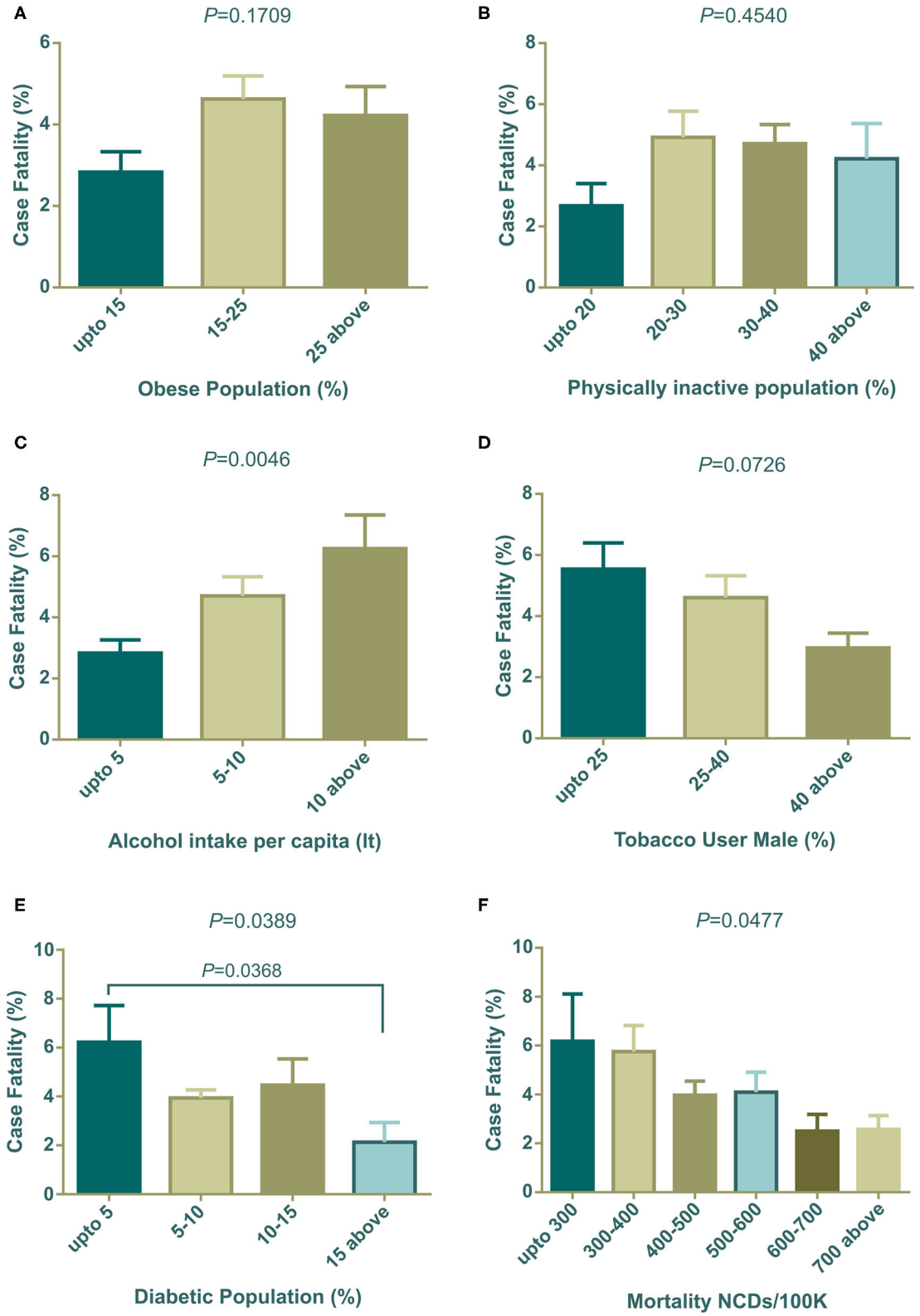

FIGURE 4 | COVID-19 case fatality (%) distribution based on countries grouped according to health indicators **(A–F)**. Alcohol intake, diabetic population, and NCDs mortality/100 K were statistically significant by one way ANOVA. Data presented are mean ± SEM.

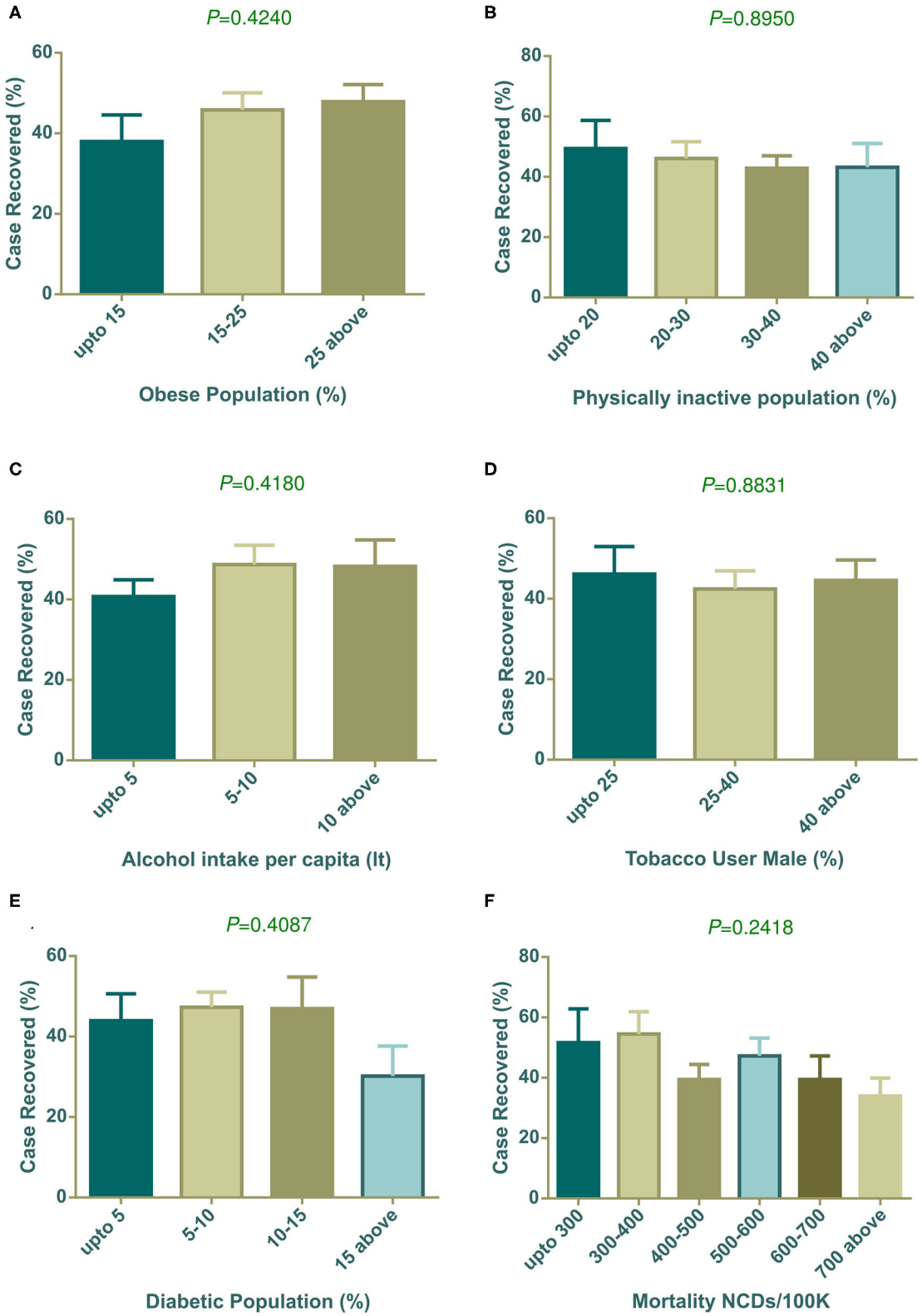

FIGURE 5 | COVID-19 case recovery (%) distribution based on countries grouped according to health indicators **(A–F)**. None of the parameters showed statistically significant variation among column means by one way ANOVA. Data presented are mean ± SEM.

TABLE 3 | Top 30 countries according to case fatality rate with their parametric distributions.

Country	Per capita GNI (PPP), USD	Population density, KM^2	Urban population Percentages	Median age, years	Per capita health expenditure, USD	Prevalence of obesity, %	Prevalence of insufficient physical activity, %	NCD mortality per 100 K people	Prevalence of diabetes, %	Alcohol consumption, per capita in litter per year	Prevalence of any kind of tobacco use by male, %
Sweden	54,030	25.00	87.43	41.10	5904.58	8.6	NA	745	22.1	0	0
Slovenia	37,450	102.64	54.54	44.90	1920.28	27.4	33.6	446.6	6.7	0.6	0
Mexico	19,340	64.91	80.16	29.30	494.68	22.1	47	452	14.2	6.5	0
Greece	29,670	83.22	79.06	45.30	1516.59	18.9	11.5	768	5.7	9.3	0
Denmark	56,410	138.07	87.87	42.00	5800.15	19.9	27.2	405.1	5.5	3.3	12.3
USA	63,690	35.77	82.26	38.50	10246.14	19.7	28.5	356.6	8.3	9.5	13.4
Brazil	15,850	25.06	86.57	33.20	928.80	29.4	28.6	291.6	7.6	8.1	14.8
Algeria	14,970	17.73	72.63	28.90	258.49	28.9	28.9	457.8	13.5	5.5	17.3
Philippines	10,740	357.69	46.91	24.10	132.90	27.8	35.9	342.4	3.9	9.8	17.3
Canada	47,590	4.08	81.41	41.80	4754.95	36.2	40	417.9	10.8	8.8	17.4
North Macedonia	15,670	82.59	57.96	39.00	328.42	NA	23.1	318.3	4.8	7.2	17.5
Hungary	29,860	107.91	71.35	43.60	981.42	25.8	33.2	560.8	9.6	0	19.4
France	46,360	122.34	80.44	41.70	4379.73	25.3	32.7	348.2	3.2	11.3	20
Ecuador	11,420	68.79	63.82	28.80	518.03	20.2	32.2	352.4	5.9	10.8	20.4
Indonesia	12,670	147.75	55.33	31.10	114.97	22.1	35.7	333.5	4.6	10.4	24.2
Ireland	67,050	70.45	63.17	37.80	4976.86	19.5	23.7	285.4	5.7	9.5	24.7
Belgium	51,740	377.21	98.00	41.60	4507.36	28.3	41.6	424.8	5.9	8.4	26
Sudan	4,430	21.30	34.64	18.30	193.79	19.9	41.4	306.4	5	7.1	26.9
Honduras	4,790	85.69	57.10	24.40	195.94	21.6	29.3	290	4.8	11.8	27.7
United Kingdom	45,350	274.83	83.40	40.60	3858.67	21.4	NA	442.4	7.3	2.9	27.8
China	18,170	148.35	59.15	38.40	440.83	23.8	26.8	297.4	6.9	8.5	28
Argentina	19,870	16.26	91.87	32.40	1324.60	26.4	38.5	580.2	6.9	10.9	28.3
Switzerland	68,820	215.52	73.80	42.70	9956.26	23.1	32.5	467.8	6.1	10.5	28.9
Netherlands	56,890	511.46	91.49	42.80	4911.44	22.5	35.4	577.2	6.9	10.4	33.1
Poland	30,010	124.04	60.06	41.90	906.82	6.4	39.7	678.3	7.1	4.5	39
Spain	39,800	93.53	80.32	43.90	2506.46	6.2	14.1	542.4	9.2	5.7	45.7
Italy	42,290	205.45	70.44	46.50	2840.13	24.9	37.7	340	4.7	6.4	49.7
Iran	21,050	50.22	74.90	31.70	475.48	32	31	826.7	17.2	0.2	56.3
Egypt	12,100	98.87	42.70	24.10	105.77	6.9	22.6	NA	6.3	0.3	82.7
Romania	27,520	84.64	54.00	42.50	555.10	NA	NA	NA	NA	NA	NA
Mean ± SEM	32520.00 ± 3573.60	125.38 ± 21.62	70.76 ± 2.91	36.76 ± 1.40	2534.52 ± 516.47	21.97 ± 1.41	31.57 ± 1.56	451.97 ± 29.21	7.81 ± 0.78	6.83 ± 0.71	24.79 ± 3.31

Data sources are mentioned in methodology and in supplements. Countries were selected decisively whose case fatality rates were above 5%. NA = Data not available.

physically inactive (**Figure 7**). About 51.85% (n = 14) of countries among the 27 consumed 5–10 liters of alcohol per capita per year and nearly half of these countries (n = 12 of 25, 48%) used fewer tobacco products (**Figure 7**). About 58.62% of countries (n = 27 of 29) were moderately diabetic (5–10% diabetic prevalence) and more than half (n = 16 of 28, 57.14%) of the countries were at risk of death, whose NCDs mortality rate per 100 K population was low to moderate (**Figure 7**).

DISCUSSION

Socioeconomic and Demographic Determinants of COVID-19 Positive Tests, Case Fatality, and Case Recovery Rate

Population density both in a country and in urban cities can have an impact on contagious disease transmission. Both contact rates and patterns in a defined geographical spatial distribution determine the mode of infectious disease transmission (47, 48).

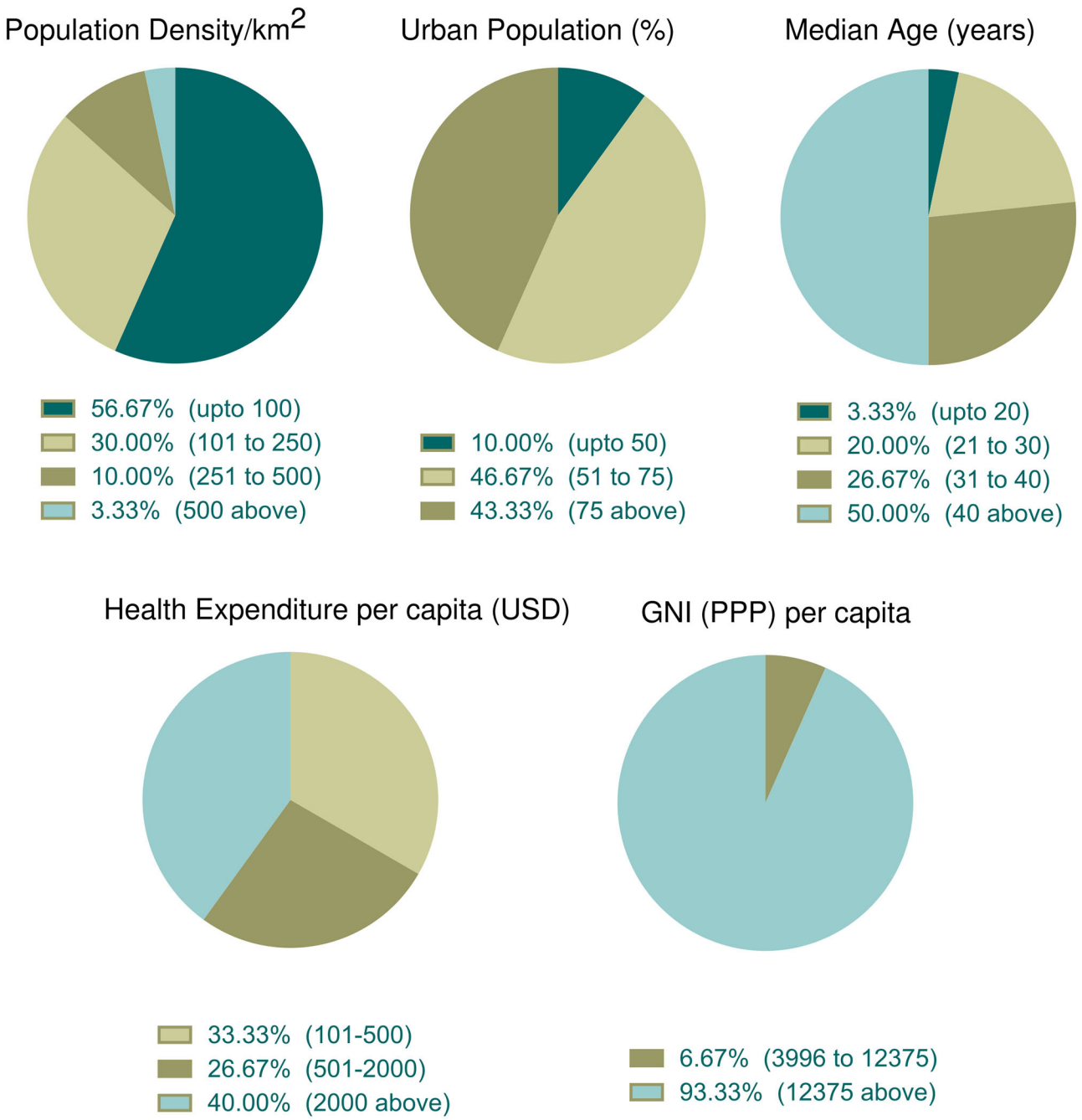

FIGURE 6 | Incidence of socioeconomic and demographic parameter distribution among the top thirty countries with high case fatality (%). Data categories with zero data are not shown in the pie chart.

The contact hours and contact number per person varies with age and number of household members. In a study in Hong Kong, the highest contact pattern was observed among school-going children, which decreased with age (48). However, the older age group with economic strength also showed elevated contact rates. We found a weak relation of population density per km^2 with the percentage of positive tests (**Figure 1A**). Generally, population density poses an increased chance of disease transmission, but the contact rate is not always determined by population density. At a low density, contact rate can increase rapidly which gets saturated in a very dense area where lack of organized social contact is evident (47). At this point, the contact rate becomes independent of population density. Although in our analysis low density countries show a slightly higher percentage of positive

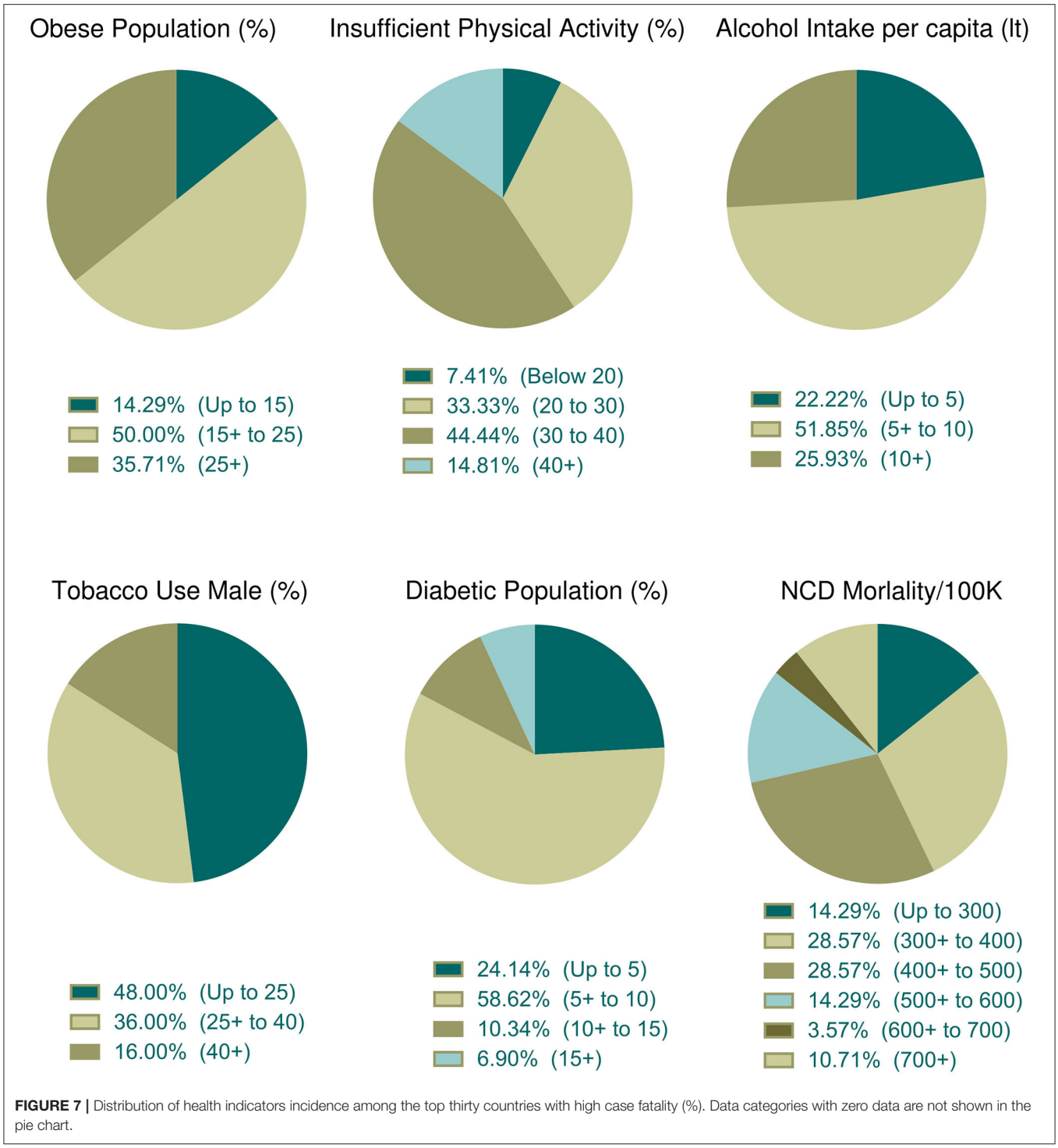

FIGURE 7 | Distribution of health indicators incidence among the top thirty countries with high case fatality (%). Data categories with zero data are not shown in the pie chart.

tests, further data analysis showed that few countries in this group e.g., Algeria, Brazil, and Afghanistan had very high positive test value as outliers as they did a very low number of tests. This inadequate testing increased their positive test rate, which agrees with our analysis in this study (**Figure 3B**).

The number of tests performed to confirm COVID-19 is a challenging issue for many countries and can affect decision making drastically. We found a statistically linear positive relation in the number of positive patients, with increasing test numbers (**Figure 3A**). The following graph depicted the number of tests per 100 K population, showing that as they increased the percentage of positive tests dropped gradually (**Figure 3B**). In a recent press conference, the WHO discussed that a high percentage of positive tests in a country indicates that

an inadequate number of tests were being performed, and they set a rough standard of 10% positive tests as an indicator of normal outcome (49). This strongly supports our data in **Figure 3B**. Countries that performed above 500 tests per 100 K population, showed around 10% positive tests. Countries with lower middle and upper middle economies performed symptom-based highly selective diagnoses due to socioeconomic, demographic, and political reasons which increased the percentage of positive tests.

Positive test rates also have close links with the urban population, median age, and income level. Among 91 countries in our study, around 74.73% ($n = 68$) of countries belonged to the high economy class, 20.88% ($n = 19$) and were from the upper middle economy and the rest were from the lower middle economy group (**Table 1**). Thus, most of the independent socioeconomic and demographic variables comply with the characteristics of developed countries. These countries possess modern health facilities, medical professionals, and cutting-edge research facilities. Despite having these benefits in health sectors they could not successfully restrict infections and deaths toll. However, these amenities certainly were advantageous in some form and contributed to patient recovery (**Figure 2D**).

Developed countries have larger city populations with a greater proportion of older inhabitants who prefer to stay at home (50, 51). The positive test rates thus were lower in groups of countries with dense urban populations (**Figure 1B**). Median age data indicated that developing countries have younger populations compared to developed countries. The young population has tendencies to go outside, making them a vulnerable age group (32). Therefore, positive test rates were high in developing countries where the median age is below 30 years (**Figure 1C**). Health expenditure per capita correlates with a higher number of tests being performed, thus countries with over 501 USD investments showed a decreased positive test rate (**Figure 1D**). As high median age belongs to countries with a strong healthcare system, case recovery was also found to be high where the median age is over 30 years (**Figure 2C**).

Case fatality and recovery from epidemics largely depend on internal and external factors along with age, presence of co-morbidities, health facilities, and the pattern of adherence at healthcare centers (25, 33, 50, 52). The effect of contagions on certain populations is also influenced by the interplay between the incubation period and the age dependent case fatality rate of the disease (53). In our study, the case fatality rate was high where the median age is over 40 years (**Figure 1C**) and in countries with more than 50% urban population (**Figure 1B**). Although increasing population density showed a gradual rise in case fatality, countries with over 500 people per km^2 density ($n = 6$), surprisingly, showed a reduced case fatality (**Figure 1A**). Most of the countries in this group have a high urban population and high median age. However, they managed to control case-fatality with remarkable success, except in the Netherlands, where they had a 12.79% case fatality. With rapid urbanization, the risks of pandemics and zoonotic diseases are increasing (54). Because it is an infectious and highly contagious disease, urban population percentages are one of the most important predictors in COVID-19 outcome. Countries with more people living in rural areas have lower case fatalities as air velocity potentially reduces disease transmission (55). In contrast, in urban medical facilities, the availability of medicines/drugs, and treatment tools accelerate disease recovery, despite a large number of patients.

Countries with high health expenditure per capita and GNI (PPP) could not restrict the pace of death as expected due to inadequate intensive care equipment and management personnel, and an overwhelming number of critical patients. Although better urban intelligence and management are partially linked with better recovery (56), as the proportion of the urban population around the globe is gradually increasing, a policy level rethink of the healthcare system is vital. We need to redesign urban intelligence, resource management, and coordination to fight the COVID-19 pandemic, in circumstances where isolation, social distancing, and quarantine are challenging (57).

Investment in health, especially toward NDCs, have shown reduced mortality/incidence ratio in cancer (58), stroke (59), and child mortality (60). Expenditure to contain the infectious disease is still a necessary component; as the controlled use of available resources can reduce the disease spreading (61). Moreover, sudden pandemic induced resource constraints can critically affect treatment and patient recovery (62). Contrary to misconceptions that higher expenditure relates to better healthcare, developed countries showed no strong negative correlation between health expenditure and case fatality (63). This could be due to the inappropriate ways in which they spend money, poorly designed policy, and political intrusions that inhibit policy and treaty implementation (64). During the COVID-19 pandemic, some countries have offered strict preventive measures and information on technology-based contact tracing to successfully contain the disease, although they fall into high risk group countries. In our study, COVID-19 related case fatality rate was comparatively higher in countries with high health expenditure per capita, which explains that the majority of health investment is insufficient or inadequate in required segments to tackle such a pandemic. In a recent study, COVID-19 incidence and case fatality was found not to be associated with health expenditure and services (34). The situation gets worse as the incidence number rises rapidly, putting pressure on healthcare capacity, as observed in some European countries (25).

Lifestyle and Health Indicators on the Case Fatality Rate

The duration of infectious disease is very important and has a direct impact on mortality (53). Clinical recovery from COVID-19 cases requires ∼14–42 days depending upon the patient's physical and clinical conditions (65). Thus, unhealthy lifestyles are important predictors of delayed recovery or death. Lifestyle includes obesity, physical activities, the pattern of drinking alcohol, tobacco usages, etc. and the prevalence of NCDs and their mortality is also linked to the raised number of case fatality rates.

Several factors including obesity, diabetes, cardiovascular disease and hypertension, cancer, and chronic respiratory diseases, have been identified as collective underlying conditions

of critical illness that can lead to poor outcomes in COVID-19 (66, 67). Recent studies have indicated that obesity and insufficient physical activity might accelerate the mortality of COVID-19 (66, 67). Our study reflects these results, as it indicated that countries with a large number of obese and insufficiently active populations had an increased number of case fatality rates. However, the Center for Disease Prevention and Control (CDC, USA), listed severe obesity (BMI over 40) as a risk factor for critical COVID-19 cases (68). Furthermore, clinical reports of critical COVID-19 patients have shown that significant numbers of patients are associated with obesity in different countries (66). Obesity and insufficient physical activity can lead to metabolic, cardiovascular disorders, and other NCDs (69). Though the relationships between obesity and NCDs are well-studied, little is known about the effect of obesity on immunity and contagious disease. Recent clinical studies on survivors and non-survivors of COVID-19 have shown that inappropriate and abnormal immunity were significantly associated with death (70). Several animal model studies showed that obesity leads to impairment of natural killer cells, reduction of macrophages, and dendritic cell activities (69), with reduced cytokine productions and weakened responses to antigen stimulations. Thus, impaired immune systems cannot fight the pathogen resulting in delayed recovery or death.

Other studies have showed that diabetes is the second top co-morbidity factor in COVID-19 case fatality after hypertension (66). Surprisingly, our analysis demonstrated that the group of countries with the lowest prevalence of diabetes observed more case fatality (**Figure 4E**). To explain this outcome, we further analyzed the group prevalence of diabetes with group median age, as diabetes is more prone to elderly people (**Supplementary 1, Table 1**). The analysis demonstrated that 55.56% ($n = 10$ of 18) countries holding up to 5% diabetes prevalence comprises the median age over 40 years, and none of the countries with 15% diabetes prevalence were from the same median age group of countries. Furthermore, cross analysis with GNI (PPP) per capita (**Supplementary 1, Table 2**) confirmed that 72.22% of developed countries were from the group with less prevalence of diabetes. This result established that diabetes is one of the key factors in case fatalities, which reflects this the findings of this study, demonstrating that case fatalities were more evident in elderly people and developed countries.

Diabetes is also characterized by chronically elevated levels of blood glucose. These raised blood sugar concentrations also increase glucose concentration in airway secretions (71). Another study also demonstrated that *in vivo* influenza virus infection and replications can be significantly increased due to the contact of pulmonary epithelial cells with raised glucose concentrations *in vitro* (72). Raised sugar levels in the blood could also weaken the anti-viral immune response and can be reverted with insulin treatment (73). Another study showed that high glucose concentration or diabetic conditions were associated with fatal outcomes in avian influenza (74). Therefore, it could be concluded that chronic diabetic conditions can elevate the case fatality rates of COVID-19.

Countries with less NCDs mortality per 100 K population faced more COVID-19 death rates (**Figure 4F**). To explain this, we again did a cross analysis of group NCDs mortality per 100 K with group median age and group GNI (PPP) per capita. Around 71.43% of the countries of the lowest NCD mortality per 100 K belong to the highest group of median age and all of them were high income group countries (see **Supplementary Table 2**). As discussed above, case fatalities were high in developed countries and the cross analysis of NCDs mortality with median age and per capita income also suggested that elderly people with NCDs were more at risk of fatal outcomes. Most incidences of mortality (60–90%) were related to preexisting one or more NCDs (75–78).

Heavy alcohol intake has been casually associated with several diseases including infectious disease (79). This could be explained by the fact that heavy intake of alcohol weakens the immune function as well as several organs including the liver and lungs, making them susceptible to microorganisms (79), and substantially lowers the adherence of antiretroviral therapy, accelerating mortality (80). Our investigations showed that case fatality was increased with per capita alcohol intake in liters (**Figure 4C**).

Tobacco smoking is generally linked with lung diseases (81) and can facilitate microbial infection (82). There is currently an ongoing scientific debate about the relationship between tobacco smoking and severity of COVID-19 (83). A recent systematic review concluded that smoking might be negatively linked to COVID-19 case fatality (84). However, another short meta-analysis stated no association between them (85). In addition, a group of French scientists have shown that tobacco smoking has negative effects of COVID-19 mortality (86) which was also reflected in our results (**Figure 4D**). Recent news (87, 88) reported that French scientists are planning a human trial to test whether tobacco can fight COVID-19. Due to the fact that this debate is ongoing, a more detailed clinical and molecular investigation is required to establish a more conclusive answer.

Limitations and Future Directions

The COVID-19 pandemic is ongoing and not yet closed, and the data used in our study reflects a snapshot of a point in time. This limits our study, which cannot capture the full view of the dynamic nature of this disease. Due to limited number of tests, many asymptomatic carriers can be left outside the diagnosis process and positive tests, case fatality and case recovery cannot be estimated accurately. However, we had to exclusively depend on the number of confirmed cases for any calculation. In addition, there are potential reliability issues in terms of the accuracy of this self-reported government data. As COVID-19 is multifactor mediated, not all factors could be included in this particular study, and integrating the molecular mechanism of the disease was beyond its scope. In the future, the nature of this molecular mechanism and its pathogenesis will gradually unfold, and more clinical data will be available, and the factors discussed in this study will be easier to interpret. Finally, the interpretation of this study could be useful in designing future studies and attempts to effectively contain such a contagious pandemic outbreak within a very short time.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AA and MMR conceived and designed the study, conducted the analysis with input from TH, equally contributed to the first draft. MMR and TH collected and sorted the data from different sources. TH added additional points and contributed to its development. After necessary corrections and suggestions from all authors, AA finalized and submitted 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/fpubh.2020.566114/full#supplementary-material

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REVIEW
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Recommendations for the Diagnosis, Prevention, and Control of Coronavirus Disease-19 in Children—The Chinese Perspectives

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Ever since SARS-CoV-2 began infecting people by the end of 2019, of whom some developed severe pneumonia (about 5%), which could be fatal (case fatality ~3.5%), the extent and speed of the COVID-19 outbreak has been phenomenal. Within 2.5 months (by March 18, 2020) over 191,127 COVID-19 patients have been identified in 161 countries. By then, over 700 pediatric patients were confirmed to have COVID-19 in China, with only about 58 diagnosed elsewhere. By now, there are thousands of children and adolescents infected. Chinese pediatricians would like to share their experience on how these patients were managed in China and the key recommendations that had guided them in meeting the evolving challenges. A group of experts were summoned by the Chinese Pediatric Society and Editorial Board of Chinese Journal of Pediatrics to extract informative data from a survey on confirmed COVID-19 pediatric patients in China. Consensus on diagnosis, management, and prevention of pediatric COVID-19 were drawn up based on the analysis of such data plus insights gained from the past SARS and MERS coronavirus outbreaks. Relevant cumulating experiences from physicians managing adult patients, expedited reports on clinical and scientific COVID-19 and SARS-CoV-2 data, and the National Health Committee guidelines on COVID-19 management were integrated into this proposal.

Keywords: COVID-19, SARS-CoV-2, pandemic (COVID-19), children, consensus recommendations, guidelines

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INTRODUCTION

In mid-December 2019, a novel and the seventh human coronavirus strain akin to SARS-CoV was identified (1–3). On January 12, 2019, the World Health Organization (WHO) temporarily named it 2019 novel coronavirus (2019-nCoV). Its high infectivity has contributed to an epidemic spreading to all parts of China and over 100 countries outside China (4). On January 21, 2019, the National Health Commission (NHC) of China defined 2019-nCoV pneumonia as a Class B Notifiable Infectious Diseases to be prevented and controlled by first-level response to major public health emergency. On January 30, 2020, the WHO declared 2019-nCoV epidemic as a Public Health Emergency of International Concern (PHEIC). On February 11, 2020, the International Committee for Taxonomy of Virus (ICTV) officially named the virus as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), while WHO named the disease SARS-CoV-2 causes coronavirus disease 2019 (COVID-19). On March 11, 2020, the WHO announced that COVID-19 outbreak has escalated to a pandemic scale.

Riding on the rising peak of COVID-19 pandemic, definitive diagnostic tests were rapidly developed and rendered available to key centers, enabling pediatricians to diagnose and manage the increasing number of COVID-19 cases (5, 6), including severe and neonatal ones (6). Meanwhile, the NHC issued "The Guideline for the Diagnosis and Management Plan for Pneumonia with New Coronavirus Infection (trial 7)," and the WHO released their recommendations via "Global Surveillance for Human Infection With Novel Coronavirus (2019-nCoV)," without much elaborations on the pediatric elements. To address the pediatric specific concerns, the Chinese Pediatric Society (CPS) has initiated a nationwide survey to systematically collect epidemiological and clinical data of pediatric COVID-19. Together with the Editorial Board of the Chinese Journal of Pediatrics, CPS also summoned a multidisciplinary group of experts to put forward recommendations for COVID-19 in pediatrics (7, 8), based on analyzing these data plus experience gained from management of both pediatric and adult cases. The current document does not attempt to assign grading of evidence to the recommendations.

CLINICAL VIROLOGY OF SARS-COV-2

Four endemic HCoV-229E, -NL63, -OC43, and -HKU1 are prevalent in the general population (9), causing usually mild clinical symptoms, but could be more serious in infants, the elderly, and the immunocompromised (10). In contrast, zoonotic MERS-CoV, SARS-CoV, and the current novel SARS-CoV-2 are opportunistic leading to unexpected outbreaks, with severe pneumonia and even fatality (11, 12). Autopsy and biopsies from adult COVID-19 patients (13) demonstrated SARS-COV-2 RNA and antigen in type II pneumocytes as compatible with *in vitro* findings demonstrating its binding to angiotensin-converting enzyme 2 (ACE2) to enter relevant cell lines. Whether and how opportunistic CoV become endemic is not yet understood, but mutations allowing selection of more adaptive variants capable of surviving better with a human host is one key conceptual mechanism. Ongoing molecular virological studies have already documented SARS-CoV-2 genetic mutants/variants in patients from China (14).

1. Infectious source and prolonged virus shedders:

   Asymptomatic (silent), presymptomatic, and convalescent patients shedding the virus are potential sources of transmission (15–18), especially for adults. Prolonged SARS-CoV-2 positivity was documented in children for at least 4 weeks after onset of symptoms and 2 weeks after being discharged home based on standard guidelines (19). This may carry clinical management implication, as prolonged virus shedding of CoV viruses were found in SARS and MERS convalescent subjects (up to weeks) as well (12), though proof of transmission by convalescent subjects has not been demonstrated.

   With respect to how children participate in the transmission chain of COVID-19, studies from Germany, the United Kingdom, and the United States had provided additional data [on top of those from China (19–23)] defining viral loads across all ages [mainly semiquantitated by the threshold cycles (Ct) data of real-time (RT)-PCR amplification of SARS-CoV-2 genes and less commonly by direct viral cultures] and linking them to rate of transmissibility (17, 24, 25). The suggested correlation of viral semiquantitation (Ct value) to viable virus isolation (26) is particularly important if proven dependable, as it offers non-culture option of determining which SARS-CoV-2-positive individual is a potential spreader of COVID-19 for more evidence-based infection prevention and control (27, 28). Analysis of epidemiological data have all pointed that children are not key primary spreaders of COVID-19 even within family clusters (29–31), which has fueled active debates and also suggestions on how school and daycare centers for children and adolescents should be reopened (32, 33) following the closures during the epidemic.
2. Infectious route

   Direct contact of the mucous membrane of the eyes, nose, and mouth with contaminated hands or infectious droplets (respiratory but also fecal or urinary) is the main mode of transmission. Aerosolization could generate microdroplets to be inhaled (3-μm droplets could remain suspended in air for up to 3 h, while submicron 0.3- to 0.5-μm aerosols could be airborne). Opportunistic transmissions are especially important in enclosed/defined space, known to be enhanced by nebulization therapy or positive pressure mechanical ventilation (5–8, 34). The SARS-CoV-2 virus has been isolated from stool and urine, in addition to respiratory secretions, of COVID-19 patients (35, 36), though fecal–oral transmission has not been definitely proven yet.
3. Epidemiological features

   No specific population is completely spared by COVID-19. As of March 18, 2020, 24:00, there is a cumulative of 81,116 confirmed cases in China, with 70,420 recovered and 3,231 deaths (37). Hubei province, whose capital city Wuhan was the epicenter of this current outbreak, was hard-hit with 67,800 confirmed cases, including 57,678 recovered

patients and 3,130 deaths. There were 110,011 confirmed COVID-19 cases reported outside China, involving 161 countries worldwide with 4,576 deaths. Patient age ranged from 36 h to 103 years old, with no gender preference, while those above 60 years or with underlying illnesses exhibited increased disease severity and mortality. Human-to-human transmission was well-illustrated by family and case clusters, together with subsequent exponential growth in cases confirmed highly infectivity of SARS-CoV-2 (5, 6, 37–39). By mid-February mass gathering clusters, including cruise and religious activities, have contributed to outbreaks beyond China.

The first 10-year-old boy confirmed with COVID-19 was driven from Wuhan to Shenzhen, Guangdong Province, from December 29, 2019 to January 4, 2020, together with five other family members. He was diagnosed and managed there since January 10, 2020 (38). More children were subsequently reported to have COVID-19 (19, 36, 40–44). By March 18, 2020, 720 pediatric confirmed COVID-19 cases have been reported in China, with 27 (all mild) outside of China, by the ongoing CPS survey.

A total of 714/720 had age data, while 682/720 had gender data. A total of 56.9% (388/682) were boys, and 43.1% were (294/682) girls. Mean age was 7.93 ± 4.26 years with neonates 1.0% (7/714), 29 days−1 year 13.3% (95/714), 1–3 years 14.1% (101/714), 4–6 years 13.2% (94/714), 7–12 years 31.9% (228/714), and 13–17years 26.5% (189/714). They were from 31 provinces, autonomic regions or municipalities in China, the top 6 provinces being Hubei (314 cases), Guangdong (58 cases), Henan (44 cases), Anhui (38 cases), Zhejiang (34 cases), and Shandong (30 cases). For 678 patients with epidemiological exposure information, 23.5% (159/678) had Wuhan-related exposure where SARS-CoV-2 had started spreading, 75.1% (509/678) had definite links to family clusters (38), and 1.5% (10/678) did not have epidemiological exposure history.

Possible occurrence of vertical maternal–fetal transmission has been supported by two recent reports (45, 46) demonstrating raised anti-SARS-CoV-2 IgM antibodies (in addition to raised IgG antibodies potentially derived from the maternal circulation) in three newborn babies out of seven deliveries from mothers with confirmed COVID-19. These newborns were all asymptomatic. However, reservation about such interpretation of these clinical scenarios has been raised, mainly based on the technical validity of antibodies employed to detect SARS-CoV-2 IgM (47). A retrospective review of nine pregnant women diagnosed in their third trimesters did not have any baby found infected after delivery (48).

On the other hand, of seven nucleic acid amplification test (NAAT)-confirmed neonatal COVID-19, five were diagnosed during the medical observations postdelivery (with throat/nasopharyngeal swab positive from 36 h to 3 days postdelivery), while the remaining two were diagnosed on postnatal day 17 (PND17) and PND19 after turning symptomatic at home.

Four early studies had been reported on COVID-19 in children (19, 49–51). One study was conducted in Guangzhou on close contacts of confirmed COVID-19 patients or members from COVID-19 family clusters. It reported a diagnostic rate of 1.3% (10 out of 745) in children based on SARS-CoV-2-specific NAAT (19) using nasopharyngeal swab, with 3.4% (111 of 3,174) in adults. Another study, carried out on all the pediatric admissions in three branches of Tongji Hospital in Wuhan between January 7 and 15, 2020, identified 1.6% (6/366) who suffered from COVID-19 based on nasopharyngeal or throat swab positivity for NAAT (49). The third was on 1,391 hospitalizations in Wuhan Children's Hospital between January 28 and February 26, 2020, which documented 12.3% ($n = 171$) COVID-19 NAAT using throat swabs (50). The fourth analyzed COVID-19 data reported to the Chinese Center for Disease Control between January 16 and February 8, 2020 identified 728 confirmed and 1,407 suspected pediatric COVID-19 cases (51).

CLINICAL FEATURES OF COVID-19 IN PEDIATRICS

The incubation period for SARS-CoV-2 causing COVID-19 is 3–7 days (medium; range 1–14). Infectious sources are predominantly respiratory secretions (lower > upper in viral load), with saliva, blood, stool, and urine from which SARS-CoV-2 RNA could be isolated. While current clinical guidelines mostly state that COVID-19 patients in clinical remission could be discharged from hospital care when two NAAT performed on relevant specimens at least 24 h apart are negative, this would be revised with more data on virus shedding status coupled with a practical way to determine the viable infectious SARS-CoV-2 biological forms become available.

1. Clinical manifestations
   From the ongoing National Survey of COVID-19 in pediatric population, clinical features were analyzed in 501. Among the

TABLE 1 | Rate (in percentage) of clinical symptoms in pediatric COVID-19 in China, Italy and USA.

	Current Chinese Ped Society	CDC MMWR*	Italy CONFIDENCE**
Number	501	2,572	100
Fever (>38°C)	57.9	44.0	54
Cough	39.9	54.3	44
Rhinorrhoea/nasal obstruction	9.8	7.2	22
Sore throat	2.6	24.4	4
Shortness of breath	1	13.4	11
Diarrhea ± vomiting	10.5	12.7	9
Headache	2.8	27.8	4
Malaise	4	NA	9
Myalgia	0.8	NA	NA
Chills or rigors	NA	NA	NA

**CDC COVID-19 response team. Coronavirus disease 2019 in children—United States, February 12–April 2, 2020. MMWR 2020, 69: 422426.*
***Parri et al. (69).*

470 patients with body temperature records, 214 cases had detailed data on dynamic changes in temperature, 324 patients of 501 had complete blood count (CBC) tested, while 371 of 501 had chest radiological imaging done.

Fever (57.9%, 272/470) and cough (39.9%, 200/501) were most frequent, whereas less frequent symptoms included stuffy nose, coryza, sneezing, headache, dizziness, malaise, myalgias, and sore throat. Notably, 53 (10.6%) presented with diarrhea and/or vomiting, a few with abdominal pain, while a 1.5-month-old infant had repeated vomiting only. These clinical symptoms mostly resolved within 1 week. Analysis of 244 consecutive COVID-19 patients admitted into Wuhan Children's Hospital, specifically comparing those with GI symptoms (13.4%) vs. those without (86.6%), showed that the former group was younger (median age 14 vs. 86 months) than the latter (52). See **Table 1** for symptom rate.

Among those with fever, most were short lasting (1–2 days), while 25.2% (54/214) lasted for 3 days or longer, 8.4% (18/214) for 3 days, 7.9% (17/214) for 4 days, 3.5% (9/214) for 5 days, 2.3% (5/214) for 6 days, and 2.3% (5/214) had fever over 7 days with the longest being 14 days in a critically sick child.

Most CBCs were normal: 5.5% (18/324) children had mild leukopenia ($2.96–3.95 \times 10^9$/L; one 2.32, another 1.8) and 6.8% (22/324) accompanied by lymphopenia ($0.36–1.48 \times 10^9$/L; five were <1.0). C-reactive protein (CRP) was normal (<20 mg/L) except transient elevation in 19/142 (9.1%, 20.9–64.6 mg/L) with 47.2 mg/L in one with mycoplasma pneumoniae coinfection and two with pyogenic tonsillitis (51.6 and 64.6 mg/L). A few had mildly elevated liver enzymes and creatine kinase (CK).

In 371 patients with chest radiological examinations, 235 (63.3%) had ground-glass or interstitial infiltration appearance (including 27 clinically asymptomatic; 4 severe/critical COVID-19, and 3 neonates), 7 (1.9%) only had increased lung markings, while 129 (34.8%) had no radiological abnormality.

Fourteen patients had evidence of coexposure/coinfection. Eleven were seropositive for mycoplasma pneumoniae IgM among which one had additional Epstein–Barr virus IgM positivity. Three were seropositive for influenza A IgM, while five had respiratory specimens positive for influenza A (2), influenza B (2), and RSV (1) antigens. One of them had prolonged fever (8 days, highest 40.2°C) whose chest CT scan revealed multiple lesions, which resolved completely 4 days later.

Seven neonates had COVID-19 confirmed. One, diagnosed postnatal day 17 (PND17), presented with sneezing, vomiting, transient fever, and diarrhea; his chest CT revealed bilateral lower lobe reticular changes (44). Another, diagnosed on PND19, was admitted for vomiting and reluctant to feed, then developed fever, cough, and diarrhea with CXR showing bilateral patchy shadows. One only had mild tachypnea and increased lung marking on CXR while another with fever only on PND5. From three reported series of infected pregnant mothers (44, 49), 3 out of the 52 newborn babies prospectively monitored right after deliveries were confirmed to have COVID-19. One was admitted into the NICU immediately (premature 31 weeks 2 days), while the other two were transferred to the NICU on PND1 and 2, respectively (53). The 31 weeker had respiratory distress syndrome, received non-invasive positive pressure ventilation after delivery, and developed disseminated intravascular coagulopathy on PND5, while all three had pneumonia documented by chest CT.

There were only four pediatric cases requiring mechanical ventilation for respiratory failure. A 13-month-old male who had known bilateral hydronephrosis started suffering from diarrhea, vomiting, and low fever. His fever remitted but resurged by day 7 together with shortness of breath requiring transfer to the ICU. He further deteriorated and evolved to acute respiratory distress syndrome (ARDS), circulatory shock, and acute renal failure (ARF). Chest radiology revealed massive consolidation with ground-glass changes predominately over the right lung. After support by mechanical ventilation and continuous renal replacement therapy (CRRT), he was extubated after 9 days and discharged 17 days after admission (41). The second one was an 8-month-old infant previously operated for congenital heart disease at 1+ months old (ventilated postoperatively for 1.5 months) and suffered from moderate malnutrition. He presented with cough for 6 days then dyspnea for 1 day. He was mechanically ventilated upon admission and successfully extubated 13 days later and eventually discharged 45 days after admission (54). The third case had acute leukemia on maintenance chemotherapy and recovered after supported by mechanical ventilation. The fourth child had background intussusception and developed multiorgan failure, complicating the course, and was the only pediatric fatality associated with COVID-19 (50).

Based on the clinical classification outlined in the Clinical classification of pediatric infection section below, 230 out of 501 confirmed cases were moderate COVID-19 (45.9%, including 27 subclinical COVID-19 with radiological changes but asymptomatic). Of these confirmed COVID-19, one was severe (0.2%), and four were critical (0.8%). A total of 210 (41.9%) suffered from mild COVID-19, while 56 (11.2%) children were totally asymptomatic with normal radiological findings.

Out of 219 children with data about their achieving NAAT negativity, 176 cases (80.4%) did so in 1–15 days postdisease onset (DPO1–15), while 29 (13.2%) turned negative by DPO16–20, and 13 (5.9%) did so by DPO21–26. One 3-month-old infant with mild COVID-19 had NAAT negativity by DPO26. The critical COVID-19 8-month-old infant had NAAT negativity of rectal swab on DPO31 and of nasopharyngeal swab on DPO-37, which became positive on DPO38 and eventually negative only by DPO49 (54).

Compared to adult COVID-19 patients (whose symptoms usually peaked 1–2 weeks after the onset of symptoms, and the duration of virus shedding may persist for 3–4 weeks or longer) (55–57), the pediatric patients had relatively mild symptoms with quick remission and shorter virus shedding duration, except for those with critical COVID-19

and perhaps the young ones. Like in SARS-CoV patients, prognosis is more optimistic in children than adults. Nonetheless, pediatricians should be vigilant and closely monitor each especially those with underlying disorders or above 12 years old, in order to identify whoever is requiring upscaled supportive treatment (51, 58).

2. Clinical classification of pediatric infection
   Based on known clinical features of established pediatric COVID-19 cases, the following classification is proposed (6):
   1) Asymptomatic infection (silent): Patients have NO notable clinical manifestations NOR any radiological abnormality, but SARS-CoV-2 infection is established by positive nuclei acid test or seroconversion.
   2) Mild infection: Patients may present with fever, fatigue, malaise, upper respiratory tract (URT) symptoms like cough, sore throat, rarely running nose and sneezing, or gastrointestinal symptoms like diarrhea, vomiting, and abdominal pain, with physical sign of URTI like pharyngeal hyperemia but not those of LRT.
   3) Moderate infection (regular): Patients have LRT features of viral bronchitis and pneumonia (bronchiolitis is rare) with no tachypnea and hypoxemia, like productive cough, and coarse or moist crepitations evolve. Wheezing is rare. Subclinical variant remains asymptomatic, yet radiologic pneumonic changes are evident (5, 6). There are suggestions that such subclinical variant should not be included (59) in the pediatric COVID-19 classification as radiological imaging (especially CT scan) should not normally be performed on asymptomatic children. Nonetheless, due to the perceived urgency in delineating whether some "asymptomatic" COVID-19 had pathological lung involvements, such investigations had actually been conducted. We opined that this variant should be kept for accurate reflection of real clinical practice, though this should not be undertaken without sound clinical judgment.
   4) Severe infection: These patients usually deteriorate by the second week after onset with respiratory distress, tachypnea, and hypoxemia with SaO_2 <92% in room air (5, 6, 60, 61); some could have only gastrointestinal symptoms at presentation.
   5) Critical infection: Rarely, a pediatric patient may develop ARDS or respiratory failure. Multiorgan failure, such as circulatory shock, encephalopathy, heart failure, disseminated coagulopathy, and acute renal failure could develop leading to fatality.

LABORATORY AND RADIOLOGY FINDINGS

1. Hematological and biochemical test
   1) CBC: Adult patients may have mild leucopenia, lowish absolute lymphocyte counts (with decreased CD4+ and CD8+ subsets), and thrombocytopenia (49, 55, 58). In pediatric patients, total WBC, and lymphocyte and platelet counts were generally normal.
   2) Inflammatory markers: C-reactive protein (CRP) was normal or slightly elevated. Procalcitonin (PCT) was mostly normal. Where available, serum/plasma cytokine and chemokine profiling may help in monitoring COVID-19 disease course and complications (7).
   3) Serum biochemical and coagulation test

   Elevated liver enzyme (ALT and ALT), CK, and myoglobin levels were observed in severe affected children. Some had hypoalbuminemia or coagulopathy reflected by increased D-dimer. Significantly elevated CRP, lactic dehydrogenase (LDH), and serum ferritin could signal or predict deterioration of COVID-19 and related complications (6).
2. Radiology findings
   1) Chest X-ray (CXR): Absence of plain CXR abnormality at surveillance and early symptomatic stage contributed to mild or subclinical COVID-19 patients being missed. In an adult patient, CXR changes are non-specific with bilateral air-space opacities and infiltrates being predominant; effusion or pneumothorax is not common. For non-severe children, CXR changes were compatible with bronchitis, as well as focal patchiness. Diffuse multiple consolidative regions could be observed bilaterally in severe cases. Significant and rapid change in CXR signals potential progression to ARDS.
   2) Chest computed tomography (CT) scan: According to the clinical course of COVID-19, CT changes could be classified into four stages:
      ① Early stage: Subpleural localized consolidation or ground-glass patchiness along the bronchovascular bundles of lung segment or subsegment, with or without concomitant interlobular septal thickening. Unilateral tiny loose opacity in peripheral or subpleural lung field may also be observed.
      ② Evolving stage: Increasing more foci extending to several lobules. Localized consolidation could coexist with ground glass or stripe opacity.
      ③ Severe stage: Bilateral diffuse consolidations, which may progress to classic air bronchogram with "white-out lung." Pleural effusion or pneumothorax was rare.
      ④ Recovery stage: Resolution of previous lesions (62, 63).

      These radiographic features are not pathognomonic of, nor specific to, CoV infection, but rather reflect the predilection of SARS-CoV-2 (like SARS-CoV and MERS-CoV) to involve LRT. While high-resolution CT scan (HRCT) is more sensitive than plain CXR in delineating pneumonic changes, such modality should be chosen for anticipated semiquantitation and pathogenetic correlation in guiding the choice of non-standard management treatment as most pediatric COVID-19 are mostly mild and self-limiting.
   3) Lung ultrasonography (LUS)
      LUS has steadily gained popularity in pediatric critical care and emergency medicine areas (64). Advantages of LUS include lack of radiation, offering real-time, and point-of-care evaluation either for diagnosing or

ongoing monitoring of evolving COVID-19 pulmonary pathologies. Though it has been used in COVID-19 management (65, 66), the urgency and unknown nature of initial COVID-19 outbreak did not allow LUS to be integrated into the Chinese NHC COVID-19 guidelines. Clearly it will eventually be more adopted in managing the ongoing COVID-19 pandemic, as suggested by colleagues in Italy and United Kingdom (67, 68) and had been used in place of CT scan in a series of COVID-19 pediatric patients (69).

DEFINITE LABORATORY DETECTION OF SARS-COV-2 AS PATHOGEN

1. Nucleic acid amplification test (NAAT) with PCR primers specific for SARS-CoV-2 genes is the primary method to detect this pathogen in clinical specimens. Target genes are usually RdRp (RNA-dependent RNA polymerase), E (envelope), and N (nucleocapsid). These tests should be conducted in a P2 health laboratory with operators stringently observing tertiary protection (5–7), but new products potentially suitable for use as points of care tests are emerging.

    1) Sample collection: respiratory specimens is most favorable, including upper respiratory tract (URT) like nasopharyngeal or oropharyngeal swab and low respiratory tract (LRT) like sputum, endotracheal aspirate, or bronchoalveolar lavage. Deep throat saliva is another proven source (20). For strongly suspected cases, a single URT negative test cannot exclude COVID-19 (5, 6, 8). Additional LRT sampling or repeating another URT is recommended. Induction of sputum should be avoided to minimize the risk of airborne transmission (6, 8). Tertiary protection with contact and airborne precautions should be strictly implemented to avoid virus transmitted by droplets from URT or aerosol from LRT (5, 6, 8).

        1.1 For a collection of nasopharyngeal swabs, a synthetic fiber swab with its plastic shaft should be inserted into the nostril parallel to the palate. Pharyngeal swabbing may provoke vomiting, anal swabbing can induce defecation, and both could create hidden environmental contamination and expose healthcare/laboratory workers to profound yet preventable risk. For uncooperative patients, it is prudent to assess whether such sampling is indispensable before committing to doing so (6). It is advisable for workers to conduct these procedures in full PPE. Other specimens such as blood (lower positive rate than respiratory secretions), stool, and urine may be collected, but their diagnostic values should be evaluated prospectively given that relevant information has not been complete (70).

        1.2 For children old enough to clear saliva from deep throat and spit it into the sample collection bottle, detailed instructions on how to do so are available (71).

        1.3 Stool and rectal swab are important samples in working up children especially that there are patients presenting primarily with gastrointestinal symptoms (21).

    2) Laboratory method: Fluorescent real-time quantitative reverse transcription polymerase chain reaction or nucleic acid sequencing to confirm and quantitate presence of SARS-nCoV-2 genome. Full viral genome sequencing is instrumental in identifying variant/mutant SARS-COV-2, which has already been reported (14).

2. SARS-nCoV-2-specific antibody-dependent tests

    1) Serological response could be determined in sera and crucial for confirmation of COVID-19 especially in asymptomatic (silent) patients and those diagnosed by clinical criteria without NAAT positivity. It also enables larger-scale epidemiological studies, screening, and detection of pediatric COVID-19 cases, which are typically mild. More serological tests have been available and used in various reports (72, 73). Similar to the standard diagnostic protocol of SARS-CoV (74), paired serum from acute and recovery phases should be tested for specific antibody titers. Seropositive conversion or greater than or equal to 4-fold elevation in antibody titers can help to retrospectively confirm the diagnosis. Sera-specific antibody-based tests will empower the confirmation of asymptomatic, epidemiological profiling, and detection of pediatric COVID-19 cases (75). As SARS-CoV-2 is a novel human coronavirus, theoretically specific immunity should be absent in unexposed population, and any suspected COVID-19 case found to have a single seropositive sample may be deemed diagnostic. Further investigation on recovery samples to measure the change in titers may strengthen and reaffirm such notion. Of note is that cross-reactivity of less specific antibodies capable of recognizing both SARS-CoV and SARS-COV-2 has actually been a demonstrated issue but could not be totally ignored when working up subjects with unknown infection (76). Given that SARS-CoV infection was not endemic, this should not be a major issue.
    2) Direct viral antigen detection by specific antibodies performed on nasopharyngeal aspirate and other tissues is another standard diagnostic approach. No clinical study has yet been reported for SARS-CoV-2 using such laboratory tests.

3. Virus culture
   The ability to culture SARS-CoV-2 in suitable cell lines (VeroE6/TMPRSS2, Caco-2, Calu-3, HEK293T, Huh-7, and LLC-MK2) (77, 78) offers direct proof whether NAAT-positive clinical samples are indeed infectious. Given the pathogenicity of SARS-CoV-2, stringent guidelines have to be followed by a laboratory doing this and, hence, are not generally carried out. With more comprehensive clinical/laboratory study addressing SARS-CoV-2 nucleic acid test and viral culture positivity, the recovery from the

SARS-CoV-2 virus by standard culture has been definitively identified in subsets of symptomatic, presymptomatic, and asymptomatic COVID-19 patients with positive NAAT (17). This corroborates with the clinical observations of asymptomatic COVID-19 patients, who could act as COVID-19 infectors (15–18). Of note is that these studies were conducted principally in adults.

DIAGNOSIS

Based on the situations of outbreak and the known clinical features of COVID-19 in pediatric patients, we propose to use risk of exposure to assign a patient for medical surveillance, suspected, and confirmed COVID-19.

1. Levels of epidemiology history (EH)
   History of exposure is crucial in early identification and diagnosis of pediatric infection. We recommend defining the history of exposure into three levels (6) within 14 days before the onset of symptoms:

   ① High risk: Those who have close contact with suspected or confirmed COVID-19 subjects, SARS-CoV-2 nucleic acid test-positive subjects, or being a member of a COVID-19 family cluster.
   ② Moderate risk: Those living in an area or community with outbreak of COVID-19 cases.
   ③ Mild risk: Living in an area without confirmed clusters or not highly epidemic.

2. Medical surveillance for COVID-19

   1) All high-risk EH children, regardless of having clinical symptoms or NOT, will be under medical surveillance and have NAAT performed on appropriate specimen(s) at designated center/hospital to avoid missing an asymptomatic patient.
   2) Children with moderate or mild-risk EH should be put under surveillance if one or more of the following are met:

      a. Fever
      b. Clinical respiratory symptom(s) or others such as fatigue, nausea, vomiting, abdomen discomfort, diarrhea, etc.
      Medical surveillance is traditionally carried out by public health personnel but innovative community networking and tele-platforms have been implemented in Hubei Province, China, to facilitate execution of home-based surveillance to meet unexpected needs (79).

3. Suspected COVID-19

   1) Any neonate born to a mother diagnosed or strongly suspected of COVID-19 (6, 80).
   2) All high-risk EH children OR ANY moderate or mild-risk EH child for whom influenza and other usual pathogens, which could cause his/her clinical symptomatology, are excluded (e.g., clinically non-responsive to oseltamivir treatment for 2 days or by appropriate laboratory tests), who meets two of the following three criteria (6):

      a. Clinical—Fever, with obvious respiratory symptom, such as tachypnea or oxygen desaturation or gastrointestinal (GI) presentations such as nausea, vomiting, abdominal discomfort, and diarrhea.
      b. Laboratory findings: any decreased WBC count, lymphopenia, and/or elevated CRP.
      c. Chest radiological changes compatible with COVID-19.
      All suspected cases need to be quarantined if not hospitalized.

4. Confirmed COVID-19
   Suspected cases will become confirmed once viral nuclei acid, exactly the same or highly homologous to the currently known SARS-CoV-2, is detected in any of the following specimens: nasopharyngeal swab, deep throat saliva, sputum, bronchial lavage, and secretion or clinical specimens from respiratory tract, blood, stool/rectal swab or urine (5–8, 34, 42, 56, 58, 81, 82).

   By mid-February 2020, in view of reported false-negative results (70) and interim limited capacity of NAAT test availability in areas where demands were overwhelming, the NHC included a clinical COVID-19 diagnostic category (ONLY for February 9th to 24th) based on positive epidemiological history plus clinical manifestations plus relevant chest radiological changes with no (or pending) virological confirmation. However, as mild pediatric COVID-19 mainly exhibits minimal, localized, and atypical lung radiological changes (63) not distinguishable from those encountered in simple viral pneumonia especially during the flu season, such clinical category had not been adopted for pediatric COVID-10 care beyond Hubei Province for the specified period.

DIFFERENTIAL DIAGNOSIS

No simple clinical constellations or syndrome is pathognomonic of COVID-19. The common COVID-19 symptoms in children are like those caused by many other viral infections. Suspecting and establishing a rare and opportunistic pathogen like SARS-CoV-2 as being responsible, especially beyond an epidemic outbreak, depend on how known etiological agents could be quickly established or ruled out.

Classic respiratory viruses include, but are not limited to, influenza, parainfluenza, adenovirus, respiratory syncytial virus, rhinovirus, human metapneumovirus, bocavirus, and other endemic human coronaviruses. Other pathogens that could cause pneumonia are mycoplasma pneumoniae, legionella, bacteria, fungus, and tuberculosis. For symptomatic patients requiring hospital admissions, tests for pathogens such as H1N1, H1N2, parainfluenza, RSV, adenovirus, bocavirus, HMPV, rhinovirus, enterovirus (such as EnV-D68), hCoV-229E, hCoV-OC43, hCoV-NL63, hCoV-HKU1, mycoplasma pneumoniae, chlamydia pneumoniae, and legionella could be performed

sequentially or even simultaneously according to good basic clinical principles (5, 6, 58, 61, 81, 82). Multiplex rapid PCR-based nucleic acid tests as respiratory or gastrointestinal panels of pathogens are, in fact, regularly used (71). For patients with underlying diseases associated with immune-deficient states, invasive fungal infection should also be considered (1, 4). In case etiological workup for infectious agent is inconclusive, non-infection diseases such as vasculitis, dermatomyositis, idiopathic interstitial pulmonary disease, and cryptogenic organizing pneumonia have to be considered and evaluated (5, 6, 61).

In the context of random emerging serious/new zoonotic viruses, which potentially lead to sinister outcomes or fatalities like H5N1, H7N9, SARS, MERS, and the current SARS-CoV-2, the demand for a wide coverage of known etiological agents is ever-increasing (83). In the community, most viral infections affecting children are self-limiting and hence not traditionally having an exact etiological agent quickly identified and confirmed. When and where resources are available and affordable, speedy point-of-care tests (e.g., rapid antigen test, respiratory pathogen panel by multiplex nucleic acid amplifications) have been increasingly integrated into primary care pediatrician's management of such possible infection(s). Technological advancements rendering these multiplex tests affordable and their clinically relevant turnaround time will help in realizing its contribution to this area (84).

On the other hand, as pediatric COVID-19 is mostly benign, the clinician must avoid being falsely reassured by a diagnosis of COVID-19 as solely or wholly responsible for the clinical symptomatology of an especially sick and toxic child. Good clinical judgment should dictate further exploration for additional cause/coinfection even in the presence of positive laboratory identification of SARS-CoV-2.

PRINCIPLE IN CLINICAL MANAGEMENT

1. Basic principle: early detection, early isolation, early diagnosis, and early treatment are the basic principle for management of at-risk children.
    1) Isolate the suspect or confirmed patients as early as possible.
    2) Choose care commensurate with the clinical severity: for asymptomatic or mild cases, especially those residing in an epidemic area where isolation facilities in local hospitals are very limited or not readily available, home isolation and management supported by structured medical surveillance and guidance is a good choice. Nonetheless, hospital care should be more appropriate for those with pneumonia, while more severe and critical cases should be admitted to PICU (6, 42, 58, 82).
2. General clinical management
    1) Isolation
        a. Hospitalized patients: Suspected patients should always be cared in a single-patient room until COVID-19 diagnosis is established or refuted. Confirmed cases could be housed together in the same room with appropriate infection control measures observed. Cases with heightened infectivity should be cared in an airborne infection isolation room (AIIR). The health care providers must be equipped according to tertiary protection standard. All biological secretion and excretion from suspected or confirmed COVID-19 patients should be handled according to disinfection guidelines (5, 6, 42, 85).
        b. Patients in the community: Condition on available local community health service (*ad hoc* or conventional), the choice of home isolation, and surveillance for a COVID-19 child should be made by the pediatrician in-charge based on the following understandings: his/her parents (or the designated guardians) will accompany the child the whole time; in addition, appropriate community supervising system and tele-medicine guidance pathways are in place and feasible. Specimens for confirmatory diagnostic tests, if deemed appropriate (e.g., deep throat saliva could be self-collected at home), could be sent to, or collected at, designated sites (5, 6, 8, 42, 85).
        c. Infants born to a mother with suspected or confirmed COVID-19: Each baby should be immediately separated from his/her mother after delivery and admitted into an isolation room for monitoring. Primary infection prevention should be adopted. Meanwhile, breastfeeding is advised to be temporarily withheld with the mother expressing milk to maintain production for continuing breastfeeding later. Indeed, positive identification of SARS-COV-2 RNA in breast milk had been reported in Italy and Germany recently (86, 87), supporting such notion. It is prudent therefore to check breast milk for NAAT negativity before resuming breastfeeding. Direct breast feeding and nursing by SARS-COV-2-positive mother should be deferred until the mother has viral shedding clearance documented.

            While continual deliberations have been raised as to whether direct contact between nursing mother and her newborn baby should be preferred in the interest of promoting maternal–child developmental health and bonding, the threat of getting moderate/severe COVID-19 is not negligible. The fact that both babies in the Ulm UMC report (87) were infected and became symptomatic (one with hypoxemia) does lend support to the current guideline of separating babies from the mothers.

            The SARS-CoV-2 nuclei acid test for the babies should be performed on respiratory tract and rectal swab samples within 24 h, 5–7 and 14 days after delivery. Any positive result should be reported immediately and further evaluation conducted to inform clinical management, especially to decide whether home isolation and observation is feasible. For home management, all household members must have their COVID-19 status documented before the eligible

neonate is discharged. Infection control and prevention measures (with masking, careful disposal of waste, and disinfection) and normal nursing routines of newborn babies will be followed.

Healthy term infants should stay for at least 7 days; if both 24 h and days 5–7 nuclei acid tests are negative, feeding pattern normal and general conditions good and stable, home isolation and surveillance could be considered. Emergence of any COVID-19 symptom during home surveillance will prompt readmission of the baby for reevaluation. Isolation (at home or in hospital) can only be discontinued at least until postnatal day 14 when NAAT is negative, as decided by the supervising doctor in charge.

Preterm infants and any term infant with birth asphyxia or other diseases should be isolated in a single room and treated accordingly (6, 80, 88–91).

2) Clinical supervision: The clinical progress and vital signs will be closely monitored, especially with the use of pulse oximetry to detect hypoxemia promptly. CBC, CRP, PCT, biochemical index (liver enzyme, myocardial kinase, pancreatic enzyme, electrolyte, and renal function), coagulation function, arterial blood gas and urinary test will be performed as deemed appropriate. Chest radiological examination will be determined based on the clinical course (6, 7).

Clinicians ought to be mindful of possible clinical deteriorations and vigilant over emergence of such clues. Persistent fever, worsening respiratory distress, change of conscious states, cardiovascular compromise, elevated inflammatory factors, and coagulation dysfunction are all important indicators of an emerging critical state, which should be managed without delay (6). Over 50% progression in chest radiological lesions/involvements in any patient should prompt the escalation of his/her care to that of severe and critical cases (see Intravenous immunoglobulin section below).

3) General and symptomatic treatment should be offered according to clinical needs. Oxygen therapy should be initiated once a patient exhibits dyspnea and/or desaturation (oxygen saturation by pulse oximetry <95%). Oxygen flow rate and mode of ventilation should be carefully chosen and adjusted to achieve sustained optimal oxygenation. Contact precautions should be observed when connecting devices to the oxygen supply (6, 8, 19, 50, 61, 82).

3. Specific therapeutic options

1) Antiviral treatment: No specific anti-SARS-CoV-2 drug has yet been proven effective. Trials of lopinavir/ritonavir (LPV/RTV), ribavirin, chloroquine, and arbidol treatment have all been proposed for compassionate use according to the Chinese NHC "Diagnosis and treatment plan of pneumonia with new coronavirus infection (trial version 7)" (7). These therapeutic options should be judiciously chosen, generally only for severe or critical cases and preferably only tried/used with informed consent (6). It is prudent for principles outlined in WHO Monitored Emergency Use of Unregistered Interventions Framework (MEURI) (92) to be adopted in choosing a specific agent by the clinical team, with emphasis on structured collection of relevant clinical data for analysis as clinical trials. Along this line, the "Guidance: multisystem inflammatory syndrome temporally associated with COVID-19" issued by the Royal College of Pediatrics and Child Health does illustrate how such proactive approach in dealing with evolving clinical problems is emphasized in the current COVID-19 pandemic (93).

The second critical COVID-19 child received LPV/RTV for 7 days (started on day 13 and day 6 after onset and admission). His fever subsided after 6 days and was successfully extubated after 7 days; however, his respiratory aspirate remained positive for SARS-nCoV-2 at that time. During therapy, serum triglyceride, total cholesterol, and lactate levels were normal, whereas significantly decreased WBC count and transient thrombocytopenia were observed 3 days after starting LPV/RTV treatment. The leukocytopenia was corrected after a dose of granulocyte-macrophage colony-stimulating factor (GM-CSF) (54).

On February 17, 2020, Chinese scientists announced that they have found that the antimalarial drug chloroquine has anti-SARS-CoV-2 virus effects (94) and immunomodulatory effects *in vitro*. Through their multicenter clinical studies, chloroquine treatment is demonstrated to be superior than non-chloroquine control by promoting a higher rate of subsidence of fever, alleviating lung pathological changes, decreasing the rate of progression to severe COVID disease, shortening the time for SARS-nCoV-2 nucleic acid positivity converting to negative, and decreasing the overall rate of SARS-nCoV-2 nucleic acid positivity (95). One observational study conducted in New York did not demonstrate either a greatly lower or an increased risk of the defined end point of intubation and death in COVID-19 patients treated with hydroxychloroquine (96). US FDA has issued a review of safety issues with the use of hydroxychloroquine and chloroquine to treat hospitalized patients with COVID-19, which identified reports that included serious heart rhythm problems and other safety issues, including blood and lymph system disorders, kidney injuries, and liver problems and failure. Hence, it has cautioned against the use of hydroxychloroquine and hydroxychloroquine outside the hospital setting or a clinical trial based on the risk of cardia arrhythmia (97).

Other medications such as arbidol are still under clinical trial (98).

Usage of antiviral treatment (see **Table 2** for dosage recommendations):

1) LPV/RTV: Extended from the treatment of HIV and dosage in children <40 kg is prescribed according to body weight (99).

TABLE 2 | Dosage of specific therapeutic agents.

	Route	Dosage	Frequency	Maximal dosage per day	Course
Lopinavir (LPV)	Oral	10 mg/kg	Twice daily	400 mg	1–2 weeks
Ritonavir (RTV)	Oral	2.5 mg/kg	Twice daily	100 mg	1–2 weeks
Kaletra (LPV/RTV)[a]	Oral	Day 14–12 months—LPV/RTV 300 mg/75 mg per m^2 16 mg/4 mg per kg	Twice daily		14 days
		>12 months−18 years LPV/RTV 300 mg/75 mg per m^2	Twice daily	800 mg/200 mg	14 days
		<15 kg−13 mg/3.25 mg per kg			
		>15–45 kg−11 mg/2.75 mg per kg			
Ribavirin	IV	10 mg/kg	8-hourly	500 mg	
Chloroquine	Oral	10 mg/kg	Twice daily	500 mg	
Arbidol	Oral	50 mg (2–6 years old) 100 mg (7–12 years old) 200 mg (>12 years old)	Three time a day		
Methylprednisolone	IV	1–2 mg/kg/day	12-hourly		3–5 days
Convalescent plasma[b]	IV	4 ml per kg	Single dose	200 ml	Single dose but may be repeated if indicated
IVIG	IV	1–2 g/kg	Single dose		May be repeated if indicated
	IV	400 mg/kg/day	Daily		5 days

[a] *Do not give Kaletra to neonates before 42 weeks postmenstrual age and postnatal age <14 days.*
[b] *Convalescent plasma should have neutralizing antibody titer >1:640.*

2) Ribavirin: Usage follows those recommended for treating adenovirus pneumonia (100) and better in combination with LPV/RTV.
3) Chloroquine: According to NHC recommendation (7).
4) Arbidol is only used in children above 2 years old, based on the Russian National Drug Formulary (98).

A combination of antiviral drugs is not recommended except for ribavirin. The antiviral agent therapy should be until 24 h following fever subsidence or up to 4 days if deemed ineffective (6). Side effects should be monitored once any antiviral medication is started, and managed or stopped accordingly.

2) Treatment for severe and critical COVID-19 cases: The guiding principles are an aggressive correction of compromised oxygenation, provision of adequate support to organ functions, and preventing complications.

1) Respiratory support: For patients with severe hypoxemia and ARDS, respiratory support should be offered by either high-flow nasal cannular or non-invasive mechanical ventilation. If deemed ineffective because of complications like recurrent apnea, ineffective breath, or cardiac arrest requiring resuscitation, invasive mechanical ventilation should be initiated. Sedation, analgesia, and muscle relaxant is required regularly. Tertiary infection control measure should be taken to prevent airborne transmission during the intubating process. Indeed, strategies for promoting health care staff safety during emergency airway management has been outlined (101). Extracorporeal membrane oxygenation (ECMO) is essential if expertise is available. Ideally, the transfer of patients to tertiary centers capable of offering ECMO should best be planned in anticipation (6–8, 36, 61, 82); hence, background organization in the local health system is often crucial.
2) Circulation support: Closely monitor vital signs like conscious level, skin color, capillary refilling time, blood pressure, and key parameters including urinary output and lactate level to ensure early identification of septic shock. Septic shock should be treated according to established pediatric guidelines. While fluid bolus therapy is accepted as first-line management, pediatricians should be cautious about potential harm from fluid overload. Preferably accurate evaluation of the patient's hemodynamic status plus careful assessment of its responsiveness following fluid loading should be considered when feasible; however, if that is deemed not feasible, an empiric 20 ml/kg of normal saline could be initiated. Vasopressors should be given early if fluid administration does not restore adequate perfusion. In patients with ARDS, stringent management of fluid intake to achieve a negative balance and treat the syndrome of capillary leakage, while maintaining a normal cardiac and renal function, are crucial. Hemodynamic status should be routinely monitored during the entire course of treatment (6–8, 36, 61, 82).
3) Multiorgan support: Intensive monitoring of patients for multiorgan function, including neuromuscular, gastrointestinal, urinary, hematological and coagulation systems, and fluid and electrolytes balance. Adopting the Sequential (sepsis-related) Organ Failure

Assessment (SOFA) score to semiquantitatively evaluate the function of various systems and determine whether intensive treatment such as CRRT are indicated (36, 61, 82).

4) Corticosteroid: Corticosteroid has been used in severe COVID-19 patients, like in SARS and MERS, with the understanding that immunopathogenesis contributing to acute lung injuries has been well-documented in macaque SARS-CoV model. New findings have even demonstrated mismatched mounting SARS-CoV spike IgG level and viral loads could be crucial or responsible for such damage (102). It is prudent to believe that rightly timed corticosteroid as an immune-modulator could be beneficial as anecdotally observed in SARS. Both pediatric and adult SARS patients had been studied showing a decrease in raised IL-6, CXCL8, IL-10, and IL-8 by corticosteroid-modulating cytokines with clinical alleviation of lung abnormalities and clinical severity (103–105). Along this line, comprehensive dynamic immune profiles may support selecting the best therapeutic window for halting or preventing life-threatening lung damages.

   In fact, the effectiveness of corticosteroid therapy in reducing mortality in adult critically ill COVID-19 patients have been demonstrated in many studies since the RECOVERY trial first published its positive results in July 2020 (106). The clinical evidence from high-quality RCT studies was evaluated in a meta-analysis led by the WHO "REACT" group (107). Meanwhile, the WHO has actually issued a formal guideline on September 2, 2020, which recommends the use of 7–10 days of systemic corticosteroid therapy in severe and critical COVID-19 patients and conditionally recommends not to do so in the non-severe COVID-19 subjects (108).

   However, as young patients have much milder clinical COVID-19 manifestations, no pediatric-specific corticosteroid therapy trial has been conducted nor reported. We recommend that corticosteroids not be considered in the early phase of disease as it may compromise the virus clearing and inhibit the host's immunity (5, 6, 8), in line with the WHO conditional recommendation. Furthermore, drawing from the experience of adult COVID-19 patients' management (109), corticosteroid may be considered in pediatric patients, based on expert opinions, if three of the following four indications are met: ① fever of more than 38.5°C, lasting for at least 3 days; ② CRP $\geq$30 mg/L; ③ serum ferritin $\geq$1,000 μg/L; ④ diffused infiltrative changes documented in both lungs. Presence of these criteria actually indicates that the disease is progressive. The overall treatment period is short, for 3–5 days, which should be discontinued once fever remits.

5) Intravenous immunoglobulin (IVIG): The efficacy of IVIG therapy in adult patients with COVID-19 is limited. With reporting of multisystemic inflammatory syndrome range of clinical problems in countries outside China, IVIG could well be indicated for confirmed COVID-19 patients with related symptoms, regardless of whether casually triggered by SARS-CoV-2 or not. Therapeutic use of IVIG has been recommended for critical pediatric infection at clinician's discretion (6).

6) Optimal empiric antimicrobials: Antibiotics should only be used when a coinfection is highly suspected or established by comprehensive microbiological evaluation. Antimicrobial prescription without a conscientious rationale should be avoided. One should ensure its dosing is pediatric appropriate and usage adjusted in accordance to observed clinical response and drug-sensitive test. For patients highly suspected of bacterial coinfection, antibiotics should be initiated immediately while samples be sent for microbiological identification (5, 6, 8, 109).

7) Traditional Chinese Medicine (TCM): Some recipes have been recommended for adult patients and could be considered by pediatricians who command reasonable knowledge of the respective benefits vs. potential risks or side effects (7). Injectable TCM preparations should be avoided in children (5, 6).

8) Other treatment (8)

   ① Plasma from COVID-19 convalescent patients with high neutralizing SARS-CoV-2 antibody tire (generally >1:640) had been used as adaptive immunotherapy in severe or critical patients with rapid disease progression (110). Recommended indications are severe or critical patients with rapid disease progression within 3 weeks of clinical illness who have evidence (laboratory or clinical) of SARS-CoV-2 viremia. No pediatric patient has received such therapy in China yet.

   ② Microecological agent: for enhancing balanced microecology of gut microbiomes and prevent emergence of secondary bacterial enteritis.

See **Table 2** for dosage recommendations.

CASE REPORTING, SURVEILLANCE, TRANSPORTATION, AND DISCONTINUATION OF ISOLATION

1. Detection and reporting

   For patients meeting the criteria to be evaluated for COVID-19, relevant public health ordinance should be followed. These usually translate into activating isolation, prevention, and control measures immediately plus notifying both infection control personnel of respective facility and the local or state health department. If other common respiratory pathogens

are undetected, the patient should be sent for SARS-CoV-2 nuclei acid test. COVID-19 may only be excluded by sequential two negative results (with 1 day apart from each other) (5, 6, 8, 42).

2. Medical observation
   A person with close contact or exposure history should be observed and assessed. Centralized or home isolation may be the solution. Duration of the medical observation may last for 14 days from the last contact with the infection source. Temperature and clinical manifestations are the major indicators for upscaling management algorithm (5, 6, 8, 42).
3. Patient transport
   Patients should be transported in a specific ambulance/vehicle with appropriate protection to health care providers. The vehicle should be disinfected after the transportation following the standard practice enlisted in "Transport of Patients with 2019-nCoV Infected Pneumonia (trial version)" (111).
4. Discontinuation of infection control isolation
   Fulfillment of all the following is recommended for discontinuation of isolation: temperature remains normal (not because of ongoing steroid) for more than 3 days, improvement of respiratory symptoms, resolution of lesion in chest radiology, and nucleic acid test for SARS-CoV-2 negative in two successive samples taken more than 1 day apart. The patients may be transferred to a non-isolation ward or discharged home based on clinical judgment (42, 58, 61, 112). Discharged patients should continue to observe home isolation and surveillance for at least another 14 days while wearing a mask, dining separately from other household members, and observe strict hand hygiene. They ought to be promptly re-assessed should any new symptom(s) appear. Follow-up for clearance of SARS-CoV-2 in clinically relevant specimens should be advised by pediatrician in-charge.

CONTROL OF HOSPITAL-ACQUIRED INFECTION

The regulations implemented by the National Health and Health Commission or other guidelines in patient transport (111–113) and usage of medical protection (42, 85, 114) should be strictly followed. Special attention should be paid to the different risk-level operational regions within a hospital or clinic and the procedures when putting on and taking off protection tools meticulously followed. Infection prevention and control professionals should be clearly designated for health care workers to get clarification of related policies, guidelines, and pragmatic supervision.

LESSONS FROM OTHER COUNTRIES

With COVID-19 spreading around the globe, more and more pediatric patients had been identified and managed worldwide. This has led to additional key issues being identified, not otherwise covered by the current guideline based mainly on the Chinese experience. An interesting report from Rome, Italy, documented a neonate born to a mother with COVID-19 infection and positive blood SARS-CoV-2 IgG antibody on PND1, but his nasopharyngeal swab NAAT only turned positive by PND14 (negative on both PND1 and 3) (86). He remained asymptomatic. Whether the presence of maternal SARS-CoV-2 had protected him from turning symptomatic remained to be speculative. Interestingly, SARS-CoV-2 had been identified in the breast milk (actually the first report in medical literature) in the early postnatal days. In this report, abstinence of breastfeeding until the maternal COVID-19 infectious status cleared was practiced, in agreement with our recommendation. In order not to deprive these newborn babies of the crucial benefits of breastmilk feeding, donor breast milk, or those from the milk bank could be considered (115). Furthermore, elaborate long-term follow-up protocol up to 1 year of age has been proposed for those newborn babies with established COVID-19, which is worth following in order to fully delineate the clinical impact of COVID-19. Meanwhile, how best should newborn babies of confirmed pregnant mothers be managed will continue to be refined with more experiences cumulated worldwide.

On the other hand, strikingly different clinical syndromes associated with COVID-19 had been reported in pediatric patients outside Asia, namely, hyperinflammatory shock syndrome, multisystem inflammatory syndrome, Kawasaki-like syndrome, atypical Kawasaki syndrome, Kawasaki disease shock syndrome, and toxic shock syndrome (116–118). This unexpected range of clinical manifestations has not been encountered/reported in China (including Hong Kong Special Administrative Region and Taiwan) even after a retrospective review of known COVID-19 patients for related features. No uniform explanations have been reached yet.

With the looming threat of missing pediatric COVID-19 patients taking acute downturn during the course of illness, Chinese pediatricians are more inclined to use CT scan to definitively delineate their possible COVID-19 pulmonary involvements speedily. Now that pediatric COVID-19 was found to be mostly taking self-limiting courses, protecting asymptomatic (mainly close contacts of confirmed cases) and mild symptomatic subjects against avoidable exposure to high radiation doses of CT scans by adopting an alternate algorithm involving expanded use of LUS, becomes very appropriate (119).

With the health care service models being widely diversified across the world, variations seen in guidelines of de-isolations were primarily linked to the respective national and state epidemic status and resources. The US CDC has reviewed the details pertaining to the evolution of the situation plus the cumulated evidence and shared on its website, e.g., the Discontinuation of Transmission-Based Precautions and Disposition of Patients with COVID-19 (120). Likewise, the European CDC has issued comprehensive documents for pragmatic guidance, e.g., discharge criteria for confirmed COVID-19 cases (121).

CONCLUDING REMARKS

Following the emergence of the third zoonotic coronavirus SARS-CoV-2 infecting humans with a clinical syndrome marked by serious pneumonia and potential fatality like SARS and MERS, over 100,000 patients have COVID-19 confirmed with over 3,600 deaths in a short 2.5 months. Despite there are yet no definitive treatment(s) proven by clinical trials to cure these coronaviral diseases, anecdotal experiences and basic scientific studies from SARS and MERS outbreaks have lined up leading agents and thoughts adopted by critical care clinicians. A few tangible antiviral and anti-inflammatory treatments were being used in severe or critically ill COVID-19 patients, with mixed claims of effectiveness. These were constantly reflected in the various updated versions of the National Health Committee guidelines for COVID-19 management.

With endemic coronaviral infections all relatively benign, the milder clinical course observed in the current pediatric COVID-19 will again not be conducive to much planned pediatric-specific studies for understanding crucial elements rendering childhood coronaviral infections unique. Arguably, the choices of management and treatment for pediatric subjects could well be distinct from adults, particularly in the rare occasions of more severe complications. For example, serious side effects previously reported in adult SARS patients from high-dose steroid usage were not experienced by the pediatric counterparts, while beneficial effects were apparent (122). Getting to better delineate what pediatric COVID-19 is like will set the stage to establishing informed management approach, allowing stakeholders to identify targeted agenda to explore for plausible differential treatments fit for our developing kids.

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

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REVIEW
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An Update on Molecular Diagnostics for COVID-19

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A novel strain of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) disease (COVID-19) has been recently identified as an infectious disease affecting the respiratory system of humans. This disease is caused by SARS-CoV-2 that was identified in Chinese patients having severe pneumonia and flu-like symptoms. COVID-19 is a contagious disease that spreads rapidly *via* droplet particles arising through sneezing and coughing action of an infected person. The reports of asymptomatic carriers changed the scenario of symptom based-diagnosis in COVID-19 and intensified the need for proper diagnosis of the majority of the population to combat the rapid transmission of virus. The diagnosis of positive cases is necessary to ensure prompt care to affected people and also to curb further spread of infection in the population. Collecting samples at the right time and from the exact anatomical site is crucial for proper molecular diagnosis. After the complete genome sequence was available, China formulated RT-PCR as a primary diagnostic procedure for detecting SARS-CoV-2. Many in-house and commercial diagnostic kits have been developed or are under development that have a potential to lower the burden of diagnosis on the primary diagnostic techniques like RT-PCR. Serological based diagnosis is another broad category of testing that can detect different serum antibodies like IgG, IgM, and IgA in an infected patient. PCR-based diagnostic procedures that are commonly used for pathogen detection need sophisticated machines and assistance of a technical expert. Despite their reliable accuracy, they are not cost-effective tests, which a common man can afford, so it becomes imperative to look for other diagnostic approaches, which could be cost effective, rapid, and sensitive with consistent accuracy. To make such diagnostics available to the common man, many techniques can be exploited among, which are Point of Care (POC), also known as bed side testing, which is developing as a portable and promising tool in pathogen diagnosis. Other lateral flow assay (LFA)-based techniques like SHERLOCK, CRISPR-Cas12a (AIOD-CRISPR), and FNCAS9 editor-limited uniform detection assay (FELUDA), etc. have shown promising results in rapid detection of pathogens. Diagnosis holds a critical importance in the pandemic situation when there is no potential drug for the pathogen available in the market. This review sums

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up the different diagnostic approaches designed or proposed to combat the crisis of widespread diagnosis due to the sudden outbreak of a novel pathogen, SARS-CoV-2 in 2019.

Keywords: Severe Acute Respiratory Syndrome Coronavirus 2, COVID-19, diagnostics, reverse transcription-PCR, SHERLOCK, CRISPR-Cas12a

INTRODUCTION

Diagnosis is a major aspect in tackling the consequences of any deadly contagious diseases. Diagnostic tests demonstrate the presence or absence of an infectious agent. Early and better diagnosis has helped in limiting fatalities due to highly infectious and contagious diseases in the past (Caliendo et al., 2013). Diagnosis has an empirical role in such diseases that are caused by any novel pathogen for which the population is not pre-immune. COVID-19 is one of such infectious diseases, highly contagious and deadly (Cascella et al., 2020). COVID-19 was found to be caused by a novel viral pathogen named as SARS-CoV-2. COVID-19 originated in Wuhan city of China and spread to almost the entire world (Rothan and Byrareddy, 2020). In January 2020, China reported the outbreak of SARS-CoV-2 that was further declared as pandemic owing to indiscriminate and rapid spread to different parts of world. Among the first 41 cases of COVID-19 reported by Huang et al., most of the patients had a history of exposure to Wuhan wild animal market. Patients had several symptoms like cough, fever, dyspnea, fatigue and radiographic indication of pneumonia (Huang et al., 2020). Whole genome sequencing of SARS-CoV-2 led scientists to design testing protocols to detect the pathogen in the affected people and also provided an insight in the phylogenetic study of the virus. It was elucidated that SARS-CoV-2 belongs to the family of beta-coronavirus, which include SARS-CoV and Middle East Respiratory Syndrome (MERS) viruses (Zhou et al., 2020a). The origin of this virus is still a matter of debate; however, bats are thought to be its primary source. SARS-CoV-2 was identified on 3rd January 2020 in the bronchioalveolar lavage fluid samples taken from a patient in Wuhan, China (Zhu et al., 2020). As the understanding regarding COVID-19 disease evolved, it was observed that SARS-CoV-2 infection spreads *via* asymptomatic carriers (Rothe et al., 2020), which further prompted health workers to increase the diagnostic frequency among the masses.

As evident from the previous outbreaks of SARS-CoV in 2003 and MERS in 2012 (Cheng et al., 2007), a sensitive, specific and rapid diagnosis for COVID-19 are crucial in identifying positive cases, tracing its contacts, finding the source of virus and finally rationalizing the measure of controlling infection. During the early period of the epidemic, complete sequencing of SARS-CoV-2 facilitated the specific primer-designing and laboratory diagnosis of COVID-19 (Lu et al., 2020). On 23rd January 2020 the first protocol of RT-PCR for COVID-19 diagnosis was published. This assay targeted viral genes related to Nucleocapsid (N), RNA-dependent RNA polymerase (RdRp), and Envelope (E) protein. RdRp was found to be most sensitive among these variants. This assay was using two probes; one probe called 'Pan Sarbeco- probe' detects SARS-CoV-2, bat-related SARS coronaviruses and other coronaviruses. However, another probe called RdRp-p2 was specific to SARS-CoV-2 only (Chan et al., 2020). Jasper Fuk-woo Chen et al. had developed a novel RT-PCR based diagnostic method for COVID-19 called RdRp/Hel, which was found to be more sensitive and specific than the previously published RdRp-p2 method (Chan et al., 2020) RT-PCR is a highly specific and sensitive diagnostic method for infectious disease detection like COVID-19. As this method is based on the detection of nucleic acids, it enables an early diagnosis of COVID-19 by detecting SARS-CoV-2 RNA in the patient sample (Corman et al., 2020). Despite being specific, sensitive and rapid, PCR-based methods lie restricted to a clinical laboratory with sophisticated equipment and trained personnel that limits its use as a Point of care diagnosis (Notomi, 2000). There is a dire need of alternative diagnostic techniques to use as point of care tests in the current pandemic. A dramatic burden on public health and society due to sudden outbreak of SARS-CoV-2 virus limited the use of sequencing and RT-PCR as a tool of rapid diagnosis because both these techniques take much time to generate test result and are affordable to a very limited section of population, thus making it imperative to develop new point of care diagnostic techniques that can compete with RT-PCR in terms of sensitivity and are easily affordable to common man. Many techniques can be exploited to make rapid and affordable testing available to common people that include Point of Care (POC) also known as bed side testing. POC testing is developing as a portable and promising tool in pathogen diagnosis (Yeh et al., 2017), a major example is the discovery of CRISPR COVID that has been shown to deliver comparable specificity and sensitivity as that of RT-PCR and sequencing based meta genomic diagnostic approaches (Hou et al., 2020). Moreover, lateral flow assay (LFA) is one of the latest POC techniques, which works on the principle of fluid flow, and it is employed in immune assays targeting protein, DNA or RNA based samples (Tang et al., 2017). According to a study conducted by Banerjee et al. in 2018, these POC based techniques can be utilized for viral detection in patient samples (Banerjee and Jaiswal, 2018). Other LFA-based techniques like SHERLOCK (Specific High Sensitivity Enzymatic Reporter unLOCKing) developed by Feng Zheng's lab is aiming to be a fast, cost-effective and sensitive technique for pathogen detection (Gootenberg et al., 2018). All-In-One Dual CRISPR-Cas12a (AIOD-CRISPR) is another promising technique known to be fast, sensitive and a very efficient system for pathogen detection (Ding et al., 2020). These techniques can be employed to lower the burden of costly diagnostic procedures as well as lowering time consumption during any pandemic disease outbreak. This review will elaborate

the diagnostic procedures, which are currently in use and will also highlight other new techniques that can be utilized for rapid and cost-effective diagnosis of COVID-19.

SAMPLE COLLECTION AND BIOSAFETY MEASURES

High level of viral loads in the upper and lower respiratory tracts have been demonstrated in COVID-19 patients within 5-6 days of the onset of symptoms (Pan et al., 2020). For an early diagnosis of COVID-19, nasopharyngeal or oropharyngeal swabs are recommended (Chan et al., 2004) (Zou et al., 2020), however, a single nasopharyngeal swab is a method of choice for health practitioners because patients can easily tolerate it and is safe for handling. To obtain a proper nasopharyngeal swab specimen, the swab must go deep into the nasal cavity eliciting tears in the patient (Druce et al., 2012). Collected swabs should be immediately transported using transport media to the diagnostic laboratory, ideally in refrigerated conditions (Druce et al., 2012). Patients with severe COVID-19 pneumonia have shown high viral loads in bronchoalveolar lavages, however, nasopharyngeal swabs were not compared in the particular study (Wang et al., 2020). These patients have also shown high viral RNA in fecal samples as well (Zhang W. et al., 2020). Thus the preferred method of collecting samples from advanced COVID-19 patients is from the stool or the rectal swabs (Cheng et al., 2004). For the safety of health practitioners and the proper processing of samples, it becomes imperative to take utmost precautions while collecting, transporting and processing the COVID-19 samples. In response to this, the health practitioners must use goggles, N95 respirators, gloves, full sleeve gowns or PPE kits in order to minimize direct contact with the COVID-19 positive patients (Karthik et al., 2020).

During the early time of COVID-19 pandemic, a simple and convenient approach of collecting patient sample was a dare need to replace the painful nasopharyngeal swab collection process. In that scenario, Rutgers Clinical Genomics Laboratory came up with an idea of developing an RT-PCR based technique, which can detect SARS-CoV-2 RNA in self collected saliva samples. They developed an assay kit, which was commercialized as TaqPath™ COVID-19 combo kit. This strategy lowered the risk of contracting infection during sample collection by the health practitioners (Afzal, 2020). Sample collection for protein-based diagnosis like IgG/IgM and LFA, requires patients' blood samples. **Figure 1** shows the schematics of specimen/sample collection for COVID-19 diagnosis as well as various nucleic acid and protein-based diagnostics approach.

RT-PCR AS A FRONTLINE DIAGNOSTIC METHOD FOR COVID-19 DIAGNOSIS

Molecular diagnostic approaches are appropriate as compared to other syndromic testing approaches because molecular diagnosis

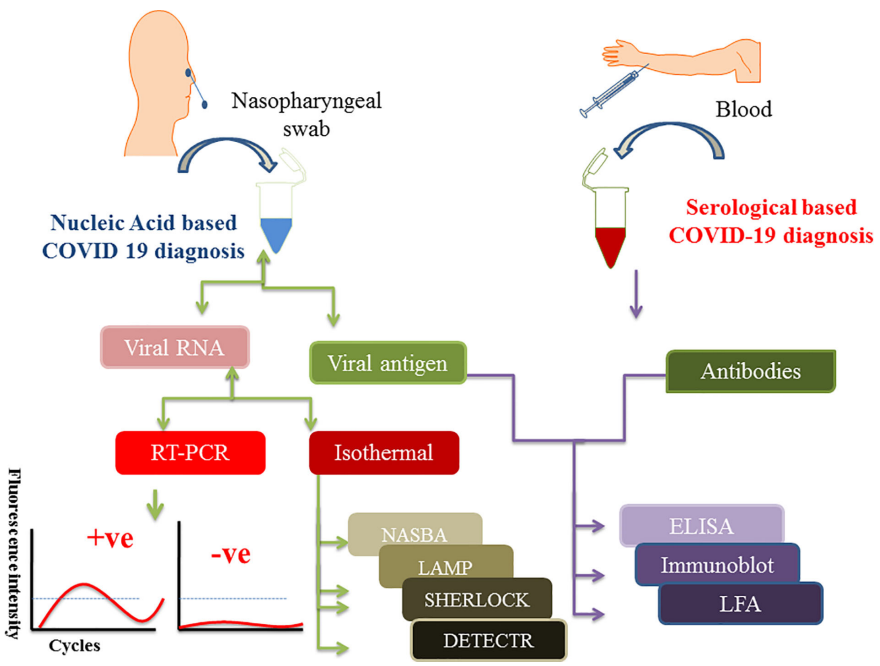

FIGURE 1 | Figure depicts the scheme of sample collection and mentions different diagnostic approaches to detect SARS-CoV-2 infection. Nasopharyngeal swabs and serum samples are drawn from the patients which are further subjected to either nucleic acid or protein-based diagnosis depending on the need. Isolated viral RNA can be used for PCR, isothermal and CRISPR based methods whereas viral antigen and antibodies (IgG/IgM) are used for protein-based diagnosis like ELISA and point of care LFA methods.

targets the genome or proteome of the pathogen thus making it a specific and reliable method of diagnosis (Zhou et al., 2020b). For a novel pathogen sequencing and diagnosis becomes imperative to recognize the nature of the pathogen and its genomic composition. Random amplification and deep sequencing strategies played a critical role in early identification of the SARS-CoV-2, which was further confirmed to be a member of the coronavirus family *via* different bioinformatics approaches (Briese et al., 2014). Using metagenomic sequencing, the first genomic sequencing was conducted for SARS-CoV-2 (Miller et al., 2019; Sheridan 2020a). On 10th January 2020, the findings were made public and the sequences submitted to the sequence repository of GenBank (Wu et al., 2020). Release of whole genome sequence of SARS-CoV-2 to public databases made it easy for scientists to design primers and probes for conducting laboratory diagnosis of COVID-19 (Corman et al., 2020). After the identification of this virus, WHO recommended real time reverse transcription polymerase chain reaction (real time RT-PCR), which is a nucleic acid-based technique, as the frontline diagnostic approach to detect SARS-CoV-2 infection in suspected patients. RT-PCR is highly sensitive and can detect infection at minute levels of pathogen present in the patient sample. It is a nucleic acid-based technique used to amplify a target gene/nucleotide present in a sample, which helps in detecting a specific pathogen and discriminating it from other related pathogens. There are usually two possible ways of performing RT-PCR including one-step assay or two-step assay. One step assay consolidates reverse transcription and PCR amplification in a single tube thus making the process of detection rapid and reproducible; however, this assay provides a lower target amplicon generation. In case of two-step assay the reactions are carried out sequentially in two separate tubes making it time-consuming, but a sensitive assay compared to the one-step assay format (Wong and Medrano, 2005).

Although eleven nucleic acid-based protocols and eight antibody detection kits have been approved by the National Medical Product Administration (NMPA) in China, PCR was considered as a preferred diagnostic technique. The US Centers for Disease Control and Prevention (CDC) uses a one-step PCR format to diagnose COVID-19 (https://www.fda.gov/media/134922/download). The assay is carried out by isolating RNA from the sample and adding to the master mix containing forward and reverse primers, nuclease-free water, reaction mixture (reverse transcriptase, polymerase, nucleotides, magnesium and other additives). A PCR thermocycler is loaded with extracted RNA and mastermix, and the temperature is set to run the PCR reaction (https://www.fda.gov/media/134922/download). Cleavage of a fluorophore quencher probe during this reaction generates a fluorescence signal that is detected by the thermocycler, and the progress of amplification is recorded. Positive and negative controls must be included whenever running any RT-PCR reaction, which makes the interpretation of results easy and stringent (Chan et al., 2020). RT-PCR and some biosensor based diagnostic kits can detect SARS-CoV-2 nucleotides in fecal samples or sewage water that can be a warning of an infectious disease outbreak in the particular area. SARS-CoV-2 can survive from hours to days in the untreated sewage water (Orive et al., 2020).

RT-PCR is a sensitive and rapid detection tool in molecular diagnostics. It can detect and amplify even a few copies of specific genomic sequence in a variety of samples, but it depends upon certain aspects to deliver reliable results like proper collection, transport, storage, and processing of samples (Afzal, 2020). It has been used for detection of diverse viruses like Adenovirus, Rotavirus, Astroviruses and many enteric viruses isolated from fecal samples (Kowada et al., 2018). A Major drawback of this technique is the need for a well-equipped laboratory and technical personnel for handling the experiment, which cannot mitigate the increased demand of rapid testing during pandemic situations like COVID-19 (Bustin and Nolan, 2004). The RT-PCR based kits are highly expensive and take much time to deliver results thus making it essential to look for other rapid and reliable diagnostic methods (Hofman et al., 2020; Sheridan 2020b).

OTHER NUCLEIC ACID-BASED DIAGNOSTIC TECHNIQUES

Nucleic Acid Sequence-Based Amplification (NASBA)

NASBA is an *in vitro* amplification process conducted in isothermal conditions. It is a two-step amplification process where the first step is the denaturation and the second step is a polymerase dependent amplification conducted isothermally (Compton, 1991). Fluorochromes are also added to the reaction in order to make it a real time-based observation. This technique has been further modified as a multiplex process called multiplex real time nucleic acid sequence based amplification (RT-NASBA), which can help in the concurrent detection of different viral infections (Mo et al., 2015). RT-NASBA has been proven to be 10–100 times more sensitive than Multiplex RT-PCR, owing to the isothermal conditions where no time is consumed in heating and cooling and production of copies is faster than RT-PCR. RT-NASBA has been previously used for detection of SARS-CoVs infections, and their sensitivity and specificity was seen to be parallel with RT-PCR diagnosis (Keightley et al., 2005). This technique can be a choice for the rapid diagnosis of COVID-19 during the current pandemic.

Loop Mediated Isothermal Amplification (LAMP)

LAMP is a diagnostic technique that is comparatively less expensive, much sensitive and rapid than RT-PCR. This technique involves the selective amplification of target nucleic acids at a constant temperature, usually 60°C. In this technique 4 to 6 specifically designed primers are used to detect distinct nucleic acid sequences, moreover there is no requirement of initial template denaturation and reaction time is minimized up to 30 minutes using strand-displacement polymerases (Kashir and Yaqinuddin, 2020). For a colorimetric based analysis LAMP

reaction mixture is added with hydroxynepthol blue (HNB) prior to amplification, thus avoiding cross contamination in the future (Malik et al., 2019). Lin Yu et al. have used another approach that combines reverse transcription with LAMP diagnostic technique (RT-LAMP) allowing direct detection of SARS-CoV-2 RNA. This technique was further coupled with a pH indicator, which helped in visual readout of the amplification reaction *via* color change in the reaction mixture (Yu et al., 2020). RT-LAMP based assay that targets S gene of SARS-CoV-2 developed by Hu et al., which showed 88.89% sensitivity and high consistency compared with RT-PCR based diagnostic methods. Time required for this assay was around an hour, considerably lower than RT-PCR (Hu et al., 2020). LAMP technique avoids the use of costly reagents and instruments, thus helping in reducing the cost of diagnosis with rapid results (Cascella et al., 2020). Various studies have highlighted the application of LAMP technique in detecting coronavirus infections in patient samples (Poon et al., 2004; Pyrc et al., 2011). It was further observed that 9 to 10 copies of viral RNA per reaction were sufficient to detect infection giving a 100 fold higher sensitivity than RT-PCR (Mori et al., 2001; Thai et al., 2004; Njiru, 2012). Many other reports are also available on the use of LAMP as a diagnostic tool which includes the work carried out by El-Tholoth et al. (2020) where they demonstrated a two stage LAMP design called COVID-19 Penn-RAMP strategy. This method can be carried out in closed tubes with either colorimetric or fluorescence detection. Their results are not only comparable to RT-PCR but also show 10 fold higher sensitivity when using purified targets (Kashir and Yaqinuddin, 2020). Penn-RAMP is based on the preliminary reaction where outer LAMP primers bind to amplify all targets concurrently through recombinase polymerase. After the first step, a second highly specific reaction initiated. The first reaction specifically uses outer LAMP primers, while the second reaction combines four other RAMP primers. This enhances the sensitivity of this method compared to normal LAMP (Song et al., 2017). For an improved diagnosis, Song's group developed a two stage isothermal Penn-RAMP double stranded DNA amplification assay that combine LAMP and Recombinase Polymerase Amplification (RPA) in a single tube thus making the assay simple and less time consuming, further this method has been integrated to a smart phone application to make it highly accessible and point of care technique (Yang et al., 2020). The challenge related to the LAMP method is the primer optimization and reaction conditions.

Point of Care Testing and COVID-19

Diagnosis of infectious diseases at the bed side of a patient where there is no need of sending patient samples to sophisticated labs is called as Point of Care testing (POC). POC has a very important role to play during community contagious infections because it enables communities to diagnose infection without the complex laboratory infrastructure. POC testing is the only option for the remote areas of any community. One of the Point-of-Care testing protocols is the LFA. Xiang J.; Yan M. et al. had used lateral flow antigen assay for COVID-19 diagnosis (Xiang et al., 2020). LFA is carried out on a strip which is a paper like membrane. The membrane has two lines coated on it among which one line contains gold nanoparticle-antibody conjugate and the other line contains a capture antibody. Patient samples are deposited on the strip. The strip draws proteins from the sample *via* capillary action. As the sample runs over the first line of the strip, antigen binds the nanoparticle-antibody conjugate. This complex then moves to the second line where the capture antibody immobilizes the complex which makes a red or blue line visible on the strip. Individual gold nanoparticles on the strip are red, however clustered gold nanoparticles in solution show a blue color. For IgM, LFA has shown 57% clinical sensitivity, 69% accuracy, and 100% specificity however LFA has shown 81% sensitivity, 86% accuracy, and 100% specificity for IgG. Clinical sensitivity equal to 82% was observed in the tests that detected both IgG and IgM (Xiang et al., 2020). These methods have comparatively less sensitivity than PCR and its variants. Researchers have tried to enhance the sensitivity of LFA-based methods by combining it with RT-LAMP and such related techniques. This combination has been used in MERS-CoV detection (Huang et al., 2018). Based on the principle of LFA, some techniques are specifically developed to detect viral pathogens. Specific High Sensitivity Enzymatic Reporter Unlocking (SHERLOCK) is one such technique developed by Feng Zheng's lab. This technique is very cost-effective and rapid in diagnosing viral pathogens. It has been used for detecting Zika and Dengue virus in patient samples (Gootenberg et al., 2017). For cold-chain and long-term storage SHERLOCK reagents are subjected to lyophilization. SHERLOCK can detect at least 200 copies/ml of serum/urine for Zika viral RNA (Gootenberg et al., 2017). A protocol has been developed by Zhang et al. for diagnosis of COVID-19 using SHERLOCK. It is a three-step diagnostic process. Isothermal amplification, detection and visual readout are the three steps of diagnosis which take less than an hour for the final results (Zhang F. et al., 2020).

CRISPR-BASED POINT OF CARE DIAGNOSIS

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) has emerged as a game changer in the molecular biology experiments. It has also reshaped the diagnostics of the current time. CRISPR are the DNA sequences found in bacteria and archaea that have been extensively used in gene editing experiments. They play an important role in antiviral defense as the sequences are derived from bacteriophages which have previously infected bacteria (Barrangou, 2015). Lots of CRISPR based techniques are currently in use or have a potential to be an option of point of care testing in pathogen diagnosis like SARS-CoV-2. All-In-One Dual CRISPR Cas12a, also termed AIOD-CRISPR is believed to be an ultrasensitive and accurate visual diagnostic technique. In order to initiate a highly specific CRISPR- based detection of nucleic acids a dual crRNA (CRISPR-RNA) is introduced in the reaction mixture (Jeon et al., 2018). All the components for nucleic acid amplification and CRISPR-based detection are mixed in a single tube and are

subjected to isothermal incubation (37°C). This limits contamination because no separate pre-amplification and amplification is needed. AIOD-CRISPR assay has been engineered for detecting SARS-CoV-2 pathogen (Jeon et al., 2018). AIOD-CRISPR system uses a pair of Cas 12a- crRNA complexes generated by two distinct crRNAs. These complexes bind to corresponding sites closer to the primer recognition sites in the target sequence. The reaction takes place in a single tube that contains: Cas12a-crRNA complex generated separately, recombinase polymerase amplification (RPA) primers, recombinase, ssDNA-FQ reporters, strand displacement DNA polymerase, ssDNA binding protein and target sequences. Binding of Cas12a-crRNA complex to the target sequence leads to the cleavage of nearby ssDNA-FQ *via* activated Cas 12a this process produces a fluorescence signal (Ding et al., 2020). In the study carried out by Xiong Ding et al. a plasmid containing 384 nucleotide N gene cDNA was used as the target to develop AIOD-CRISPR assay. This assay could detect 1.3 copies of SARS-CoV-2 N plasmid in both visual and real time detection within 40 minutes. AIOD-CRISPR has the benefit of being used as a Point of Care diagnosis for pathogenic diseases like COVID-19, owing to its simple use and visual detection by fluorescence or color change (Ding et al., 2020). Broughton and group had come up with a CRISPR Cas12 based detection method which they claim to be the most rapid (<40) minute technique among isothermal nucleic acid based POC technique. This CRISPR Cas12-based technique is used to detect SARS-CoV-2 from extracted RNA samples of suspected patients (Chen et al., 2018; Chiu, 2018). This technique has been designed to perform simultaneous reverse transcription and isothermal amplification using RT-LAMP to the RNA extracted from nasopharyngeal swabs followed by Cas12-mediated detection of virus. The technique has been named DNA endonuclease targeted CRISPR trans reporter (DETECTR) assay (Broughton et al., 2020). DETECTR assay showed a comparable accuracy related to RT-PCR. Some key advantages of this assay are isothermal amplification thus avoiding the need of thermocycling, easy to use systems like lateral flow strips, avoiding the use of complex laboratory infrastructure. This technique can be easily mobilized to the hot spots of COVID-19 transmission to ease the diagnosis process. A distinct approach in response to the increasing demand of global rapid testing for detecting SARS-CoV-2 infection was showcased by Rauch et al. which is CRISPR Cas13 based diagnostic method. The test has been named as CREST (Cas13-based, Rugged, equitable, scalable testing) (Rauch et al., 2020). This method is sensitive and a field deployable procedure to combat diagnosis crisis in pandemic situations. The method is easy and can be used at minimal infrastructure sites. CREST uses easily available protein, low cost thermocyclers and easy to use fluorescent visualizers which makes it a very low cost and easily affordable diagnostic technique. An another portable, accurate and mobile phone based diagnostic assay was reported as an amplification free assay using Cas13a for directly detecting SARS-CoV-2. The assay utilizes patient nasal swab to detect SARS-CoV-2 infection which can be analyzed with the help of a smart phone, this technology has made testing portable and affordable in the low resource areas. It has exhibited limit of detection in 10 fM of RNA target. This technique is much advanced than CRISPR COVID (Hou et al., 2020) as previously mentioned in this manuscript, which provides qualitative results using isothermal amplification. The sensitivity of other CRISPR based tests have been taken into consideration while developing this assay. Using best combination of crRNAs for entire viral genome makes this assay best fit for point of care diagnosis (Fozouni et al., 2020). A highly accurate, single nucleotide variant detection system developed by IGIB India employs *Francisella novicida* (Fn-Cas9) based enzymatic readout for nucleotide detection and nucleobase identification. They have named this approach as FnCas9 Editor Linked Uniform Detection Assay (FELUDA) (Azhar et al., 2020).

PROTEIN-BASED COVID-19 DIAGNOSIS

Viral proteins as antigens or antibodies generated in response to viral infection can serve as the means of diagnosis in viral infectious diseases like COVID-19. Relying on viral proteins for detection is cumbersome as the viral load fluctuates during the course of infection, so antibodies can better serve the diagnosis process (To et al., 2020). It has been an age old practice to detect specific antigens by antibodies that are directed against these antigenic epitopes using immunoblot assays (Hartstein et al., 1989). COVID-19 can be diagnosed indirectly by detecting antibodies generated in the patient's blood in a certain window period. However, there is a challenge of cross-reactivity between antibodies generated against SARS-CoV-2 and antibodies against other coronaviruses. A high frequency of cross reactivity was observed by a study carried out by Lv et al. where they had tested plasma samples taken from fifteen COVID-19 patients (Lv et al., 2020). According to a study carried out by Bin Ju et al. where the antibody response was characterized in eight COVID-19 patients and around 206 mAbs specific to SARS-CoV-2 receptor binding domain were also isolated. They observed the diverse antibody generation in the set of patients and proposed that such antibodies can serve as prophylactic and therapeutic strategies against COVID-19 (Ju et al., 2020). Liu et al. used SARS-CoV-2 IgG/IgM antibody test kit that was manufactured by a Chinese Biotechnology company and further approved by the China Food and Drug Administration. The protocol used around 5 ml of fasting blood of every participant. After collecting serum, the SARS-CoV-2 IgG/IgM were detected. The kit consists of three detection lines, control (C) line/zone, G zone and M zone. C line appears when the sample is flushed over it. A red test line in G and M zones will indicate the SARS-CoV-2 IgG/IgM in the samples. The test is to be repeated if the C line doesn't appear on the strip (Liu et al., 2020). A schematic diagram showing the general protocol of IgG/IgM rapid and Point of Care COVID-19 diagnosis is depicted in **Figure 2**. Study conducted by Zhao et al. who had used enzyme linked immunosorbent assay (ELISA) kit developed by Baijing Wantai pharmaceutical company. The kit works on the principle of double antigen sandwich assay. A recombinant antigen containing receptor-

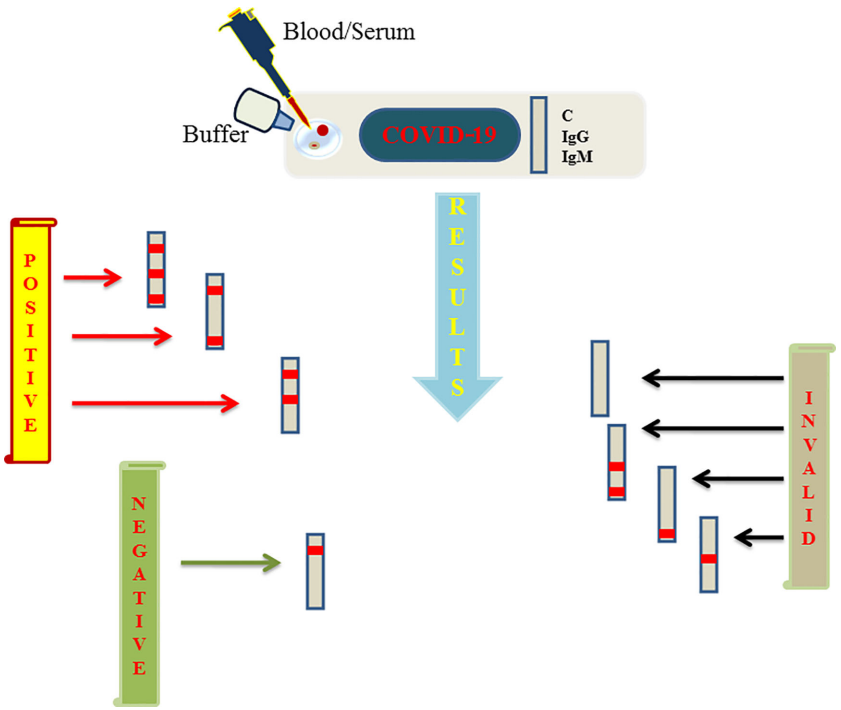

FIGURE 2 | Figure represents schematics regarding the rapid and point of care test based on lateral flow assay (LFA) launched by various biotechnology companies and further approved by FDA to aid the COVID-19 testing. A sample well is flushed with blood/serum and buffer on the strip. Strip consists of three lines among which two are G and M detection lines representing IgG and IgM respectively and a quality control line represented as C line. If a red line appears on control, G and M or control and one among G/M, the test is said to be positive. Negative test shows only single redline on C line only. If C line is not visible the results are considered as invalid which needs to be repeated again.

binding domain (RBD) of the spike protein of SARS-CoV-2 expressed in mammalian cells was used as an immobilized horseradish peroxidase (HRP)-conjugate antigen. To detect IgM in the patient samples, IgM μ-chain capture method was used, however IgG was detected by using indirect ELISA kit based on recombinant nucleoproteins. They have claimed around 99% sensitivity of IgM and IgG antibodies for this assay (Zhao et al., 2020). Elevated levels of C reactive-protein and D-dimer and low levels of lymphocytes, blood platelets and leukocytes were shown by Guan et al. in the SARS-CoV-2 infected patients. The challenge in using them as biomarkers is that such markers are also found in different other ailments and abnormalities (Guan et al., 2020). Based on different techniques, there are various FDA approved diagnostic kits or kits with an emergency approval in the market. **Table 1** summarizes various such diagnostic kits used for diagnosis of COVID-19. A biosensor-based point of care test strategy was also reported by some research groups where they are claiming rapid antigen detection in saliva of COVID-19 positive patients. The strategy includes developing of a U bent Fiber optic probe over which the gold nanoparticles are immobilized. The anti-N-protein antibody (monoclonal) covalently conjugates with immobilized nanoparticles *via* thiol-PEG-NHS based binding. This biofunctionalized system are used to detect COVID-19 by applying saliva samples. The device is named as Fiber -Optic Biosensor device and works on the principle of monitoring an optical power loss in light (Murugan et al., 2020). Another setting of biosensor development for COVID-19 as highlighted by Pooja Nag et al. (2020) is an antibody immobilization on either polyaniline or gold nanoparticle coated optical fibers for specific detection of viral proteins in the sample, viral binding with the immobilized antibodies will change the refractive index in the local environment, thus causing change in the light intensity or absorption. Viral surface protein is also immobilized on the surface of optical fiber for the detection of IgG/IgM in the patient samples. The limit of detection claimed by the researchers in such setting was 100 U/ml in an hour (Nag et al., 2020). The sensitivity of such biosensor-based settings cannot be compared with RT-PCR sensitivity for a reason that antigen antibody interaction or immune response cannot be considered as precise indicators of pathogen infection or disease propagation. Another reason is that two types of pathogens can elicit same type of immune response which is a drawback of this setting in specificity.

DISCUSSION

During the pandemic, the only way to tackle with the pathogen is to limit its spread which is only possible if the affected people get detected and separated at the earliest. This review has tried to compile the different diagnostic approaches used by academic labs and clinicians to diagnose COVID-19 disease since the

TABLE 1 | FDA approved diagnostic kits or kits with an emergency approval in the market to be used for the detection of COVID-19.

S/No	Name of testing kit	Technology used	Result time	Samples used	Authorization
01	InBios Smart Detect SARS-CoV-2 rRT-PCR Kit	RT-PCR	4 h	Nasal/Nasopharyngeal swabs	EUA
02	CO-DIAGNOSTICS INC. Logix Smart Coronavirus 2019 (COVID-19) Kit	RT-PCR	Not mentioned	Respiratory tract samples/ serum	EUA
03	Gnomegen COVID-19 RT-Digital PCR Detection Kit	RT-digital PCR	Not mentioned	Not mentioned	EUA
04	ScienCell SARS-CoV-2 Coronavirus Detection Kit	RT-qPCR	Not mentioned	Respiratory specimen/serum	EUA
05	Luminex ARIES SARS-CoV-2 Assay	RT-PCR	2 h	Nasopharyngeal swabs	EUA
06	Luminex NxTAG CoV Extended Panel	RT-PCR	>2 h	Nasopharyngeal swabs	EUA
07	BD BioGX SARS-CoV-2 Reagents for the BD MAX™ System	RT-PCR	Not mentioned	Nasopharyngeal/ Oropharyngeal swabs	EUA
08	BD SARS-CoV-2 Reagents for the BD MAX™ System	RT-PCR	Not mentioned	Nasopharyngeal/ Oropharyngeal swabs	EUA
09	QIAGEN QIAstat-Dx Respiratory SARS-CoV-2 Panel	RT-PCR	1 h	Nasopharyngeal swabs	EUA
10	Abbot ID NOW™ COVID-19 Assay	Point of care/isothermal	15 min	Nasopharyngeal/ Oropharyngeal swabs	EUA
11	BGI Genomics Real-Time Fluorescent RT-PCR Kit	Taq Man RT-PCR	3 h	Throat swabs	EUA
12	Avellino CoV-2 Test	RT-PCR	1 h	Nasopharyngeal/ Oropharyngeal swabs	EUA
13	Avellino CoV-2 Test	PCR/point of care	30 min	Throat and nasal swabs	EUA
14	BioFire Diagnostics BioFire® COVID-19 Test	Nested/Multiplexed/RT-PCR	1 h	Throat and nasal swabs	EUA
15	Primerdesign Ltd. COVID-19 genesig Assay	RT-PCR	<1 h	Nasopharyngeal/ Oropharyngeal swabs	EUA
16	ThermoFisher Scientific TaqPath COVID-19 Multiplex Diagnostic Solution	RT-PCR	<2 h	Nasopharyngeal/ Oropharyngeal swabs	EUA
17	The Centers for Disease Control and Prevention (CDC) 2019-nCoV Diagnostic Panel	RT-PCR	>2 h	Upper/lower respiratory specimen	EUA
18	Cellex qSARS-CoV-2 IgG/IgM Rapid Test	LFA	15-20 min	Blood/serum	EUA
19	TaqPath™ COVID-19 Combo Kit	RT-PCR	<1 h	Self-collected saliva	FDA

These approved kits are for the test of SARS-CoV-2 under Emergency Use Approval (EUA) and the information is available till April 9, 2020 which is mentioned here. It includes technique by which the testing kit operates, time of result and sample collection (https://hitconsultant.net/2020/04/23/in-depth-32-fda-approved-covid-19-testing-kits/#.XrZnkUQzbIV).

identification of SARS-CoV-2 till now. Identification of SARS-CoV-2 in Wuhan, China using sequencing techniques was a major breakthrough (Rothan and Byrareddy, 2020) because its identification led scientists to progress for its diagnosis and therapeutic studies. The first recommended technique for its diagnosis by the CDC China was RT-PCR. This technique is much used during the current COVID-19 pandemic (Corman et al., 2020). It is the same technique that was used to diagnose SARS-CoV in 2002. The lessons learned from SARS-CoV outbreak has guided the very early identification of SARS-CoV-2 infection using sequencing and RT-PCR techniques. During the course of time, since its identification, there has been an immense study on developing rapid nucleic acid-based tests to detect COVID-19 disease among which SHERLOCK, CRISPR and other lateral flow based diagnostic kits are important to mention. These diagnostic approaches are parallelly competing in diagnostic accuracy with the RT-PCR-based diagnosis, however the biosensor related diagnosis needs more understanding and optimization to make them fit for pathogen diagnosis without the need of further confirmations using RT-PCR based -tests. Another approach was the establishment of serological-based diagnostic tests which are comparatively easy to handle and don't need sophisticated machines or trained personnel like RT-PCR and can be easily used in the home settings so as to decrease exposure of health practitioners who are at high risk of encountering an infection during this pandemic. This manuscript has tried to bring in light various serological-based diagnostic approaches as depicted in **Figure 2** that is based on IgG/IgM antibodies. Asymptomatic spread of COVID-19 as reported by some research groups, made it crucial to develop multiplex and Point-of-Care techniques like isothermal amplification, CRISPR-based techniques and microfluidic techniques, so that they can be used to test the majority of the population and isolate infected persons mostly in remote areas, quarantine centers, in developing countries which lack enough resources and skills

Data on COVID-19 is evolving very rapidly, and there is no doubt that some of the specifics of this article may change as more studies become available. This review will help a reader to understand the established and other promising techniques in managing pandemic diseases like COVID-19.

AUTHOR CONTRIBUTIONS

JI and KUI conceived the idea. KUI wrote the review article. JI has read, corrected the write up, and finalized. All authors contributed to the article and approved the submitted version.

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Critical Care Response to the Outbreak of COVID-19: The Experience From Guangdong Province, China

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In December 2019, human infection with a novel coronavirus, known as SARS-CoV-2, was confirmed in Wuhan, China, and spread rapidly beyond Wuhan and around the world. By 7 May 2020, a total of 84,409 patients were infected in mainland China, with 4,643 deaths, according to a Chinese Center for Disease Control and Prevention report. Recent studies reported that critically ill patients were presented with high mortality. However, the clinical experiences of patients with coronavirus disease 2019 (COVID-19) have not been described in Guangdong Province, where by 7 May 2020, 1,589 people had been confirmed as having COVID-19 but with a very low mortality of 8 death (0.5%). Here, we describe the experience of critical care response to the outbreak of SARS-CoV-2 in Guangdong Province in the following points: Early intervention by the government, Establishment of a Multidisciplinary Working Group, Prompt intensive care interventions, Adequate ICU beds and Human resource in ICU, Infection control practices.

Keywords: ICU–intensive care unit, COVID-19, Guangdong Province, mortality, critical response

INTRODUCTION

In December 2019, human infection with a novel coronavirus, known as SARS-CoV-2, was confirmed in Wuhan, China, and spread rapidly beyond Wuhan and around the world (1, 2). By May 7, 2020, a total of 84,409 patients were infected in mainland China, with 4,643 deaths, according to a Chinese Center for Disease Control and Prevention (CCDC) report (3). Recent studies reported that critically ill patients were presented with high mortality (4–8).

Sharing experiences of the management of coronavirus disease 2019 (COVID-19) patients from different centers are of vital importance for combating the pandemic. Fangcang shelter hospitals were rapidly built and responded to COVID-19 emergencies in Wuhan, China (9). Increasing intensive care unit (ICU) capacity and forecasting ICU demand were performed for more critically ill patients in the epicenter of Lombardy, Italy (10). However, the clinical experiences of patients with COVID-19 have not been well-described outside of the epidemic centers. Here, we introduce the critical care response to the outbreak of SARS-CoV-2 in Guangdong Province, where 1,589 people had been confirmed with COVID-19 but had a very low mortality of 8 deaths (0.5%) by May 7, 2020 (3).

EARLY INTERVENTION BY THE GOVERNMENT

Guangdong Province had initiated a level-one response to the public health emergency in January 2020. Under the coordination of the Government of Guangdong Province, the traffic and movement in the regions of Guangdong were restricted. Furthermore, early case detection, early reporting, early isolation, and contact quarantine, and early supportive care were conducted and led by the Government. Thus, the infected patients were effectively reduced, and the pandemic was effectively contained in Guangdong in a relatively short period.

THE ESTABLISHMENT OF A MULTIDISCIPLINARY WORKING GROUP

At the very beginning of the SARS-CoV-2 outbreak in Guangdong, a COVID-19 study group and an intensivist-led multidisciplinary team consisting of respiratory physicians, infectious disease specialists, and radiologists were established and participated in combatting the COVID-19 pandemic under the coordination of the Government of Guangdong Province. In addition, to help other cities in Guangdong, which faced challenges but had limited ICU resources, Guangdong released a "pairing-up support" plan. An expert team from each tertiary hospital in Guangzhou, the capital city of Guangdong Province, was paired up and sent to each designated hospital in remote areas across Guangdong. In a designated hospital, the intensivists made their rounds twice every day in the isolation wards and provided consultation for mild patients in these isolation wards. A rapid response team consisting of frontline health staff was also established for the care of patients with sudden clinical deterioration. All confirmed patients were screened for high-risk cases by the following measurements: (1) 5–7 days after onset; (2) age >50 years; (3) overweight patients, pregnant women, and children; (4) underlying health condition (e.g., diabetes, cardiovascular diseases, etc.); (5) persistent fever; (6) large lesion and/or more than two lesions in lungs; (7) fast decline in lymphocytes; (8) respiratory rate > 25/min and /or SpO2 <95% (during rest and breathing room air); (9) hypoxemia with normal heart rate; (10) altered mental status; (11) decreased appetite; and (12) dysfunction of extrapulmonary organs.

The above measures enabled the frontline health staff to identify patients with suddenly deteriorated statuses and intervene early to stabilize the patient. If patients presented with a respiratory rate >30/min, PaO_2/FiO_2 <250 mmHg (during rest and room air) or any signs of organ failure, they would be transferred to ICU for further monitoring and an intensivist-led multidisciplinary team would take over their treatment in a timely manner. In addition, the frontline health staff in the designated hospital would collect all new medical data of severe and critical cases every day and send them to the COVID-19 Data Center in Guangzhou. The data would be screened and discussed by the senior experts in the multidisciplinary team. A well-designed web-based video consultation system for critically ill patients with COVID-19 was established and applied across different cities in Guangdong Province. This allowed the multidisciplinary team to share their experience with the frontline health staff and help with the management of critical illness remotely across hospitals.

PROMPT INTENSIVE CARE INTERVENTIONS

A research letter published in JAMA Network Open described 168 patients who died of COVID-19 from 21 hospitals in Wuhan which was the epicenter of the COVID-19 outbreak in China. The results showed that only approximately one-fifth of non-survivors with COVID-19 received invasive mechanical ventilation and further aggressive respiratory support prior to death, indicating that many patients had delayed intubation (11). The authors attributed the low proportion of invasive mechanical ventilation in non-survivors to the lack of invasive mechanical ventilators and patient management by a non-intensivist dominated medical team (11). Distinct from the characteristics of critically ill COVID-19 patients reported in Wuhan and other epicenters of the outbreak (4–7), patients in Guangdong were managed by a group of trained intensivists and specialist nurses with sufficient ventilators, extracorporeal membrane oxygenation (ECMO) equipment, and personal protective equipment. Moreover, as shown in our preprint data (12), the relatively low APACHE II and SOFA scores (the median APACHE II and SOFA scores was 14 and 4.0, respectively), of all patients at ICU admission indicated that fewer critically ill patients were admitted to the ICU to receive high intensity monitoring and prompt intensive care interventions. Besides, in our preprint study, 9 patients received ECMO due to a low but not too low PaO_2/FiO_2 ratio [85.2 (58.3–103.4) mmHg] or a high but not too high $PaCO_2$ level [59.2 (53.5–68.9) mmHg], indicating early ECMO was initiated in patients presenting rapidly progressive respiratory failure (criteria for ECMO initiation: PaO_2/FiO_2 from 120 to 100 mmHg and/or $PaCO_2$ from 50 to 55 mmHg, <4 h).

ADEQUATE ICU BEDS AND HUMAN RESOURCES IN THE ICU

The outbreak has led to a significant increase in the need for ICU beds. All designated hospitals in Guangdong actively prepared more ICU beds for the potential surge of critically ill patients with COVID-19. Meanwhile, the doctor/nurse-to-patient ratio was increased to intensively monitor the critically ill patients. Collectively, these measures may have contributed to the low mortality rate in Guangdong, which may reflect a lower mortality of the disease if there are adequate ICU beds, intensivists, and special nurses.

INFECTION CONTROL PRACTICES

Strict isolation and protection measures were a top priority. Generally, confirmed patients were first isolated in single rooms with negative pressure. If more patients arrived, they were

placed and treated in a closed unit. Medical staff were well-trained with handwashing, environmental cleaning, and had adequate personal protective equipment. Standard practices were established during airway management, including non-invasive mechanical ventilation (NIV), intubation, and bronchoscopy. According to the Chinese management guideline for COVID-19 (version 7.0), NIV can be considered for patients if standard oxygen therapy has failed (13). In our practices, for patients with NIV, they were monitored closely and cared for in a single room with negative pressure where intubation could be facilitated in the event of decompensation. In addition, an advanced, highly hydrophobic bacterial/viral filter (Iso-Gard HEPA Light, Item #: 28022) was connected to an NIV mask to reduce the production of aerosol generation. For intubation, an expert who specialized in the procedure was recommended. Endotracheal intubation was done in an airborne infection isolation room. The use of bronchoscopy was recommended for subglottic secretion drainage and tracheal aspirate in intubated patients. Personal protective equipment, including protective clothing, head coverings, double-gloving, N95 respirators, and eye protection, were provided during intubation and bronchoscopy. No medical staff were infected when dealing with patients with COVID-19 in Guangdong Province.

CONCLUSION

The high mortality of patients in recent reports (4–8) may reflect the crisis of critical care medicine rather than the nature of COVID-19. Our experiences in Guangdong in the management of mild and critically ill patients with COVID-19 are of vital importance for intensive care colleagues in the community all around the world, who need to be prepared for any outbreaks in the future as it may became a seasonal threat for public health.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

XL, YoX, ZX, NZ, TQ, and YL: conception and design. All authors: administrative support, provision of study materials or patients, collection and assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript.

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Clinical Characteristics of Patients With Progressive and Non-progressive Coronavirus Disease 2019: Evidence From 365 Hospitalised Patients in Honghu and Nanchang, China

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Background: Coronavirus disease (COVID-19) has swept around the globe and led to a worldwide catastrophe. Studies examining the disease progression of patients with non-severe disease on admission are scarce but of profound importance in the early identification of patients at a high risk of deterioration.

Objectives: To elucidate the differences in clinical characteristics between patients with progressive and non-progressive COVID-19 and to determine the risk factors for disease progression.

Study design: Clinical data of 365 patients with non-severe COVID-19 from 1 January 2020 to 18 March 2020 were retrospectively collected. Patients were stratified into progressive and non-progressive disease groups. Univariate and multivariate logistic regression analyses were performed to determine the independent risk factors for disease progression.

Results: Compared with patients with non-progressive disease, those who progressed to severe COVID-19 were older and had significantly decreased lymphocyte and eosinophil counts; increased neutrophil and platelet counts; lower albumin levels; higher levels of lactate dehydrogenase, C-reactive protein (CRP), creatinine, creatinine kinase, and urea nitrogen; and longer prothrombin times. Hypertension, fever, fatigue, anorexia, bacterial coinfection, bilateral patchy shadowing, antibiotic and corticosteroid administration, and oxygen support had a significantly higher incidence among patients with progressive disease. A significantly longer duration of hospital stay was also observed in patients with progressive disease. Bilateral patchy shadowing (OR = 4.82,

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95% CI: 1.33–17.50; $P = 0.017$) and elevated levels of creatinine (OR =6.24, 95% CI: 1.42–27.40; $P = 0.015$), and CRP (OR = 7.28, 95% CI: 2.56–20.74; $P < 0.001$) were independent predictors for disease progression.

Conclusion: The clinical characteristics of patients with progressive and non-progressive COVID-19 were significantly different. Bilateral patchy shadowing and increased levels of creatinine, and CRP were independent predictors of disease progression.

Keywords: coronavirus disease 2019, disease progression, severe acute respiratory syndrome coronavirus 2, bilateral patchy shadowing, creatinine kinase, creatinine, C-reactive protein

INTRODUCTION

Coronavirus disease (COVID-19) has swept around the world, with more than 2.9 million confirmed cases resulting in 204,000 deaths as of 27 April 2020. Its pathogen, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), can spread rapidly between humans, causing the number of confirmed cases to increase quickly. The severity spectrum of COVID-19 ranges from mild to critical, but the majority of infections have not been severe (1–6). Eighty-one percent of confirmed cases have been mild to moderate, including those with and without pneumonia; 14% had severe disease (dyspnoea, blood oxygen saturation $\leq$ 93% at rest, PaO_2/FiO_2 ratio $\leq$ 300 mmHg, respiratory rate $\geq$ 30 breaths/min, and lung infiltrates appearing within 24–48 h in $>$ 50% of the lung field); and 5% have presented in critical condition (septic shock, respiratory failure, and/or multiple organ dysfunction) (7). It has been reported that mild symptoms may become severe within 5–7 days (1, 7), suggesting that patients initially hospitalised with mild or moderate disease are still at a risk of severe or critical illness. If the treatment of severely ill patients lies "downstream" to the control of COVID-19, then it is the "upstream" strategy to identify patients who are at a risk of progressing to severe disease. Early identification of these patients coupled with early intervention could save lives and alleviate the burden on the health care system.

Previous studies placed huge significance on transmission dynamics (8, 9), the clinical manifestations of COVID-19 (3, 4, 7), characteristics of patients with severe/critical disease (6, 10), and the differences between patients with severe and non-severe disease (11, 12). However, studies shedding light on patients with non-severe disease on admission and tracking their disease progression are scarce but of profound importance. Therefore, in this study, we tracked patients admitted with mild or moderate COVID-19 and probed the discrepancies between progressor and non-progressor patients to determine the risk factors for disease progression. In this way, we hope to guide the development of effective early predictive tools to identify patients at a high risk for developing severe disease.

MATERIALS AND METHODS

Study Design and Patient Cohort

This retrospective study was approved by the Medical Ethics Committee of Nanfang Hospital of Southern Medical University. Written informed consent from all participating patients was obtained.

We retrospectively collected data from 690 patients with confirmed COVID-19 diagnosed between 1 January 2020 and 18 March 2020 from hospitals in Honghu and Nanchang. Patients were diagnosed according to the guidelines of the Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia (Trial Version 7) (13). SARS-CoV-2 nucleic acid tests were positive in all participants, and all diagnostic criteria were met according to the guidelines. After excluding patients who had incomplete clinical data, 442 patients were retained, among whom 365 patients with mild or moderate disease were included in the final analysis. Of the 365 patients, 285 were from the People's Hospital of Honghu, while 80 were from the First Affiliated Hospital of Nanchang University.

Definition

All patients were diagnosed and typed under the guidance of the Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia (Trial Version 7) (13) developed by the Chinese National Health Commission and the State Administration of Traditional Chinese Medicine. Patients with mild symptoms and no signs of pneumonia on imaging were regarded to have mild disease, while fever and respiratory symptoms were indicators of moderate disease. Patients who suffered from respiratory distress, arterial partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2) $\leq$ 300 mmHg, oxygen saturation $\leq$ 93% at rest, or with obvious lesion progression on chest imaging within 24–48 h of $>$50%, were considered to have severe disease. When respiratory failure, shock, or other organ failure appeared, patients were diagnosed with critical disease. In this study, patients who maintained mild or moderate symptoms during the entire hospital stay were assigned to the non-progressive disease group, and those with mild or moderate disease on admission who later progressed to a severe or critical status were assigned to the progressive disease group.

Data Collection and Outcome Evaluation

Clinical electronic medical records, nursing records, laboratory findings, and radiological reports for all included patients with COVID-19 were reviewed. For each patient, detailed admission data were collected, including demographic information, signs and symptoms, comorbidities, imaging reports, and laboratory test results. After admission, the treatment, disease severity

state, outcomes, and length of hospital stay were also recorded. The treatments were conducted before disease progression. Two researchers independently checked the electronic medical reports and recorded the daily assessment of the disease severity. The progression from non-severe to severe disease was monitored.

Statistical Analysis

Continuous variables are presented as mean and standard deviation (SD) while normally distributed and otherwise as medians and interquartile ranges (IQR). Categorical variables are presented as frequencies and percentages. For continuous variables, the comparisons between progressive and non-progressive disease groups were performed using the Student's *t*-test or the Mann–Whitney U test, as appropriate. For categorical variables, comparisons were conducted using the Pearson's chi-square test or Fisher's exact test, as appropriate. To investigate the risk factors associated with disease progression, univariate and multivariate logistic regression models were used. In the univariate logistic regression analyses, variables with $P < 0.05$ were regarded as potential risk factors and included in multivariate regression analysis by a backward elimination procedure (likelihood ratio test and elimination if $P > 0.1$). Statistical analyses were conducted with the SPSS software version 25.0 and R version 4.0.2. The pROC package was employed to draw the receiver operating characteristic (ROC) curves and calculate the area under the curve (AUC). All statistical tests were two-sided, and $P < 0.05$ was regarded as statistically significant.

RESULTS

By 18 March 2020, a total of 690 patients with confirmed COVID-19 were recruited, of whom 365 patients from the People's Hospital of Honghu and the First Affiliated Hospital of Nanchang University with complete medical records and who were diagnosed with mild or moderate COVID-19 on admission were included in this study.

The demographics and baseline clinical characteristics of these patients are shown in **Tables 1**, **2**. The patients' mean age was 46.8 years [SD 15.5], 74 patients (20.3%) were older than 60 years, and 176 (48.2%) were men. Only 7 (1.9%) were current smokers. Hypertension (10.7%) was the most commonly observed comorbidity, followed by diabetes mellitus (4.7%), chronic liver disease (2.5%), and cardiovascular disease (1.6%). Among these patients, 221 (60.5%) had a fever, 194 (53.2%) had a cough, 253 (69.3%) had multi-lobular infiltration, and 228 (62.5%) had bacterial co-infection. The patients' median temperature on admission was 36.8 degrees Centigrade (IQR 36.5–37.1). As for treatment, 352 (96.4%) received antiviral therapy, 251 (68.8%) underwent antibiotic therapy, 131 (35.9%) were treated with corticosteroids, and 149 (40.8%) required oxygen support. In terms of the outcomes, 363 patients (99.5%) were ultimately discharged from the hospital, and 2 patients (0.5%) died during their hospital stay. The median length of hospital stay was 14 days (IQR 10–20).

The patients were divided into progressive and non-progressive disease groups, depending on whether their disease became severe after admission. The results of the comparisons between groups are shown in **Tables 3**, **4**. Compared with patients with non-progressive disease, those who progressed to severe COVID-19 were older (59.3 years [SD 13.2] vs. 45.9 years [SD 15.3], $P < 0.001$). The proportion of patients with hypertension, fever, fatigue, anorexia, bacterial co-infection, or bilateral patchy shadowing was significantly higher among patients with progressive disease. From the perspective of laboratory findings, we found significantly decreased lymphocyte

TABLE 1 | Demographics and baseline characteristics of patients with non–severe COVID-19.

Characteristic	All patients ($n = 365$)
Age, years	46.8 ± 15.5
≥60	74 (20.3)
Male	176 (48.2)
Laboratory findings	
Red blood cell count, $\times 10^9$/L	4.5 (4.1–4.9)
White blood cell count, $\times 10^9$/L	6.0 (4.3–6.9)
<10	346 (94.8)
Lymphocyte count, $\times 10^9$/ L	1.5 (1.1–1.8)
<0.8	36 (9.9)
Neutrophil count, $\times 10^9$/L	4.0 (2.5–4.7)
>6.4	32 (8.8)
Eosinophil counts, $\times 10^9$/L	0.1 (0.0–0.1)
Hemoglobin, g/dL	135.3 (125.0–147.0)
Platelet count, $\times 10^9$/L	241.4 (178.0–293.5)
<100	9 (2.5)
Albumin, g/L	42.7 (38.1–44.9)
<34	27 (7.4)
Total bilirubin, μmol/L	11.1 (7.3–13.4)
>17.1	44 (12.1)
Direct bilirubin, μmol/L	3.4 (2.2–3.8)
Alanine aminotransferase, U/L	30.6 (13.0–34.5)
>40	66 (18.1)
Prothrombin time, s	12.6 (11.9–13.2)
≥16	4 (1.1)
Creatinine, μmol/L	65.6 (50.9–74.2)
≥133	10 (2.7)
Urea nitrogen, mmol/L	4.6 (3.3–5.1)
Lactate dehydrogenase, U/L	219.4 (167.5–246.0)
>245	92 (25.2)
Creatinine kinase, U/L	94.0 (44.5–103.0)
>200	27 (7.4)
C-reactive protein, mg/L	16.3 (0.5–15.2)
≥10	109 (29.9)
Abnormalities on chest CT	
Ground-glass opacity	181 (49.6)
Local patchy shadowing	55 (15.1)
Bilateral patchy shadowing	200 (54.8)
Interstitial abnormalities	11 (3.0)

Continuous variable data are presented as the mean (standard deviation) or median (interquartile ranges, IQR). Classified variable data are presented as n (%).

TABLE 2 | Smoking history, comorbidity, signs and symptoms, treatment and clinical outcomes of patients with non-severe COVID-19.

Characteristic	All patients ($n = 365$)
Smoking history	
Current smokers	7 (1.9)
Ex-smokers	0 (0.0)
Comorbidity	
Hypertension	39 (10.7)
Diabetes mellitus	17 (4.7)
Cardiovascular disease	6 (1.6)
Cerebrovascular disease	3 (0.8)
Chronic liver disease	9 (2.5)
COPD	2 (0.5)
Asthma	1 (0.3)
Renal disease	2 (0.5)
Cancer	2 (0.5)
Signs and symptoms	
Fever	221 (60.5)
Temperature on admission (°C)	36.8 (36.5–37.1)
Highest temperature (°C)	37.4 (36.6–38.0)
<37.3	192 (52.6)
37.3–37.9	61 (16.7)
38–38.9	92 (25.2)
≥39	20 (5.5)
Cough	194 (53.2)
Sputum production	52 (14.2)
Nasal congestion	6 (1.6)
Fatigue	77 (21.1)
Headache	17 (4.7)
Sore throat	37 (10.1)
Shortness of breath	28 (7.7)
Dyspnea	13 (3.6)
Anorexia	27 (7.4)
Diarrhea	15 (4.1)
Nausea	13 (3.6)
Myalgia or arthralgia	1 (0.3)
Combination of bacterial infection	228 (62.5)
Treatment	
Antiviral therapy	352 (96.4)
Antibiotic therapy	251 (68.8)
Use of corticosteroid	131 (35.9)
Oxygen support	149 (40.8)
Clinical outcomes	
Discharge from hospital	363 (99.5)
Length of hospital stay	14.0 (10.0–20.0)
Death	2 (0.5)

Continuous variable data are presented as the mean (standard deviation) or median (interquartile ranges, IQR). Classified variable data are presented as n (%). COPD, Chronic obstructive pulmonary disease.

and eosinophil counts; increased neutrophil counts; lower albumin levels; higher levels of lactate dehydrogenase, C-reactive protein (CRP), creatinine, creatinine kinase, and urea nitrogen; and a longer prothrombin time in patients with progressive disease than in patients with non-progressive disease. Notably, a significantly higher ratio of patients with progressive disease received antibiotic therapy and had corticosteroids and oxygen support administered. A significantly longer length of hospital stay was also observed in patients with progressive disease.

To clarify the risk factors for disease progression, univariate and multivariate logistic analyses were conducted (**Table 5**). Univariate logistic regression identified 13 potential risk factors for disease deterioration. Next, these clinical factors were analysed by multivariate logistic regression, and three variables were retained, suggesting that bilateral patchy shadowing (OR = 4.82, 95% CI: 1.33–17.50; $P = 0.017$) and elevated levels of creatinine (OR = 6.24, 95% CI: 1.42–27.40; $P = 0.015$), and CRP (OR = 7.28, 95% CI: 2.56–20.74; $P < 0.001$) were independent predictors of disease progression. Creatine kinase was statistically significant in the univariate analyses but insignificant in the multivariate analyses. Moreover, ROC curves were used to evaluate the discrimination and predictive ability of creatinine kinase, creatinine, C-reactive protein and bilateral patchy shadowing (**Supplementary Figure 1**). Results showed that bilateral patchy shadowing, creatinine and CRP obtained AUCs of 0.681, 0.766, 0.806, respectively. Sensitivity and specificity of these parameters at the clinical threshold were also presented. According to ROC analyses, creatinine, C-reactive protein and bilateral patchy shadowing performed well to categorise the patients into severe and non-severe groups.

DISCUSSION

The majority of patients were diagnosed with mild or moderate COVID-19 upon initial hospitalization. Although they had mild symptoms on admission, they were still at a risk of illness deterioration. There is a medical need to clarify the differences between patients with progressive and non-progressive disease in order to predict which patients are at risk of exacerbation and to conduct early interventions to reduce mortality. In this study, we assessed 365 patients with mild or moderate confirmed COVID-19 on admission. Twenty-six (7.1%) patients progressed to severe or critical disease after admission and were assigned to the progressive disease group, while the others were assigned to the non-progressive disease group. We elucidated the discrepancies in the clinical characteristics between patients with progressive and non-progressive disease and investigated the independent risk factors that could influence disease deterioration, with the purpose to enhance the early identification of at-risk populations.

Many studies have investigated the risk factors associated with disease severity by comparing patients with non-severe COVID-19 and those with severe COVID-19. Liu et al. (10) reported that age, fever, SpO_2, and cough were linked to severe or critical infections in patients with COVID-19. Zhou et al. (14) proposed that lymphopenia and increased CRP levels were independent risk factors for disease severity. Gong et al. (12) revealed that age, increased CRP, serum lactate dehydrogenase, the coefficient of variation of red blood cell distribution width, albumin, blood urea nitrogen, and direct bilirubin were related to severe COVID-19 and established a predictive tool to distinguish individuals

TABLE 3 | Demographics and clinical characteristics of progressive and non-progressive COVID-19 patients.

Characteristic	Disease progression		
	Non-progressor patients ($n = 339$)	Progressor patients ($n = 26$)	*P*-value
Age, years	45.9 ± 15.3	59.3 ± 13.2	< 0.001
≥60	63 (18.6)	11 (42.3)	0.004
Male	157 (46.3)	19 (73.1)	0.008
Laboratory findings			
Red blood cell count, $\times 10^9$/L	4.5 (4.1–4.8)	4.5 (3.9–5.0)	0.857
White blood cell count, $\times 10^9$/L	5.5 (4.3–6.8)	6.1 (4.3–7.6)	0.352
<10	323 (95.3)	23 (88.5)	$p = 0.131$
Lymphocyte count, $\times 10^9$/ L	1.47 (1.1–1.6)	0.9 (0.7–1.5)	< 0.001
<0.8	29 (8.6)	7 (26.9)	0.001
Neutrophil count, $\times 10^9$/L	3.4 (2.4–4.6)	4.0 (2.7–6.5)	0.033
>6.4	25 (7.4)	7 (26.9)	0.001
Eosinophil counts, $\times 10^9$/L	0.05 (0–0.1)	0.01 (0–0.1)	0.002
Hemoglobin, g/dL	135.0 (125.0–147.0)	141.0 (120.8–154.5)	0.445
Platelet count, $\times 10^9$/L	236.0 (180.0–297.8)	197.0 (141.8–233.5)	0.004
<100	7 (2.1)	2 (7.7)	0.075
Albumin, g/L	42.0 (38.5–45.0)	38.2 (34.3–42.8)	0.004
Total bilirubin, μmol/L	9.8 (7.3–13.3)	11.3 (7.2–15.4)	0.315
>17.1	38 (11.2)	6 (23.1)	0.108
Alanine aminotransferase, U/L	20.0 (13.0–35.0)	29.0 (17.0–35.0)	0.085
>40	63 (18.6)	3 (11.5)	0.596
Prothrombin time, s	12.5 (11.8–13.1)	13.0 (12.4–13.8)	0.003
≥16	3 (0.9)	1 (3.8)	0.257
Creatinine >133μmol/L	6 (1.8)	4 (15.4)	< 0.001
Lactate dehydrogenase, U/L	199.0 (167.0–241.0)	248.5 (208.0–342.8)	< 0.001
>245	78 (23.0)	14 (53.8)	< 0.001
Creatinine kinase, U/L	67.0 (44.0–95.0)	108.0 (60.3–192.3)	0.011
>120	56 (16.5)	11 (42.3)	0.003
C-reactive protein, mg/L	2.0 (0.5–12.3)	37.7 (9.6–59.9)	< 0.001
≥10	89 (26.3)	20 (76.9)	
Abnormalities on chest CT			
Ground-glass opacity	166 (49.0)	15 (57.7)	0.391
Local patchy shadowing	53 (15.6)	2 (7.7)	0.397
Bilateral patchy shadowing	177 (52.2)	23 (88.5)	< 0.001
Interstitial abnormalities	11 (3.2)	0 (0)	> 0.999

Continuous variable data are presented as the mean (standard deviation) or median (interquartile ranges, IQR). Classified variable data are presented as n (%).

with severe disease. However, in our study, we compared patients with progressive and non-progressive disease, all of whom had mild or moderate COVID-19 on admission. Our study may provide a new perspective on the initial characteristics of patients who will develop severe COVID-19.

Bilateral patchy shadowing and increased levels of creatinine, and CRP were found to be independent predictors of disease progression in this study. Bilateral patchy shadowing is a typical abnormality on chest computed tomography in patients with COVID-19 and has been frequently reported in the literature (15–17). A systematic review of imaging findings in 919 patients with COVID-19 revealed that bilateral involvement was one of the most commonly reported CT findings in severely ill patients (18). Another meta-analysis of radiography findings in patients with SARS, the pathogen of which shares 79.5% of its sequence homology with SARS-CoV-2 (19), found that bilateral involvement of the lungs was the main radiological finding in cases of mortality. These studies verified the role of bilateral patchy shadowing in predicting disease progression to some extent. Elevated creatinine levels were reported to closely correlate with severe COVID-19 (17) or, even worse, with death (1). In another study that attempted to clarify the association between kidney disease and in-hospital death of patients with COVID-19, increased creatinine levels, suggesting kidney injury, were found to represent a higher risk of disease progression in patients with COVID-19 and a greater likelihood of being admitted to the intensive care unit and undergoing mechanical ventilation (20). CRP functions as a non-specific

TABLE 4 | Smoking history, comorbidity, signs and symptoms, treatment and clinical outcomes of progressive and non-progressive COVID-19 patients.

Characteristic	Disease progression		
	Non-progressor patients ($n = 339$)	Progressor patients ($n = 26$)	*P*-value
Smoking history			
Current smokers	5 (1.5)	2 (7.7)	0.082
Ex-smokers	0 (0)	0 (0)	
Comorbidity			
Hypertension	33 (9.7)	6 (23.1)	0.046
Cardiovascular disease	4 (1.2)	2 (7.7)	0.061
Cerebrovascular disease	2 (0.6)	1 (3.8)	0.199
Chronic liver disease	8 (2.4)	1 (3.8)	0.49
COPD	2 (0.6)	0 (0)	> 0.999
Asthma	1 (0.3)	0 (0)	> 0.999
Renal disease	2 (0.6)	0 (0)	> 0.999
Cancer	2 (0.6)	0 (0)	> 0.999
Signs and symptoms			
Fever	198 (58.4)	23 (88.5)	0.003
Temperature on admission (°C)	36.7 (36.5–37.1)	37.1 (36.7–38.0)	0.001
Highest temperature (°C)	37.1 (36.6–38.0)	38.3 (37.7–38.6)	< 0.001
<37.3	188 (55.5)	4 (15.4)	< 0.001
37.3–37.9	57 (16.8)	4 (15.4)	
38–38.9	79 (23.3)	13 (50.0)	
≥39	15 (4.4)	5 (19.2)	
Cough	179 (52.8)	15 (57.7)	0.63
Sputum production	48 (14.2)	4 (15.4)	0.775
Nasal congestion	4 (1.2)	2 (7.7)	0.061
Fatigue	67 (19.8)	10 (38.5)	0.024
Headache	16 (4.7)	1 (3.8)	> 0.999
Sore throat	35 (10.3)	2 (7.7)	> 0.999
Shortness of breath	25 (7.4)	3 (11.5)	0.437
Dyspnea	12 (3.5)	1 (3.8)	> 0.999
Anorexia	22 (6.5)	5 (19.2)	0.033
Diarrhea	13 (3.8)	2 (7.7)	0.29
Nausea	12 (3.5)	1 (3.8)	> 0.999
Myalgia or arthralgia	1 (0.3)	0 (0)	> 0.999
Combination of bacterial infection	205 (60.5)	23 (88.5)	0.005
Treatment			
Antiviral therapy	326 (96.2)	26 (100.0)	0.611
Antibiotic therapy	228 (67.3)	23 (88.5)	0.025
Use of corticosteroid	109 (32.2)	22 (84.6)	< 0.001
Oxygen support	125 (36.9)	24 (92.3)	< 0.001
Clinical outcomes			
Discharge from hospital	338 (99.7)	25 (96.2)	0.138
Length of hospital stay	14.0 (9.0–20.0)	21.0 (16.0–27.3)	< 0.001
Death	1 (0.3)	1 (3.8)	0.138

Continuous variable data are presented as the mean (standard deviation) or median (interquartile ranges, IQR). Classified variable data are presented as n (%). COPD, Chronic obstructive pulmonary disease.

inflammatory marker and is linked to the state of infection and inflammation in patients with severe COVID-19 (21). Prior to CT findings, CRP increased at the early stage of severe COVID-19. CRP level was reported to be associated with CT scores and disease deterioration of COVID-19 (22). The predictive role of CRP for severe COVID-19 has been previously reported (22–24). Increased creatine kinase levels obtained statistical significance in the univariate analyses but lost its position in the multivariate analyses, suggesting that increased creatine kinase levels may be not an independent predictor for disease

TABLE 5 | Risk factors associated with disease progression among patients with non-severe COVID-19.

	Univariate			Multivariate		
Characteristic	**OR**	**95%CI**	***P*-value**	**OR**	**95%CI**	***P*-value**
Age (≥ 60 years)	3.213	1.408–7.329	0.006			
Gender	0.318	0.130–0.776	0.012			
Hypertension	2.782	1.044–7.415	0.041			
Fatigue	2.537	1.102–5.843	0.029			
Anorexia	3.431	1.181–9.969	0.024			
Bilateral patchy shadowing	7.017	2.068–23.812	0.002	4.822	1.329–17.503	0.017
Lymphocyte count ($<0.8\times10^9$/L)	4.677	1.779–12.291	0.002			
Neutrophil count ($>6.4\times10^9$/L)	4.627	1.776–12.055	0.002			
Platelet count ($<100\times10^9$/L)	0.353	0.145–0.859	0.022			
Creatinine (≥133μmol/L)	10.091	2.651–38.410	0.001	6.241	1.422–27.399	0.015
Lactate dehydrogenase (>245 U/L)	3.904	1.734–8.788	0.001			
Creatinine kinase (>200 U/L)	4.543	1.649–12.517	0.003			
C-reactive protein (≥10 mg/L)	9.363	3.644–24.062	< 0.001	7.284	2.558–20.740	< 0.001

OR, odds ratio; CI, confidence interval.

progression. They are often accompanied by elevated levels of creatine kinase-MB (CK-MB), one of the three forms of creatine kinase and serving as a specific indicator of myocardial injury. The underlying mechanism of how SARS-CoV-2 causes acute myocardial injury is speculated to be related to angiotensin-converting enzyme 2 (25). Interestingly, in a report involving 138 patients hospitalised with COVID-19 in Wuhan, 36 patients who presented with severe manifestations and were treated in the intensive care unit had significantly higher levels of CK-MB than those with non-severe symptoms (1), suggesting that severely ill patients were likely to suffer from acute myocardial injury.

Several limitations existed in the present study. Only 26 patients were included in the group with progressive disease. The relatively small sample size may have had some impact on the results of our study. However, the total population from which these patients were sampled was large at 690. In addition, some clinical indicators such as cytokines were not included because of data unavailability. The role of these indicators may have been underestimated. Moreover, the retrospective nature of the data collection and the risk of potential attribution bias, since the reviewers were not blinded to the final clinical status when they attributed the initial clinical status, remain intrinsic limitations of this study. However, when considering that it is extremely difficult to undertake a prospective collection of data in the context of a COVID-19 outbreak, which still poses a threat to the global community, this method seems appropriate. Additionally, since some previous studies exist in this field, the novelty of this report is limited. Despite these limitations, we still hope our study can contribute to improving the clinical management of disease and benefit patients with COVID-19, especially since so much remains unknown regarding the progression of this disease. More clinical data and further work are urgently needed to define the relevant thresholds and predictive values for the three main variables. Development of predictive tools based on these results to stratify initially hospitalised patients into groups with non-progressive and progressive disease holds great promise and calls for further comprehensive research work.

In conclusion, the patients with progressive disease were significantly different from those with non-progressive disease in terms of demographic features, clinical manifestation, laboratory findings, imaging reports, and clinical outcomes. Bilateral patchy shadowing and elevated levels of creatinine and CRP were found to be independent predictors of disease progression. We hope our study can help elucidate the pathogenic characteristics of patients with COVID-19 and help develop effective biomarkers and further therapeutic strategies for patients with COVID-19.

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 Medical Ethics Committee of Nanfang Hospital of Southern Medical University and the institutional ethics review boards of all participating hospitals. The requirement for informed consent was waived by the ethics committees.

AUTHOR CONTRIBUTIONS

YZ and L-sX designed the research study, analysed the data, and wrote the paper. YZ, L-sX, HongbZ, and PL contributed with literature search and tables. CH and W-FZ contributed with data analysis and writing of the paper. Q-cS, M-yS, S-sL, and W-lZ contributed with data collection. H-yZ and MG performed statistical analyses. LL, Y-LH, and HongZ designed the research study, analysed clinical data, and wrote the paper. 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.2020.556818/full#supplementary-material

Supplementary Figure 1 | ROC curves for assessing the predictive value of creatinine kinase, creatinine, C-reactive protein and bilateral patchy shadowing for disease progression. ROC, receiver operating characteristic curve; AUC, area under the ROC curve.

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Analysis of Peripheral Blood IL-6 and Leukocyte Characteristics in 364 COVID-19 Patients of Wuhan

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SARS-CoV-2, the pathogen of COVID-19, is spreading around the world. Different individuals infected with COVID-19 have different manifestations. It is urgent to determine the risk factors of disease progress of COVID-19. 364 patients diagnosed with COVID-19, who were admitted to Wuhan Pulmonary Hospital from February 3, 2020 to March 16, 2020, were divided into mild, ordinary, severe, and critical groups, according to Chinese novel coronavirus pneumonia diagnosis and treatment plan. Peripheral blood IL-6 and leukocyte characteristics were analyzed, to evaluate the correlation with the severity of COVID-19. The levels of peripheral blood IL-6 were 2.35 ± 0.46 pg/ml (mild), 6.48 ± 1.13 pg/ml (ordinary), 20.30 ± 5.15 pg/ml (severe), and 123.48 ± 44.31 pg/ml (critical). The leukocytes were 5.70 ± 0.41×10^9/L (mild), 6.21 ± 0.14×10^9/L (ordinary), 6.37 ± 0.26×10^9/L (severe), and 10.03 ± 1.43×10^9/L (critical). The lymphocytes were 1.46 ± 0.19×10^9/L (mild), 1.89 ± 0.14×10^9/L (ordinary), 1.26 ± 0.07×10^9/L (severe), and 1.17 ± 0.23×10^9/L (critical). The neutrophils were 3.63 ± 0.36×10^9/L (mild), 3.78 ± 0.11×10^9/L (ordinary), 4.47 ± 0.25×10^9/L (severe), and 7.92 ± 1.19×10^9/L (critical). The monocytes were 0.42 ± 0.05×10^9/L (mild), 0.44 ± 0.01×10^9/L (ordinary), 0.46 ± 0.02×10^9/L (severe), and 0.78 ± 0.25×10^9/L (critical). Conclusively, increase of peripheral blood IL-6 and decrease of lymphocytes can be used as the indicators of severe COVID-19. The increase of neutrophils and monocytes was noticed in critical cases of COVID-19, suggesting that the increase of neutrophils and monocytes should be considered as risk factors of critical cases of COVID-19. Peripheral blood IL-6 and leukocyte characteristics were also analyzed in different age groups. The increase of serum IL-6, decrease of lymphocytes, and increase of neutrophils were noticed in patients over 60 years old.

Keywords: 2019 coronavirus disease, SARS coronavirus 2, IL-6, leukocytes, lymphocytes, neutrophils, monocytes

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INTRODUCTION

Since the outbreak of 2019 coronavirus disease (COVID-19) in Wuhan at the end of December 2019, the epidemic has spread rapidly, with confirmed and fatal cases in many countries around the world. As of April 19, 2020, a total of 2,241,778 cases have been confirmed globally, with a total of 152,551 deaths (1). The pathogen causing COVID-19 was a novel coronavirus (2019 novel coronavirus, 2019nCoV) (2), lately named as SARS coronavirus 2 (SARS-CoV-2) (3), which was confirmed by viral genome sequencing in alveolar lavage fluid of three COVID-19 patients in Jinyintan Hospital, Wuhan city (2). SARS-CoV-2 is a betacoronavirus and the genome is positive single-stranded RNA, with an envelope on which the mushroom protein spines make the virus shape like a crown (2).

Different individuals infected with COVID-19 have different manifestations. It is urgent to determine the risk factors of disease progress of COVID-19. Further research is being carried out on the pathogenesis of COVID-19 caused by SARS-CoV-2 infection. According to the latest research findings, as humans are infected with SARS-CoV-2, $CD4^+$ T cells are rapidly activated, proliferate and differentiate into Th1 cells, and produce granulocyte-macrophage colony stimulating factor (GM-CSF), which further induces inflammatory $CD14^+CD16^+$ monocytes to over-express IL-6 and other factors, promoting inflammatory responses (4). It is speculated that GM-CSF and IL-6 may be the key factors inducing cytokine storm. In addition, peripheral lymphocytopenia in patients with COVID-19 was revealed by some retrospective studies (5–8). Therefore, we suspected that in the pathogenesis of COVID-19, both lymphocyte injury and cytokine storm might be involved. Three hundred sixty-four patients diagnosed with COVID-19, who were admitted to Wuhan Pulmonary Hospital from February 3, 2020 to March 16, 2020, were divided into mild, ordinary, severe, and critical groups, according to Chinese novel coronavirus pneumonia diagnosis and treatment plan. Peripheral blood IL-6 and leukocyte characteristics were retrospectively analyzed, to evaluate the correlation of these laboratory indicators with the severity of COVID-19.

MATERIALS AND METHODS

Patients

Three hundred sixty-four patients diagnosed with COVID-19, who were admitted to Wuhan Pulmonary Hospital from February 3, 2020 to March 16, 2020, were divided into mild, ordinary, severe, and critical groups, according to Chinese novel coronavirus pneumonia diagnosis and treatment plan: mild type, with slight clinical symptoms but no imaging presentation of pneumonia; ordinary type, with fever, respiratory tract and other symptoms, imaging findings of pneumonia; severe type, with any of the following conditions: respiratory distress, respiratory frequency ≥30 times/minutes, finger oxygen saturation at rest ≤93%, or oxygenation index [PaO2/FiO2] ≤300 mmHg (1 mmHg = 0.133 kPa); critical type, with any of the following conditions: respiratory failure requires mechanical ventilation, shock, combined with other organ failure that requires intensive care unit care and treatment (8). All the patients were diagnosed through the positive detection of SARS-CoV-2 nucleic acid from throat swabs or sputum samples.

Detection of SARS-CoV-2 Nucleic Acid

Samples of pharyngeal swabs and sputum from patients were collected. RNA was extracted using RNA extraction kit (2020031, Da 'an, China) and Real-Time PCR was performed to detect the sequence of N protein and ORF1ab of SARS-CoV-2 using quantitative PCR kit (DA0930, Da 'an, China) with Real-Time PCR apparatus (7500, ABI, USA).

Routine Blood Test

2 ml of venous blood of patients was collected in the EDTA-2K anti-coagulant tube and routine blood test was detected (BC-6900, Mindray, China). The data of routine blood test collected in this study were all the analysis of the first test results after admission.

Detection of Serum IL-6

The peripheral blood of patients was collected and centrifuged at 4,000 rpm for 4 min. The serum concentration of IL-6 was measured (Cobas 8000, Roche, USA). All the data of IL-6 collected in this study were the first detection after the patient was admitted to the hospital.

Statistical Analysis

Categorical variables were described as percentages, continuous variables were described as mean, standard errors of the mean, and interquartile range (IQR) values. A nonparametric test (kruskal-wallis rank sum and unpaired, two-sided Mann–Whitney U-test) was used to compare the means of continuous variables. All statistical analyses were performed using SPSS 19.0 software (SPSS Inc). $P < 0.05$ was considered statistically significant.

RESULTS

Patients' Characteristics

Three hundred sixty-four patients diagnosed with COVID-19, who were admitted to Wuhan Pulmonary Hospital from February 3, 2020 to March 16, 2020, were divided into mild, ordinary, severe and critical groups, according to Chinese novel coronavirus pneumonia diagnosis and treatment plan. All the patients were diagnosed through the positive detection of SARS-CoV-2 nucleic acid from throat swabs or sputum samples. The average age of the 364 patients is 62 years old. In the mild group, there were 11 cases (3.02%), with an average age of 56 years (IQR, 45–70, range, 40–78 years). In the ordinary group, there were 268 cases (73.63%), with an average age of 58.5 years (IQR, 47–67, range 18–96 years). There were 67 patients (18.40%) in the severe group, with an average age of 69 years (IQR, 58–77, range of 31–94 years). There were 18 patients (4.95%) in the critical group, with an average age of 67.5 years (IQR, 55.25–73.5, range of 36–84 years) (**Table 1** and **Figure 1A**). Age distribution of the patients was analyzed. There were 3 patients under the age of 20 years (0.82%), 51 patients between the ages of 20 and 40 years (14.01%), 115 patients between the ages of 40 and 60 years

TABLE 1 | Patient characteristics.

	Total (N = 364)	Mild (N = 11)	Ordinary (N = 268)	Severe (N = 67)	Critical (N = 18)
Age, median (IQR)	62 (49–69.75)	56 (45–70)	58.5 (47–67)	69 (58–77)	67.5 (55.25–73.50)
Sex					
Female (%)	203 (56)	6 (55)	156 (58)	34 (51)	7 (39)
Male (%)	161 (44)	5 (45)	112 (42)	33 (49)	11 (61)

Three hundred sixty-four patients diagnosed with COVID-19, who were admitted to Wuhan Pulmonary Hospital from February 3, 2020 to March 16, 2020, were divided into mild, ordinary, severe, and critical groups, according to Chinese novel coronavirus pneumonia diagnosis and treatment plan. Information of the patients including age and gender in each group was analyzed. Statistical analysis was performed and data of age were expressed as mean (IQR).

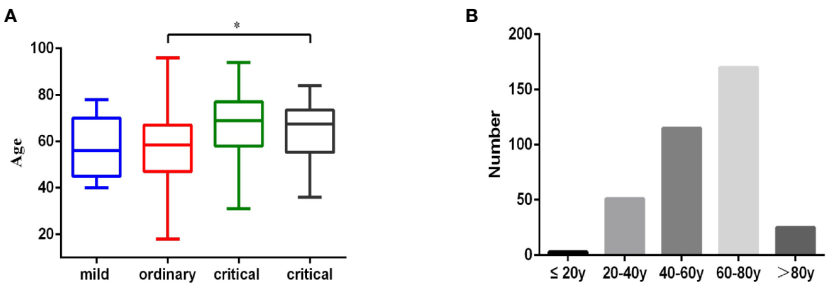

FIGURE 1 | Age characteristics of 364 COVID-19 patient. **(A)**. 364 patients diagnosed with COVID-19, who were admitted to Wuhan Pulmonary Hospital from February 3, 2020 to March 16, 2020, were divided into mild (n = 11), ordinary (n = 268), severe (n = 67), and critical (n = 18) groups according to Chinese novel coronavirus pneumonia diagnosis and treatment plan. Age information of the patients in each group was analyzed. Statistical analysis was performed using a nonparametric test (kruskal-wallis rank sum) and data of age were expressed as mean (IQR), *p < 0.05. **(B)**. The age distribution of all 364 patients.

(31.59%), 170 patients between the ages of 60 and 80 years (46.70%), and 25 patients over the age of 80 years (6.86%) (**Figure 1B** and **Table 4**). Similar to other retrospective studies, COVID-19 patients admitted to Wuhan Pulmonary Hospital had the largest number of patients in the ordinary group, followed by patients in the severe group, and fewer patients in mild and critical groups (9, 10).

Characteristics of IL-6 in Peripheral Blood of Patients With COVID-19

The reference value of IL-6 is 0–7 pg/ml. Increase of serum IL-6 was not observed in mild patients. The percentage of patients with increased serum IL-6 was 15.30% in the ordinary group, 49.25% in the severe group and 83.33% in the critical group (**Table 2**). The concentration of serum IL-6 of mild, ordinary, severe, and critical patients was 2.35 ± 0.46 pg/ml, 6.48 ± 1.13 pg/ml, 20.30 ± 5.15 pg/ml, and 123.48 ± 44.31 pg/ml, respectively. Among them, the increase of IL-6 in severe and critical patients was significant, as compared with mild and ordinary patients ($P < 0.05$) (**Figure 2**). Conclusively, increase of serum IL-6 is common in severe and critical forms of COVID-19, especially in critical cases.

Characteristics of Peripheral Blood Leukocytes in Patients With COVID-19

The reference value of leukocytes is 3.5–9.5×10^9/L. According to the blood count of the patients, over 90% of patients in the mild and ordinary groups, had the normal leukocyte count. In the severe group, 88.06% patients had the normal leukocyte count. However, in the critical group, leukocyte count was normal in only 55.56% patients, while 38.89% patients had the increased leukocyte count and 5.56% patients had the decreased leukocyte count (**Table 3**). The mean leukocyte count of the patients in the mild, ordinary, severe and critical groups was 5.70 ± 0.41×10^9/L, 6.21 ± 0.14×10^9/L, 6.37 ± 0.26×10^9/L, and 10.03 ± 1.43×10^9/L, respectively (**Figure 3A**). The increase of leukocyte count in critical patients was significant, as compared with ordinary patients ($P < 0.05$) (**Figure 3A**).

The count of lymphocytes, neutrophils, and monocytes was further analyzed. The reference count of lymphocytes is 1.1–3.2×10^9/L. According to the results, 27.27% patients in the mild group, 14.93% patients in the ordinary group, 35.82% patients in the severe group, and 61.11% patients in the critical group, had decreased lymphocyte count (**Table 3**). The lymphocyte counts were 1.46 ± 0.19×10^9/L in the mild patients, 1.89 ± 0.14×10^9/L in

TABLE 2 | Characteristics of serum IL-6 in patients with COVID-19.

		mild group	ordinary group	severe group	critical group
IL-6	within normal values (n,%)	11(100.00)	227(84.70)	34(50.75)	3(16.67)
	above normal values (n,%)	–	41(15.30)	33(49.25)	15(83.33)

The peripheral blood of patients was collected and centrifuged at 4,000 rpm for 4min. The serum concentration of IL-6 was measured. All the data of IL-6 collected in this study were the first detection after the patient was admitted to the hospital. The percentage of patients with elevated IL-6 was calculated in mild, ordinary, severe, and critical groups, according to the reference of serum IL-6 concentration (0–7 pg/ml).

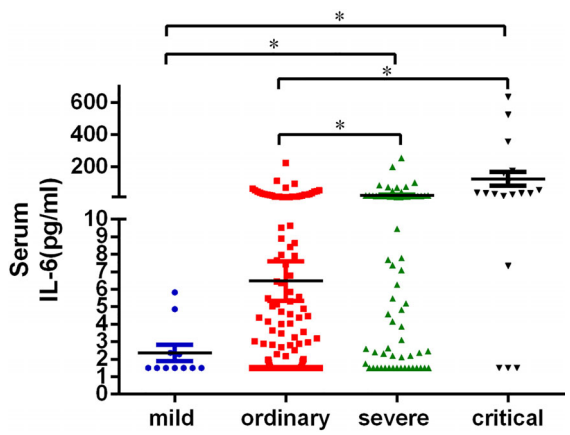

FIGURE 2 | Serum IL-6 level in patients with COVID-19. The peripheral blood of patients was collected and centrifuged at 4,000 rpm for 4min. The serum concentration of IL-6 was measured. All the data of IL-6 collected in this study were the first detection after the patient was admitted to the hospital. Statistical analysis of the serum IL-6 of the patients in mild (n = 11), ordinary (n = 268), severe (n = 67), and critical (n = 18) groups was performed using unpaired, two-sided Mann-Whitney U-test and data were expressed as mean ± SEM, *p < 0.05.

the ordinary patients, 1.26 ± 0.07×10^9/L in the severe patients, and 1.17 ± 0.23×10^9/L in the critical patients, respectively. There was a significant decrease of lymphocytes in the severe and critical groups, as compared with ordinary group ($P < 0.05$) (**Figure 3B**).

The reference count of neutrophils is 1.8–6.3×10^9/L. No increase of neutrophils was observed in the mild group. While 7.09% patients in the ordinary group, 14.93% patients in the severe group, 50.00% patients in the critical group, had the increased neutrophils (**Table 3**). The count of neutrophils was respectively 3.63 ± 0.36×10^9/L in the mild patients, 3.78 ± 0.11×10^9/L in the ordinary group, 4.47 ± 0.25×10^9/L in the severe group, and 7.92 ± 1.19×10^9/L in the critical group (**Figure 3C**). The increase of neutrophil count in critical patients was significant, as compared with ordinary patients ($P < 0.05$) (**Figure 3C**).

The reference count of monocytes is 0.1–0.6×10^9/L. According to the results, 18.18% patients in the mild group, 13.92% patients in the ordinary group, 19.40% patients in the severe group, and 33.33% patients in the critical group, had increased monocyte count (**Table 3**). The count of monocytes was respectively 0.42 ± 0.05×10^9/L in the mild patients, 0.44 ± 0.01×10^9/L in the ordinary group, 0.46 ± 0.02×10^9/L in the severe group, and 0.78 ± 0.25×10^9/L in the critical group (**Figure 3D**). There was a significant increase of monocytes in the critical group, as compared with ordinary and severe groups ($P < 0.05$) (**Figure 3D**). Taken together, lymphocyte decrease and neutrophil increase are common leukocyte characteristics in critical cases of COVID-19. In addition, increase of monocytes was observed in one third of critically ill patients.

Analysis of Serum IL-6 and Peripheral Blood Leukocyte Characteristics in Different Age Groups

Peripheral blood IL-6 and leukocyte characteristics were also analyzed in different age groups. The increase of serum IL-6, decrease of lymphocytes were also noticed in patients over 60 years old. The concentration of serum IL-6 in different age group was 1.50 ± 0.00 pg/ml (≤ 20y), 3.76 ± 0.92 pg/ml (20 < Age ≤ 40), 5.65 ± 1.19 pg/ml (40 < Age ≤ 60), 22.16 ± 5.66 pg/ml (60 < Age ≤ 80), and 29.29 ± 11.04 pg/ml (Age > 80), respectively (**Table 4**). Since there were only 3 patients under the age of 20 years, statistical analysis was performed to compare the difference of other age groups with the group between the ages of 20 and 40 years. The level of serum IL-6 increased significantly in elderly patients over 60 years old, as compared with 20–40-year patients ($p < 0.05$) (**Figure 4A**). No difference of leukocyte count was observed in different age groups (**Figure 4B**). Significant decrease of lymphocytes was observed in groups over 60 years old, as compared with 20–40-year group ($p < 0.05$) (**Figure 4C**). Increase of neutrophils was only observed in the group over 80 years old (**Figure 4D**). No difference of monocyte count was observed in different age groups (**Figure 4E**). Conclusively, the increase of serum IL-6, decrease of lymphocytes and increase of neutrophils should be also paid attention in older patients infected of COVID-19.

TABLE 3 | Characteristics of peripheral blood leukocytes in patients with COVID-19.

		mild group	ordinary group	severe group	critical group
Leukocyte count	below normal values (n,%)	1(9.09)	10(3.73)	2(2.99)	1(5.56)
	within normal values (n,%)	10(90.91)	244(91.04)	59(88.06)	10 (55.56)
	above normal values (n,%)	–	14(5.22)	6 (8.96)	7(38.89)
Lymphocyte count	below normal values (n,%)	3(27.27)	40(14.93)	24(35.82)	11(61.11)
	within normal values (n,%)	8(72.73)	215(80.22)	43(64.18)	6(33.33)
	above normal values (n,%)	–	13(4.85)	–	1(5.56)
Neutrophil count	below normal values (n,%)	1(9.09)	14(5.22)	1(1.50)	1(5.56)
	within normal values (n,%)	10(90.91)	235(87.69)	56(83.58)	8(44.44)
	above normal values (n,%)	–	19(7.09)	10(14.93)	9(50.00)
Monocyte count	below normal values (n,%)	–	–	–	1(5.56)
	within normal values (n,%)	9(81.82)	234(98.73)	54(80.60)	11(61.11)
	above normal values (n,%)	2(18.18)	33(13.92)	13(19.40)	6(33.33)

Information of the routine blood test of patients in mild, ordinary, severe, and critical groups was analyzed. All the data of routine blood test collected in this study were the first detection after the patient was admitted to the hospital. The percentage of patients with abnormal leukocyte count, lymphocyte count, neutrophil count, and monocyte count was calculated in each group, according to the reference (leukocyte count: 3.5–9.5×10^9/L, lymphocyte count: 1.1–3.2×10^9/L, neutrophil count: 1.8–6.3×10^9/L, monocyte count: 0.1–0.6×10^9/L).

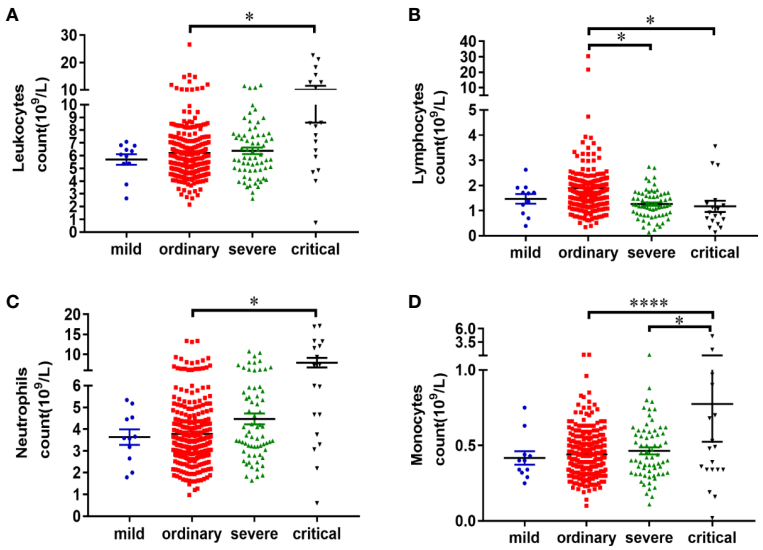

FIGURE 3 | Characteristics of peripheral blood leukocytes in patients with COVID-19. Information of the routine blood test of patients was analyzed. All the data of routine blood test collected in this study were the first detection after the patient was admitted to the hospital. Statistical analysis of the leukocyte count **(A)**, lymphocyte count **(B)**, neutrophil count **(C)**, and monocyte count **(D)** of the patients in mild (n = 11), ordinary (n = 268), severe (n = 67), and critical (n = 18) groups was performed using unpaired, two-sided Mann-Whitney U-test and data were expressed as mean ± SEM, *p < 0.05, ****p < 0.0001.

TABLE 4 | Analysis of serum IL-6 and peripheral blood leukocyte characteristics in different age groups.

	Age ≤ 20	20<Age ≤ 40	40<Age ≤ 60	60<Age ≤ 80	Age>80
Patiens Number	3(0.82%)	51(14.01%)	115(31.59%)	170 (46.70%)	25(6.86%)
Serum IL-6 (pg/ml)	1.50 ± 0.00	3.76 ± 0.92	5.65 ± 1.19	22.16 ± 5.66	29.29 ± 11.04
Leukocyte count (10^9/L)	6.14 ± 0.43	6.35 ± 0.27	6.14 ± 0.22	6.52 ± 0.24	7.06 ± 0.49
Lymphocyte count (10^9/L)	1.96 ± 0.36	2.57 ± 0.57	1.68 ± 0.06	1.56 ± 0.13	1.28 ± 0.14
Neutrophil count (10^9/L)	3.67 ± 0.27	3.71 ± 0.21	3.80 ± 0.20	4.30 ± 0.19	5.13 ± 0.51
Monocyte count (10^9/L)	0.42 ± 0.07	0.43 ± 0.02	0.43 ± 0.02	0.48 ± 0.03	0.49 ± 0.04

Three hundred sixty-four patients diagnosed with COVID-19, who were admitted to Wuhan Pulmonary Hospital from February 3, 2020 to March 16, 2020, were divided into different age groups (Age ≤ 20, 20 < Age ≤ 40, 40 < Age ≤ 60, 60 < Age ≤ 80, Age > 80). Information of serum IL-6 and the routine blood test of patients in each group was analyzed. Statistical analysis was performed and data were expressed as mean ± SEM.

DISCUSSION

A total of 364 patients diagnosed with COVID-19, who were admitted to Wuhan Pulmonary Hospital from February 3, 2020 to March 16, 2020, were included in this study and divided into mild, ordinary, severe and critical forms, according to Chinese novel coronavirus pneumonia diagnosis and treatment plan. It was reported that most of the patients infected with COVID-19 were mild cases. However, Wuhan Pulmonary Hospital was a designated hospital for COVID-19 treatment and mainly received patients of non-mild form. Therefore, COVID-19 patients in this study, had the largest number of patients in the ordinary group (73.63%), followed by patients in the severe group (18.40%), and fewer patients in mild (3.02%) and critical (4.95%) groups, similar to other retrospective studies (9, 10).

It was observed that serum IL-6 level was not significantly increased in patients in mild and ordinary groups without significant lung injury indicated by lung CT. However, 49.25% of patients in the severe group and 83.33% of patients in the critical group had the increased serum IL-6, indicating the severity of the disease is accompanied by inflammatory reaction. In current study, the increase of leukocyte count and neutrophil count was also observed, especially in critically ill patients. It has been recently reported that the counts of leukocytes and neutrophils of COVID-19 victims were higher than those of the survivors (11). Increase of neutrophils might be related with the secondary bacterial infection due to immunosuppression caused by virus infection. We also suspected that neutrophils might be involved in inflammation of COVID-19 pathogenesis, since some patients with COVID-19 had obvious increase of neutrophils in the early stage of the disease, without apparent sign of bacterial infection. Recently, it was reported that neutrophils played an important role in inflammatory reaction (12, 13). Upon COVID-19 infection, neutrophils released higher levels of neutrophil extracellular traps (NETs), which act as important mediators of tissue damage in inflammatory reaction (14).

Circulating monocytes in peripheral blood express a broad repertoire of plasma membrane and intracellular receptors,

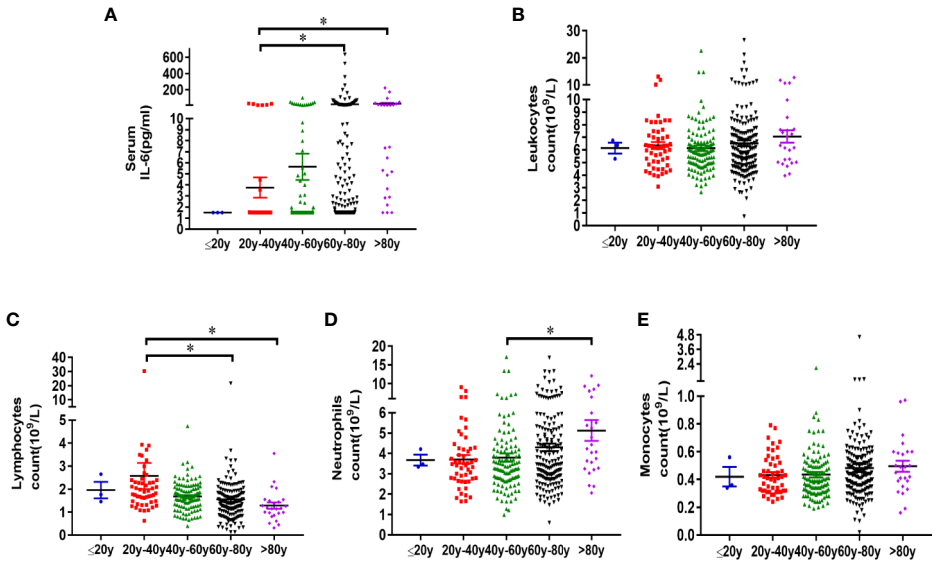

FIGURE 4 | Analysis of serum IL-6 and peripheral blood leukocyte characteristics in different age groups. Three hundred sixty-four patients diagnosed with COVID-19, who were admitted to Wuhan Pulmonary Hospital from February 3, 2020 to March 16, 2020, were divided into different age groups (Age ≤ 20, n = 3; 20 < Age ≤ 40, n = 51; 40 < Age ≤ 60, n = 115; 60 < Age ≤ 80, n = 170; Age > 80, n = 25). Information of serum IL-6 (a), leukocyte count **(B)**, lymphocyte count **(C)**, neutrophil count **(D)** and monocyte count **(E)** of patients in each group was analyzed. Statistical analysis was performed using unpaired, two-sided Mann-Whitney U-test and data were expressed as mean ± SEM, *p < 0.05.

serving as sensors of micro-organisms, dying cells and soluble products, to mediate innate immune response. Monocytes differentiate into resident macrophages, following tissue inflammation, infection, or malignancy. Monocyte-derived macrophages are reported to be the main generators of inflammation in COVID-19 (15, 16). In this study, the increase of monocytes was noticed in critical group. The increase of neutrophils and monocytes, two kinds of important cells in innate immunity, indicated the activation of innate immunity and the inflammatory reaction, which promotes the elimination of virus by adaptive immune mechanism.

However, lymphocytopenia is a common feature in critically ill patients. In the critical group, 61.11% of the patients had decreased lymphocytes, while in the severe group, about one third of the patients had decreased lymphocyte count. A number of retrospective studies have reported that lymphocytopenia is common in COVID-19 patients (5–8) and lymphocytopenia can be regarded as an indicator of the progression of COVID-19 (17). At present, there is no clear understanding about the mechanism of lymphocytopenia in COVID-19. Some studies have pointed out that host factors may be involved in the reduction of lymphocytes (18). It was reported that the patients with severe and critical COVID-19 infection are generally older, as compared with patients with mild and ordinary form, and have a higher proportion of underlying diseases such as hypertension, diabetes, cardiovascular or cerebrovascular diseases (10). From the analysis of the patients' information in this study, we have also observed significant decrease of lymphocytes in groups over 60 years old. Aging and chronic diseases are more likely to lead to chronic endothelial dysfunction, which causes the destruction of the cell-cell connection, death of endothelial cells, destruction of the blood-tissue barriers, enhancement of leukocyte adhesion and exudation (13), which may help us explain the lymphopenia in peripheral circulation in elderly patients, in severe and critical COVID-19 cases. Increase of neutrophils and monocytes, along with decreased lymphocytes in critically ill patients of COVID-19, demonstrated dysregulation of innate and acquired immunity. The detailed mechanisms, why activated innate immunity cannot effectively promote the acquired immunity to clear the virus, but cause immunopathology, including lymphocytopenia, inflammatory tissue damage, should be further researched in the pathogenesis of COVID-19.

Different individuals infected with COVID-19 have different manifestations and different outcomes. With the spread of SARS-CoV-2 in many countries around the world, it is urgent to determine the criteria for severe diseases, especially critical cases. Detection of inflammatory cytokines represented by serum IL-6 and routine blood tests can be quickly and easily performed. Through the study, we found that increased serum IL-6, decreased lymphocytes, and increased neutrophils and monocytes, can help us to assess critical patients with poor clinical outcomes.

Conclusively, this retrospective study analyzed the correlation of serum IL-6 and leukocyte characteristics with the severity of COVID-19. Peripheral blood IL-6 and leukocyte characteristics were also analyzed in different age groups. The increase of serum IL-6, decrease of lymphocytes, and increase of neutrophils were also noticed in patients over 60 years old, which help us understand why COVID-19 is more dangerous for the elder population.

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 Wuhan pulmonary hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QL, MZ, BY, HH, YH, and YW performed the experiments in this study. QL, CZ, ST, and YX analyzed and interpreted the data. CZ performed statistical analysis. QL, YX, MZ, and CS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Artificial Intelligence

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Machine Learning Approaches Reveal That the Number of Tests Do Not Matter to the Prediction of Global Confirmed COVID-19 Cases

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Coronavirus disease 2019 (COVID-19) has developed into a global pandemic, affecting every nation and territory in the world. Machine learning-based approaches are useful when trying to understand the complexity behind the spread of the disease and how to contain its spread effectively. The unsupervised learning method could be useful to evaluate the shortcomings of health facilities in areas of increased infection as well as what strategies are necessary to prevent disease spread within or outside of the country. To contribute toward the well-being of society, this paper focusses on the implementation of machine learning techniques for identifying common prevailing public health care facilities and concerns related to COVID-19 as well as attitudes to infection prevention strategies held by people from different countries concerning the current pandemic situation. Regression tree, random forest, cluster analysis and principal component machine learning techniques are used to analyze the global COVID-19 data of 133 countries obtained from the Worldometer website as of April 17, 2020. The analysis revealed that there are four major clusters among the countries. Eight countries having the highest cumulative infected cases and deaths, forming the first cluster. Seven countries, United States, Spain, Italy, France, Germany, United Kingdom, and Iran, play a vital role in explaining the 60% variation of the total variations by us of the first component characterized by all variables except for the rate variables. The remaining countries explain only 20% of the variation of the total variation by use of the second component characterized by only rate variables. Most strikingly, the analysis found that the variable number of tests by the country did not play a vital role in the prediction of the cumulative number of confirmed cases.

Keywords: COVID-19 disease, cluster analysis, machine learning, principal component analysis, regression tree

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1. INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an infectious disease that first emerged in December 2019 in Wuhan, the capital of China's Hubei province (Roosa et al., 2020). It has spread to nearly 213 countries and territories and has infected more than 2.3 million people as of April 17, 2020, has killing approximately 155,000 people worldwide (Max Roser and Ortiz-Ospina, 2020) (also see **Figure 3**). As of April 17, 2020, the highest crude fatality rate was observed in Belgium

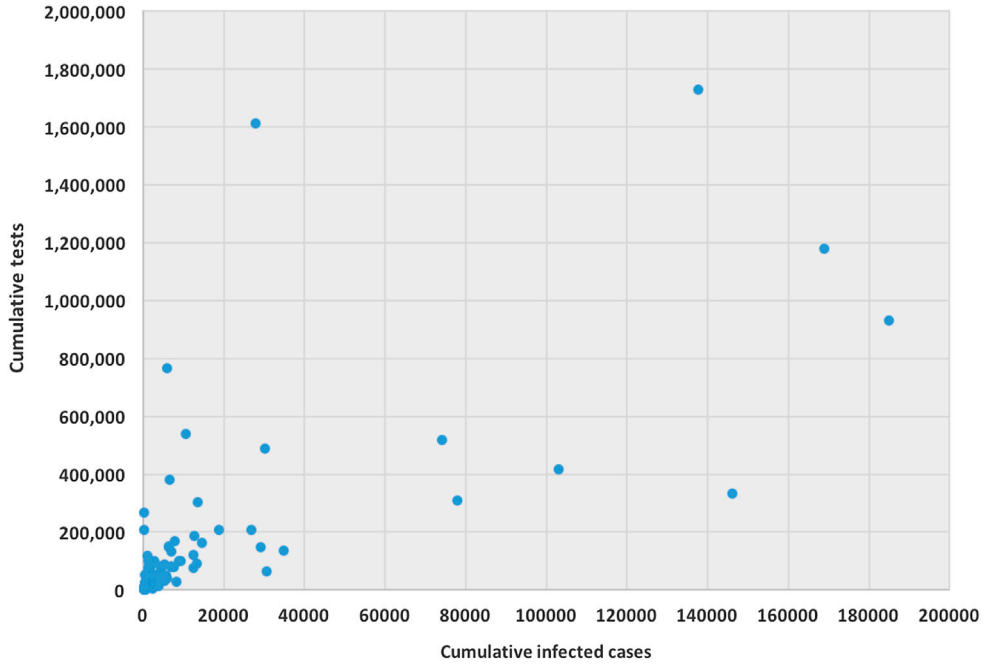

FIGURE 1 | Scatter plot between cumulative tests and cumulative cases for 132 countries (except United States).

(nearly 485 per million), followed by Spain (nearly 435 per million), and Italy (nearly 390 per million) (Max Roser and Ortiz-Ospina, 2020). However, the highest number of deaths took place in United States (over 38,000), followed by Italy, Spain, and France. The countries most affected have conducted a large number of tests. As of April 17, 2020, the United States has conducted more than 3.7 million tests, followed by Russia (over 1.8 million), Germany (over 1.6 million), and Italy (approximately 1.3 million). The number of active cases is growing as the number of cases is growing. As of April 17, 2020, globally, nearly 67% of the total cases are active cases, and hence 23% are recovered (Max Roser and Ortiz-Ospina, 2020).

Most of the affected countries have been maintaining social distancing, closing educational institutes, offices, and markets to reduce the rate of spread; these methods have not had universal reach, however, and there are many countries where people are commuting in crowded public transport or even living in close quarters in urban slums (Hui et al., 2020). Also, in many countries, the public healthcare systems are insufficient and overburdened, and this poses a potentially dangerous threat to public health (Khan and Hossain, 2020b). According to World Bank data (World Bank, 2020), in 2015, Bangaldesh had 0.8 hospital beds per 1,000 people, India had 0.7 (2011), Pakistan had 0.6 (2012), and the United States had 2.9 (2012), whereas China had 4.2 (2012) beds per 1,000 people. It is recommended that intensive care unit (ICU) practitioners, hospital administrators, governments, and policymakers must prepare for a substantial increase in critical care bed capacity, with a focus not just on infrastructure and supplies but also on staff management (Phua et al., 2020).

The ability for testing for COVID-19 varies from country to country. Testing ability is one of our most important tools for slowing down and reducing the spread and impact of the virus, but it is also dependent on a country's financial capability, laboratory capacity, and access although it. Low- and middle-income countries may have to battle their COVID-19 pandemic with scarcer resources. Tests allow us to identify infected individuals, guiding the medical treatment that they receive. They also enable the isolation of those infected and the tracing and quarantining of anyone they have been in contact with (Hellewell et al., 2020). As of April 17, 2020, the United States have administered the highest no. of tests, approximately 3.4 million, which is almost 20% of global test total, followed by Germany (over 1.7 million), Russia (over 1.6 million), and Italy (approximately 1.2 million). **Figure 1** is a scatter plot of the cumulative cases and cumulative tests for 132 countries. The United States was discarded for this graph since the United States have had an exceptionally high number of tests performed. We found that the correlation coefficient between these two variables for 132 countries is 0.71, which indicates a strong positive correlation; if including United States, the coefficient is 0.88, which indicates a very high positive correlation.

Artificial intelligence (AI) and machine learning expertise are needed in order to help experts within public health and epidemiology. For example, Muzammal et al. (2020) used a multi-sensor data-fusion-enabled ensemble approach for medical data. Pirbhulal et al. (2019) used machine learning tools to enable a security framework for IoT-based healthcare. AI provides a useful tool that can help in computing risk factors, classification, even drug analysis, and it can also responding to crises, according to health data specialists. Because of the increase in COVID-19 patients and the overall lack of sufficient equipment to receive all patients, difficult choices must be made. The necessary medical care is thus applied

only to patients that have a higher probability of survival. Calculating the probability to survive and the effect of each feature, such as symptoms in our case, on survival probability is done using survival analysis. In the presence of massive epidemic data, the machine learning techniques help to identify the epidemic patterns so that early action can be planned to stop the spread of the virus. AI and big data can be found in a lot of applications in various fields, e.g., AI in computer science, AI in banking, AI in agriculture, and AI in healthcare. These technologies have established roles in these fields, and they currently play important roles in the global battle against the COVID-19 pandemic.

There are a number of research works where machine learning tools have been used for global and local COVID-19 data analysis. Recently, Chuanyu et al. (2020) used several machine learning tools, including elastic net, random forest, and bagged flexible discriminant analysis, for predicting the mortality risk of COVID-19 patients. This work is completely different from other COVID-19-related works since we have focused on the classification and prediction of a cumulative number of confirmed COVID-19 cases. To our knowledge, there is no work so far that has used such machine learning techniques to predict confirmed COVID-19 cases. Magdon-Ismail (2020) presented a robust data-driven machine learning analysis of the COVID-19 pandemic from its early infection dynamics. McCall (2020) discussed how artificial intelligence protects healthcare workers and helps curb the spread of COVID-19. Waiker (2020) discussed possibilities of identifying and evaluating the virus with technology, AI, and analytics. Waiker (2020) used deep learning methods to review and critically appraise published and preprint reports of prediction models for COVID-19 patients. In particular, several study works (Afshar et al., 2020; Asnaoui et al., 2020; Corman et al., 2020; Fomsgaard and Rosenstierne, 2020; Forbes, 2020; Ghoshal and Tucker, 2020; Gozes et al., 2020; Hall et al., 2020; Healthitanalytics, 2020; Hu et al., 2020a; Hu et al., 2020b; IBM, 2020; Loey et al., 2020; Maghdid et al., 2020; Narin et al., 2020; Pal et al., 2020; Pham et al., 2020; Qi et al., 2020; Rao and Vazquez, 2020; Satu et al., 2020; Sodhro et al., 2019; Yan et al., 2020; Zhang et al., 2020; Zheng et al., 2020) have used machine learning techniques, including big data techniques, to process COVID-19 data to determine the spread of disease, predict the risk of disease, and to assess the diagnosis of disease, number of incidences, and healthcare facilities.

The above studies mainly focused on the occurrence of confirmed, recovered, and fatal cases in Wuhan and the rest of the world to understand the suspected threats and plan for subsequent containment actions. To better understand and work to alleviate the COVID-19 pandemic, many papers and preprints, as outlined above, have been published online in the last 78 months. Our main purpose is to show the effectiveness of machine learning approaches to fight against the COVID-19 pandemic and review state-of-the-art solutions using these technologies. In this paper, however, we use machine learning approaches to explore whether the global cumulative number of infected people can be predicted using the data provided by Worldometer (Max Roser and Ortiz-Ospina, 2020) as of April 17, 2020. We believe that machine learning-based approaches are useful when trying to understand the complexity behind the spread of the disease and how to contain the spread of such outbreaks effectively. As the outbreak of the COVID-19 has become a worldwide pandemic, a real-time analyses of epidemiological data is needed to prepare society with better action plans to combat the disease. We also demonstrate useful approaches when using unsupervised machine learning techniques to explore the nature of propagation in different countries.

This analysis is expected to bring useful findings, as countries with poor health infrastructure, a lack of smart strategies for testing, and a lack of health care for patients could descend into a rapid spread of disease and later stages of infection. It is therefore important to use unsupervised and supervised methods to classify countries in terms of disease spread and prediction of the global number of cumulative cases of COVID-19. A number of variables are considered for this study, including the country, number of new cases, total number of active cases, total number of deaths, total number of recovered patients, total number of serious cases, total number of tests, deaths per million, cases per million, and tests per million. We are also interested in identifying what the total number of tests that are vital to predict the total number of infections for countries. We will further investigate whether the countries are clustered on the basis of these covariates. Finally, whether the total variations can be explained with some latent groups which are uncorrelated each other.

2. METHODOLOGY

The data used for the current study have been collected from real-time COVID-19 data from the Worldometer website (Max Roser and Ortiz-Ospina, 2020) as reported as of April 17, 2020. The Worldometer is a data repository and a free reference website that is trusted by the likes of the United Kingdom Government, Johns Hopkins CSSE, etc. For the current study, we collated the information obtained on 133 countries that have crossed the 100 number of confirmed COVID-19 cases.

For each country we collected information on a total of 10 variables: the cumulative confirmed cases, new confirmed cases, cumulative deaths, cumulative recovered patients, cumulative active cases, cumulative seriously critical patients, infection rate in million, death rate in million, cumulative tests conducted, and test rate in millions. These numbers and rates are provided by the respective countries and then stored on the Worldometer website (Max Roser and Ortiz-Ospina, 2020). New confirmed cases are the confirmed cases reported on April 17, 2020. The definition of recovery and serious cases vary from country to country. According to Max Roser and Ortiz-Ospina (2020), the recovered number is not very accurate, as reports can be missing, incomplete, incorrect, and be based on different definitions or dates (or a combination of all of these) for many governments, both at the local and national level, and there may also be differences between states within the same country or counties within the same state. We considered the data that represent the rates of cases, deaths, tests per million, etc. in our analysis since these are the vital statistics that represent the proxy of the respective population size. We found a number of missing values for each variable except for the cumulative number of infected patients. There are some countries that did not provide information on the number of domestic tests performed, such as China, Kuwait,

Algorithm 1 | CART algorithm

Procedure CART

1. Start at the root node
2. For each ordered value of X, convert it to an unordered variable $\tilde{X}$ by grouping its values in the node into a small number of intervals,
if X is unordeered, **then return** $\tilde{X} = X$
3. Perform a chi-squared test of independence for each $\tilde{X}$ variable vs. Y on the data in the node and compute its significance probability
4. Choose the variable X^* associated with the $\tilde{X}$ that has the smallest significance probability
5. Find the split set $\{X^* \in S^*\}$ that minimizes the sum of gini indexes and use it to split the node into two child nodes
6. **if** a stopping criteria is reached, **then return** exit

Otherwise, apply steps 2–5 to each child node

7. Prune the tree with the CART method

Algorithm 2 | Random forest algorithm

Procedure RF

1. Randomly select M features from the feature set.
2. For each X in M,

a. Calculate the information gain

$$Gain(t,X) = E(t) - E(t,X)$$
$$E(t) = \sum_{i=1}^{c} -P_i \log_2 P_i$$
$$E(t,X) = \sum_{c \in X} P(c)E(c),$$

Where $E(t)$ is the entropy of the two classes, and $E(t,X)$ is the entropy of feature X

b. Select the node d that has the highest information gain

c. Split the node into sub-nodes

d. Repeat steps *a*, *b*, and *c* to construct the tree until the minimum number of samples required to split is reached

3. Repeat steps 1 and 2 for N times to build forest of N trees

Oman, Cameroon, and Afghanistan. Before implementing any unsupervised machine learning techniques, such as principal component analysis (PCA), random forest, cluster analysis, and regression tree using the Classification And Regression Tree (CART) method Breiman et al. (1984) and the R package caret Kuhn (2020), we imputed all missing values with the Expected-Minimization algorithm technique, as suggested in Dray and Josse (2020). All 10 features were used for both PCA and cluster analysis. For CART and random forest analysis, however, the cumulative number of cases was used as a (Y), but all of the 10 variables were used as independent features (X). The pseudocode of CART and random forest methods are given below. Methods used for this study are displayed in a flowchart as displayed in **Figure 2**.

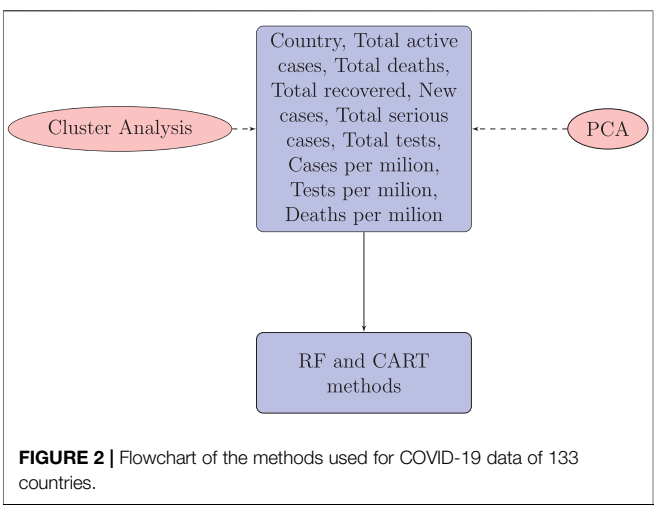

FIGURE 2 | Flowchart of the methods used for COVID-19 data of 133 countries.

3. ANALYSIS

Figure 3 displays of most of the COVID-19 cases and deaths are from the United States and European countries. We found that the United States and European countries, such as Germany, Russia, Italy, Spain, the United Kingdom, and France, administered a very high number of tests. The average number of tests among 133 countries is found to be nearly 156,500. The United States performed the highest at 3,398,140 and San Mario the lowest at 846 tests as of April 17, 2020.

All variables except for the country are correlated in this study. We standardized the data and imputed the missing value use of the Expectation-Maximization (EM) algorithm, according to (Dray and Josse, 2020), prior to performing the principal component analysis. We found the principal components through orthogonal transformation by converting the 10 correlated variables of the 133 countries into a set of values that are linearly uncorrelated variables. This exploratory data analysis is useful for making predictive models. This unsupervized machine learning technique will give the patterns of similarity in the countries and those orthogonal variables found. **Figure 4** shows such pattern where the first two principal components are displayed. We found that most of the variance (80%) is explained by the first two principal components.

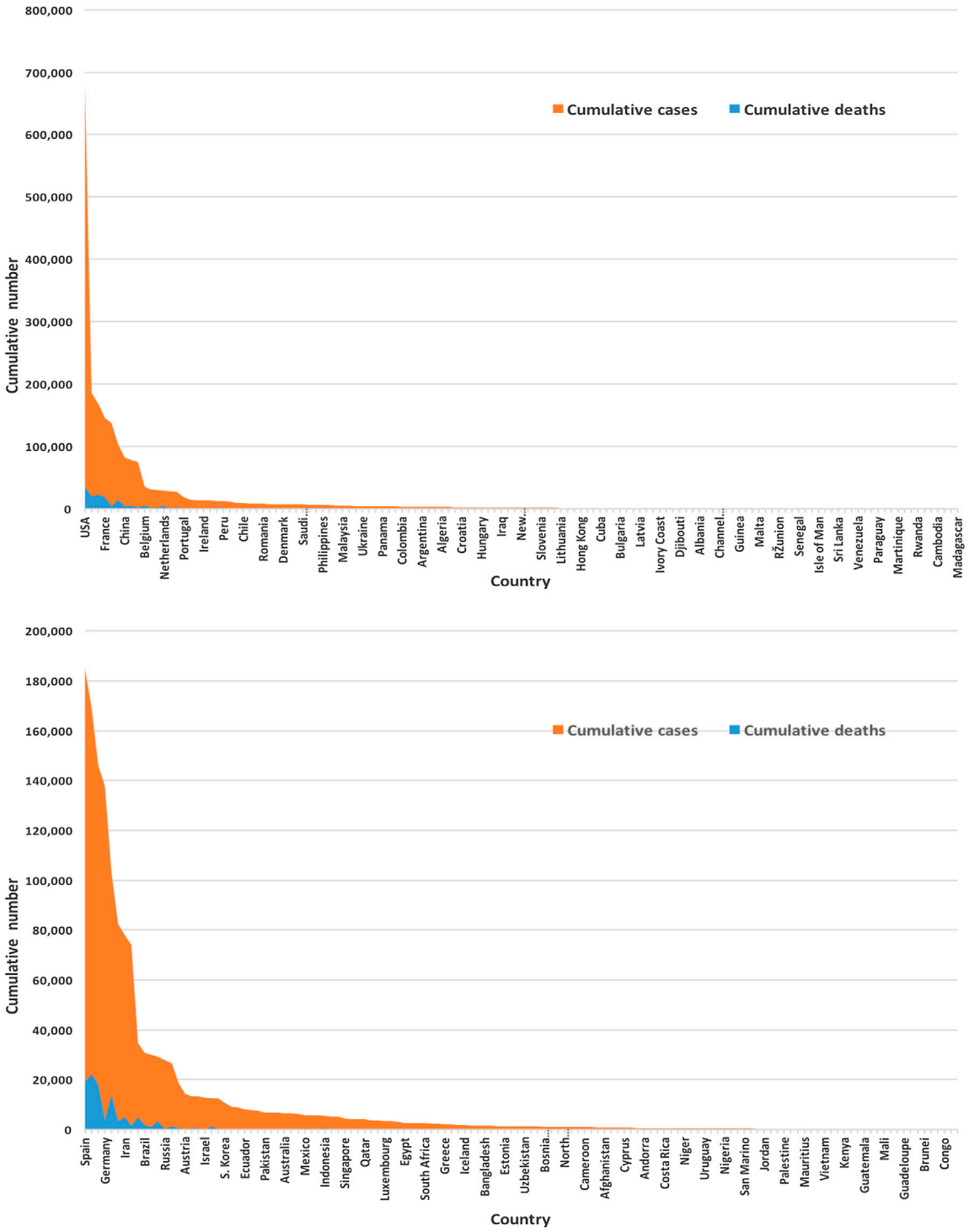

FIGURE 3 | Global infected cases and deaths of COVID-19 for 133 countries **(upper panel)** and without the United States **(lower panel)** as of April 17, 2020.

The main results are reflected in the graph of the scores in **Figure 4**, where we show the countries in the axes formed by the first two principal components. The cloud of individual points is centered at the origin to facilitate the data analysis. The first principal component is characterized by the variables: cumulative infected cases, cumulative deaths, active cases, cumulative recovered cases, cumulative serious cases, new cases, and cumulative tests. The countries that are vital to explaining the 60% variation of total variations by the first component include the United States, Spain, Italy, France, Germany, the United

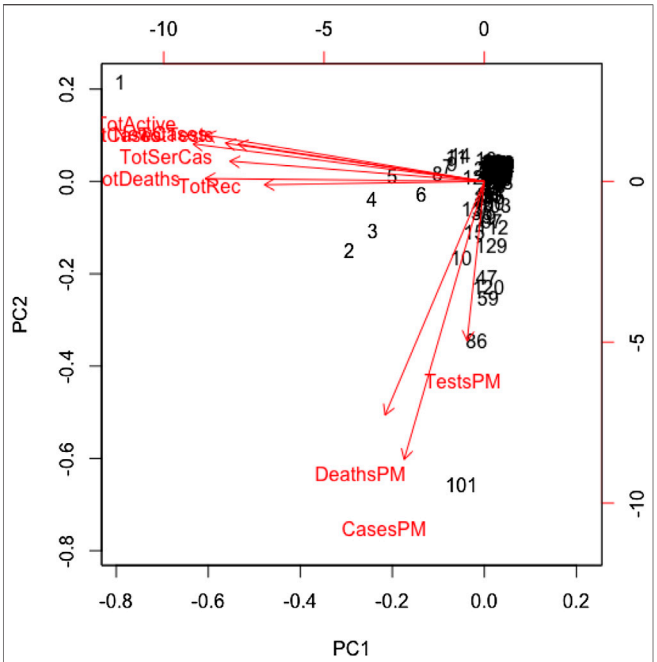

FIGURE 4 | Principal component analysis results for global COVID-19 data of 133 countries.

population size is the proxy of these rates, playing a vital role in the second principal component, which explains 20% of the total variations.

We used the cluster analysis technique for the imputed and standardized data, as used in the principal component analysis. The heatmap of the hierarchical cluster analysis, as shown in **Figure 5**, reveals that there are two clusters among the variables and four clusters among the countries. Three rate variables together–tests, cases, and deaths per million form one cluster while the remaining seven variables together form the second cluster. It is mentioned that the rate variables under the first cluster together address the population number. Population is a significant factor when assessing a country's COVID-19 response. However, we observed four major clusters among the countries.

Table 1 shows the full list of the clusters. The first cluster contains all the countries that contributed to the first principal component's variation in the PCA analysis along with China. The PCA also suggests that we validate this clustering because the heatmap in **Figure 5** reveals that these countries are clustered based on the maximum variation directed by the all seven variables. It is observed, from the data collected, that these nine countries were the most affected countries. Additionally, the economic conditions and medical facilities of these countries are among the best in the world. The second cluster contains 43 countries which are clustered according to all variables except for

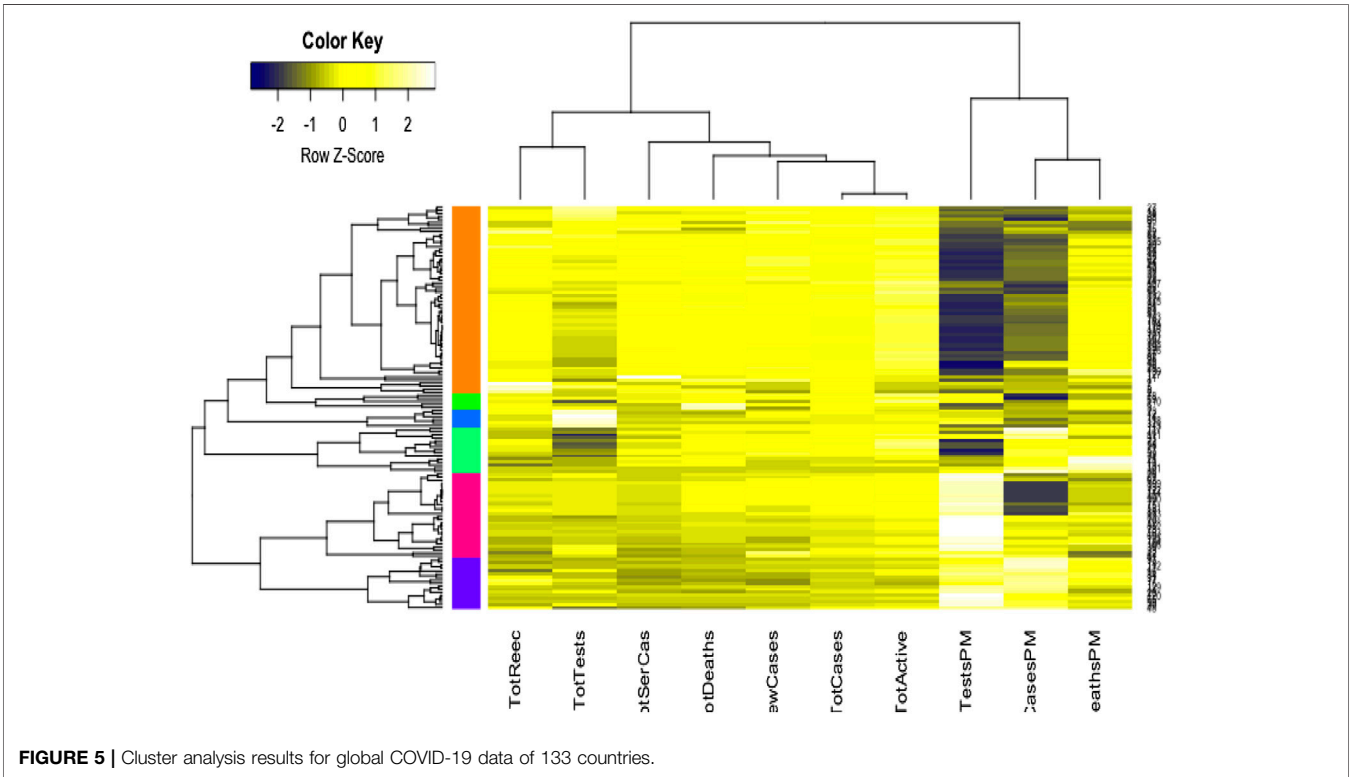

FIGURE 5 | Cluster analysis results for global COVID-19 data of 133 countries.

Kingdom, and Iran. The second principal component is characterized by the remaining variables: rate of deaths, rate of infected cases, and rate of tests per million. A country's

the test and case rates per million. Most of the 43 countries are middle income countries and have moderated health facilities. The third cluster consists of 14 countries that are clustered based

TABLE 1 | Cluster-wize country lists for 133 countries.

Cluster	Country
Cluster 1 (n = 8)	United States, Spain, Italy, France, Germany, United Kingdom, China, Iran
Cluster 2 (n = 43)	Afghanistan, Australia, Austria, Belarus, Brazil, Brunei, Burkina Faso, Cameroon, Canada, Congo, Cyprus, Czechia, Denmark, Diamond Princess, DRC, Estonia, Finland, Guadeloupe, Guinea, Hong Kong, Israel, Ivory coast, Kuwait, Latvia, Lithuania, Madagascar, Mali, Martinique, Netherlands, New Zealand, Norway, Oman, Portugal, Qatar, Russia, Runion, S. Korea, Senegal, Singapore, Slovenia, Sweden, Turkey, Venezuela
Cluster 3 (n = 14)	Andorra, Bahrain, Belgium, Channel Islands, Faeroe Islands, Gibraltar, Iceland, Ireland, Isle of Man, Luxembourg, Malta, San Marino, Switzerland, UAE
Cluster 4 (n = 68)	Albania, Algeria, Argentina, Armenia, Azerbaijan, Bangladesh, Bolivia, Bosnia and Herzegovina, Bulgaria, Cambodia, Chile, Colombia, Costa Rica, Croatia, Cuba, Djibouti, Dominican Republic, Ecuador, Egypt, El Salvador, Georgia, Ghana, Greece, Guatemala, Honduras, Hungary, India, Indonesia, Iraq, Jamaica, Japan, Jordan, Kazakhstan, Kenya, Kyrgyzstan, Lebanon, Malaysia, Mauritius, Mayotte, Mexico, Moldova, Montenegro, Morocco, Niger, Nigeria, North Macedonia, Pakistan, Palestine, Panama, Paraguay, Peru, Philippines, Poland, Romania, Rwanda, Saudi Arabia, Serbia, Slovakia, South Africa, Sri Lanka, Taiwan, Thailand, Trinidad and Tobago, Tunisia, Ukraine, Uruguay, Uzbekistan, Vietnam

on all variables other than death rate per million. These countries have much fewer deaths. These 14 countries are rich and may have well-developed health facilities available. The final cluster consists of the highest number, 68 countries, and these are clustered mainly based on the test and case rates variable, though other variables were also used in this study. Most of these 68 countries are poor, and they may thus have very poor conditions for treatment and healthcare facilities.

We implemented the regression tree using CART to predict the cumulative number of infected people. The main purpose of implementing the regression tree is to see whether the global cumulative number of infected people can be predicted accurately using the 10 variables in this study. Results are presented in **Table 2**, which shows the weights, including their percentage of importance, for all 10 variables. It revealed from the results that country and cumulative active cases appeared to be the most important variables to predict the cumulative number of infected people, and these were followed by the cumulative deaths, cumulative recovered cases, new case, and cumulative serious cases. Most strikingly, however, we found that the cumulative tests appeared as one of the most unimportant variables to predict the cumulative number of infections.

We also implemented the random forest to predict the cumulative number of infected people. The random forest is a model made up of many decision trees that are then transformed into a single ensemble model. This model uses two key concepts–random sampling of training data points when building trees and random subsets of features considered when splitting nodes. The decision tree is prone to overfitting when we do not limit the maximum depth, and this is due to its unlimited flexibility. As an alternative to limiting the depth of the tree, which reduces variance and increases bias is the random forest. Results are presented in **Table 2**, which shows the weights, including their percentage of importance, of all 10 variables. The weight is the total decrease in node impurities, measured by the Gini Index from splitting the variable, averaged over all trees. We found very similar results for the regression tree using CART. That is, country and cumulative active cases appeared to be the most important variables with which to predict the cumulative number of infected people. And the cumulative tests appeared to be one of the unimportant variables with which to predict the cumulative number of infections. This is a striking finding obtained by using both the CART and random forest methods. We have not found any other studies that have obtained a similar result.

The prediction accuracy for both methods, regression tree and random forest, has been measured with the root mean square log error (RMSLE). The RMSLE is calculated as

$$\text{RMSLE}\ (\hat{y}_i, y_i) = \sqrt{\frac{1}{n}\sum_{i=1}^{n}\left[\log(\hat{y}_i + 1) - \log(y_i + 1)\right]^2}$$

where n is the total number of observations, $\hat{y}_i$ is the predicted value, and y_i is the actual value for the ith cases. Here, $\log(y_i)$ is the natural logarithm of y_i. **Table 2** shows that the random forest method can predict more efficiently than the regression tree method. This suggests that random forest, as expected, is better when predicting the global cumulative cases of COVID-19.

TABLE 2 | Importance of variables by regression tree and random forest.

	Regression tree	Random forest
Variable names	**Percentage of importance (weights)**	
Country	25 (454.2)	19.1 (31.4)
Total active cases	24 (421.8)	16.1 (26.5)
Total deaths	16 (332.7)	15.7 (25.8)
Total recovered	14 (255.2)	13.2 (21.7)
New cases	10 (174.7)	14.0 (23.0)
Total serious cases	8 (149.0)	13.3 (21.9)
Total tests	1 (17.4)	7.2 (11.9)
Cases per million	1 (17.3)	0.3 (0.5)
Tests per million	0 (1.0)	0.2 (0.4)
Deaths per million	0 (0.0)	0.9 (1.4)
RMSLE	0.339	0.287

4. DISCUSSIONS AND CONCLUSIONS

In this paper, we demonstrated how to implement the basic machine learning techniques–principal component, cluster analysis, and regression tree to analyze global COVID-19 data that was extracted from the Worldometer website (Max Roser and Ortiz-Ospina, 2020) as of April 17, 2020. We considered 10 variables for each of the 133 countries. Through use of PCA analysis found that there are two latent variables that are characterized by the 10 variables we considered. The first principal component explains the 60% variation of the total variations, and this is characterized mainly by seven variables. These are the total infected cases, deaths, active cases, recovered

cases, serious cases, new cases, and total tests. The majority of the total variations is made up of all variables except for the rate variables. The remaining three variables—case, death, and test rates (measured in per million)—characterize the second principal component, which accounts for the 20% variation of the total variations. The latent factor behind this appears to be the country's population size, as all these three variables representing their population size. None of the populations (nor the population densities) of the 133 countries are. We believe that country's population size or indirectly the associated population density is responsible for the 20% variation of the total variations.

The cluster analysis found four major clusters among the countries but two clusters among the 11 variables. The analysis reveals that the countries are clustered based on the variation among the variables. We found that the eight countries that have the highest number of cases form a cluster, while 43 countries form another cluster based on all the variables except for the case and test rates. The eight countries are the United States, Spain, Italy, France, Germany, the United Kingdom, China, and Iran, and they are all homogeneous in term of cumulative cases, deaths, active cases, and tests. Most of them were/are the epicenter of the pandemic. However, we found that 14 countries with very low rates of death form one cluster and 68 countries with higher test and case rates, along with the significant effect of the other eight variables, form the fourth cluster. Countries and territories with low death rates include Bahrain, Belgium, Channel Islands, Faeroe Islands, Gibraltar, Iceland, Ireland, Isle of Man, Luxembourg, Malta, San Marino, Switzerland, and the UAE.

We found from both the regression tree and random forest analyses that country, total active cases, total deaths, total recovered cases, new cases, and total serious cases are very important variables with which to predict the cumulative number of cases. The number of tests (including the three rate variables) is not an important variable. As stated, global data analysis indicates that the cumulative number of tests is not significant when predicting cumulative cases, but it is quite important to consider a specific country in terms of situation and context. Besides, the policies on testing differ from country to country, region to region, or even city to city. It mainly depends on what stage a specific country or community has reached in terms of the pandemic curve or the level of preparedness in terms of lab facilities, lab staff, sample collection strategies, etc. When resources are limited and the healthcare system is overloaded, widespread testing, such as that suggested by the World Health Organization (WHO), may not be implemented. This is a reality for many of the low- and middle-income countries on our list of 133. The number of tests is important for many countries to limit the spread in the early stages (or even in any stage of spread), as this affects the ability to identify cases and isolate them and their contacts. However, global COVID-19 data analysis results reveal that cumulative tests are not at all important determinants with which to predict the cumulative number of tests for the country.

The world grapples with the containment of the COVID-19 outbreak, and countries are trying to reduce virus spread by performing tests for detecting and then isolating the infected people and quarantining the susceptible people. Besides, continuing the lockdown and social distancing is expected to help in reducing the spread considerably. However, this paper found that the countries are clustered with respect to underlying effects of the covariates, though the countries are fighting independently against this virus war. Similarly, variables related to rates form a cluster together while other variables form another cluster. Most strikingly, we found that the cumulative tests appeared as an unimportant variable when predicting the cumulative number of infected people.

This study was conducted to assess how the countries are clustered in terms of the covariates. Implementation of unsupervized and supervised methods revealed that the classification of countries is important, as it might help when analyzing the spread of disease and predicting the global cumulative cases of COVID-19. However, the countries in each cluster might have different strategies and policies with which to control the epidemic outbreak. They should all depend on data-dependent strategies, such as tracing and tracking the reproduction number of COVID-19, when developing methods with which to control the outbreak. Some early studies with data from Wuhan revealed the importance of exploring the reproduction number of COVID-19 (Kenji et al., 2020; Qun et al., 2020). Also, countries within the cluster need to evaluate several health facilities and their preparedness. So, the unsupervised learning could be useful when learning about the shortcomings of health facilities in the groups where infection is higher and when assessing what strategies are necessary when trying to prevent the spread of infection within or outside the country. Besides, classification and grouping based on the underlying latent feature could be useful to countries when trying to control the epidemic outbreaks through common remedial measures.

We used the CART and random forest methods, although random forest has better predictive power and accuracy than a single CART model due to the lower variance exhibited by the random forest. Our main goal was to know whether the independent features are significantly associated with the dependent variable "cumulative cases" rather than predictive accuracy. However, the CART has advantages: the rules are easily interpretable and it offers automatic handling of variable selection, missing values, outliers, local effect modeling, variable interaction, and non-linear relationships. Although the definition of recovery may vary from country to country, this study has used the number of recovered people, without knowing the actual definition of the recovery from COVID-19, for the respective country. This is a limitation of this study. One of the future directions could be the comparison of results of regression tree using CART and random forest methods with other machine learning counterparts, such as the support vector machine (SVM) and deep learning methods.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analysed for this study. The data used for the current study has been collected from the real time COVID-19 data from the Worldometer website (Max Roser and

Ortiz-Ospina, 2020) until April 17, 2020. The working data set used for this study has been submitted to the journal as additional supporting file.

AUTHOR CONTRIBUTIONS

MK carried out the statistical analysis and contributed to draft the manuscript. AH arranged the datasets and contributed to finalize the manuscript.

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This manuscript has been released as a pre-print at medRxiv (https://www.medrxiv.org) (Khan and Hossain, 2020a).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frai.2020.561801/full#supplementary-material

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REVIEW
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An Italian Guidance Model for the Management of Suspected or Confirmed COVID-19 Patients in the Primary Care Setting

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An outbreak of Severe Acute Respiratory Syndrome Coronavirus 2 started in China's Hubei province at the end of 2019 has rapidly become a pandemic. In Italy, a great number of patients was managed in primary care setting and the role of general practitioners and physicians working in the first-aid emergency medical service has become of utmost importance to coordinate the network between the territory and hospitals during the pandemic. Aim of this manuscript is to provide a guidance model for the management of suspected, probable, or confirmed cases of SARS-CoV-2 infection in the primary care setting, from diagnosis to treatment, applying also the recommendations of the Italian Society of General Medicine. Moreover, this multidisciplinary contribution would analyze and synthetize the preventive measures to limit the spread of SARS-CoV-2 infection in the general population as well as the perspective for vaccines.

Keywords: COVID-19, primary health care, general practitioners, severe acute respiratory syndrome, severe acute respiratory syndrome coronavirus 2, SARS virus, coronavirus infections

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INTRODUCTION

An outbreak of unexplained low respiratory infections cases detected in Wuhan (the largest metropolitan area in China's Hubei province accounting for 11 million inhabitants), was first reported to the WHO Country Office in China, on December 31, 2019 (1). The etiology of this illness was attributed to a novel virus belonging to the coronavirus (CoV) family, named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), originated from a reservoir of bats and unknown intermediate hosts (2–4). Previous outbreaks of coronaviruses (CoVs) such as the severe acute respiratory syndrome (SARS-CoV) in 2002 and the Middle East respiratory syndrome (MERS-CoV) in 2012 have been recorded previously and properly contained by Public Health Authorities in the past (5).

SARS-CoV-2 is a member of subgenus Sarbecovirus (previously lineage b) in the family Coronaviridae, genus Betacoronavirus, with high (87.6–87.8%) genome sequence identities to SARSr-Rp-BatCoV-ZXC21/ZC45, detected in Rhinolophus pusillus bats from Zhoushan, China, during 2015 (4). The outbreak of SARS-CoV-2 was declared a Public Health Emergency of International Concern on 30th of January 2020, when counted over 7,800 confirmed cases in China and 82 cases outside China (6). At that time, in Italy, only three imported cases (a couple of Chinese tourists and a University researcher back from the Hubei Province) were laboratory-confirmed and hospitalized at the "Lazzaro Spallanzani" Hospital in Rome (7). On 11 February 2020, WHO specified a new name for the disease caused by SARS-CoV-2: COVID-19 (2). Due to the characteristics of the novel Coronavirus (contagious for a long time, also in asymptomatic hosts) and to the extraordinary spread capacity (estimated R0 from 2.5 to 5.5), SARS-CoV-2 has reached the necessary epidemiological criteria to be declared a pandemic by the WHO on 11th March 2020, when more than 400,000 people in at least 150 countries had already been infected (8).

In Italy, the epidemic outbreak was in northern Italy, and this is probably due to the factors that may have influenced the sharp increase in the outbreak, including higher pollution and the presence of airports with international connections (9, 10). The rate of patients in home isolation without symptoms or with mild symptoms in Italy has progressively increased from 35% on March 11th when over 10,000 people were infected by SARS-CoV-2, to >95% on August 31st with more 268,000 infected people (11). Therefore, the role of the general practitioner in the context of the SARS-CoV-2 outbreak is crucial both for early detection of suspected patients and for the high rate of infected patients that are in home isolation and can be safely managed in the primary care setting. The aim of this manuscript is to provide a model for the management of suspected, probable, or confirmed cases of SARS-CoV-2 in the primary care setting, ranging from diagnosis to treatment.

Moreover, this multidisciplinary contribution analyze and synthetize the evidence based preventive measures that could limit the spread of SARS-CoV-2 infection in the general population as well as the perspective for vaccines.

COVID-19: INFECTION, TRANSMISSION AND DEFINITION OF CASES

SARS-CoV-2 is an RNA betacoronavirus and belongs to the coronaviridae family, which is considered an important human and animal pathogen. SARS-CoV-2 presents genetic regions similar to the SARS-Cov_1 and uses angiotensin-converting enzyme 2 as a cellular input receptor (12).

Compared to SARS-CoV-1 that replicates mainly in the lower respiratory tract, SARS-CoV-2 is highly present in the upper respiratory tract—even in asymptomatic patients—and this explains the greater transmission and spread caused by SARS-CoV-2 (13).

SARS-CoV-2 is transmitted via droplets and fomites during close unprotected contact, especially in enclosed places (14). Airborne transmission has not been reported for SARS-CoV-2 and it is not considered a way of contagion (15), although certain aerosol-generating procedures (i.e., orotracheal intubation) conducted in healthcare facilities could represent a way of airborne transmission (16).

However, patients undergoing procedures as noninvasive ventilation at home may be source of airborne transmission (17).

Fecal shedding has been demonstrated by some patients, but the fecal-oral route does not seem to be a possible way of SARS-CoV-2 transmission (14). The incubation period is approximately 14 days with most patients manifesting infection 4–5 days after exposure (18, 19).

Covid-19 cases are distinguished by WHO into suspected, probable and confirmed cases (20) as follows:

- Suspected cases:
  - a patient with an acute respiratory tract infection presenting one of the following signs: cough, fever, shortness of breath AND no other etiology that fully explains the clinical presentation AND a history of travel or residence in a country/area reporting local or community transmission during the 14 days prior to symptom onset; OR
  - a patient with any acute respiratory illness AND a close contact with a confirmed or probable COVID-19 case in the last 14 days prior to the onset of symptoms; OR
  - a patient with severe acute respiratory infection with fever and at least one sign/symptom of respiratory disease (e.g., cough, fever, shortness breath) AND requiring hospitalization AND no other etiology that fully explains the clinical presentation.
- Probable Case: a suspected case testing for SARS-CoV-2 with inconclusive or positive results for a pan-coronavirus assay.
- Confirmed Cases: a case with laboratory confirmation of SARS-CoV-2 infection, irrespective of clinical signs and symptoms.
  Two main reason could be associated with a negative swab followed by a positive one after some days or weeks. The first one is associated with the low sensitivity of oro- and naso- pharyngeal swabs with possible false negative results. The second one could be related with the timing of the swab execution. SARS-CoV-2 demonstrate a time range of incubation that varied, 2–14 days but in some cases a positive swab was observed also 20 or 30 days after the suspect contact with a COVID-19 patient (21).

CLINICAL SYNDROME AND APPROACH TO SUSPECTED OR CONFIRMED COVID-19 PATIENTS IN THE PRIMARY CARE SETTING

The majority of people infected with SARS-CoV-2 (from 50 to 75%) are asymptomatic (22, 23), but the exact frequency of asymptomatic patients is difficult to establish yet. Asymptomatic

confirmed cases may have lung abnormalities on chest CT and could represent "a formidable source" of contagion if adequate physical distancing measures lack (24–26). Interestingly, in a cohort of 58 asymptomatic patients admitted to hospital with an history of exposure to SARS-CoV-2, no symptoms and no alteration in laboratory findings, lung CT showed ground-glass opacity in about 95% of cases, and after short-term follow-up, 28% of them presented symptoms (27).

Symptomatic infection can be associated with different clinical illness (mild to critical form) (27). Most patients presenting to the general practitioner generally complain fever (body temperature > 37.5 in 89%), dry cough, shortness of breath (68%), anorexia (40%), productive cough (34%), myalgias (15%) or other suspected symptoms for COVID-19, such as olfactory and taste disorders (28–30). However, there is a major overall of symptoms of COVID-19 with other respiratory infections (e.g., flu from influenza virus) which makes it difficult to distinguish SARS-CoV-2 infection from them at an early stage. Another challenge for the general practitioner is the identification of cases at high risk of progression that may require hospitalization, considering that these patients are usually monitored through phone calls. Therefore, the general practitioner and physicians working in the first-aid emergency medical service coordinate the network between the territory and hospitals. For the abovementioned reasons, the Italian Society of General Medicine & Primary Care (S.I.M.G.), has published a guidance document (version 1.5 updated on April 29th, 2020) intended for general practitioners for the management of patients with suspected, probable or confirmed diagnosis of SARS-CoV-2 infection in the primary care setting (30). Herein, we illustrate the "ISVaMPIT" (i.e., Identify, Signal/report, eValuate, Monitor, Plan, Initiate Therapy) approach to these patients according to the guidance document, updated with information from current literature (30).

Patients with symptoms suspicious for COVID-19 should call their general practitioner and avoid going to his office, to first-aid emergency medical service, or the emergency department of hospitals. The general practitioner collects information regarding symptoms and epidemiological links with COVID-19 patients through phone calls and identifies suspected cases of COVID-19 (i.e., Identify of ISVaMPIT approach). Suspected cases of COVID-19 need to be reported to the local public health and hygiene service (i.e., Signal/report of ISVaMPIT approach) and appropriate precautions must be adopted for these suspected cases. Specifically, if the patient is asymptomatic the general practitioner should arrange fiduciary home isolation for him and his family for 14 days, and this quarantine can only be stopped in the presence of negative swab. In case of symptomatic patients the general practitioner will perform a telephone triage through a standardized form that allows to evaluate the severity of the symptoms and the presence of important comorbidities and risk factors and to decide whether to proceed with fiduciary home isolation, eventually request a swab and contact the special care continuity units (USCA), or to send the patient to the hospital for hospitalization by contacting the emergency/urgency service (30). If the symptomatic patient will not be hospitalized or in case of confirmed cases of COVID-19 not requiring hospitalization, the general practitioner should arrange fiduciary home isolation for him and his family for 14 days, and this quarantine can only be stopped in the presence of two negative swabs and clinical remission.

For the outpatient management of the COVID-19 or suspected COVID-19 patient by the general practitioner, personal protective equipment must be available in full (including the full suit or disposable gown, visor and, footwear as well as FFP2/FFP3 masks) (30, 31). If the personal protective equipment is not available, the general practitioner should not carry out the physical examination in his office or at patient home, but clinically evaluate and monitor the patient through telephone calls and/or, if possible, through video consultation (i.e., eValuate and Monitor of ISVaMPIT approach) (30).

Monitoring of clinical conditions includes daily monitoring of temperature, blood pressure (values $\leq$100 or $\geq$ 200 mmHg systolic indicate the severity of the disease), blood oxygen saturation, patient's state of consciousness, heart rate, and respiratory rate. Useful and practical tools for patient telephone monitoring of suspected or confirmed COVID-19 patients and prognostic evaluation include:

- the Mews score scale (Modified Early Warning Score), that gives a score based on the evaluation of five parameters (heart rate, body temperature, respiratory rate, alteration of consciousness, blood pressure) establishing the stability of the patient clinical condition (32);
- the "walking test," which is performed by inviting the patient to walk for about 5 min with a finger pulse oximeter and then evaluating saturation at intervals of about 1 min; this test can reveal occult alterations in blood oxygen saturation in patients who do not experience desaturation at rest;
- CURB-65 score, which is an acronym for each of the risk factors measured (i.e., confusion of new-onset, blood urea nitrogen, respiratory rate, blood pressure, and age > 65 years old) and estimates mortality of community-acquired pneumonia (33, 34).
- Single breath counting (SBC) is an alternative test to assess pulmonary function. SBC is measured by asking patients to take a deep breath and count as far as possible in their normal speaking voice without taking another breath (35, 36).

In addition to monitoring the clinical status, the general practitioner will verify the adherence to preventive measures, the psychological status as well as the basic social and welfare patient conditions during these phone calls or video consultations.

For the outpatient management of suspected or confirmed COVID-19 patients who cannot be handled over the phone by the general practitioner or sent to the hospital, general practitioners can require the involvement of Special Units for Continuous Healthcare (USCA) or may send the patient to the special tents of the emergency departments of the hospital (i.e., Plan and Monitor of ISVaMPIT approach and as suggested by ministerial guidelines). USCAs have been set up in Italy to support primary care assistance in the setting of COVID-19 (37). Once activated by the general practitioner, the USCA team—using the suitable personal protective equipment—goes to patients' homes to perform a physical examination, assessment

of blood oxygen saturation, eventual swab if needed, medical drug prescriptions, and, some first-level diagnostic tools (i.e., portable ultrasound, portable ECG) if available (38).The use of portable ultrasound in SARS-CoV-2 patients has emerged in the front lines of the SARS-CoV-2 epidemic in Italy (39). For instance, the predilection of lung pulmonary findings in subpleural regions and the availability of wireless ultrasound probes prompted to the possibility of using portable ultrasound as a potential triage and diagnostic tool in primary care setting after an adequate lung ultrasound training (37). Although there is limited experience at this time on US in SARS-CoV-2 patients, the main imaging findings in SARS-CoV-2 patients include thickening of the pleural line with pleural line irregularity, B lines in a variety of patterns including focal, multifocal, and confluent, consolidations in a variety of patterns including multifocal small, non-translobar, and translobar, A lines during the recovery phase, while pleural effusions were uncommon (38, 40–42).

Finally, in case of accidental or scheduled access to the general practitioner medical office of suspected cases of COVID-19, it is necessary to perform the sanitation of the environment according to the procedures indicated by the Italian National Institute of Health (Istituto Superiore di Sanità, ISS) (43).

THE ROLE OF SWAB IN THE PRIMARY CARE SETTING

In Italy, there have been regional differences in the execution of the swabs. For instance, in the municipality of Vo' Euganeo in the Veneto region swab was performed to the entire local population based on the North Korean model, while in the remaining Italian regions, swab was performed only in some suspected cases of COVID-19 (44). On April 3, 2020, in Italy, the Ministry of Health indicated that swab should be reserved primarily to symptomatic/paucisymptomatic suspected cases of COVID-19 and symptomatic contacts of the suspected case (i.e., symptomatic contacts of the suspected case were defined as those who had contact with the suspected or confirmed case of COVID-19 in the 48 h before the onset of symptoms), health care professionals at high risk (45).

The general practitioner in the case of early/mild symptomatic suspected cases can request the swab by filling-in the telephone monitoring form and sending it to the territorial public authority that will take charge of the request and will perform the swab at the patient home (30). Based on the indication of the Italian Ministry of Health (45), some Italian regions have created "drive-through" COVID-19 checks in which people are referred to these checks by their local health authorities, queue in their cars to get tested and the swab is then performed from the relative safety of people's cars (46).

These testing strategies are generally preferred for patients who no longer have symptoms of COVID-19 and have to undergo the two consecutive negative laboratory PCR tests to be declared definitively "non-infecting," in accordance with Italian guidelines of the Ministry of Health (47).

The CDC's criteria for patients to clear isolation, is based on two main strategies (48, 49):

1) Test-based strategy that represent the initial recommendation but still recognized (published on 12 January 2020), based on knowledge and experience with similar coronaviruses:
   - Resolution of fever without the use of fever-reducing medications AND
   - Improvement in respiratory symptoms (e.g., cough, shortness of breath), AND
   - Negative results of an FDA Emergency Use Authorized COVID-19 molecular assay for detection of SARS-CoV-2 RNA from at least two consecutive nasopharyngeal swab specimens collected $\geq$24 h apart (total of two negative specimens).
2) Non-test-based strategy, new WHO recommendation (published on 27 May 2020) that proposed:
   - For symptomatic patients: 10 days after symptom onset, plus at least three additional days without symptoms (including without fever and without respiratory symptoms)
   - For asymptomatic cases: 10 days after positive test for SARS-CoV-2.

These criteria can apply to all COVID-19 cases despite the type of specimen or disease severity and reflect recent findings that patients who no longer have symptoms sometimes stay positive for the COVID-19 virus (SARS-CoV-2) by molecular testing for many weeks. Regardless of the positive test result, these patients are unlikely to be infectious and transmit the virus. Although the risk of transmission after symptom resolution is likely to be minimal, it cannot be completely ruled out.

In symptomatic patients with a severe disease for prolonged periods of time, a laboratory-based approach measuring viral load and neutralizing antibody (or proven equivalent antibody) levels might be useful to decide for prolonged isolation.

At the moment, it is possible to use test-based strategy and in this case the initial recommendation of two negative PCR tests at least 24 h apart can be applied.

According to Italian guidelines, a Covid-19 patient is considered healed only after the resolution of symptoms and two negative consecutive tests for SARS-CoV-2 at 24-h intervals. Even if patients recover clinically before 7 days of onset of symptoms, it is still recommended to wait a 7-day interval between the first and last test. In asymptomatic patients, a negative SARS-CoV-2 RNA test at 14 days after the first test is needed (end of the quarantine period). For virus clearance it is defined as a negative viral RNA from body fluids of symptomatic and asymptomatic patients, accompanied by appearance of specific IgG (47). The discharge criteria do not allow the discontinuation of preventive measures, therefore, despite the negativity of the laboratory PCR test, the patient at home will still have to observe an isolation period for a total of 14 days. The optimum would be represented by the execution of a serological test that determines the presence of IgG. There is still no consensus on the need to repeat the serological test 30 days after the first determination.

Different approaches are provided in other countries. As an example, in France, a Covid-19 patient must remain in home isolation for at least 7 days since the onset of symptoms, and may leave home wearing a mask and respecting social distancing after these 7 days if he feels healthy; in case of persisting symptoms at the end of these 7-day quarantine, the patient must remain in home isolation and may leave home only 2 days after complete remission of symptoms, without the need for a new test for SARS-CoV-2 (50). In Germany, a positive test result means that the person concerned must self-isolate for 10 days; in most States of Germany, testing negative means home quarantine is no longer required, but in specific German States, a repeat test a number of days later may be necessary (51).

PREVENTIVE MEASURES OF SARS-COV-2 INFECTION IN THE GENERAL POPULATION

According to the WHO guidelines, some preventive measures should be carried out by general population to reduce the risk chances of being infected or spreading SARS-CoV-2 and the general practitioner should indicate them to his patients (52).

In particular, the following safety measures are considered essential in the prevention of the SARS-CoV-2 infection:

1. Regularly and thoroughly handwashing with soap and water or with an alcohol-based hand rub. If gloves are used, they should be change after usage or frequently. Hand washing remains the better way to prevent the virus spread;
2. Physical distancing (at least 1 meter or 3 feet distance between people) represents a second key point to prevent SARS-CoV-2 diffusion. For instance, when someone coughs, sneezes, or speaks they spray small liquid (called "droplets") from their nose or mouth which could contain the virus, also if asymptomatic;
3. Avoid crowded places or events because it is difficult to avoid close contact with other people;
4. Avoid touching eyes, nose, and mouth with the hands. Avoid also to touch face if you are wearing gloves. Hands usually touch many surfaces and can pick-up viruses;
5. Follow correct respiratory hygiene such as covering mouth and nose with bent elbow or tissue when coughing or sneezing and after washing hands immediately;
6. Self and home isolation even with minor symptoms such as cough, headache, mild fever is necessary until a diagnosis of SARS-CoV-2. In case of necessity to leave the house wearing a mask is mandatory;
7. The use of personal protective equipment such as medical mask (without filters) is useful mainly in closed places such as markets, stores, shops, medical clinics, post offices, and bank offices.
8. It should be aware that coronaviruses can remain infectious on inanimate surfaces like metal, glass or plastic for up to 9 days. It is of utmost importance that surfaces and equipment like ventilators used for SARS-CoV-2 infected children should be carefully disinfected with about 62–71% ethanol, 0.5% hydrogen peroxide or 0.1% sodium hypochlorite within 1 min (53).

TREATMENT FOR COVID-19 IN THE PRIMARY CARE AND HOSPITAL SETTING

Regarding the therapeutic management of the patient to date, no therapies have been shown effective against SARS-CoV-2 and all treatments—although potentiality effective against COVID-19—need either appropriate drug development or clinical trial to be suitable for clinical use (54, 55). Therefore, the Italian Drug Agency (i.e., AIFA) has opened a section of its website where it constantly updates the therapies under investigation for Sars-CoV-2 infection (56).

There are few drugs available in the primary care setting.

In case of asymptomatic suspected or confirmed cases, no therapy is performed but only clinical monitoring. The potential role of probiotics in the treatment of SARS-CoV2 infection has been suggested by some studies as seem to be involved in reducing the expression of proinflammatory cytokines and in the restoration of microbial flora preventing secondary infections; however, products available for bacteriotherapy are not the same and have different potential effects (57, 58). In case of paucisymptomatic patients but with a negative swab or in the absence of swab, it is possible to start only a symptomatic therapy with paracetamol, which currently remains the first choice as antipyretic, cough sedatives and hydration and, if necessary, home oxygen therapy. Nevertheless, there are no clear indication for the amount of oxygenation to deliver at home. Patient develop silent hypoxia, namely hypoxia without dyspnea, in up to 18.7% of cases (19), and, therefore, ma refuse the use of oxygen.

If the patient is symptomatic and has a positive swab, it is important to discourage widespread use of antibiotics as their use may lead to higher bacterial resistance rates. Indeed, recently AIFA in the technical data sheet of azithromycin has discouraged its use if not in the presence of bacterial superinfection also because of its potential side effects (56).

The following therapies are instead mainly used in a hospital environment (59). First, both AIFA and FDA revoked the authorization for the use of hydroxychloroquine in COVID-19 patients because the potential risks, as i.e., prolongation of QTc, were greater than the possible beneficial effects (56, 60). It seems that therapy with corticosteroids is to be reserved for patients with more severe symptoms who require oxygen support, at a dose of 6 mg daily for 10 days or until discharge.

On June 2020, the European Medicines Agency first and then AIFA approved the use of Remdesivir—a novel nucleotide analog—as Emergency Support Instrument for adult and pediatric hospitalized COVID-19 patients with more severe clinical conditions (61, 62).

However, it is important to emphasize that Remdesivir is an investigational drug and that the authorization could be revoked on the basis of data that would demonstrate its non-safety. The suggested adult dose is 200 mg intravenously on day

1 followed by 100 mg daily for 5 days total (61, 62). The drug is not recommended for alanine aminotransferase values > 5 times the normal value and is not recommended in patients with glomerular filtrate < 30 ml/min unless there is a benefit greater than the risk of administering it (61, 62).

Another therapeutic strategy, initially tested in China, could be hyperimmune plasma containing specific antibodies against the virus, obtained by convalescent or hospitalized subjects, when there are no therapeutic alternatives. In Italy, the study TSUNAMI (TranSfUsion of coNvaleScent plAsma for the treatment of severe pneuMonIa due to SARS.CoV2) was authorized in May by the Ethics Committee of the Spallanzani Hospital in Rome aimed at evaluating the effectiveness of this experimental treatment which involved centers distributed in 12 regions on the Italian national territory (63).

Considering the risk of thromboembolic events, recently AIFA has also authorized a study on heparin (mainly with low molecular weight heparin, LMWH), in the light of recent evidence showing a better prognosis of patients treated with this drug, particularly in those with high D-dimer, as thrombotic processes in the vessels induced by the inflammatory cascade is described (56, 64, 65). However, long-term treatment with this drug could lead to important side effects, so the benefits of such a therapeutic protocol are still being evaluated. In patients with severely compromised clinical situations, home management should be focused on supportive and palliative therapy (66).

VACCINATION AGAINST SARS-COV-2: WHERE DO WE STAND?

The quick diffusion of COVID-19 across the world requires the development of effective therapeutic options against the disease caused by SARS-CoV-2. The absence of licensed vaccines or antiviral drugs forced clinicians to setup the treatment of COVID-19 disease mainly on their experience (67).

Usually, a vaccination exposes the body to an antigen that is unable to cause a disease, but that should elicit the immune response to limit or block the infection. Some of the technologies tested by researcher were previously used in vaccines licensed and offered to the general population. At the same time, different strategies that have not been used before in the production of vaccines are currently under trials (68).

At least six research groups have already begun phase 1 of the trial, injecting the first dose of the new vaccine developed into healthy volunteers, while other groups are experimenting the safety of the vaccine precursors on animals, as reported in a guide published on *Nature* in the month of April 2020 (69).

Several strategies are being tested to develop a vaccine against SARS-CoV-2 that could be resumed as follows (68–70):

a. attenuated (live-attenuated) or inactivated virus vaccines: a type of vaccination that contain the SARS-CoV-2 in a weakened or inactivated form, that especially for live-attenuated vaccines requires several safety testing (71);
b. viral-vector vaccines: engineering of other viruses (such as adenoviruses), replicating or not-replicating in a weakened form, to produce SARS-CoV-2 proteins, such as spike proteins (72);
c. nucleic acid vaccines: these vaccines will encode the immunogenic proteins of SARS-CoV-2 (such as spike proteins) using the DNA or the RNA (73);
d. protein-based vaccines: use of a fragment of proteins (protein subunits) or viral-like particles (VLP) of the SARS-CoV-2 to inject directly into the human body;

As of 31 July 2020, 7 months after the beginning of the SARS-CoV-2 pandemic, more than 165 vaccines are being developed worldwide and at least 19 vaccine candidates have, entered clinical trials, including phase 2 and 3 trials (74). Regarding the new vaccines against SARS-CoV-2, the major challenges for scientific community are the clinical safety and the effectiveness on a large scale, but also the construction of adequate platforms that could support vaccine production on a large scale (75).

In order implement vaccination plans and to guarantee the largest acceptance of new SARS-Cov-2 vaccines among at-risk categories such as subject with comorbidities, elderly or healthcare workers, previously studies conducted analyzed evidence-based strategies on how to reduce vaccine hesitancy (76–79).

To conclude, the SARS-CoV-2 outbreak is changing its face in Italy with an increasing number of patients managed in the primary care setting. The general practitioner and physicians working in the first-aid emergency medical service coordinate the network between the territory and hospitals. The role of the general practitioner in the context of the SARS-CoV-2 outbreak is crucial both for early detection of suspected patients and for the high rate of infected patients that are in home isolation and can be safely managed in the primary care setting. This article reviews the Italian model for the management of patients with suspected, probable, or confirmed diagnosis of SARS-CoV-2 infection in the primary care setting, providing insights from different specialties that may help in guiding patient management.

AUTHOR CONTRIBUTIONS

GL, NL, FV, CC, and CI: literature review and manuscript drafting. SS, FD, and CG: manuscript reviewing. All authors contributed to the article and approved the submitted version.

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Foundation and Clinical Evaluation of a New Method for Detecting SARS-CoV-2 Antigen by Fluorescent Microsphere Immunochromatography

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Purpose: To develop a rapid detection reagent for SARS-CoV-2 antigen for the auxiliary diagnosis of new coronary pneumonia (COVID-19), and perform the methodological evaluation and clinical evaluation of the reagent.

Method: SARS-CoV-2 N-protein test strip was created by combining fluorescent microsphere labeling technology and immunochromatographic technology, based on the principle of double antibody sandwich. Then we evaluated the analytical capability and clinical application of the strips.

Result: The limit of detection of the strips for recombinant N protein was 100 ng/ml and for activated SARS -CoV-2 virus was 1×10^3 $TCID_{50}$/ml. The strips also have high analytical specificity and anti-interference capability. According to the predetermined cut-off value, the specificity of the test strip in healthy controls and patients with other respiratory disease was 100.00 and 97.29%, the sensitivity in COVID-19 cases at progress stage and cured stage was 67.15 and 7.02%. The positive percentage agreement and negative percentage agreement of antigen strip to RNA test were 83.16 and 94.45%.

Conclusion: SARS-CoV-2 fluorescence immunochromatographic test strip can achieve fast, sensitive and accurate detection, which can meet the clinical requirements for rapid detection of viruses on the spot.

Keywords: SARS-CoV-2, COVID-19, clinical evaluation, antigen detection, immunochromatographic

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INTRODUCTION

In December 2019, a number of cases of viral pneumonia were found in Wuhan, China, and the initial cases were related to the exposure in Wuhan seafood market (Jiang et al., 2020; Wu et al., 2020). On February 11, 2020, the International Committee on Taxonomy of Viruses (ICTV) announced the official name of the new coronavirus: severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); simultaneously, the World Health Organization (WHO) announced that the new coronavirus-infected pneumonia was officially named "COVID-19" (Munster et al., 2020; Zhu N. et al., 2020). The COVID-19 has spread to 206 countries and regions in the world by April 4th, 2020, accounting for 88.4% of the total. More than 1 million cases of covid-19 have been confirmed worldwide, and more than 50,000 cases have died.

Coronavirus is a common positive-strand RNA virus that causes respiratory diseases, existing widely in nature. Humans, vertebrates and invertebrates can be their parasitic host (Guo et al., 2020). So far, 7 kinds of infectious coronavirus have been found, i.e. HCoV-229-E, HCoV-OC43, SARS-CoV, HCoV-NL63, HCoV-HKU1, MERS-CoV and the recently discovered SARS-CoV-2 in Wuhan. Among those, 229E, NL63, OC43 and HKU1 can cause common cold symptoms, and the remaining three are highly pathogenic SARS-CoV (atypical pneumonia), MERS-CoV (Middle Eastern Respiratory Syndrome Coronavirus), and the newly discovered SARS-CoV-2.

According to the analysis of the SARS-CoV-2 database officially released by the National Genomics Science Data Center on January 22, the whole genome sequence of SARS-CoV-2 is 29903nt, which mainly includes the genes ORF1a and 1b encoding non-structural proteins and S, E, M, N encoding structural proteins. The M and E protein play a critical role in coordinating virus assembly and forming mature viral envelopes, while the N protein binds to the viral RNA and is involved in the transcription and replication of viral RNA, as well as packaging of the encapsidated genome into virions (Ashour et al., 2020; Nishiga et al., 2020; Zhu C. et al., 2020). Research shows that both SARS-CoV-2 in 2019 and the SARS outbreak in 2003 probably originate from the bat, and genome sequence similarity is up to 80%. Moreover, SARS-CoV-2 infection path and the pathogenesis are similar to SARS (Sun et al., 2020; Zhou et al., 2020), namely, the S-protein of SARS-CoV-2 and Angiotensin converting enzyme gene 2 (Angiotensin-converting enzyme 2, ACE2) interacting invades into the host cell, and then complete the replication of the virus (Prompetchara et al., 2020).

Pathogens are usually tested by molecular diagnosis and immunodiagnosis. The N protein can be exposed in the process of virus assembling, which make it become one of the targets of clinical detection. An article published reported detect the N protein of MERS-CoV with antigen detection method was feasible (Chen et al., 2015). In the early stage of the SARS-CoV-2 outbreak, the fluorescence PCR method was adopted in preference. The results of this method were accurate, but there were also some problems such as complicated operation and susceptibility to environmental factors. Therefore, the rapid immunodiagnostic reagents can be used for screening in the middle and late stages of the epidemic prevention. In the process of developing immunoassay reagents, the specific and conserved sequence of viral N protein was selected through the published genome sequences, a large number of antibodies were screened, and then the viral antigens were detected by the method of double-antibody sandwiched antigens.

MATERIALS AND METHODS

Patients and Samples

Nasal/oropharyngeal swabs of a total of 990 samples from January 2020 to April 2020 were collected and tested in this study, including 247 COVID-19 patients, 443 patients with other respiratory diseases and 300 healthy people. Nasal/oropharyngeal swab samples were collected from the patients/healthy people according to standard operation, stored and transported within a single tube of Hanks virus preservation solution (ph7.4–7.6) (Beijing Youkang Technology Co., Ltd) to prevent viral RNA/protein degradation.

Reagents and Equipment

Mouse anti-SARS-CoV-2 N protein monoclonal antibody-1 and mouse anti-SARS-CoV-2 N protein monoclonal antibody-2 were purchased from Beijing Biosynthesis Biotechnology Co., Ltd.; The recombinant N protein of SARS-CoV-2 were donated by Tianjin University; The rabbit IgG was purchased from Beijing Mingchaoxi Technology Co., Ltd. Company; The sheep anti-rabbit secondary antibody was purchased from Kema Biotechnology (Beijing) Co., Ltd.; The latex fluorescent microspheres were purchased from Ocean Nanotech (USA); The nitrocellulose membrane was purchased from Merck Chemical Technology (Shanghai) Co., Ltd.; The glass cellulose membrane was purchased from Shanghai Joey Biotechnology Co., Ltd.; The PVP bottom plate was purchased from Shanghai Kinbio Biotechnology Co., Ltd.; The N-hydroxysuccinimide (NHS), Carbonized (EDC) and the biological preservatives were purchased from Sigma-Aldrich (Shanghai) Trading Co., Ltd. The Savant-100 fluorescent immunochromatography analyzer was purchased from Beijing Savant Biotechnology Co., Ltd. The Symphony-100 fluorescence immunoanalyzer was purchased from Tianjin Boomscience Technology Co., Ltd. Beijing Savant Biotechnology Co., Ltd. is responsible for the small batch production of SARS-CoV-2 antigen test strips and packaging materials.

Method

The design and procedure are illustrated schematically in **Figures 1** and **2**.

(1) Recombinant SARS-CoV-2 N protein

According to the sequence of SARS-CoV-2 (Wuhan, Accession: QHD43423.2), the gene of N protein was synthesized and inserted into pET28a vector. The recombinant SARS-CoV-2 N protein was obtained by inducing expression in E. coli and purified by Ni affinity column.

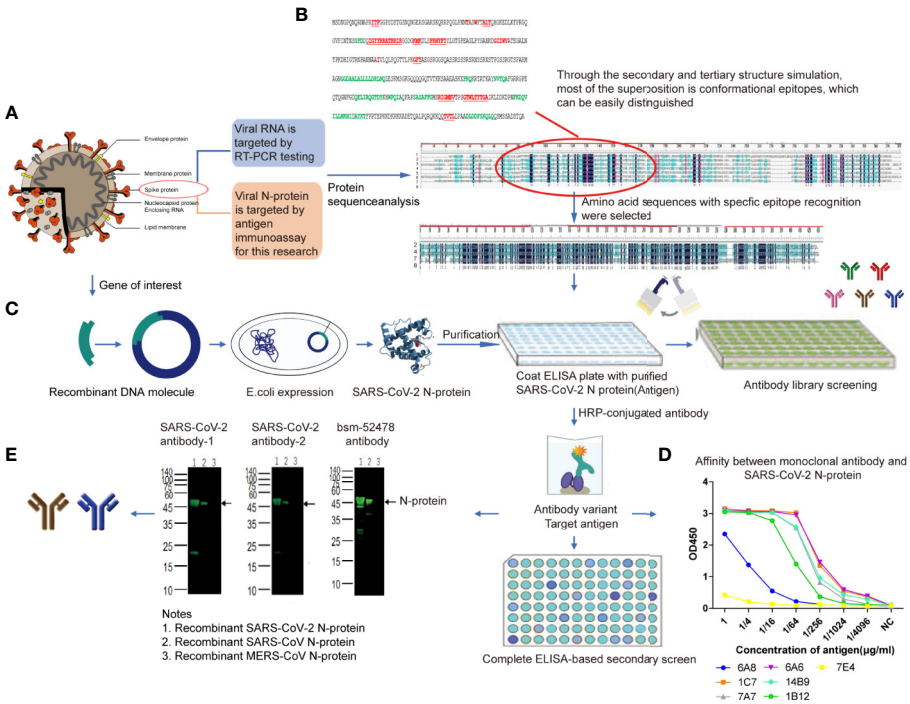

FIGURE 1 | SARS-CoV-2 N-protein specific antibody selection and cross reaction **(A)**. SARS-CoV-2 N-protein was chose for antigen immunoassay **(B)**. The sequence comparison between SARS-CoV-2 N-protein and that of the other 6 kinds of coronaviruses. From the top to the bottom of the list: 1, HCoV-229E (AOG74787.1_N); 2, SARS-CoV-2 (YP009724397.2_N); 3, HCoV-HKU1(AGW27885.1_N); 4,MERS-CoV (AHC74105.1_N); 5, HCoV-NL63 (AFV53152.1_N); 6, HCoV-OC43 (QDH43730.1_N); 7, SARS-CoV ShanghaiQXC1(AAR86795.1_N); 8,consensus sequence, the black area **(C)**. The expression of SARS-CoV-2 N-protein **(D)**. Affinity determination between monoclonal antibody and SARS-CoV-2 N-protein. 6A8, 6A6, 7e4, 1c7, 14b9, 7a7, and 1B12 represent seven monoclonal antibodies selected from antibody library of Beijing Biosynthesis Biotechnology Co. Ltd **(E)**. Cross-reaction result. Anti-SARS-CoV-2 N-protein monoclonal antibody-1 and antibody-2 were selected as immune target.

(2) Preparation of test strips

Firstly, 1 ml of latex fluorescent microspheres, 1 mg EDC and 1 mg NHS were mixed and stirred at room temperature for 3 h. Then 0.1 mg mouse anti-SARS-CoV-2 N protein monoclonal antibody-2 was added and stirred at room temperature for 1 h. Following that, 10 mg BSA blocking solution was added and stirred for 1 h. The centrifugation was performed at 2–8°C for 30 min at 11,000 r/min to remove the supernatant. Finally, the solid precipitate was redissolved to 1 ml of phosphate buffer solution (1 mol/L, pH = 7.4), 1 μL of Proclin 300 was added, and the mixture was stored at 4°C for use. In the same way, the rabbit IgG was labeled with latex fluorescent microspheres and stored at 4°C for use.

(3) Preparation of coating pads

The mouse anti-SARS-CoV-2 N protein monoclonal antibody-1 and the sheep anti-rabbit secondary antibody were diluted with phosphate buffer solution, respectively. The above solution was coated on the NC membrane at a concentration of 1 mg/ml, using a gold film sprayer. The line with mouse anti-SARS-CoV-2 N protein monoclonal antibody-1 was set as the test line (T line), and the other with sheep anti-rabbit secondary antibody was set as the quality control line (C line). The as-prepared pads were dried at 37°C with humidity <30% for 4 h.

(4) Preparation of marker pads

The latex fluorescent microsphere-labeled mouse anti-SARS-CoV-2 N protein monoclonal antibody-2 and latex fluorescent microsphere-labeled rabbit IgG were mixed in a volume ratio of 1:1 and sprayed on glass cellulose membrane at a rate of 10 μL/cm. Then the marker pad was dried at 45°C for 4 h.

(5) Test strip assembly

First, the coating pad was pasted on the PVC base. Then the absorbent pad near the C line on the NC film and the marker pad on the end near the T line were connected and cut into 4 ± 0.1 mm test strip with a slitter.

Detection

Antigen detection method with test strips was described as follows (1): Take out the test card at room temperature, cut the package and lay it on the table for later use (2). Take out the standard card in the kit, read the curve information in the standard card at the IC card sensing position of the fluorescence immunochromatograph and store it in the analyzer (3); Before testing, select the curve information consistent with the item and batch number (4); Add 60 μL of sample solution to each test strip, leave it at room temperature for 15 min and then insert it into the

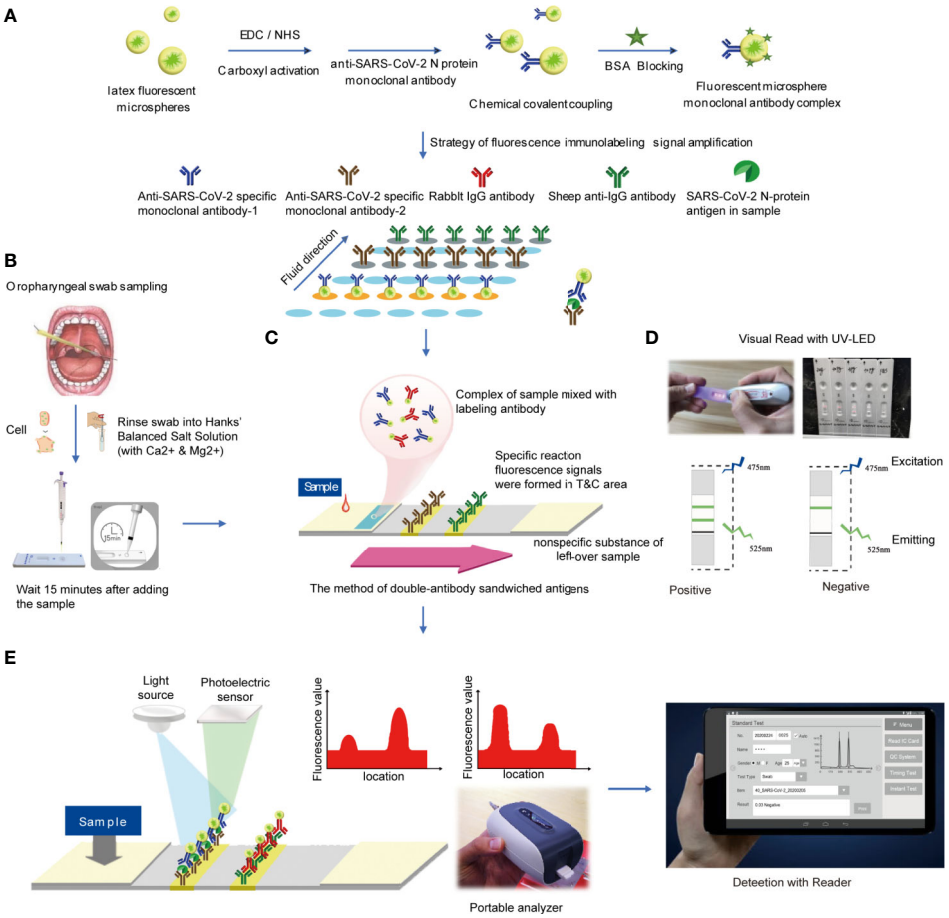

FIGURE 2 | Reagent preparation and detection process **(A)**. Preparation of antibody detection reagent strip **(B)**. Oropharyngeal swab sampling **(C)**. The method of double-antibody sandwiched antigens **(D)**. Read the result using UV-LED **(E)**. Analyzed by the detector.

fluorescence immunochromatography analyzer for detection. The instrument will automatically calculate the sample concentration value (**Figure 2E**).

Detection of SARS-CoV-2 nucleic acid RNA was performed by fluorescence quantitative PCR: nucleic acid extraction was operated according to the literature (Mollica, 2010), and the fluorescence quantitative PCR process was operated according to the instructions.

Data Analysis and Statistics

Statistics analysis was performed with the software SPSS 20.0, and nonparametric test and two-side $\chi 2$ test were used to compare the differences between the two groups. A receiver operating characteristic (ROC) analysis was constructed to determine the best cut-off value to predict the outcome. The probability was calculated using a logistic regression model, and the estimated probabilities were used in a ROC analysis to calculate the area under curve (AUC) for different models. P value < 0.05 will be considered statistically significant.

RESULTS

Limit of Detection (LoD) of Recombinant N Protein

SARS-CoV-2 recombinant N protein (concentration: 0.5 mg/ml) was used to prepare samples at concentration of 50, 100, 200, 500, and 1 μg/ml. We detected samples at each concentration 20 times by test strips at three batches, and defined the lowest concentration with positive results over 19/20 replicates as the limit of detection. As shown in **Table 1**, 100 ng/ml was defined as the limit of detection for the strips.

LoD of Activated SARS -CoV-2 Virus

The LoD for the activated SARS-CoV-2 virus (IVCAS 6.7512, Wuhan Institute of Virology. CSA.) was tested, in both the sample preservation solution and real clinical matrix (sample preservation solution mixed with orophyngeal swabs samples of healthy people). The experiments were conducted in the P3 Biosafety Protection Laboratory. Initially, the activated SARS-CoV-2 culture medium (host cell: Vero E6 cell line,

TABLE 1 | Limit of detection.

	50 ng/ml*			100 ng/ml*			750 $TCID_{50}$/ml**			1000 $TCID_{50}$/ml**		
	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3
No. tested	20	20	20	20	20	20	20	20	20	20	20	20
Positive results	18	18	16	20	20	20	15	20	16	20	20	20
Positive ratio		88.33%			100%			85.00%			100%	

*tests used with recombinant protein-N of SARS-CoV-2; **tests used with culture fluid of SARS-CoV-2. The results were detected by a Savant-100 Fluorescence immunochromatographic analyzer.

ATCC CRL-1586 ™) was diluted at gradients of 1:1, 1:2, 1:20, 1:400 and 1: 2,000, each sample was tested at least twice, and the dilution at 1:2,000 was tested for 13 times. Then, the concentration of 1:2,000 (100% positive,13/13) was used as the LoD range to expand the testing for the LoD. Dilutions with concentrations of 2×10^3, 1×10^3, and 7.5×10^2 $TCID_{50}$/ml were tested for 20 times, respectively. These results revealed a 100% (20/20) positive result at 1×10^3 $TCID_{50}$/ml concentration. Hence, 1×10^3 $TCID_{50}$/ml was set as the LoD (**Table 1**).

Anti-Interference

The experiment investigated the interference to the test results of common interference substances in samples such as mucin, blood, nasal cavity drugs, antiviral drugs, antibiotics, and so on. The samples of the same antigen concentration were mixed with different interfering substances, and then the strips in same batch were used for the test. As shown in **Table 2**, the test results had no deviation from the detection results of the SARS-CoV-2, which indicated that the common interfering substances in the samples did not interfere with the experimental results.

TABLE 2 | Concentration table of interfering substances.

Substances	Concentration	Result
Full blood	2.2%	Negative
Mucin	250 μg/mL	Negative
Guaifenesin	20 mg/ml	Negative
Ribavirin	10 mg/ml	Negative
(R)-(-)-Phenylephrine hydrochloride	200 mg/ml	Negative
Chlorpheniramine Maleate	25 mg/ml	Negative
Levofloxacin	20 mg/ml	Negative
Tobramycin	5 mg/ml	Negative
Lopinavir	20 mg/ml	Negative
Oseltamivir phosphate	20 mg/ml	Negative
Ritonavir	2.5 mg/ml	Negative
Peramivir Trihydrate	0.2 mg/ml	Negative
Cephradine	20 mg/ml	Negative
Zanamivir	20 mg/ml	Negative
Flunisolide	5 mg/ml	Negative
Fluticasone propionate	15 mg/ml	Negative
Dexamethasone	10 mg/ml	Negative
Mometasone Furoate	10 mg/ml	Negative
Beclomethasone	2 mg/ml	Negative
Triamcinolone acetonide	2 mg/ml	Negative
Fluocinolone Acetonide	10 mg/ml	Negative
Azithromycin	20 mg/ml	Negative
Meropenem trihydrate	5 mg/ml	Negative
Ceftriaxone Sodium Trihydrate	25 mg/ml	Negative
Budesonide	40 mg/ml	Negative
Arbidol hydrochloride	10 mg/ml	Negative
HUMAN IFN-ALPHA A	0.2 mg/ml	Negative

Cross-Reaction

Affinity experiment between monoclonal antibody (From antibody library of Beijing Biosynthesis Biotechnology Co. Ltd.) and SARS-CoV-2 N protein showed the optimum working concentration of selected antibody (**Figure 1D**). Western blotting (WB) (Algenas et al., 2014) cross-reaction experiments were conducted between two monoclonal antibodies of SARS-CoV-2 recombinant N protein and recombinant N protein of SARS-CoV and MERS-CoV, in order to verify the specificity of antibodies (**Figure 1E**). The results of cross-reaction detection showed that the antibody had cross-reactivity towards recombinant N protein of SARS-CoV and no cross-reactivity towards that of other 7 kinds of coronavirus (**Table 3**), which was consistent with other studies (Saahir Khan et al., 2020). For the SARS-CoV N protein which produced a positive response, we diluted the sample into concentrations of 1, 500, 200, 100, and 50 ng/ml. The results showed when SARS-CoV N protein was diluted to concentration of 50 ng/ml, there was no cross-reactivity (**Table 3**).

Precision Verification

According to the requirements of EP15-A3, the negative(N1\N2), weakly positive (S1\S2) and strong positive (P1\P2) samples were tested 5 times a day for 5 days, and the total precision of all samples was calculated for positive or negative percentage agreement. The results in **Table 4** showed that except the positive percentage agreement of S2 samples was 96%, the percentage agreements of other samples were all 100%. All antigen kits in test showed uniform fluorescence distribution under UV-LED excitation.

Cutoff Value Determination

To investigate how sensitive and specificity the assay is, in our primary experiment, 306 samples (age range 2–86, average 40.12 ± 14.33) from 219 healthy controls and 87 diagnosed COVID-19 cases were detected and used to determine the cutoff value. As shown in **Figure 3A**, A p value of < 0.05 was considered statistically significant for all tests. AUC was 0.932 (95% CI: 0.894–0.970). The max Youden Index (Youden Index = Sensitivity + Specificity -1) was 0.846, with a cutoff of 0.0495, a sensitivity of 90.0%, and a specificity of 94.7%. Therefore, T/C 0.05 was set as cutoff value.

Clinical Application and Evaluation

This part study was a randomized controlled and single-blind experiment. Clinical samples from 247 confirmed COVID-19 patients (male, n = 102, female, n = 145; age range 2–68, median 48), 443 patients with other respiratory diseases (male, n = 222, female, n = 221; age range 0–93, median 35) and 300 healthy people (male, n = 159, female=141; age range 20–58, median 32) were enrolled and detected by the antigen strips and the

TABLE 3 | Results of different pathogen samples.

Item	Substance	Source	Concentration	Test Result
1	Dilution	Hanks' balanced salt solution	–	Negative
2	HCoV-HKU1 N protein	Genetically engineered virus recombinant N protein	1 μg/mL	Negative
3	HCoV-OC43 N protein	Genetically engineered virus recombinant N protein	1 μg/mL	Negative
4	HCoV-229E N protein	Genetically engineered virus recombinant N protein	1 μg/mL	Negative
5	HCoV-NL63	Genetically engineered virus recombinant N protein	1 μg/mL	Negative
6	HKU8 N protein (from bat)	Genetically engineered virus recombinant N protein	1 μg/mL	Negative
7	HKU10 N protein (from bat)	Genetically engineered virus recombinant N protein	1 μg/mL	Negative
8	MERS-CoV	Genetically engineered virus recombinant N protein	1 μg/mL	Negative
9	SARS-CoV N protein	Genetically engineered virus recombinant N protein	1 μg/mL	Positive
	SARS-CoV N protein	Genetically engineered virus recombinant N protein	500 ng/ml	Positive
	SARS-CoV N protein	Genetically engineered virus recombinant N protein	200 ng/ml	Positive
	SARS-CoV N protein	Genetically engineered virus recombinant N protein	100 ng/ml	Positive
	SARS-CoV N protein	Genetically engineered virus recombinant N protein	50 ng/ml	Negative

TABLE 4 | Precision.

	Negative		Weakly Positive		Strong Positive	
Sample	N1	N2	S1	S2	P1	P2
NPA*	100%	100%	/	/	/	/
PPA*	/	/	100%	96%	100%	100%

NPA respond to Negative Percentage Agreement, PPA respond to Positive Percentage Agreement.

fluorescence PCR method, respectively. Finally, we evaluated the sensitivity and specificity of detection base on their clinical diagnosis after unblinding. Among the 247 COVID-19 patients, there were 137 patients (male, n = 47, female, n = 90; age range 2–86, median 49) at the disease progression stage (day 0–44 after onset of fever, average day 25), and 110 patients (male, n = 55, female, n = 55; age range 4–86, median 48) at the cured stage (days 19–55, average day 37). According to the discharge standard of COVID-19 by the National Health Committee, at the cured stage here means that the body temperature is normal for more than three days, the symptoms of respiratory tract are obviously improved, the CT image shows that the inflammation is obviously absorbed, and the nucleic acid test of respiratory tract pathogen is negative for two consecutive times. As shown in **Figures 3B–D** and **Table 5**, the results showed that the specificity of the test strip in healthy controls and patients with other respiratory disease was 100.00 and 97.29%, the sensitivity in cases at progress stage and cured stage was 67.15 and 7.02%. Compared to the results of RNA test, which is the most commonly used technology in clinic, the positive percentage agreement (PPA) and negative percentage agreement (NPA) of antigen strip were 83.16% (79/95) and 94.45% (562/595) (**Table 5**).

Among the 443 patients with other respiratory diseases, there were 68 influenza cases infected with Influenza A H1N1, Influenza A H3, Influenza B Victoria and Influenza B Yamagata. The strip has great identification ability, with a false positive rate of 1.5% (1/68). For the cases with COVID-19, we observed the antigen detection rate at different time of onset. As shown in **Figure 3C**, the detection rate of antigen was higher than 48% within 35 days after onset of fever. Its trend overall the development of disease was similar to that of RNA detection. The detection rates of both of the two methods were significantly reduced after 35 days, which were consistent with the course of disease.

If the antigen test was used to supple RNA detection for auxiliary diagnosis, the diagnostic value of combined evaluation increased to AUC of 0.865(95% CI: 0.822–0.909) (**Figure 3D**), while the diagnostic value of single antigen test and RNA test was 0.802(95% CI: 0.752–0.852) and 0.825(95% CI:0.776–0.874).

DISCUSSION

At present, the detection of SARS-CoV-2 mainly includes nucleic acid detection and immune detection. Nucleic acid testing, or molecular testing, is the gold standard for diagnosing infectious diseases by detecting the genetic material of viruses. However, this method is mainly performed by fluorescence quantitative PCR, which has the problems of complicated extraction process, high requirements of experimental environment and conditions, long detection time, and extremely high rate of missed detection. In addition, research has shown that the SARS-CoV-2 is a new type of RNA virus, and RNA is easily degraded during the extraction process (Landolt et al., 2016; Nwokeoji et al., 2016; Le Bleu et al., 2017), which will lead to the false negative results. Therefore, RNA cannot be adapted to the requirements of rapid test and a large number of suspected case screening. According to the experience obtained from the immunoassay of SARS virus, immunoassay can be used as a supplement to the nucleic acid detection of SARS-CoV-2, especially for suspected cases with similar clinical symptoms and negative nucleic acid test, which has a great complementary diagnostic effect (Li et al., 2003; Shi et al., 2003).

Immunoassay is based on the specific response between antigens and antibodies, by detecting viral proteins (antigens) in the body, or antibodies specific to viral proteins in the body to make a diagnosis. Immunoassay includes antigen detection and antibody detection. The antigen detection kit based on immunofluorescent microspheres only needs to add the sample to the lysate to lyse out the antigen in the sample for detection, which can avoid the tedium of quantitative PCR. Though the RNA extraction process greatly simplifies the experimental operation process and shortens the detection time, there still remain missing test. On the other hand, RNA detection is aimed at the detection of

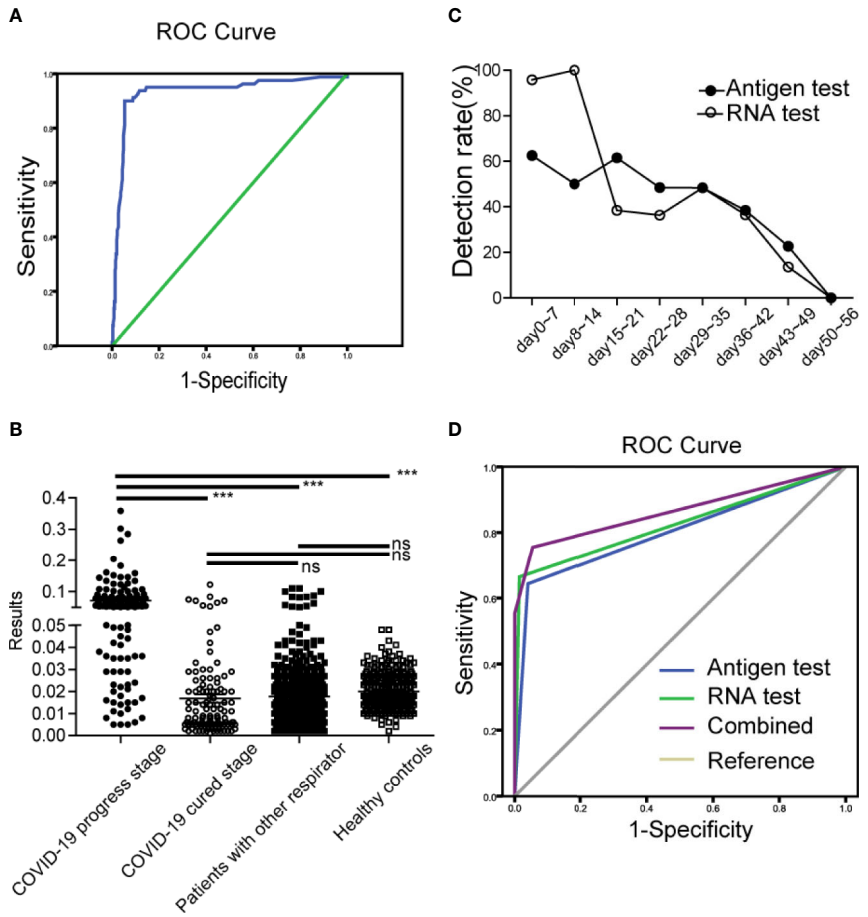

FIGURE 3 | Clinical application and evaluation of antigen strips **(A)**. ROC curve for the cutoff determine of antigen strips **(B)**. The results of antigen strips in COVID-19 cases (progress stage, ●; cured stage, ○), patients with other respiratory diseases (■), and healthy controls (□). ***$p < 0.001$; ns, nonsense **(C)**. The antigen detection rate at different time of development of COVID-19. Antigen test (●), RNA test (○) **(D)**. Diagnosis value of antigen strips, RNA detection and combined evaluation.

TABLE 5 | The specificity and sensitivity of test strip.

Study group	No.test	Antigen Strip				RNA test			
		Positive	Nagetive	Sensitivity (%)	Specificity (%)	Positive	Nagetive	Sensitivity (%)	Specificity (%)
Healthy controls	300	0	300	–	100	–	–	–	–
Other respiratory disease	443	12	431	–	97.29	0	443	–	100.00
SARS-CoV-2 Samples	247	100	147	40.49	–	95	152	38.46	–
Progress stage	137	92	45	67.15	–	95	42	69.34	–
Cured stage	110	8	102	7.02	–	0	110	0	–
Total (without healthy controls)	690	112	578	–	–	95	595	–	–
No. agreement with RNA test	–	79	562	–	–	–	–	–	–
Percentage agreement with RNA test	–	83.2%*	94.5%**	–	–	–	–	–	–

**Positive Percentage Agreement, **Negative Percentage Agreement.*

live virus, while antigen detection is aimed at virus protein. In principle, protein fragments can also be detected after virus rupture. Therefore, it is particularly important to perform antibody testing after infection as a complement to the deficiency.

By comparing several detection methods of the SARS-CoV-2, the detection time of nucleic acid is earlier, antigen detection is more convenient and faster, and the combined use can better meet the requirements of SARS-CoV-2 detection. In this study, the diagnostic value of combined evaluation increased to AUC of 0.865(95% CI: 0.822–0.909), while the diagnostic value of single antigen test and RNA test was 0.802(95% CI: 0.752–0.852) and 0.825 (95% CI: 0.776–0.874) (**Figure 3D**).

In order to improve the sensitivity of this method, high specific monoclonal antibodies with enough affinity were selected for the specific spatial structure (antigenic determinant) of the SARS-CoV-2 N protein. Then, the method of fluorescence immunolabeling combined with fluorescence photometric signal detection was chosen, which could improve the sensitivity of the analysis method by 1-2 orders of magnitude, when compared to the traditional colloidal gold particle labeling method. Data from multicenter clinical trials showed that the sensitivity of antigen strip in COVID-19 cases at progress stage was 67.15%, but the results were close to RNA test, with a PPA and NPA of 83.16 and 94.45%. On another hand, the detection rate of antigen strip in COVID-19 cases at cured stage 7.02%, except for the possibility of false positives, this result also indicated that antigen fragments may temporarily store in the body of the cured patients or antigen test may assist RNA detection as a discharge index. The product "New Coronavirus (SARS-CoV-2) N Protein Detection Kit (Fluorescence Immunochromatography)" have been certified by European Conformity (Mar. 13, 2020; certificate no. HKT-20200313-001) and obtained the Provisional Authorization issued by health science authority of Singapore (July 13, 2020; MDPA2020-98).

In the present study, the validation experiment for the activated SARS-CoV-2 virus was conducted in the P3 laboratory, and the clinical experiments were performed in the P2+ laboratory laboratories (medical staff using P3 protection). The test strips exhibited good performance in detecting the live virus. How about the detection of inactivated virus? The results before and after the inactivation were compared, and it was found that the protein destruction after inactivation would affect the detection results (data shown in another study). The average test result (T/C value) of 450 ng of purified N protein was 2.89 (positive) before inactivation, and 0.041 (negative) after inactivation (56°C treatment). Then, the N protein was mixed with the throat swab (obtained from healthy people) and nasal swab (obtained from healthy people), respectively. The detection results were significantly reduced after the sample inactivation. Although the activated virus was a limitation in the present study, this ensured the sensitivity and accuracy of the detection. Therefore, the test should be operated in the P2+ laboratory, which can much faster provide a preliminary result, and cooperate with the nucleic acid detection, thereby improving the efficiency and accuracy. On the other hand, the antigen test does not require trained specialists or large-scale equipment, making it possible to be easily used in healthy population screening, and the self-monitoring of patients after cure. We have done comparison tests in terms of simplicity and feasibility. An UV-light can be used as a detection instrument for the antigen strips with a high sensitivity. The detection results of UV-light (LoD $1 \times 10^3 TCID_{50}$/ml) were consistent with that of Savant-100 Fluorescence immunochromatographic analyzer, which was used in the study (the data of detection and clinical trials were submitted to CE certification and Singapore HAS certification).These advantages would meet the need of "rapid, instrument-free antigen test" recommended by Mina and colleagues in a recent perspective paper (Mina et al., 2020). The commentary recommends that in addition to current RT-PCR fluorescent testing, lateral-flow testing methods that are rapid and instrument-free should be developed for use in school, airports, and even at home. Such rapid test methods would be able to diagnosis COVID-19 earlier before infected patients are able to transmit their infections to others. The investigators will continue to improve the method in further research, in order to increase the sensitivity and specificity of the strips for inactivated virus. Furthermore, the investigators hope to develop portable equipment, reagents and standards suitable for home use, similar to those used as a blood glucose meter.

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 Ethics Committee of Chinese PLA General Hospital, No. 2020-001; Ethics Committee of Wuhan Huoshenshan Hospital, No. 2020-003; Ethics Committee of Chongqing Public Health Medical Center, No.2020-007-01. The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

KZ and KH designed the study. SL and KD established methods. LZ, YZ, LC, JH, and FL collected the clinical information and samples. YL, HL, JL, PS, YX, and JYW performed the detection. CZ and KH performed data analysis and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Patterns of Deterioration in Moderate Patients With COVID-19 From Jan 2020 to Mar 2020: A Multi-Center, Retrospective Cohort Study in China

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Background: Around the globe, moderate cases account for the largest proportion of all coronavirus disease 2019 (COVID-19) patients, and deteriorated moderate patients contribute the most in mortality. However, published articles failed to address the deterioration details of moderate cases, especially on when and how they deteriorated.

Methods: All moderate COVID-19 patients hospitalized in Guangdong Province from January 14 to March 16, 2020, were included in this multicenter retrospective cohort study and were divided into deteriorated and non-deteriorated groups according to clinical status. Symptoms and demographic, therapeutic, and laboratory test result characteristics were collected to explore the features of disease deterioration.

Results: Of 1,168 moderate patients included, 148 (13%) deteriorated to severe (130 cases) or critical (18 cases) status. Over 20% of the older subgroup (>50 years old) showed deterioration. The median time for deterioration was 11 days after onset [interquartile range (IQR) 9–14 days]. In addition, 12.2% severe cases could further develop to critical status after 3 days (IQR 2–6.5 days) of having a severe condition. Respiratory dysfunction and hypoxia were the major manifestations as disease deterioration, while 76 cases (52.1%) showed respiratory rate >30 breaths/min, 119 cases (80.4%) showed SaO_2 <93%, 100 cases (67.5%) had 201 < PaO_2/FiO_2 < 300, and 27 cases (18.9%) had blood lactic acid >2.0 mmol/L. In view of multiple organ dysfunction, 87.8% of acute respiratory distress syndrome (ARDS), 20.2% of acute kidney injury (AKI), 6.8% of coagulopathy, 4% of acute heart failure (AHF), 3.4% of acute hepatic injury (AHI), and 5.4% of shock occurred in deteriorated patients, while organ injury occurred in the following sequence: ARDS, AKI, AHF, coagulopathy, AHI, and shock.

Conclusions: The deteriorated pattern of moderate COVID-19 patients is characterized as the 11th day from onset (IQR 9–14 days) being an important time point of disease deterioration with further exacerbation to critical condition in 3 days (IQR 2–6.5 days), A RDS followed by AKI being the typical modes of sequential organ damage.

Keywords: COVID-19, moderate, deterioration, time point, pattern

INTRODUCTION

Up to October 26, 2020, the rapid spread of coronavirus disease 2019 (COVID-19) has posed a great challenge to one's health and epidemic prevention in many countries and regions, and the number of infected people has exceeded 40 million (1). According to the existing reports and our practice, the largest proportion (84.25%) of diagnosed cases only had moderate symptoms at the beginning of admission, but their condition would worsen during hospitalization or even developed to critical status, which contributes most to mortality (2, 3). Therefore, avoiding death from disease deterioration in moderate patients is the most crucial strategy for COVID-19 treatment (4–6). There have been many researches on the deterioration of COVID-19 patients, but most of them were aimed at exploring the risk factors of deterioration (7–10). The population of those articles had not been clearly classified, and the clinical details for deterioration of COVID-19 have not been revealed either; hence, the guiding value of those studies for clinical treatment was very limited.

In view of the above, a multicenter observational study for the moderate population was carried out to describe the clinical features of moderate cases and determine time points of disease deterioration and the sequence of organ disorders explaining the following key issues: 1. What are the clinical characteristics of disease deterioration in moderate cases? 2. What is the time window and risk factors for disease deterioration in this population? 3. What is the order and degree of the main organ damage when patients deteriorate? These findings would provide specific clinical evidence for effective prevention and treatment of COVID-19.

METHODS

Study Design

A multicenter retrospective cohort study was carried out at 32 hospitals in Guangdong Province designated to treat COVID-19 patients. All patients who were diagnosed with COVID-19 were screened for eligibility in our study. We retrospectively analyzed moderate patients from January 14, 2020, to March 16, 2020. The Guangdong COVID-19 Prevention and Control Headquarters was set up in Guangdong Province to direct and coordinate the treatment of COVID-19 patients across the province. An electronic medical information reporting system (E-System) was put together by the Guangdong Health Commission for the entire provincial medical data collection.

Data Collection

Medical records for 1,315 COVID-19 patients in Guangdong province who had completed hospitalization by March 16, 2020, were extracted from the E-System. Medical records covered epidemiology, demographics, clinical symptoms, comorbidities, laboratory tests, and illness severity, as well as the main treatments during hospitalization and key dates in disease development. All patient data were reviewed by two researchers, and a third researcher adjudicated differences in interpretation if applicable.

Definitions

Disease deterioration was defined as the development to severe or critical symptoms in moderate cases.

Moderate symptoms: fever and mild respiratory symptoms (cough, sore throat, runny nose, etc.), multiple patchy shadowing and ground-glass opacity in lung CT, and normal range of vital signs.

Severe symptoms: respiratory distress [respiratory rate (RR) $\geq$ 30 breaths/min and/or SaO_2 $\leq$93% and/or arterial oxygen tension/inspiratory oxygen fraction (PaO_2/FiO_2) $\leq$300 mmHg under resting condition] (1 mmHg = 0.133 kPa) and/or radiology findings showing that the range of pulmonary lesions increased by more than 50% within 24–48 h, but no mechanical ventilation is required, and no organ failure.

Critical symptoms: Severe acute respiratory distress syndrome (ARDS) (PaO_2/FiO_2 $\leq$100 mmHg) and requiring mechanical ventilation and/or shock occurs and/or presence of organ failure.

Patients with moderate, severe, and critical symptoms were defined as moderate patients, severe patients, and critical patients, respectively.

Criteria of diagnosis and discharge: Patients were diagnosed with COVID-19 according to the Chinese management guidelines for COVID-19 (11). Laboratory procedures for high-throughput sequencing and real-time reverse transcriptase–polymerase chain reaction (RT-PCR) assays have been reported elsewhere (12, 13). Criteria for discharge were the absence of fever for at least 3 days, substantial improvement in lung CT, clinical remission of respiratory symptoms, and two negative severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) assays (throat swab samples) obtained at least 24 h apart.

ARDS was diagnosed according to the Berlin Definition (14). Acute renal injury [acute kidney injury (AKI)] was diagnosed

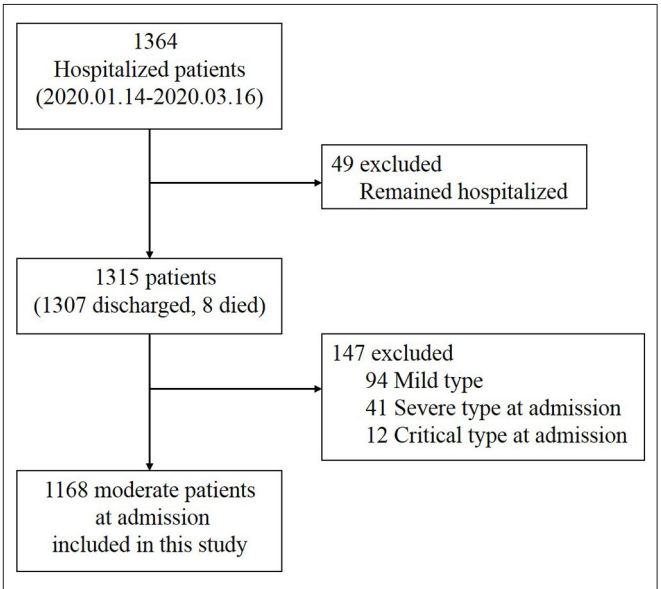

FIGURE 1 | Selection of coronavirus disease 2019 (COVID-19) cases for inclusion in the study.

according to the Kidney Disease Improving Global Outcomes (KDIGO) clinical practice guidelines (15). Coagulopathy was defined as a 3-s extension of prothrombin time or a 5-s extension of activated partial thromboplastin time. Acute heart failure (AHF) was diagnosed according to the European Society of Cardiology (ESC) (16). Acute hepatic injury (AHI) was diagnosed according to the European Association for the Study of the Liver (EASL) (17). Shock was defined according to the 2016. Third International Consensus Definition for Sepsis and Septic Shock (18). Multiple organ dysfunction syndrome (MODS) was defined as the simultaneous or sequential dysfunction of two or more organs in the course of acute diseases (19).

Statistical Analysis

Continuous variables were presented as median with interquartile range (IQR) and were compared using Mann–Whitney U-test. Categorical variables were summarized as counts and percentages, and the two groups (showing deteriorated, showing non-deteriorated) were compared using chi-square tests or Fisher's exact tests. Missing data were not imputed. Univariate and multivariate logistic regression analyses were performed to explore pre-hospital risk factors associated with disease progression. The parameters of the logistic model are estimated using the maximum likelihood method. A two-sided α of < 0.05 was considered statistically significant. All analyses were performed using IBM SPSS software (version 25.0).

TABLE 1 | Demographics and clinical characteristics of the 1,168 coronavirus disease 2019 (COVID-19) patients included in this study.

Demographics and clinical characteristics	Total (n = 1,168)	Non-deteriorated (n = 1,020)	Deteriorated (n = 148)			p-value
			Severe patients (n_1 = 130)	Critical patients (n_2 = 18)	Total	
Age, years	43.5 (32–57)	41 (31–56)	58 (48–64)	60.5 (48.25–69.5)	58 (48.25–64)	<0.001*
Age (≥50 years)	472 (40%)	363 (36%)	96 (74%)	13 (72%)	109 (74%)	<0.001*
Male	560 (48%)	470 (46%)	76 (56%)	14 (78%)	90 (61%)	0.001*
Imported case	915 (78%)	802 (79%)	98 (75%)	15 (83%)	113 (76%)	0.36
	(n = 1,155)	(n = 1,007)			(n = 148)	
Comorbidity						
Any	245 (21%)	172 (17%)	57 (44%)	16 (89%)	73 (49%)	<0.001*
Diabetes	50 (4%)	27 (3%)	19 (15%)	4 (22%)	23 (16%)	<0.001*
Hypertension	110 (9%)	74 (7%)	26 (20%)	10 (56%)	36 (24%)	<0.001*
Cardiovascular disease	33 (3%)	16 (2%)	14 (11%)	3 (17%)	17 (12%)	<0.001*
Cerebrovascular disease	10 (1%)	6 (1%)	3 (2%)	1 (6%)	4 (3%)	0.028*
Chronic kidney disease	11 (1%)	4 (0%)	5 (4%)	2 (11%)	7 (5%)	<0.001*
Chronic lung disease	33 (3%)	27 (3%)	6 (5%)	0	6 (4%)	0.30
Chronic liver disease	39 (3%)	31 (3%)	6 (5%)	2 (11%)	8 (5%)	0.14
History of Cancer	11 (1%)	8 (1%)	3 (2%)	0	3 (2%)	0.15
Symptoms						
Fever	807 (69%)	676 (66%)	114 (88%)	17 (94%)	131 (89%)	<0.001*
Cough	575 (49%)	475 (47%)	87 (67%)	13 (72%)	100 (68%)	<0.001*
Fatigue	132 (11%)	109 (11%)	19 (15%)	4 (22%)	23 (16%)	0.081
Myalgia	93 (8%)	80 (8%)	11 (9%)	2 (11%)	13 (9%)	0.69
Diarrhea	23 (2%)	20 (2%)	2 (2%)	1 (6%)	3 (2%)	>0.99
Dyspnea	78 (7%)	55 (5%)	17 (13%)	6 (33%)	23 (16%)	<0.001*
Treatments during prodromal stage						
Antibiotic	252	215 (21.1%)	32	5	37 (25.0%)	0.278
Antivirals	1,042	915 (89.7%)	115	12	127 (85.8%)	0.153
Oxygen therapy	1,106	959 (94.0%)	121	16	137 (92.6%)	0.492
Incubation, days	8 (5–13)	9 (5–13)	6 (3.25–9)	9 (5–12)	6 (4–10)	<0.001*
	(n = 748)	(n = 633)		(n = 115)		
Incubation (<8 days)	331 (28%)	261 (26%)	64 (49%)	6 (33%)	70 (47%)	<0.001*
	(n = 748)	(n = 633)		(n = 115)		
Disease onset to admission, days	3 (1–6)	3 (1–6)	4 (2–6.25)	3.5 (2.75–5)	4 (2–6)	0.004*
Length of hospitalization	18 (14–24)	17 (13–23)	23 (18–32.25)	38.5 (29.25–46)	24 (19–34)	<0.001*

*Data are median values [interquartile range (IQR)], n (%), or n/N (%). In comparison between deteriorated and non-deteriorated groups, p-values were calculated using the Mann–Whitney U-test, chi-square test, or Fisher's exact test, as appropriate. * A two-sided α of < 0.05 was considered statistically significant.*

RESULTS

Demographic and Basic Clinical Characteristics of the Study Population

Of 1,364 patients diagnosed with COVID-19, 1,315 patients were enrolled in our study by excluding 49 patients who were still hospitalized. We further excluded 147 cases, including 94 mild cases that showed no signs of pneumonia on chest CT scans/X-rays throughout their hospital stay and 53 cases that presented with severe (41 cases) or critical (12 cases) symptoms at admission (**Figure 1**).

A total of 1,168 moderate cases at admission were included in the final analysis. Of these, 1,167 patients were successfully treated, and one patient died. Among them, 148 patients (13%) deteriorated during their hospital stay and developed severe (130 cases) or critical symptoms (18 cases). Compared with the non-deteriorated group, older median age [58.0 years (IQR 48.25–64) vs. 43.5 years (IQR 32–57), $p < 0.001$], higher proportion of male (46 vs. 61%, p = 0.001), higher proportion of comorbidities as diabetes (16 vs. 3%, $p < 0.001$), hypertension (24 vs. 7%, $p < 0.001$), cardiovascular disease (12 vs. 2%, $p < 0.001$), and cerebrovascular disease (3 vs. 1%, $p = 0.028$), and higher proportion of pre-admission symptoms as fever (89 vs. 66%, $p < 0.001$), cough (68 vs. 47%, $p < 0.001$), and dyspnea (16 vs. 5%, $p < 0.001$) were found in the deteriorated group (**Table 1**, **Figure 2**).

Pre-Hospital Risk Factors Associated With Coronavirus Disease 2019 Deterioration

As shown in **Table 2**, multivariate regression result suggested that age ≥50 years [odds ratio (OR) = 6.41, 95% CI 3.78–10.88, $p < 0.0001$], male (OR = 2.28, 95% CI 1.40–3.70, $p = 0.01$), incubation period <8 days (OR = 1.93, 95% CI 1.20–3.10, $p = 0.007$), diabetes (OR = 2.85, 95% CI 1.10–7.41, $p = 0.032$), hypertension (OR = 2.04, 95% CI 1.01–4.15, $p = 0.048$), fever (OR = 4.39, 95% CI 1.98–9.70, $p < 0.0001$), cough (OR = 3.49, 95% CI 2.10–5.80, $p < 0.0001$), and dyspnea (OR = 5.94, 95% CI 2.73–12.91, $p < 0.0001$) were pre-hospital risk factors of statistical significance based on filtrated items ($p < 0.05$) analyzed by univariate regression, while cardiovascular, cerebrovascular, and chronic kidney diseases were ruled out as pre-hospital risk factors. Forest plot displayed each pre-hospital factor for deterioration in moderate cases (**Figure 3**).

Time Evolution for Deterioration in Moderate Patients

The clinical course of each stage was displayed in a schematic diagram as **Figure 4**. For the non-deteriorated group, patients experienced 9 days (IQR 7–12) of onset stage and entered 9 days (IQR 9–14) remission stage. For the deteriorated group, patients worsen on the 11th day (IQR 9–14) after onset, and the deterioration period lasted for 7.5 days (IQR 4–12) (**Figures 4A,B**). In subgroup analysis, 130 cases only deteriorated

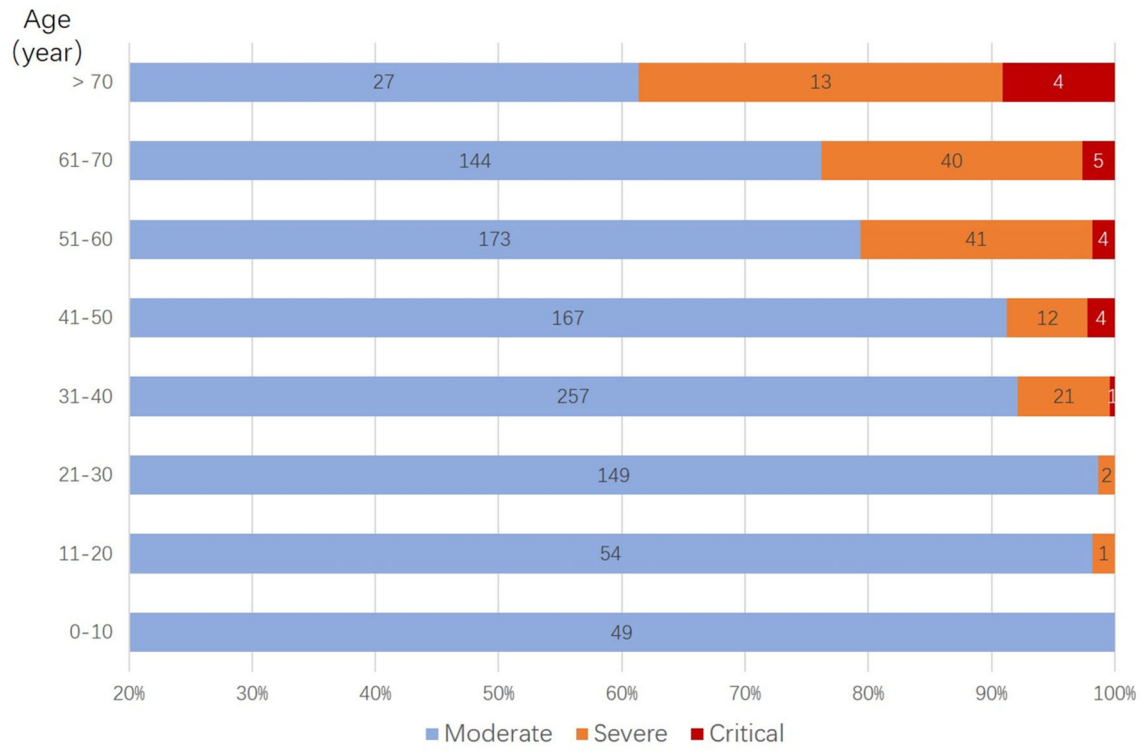

FIGURE 2 | Distribution of deteriorated patients in different age groups. Stratified by age, more deterioration was observed with older age groups, in which cases older than 50 years have a significantly higher percentage of deterioration case ($p < 0.001$).

TABLE 2 | Pre-hospital risk factors associated with deterioration in moderate coronavirus disease 2019 (COVID-19) cases.

Variables	Univariate OR (95% CI)	*P*-value	Multivariate OR (95% CI)	*p*-value
Age ≥50 years (vs. <50 years)	5.06 (3.43–7.45)	<0.0001*	6.41 (3.78–10.88)	<0.0001
Male (vs. female)	1.82 (1.28–2.58)	0.001*	2.28 (1.40–3.70)	0.001
Incubation <8 days (vs. ≥8 days)	2.22 (1.48–3.33)	<0.0001*	1.93 (1.20–3.10)	0.007
Disease onset to admission, days	1.05 (1.00–1.10)	0.032*	–	–
Diabetes	6.77 (3.77–12.16)	<0.0001*	2.85 (1.10–7.41)	0.032
Hypertension	4.11 (2.64–6.41)	<0.0001*	2.04 (1.01–4.15)	0.048
Cardiovascular disease	8.14 (4.02–16.51)	<0.0001*	–	–
Cerebrovascular disease	4.69 (1.31–16.84)	0.018*	–	–
Chronic kidney disease	12.61 (3.65–43.62)	<0.0001*	-	-
Fever	3.92 (2.33–6.61)	<0.0001*	4.39 (1.98–9.70)	<0.0001
Cough	2.39 (1.66–3.45)	<0.0001*	3.49 (2.10–5.80)	<0.0001
Dyspnea	3.23 (1.92–5.44)	<0.0001*	5.94 (2.73–12.91)	<0.0001

*OR, odds ratio. *Variables significant at the 0.05 level in univariate analyses were considered.*

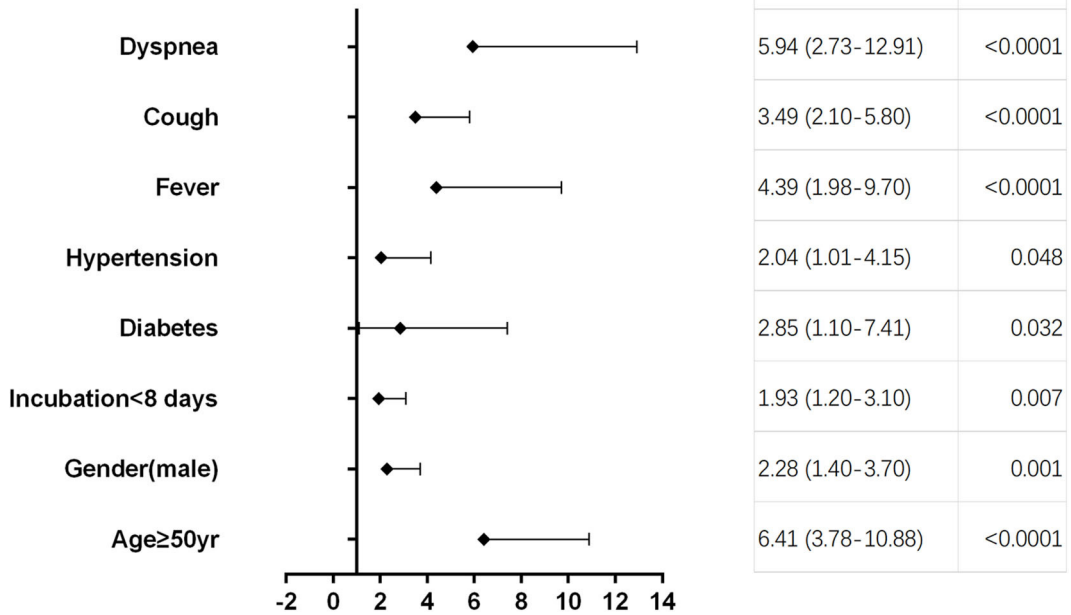

FIGURE 3 | Forest plot of pre-hospital factors. Forest plot displaying the odds ratio (OR) and 95% confidence interval (95% CI) of each pre-hospital factor for deterioration in moderate cases.

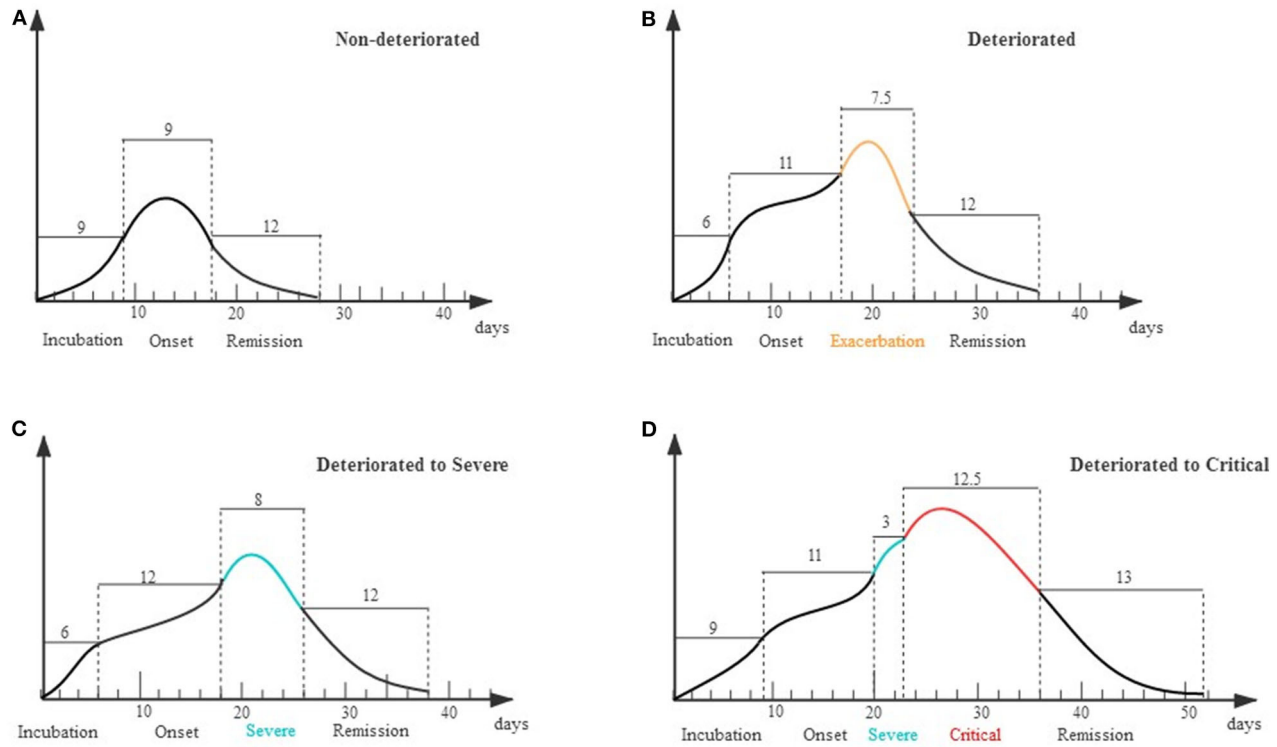

FIGURE 4 | Schematic diagram for evolution of deterioration in moderate patients. X axis represents different stages of the clinical course that patients experienced; Y axis represents the severity of illness. **(A)** Non-deteriorated group. Patients typically had a 9-day disease incubation period [median, interquartile range (IQR) 5–13, $n = 633$] and a 9-day onset stage (IQR 7–12, $n = 1,020$) before entering a 12-day remission stage (IQR 9–17, $n = 1,020$). **(B)** Deteriorated group. Patients typically had a 6-day disease incubation period (IQR 4–10, $n = 115$) and an 11-day onset stage (IQR 9–14, $n = 148$) before deteriorating and entering an exacerbation stage of 7.5 days (IQR 4–12, $n = 144$). **(C)** Deteriorated to Severe. Patients had a 12-day onset stage (IQR 9–14, $n = 130$) before deteriorating to an 8-day exacerbation stage (IQR 4–12, $n = 130$). **(D)** Deteriorated to Critical. Patients deteriorated to a 3-day severe stage (IQR 2–6.5, $n = 14$) on the 11th day (IQR 8–12) from illness onset and further stepped into critical stage (IQR 6.75–25.75, $n = 18$).

to severe condition, of which deterioration happened on the 12th day (IQR 9–14) after onset. The other 18 cases deteriorated on the 11th day (IQR 8–12), while they suffered a second deterioration stage after 3 days (IQR 2–6.5) in severe condition (**Figures 4C,D**).

Evaluation of Organ Injury Severity in Deteriorated Coronavirus Disease 2019 Patients

Of 148 cases in the deteriorated group, 87.8% ($n = 130$) has progressed to severe condition while 12.2% ($n = 18$) to critical condition. When deterioration happened, respiratory dysfunction and hypoxia were major threats in our patients, with 76 cases (52.1%) having anRR >30 breaths/min, 119 cases (80.4%) SaO_2 <93%, 100 cases (67.5%) 201 ≤ PaO_2/FiO_2 <300, 27 cases (18.9%) serum lactate (Lac) >2.0 mmol/L, and 25 cases (17.3%) serum creatinine (Scr) >133 μmol/l (**Table 3**, **Figure 5A**).

In patients in critical condition, their lung function and hypoxia were even worse, with 18 cases having SaO_2 <93%, PaO_2/FiO_2 <200, of which 44.4% cases had PaO_2/FiO_2 ≤100, 83.3% cases Lac >2.0 mmol/L. Meanwhile, AKI were tensed, with 66.7% cases Scr >133 μmol/l, 38.9% cases urine output <30 ml/h. Acute circulatory failure and multiple organ dysfunction happened, with 44.4% cases having systolic blood pressure (SBP) <90 mmHg, 33.3% cases ejection fraction (EF) <45%, 11.1% cases total bilirubin >34 μmol/l, 33.3% ($n = 6$) prothrombin time (PT) <17 s (**Table 3**, **Figure 5B**).

Sequential Organ Injury in Deteriorated Coronavirus Disease 2019 Patients

In view of multiple organ dysfunction (**Figure 6A**), 87.8% ($n = 130$) of ARDS, 20.2% ($n = 30$) of AKI, 6.8% ($n = 10$) of coagulopathy, 4% ($n = 6$) of AHF, 3.4% ($n = 5$) of AHI, and 5.4% ($n = 8$) of shock occurred in 148 deteriorated patients. Among them, the organ injuries in critical condition appeared on the following time sequence (**Figure 6B**): ARDS ($n = 18$) occurred on day 10 (IQR 9–12) from onset, AKI ($n = 15$) occurred on day 12 (IQR 10–15) from onset, AHF ($n = 6$) occurred on day 14 (IQR 13–15) from onset, coagulopathy ($n = 7$) occurred on day 15 (IQR 13–16) from onset, AHI ($n = 4$) occurred on day 15.5 (IQR 15–17) after onset, and shock ($n = 8$) on day 17 (IQR 16–18) from onset.

TABLE 3 | Clinical features of 148 deteriorated moderate cases.

Clinical variables	Severe	Critical
RR (breaths/min)	33 (25–35) ($n = 143$)	33 (35–43) ($n = 18$)
>30, n (%)	76 (52.1)	15 (83.3)
SaO_2 (%)	92 (90–92) ($n = 148$)	88 (85–91) ($n = 18$)
<93, n (%)	119 (80.4)	18 (100.0)
PaO_2/FiO_2 (mmHg)	230 (213–267) ($n = 148$)	109 (89.7–123) ($n = 18$)
>300, n (%)	37 (25.1)	0
201–300, n (%)	100 (67.5)	0
101–200, n (%)	11 (7.4)	10 (55.6)
≤100, n (%)	0	8 (44.4)
SBP (mmHg)	121 (112–145.5) ($n = 142$)	114 (87.7–133.7) ($n = 18$)
<90, n (%)	0	8 (44.4)
EF (%)	65 (56–67) ($n = 53$)	55.5 (41–62.5) ($n = 18$)
<45, n (%)	3 (5.6)	6 (33.3)
Serum lactate (mmol/l)	1.7 (1.4–1.9) ($n = 143$)	3.3 (2.3–4.4) ($n = 18$)
>2.0, n (%)	27 (18.9)	15 (83.3)
Scr (μmol/l)	98 (88–123) ($n = 144$)	209.5 (128.7–323) ($n = 18$)
>133, n (%)	25 (17.3)	12 (66.7)
Urine output (ml/h)	90 (78–100) $n = 63$	45 (23.6–58.2) ($n = 18$)
<30, n (%)	0	7 (38.9)
Total bilirubin (μmol/l)	21.1 (19.2–24.6) $n = 148$	24.2 (19.7–32) ($n = 18$)
>34, n (%)	4 (2.7)	2 (11.1)
PT (s)	15 (14–16) ($n = 145$)	14 (13–19) ($n = 18$)
>17, n (%)	10 (6.9)	6 (33.3)

Data are median values (IQR), n (%), or n/N (%).
RR, respiratory rate; SBP, systolic blood pressure; SaO_2, blood oxygen saturation; PaO_2/FiO_2, arterial oxygen tension/inspiratory oxygen fraction; Scr, serum creatinine; EF, ejection fraction; PT, prothrombin time.

DISCUSSION

It was shown in our data that moderate cases accounted for the most (88.8%; 1,168/1,315) patients with COVID-19 in Guangdong Province. Moreover, the majority of severe and critical cases resulted from the deteriorated moderate cases (76.0%; 130/171 and 60.0%; 18/30, respectively). The results indicated that moderate cases were the main component of COVID-19 patients. Although several studies have elucidated the risk factors for deterioration in moderate patients, few information was provided on when and how disease deterioration happens (5–8).

Our data revealed that 148 patients, accounting for 13% of moderate cases, deteriorated to severe or critical condition. These patients had the marked characteristics of age >50 years old, male, and high proportion of combined chronic diseases, suggesting that these characteristics were related to disease deterioration. As reported by previous studies, these characteristics were the risk factors for mortality (20, 21). Thus, as these features were the inherent background of patients, they were worthy of clinicians' concern throughout the entire clinical process.

In our study, patients in the non-deteriorated group shifted into remission stage on 9 days (IQR 7–12) after symptom onset; however, patients in the deterioration group exacerbated on the 11th day (IQR 9–14) from onset. For the 18 patients who further exacerbated into critical status, only 3 days (IQR 2–6.5) were spent for exacerbation from severe condition. Data above were plotted in a schematic diagram to display. This suggested that the 2nd week from onset was the time window for deterioration, and it may further exacerbate to critical condition in 3 days since that. Study suggested that timely oxygen therapy or even humidified high-flow nasal cannula therapy be implemented to prevent deterioration (22). Hence, clinicians should pay more attention to the time points for deterioration—interfere in advance to prevent organ function disorders.

Regression results showed that age ≥50 years, dyspnea, fever, cough, diabetes, male gender, hypertension, and an incubation period <8 days were pre-hospital risk factors for deterioration in moderate COVID-19 cases. Several studies (20, 21, 23) had revealed that the prognosis was worse in older COVID-19 patients and those with more clinical symptoms like fever (?38.5°C), cough, and shortness of breath. Furthermore, they found that these COVID-19 patients had lower levels of lymphocyte and serum albumin, higher levels of lactate dehydrogenase, B-type natriuretic peptide, and D-dimer, which might be correlated with severity of illness. In addition, it is reported that a shorter incubation period for patients with acute respiratory syndrome coronavirus infection could be indicative of a higher infective dose, leading to faster/greater pathogen replication, outrunning adaptive immune responses, or leading to a more aggressive and damaging inflammatory response, therefore causing more severe disease (24). Hence, the above reasons could partly explain why the moderate COVID-19 patients with pre-hospital risk factors mentioned above were inclined to deteriorate. Although previous study had indicated that male gender might be prone to being affected (25), why disease deterioration occurred more in male COVID-19 patients is still unclear and more research is needed. Stated thus, it is important that clinicians should record patient symptoms and medical history in detail at the earliest opportunity. Patients with moderate COVID-19 symptoms and any of the above risk factors should be admitted to the hospital preferentially without delay and receive careful attention throughout the whole treatment process.

Then, what is the order and degree of the major organs involved during disease deterioration?

Pulmonary system was the primary target attacked by SARS-CoV-2. Our study showed that almost all deteriorated patients

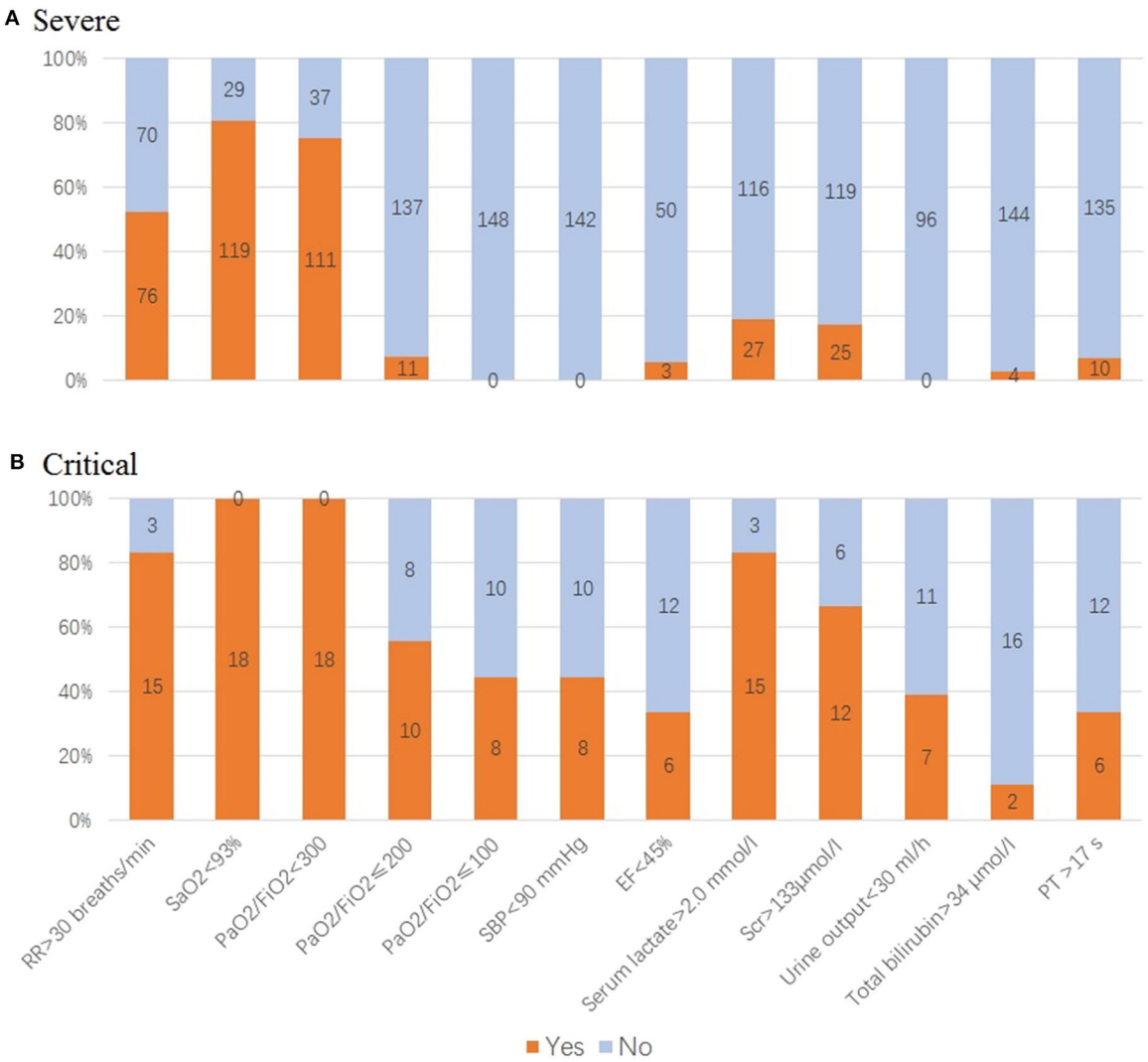

FIGURE 5 | Evaluation of organ function in deteriorated coronavirus disease 2019 (COVID-19) patients. **(A)** For patients in severe condition: 76 cases with respiratory rate (RR) >30 breaths/min, 119 cases SaO_2 <93%, 111 cases PaO_2/FiO_2 <300, 11 cases PaO_2/FiO_2 ≤200, 3 cases ejection fraction (EF) <45%, 27 cases serum lactate >2.0 mmol/l, 25 cases serum creatinine (Scr) >133 μmol/l, four cases total bilirubin >34 μmol/l, and 10 cases prothrombin time (PT) <17 s. **(B)** For patients in critical condition: 18 cases SaO_2 <93%, PaO_2/FiO_2 <200, of which 44.4% cases PaO_2/FiO_2 ≤100, 83.3% cases serum lactate >2.0 mmol/L. Of those, 66.7% cases Scr >133 μmol/l, 38.9% cases urine output <30 ml/h. Acute circulatory failure and multiple organ dysfunction happened, with 44.4% cases having systolic blood pressure (SBP) <90 mmHg, 33.3% cases EF <45%, 11.1% cases total bilirubin >34 μmol/l, 33.3% cases PT <17 s.

primarily appeared with shortness of breath, decreasing of SaO_2 and oxygenation index, as well as increasing of serum lactate level, indicating that acute lung injury was the jumping-off point for disease deterioration in moderate COVID-19 patients. It has been reported that the viral spike glycoprotein of SARS-CoV-2 would specifically combine with angiotensin-converting enzyme-2 on the membrane of pulmonary epithelial cells when the virus invaded the lungs *via* the respiratory tract (26). Furthermore, histological examination after autopsy showed typical ARDS pathological manifestations in lung tissue of dead COVID-19 patients, such as bilateral diffuse alveolar damage accompanied by fibrous mucous exudates, exfoliation of pulmonary cells, and formation of hyaline membrane (27). In addition, a recent study found that the bronchoalveolar lavage fluid in severe/critical patients was enriched with macrophages derived from monocytes that produced multiple cytokines [interleukin (IL)-8, IL-6, and IL-1β] associated with inflammatory storm, and in the meantime, $CD8^+$ T cells/proliferating T cell value apparently decreased (28). These immune disorders not only caused abnormal inflammatory response in the lung but also damaged the microcirculation of important organs besides the lung, even inducing disseminated intravascular coagulation (DIC) (29). These basic studies finely explained clinical phenomenon we observed and supported the treatment strategy of adequate oxygen.

Kidney was the second main target organ according to our data, as AKI accounted for 20.2% in deteriorated moderate cases and even 66.7% in critical patients. Through the scRNA-seq

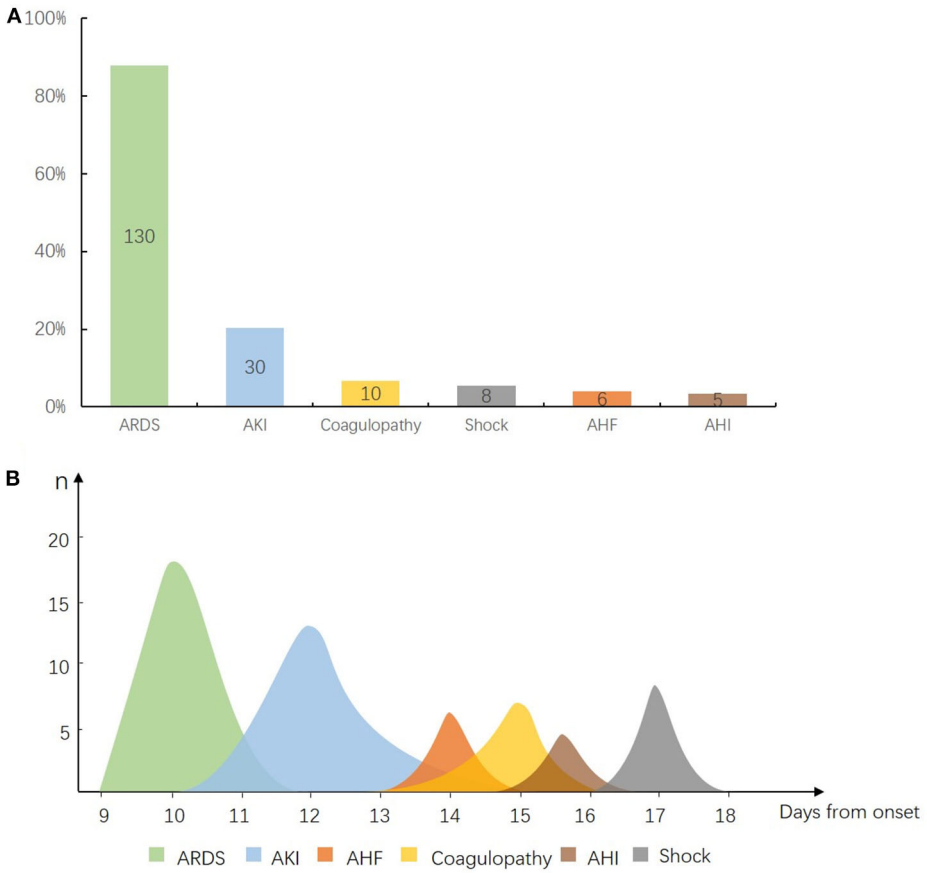

FIGURE 6 | Sequential organ injury in deteriorated coronavirus disease 2019 (COVID-19) patients. **(A)** Composition of organ dysfunction in 148 deteriorated patients. X axis for each kind of organ injury and Y axis for the ratio of patients who developed each kind of organ injury. Out of 148 cases, 87.8% ($n = 130$) of acute respiratory distress syndrome (ARDS), 20.2% ($n = 30$) of acute kidney injury (AKI), 6.8% ($n = 10$) of coagulopathy, 4% ($n = 6$) of acute heart failure (AHF), 3.4% ($n = 5$) of acute hepatic injury (AHI), and 5.4% ($n = 8$) of shock. **(B)** Time windows for occurrence of organ injury in 18 critical patients. X axis for time from symptom onset to start of each kind of organ injury and Y axis for the number of patients who developed organ injury of each kind. ARDS occurred on day 10 [interquartile range (IQR) 9–12] from onset, AKI ($n = 15$) on day 12 (IQR 10–15), AHF ($n = 6$) on day 14 (IQR 13–15), coagulopathy (n = 7) on day 15 (IQR 13–16), AHI ($n = 4$) on day 15.5 (IQR 15–17), and shock ($n = 8$) on day 17 (IQR 16–18).

analysis, researchers found that podocytes and proximal tubules were potential host cells targeted by SARS-CoV-2, which caused AKI under the joint action of systemic inflammatory response induced by hypoxia (30, 31).

Furthermore, in this study, AHF, coagulation dysfunction, liver dysfunction, and circulatory failure occurred successively. These typical scenes of MODS in the intensive care unit (ICU) followed by further development of lung injury and tissue hypoxia (further reduced oxygenation index and increased lactate level), as well as renal injury, continuously worsened.

Therefore, from the overall clinical process, lung injury and AKI sequential development is an important pattern of COVID-19 progression, suggesting that clinicians should focus on hypoxia improvement and kidney protection at the early stage to prevent disease from developing MODS.

Our study has some limitations. First, by excluding patients who remained in the hospital on the cutoff date, we may have underestimated the effect of potential risk factors for predicting deterioration. Second, because a small number of patients cannot recall the date accurately of contacting with the transmission source, their exact incubation period is uncertain. Third, the lack of data in partial severe patients, like left heart EF, coagulatory function, urine volume, as well as deficiency of baseline organ function level in severe and critical patients prevents organ injury degree measuring, which is an inevitable flaw in a retrospective study. Further studies of cohort-based design will be more helpful for clinicians to understand the deterioration process.

In conclusion, our study revealed that COVID-19 patients with deterioration had the characteristics of older age, male, and more basic diseases. The deteriorated pattern of moderate COVID-19 patients was characterized as [1] the 11th day from onset (IQR 9–14) was an important time point of disease deterioration, and it may further exacerbate to critical condition in 3 days (IQR 2–6.5); [2] organ damage induced by COVID-19

with a certain sequence, and ARDS followed by AKI was the common mode of deterioration.

DATA AVAILABILITY STATEMENT

The data analyzed in this study was obtained from Guangdong Health Commission and is restricted to protect confidentiality. Requests to access these datasets should be directed to the corresponding author, and will only be provided with approval from the Guangdong Health Commission.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Institutional Review Board of Guangdong Health Commission and Guangdong Provincial People's Hospital (No. GDREC2020028H). 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

MF and XL had the idea for and designed the study. S-lC, HX, H-yF, and S-sH had full access to all data in the study and took responsibility for the integrity of the data and data analysis. S-sH, J-fS, J-lH, W-lS, and R-jW were responsible for data collection. MF, S-lC, H-yF, S-sH, J-fS, and LZ contributed to writing of the report. All authors contributed to data acquisition and data interpretation, reviewed, and approved the final version. All authors contributed to the article and approved the submitted version.

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frontiers in Immunology

HYPOTHESIS AND THEORY
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A Potential Bioelectromagnetic Method to Slow Down the Progression and Prevent the Development of Ultimate Pulmonary Fibrosis by COVID-19

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Introduction: Right now, we are facing a global pandemic caused by the coronavirus SARS-CoV-2 that causes the highly contagious human disease COVID-19. The number of COVID-19 cases is increasing at an alarming rate, more and more people suffer from it, and the death toll is on the rise since December 2019, when COVID-19 has presumably appeared. We need an urgent solution for the prevention, treatment, and recovery of the involved patients.

Methods: Modulated electro-hyperthermia (mEHT) is known as an immuno-supportive therapy in oncology. Our proposal is to apply this method to prevent the progression of the disease after its identification, to provide treatment when necessary, and deliver rehabilitation to diminish the fibrotic—often fatal—consequences of the infection.

Hypothesis: The effects of mEHT, which are proven for oncological applications, could be utilized for the inactivation of the virus or for treating the fibrotic consequences. The hypothesized mEHT effects, which could have a role in the antiviral treatment, it could be applied for viral-specific immune-activation and for anti-fibrotic treatments.

Keywords: SARS-CoV-21, rehabilitation, electric field, immune-effect, heat-shock protein, modulated electro-hyperthermia

INTRODUCTION

The present global pandemic is a disease named COVID-19. It is caused by a coronavirus (SARS-CoV-2), that belongs to the order of Nidovirales, the family of Coronaviridae, and the subfamily of Coronavirinae (1). There are nearly 30 recognized coronaviruses (CoVs) that infect humans, mammals, fowls, and other animals (2). It is the third known zoonotic CoV disease after SARS-CoV-1 and the Middle East respiratory syndrome (MERS). The genome sequence of SARS-CoV-2 did not bear a close relationship to any of the previously identified CoVs (3, 4). The epidemiology research shows the validity of the complexity (long-tail distribution): SARS-CoV-2 characterizes by

the 80/20 law (5); which means 80% of transmissions happen by 20% of the infected individuals, "superspreaders" (6). The SSs are not only spreading intensively, but it seems like they have a high mutation-activity too. The large dataset of genome assemblies show how genomic diversity was evolved from one common accessor (7), but rapidly mutated, and four major SSs developed during the spreading of the pandemic, different ones in different continents (8). Importantly, genetic diversity studies show that the mutations in the viral RNA sequence allow the interaction of the virus with the cells of the host's immune system associated with CD8 T-cell and CD4 T-cell responses (9).

The COVID-19 is more serious than other viral infections starting with an upper respiratory infection (URI) because SARS-CoV-2 has multiple "faces"; it is a "great imitator" (10). It can have simple symptoms like a common cold with a runny nose, but it can have much more severe symptoms, like loss of smell, fatigue, vomiting, diarrhea, abdominal pain, muscle aches, even whole-body symptoms as rashes or spots of redness. Severe organ damage is also possible (heart failure, kidney damage, liver damage, etc.), which usually happens as a complication after the COVID period of the disease.

In contrast to earlier CoV infections, the patient who has SARS-CoV-2 may remain asymptomatic in the first stage, which may progress into severe pneumonia (11), dyspnea, renal insufficiency, and even death (12). This long period of incubation without symptoms accelerates the pandemic because the virus carrier person is unknown for an extended period. The long incubation period and mild URI symptoms do not alert the patient, and many times, when the disease becomes severe, it is already too late to avoid the damages. The highly uncontrolled spreading among the population increases the total cases of the global epidemic, and contrary to the mortality observed in the cases of SARS-CoV-1 or MERS-CoV, the total number of deaths is significantly higher (13).

A highly possible hypothesis (14) speculates that the COVID-19 pandemic is completely underdiagnosed, and in consequence, the infection spreads silently across the entire globe. The conditions could develop clusters of severe infections among endangered subjects and randomly promote only lately recognized spots of death cases. However, it is another possibility that a significant number of individuals develop immunity against the virus, and the current danger naturally falls into a level of seasonal flu. Due to the uncertain future of the development, implementing reasonable measures to gain control over the development of the disease is the primary goal. The network analysis for blocking the slink spread of the virus may bear immense potential from public health perspectives (15). The fatality rate is very different by countries, ranging between 1%-14%, highly depending on the age of the infected population and comorbidities (16). The severity of the disease depends of the dose of infection (14). About 80% of the confirmed cases are asymptomatic or mild, 13.8% are severe, with symptoms including pneumonia and shortness of breath, and 4.7% of the patients get in critical conditions (17). All three human CoVs' induced pneumonia. Severe lung involvement can be formed by excessive and aberrant non-effective host immune responses; furthermore, multiorgan failure could be developed, and the situation can easily become lethal (18–20).

In addition to the medical issues, social and global economic challenges are also incredible. Heavy social load appears with the suddenly overloaded medical capacities, which cannot adequately suppress the further escalation of the disease and serve the hospitalized patients who fall back upon critical care and need special technical conditions.

MEDICAL CHALLENGES

Medical questions are complex and depend on multiple personal and conditional factors. The central question that needs to be answered for a step toward successful clinical therapy is the precise understanding of the mechanism of the infection, considering its complexity determined by the interconnected physiological feedback mechanisms of human homeostatic regulation. The human organism has developed defensive mechanisms to prevent the propagation and proliferation of invading pathogens. One of the most ancient types of immune reactions is the elimination of bacterial pathogen-infected cells by apoptosis (21). In plants, pathogens trigger a hypersensitive response (HR), inducing systemic acquired resistance (SAR) (22) as a kind of apoptosis. In animals and humans, homeostatic dynamic processes control the activation of apoptosis in a highly controlled manner. In both animals and plants, apoptosis is promoted by producing pro-inflammatory molecules associated with tissue development and homeostasis (22, 23). Despite the differences, both types of apoptosis are associated with the induction of similar morphological features, including membrane blebbing, cytosolic fragmentation, nuclear condensation, and fragmentation as well as biochemical events such as the degradation of genomic DNA, proteolysis, and redistribution of membrane lipids (24, 25). These changes are primarily due to the activation of caspases, a family of cysteine proteases (26, 27). Moreover, infected host cells produce defensins, host defense peptides of innate immunity (28), and induce pro-inflammatory cytokines with an important role in the clearance of the invading pathogen.

Similar to patients with SARS-CoV and MERS-CoV, some patients with SARS-CoV-2 develop acute respiratory distress syndrome (ARDS) with characteristic pulmonary ground-glass opacity on computer tomography (CT) images. In most patients with advanced disease, the SARS-CoV-2 infection is also associated with a cytokine storm, which is characterized by increased plasma concentrations of interleukins 2, 7, and 10, granulocyte-colony stimulating factor, interferon-γ-inducible protein 10, monocyte chemoattractant protein 1, macrophage inflammatory protein 1α, and tumor necrosis factor α (18–20, 29, 30).

At present, the clinical manifestations and severity of the COVID-19 epidemic are similar to SARS-CoV-1 (31) in inducing cytokine storm. The SARS-CoV-2 shares 79.5% of the gene sequence with the SARS-CoV-1 (32). Similarly to SARS-CoV-1, SARS-CoV-2 invades the alveolar epithelial cells by

binding to the human spike protein recognition, angiotensin-converting enzyme 2 (ACE2) receptor (33, 34). The investigation of cell-membrane trafficking could identify the biological mechanisms behind the SARS-CoV-2 infection. The inhibitor of predominant cellular receptor ACE2 is a vasoconstrictor, which protects against organ damages; thus, it is an effective treatment option in hypertension, diabetes, and cardiovascular disease. ACE2 inhibitor, therefore, has a protective effect on ARDS, in which respiratory failure has a rapid onset of widespread inflammation in the lungs. The current statistical data shows poor prognosis related to the male gender, elderly ages, and comorbidities like hypertension, diabetes, cardiovascular diseases, which are also connected to ACE2 (35).

The other central player of the invasion of viral infection to the host cell is the extracellular matrix metalloproteinase inducer CD147 protein on the cell surface (36), extending the action of infection promoter ACE2 receptor. The invasion of the virus to the host cell *via* CD147 is a route that is added to the ACE promotion. The activity of CD147 grows under hypoxic conditions (37), which is usually generated by the massive ATP use of the viral infection of the cell. Blocking the CD147 protein could be a useful strategy for the prevention or first-phase treatment of SARS-CoV-2 viral infection, suppressing the possibility of developing the disease to a severe phase. It is well-known that CD147 appears in most malignant processes (38) involved in vascular endothelial growth factor production, and it could promote the tumor cell invasion and metastasis. The CD147 protein appears like a hallmark of cancer with metabolic reprogramming. CD147 enhances glucose metabolism (39), and it contributes to the immunosuppression by inhibiting the p53-dependent signaling pathway (40).

An appropriate blocking of the ACE2 and CD147 silences the common cell-entries of viruses and could give a solid base for successful therapy. However, their involvement in many independent signal pathways could cause a serious imbalance of the physiological regulations. This "double-edged sword" phenomenon makes the treatment very complicated. The infection by SARS-CoV-2 CoV shows the same complex phenomenon as the self-organized living organism in general (41). It means that processes in the development of the diseases are stochastic (time-dependent probabilities of the events) have promoters and suppressors, and their balances decide the infection's fate. The cumulative dose of viral exposure and the efficacy of the local innate immune response (natural IgA and IgM antibodies, mannose-binding lectin) form the most important balance in the first 10–15 days of the infection (42). When the virus is able to block the defending primary innate immunity, it could rapidly spread from the upper airways to the alveoli, replicating itself without local protection. This phenomenon causes pneumonia and induces high antigen concentration clinically. The delayed and strong adaptive immune response (high-affinity IgM and IgG antibodies) that follows causes unstoppable inflammation and generates cytokine storm, mostly requiring intensive care and being fatal in some cases (30, 31). The balance of physical activity also has an effect: a low or moderate physical activity could be helpful, but an extreme one could facilitate the spread of the virus. The same problem could arise in the case of oral breathing with hyperventilation during the incubation processes. The virus bypasses the immune barrier and determines the rapid development of the disease. This well-presented spatio-temporal harmony (balancing the viral-load, repetitive infection, the timing of the immune actions, concurrent effects of personal immune regulations, etc.) of the development of infection decides the fate of the patient (42). Consequently, understanding the complex process of the development of the infection is crucial for the medical attention, prevention, treatment, and rehabilitation of the patients. The COVID-19 disease has many unusual aspects compared to other respiratory viral infections: severe lymphopenia, causing a deficiency in immune regulatory processes; a cytokine storm with an extensive activation of cytokine secreting cells with innate and adaptive immune mechanisms, leading to acute respiratory distress syndrome and multiorgan failure (43). Laboratory evidence of clinical patients showed that a specific T-cell response against SARS-CoV-2 is important for recognizing and killing infected cells (44), and the measurement of these could inform the design of prophylactic vaccines and immunotherapy for the future.

The host immunity in COVID-19 patients with different severity of the illness was compared after patient admission (45). Several laboratory values, such as ferritin, lactate dehydrogenase, and D-dimer, were increased in the tests of patients in severe and extremely severe conditions. The absolute numbers of CD4+ T cells (helper T-cells), CD8+ T cells (killer T-cells), and B cells significantly decreased, and the NK-cells significantly increased with the escalation of infection, while their percentage proportion did not change so markedly. The ratio of CD4/CD8 cells did not change, while the activation markers of CD4+ and CD8+ T cells (HLA-DR and CD45RO) increased, and the co-stimulatory CD28 decreased by the aggravation of the disease. The percentage of natural regulatory (suppressor) T-cells (Tregs) was decreased in extremely severe patients. The percentage of interferon-gamma (IFN-γ), a soluble cytokine, which is critical for innate and adaptive immune reactions, is produced predominantly by NK-cells and promoting the development of CD8+ and CD4+ T cells, increased by the development of the illness. Cytokines IL-2R, IL-6, and IL-10 were all increased in extremely severe patients, while the activation of dendritic cells (DCs) and B cells was decreased in extremely severe stages of the patients. Interestingly, the age of the patients in severe and extremely severe stages of the disease had no significant influence. Another remarkable observation is that the CD4+ and CD8+ T cells are involved in the pathogenesis of an extremely severe viral infection (45).

The early pathological changes made by SARS-CoV-2 show viral interstitial pneumonia, and diffuse alveolar damage in the lung, as well as pulmonary edema, is also observed frequently (46). It suggests that for patients with early-stage mild SARS-CoV-2 infection, even after their condition improves and is discharged from the hospital, there is a potential—hidden—the risk of further progression to pulmonary fibrosis (47). Viral pneumonia often causes acute idiopathic pulmonary fibrosis (IPF), and the exact mechanism is not yet evident, often leading to irreversible restrictive lung function deterioration and death,

practically *via* suffocation. In the cases of those who survive intensive care, these aberrant and excessive immune responses lead to long-term lung damage and fibrosis, resulting in functional disability and reduced quality of life (48, 49). According to a meta-analysis of 50 466 COVID-19 hospitalized patients, 14.8% of COVID-19 patients developed ARDS (50), which is lower than the observed 20% of SARS patients; ARDS survivors often had pulmonary fibrosis, and 36% and 30% of SARS patients develop IPF 3 and 6 months after infection (51). Therefore, patients overgone by SARS-CoV-2 infection have the potential to progress to IPF, irrespective of how severe the disease was.

A large number of COVID-19 patients require mechanical ventilation to maintain respiratory function. This forceful mechanical interaction could cause ventilation-related lung injury (52). Extensive ventilation could facilitate the direct penetration of the high concentration of SARS-CoV-2 in the lower airways, escaping the impact on the mucosae with neutralizing antibodies (42). The adverse effects of mechanical ventilation are mediated by the systemic release of local inflammatory cytokines and the cellular, molecular mechanisms involved in lung injury caused by mechanical stress. The lung damage caused by mechanical ventilation can become an additional acute lung injury (ALI) (53). These changes' underlying mechanisms may be an epithelial-mesenchymal transition (EMT) and the release of pro-fibrotic mediators caused by cell stretching and mechanical ventilation (54). The aggravating ALI also could induce pulmonary fibrosis.

PATHOLOGICAL NEEDS

The recent understanding and the basic messages from the available literature of the COVID-19 research show how to continue the systemic investigations:

1. Many details of the development of the disease are unclear, first of all, the role of the innate and adaptive immune actions through the development of the severity of the disease.
2. Post-disease immunity and rehabilitation need more research data.
3. The spatio-temporal order of the infection looks significant, but it is not entirely understood. The dose and the repeated reinfection of the virus and the time development of the stages of the symptoms have more to investigate.
4. The importance of the spatio-temporal order is clearly shown by the request of the strong immune possibility at the beginning of the infection. However, the opposite happens: it suppresses the immune reaction while avoiding the cytokine storm when the severity of the disease develops.

THE HYPOTHESIS

Our hypothesis is that the treatment of SARS-CoV-2 infected subjects could be successfully supplemented with modulated electro-hyperthermia [mEHT (trade name oncothermia (55))]. Our proposal focuses on the electromagnetic impact, which combines the effects caused by heat and electric field, using their strong synergy (56). The temperature-dependent and non-temperature dependent factors are combined for optimal treatment (57). The mEHT uses the biophysical differences between the malignant and healthy cells to select them and induce apoptotic signals (58); and immune-mediated abscopal effect (59–61). The technical details (62) and clinical achievements (63) are published elsewhere. The method has a long, successful history; it is applied in oncology worldwide (64). The inhibitory effect of mEHT to COVID-19 is hypothesized due to its immune effects and the biophysically driven selection of cells that create apoptotic signal transduction in the infected cells.

The mEHT could be used for three intentions, encompassing the entire medical activities used in the epidemic. The method could be applied:

1. for the treatment of patients suffering from mild and severe symptoms;
2. for the convalescence, recovering period, when the individual is discharged from the hospital but needs care for rehabilitation.

Our proposal is to use proper electromagnetic treatment, which may solve numerous challenges in SARS-CoV-2 viral infection and its consequences, based on the results achieved by mEHT applications that were proven successful in malignant diseases (63). We propose the application of a wide set of mEHT actions for the treatment of SARS-CoV-2 infection and its post-treatment syndrome (**Figure 1**).

SELECTION OF INFECTED CELLS

SARS-CoV-2 infected cells have a higher metabolic rate, activating (65) and hijacking (66, 67) the energy from the host cell. The higher metabolic rate creates high ionic concentration in the near vicinity of the viral-infected cells. The high ionic concentration creates better conductivity in the cell's microenvironment, which enhances the electric current in the area. This is the selection factor, which is extensively used by mEHT (68, 69). The selected cells will be the target of the electrothermal effect (70), which precisely attacks the targets only (71). The non-isothermal, certainly heterogenic heating process by mEHT selects the high conductivity cellular microenvironments (72). The high metabolic rate forms the change of the electric impedance. The enhanced metabolism causes high conductivity in the vicinity of the infected cells. Due to the intensive transfer of metabolites and other produced molecules, the concentration of the SARS-CoV-2 viruses is relatively high in the microvolume around the infected cells. Therefore, due to the relatively low electric impedance of the microenvironment of the infected cells, the mEHT selects them in the depth of the body like it does in the malignancy (73). The applied radiofrequency (RF) current drives the mEHT effect to

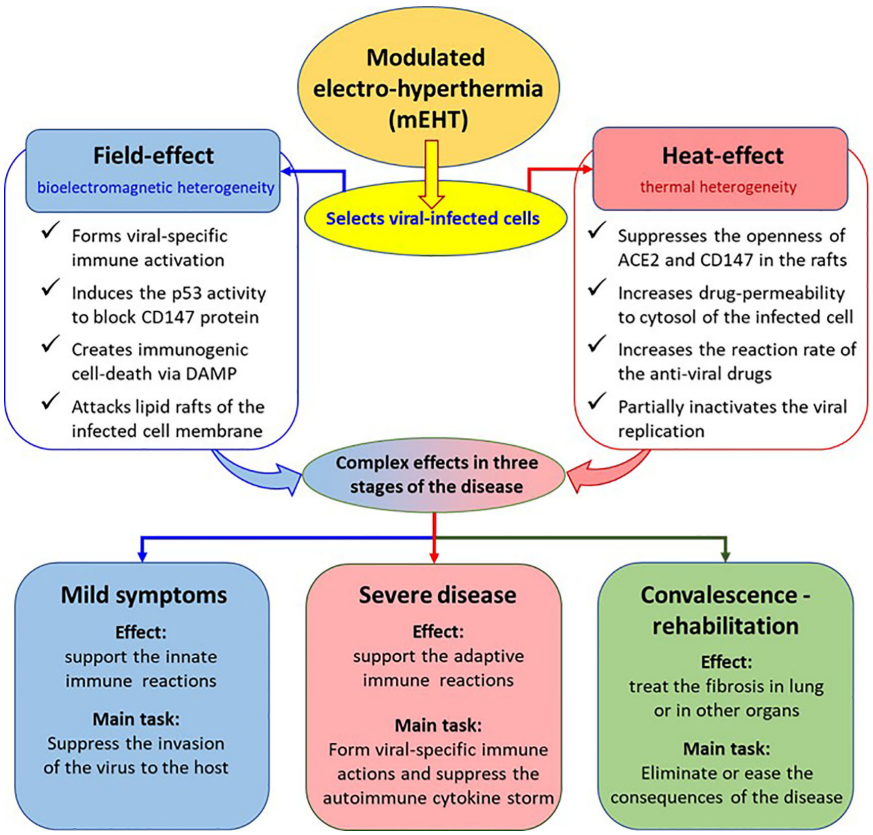

FIGURE 1 | The possible action of mEHT treatment in COVID-19. The effects are deduced from results observed in oncology.

the selected active infected cells (**Figure 2**). The treatment could break to the slower speed of the viral replication, which could be essential in processes when the adaptive immune defense is prepared. The slower replication activity could keep mild disease, which does not transfer to the severe stages.

The lipid rafts of the cell-membrane are involved in the actions of SARS-CoVs (75–78). A hypothesis had been published that presumes the active reduction of SARS-CoV-2 infectivity by lipid rafts, inhibiting the lipid-dependent binding to the host cells (79). Instead of the chemical reagents, mEHT could directly target the lipid rafts of the selected viral-infected cells, as it happens in a tumorous cell with higher metabolic activity (80) (**Figure 3**). The high energy absorption by selected rafts is supported well by in-silico models, too (81).

THE EFFECT OF TEMPERATURE

Unfortunately, the mitochondrial way of apoptosis in SARS-CoV-2 infected cells relates to the induction of viral

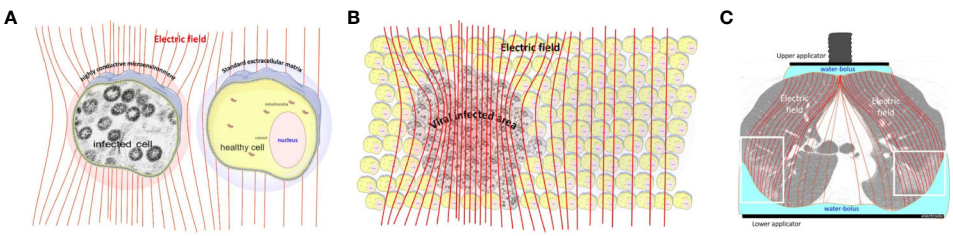

FIGURE 2 | The radiofrequency current flows into the direction of the lower impedance. **(A)** The increased metabolic activity of the infected cells allow lower impedance (higher conductance) in their microenvironment than in the healthy cells. **(B)** The electric field selects the volumes with a high concentration of the infected cells. **(C)** the lung-lesions are loaded more with the radiofrequency current than the non-infected parts [The CT picture is from (74)].

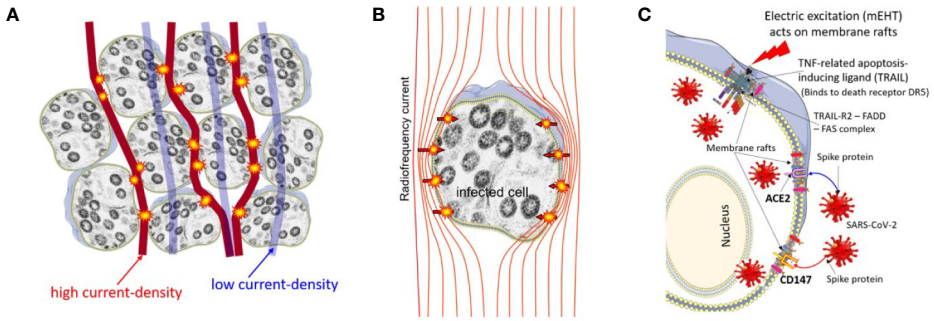

FIGURE 3 | The high current density of the applied radiofrequency power excites the rafts at the cell membrane of the infected cells, which are selected by impedance differences. **(A)** The current flows dominantly in the extracellular matrix. **(B)** The current (energy absorption) excites the membrane rafts. **(C)** The ACE2, CD147, and DR5 death receptors are involved in the rafts.

pathogenesis, with a positive correlation between apoptosis and virus replication (82, 83). The observation of apoptosis in various SARS-CoV-2 infected tissues also suggested that the induction of caspase-dependent mitochondrial apoptosis could be vital for viral pathogenesis (84). The main action of apoptosis by mEHT uses an external apoptotic pathway, exciting the death receptors on the membrane surface (85); as well as some of the pathways are caspase-independent (86), which creates different apoptotic processes from the mitochondrial signal transfer, and could helpfully suppress the viral replication. The observation of Jun N-terminal kinases (JNK) as a dominant factor to induce apoptotic cell death in mEHT (87) supports the expectations of suppressing the viral replication process.

Nobel-laurate A. Lwoff had shown in extensive researches that viruses are usually sensitive to temperature at a cellular level in living conditions. Studying the poliovirus, he had shown an optimal interval of the temperature where viruses are active, introducing infraoptimal and supraoptimal threshold temperatures when the yield of virus-replication decreases by 94% (88). For polioviruses, the infraoptimal (t^+) and supraoptimal (t^-) thresholds are: t^+=38.5°C, t^-=30.5°C. He had shown no viron production at 39.5°C He also studied the pH dependence of viral reproduction, which shows a strong inactivation of the virus in the acidic environment (89). These observations were made much before the recognition of human CoVs. In the SARS-CoV-2 infection, the thermal aggregation of membrane proteins happens (90). In an earlier study (91), the temperature of the infected cells increased with higher metabolism. During the invasion of the host cell, the virus uses ACE2 and binding sialic acid on the cell membrane through hemagglutinin (HA) (92). The virus infection uses several metabolic systems in the host, using large amounts of energy, developing heat as well, which could increase the temperature of the host-cells by approximately 4°C–5°C, while the amount of available ATP decreases as soon as 3 h after the invasion (91). This intensive ATP consumption in a short time causes a sudden temperature increase. In light of the recent literature, the additional heating to the viral infection could create complications. The SARS-CoV-2 virus is stable at a higher temperature, while other coronaviruses are thermosensitive. Heating the body in a wet environment (like a sauna) could be the solution by the inhalation of air with a high temperature (93). The complete inactivation of the virus needs a higher temperature (94); with a phase-transition-like phenomenon at 56°C, which is well over the physiological limit of the body temperature. The viral load measurements in the sputum show the partial inactivation of the viral load at 42°C for 15 min (95) (**Figure 4**).

The partial inactivation is time-dependent and accompanied by the long-existing fever of the infected patient; it could promote other effects in the complex homeostatic regulation of the human body, while the electromagnetic interactions eliminate the load (96, 97) (**Figure 5**).

The thermal aggregation of the SARS-CoV membrane protein may be one of the reasons for the inactivation of this virus by heat (98). The mEHT is also a partly thermal method (99), which locally heats the membrane rafts of the targeted cells in-depth (100).

When cells are exposed to heat-shock, cells develop chaperone proteins (heat-shock proteins, HSPs) protecting the cells from the negative effects of the heat (101). In general, any stress of the cell develops these very conservative proteins as the most effective general protection against stresses. HSPs are involved in the repairing mechanisms of stress-induced damages, as well as participate in multiple signal-pathways, including many apoptotic signal transmissions. Several HSPs (denoted by their molecular weight in kD) are developed in the cell, involving different functions in the combat with the stress. The most important HSP homilies are the 60, 70, and 90 kD proteins (HSP60, HSP70, and HSP90). HSP chaperones have a wide range of applicabilities in medicine (102): thermal stress induces the most HSP70 (103); respiratory hyperthermia induces cytoprotective heat shock response in vesicular stomatitis virus (104) and rhinovirus-infected cells (105); the selective stress of mEHT method builds up HSP for supporting cell-resistance (106) and immune effects (107). HSP70 has an antiviral effect, so we expect that the heat and electric shock presented by mEHT induces a protective chaperone expression against the viral invasion in uninfected cells. The general antiviral effect of

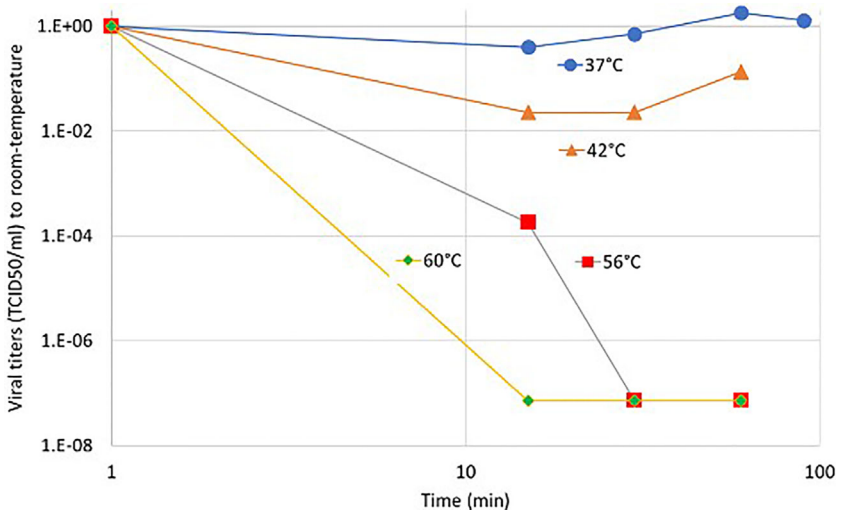

FIGURE 4 | The relative variation of the viral titers (TCID50) of sputum by temperature compared to room-temperature.

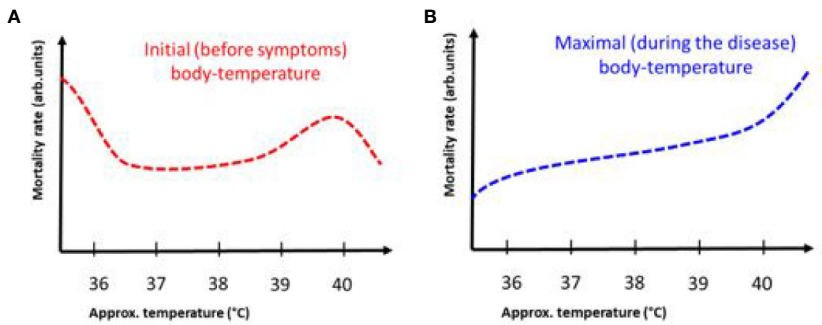

FIGURE 5 | The dependence of mortality on body temperature before and during COVID-19 infection. The trend between the two measures is different. **(A)** Mortality rate as a function of maximum body temperature at the onset of symptoms. The 40°C bulge is probably due to an independent inflammatory effect, increasing mortality at the end of the day. **(B)** The mortality rate versus the maximum body temperature during the disease has a permanent increase.

hyperthermia is also shown (108). HSP70 does essential information transfer for virus-specific immune action by antigen-presenting cells (APCs) (see below).

THE IMMUNE STIMULATIVE EFFECTS

mEHT is an immune-stimulatory treatment (109–111). Immune effects are important factors against viral invasion (112). The desired effect may be induced by an appropriate HSP expression (113), like the immune action, it suppresses the human papillomavirus (114), and even human immune deficiency virus (115). We expect the immune-responses presented earlier in the case of other viruses also for patients suffering in COVID-19 (116). This expectation induces recommending the same therapy as well (117). Using the immunostimulatory effects of the autoimmune reactions (118) and fever (119) could be a helpful therapeutical strategy when it is controlled and does not allow an overreacted stormy feedback (120, 121). The clinical statistics of SARS-CoV-2 infected patients show that significantly more patients had fever among the cases where no critical care was necessary (122). A sign of the advantage of hyperthermia could also be that ACE2 inhibition was proven in preclinical experiments (123).

The mEHT effects could be divided into two categories: heat effects and field actions (124). Heating increases the intracellular pressure (70) and could damage the membrane, causing the direct necrosis of the cell, blocking the further replication of the virus by the nucleus. Importantly, the drug permeability to the cell is promoted by combined heat and field actions (125), which could be an important factor for the newly approved drug Remdesivir, which blocks RNA-dependent RNA polymerase (RdRp) in the nucleus (126). The field effects are mostly molecular and—in consequence of the molecular changes—

immunological (127). The absorbed energy triggers extrinsic apoptotic signals, which are going through their pathway and could excite the variants of the apoptotic processes (107). A damage-associated molecular pattern (DAMP) is formed, which —when properly appears in time and localization—could complete the apoptosis as immunogenic cell death (ICD) (128). In the ICD process, the freed special DAMP molecules - like the 70 kDa heat-shock proteins (HSP70), calreticulin, HGMB1, and ATP —provide "info signal", "eat me signal", "danger signal", and "find me signal", respectively. These molecules provide infection-specific information for immune actions (129). The information carrier HSP in the extracellular electrolyte could serve as the surveillance information for the immune-system (130). The DAMP-induced ICD forms antibody presenting cells (APC) by the maturation of the dendritic (DC) producing immune actions by forming a virus-specific killer (CD8+) and helper (CD4+) T-cells directly (131). The immune-effects are well-proven in preclinical (132) and clinical (133) practice (**Figure 6**). The virus adapts to the immune system of its host (the human population) by natural selection, but the *in situ* produced genetic information to the APC production could compete with the adaption and stop, or at least suppress the viral replication.

The virus infects the immune cells due to its cross-immunity with common-cold immune reactions (9). The viral-load affects the interaction with the cellular immune response helper (CD4+) and killer (CD8+) T-cells in cooperation with the B-lymphocytes. These cells produce different cytokines: the helper T-cells produce interferons and interleukins, while the killer T-cells damage the viral-loaded cells by cytolysis and necrotizing cytokines. The starting immune reactions to SARS-CoV-2 have a competition between the infecting viral load and the innate immune system's activity, especially the NK-cells, the already primed CD8+ cytotoxic T-cells and their activity and nonspecific or other CoV-related mucosal IgA levels with their T-cell interactions. An increased cytokine level [tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)], as well as chemokines secreted in correlation with the migration of NK cells and macrophages in the virally infected area. A problem arises when the viral load dose is too high or the innate immune system is too weak. Due to the longer time to develop the adaptive immune system, the immune reply is not immediate. During the time of missing the complete adaptive reaction, the viral expansion could intensify, and the task for an adaptive immune system becomes more difficult. A second wave of the cytokine production starts [TNF-α, IL-6, gamma interferon (IFN-γ), IL-2, and IL-5], which may cause a cytokine storm, and could coincide with pneumonitis, developing a high risk of an extremely severe illness. Therefore, it is indicated (134) that leukocyte-mediated antiviral responses have a double-edge sword role that may contribute to the clearance of SARS-CoV but pneumonitis as well. The viral dose and the timing in the infective development have a decisional role in the actual conformation of the disease's status and severity. The clinical intention is the prevention of the development of severe disease when the patient only has mild symptoms of the infection. An immunological way is a perfect option when the T and B cell immunity with virus-specific antibodies could inactivate or slow down the development of the diseases (43). The mild disease develops these virus-specific immune cells in the right phase of the infection when these could successfully fight against the viral load. However, when the immediate action of the innate immune system is not able to compensate the viral load, the developed cytokine storm and the viral load together becomes too extensive, and the adaptive immune reaction is late; therefore, the cytokine storm develops further, and the disease reaches a severe form.

The mEHT induces the p53 activity and causes apoptosis (125), which could arrest the accelerated viral load by arresting the invasion CD147 by the active p53. The present clinical studies with the CD147 blocking drug Meplazumab are feasible and can prevent SARS-CoV-2 spike binding and the subsequent infection (135). It may also have beneficial effects on other COVID-19 treatments (136). The mEHT could be a significant addition as a complementary application with Meplazumab. Supporting the innate immunity and preparing the action of the adaptive answer at the appropriate time and force with mEHT could be crucial in blocking the infection from turning into a severe phase.

TREATMENT OF FIBROSIS

Here we provided a theoretical basis for the utilization of modulated electro-hyperthermia against COVID-19 and other coronaviridae.

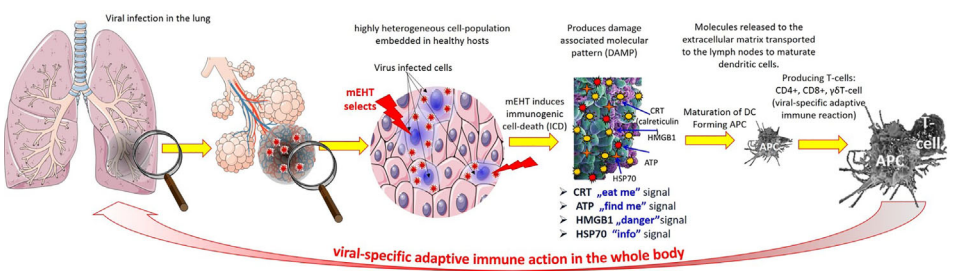

FIGURE 6 | The antiviral immune-effect of mEHT: the mEHT selects the infected cells which release a damage-associated molecular pattern through the process of immunogenic cell-death. The DAMP provides viral genetic information to maturate the dendritic cells producing antigen presentation for T-cells, which turn to viral specific CD4+ CD8+ form. The viral-specific T-cells attack the infected cells in all over the body.

The preventive effect is both primary (antiviral) and secondary (anti-fibrotic). In the long run, the latter will be responsible for decreased pulmonary capacity and eventual fatal outcome.

Therefore, in CoV pneumonia cases, it is important to control cytokine production and inflammatory response, given that they are responsible for the accumulation of cells and fluids. This strategy is challenging as we have not yet clearly identified any features in an immune response that can be inhibited specifically without compromising the beneficial host defense (12).

The mEHT is a good tool to treat fibrotic damages of patients during and after a viral infection and ALI, which releases mediators for the fibrosis (53). The EMT promotes the fibrotic processes (54, 137). The mEHT is a good tool to moderate the EMT by the electric field (138). Furthermore, HSP development is also a great possibility to reduce the effects of fibrosis, which is suppressed by the presence of these chaperones (139). The effect of HSP on fibrotic lesions is shown by the cure of warts too (140, 141). The high-dose vitamin C mEHT is inhibitory of the fibrosis in consequence of viral infection (142). The mEHT with low-frequency modulation on a high-frequency carrier (62) allows a deep penetration that reaches the infected cells in the lung. The demodulated signal promotes re-establishment of the homeostatic balance of the cells by charging the redistribution (143) and by the effective intracellular reordering of the cytoskeleton (144), giving less opportunity of the cytoskeleton components (structural: tubulin, actin; and dynamical: dynein, kinesin, and myosin) (145) providing intracellular transport to the correct location for the replication of the virus. Patients with penile fibrosis (Peyronie's disease) (146), and palm fibrosis (Dupuytren's contracture) (147) were successfully treated with mEHT. Furthermore, the mEHT treatment on malignant fibrosarcoma also showed a great benefit for the patient (148). The mEHT is successfully applied as a complementary treatment for lung cancer with high-dose vitamin C (149). Lung effusion is also one of the patient's remaining negative states cured of the viral disease of SARS-CoV-2. mEHT offers a solution to this problem, too (150). Note that RF-current is widely used for cellulite fibrosis (151) and skin laxity (152), but only for near-surface areas. The mEHT is active in-depth (73), so the usual activity against the fibrotic structures is anyway expected.

The allometric fractal considerations help the solution of structural and metabolic problems of SARS viruses (153). The mEHT applies time-fractal modulation, which drives the processes to the direction of the healthy homeostatic balance, promoting the immune-system for surveillance (154). Mechanistic investigations of mEHT effects on related lung fibrotic animal models can be effectively conducted using *in vivo* molecular imaging outcome measures (155) and fractal dimension analysis of X-ray computed tomography (156). The mEHT acts on the fractal complexity (157). It uses an allometric approach (158) for self-similarity (41) of homeostatic systems (159), which could be an additional support to its action against forming IPF. The mEHT method, with its fractal-physiology considerations, could be a help to find the best treatment option. We are convinced that the disease's complexity could be handled only by the complexity of the treatment. A miracle single curing effect is unrealistic.

The adverse effects of the mEHT in oncological applications are few, and it may reduce the side effects of the other therapies (160), and it has pain-reduction possibility too (161).

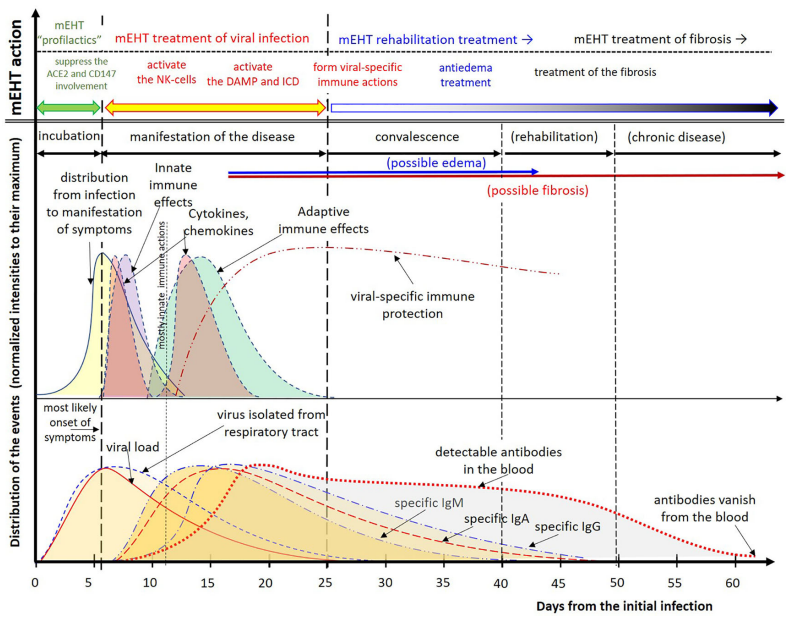

FIGURE 7 | Summary of the viral effects of SARS-CoV-2 and the hypothesis based proposal for treatment. Due to the considerable individual differences, the distributions are normalized to their maximum. The intervals are approximate. The distributions are composed to their most likely form using the results (71, 107, 162–169). The disappearance of antibodies to SARS-CoV-2 was measured too (170), but others measured the opposite, existing antibody protection at least for two years (171).

SUMMARY

The above hypothesis is comprehensively summarized in **Figure 7**. The distributions are not symmetrical (not normal distribution), having a longer "tail". The tail is a typical fingerprint of the complexity (multiple feedback interactions with stochastic results) (172). The complexity depends on the patient and the disease's general conditions, so the distribution curves are only orienting the reader; they could not be exact. The intensities of the effects also have individual dependencies, so all are normalized to their maximal values.

We conclude that the hypothesis that mEHT could be a helpful treatment in all phases of the COVID-19 has feasible references. Experimental and clinical data are mandatory and warranted.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All named authors take responsibility for the integrity of the work as a whole and have given their approval for this version to be published. All authors contributed to the article and approved the submitted version.

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REVIEW
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Multilevel Engagements of Pharmacists During the COVID-19 Pandemic: The Way Forward

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Severe acute respiratory syndrome caused by the novel coronavirus (SARS-CoV-2) was first reported in China in December 2019 which was later declared to be a public health emergency of international concern by the World Health Organization (WHO). This virus proved to be very contagious resulting in life-threatening respiratory intricacies posing overall public health and governance challenges. Amid the coronavirus pandemic and the unprecedented increase in healthcare demands, only inventive and adaptive practice among healthcare professionals is the need of the hour. Pharmacy services are an important mainstay in the public health and have considerable potential to combat the coronavirus disease 2019 (COVID-19) pandemic. Pharmacists working in several localities and health facilities are linked to patients either directly or indirectly. They can act swiftly in public health response such as drafting professional service guidance to pharmacists working in various healthcare facilities, ensuring effective medicine supply system, monitoring and resolving drug shortage issues, establishing and promoting remote pharmacy services, counseling the public on infection prevention basics, educating about proper use of personal protective equipment, discouraging self-medication, participating in clinical trials, small-scale manufacturing of sanitizers and disinfectants, busting the prevailing myths, and conducting drug evaluation and active surveillance. These interventions will help ease unprecedented burden on healthcare facilities during the ongoing pandemic and eventually will add value to patients and the healthcare system. The current manuscript accentuates the potential roles and activities that pharmacists can initiate in various healthcare facilities to help in relieving pressure on the overwhelmed healthcare system. The information and suggestions offered in this review could help in the restructuring of existing pharmacy services by governments, public health bodies, and policy makers in response to the COVID-19 pandemic. Moreover, this manuscript will underscore any unrealized potential among pharmacists working in various sectors including community, hospital, industry, and drug regulatory authorities.

Keywords: COVID-19, coronavirus, pharmacist, clinical pharmacist, community pharmacist, industrial pharmacist, pharmacy services, pandemic

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BACKGROUND

Pharmacists are one of the most trusted professions worldwide alongside firefighters, nurses, teachers, and doctors (1, 2). During the current pandemic when the healthcare system is collapsing amid the unprecedented number of coronavirus disease 2019 (COVID-19) cases, pharmacists can play a pivotal role in disease prevention, management, and containment (3). They work in several localities and are linked to the patients, either directly or indirectly (4). Health authorities across various countries are recognizing the value of community pharmacists in the healthcare system due to their availability and accessibility to the public. However, pharmacy services in infectious disease control and pandemic are least appreciated.

During the ongoing COVID-19 crisis where clinicians and nurses are overburdened, pharmacists are well-situated to offer collaborative and complementary expertise alongside current models of care. However, the capabilities of pharmacists are under-recognized both by patients and physicians. Practitioners report strong mutual respect for pharmacists as allied health professionals, but communication between them could be strengthened (2). Moreover, there is a dearth of investigations to ascertain the impact of pharmacy services in controlling infectious diseases. There is a dire need to potentially utilize the services of pharmacists working in community, hospital, industry, and drug regulatory authorities. In addition, integrated efforts of pharmacists working in various settings with clinicians, nurses, and public health officials will strengthen the ongoing maneuvers to contain COVID-19. The purpose of this manuscript is to underscore the pivotal contributions of pharmacists which could potentially assist to curb the growing encumbrance of the disease.

Since community pharmacies, hospitals, pharmaceutical industries, and drug regulatory authorities are exempted from the current lockdown which is being observed by most of the countries, effective utilization of pharmacist's services will be of paramount importance to tackle the quadruple burden associated with the pandemic. A pharmacist, being a most trusted and accessible healthcare professional, can be utilized to combat the chaos attributed to the pandemic (5, 6).

METHODS

To provide a summary of the potential roles and responsibilities of pharmacists during the COVID-19 pandemic, published data from scholarly articles, organizations, and stakeholders were reviewed. Literature was searched by four independent reviewers (THM, AL, AA, YHK) using various electronic databases from inception to July 2020. The end date of the review time generally coincides with the generally accepted end of the first wave of COVID-19 in the Northern Hemisphere.

Selection Criteria for Studies

The current review describes the various roles of pharmacists that can be performed during the COVID-19 pandemic to leverage all possible resources in the best interest of patient care and management. Moreover, data from studies providing prospective implications of pharmacy services in the management of COVID-19 pandemic were also included to give insight of the possible roles of pharmacists in combating COVID-19. Relevant data was collected via electronic search of different scientific sources including PubMed (https://www.ncbi.nlm.nih.gov/pubmed), Science Direct (https://www.sciencedirect.com/), Google Scholar (https://scholar.google.com/), Scientific Electronic Library Online (SciELO) (http://www.scielo.org/), Cochrane Library (https://www.cochranelibrary.com/), and Web of Science (http://www.webofknowledge.com/). The study databases were selected on account of their score for health and pharmaceutical journals. These databases covered articles of peer-reviewed journals, books, and supplementary reports covering multilevel engagement of pharmacists in COVID-19.

Search Strategies

The search strategies utilized a combination of the following terms: "COVID-19," "Corona virus disease," "pharmacist," "community pharmacist," "industrial pharmacist," "hospital pharmacist," "drug regulatory authorities," "regulatory pharmacists," "pharmaceutical industry," "pharmacy department," "SARS-CoV-2," "pharmacy management," "pharmaceutical care," "clinical pharmacist," "pandemic," and "outbreak."

Inclusion/Exclusion Criteria

Studies on the functions and roles of pharmacists during the COVID-19 pandemic published during the period from inception to July 2020 were included in this review. Moreover, this review also included the studies describing the various fundamental responsibilities of pharmacists which can be utilized during the ongoing health crises. Only research studies, review articles, case studies, books, short reports, position papers, perspectives, organizational recommendations, and authentic guidelines on management and containment of COVID-19 were considered. Abstracts, scientific correspondence, posters, advertisements, thesis, web pages, and news were excluded. The studies published in a language other than English were not included in this review.

Data Extraction

Following the introductory search, retrieved articles were imported to EndNote X7 to remove the duplications. The eligibility of the each study was assessed by all authors through screening of the title and abstract. After an initial screening, a full-text evaluation was carried out for the final selection of articles. Any disagreement regarding the suitability of studies among authors was resolved through mutual agreement and discussion. Only studies for which consent was provided by all authors were included in the manuscript. The studies related to the potential role of pharmacists for COVID-19 were selected and carefully analyzed for this review. With the information assembled through these studies, the difference between the traditional and the expanded role of pharmacists was pointed out. Major suggestions are offered on how to utilize the contemporary

roles of pharmacists to effectively employ all available resources thus easing burden on other healthcare professionals.

ROLE OF PHARMACISTS IN PREVIOUS OUTBREAKS

Pharmacists have been actively involved in various infectious disease outbreaks. During epidemics, the role of pharmacists broadened from routine duties to preventive activities such as disease monitoring and surveillance, immunizations, and diagnostic testing (7). Pharmacist associations around the globe have played a crucial role in pandemics to plan beforehand in order to leverage all the resources to their maximum extent (8). According to the Canadian Pharmacists Association (9), the role of pharmacists was designed in pandemic preparedness plan for combating H1N1 influenza (9). To combat the shortage of antivirals, pharmacists compounded oseltamivir in the hospital pharmacy during the influenza (H1N1) pandemic (10). Pharmacists played their role in effectively promoting an alternative method of supply and dispensing of antivirals from public stockpiles during the flu pandemic (11). Nigerian pharmacists helped during the Ebola outbreak by promoting infection control measures and educating the public on how to avoid the disease spread (12). Pharmaceutical care, medication therapy, infection control, and immunization are among the top listed duties delivered by the pharmacists during the Ebola epidemics (13). Effective communication between health departments and community pharmacies was proved to be an effective response to the influenza pandemic (7).

The role of pharmacists is well-appreciated during the outbreaks of vaccine-preventable diseases. The education of patients and the public regarding the importance of vaccines resulted in increased vaccination rates (14). Pharmacists played a valuable role in the protection of high-risk individuals through vaccination programs (15).

Pharmacists are known for reducing the work load of public health professionals during the measles outbreak. Being an easily available, accessible, and trustworthy professional, pharmacist ran vaccination plans and increased the vaccine awareness which ultimately resulted in vaccination acceptance rates (16). Pharmacists provided patient-centered therapy with stringent infection control measures during the severe acute respiratory syndrome (SARS) outbreak. They delivered the medicines not only in wards but also in quarantine areas. Pharmacists have proved themselves as a valuable member of the team in health crisis by providing information services and patient care (16, 17).

Keeping in view the previous contributions and current extended pharmacy services, pharmacists can be involved at different levels in disease control and prevention, patient care, and treatment during the COVID-19 turmoil. **Figure 1** describes the potential contributions of pharmacists working at different levels of the healthcare system.

Role of Community Pharmacists

Community pharmacies have remunerated their roles from traditional dispensing to more comprehensive clinical services in recent years. Community pharmacies are considered the first entry point in both outbreak-affected and unaffected areas during the COVID-19 pandemic (18), where pharmacists are most accessible and underutilized healthcare professionals to cope with COVID-19 havocs (6, 19). Community pharmacists can provide breadth of public health and clinical pharmacy services during the ongoing pandemic to empower people to self-manage their health. Following are the potential roles of the pharmacist at the community level to serve the public in the current circumstances.

Disease Education and Counseling

The COVID-19 pandemic is accompanied by several misleading narratives which raised confusions and uncertainties among the general public (20). The importance of public awareness and education for COVID-19 cannot be disregarded during the current pandemic. Pharmacists being frontline healthcare providers can guide people regarding the disease, its causes, and routes of transmission, thus assisting to neutralize the confusions in the general community (21). Counseling of patients regarding the onset of sign and symptoms after contracting the virus is very crucial as the patient can remain asymptomatic for 2–14 days following virus exposure (22). The median incubation time for coronavirus is 5.1 days, suggesting the appropriateness of quarantine time of 14 days (23). Pharmacists should be well aware and provide sound advice to the public about the clinical manifestations of the disease. Moreover, they can provide advice to suspects on whether there is a need to be quarantined or not. Since the virus can spread through airborne droplets, coming in contact with infected persons, and by touching contaminated material surfaces (24), the disease education carries utmost importance to halt the transmission chain (25).

With the rise in the number of COVID-19 cases, there is unprecedented burden on the healthcare system overwhelming hospitals and healthcare facilities (22). In this critical time, pharmacists can provide proper disease education at the community level to avoid the unnecessary scares (3). Symptoms-related counseling will help the patient to understand the point or time when medical attention is needed. As most of the suspects are visiting the emergency department even due to cough or flu which is adding unnecessary workload on the hospitals (26), community pharmacists can educate the public about the differentiating symptoms, thereby reducing the unnecessary visits to the emergency departments.

Likewise, during the current pandemic, self-medication is becoming prominent due to amplified information on drugs floating on social media and news channels. Drug repurposing is improving the clinical conditions of patients but at the same time portending substantial risks of self-medication (27). Various reports have indicated chloroquine poisoning and shortage following the announcements related to its effectiveness on electronic media (28, 29). Community pharmacists must observe any kind of unusual drug use among the public or any irrational prescribing pattern among physicians in their localities.

Education on Hand and Respiratory Hygiene

Pharmacists can utilize several methods of education to enhance the public understanding of disease (30). Educational

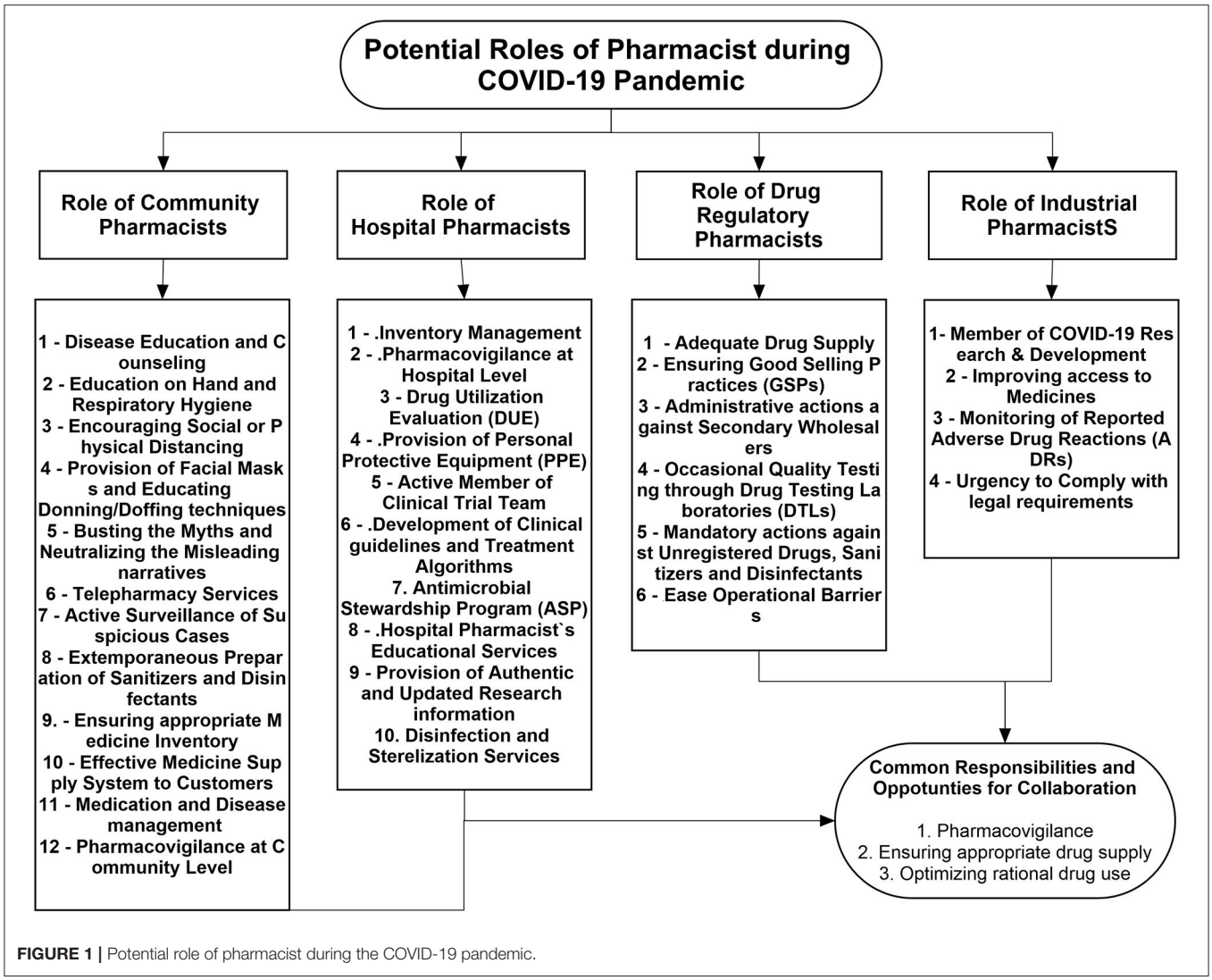

FIGURE 1 | Potential role of pharmacist during the COVID-19 pandemic.

presentations in video formats, posters, flyers, or hangings could prove very impactful in educating the masses coming to the pharmacy. Pharmacists can arrange hand sanitizers at all patient counters and should practically demonstrate the correct method of hand sanitization. Similarly, considerable attention should also be paid to good hygiene practices among pharmacy staff. Isolation of pharmacy staff from the customers through a glass screen or polythene material would not only give a message to the customers about the importance of physical distancing but will also ensure the protection of the staff. Appropriate practical gesture will have long-lasting impact on customers about the seriousness of good hygiene (23).

Encouraging Social or Physical Distancing

Teaching people about social distancing would be easier if all the pharmacy staff is also practically demonstrating it to the customers. There should be at least 1 m (3 ft) distance between every pharmacy personnel. The floor of the pharmacy can be marked with the signs of 1 m distance to avoid any close contact among the customers. This method will provide actual sense of physical distancing to the customers. The minimum safe distance to avoid viral contract should be displayed at every counter. Customers should be encouraged to maintain at least minimum distance while handling the routine matters. Several studies have suggested to maintain at least 2 m (6 ft) distance to avoid any viral contraction (31–35). The public should appropriately be counseled to avoid any contact with the surfaces frequently used by the people such as door knobs or handles, and in case if they have come in contact with such surfaces, they should follow immediate hand wash technique and should avoid touching their face. Since pharmacies have become frequently visited places during the pandemic, effective utilization of an educational tool will help to provide authentic and useful information to the general public (36).

Provision of Facial Masks and Educating on Donning/Doffing Techniques

The use of masks is a basic protective measure to avoid viral contract from the patients (37). However, its effectiveness can only be ensured following the appropriate methods of use.

Pharmacies are authentic selling points for masks and can be served as an educational platform for the public to get awareness regarding the types of masks and their correct methods of use. Incorrect use of masks and inauthentic brands will not only increase the risks of disease contraction but will also pose economic burden to the public and may lead to shortage of masks for high-risk users (38). Pharmacists should use visual aids to educate the public about the donning and doffing techniques and should ensure that people are using authentic masks dedicated for viral protection. Correct donning and doffing of masks and their appropriate disposals are among most effective measures stated by various health authorities around the globe (37).

Busting the Myths and Neutralizing the Misleading Narratives

The COVID-19 pandemic is accompanied by rapidly prevailing myths on treatment and prevention. Moreover, several conspiracy theories and misleading narratives have sprouted and proliferated in many countries, posing substantial challenges for health officials to contain the disease. Since community pharmacists are considered trusted professionals, they can play a pivotal role in busting these myths by providing reliable information to the public. Pharmacies can make pamphlets on prevailing myths and can distribute to each customer attending the premises. They may ask questions to the customers in order to ascertain their perception toward the disease and can clarify any confusion. The WHO has issued various videos and images to eradicate the myths and the same can be utilized in the pharmacy premises (39). Moreover, it is pertinent to mention that every news channel or agency is efficiently engaged to break any new findings or studies related to the treatment and prevention of COVID-19. Such news reports may influence the people who are searching for appropriate measures to save themselves from the virus. Pharmacists at the community level can provide timely intervention to bust such claims and prevent the public from self-medicating. Any sort of self-medication during the current pandemic can be devastating and may aggravate the ongoing health crises for which none of the country is readily prepared.

Telepharmacy Services

COVID-19 has changed the use of informational technology in the healthcare system. Telemedicine provides electronic consultations and has reduced the risk of transmission by reducing in-person contact among people. Telepharmacy is one of the practical aspects of telemedicine that refers to providing pharmaceutical services within the scope of a pharmacist's responsibilities, with a temporal and spatial distance between patients as the consumers of health services and healthcare providers. This service will not only be useful for COVID-19 patients but also for chronic patients and the general community experiencing the restricted movements amid lockdown. Moreover, telepharmacy will also reduce the volume of patients seeking care at health facilities (40). Though the telepharmacy program would not solve all the health problems, it is well-suited as a solution to make effective connection between pharmacists, patients, and other healthcare professionals. Moreover, community pharmacists can consider this service to follow COVID-19 patients after discharge to ascertain the recovery pattern. For routine chronic patients, pharmacies can use their webpages, message services, or social networking links to respond to the queries.

Active Surveillance of Suspicious Cases

Early detection and referral of suspected cases are vital to prevent large-scale community transmission. Community pharmacists, therefore, must remain very vigilant and be able to screen the patients for necessary referrals. The Centers for Disease Control and Prevention (CDC) and the International Pharmaceutical Federation (FIP) have provided guidelines for the investigation of suspected COVID-19 cases (21). There are several other risk assessment scales available to identify the suspected cases such as CDC's coronavirus self-checker (41). Pharmacists should arrange sensitive thermometers at the pharmacy to identify the suspects. Any suspected case must immediately be notified to the designated health authorities in order to avoid any further disease spillover. Since most of the people are hiding their symptoms due to the fear of being quarantined or due to the phenomenon called disease denial, pharmacist can be a facilitator for national disease surveillance unit. Community pharmacies along with educating the public can play a crucial role in case identification and reporting.

Extemporaneous Preparation of Sanitizers and Disinfectants

The COVID-19 pandemic has caused shortage of hand sanitizers in many developing and developed country, either due to increased consumption or disruption of raw material supply. Community pharmacists can employ their expertise of compounding and ensure the availability of hand sanitizers and disinfectants all the time at an affordable cost. The WHO recommended the use of ethanol (80%) and isopropyl alcohol (75%) in all kinds of hand rub formulations (23). Pharmacists can also prepare disinfectants according to national legal provisions. Moreover, all surfaces of pharmacies should also be disinfected. A simple formulation of 10 ml bleach in 990 ml water can be used as an effective disinfecting solution (31).

Ensuring Appropriate Medicine Inventory

During these unprecedented times, the global drug supply could severely get impacted by this pandemic, and the results of this shortage could be catastrophic and may last for an extended period, primarily due to the global economic disruption at unprecedented speed and scale. It must be noted that drug shortage could lead to serious consequences when it comes to patient outcomes (42). Pharmacists can play an important role in the mitigation of emerging drug shortages related to the pandemic. Pharmacists should aim at procuring and stocking the right amount of medicine to guarantee the supply round the clock. The regulatory body of pharmacy or the whole department should work in collaboration and devise shortage and mitigation plan beforehand (43). Drug demand analysis to identify medications of interest should also be conducted in a parallel manner (44). One pharmacist can be designated

specifically for procuring to ensure the supply of medicine and to avoid any possible shortage. Meanwhile, they should also use their pharmacologic and pharmacokinetic knowledge to design algorithms to successfully implement medication-sparing strategies (44). Vigilant observation of any medicine's unusual selling trend and timely reporting can prevent drug shortage. Pharmacists must use their compounding abilities and utilize alternate ingredients to ensure proper supply of medications, sanitizers, and disinfectants (43). Since ensuring the availability of all medicines during the current state of unrest becomes quite difficult, community pharmacies can make a priority list according to the regional need and should ensure the availability of these medicines, particularly those required for COVID-19 management (44). Moreover, the pharmacist is able to navigate alongside other members of the healthcare team alternative therapeutic options until the shortages are resolved.

Effective Medicine Supply System to Customers

In order to ensure appropriate supply of medications especially in small towns where local pharmacies may have closed, community pharmacists can arrange home deliveries or an electronic prescription refill system. Home delivery services will prove to be very helpful for people in quarantine as well as for those with weak immunity, i.e., the elders (25). Pharmacists can be authorized by governing bodies to use their judgment to refill prescription for at least 30 days to avoid unnecessary patient visits. Similar practice has been adopted in the USA during the time of Hurricane Katrina (45). In order to avoid pharmacy visits, early refills for maintenance medicines would also be beneficial for the patients with chronic illnesses (45). Moreover, pharmacists should also ensure that medicines are being sold for the purpose of use rather than for stocking.

Medication and Disease Management

It is a critical time to convert the traditional role of pharmacists into the extended role to allow them provisional prescribing of medications in collaboration with a physician within some jurisdiction (19). This function would be critical in areas where healthcare providers are busy with COVID-19 management and deployed from their routine practice sites to other areas. It will ultimately increase the dependence of these healthcare professionals on pharmacists as the medication experts on the team (44). Patients with chronic diseases can benefit from this service where they do not have to visit the hospital for their prescription refill (45). Moreover, pharmacists can arrange on-call meetings with physicians to seek any guidance regarding prescription refills. These extended pharmacy services (EPS) will possibly reduce the mobility of patients with chronic illnesses. It must be noted that chronic patients have portended higher mortality rate during the COVID-19 pandemic. Moreover, recent data has indicated the high ability of chronic patients to contract the virus and to have severe symptoms of COVID-19 (46). Therapeutic switching is a major challenge for clinicians due to drug shortage during the pandemic. Pharmacists should intimate local clinicians regarding the availability of therapeutic alternatives. Examples here include the switching between the intravenous analgesic fentanyl to remifentanil. Since the supply of intravenous medications is substantially high during the pandemic, pharmacists should educate other members of the healthcare team about the use of adjuvant medications such as oral and transdermal formulations.

Pharmacovigilance at the Community Level

Since the healthcare system is overwhelmed with the increasing number of COVID-19 cases, any drug-related problems (DRPs) might easily be neglected by healthcare professionals. These DRPs are either related to the ongoing use of chronic medications or self-medication and may associate with adverse outcomes. Pharmacists must keep a vigilant observation on the safety profile of these drugs. As most of the healthcare authorities are engaged in containing the virus, any untoward effect of the drug will get unnoticed (25). It is a fundamental responsibility of community pharmacists to ensure the safe use of drugs, particularly among the chronic patients. Moreover, community pharmacists should efficiently monitor the potential side effects of repurposed drugs employed in the prevention and treatment of COVID-19.

Role of Hospital Pharmacists

In public health emergencies, pharmacists play a distinguished role in reducing the burden of disease. Hospital pharmacists provide pharmaceutical care and services to both in-patient and out-patient (7). During the COVID-19 pandemic, their duties expanded from routine activities to the focused care for COVID-19 hospitalized patients. Pharmacists became an important part of the medical team to improve the therapeutic outcomes and ultimately the pandemic control (47). They also ensure the adequate supply and stock of requisite drugs and other medical products in accordance to the patients' demand (48).

Inventory Management

Hospital pharmacists are primarily responsible to ensure the timely provision of medications to the patients (49). Pharmacists can play a vital role to identify and alleviate possibilities of drug shortages. Drug shortages in the current scenario can compromise or adversely affect the patient medication therapy. Moreover, pharmacists should identify the factors in the supply chain contributing to the drug shortage. The pharmacy staff can contact pharmaceutical manufacturers, distributors, community pharmacies, and the regulatory agencies to inquire about the cause and duration of the shortage (50). Due to limited trade and closure of various pharmaceutical plants, disruptions in the pharmaceutical supply chain are observed resulting in increased prices and drug shortages globally (51). In this inevitable situation, pharmacists can find therapeutic alternatives to avoid any hindrance in the provision of therapy. They should develop a proactive attitude through quantitative assessment of the inventory and estimation of the drug shortage period. Alternatives should be inventoried to ensure sufficient supply to meet the increasing demand (52). Moreover, provision of drugs to prioritized patients is the need of the hour. It would be beneficial to reserve repurposed drugs for COVID-19 patients (53). Hospital pharmacists can assist clinicians in drug switching and adjunctive therapy to cope with the drug shortages.

Pharmacovigilance at the Hospital Level

Effective pharmacovigilance results in reduced cost of care while improving therapeutic outcomes, which are the need of the hour during the ongoing pandemic (54). Currently, numerous drugs are being tested for COVID-19 and few of them are linked to serious adverse effects. Chloroquine and its derivative hydroxychloroquine pose risks of QT prolongation and require caution among patients with G6PD deficiency and diabetes. Lopinavir and ritonavir are associated with the risks of cardiac arrhythmias due to QT prolongation, and careful monitoring is required among patients with hepatic problems. Corticosteroids are considered for patients with respiratory distress syndrome or refractory shock and are not recommended for viral pneumonia (55). Baricitinib should be used with extreme caution in susceptible patients with ongoing pneumonia associated with SARS-CoV-2 (56). During the current phase of drug repurposing, the hospital pharmacists are keenly monitoring drug safety by detecting, investigating, and reporting drug-related problems among COVID-19 patients (57). Moreover, hospital pharmacists should review safety data of published studies which are desperately needed by healthcare professionals. Despite the unprecedented global challenge posed by the COVID-19 pandemic, the importance of patient safety should not be disregarded.

Drug Utilization Evaluation (DUE)

Drug repurposing for COVID-19 is improving the clinical conditions of patients but at the same time posing substantial risks of drug-related problems. The use of these drugs is subjected to careful assessment only if the desired effects overshadow the risks. Pharmacists provide accurate clinical information to the healthcare professionals regarding the drug safety, interactions, and adverse effects (53). Optimizing the rational use of repurposed drugs is the need of the hour. A study from China indicated that the use of ribavirin for COVID-19 widely varies in hospital in terms of duration and timing of treatment (58). Pharmacists should ensure the appropriate use of these medications in the hospital, particularly in the vulnerable population such as the elderly, immune-compromised patients, and pregnant women. These population should be considered for targeted drug use evaluation (59). Moreover, routine DUE should not be neglected during the current pandemic.

Active Member of the Clinical Trial Team

Since most of the hospitals are conducting clinical trials on the treatment of COVID-19, pharmacists can improvise their roles in the provision of the right administration of drugs and with appropriate documentation. Moreover, pharmacist involvement in these trials will strengthen the findings and aid to minimize the potential bias. The primary areas in which pharmacists can work include safety and efficacy evaluation, provision of drug or placebo, and follow-up of patients to ensure optimal therapy (49, 50). Being a core member of the antimicrobial stewardship team, pharmacists can facilitate the use of investigational drugs, where the antivirals with established efficacy are being evaluated for prophylaxis and treatment of COVID-19 (53).

Development of Clinical Guidelines and Treatment Algorithms

In the absence of specific treatments for COVID-19, there is a dire need of clinical guidelines and treatment algorithm to manage the patients. Pharmacists, physicians, and other healthcare professionals can work together to develop these guidelines. Since clinical information is rapidly changing and evolving with ongoing research, these guidelines must follow evidence-based practice. Several organizations have provided the stepwise management of patients with COVID-19 (46, 50, 60–63). The primary care consists of symptomatic management and oxygen support (64). Pharmacists can play a vital role in the formulation of dosing regimen and monitoring of safety and efficacy of the drugs (65). The FIP has also formulated a list of medicines being used in COVID-19 to help pharmacists around the globe (50). Hospital pharmacists can prepare dosage guidelines, precautionary notes, and list of potential drug interactions, adverse drug reactions, and contraindications.

Antimicrobial Stewardship Program

Increasing antimicrobial resistance is an important public health problem. Pharmacists play an integral role in the team to implement antimicrobial stewardship programs (ASPs) along with physicians and nurses. The program typically focuses on the use of evidence-based data and monitors the antibiotic consumption and sensitivity patterns in the patient population to highlight and promote rational antibiotic prescribing (66). The pharmacists provide individualized patient treatments, improve therapeutic outcomes, and contribute in the rational prescribing of antibiotics as per antimicrobial stewardship guidelines, ultimately reducing the development of resistance (67). During the COVID-19 pandemic, implementation of ASPs has assisted pharmacists in the formulation of treatment protocols for repurposed antiviral drugs and improving therapeutic interventions for patients (68). ASPs have a potential role as the gatekeeper for appropriate use of COVID-19 drugs in order to optimize patient's selection and to minimize antibiotic misuse. Moreover, ASPs will also assist to curb the potential threats of shortage of repurposed drugs, i.e., hydroxychloroquine, especially for patients with prime indications such as rheumatological disorders. Formulary restrictions and preauthorization through ASPs will ensure proper allocation of medications for patients in high need. Since there is a high concern that antimicrobials may be overused among COVID-19 patients, hospital pharmacists should focus their efforts to establish an effective ASP in collaboration with other healthcare professionals (69).

Hospital Pharmacists' Educational Services

Hospital pharmacists interact with patients and their caregivers at the time of hospital discharge to provide advice on the appropriate use of medications. This service can be utilized to educate patients regarding precautionary measures in order to prevent the acquisition of infection. As family members of COVID-19 patients are at high risk of contracting the infection, their education and awareness carries paramount importance. Hospital pharmacists can continue their clinical services for

chronic patients through telepharmacy in order to avoid the contact of the patients to the hospitals (49). Pharmacists can also provide education to nurses and paramedic staff on the appropriate drug administration, use of protective equipment, and reporting of adverse events. These educational series will not only optimize patient care but will also reduce the burden on clinicians working at the frontline during the pandemic. Hospital pharmacists should ensure that nurses and allied health staff are well-equipped with the necessary knowledge and skills required to combat COVID-19. Pharmacist can educate hospital staff regarding the standard operating procedures to deal with the patient's samples, belongings, and waste material (53). In the setting of the current pandemic, ASPs can be actively involved in educating providers on local COVID-19 treatment protocols, especially if ASPs are involved in developing treatment guidelines. As discussed earlier, education is a core activity of ASPs and can be utilized to increase the awareness among other healthcare professionals regarding the drug toxicities associated with COVID-19 treatments.

Provision of Authentic and Updated Research Data

As most of the physicians and nursing staff are overburdened amid unprecedented cases, pharmacists could act as authenticated and updated source of information. The research on COVID-19 is quite dynamic as numerous new findings are originating every day. As hospital pharmacists are also experts in research interpretation, they can timely disseminate information on the recent advancements in the COVID-19 management and prevention. Moreover, pharmacists can communicate to healthcare providers regarding potential medication-related problems (MRPs) of repurposed drugs in order to ensure the drug safety and optimal therapy (53).

Disinfection and Sterilization Services

Hospital pharmacists are responsible and strictly advised to adhere to the regulations for infection prevention and control in hospitals and medical institutes. Since COVID-19 demonstrated rapid transmission, disinfection, and sterilization services are of utmost importance to contain the virus. Hospital pharmacists along with the hospital's infection prevention team should ensure the disinfection of all surfaces within the hospital premises (49, 50, 53). These safety measures aid to protect both the pharmacy staff and patients from infections (65).

Role of Industrial Pharmacists

The COVID-19 pandemic caused increased demand of drugs, surgical supplies, personal protective equipments (PPEs), and supportive care appliances. With many countries introducing lockdowns and travel restrictions, the global network for manufacturing and delivering medicines is widely affected. Pharmaceutical industries are experiencing major challenges in securing deliveries of medicines, not only for COVID-19 but also for other diseases. In this context, the responsibilities of industrial pharmacists have much increased. The continuous and timely production of key drugs being utilized during the current pandemic is of paramount importance and contributes substantially to alleviate the disease burden (49, 50). Industrial pharmacists are key players during the current battle against the virus and can ensure following tasks.

Member of COVID-19 Research and Development

The worldwide COVID-19 outbreak has highlighted the importance of the timely development of not only vaccines but also broad-spectrum antiviral drugs (70). Currently, most of the pharmaceutical organizations are engaged to sponsor or conduct clinical trials on vaccines and drugs (71). Numerous research experiments and trials are undergoing to accelerate vaccine and drug development (72). Industrial pharmacists should ensure the appropriate and safe use of medications during the clinical trials. Moreover, pharmacists working in industries are also responsible to ensure the ethical aspect and effective resource utilization during clinical research conducted by the industry (49, 50).

Improving Access to Medicines

Pharmacists working in the industries are well aware of the significance of timely distribution of medications to patients, particularly life-saving and essential drugs during the current emergency circumstances (73). Unavailability of medicines creates frustration for everyone including pharmacists, physicians, nurses, and patients. Various factors such as trade restrictions, strict registration, and regulatory compliance may result in drug shortages (52). Industrial pharmacists must be fully prepared and should develop a contingency plan to avoid any sort of shortages. However, various health regulatory agencies have spared and relaxed the manufacturers from the fulfillment of conventional regulations and granted the priority approvals for the repurposed and experimental drugs (73–75). In such circumstances, the responsibilities of pharmacists are enhanced to ensure the standards, quality, and ethics.

Monitoring of Reported Adverse Drug Reactions (ADRs)

Industrial pharmacists are vigilantly working in the pharmacovigilance centers as the qualified person for pharmacovigilance (QPPV). They receive information from the healthcare authorities and other pharmacists dealing with COVID-19 patients about any adverse events of the proprietary drug. Moreover, it is also the fundamental responsibility of the pharmacist to update these events in the National Pharmacovigilance Database. Pharmaceutical organizations producing investigational drugs should ensure safety reporting according to national legal requirements. Industrial pharmacists are also responsible to collect safety data of their drugs by all means including personnel visit, email, phone, or fax. Pharmacists should develop a smooth and convenient reporting tool for the adverse events (76). Moreover, any updated information regarding the safety and efficacy of the drug should be provided to the healthcare professionals in a timely manner. Marketing departments of the pharmaceutical organizations can be an important source of feedback from the healthcare professionals.

Urgency to Comply With Legal Requirements

The impact of COVID-19 on pharmaceutical companies has been unique, as organizations had setup emergency management systems to continue their operations. Moreover, healthcare professionals and the general public are also expecting significant contribution of industries against COVID-19. During these unprecedented times, industrial pharmacists need to adopt proactive and active measures to fulfill the legal requirements of production and supply. Since regulatory authorities in many countries provided legal flexibilities (77), pharmacists should ensure that drug supply should not be disrupted merely due to legal procedures.

Role of Drug Regulatory and Administrative Pharmacists

The responsibilities of drug regulatory and administrative authorities have increased amid the sharp increase in the demand of medications and drug repurposing applications. A quick response from these authorities is of utmost importance to ensure the appropriate use of medications in the current state of unrest. They can ensure that pharmacists performing in community pharmacies, hospitals, clinics, or in industrial sectors are fully equipped and authorized to respond to the COVID-19 emergency plan (43, 78). A rapid increase in drug demand requires vigilant monitoring of supply, procurement, and storage. The regulatory department can ensure appropriate, timely, and necessary availability of the drugs at the points in high demand. Following is the list of domains in which drug regulatory authorities can effectively respond.

Adequate Drug Supply

Since the COVID-19 pandemic continues to put strain on the healthcare system, pharmacists at administrative levels can develop a COVID-19 emergency preparedness plan to mitigate the disruptions in supply which could lead to drug shortages (21). Specific committees can be designed to estimate the quantities of essential patient care medications, equipment, and PPE at varying patient care facilities. Such kind of forecasting and planning would help in conserving medicines, equipment, and supplies before they go black in the market. In addition to the forecasting analysis, quick and smart response is the need of the hour. The administration can ask federal health authorities to safeguard the drug supply which will help in maintaining transparency in the supply chain (79). Moreover, reporting of the causes of drug shortage and its expected duration will aid in institutional planning to manage the alternative sources of drug procurements. Drug administrative authorities should highlight alternative vendors to make sure the availability of life-saving drugs. Priority supplies to tertiary care hospitals particularly those dealing with COVID-19 patients will aid to avoid any drug shortages in these facilities.

Ensuring Good Selling Practices (GSPs)

Pharmacists are well-positioned to reduce risks of further medication shortages arising from COVID-19 by reassuring patients and members of the public of the continued availability of OTC and prescription medications based on rational levels of demand and implementing policies to prevent unnecessary stockpiling (45). Recent reports have indicated the shortage of chloroquine (CQ) and hydroxychloroquine (HCQ) which would create problems for the patients with systematic lupus erythematosus and rheumatological and dermatological disorders (80). The probable causes of shortage are stocking of drugs by patients, hospital administration, drug retailers, and physicians (81). Drug regulatory pharmacists must observe any unusual sale of a particular medicine or equipment in order to make strict oversight against parties involved in price gouging and those taking advantage of the heightened demand for supplies. Similarly, pharmacists at the administrative levels should make sure the pharmaceutical companies and other stakeholders realize their duty and make sure the availability of the drugs to those who need them the most. A combined sensible effort of community pharmacists along with drug regulatory and administrative authorities could definitely help through this time of crisis.

Administrative Actions Against Secondary Wholesalers

In the best interest of patient care, pharmacists and clinicians must be able to access the medication and supplies they need during the current outbreak. Personal protective equipment commonly employed in healthcare facilities are now a scarce commodity. The heightened price of PPE including masks, gloves, respirators, goggles, face shields, and gowns is recorded in the USA and in many other countries around the globe (82). The demand for specific PPE used in response to COVID-19 has increased about 1,000- to 2,000-folds. Moreover, N95 respirators are also experiencing the largest constraints. The procurement of sanitizer has largely been affected both in terms of purchasing and delivery (83). These issues must be addressed in haste to quell the further deterioration of the drug supply system. We suggest the following actions that must be taken at the administration level to mitigate the stock hoarding and price gouging practices during a pandemic:

- Active surveillance to identify the secondary wholesalers involved in stockpiling and price surging activities.
- Increase supply chain security by working with manufacturers.
- Shortage of personal protective equipment (PPE) can be prevented at the federal level by identifying and reporting the agents involve in hoarding and should be dealt with an iron hand (79). Moreover, guidance in making PPE from alternative resources can be provided to pharmacists that can effectively prevent their shortage.
- Monitoring of purchasing trends by the pharmacies and frequent follow-up to ensure the availability of the stock in the premises.
- Administrative authorities should contact reputable manufacturers and supplier to ramp up production and meet the increased demand needs.

Occasional Quality Testing Through Drug Testing Laboratories (DTLs)

Relaxing regulatory requirements can effectively ease the manufacturers to increase the supply of medicine and equipment needed during the current pandemic. Considering the occasional

testing of medications through drug testing laboratories will shorten the time span of drug availability in the market. Such sort of regulatory relaxations will be beneficial for alleviating the shortage of medicines which are being used in supportive care of COVID-19 (79). However, additional caution must be practiced for the drugs for which provisional quality approval is granted. It must be noted that relaxation of regulatory conditions should not compromise the quality of production and supply.

Mandatory Actions Against Unregistered Drugs, Sanitizers, and Disinfectants

As part of the surveillance team and contingency planning, administration can stem out the false and misleading claims about products purporting to treat or prevent COVID-19 (79, 84). Drug inspectors and drug monitoring teams should be authorized to seize and impede the sale of any unregistered drug and report bad actors. Similarly, shortage of hand sanitizers and disinfectant can also be dealt in same way to stop their hoarding and stockpiling (79). Meanwhile, proper training can be provided to all pharmacists working in any sector for small-scale manufacturing of sanitizers and disinfectants from alternative sources.

Ease Operational Barriers

Easing operational barriers for pharmacists working in any health facility will help in their effective engagement in COVID-19 response (85). Drug administrative authorities can allow a grace period in the renewal of licensures during emergency period. They should also waive some restrictions of good manufacturing practices thus making sure the timely availability of the medicines.

INTERPROFESSIONAL COLLABORATION IN THE HEALTHCARE SYSTEM

It is important to recognize the pharmacists' engagements through interprofessional collaborations, which involves pharmacists and other healthcare professionals from various

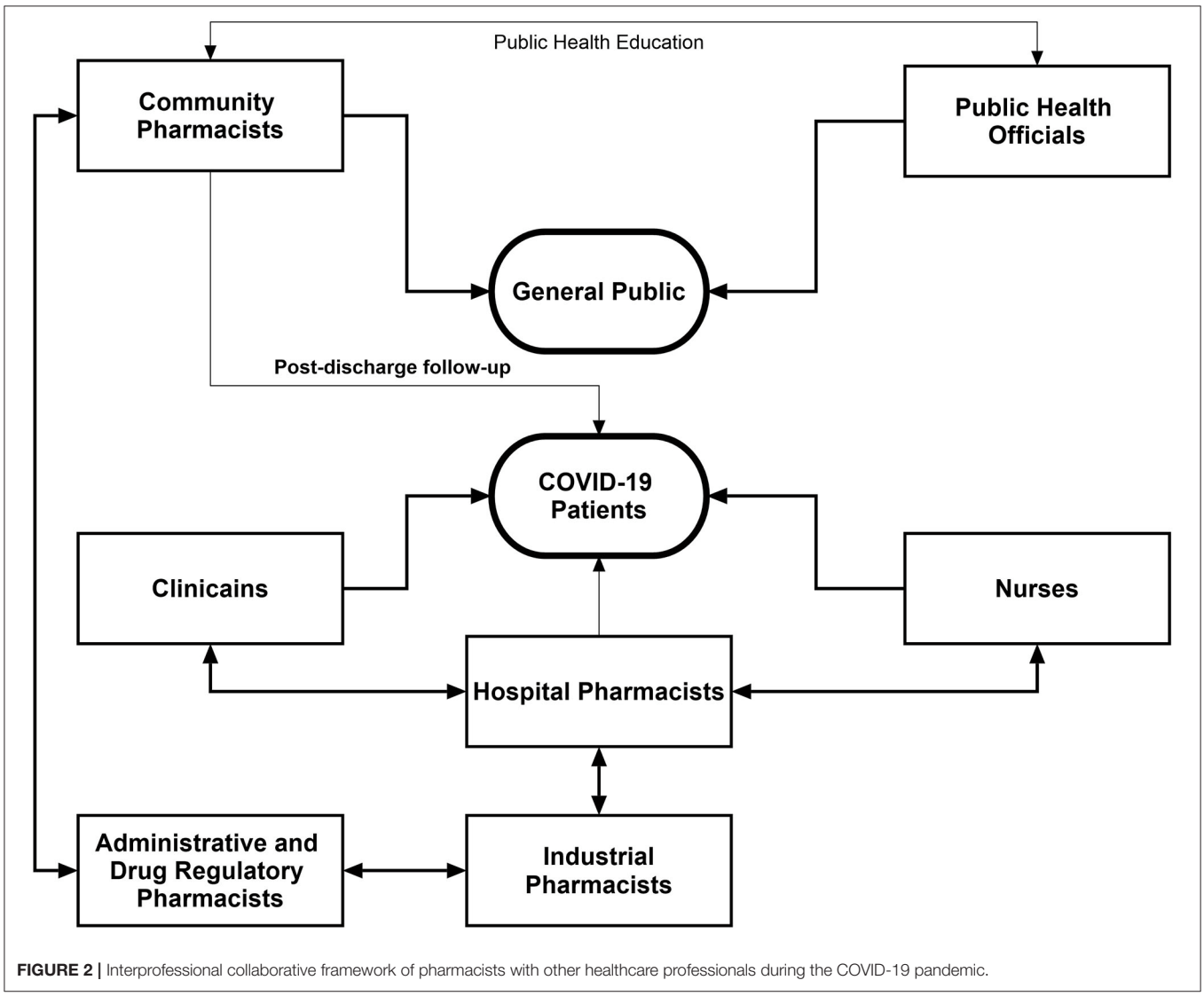

FIGURE 2 | Interprofessional collaborative framework of pharmacists with other healthcare professionals during the COVID-19 pandemic.

disciplines working together with shared goals, mutual interest, respect, and understanding about each other's roles, along with the acceptance that patients are team members (86). It is pertinent to mention that there is a substantial relationship between the extent of interprofessional collaboration and patient safety (87). We believe that the battle against COVID-19 can only be won through collaborative maneuvers. **Figure 2** describes the summary of the collaborative framework of different healthcare professionals to combat the pandemic. Community pharmacists can assist drug regulatory authorities in identifying the unusual practices such as irrational use, stocking, and misconception among the general public related to the repurposed drugs. Keeping in view the easy accessibility of community pharmacists, they can work with public health officials to ensure that the population is complying with the preventive measures and to neutralize the myths related to COVID-19. The public health department should consider the potential of pharmacists in this regard.

Community pharmacists along with clinicians can effectively arrange the postdischarge follow-up of COVID-19 patients which could provide greater insight into the recovery pattern of such patients. Hospital pharmacists can optimize patient outcomes through working collaboratively within multidisciplinary teams to achieve the responsible use of medicines. In addition, hospital pharmacists should aid overburdened clinicians and nurses through medication reconciliation in order to minimize DRPs. Furthermore, integrated maneuvers of hospital and industrial pharmacists can assist to solve the issues related to drug shortage and supply. A vigilant contact of industrial pharmacists with hospital administration will be of paramount importance to ensure the adequate and continuous supply of medications. During the current health crisis, drug and health regulatory authorities hold greater responsibilities as the pandemic is accompanied by various malpractices on drug use and disease management. Drug regulatory pharmacists should ensure adequate production of drugs in the industry, good selling practices, rational use of drugs, and quality and standards of medication supply and use. These activities can be effectively accomplished through intra- and interprofessional collaboration of drug regulatory pharmacists with other pharmacists and healthcare professionals. The integration of pharmacists into core healthcare teams during the current pandemic would facilitate positive patient outcomes, better team decision-making around drug therapy, improved continuity of care, and improved patient safety.

CONCLUSIVE REMARKS

During the current crisis, innovative and adaptive methods of practicing will be required across all health professions. The roles and activities underscored in the current manuscript are not exhaustive but serve to illustrate a range of areas in which pharmacists at different levels could make substantial contributions. These are currently implemented to varying extents across different countries. The activities of pharmacists during the COVID-19 pandemic widely differ from those have been described in previous pandemics and outbreaks during which pharmacists were primarily engaged in vaccination and disease education. This pandemic put more responsibilities on pharmacists due to disease novelty, its rapid transmission, associated morbidity and mortality, massive infodemic, misleading narratives, lack of vaccine or specific drugs, lack of treatment guidelines, and overwhelmed healthcare system.

Pharmacists can give full play to their professional expertise; analyze the current situation rationally; test, treat, and immunize; formulate telehealth policies expeditiously; and guarantee medication safety and rational use of drugs (43, 85, 88). In restructuring existing health services to respond to the current public health crisis, it is important that governments, public health bodies, and policy makers review existing services and make full use of any unrealized potential among pharmacists working in various sectors. In short, pharmacists could readily play a role in ramping up COVID-19 testing and treatment and, eventually, when available, providing the vaccine. Relaxing state phlebotomy laws could yield additional benefits, as drawing blood may be necessary in efforts to search for antibodies for COVID-19. Any restrictions on the ability of pharmacists to immunize using FDA-approved vaccines should also be reconsidered.

Since the impact of traditional and extended pharmacy services is not evaluated during the pandemic, well-structured and controlled studies are needed in this regard. Moreover, the extent of preparedness among pharmacists for any future outbreak is required to be ascertained. This review also identifies the poor coordination and collaboration of pharmacists working in different sectors with other frontline healthcare professionals dealing with COVID-19. These shortcomings should be considered while designing future research and implementing health policies for infectious diseases.

AUTHOR CONTRIBUTIONS

TM, YK, and NA: conception or design of the work. AAl, AL, and AAb: analysis or interpretation of studies for the work. TM, AL, AAb, and NT: drafted the work. NA, YK, AAl, and NT: revised the manuscript critically for important intellectual content. TM, YK, AL, and AAb: provided approval for publication of the content. All authors contributed to the article and approved the submitted version.

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frontiers in Immunology

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Early Phases of COVID-19 Are Characterized by a Reduction in Lymphocyte Populations and the Presence of Atypical Monocytes

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Background: Severe acute respiratory syndrome coronavirus 2 is a recently discovered pathogen responsible of coronavirus disease 2019 (COVID-19). The immunological changes associated with this infection are largely unknown.

Methods: We evaluated the peripheral blood mononuclear cells profile of 63 patients with COVID-19 at diagnosis. We also assessed the presence of association with inflammatory biomarkers and the 28-day mortality.

Results: Lymphocytopenia was present in 51 of 63 (80.9%) patients, with a median value of 720 lymphocytes/μl (IQR 520-1,135). This reduction was mirrored also on CD8+ (128 cells/μl, IQR 55-215), natural killer (67 cells/μl, IQR 35–158) and natural killer T (31 cells/μl, IQR 11–78) cells. Monocytes were preserved in total number but displayed among them a subpopulation with a higher forward and side scatter properties, composed mainly of cells with a reduced expression of both CD14 and HLA-DR. Patients who died in the 28 days from admission (N=10, 15.9%), when compared to those who did not, displayed lower mean values of CD3+ (337.4 cells/μl vs 585.9 cells/μl; p=0.028) and CD4+ cells (232.2 cells/μl vs 381.1 cells/μl; p=0.042) and an higher percentage of CD8+/CD38+/HLA-DR+ lymphocytes (13.5% vs 7.6%; p=0.026).

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Discussion: The early phases of COVID-19 are characterized by lymphocytopenia, predominance of Th2-like lymphocytes and monocytes with altered immune profile, which include atypical mononuclear cells.

Keywords: COVID-19, SARS-CoV-2, peripheral blood mononuclear cells, immune profiling, inflammation, monocytes

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a new beta coronavirus identified in China in December 2019 which is responsible of coronavirus disease 2019 (COVID-19) (1). The virus rapidly spread worldwide, and it is responsible of a pandemic which is exerting a tremendous pressure on national health systems (2).

Given the novelty of this virus, how the immune system deals with it is largely unknown. Preliminary studies from China highlighted a reduction of lymphocytes count, particularly of CD4+ T and CD8+ T cells, an increase of the neutrophils-to-lymphocytes ratio (NLR), a concomitant decrease in interferon gamma (IFN-γ) production and an association with elevated inflammatory markers (3–5). An excessive pro-inflammatory cytokines release profile was confirmed also by a transcriptomic study performed on peripheral blood mononuclear cells (PBMC) (6). Also innate immunity cells are involved. Indeed, Zheng and colleagues demonstrated an upregulation of the inhibitory receptor NKG2A expression on natural killer cells (NK) and cytotoxic lymphocytes (CTLs) in COVID-19 patients with a compromised degranulation capacity and a reduced production of IFN-γ, IL-2, granzyme B and tumor necrosis factor α (TNF-α) (7). Finally, preliminary unpublished reports described the presence of peripheral blood monocytes with morphologic and phenotypic changes displaying macrophage markers. The severity of the alterations occurring to these atypical monocytes was correlated with patient outcome (8).

A better knowledge of the immune response against the virus is mandatory, since it has been postulated that severe forms of the disease are associated with a cytokine storm, where monocytes/macrophages play a central role (9–12). As a consequence, several trials evaluating the efficacy of immunosuppressive or immunomodulatory drugs (tocilizumab, anakinra, sarilumab, eculizumab, ruxolitinib, fingolimod, emapalumab, tofacitinib, meplazumab) are currently undergoing worldwide (13).

In attempt to provide our contribution to the field, we designed the present study describing PBMC's characteristics and basic inflammatory markers values in a cohort of Italian patients who were diagnosed of SARS-CoV-2 infection and were hospitalized.

MATERIALS AND METHODS

Study Population

We enrolled patients consecutively admitted to the Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, a University Hospital in Milano, Italy, diagnosed with COVID-19 in the period from March 17 to April 3, 2020. Diagnosis of COVID-19 was defined by the presence of compatible signs/symptoms (fever, cough, coryza, myalgia, diarrhoea, dyspnoea, tachypnoea, ageusia, anosmia) and a positive nasopharyngeal swab for SARS-CoV-2 detected through real-time reverse transcription-polymerase chain reaction (rRT-PCR). Serum levels of inflammatory biomarkers were assessed as standard clinical practice. Flow cytometry analysis was performed on fresh blood left-over of samples collected at hospital admission for clinical purposes. We analyzed only blood samples collected in the first 48 h since diagnosis. Samples of fresh blood left-over from local blood donor bank were also employed as healthy controls. The study was approved by the Institutional Review Board Milano Area 2 (#358_2020) and was conducted in accordance with the Helsinki Declaration.

Inflammatory Biomarkers Assessment and Clinical Data Collection

Procalcitonin (PCT), ferritin and interleukin-6 (IL-6) were measured with electro-chemiluminescent immunoassays (ECLIA) on a Roche Cobas e801 instrument (Roche Diagnostics, Monza, Italy). C-reactive protein (CRP) was measured with an immunoturbidimetric method and lactate dehydrogenase (LDH) with the International Federation of Clinical Chemistry (IFCC) optimized method on a Roche Cobas c702 instrument (Roche Diagnostics, Monza, Italy).

For each patient, the P/F ratio [partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2)], was calculated at the admission to the hospital. The 28-day mortality was collected from electronic medical records.

SARS-CoV-2 Detection

Two different methods were used for viral detection. The first one consisted in Seegene Inc reagents (Seoul, Korea), RNA extraction with STARMag Universal Cartridge kit on Nimbus instrument (Hamilton, Agrate Brianza, Italy) and amplification with Allplex® 2019-nCoV assay, while the second employed a GeneFinder® COVID-19 Plus RealAmp Kit (OSANG Healthcare, Anyangcheondong-ro, Dongan-gu, Anyang-si, Gyeonggi-do, Korea) on ELITech InGenius® instrument (Torino, Italy). Both assays identify the virus by multiplex rRT-PCR targeting three viral genes (E, RdRP and N).

Flow Cytometry Analysis

Fresh peripheral blood samples were processed within 24 h from collection for the following evaluations: i) classical lymphocyte subpopulations count, ii) CD4+ T-cells polarization (14–16), iii) lymphocytes activation status, iv) monocyte subpopulations, and v) NK-cell subsets. For this purpose, we employed the

monoclonal antibodies (BD Biosciences, San Jose, CA) reported in **Supplementary Table 1**. **Supplementary Figures 1 to 5** show the surface markers and gating strategy employed to identify different immune cells. Each antibody was titrated individually and the optimal dilution for a given staining of 100 µl volume of whole blood was determined by comparison with the isotype matched controls. After the cell staining, erythrocytes were lysed with lysis buffer (Pharm Lyse, BD Biosciences). Following the washing, samples were acquired using a BD FACSLyric flow cytometer equipped with three lasers: a 405 nm violet laser, a 488 nm blue laser, and a 647 nm red laser. For each tube we set a stopping gate criterion of 50,000 events in the lymphocyte gate, forward scatter (FSC) versus side scatter (SSC), and the data were analysed using FACSSuite and FlowJo softwares (BD Biosciences). An automatic standard compensation was applied for each acquisition. Internal quality assurance procedures included BD cytometer setup and tracking beads, according to the manufacturer's instructions.

Statistical Analysis

Descriptive statistics were performed for all the variables assessed in the study population. The Spearman test and linear regression were used to examine correlations. The Friedmann test followed by Dunn's correction for multiple comparisons and multiple t test followed by Holm-Sidak correction for multiple comparisons were employed to assess differences among groups. Parametric tests were employed for continuous variables, whereas non-parametric test for those not-normally distributed (Kolmogorov-Smirnov test). A p value <0.05 was deemed statistically significant. All the analysis was performed with GraphPad Prism 8 (GraphPad Inc, USA).

RESULTS

Demographic and Clinical Characteristics and Biochemical Values

Sixty-three patients with confirmed SARS-CoV-2 infection were enrolled, on average 5.3 days (SD 2.9) after symptoms onset. The median P/F ratio at admission was 170 mmHg. Hypoxemia was severe (P/F < 100 mmHg) in 13 patients (20.6%), moderate (P/F 100–200 mmHg) in 13 (20.6%) and mild (P/F 200–300 mmHg) in 23 (36.5%), while the remaining 14 patients had a P/F > 300 mmHg. Overall, the 28-day mortality rate was 15.9% (10/63). Demographic and clinical characteristics and inflammatory biomarker values are shown in **Table 1**. Overall, all the inflammatory markers considered were above the reference intervals.

White Blood Cells Total Count and Subpopulations

At diagnosis of SARS-CoV-2 infection, median values of white blood cell (WBC), monocyte and neutrophil were within the reference intervals employed in our Centre. Instead, median lymphocyte count was below the lower reference limit. Overall, lymphocytopenia was found in 51 of the 63 patients (80.9%). Consequently, the NLR was oriented toward high values, well above the reference intervals of 0.78–3.53 (**Table 2**) (17).

Lymphocyte Subpopulations

Lymphocyte subpopulations mirrored the general feature of lymphocytopenia. Median values of CD3+ and CD8+ lymphocyte counts were below the lower reference limit (**Table 2**). Likewise, the two groups of cytotoxic lymphocytes (CTLs) belonging to the innate immunity, NK (CD3-CD16+CD56+) and NKT (CD3+CD16+CD56+) cells, were below the lower reference limits (**Table 2**). Regarding the frequencies of NK subsets, we did not detect any statistical difference of $CD56^{bright}CD16-$ (immature) and $CD56^{dim}CD16+$ (mature) NK cells among total lymphocytes between patients and healthy donors (CTR) (**Figure 1A**).

Concerning T lymphocytes activation, assessed through the expression of CD38 and HLA-DR on CD4+ (**Figure 1B**) and CD8+ cells (**Figure 1C**), we found a significant increase of activated CTLs (6.8% IQR 3.9–10.1 vs 2.9% IQR 1.5–5.7) in COVID patients.

Figures 1D, E show CD4+ cell polarization The relative median frequencies of Th1-like (CXCR3+CCR6-), Th2-like (CXCR3-CCR6-), Th17-like (CXCR3-CCR6+), and Treg (CD25++CD127low) lymphocytes were 13.1% (IQR 10.8–17.6), 27.6% (IQR 21.7–36.7), 7.2% (IQR 3.6–12), and 5.1% (IQR 3.6–6.8), respectively. Reference values for the same cell populations were 21.8% (IQR 20–24), 21.6% (IQR 19–25), 11.6% (IQR 9–15),

TABLE 1 | Demographic and clinical characteristics and inflammatory biomarker levels of 63 patients with COVID-19 at diagnosis.

Variable	Value	Reference values	
Men, n (%)	48 (76.2)		
Age, mean (SD)	59.1 (13.7)		
Days since symptoms appearance, mean (SD)	5.3 (2.9)		
P/F ratio (mmHg)	170 (107–290)	>300	*
CRP (mg/dl)	11.95 (7.43–19.02)	<0.5	*
Ferritin (µg/L)	1,503 (724–2,887)	30–400	*
LDH (U/L)	335 (273–424)	135–225	*
IL-6 (ng/L)	81.95 (28.9–112.8)	0–10	*
PCT (µg/L)	0.33 (0.16–1.46)	0.02–0.06	*
Fibrinogen (mg/dl)	571 (470–722)	165–300	*
D-dimer (µg/L)	1,479 (678–2,529)	<500	*
ALT (U/L)	41 (28–69)	9–59	

*All data are reported as median with interquartile range (IQR) except when stated otherwise. *Values outside of normality range. [P/F ratio, partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2); CRP, C-reactive protein; LDH, lactate dehydrogenase; IL-6, interleukin 6; PCT, procalcitonin; ALT, alanine aminotransferase].*

TABLE 2 | White blood cell count, leukocyte subpopulations and reference values employed in our center.

Variable	Value	Reference values	
White blood cells (cells/μl)	6,520 (5,015–9,200)	4,800–10,800	
Monocytes (cells/μl)	330 (195–510)	300–600	
Lymphocytes (cells/μl)	720 (520–1,135)	1,200–3,400	*
Neutrophils (cells/μl)	5,070 (3,785–8,105)	1,500–6,500	
Neutrophils/Lymphocytes ratio	7.5 (4.39–14.02)	0.78–3.53	*
CD3+ lymphocytes (cells/μl)	500 (273–728)	700–2,100	*
CD4+ lymphocytes (cells/μl)	342 (192–451)	300–1,400	
CD8+ lymphocytes (cells/μl)	128 (55–215)	200–900	*
CD4+/CD8+ ratio	2.93 (1.9–3.7)	1–3.6	
CD19+ lymphocytes (cells/μl)	120 (54–182)	100–500	
Natural killer cells (cells/μl)	67 (35–182)	90–600	*
Natural killer T cells (cells/μl)	31 (11–73)	143 (median value)	*
Platelets (10^3 cells/μl)	247 (162–318)	130–400	

*All data are reported as median with interquartile range (IQR). * Values outside reference intervals.*

6.2% (IQR 5–7) of CD4+ cells for Th1-like, Th2-like, Th17-like, and Treg lymphocytes, respectively. COVID-19 patients showed a statistically significant reduction in percentage of Th1-like and Th17-like cells. The Th1/Th2 ratio (0.47, IQR 0.36–0.65 vs. 1.0, IQR 0.9–1.1) was altered, whereas the Treg/Th17 ratio was not influenced (0.62, IQR 0.5–1.2 vs 0.8, IQR 0.5–0.9).

Monocytes

The median frequencies of monocyte subpopulations are displayed in **Figure 1F**. Overall, relative subpopulations were 3.3% (IQR 1.66–5.7), 3.7% (IQR 0.8–8.7), and 77.2% (IQR 70.28–85.9) for non-classical ($CD14^{dim}$CD16+), intermediate (CD14+CD16+), and classical monocytes (CD14+CD16-), respectively. Reference intervals for the above monocyte subpopulations were 7.7% (IQR 5–10), 5.3% (IQR 4–7), and 82.6% (IQR 80–84) for non-classical, intermediate and classical monocytes, respectively. Finally, the median percentage of HLA-DR+ monocytes was 83.4% (IQR 75.2–92.3), below our internal control reference value of 96% (IQR 94–98).

Morphological identification of monocytes in COVID-19 patients became challenging due to altered scatter properties. Furthermore, we noticed distinct $CD4^{dim}$ cell population with high FSC and SSC (**Figure 2A**). Blood smear examination confirmed the presence of atypical monocytes with vacuoles in their cytoplasm (**Figure 2B**). Therefore, we modified flow cytometer parameters with reduced photomultiplier tube values in order to analyze better these peculiar monocytes (high-SC). We performed this analysis in a subgroup of 14 patients and we considered significant a value of high-SC > 2% of total monocytes. Overall, the high-SC population was present in 11/14 (79%) patients where they represented a variable fraction of total monocytes (range 2%–14%).

Moreover, to better understand the differences in the phenotypic signature between normal and high-SC monocytes in COVID-19 patients and in healthy donors (n=10), we compared the Median Fluorescence Intensity (MFI), a parameter proportional to antigen density, of CD14, CD16, and HLA-DR in these monocyte populations. Overall, the MFI of CD14 and HLA-DR on high-SC monocytes was lower than those of healthy donors ($p<0.05$). Regarding HLA-DR, we found a significant reduction on COVID-19 patients' monocytes with normal scatter properties ($p<0.05$). For a better characterization of these atypical cells, we compared their CD14 and HLA-DR expression with normal monocytes and granulocytes of the same patient. As shown in **Supplementary Figure 7**, high-SC monocytes expressed similar marker profile of non-classical and classical monocytes.

Finally, we observed a rising trend of CD16 MFI, although not significant, in high-SC monocytes compared to normal monocytes from COVID-19 patients and healthy donors (**Figure 2C**).

Correlations With Inflammatory Biomarkers

We also assessed the relationship between the different variables evaluated in our cell populations and inflammatory biomarkers. Interestingly, the IL-6 values of and the monocyte counts were not correlated (R=0.01; $p=0.60$). (**Supplementary Figure 6**). Also, all other variables did not show any significant correlation.

Variables Associated With Death

Finally, we stratified all the results according to 28-day mortality. Patients who died within 28 days from admission had higher age, LDH values, CD4/CD8 ratio and percentage of CD38+/HLA-DR + CTLs compared to those alive (**Table 3**). Instead, they had lower P/F ratio, CD3+ and CD4+ lymphocyte counts. All other variables did not show any significant difference.

DISCUSSION

In our cohort of patients hospitalized for COVID-19, we observed a reduction of circulating lymphocytes, both in terms of total count and specific subpopulations. Precisely, all cytotoxic lymphocytes (CTLs, NK, and NKT cells) were below the lower reference limits. Moreover, activated CD8+ T cells were increased, whereas CD4+ lymphocytes were polarized toward a Th2-like phenotype. Intriguingly, monocytes, although not modified in cell number and distribution, displayed morphological and phenotypical alterations. IL-6 levels were not correlated to monocyte counts. Finally, patients who died within 28 days from admission presented lower counts of CD3+ and CD4+ cells and a higher percentage of activated CTLs.

Our results are in line with those recently reported by several groups regarding a profound reduction of lymphocytes in the

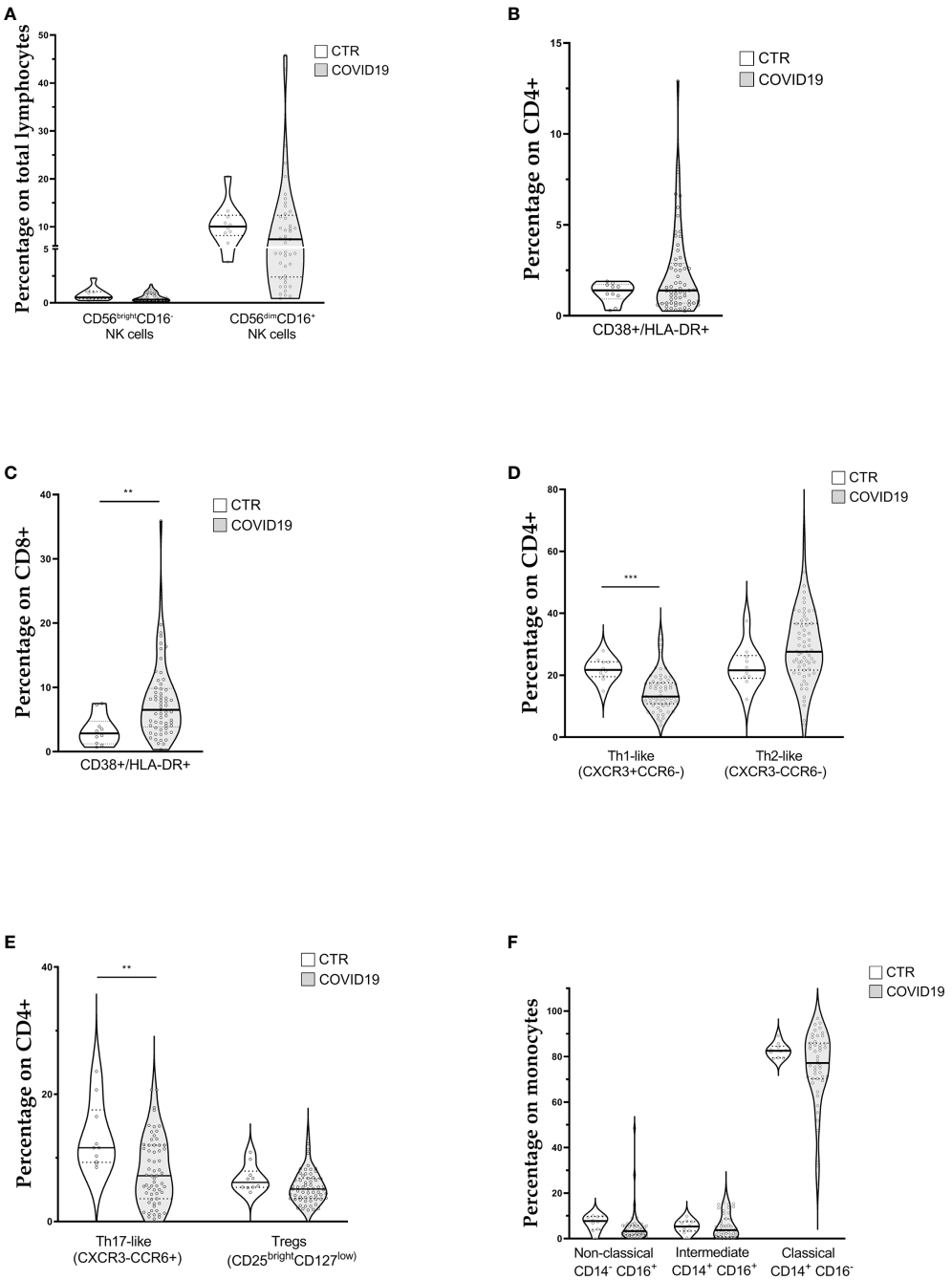

FIGURE 1 | Violin plots showing lymphocyte and monocyte subpopulations (reported as median value) assessed in 63 patients with COVID-19 at diagnosis and 10 controls (CTR). **(A)** Natural killer cell subpopulations **(B)** CD4+ cells activation; **(C)** CD8+ cells activation **(D, E)** CD4+ cells polarization; **(F)** Monocytes subpopulations. [Th1-like (CXCR3+CCR6-): Type 1 T helper-like cells; Th2-like (CXCR3-CCR6-): Type 2 T helper-like cells; Th17-like (CXCR3-CCR6+): T helper 17-like cells; Tregs: regulatory T cells; NK: natural killer cells]. t-test was used to calculate statistical difference. (** p value <0.01; *** p value <0.005).

context of a preserved total WBC count (4, 10, 18). Of note, in our cohort lymphocytopenia was present in 81% of the enrolled patients, a higher percentage compared to those observed in the works by Chen (72% of severe cases, 10% of moderate cases) and by Huang (63% of all cases) (4, 19). The reduction of cytotoxic lymphocytes, both of innate and adaptive immunity, is a relevant finding. Indeed, in the context of several rheumatologic diseases, cytolytic cells may induce apoptosis in activated macrophages and T cells controlling the inflammatory response (20). An impairment in the cytolytic compartment may result in a overstimulation of the immune system leading to the multiorgan failure and this defect has been linked to elevated levels of IL-6 (21).

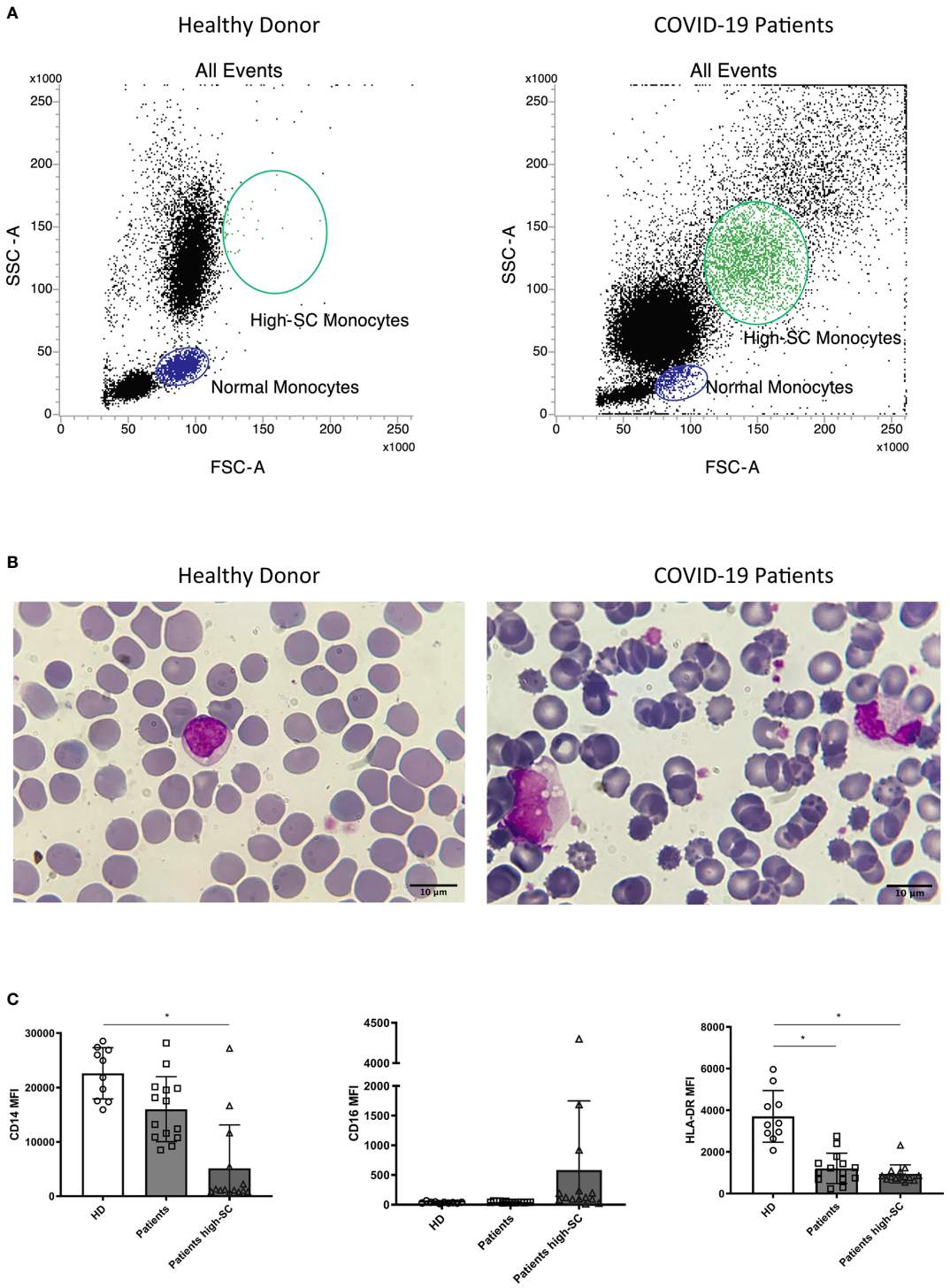

FIGURE 2 | Morphologic and phenotypic differences between monocytes from a healthy donor (left) and a COVID-19 patient (right): **(A)** flow cytometry dot plot showing normal and high-SC monocytes; **(B)** monocytes from May-Grunwald Giemsa stained blood smears (100X magnification, Leica Microscope DMLS); **(C)** histograms showing median fluorescence intensity (MFI) of CD14, CD16, and HLA-DR on monocytes from healthy donors (HD, n=10), normal and atypical monocytes from COVID-19 patients (n=14). Non parametric Kruskal Wallis test was used to determine significant difference. Kolmogorov-Smirnov test was used to assess normal distribution of samples. (* p value <0.005).

TABLE 3 | Variables with significant differences between those who died in the 28 days from admission and those who did not.

	p value	Mean dead	Mean alive	Difference	SE of difference	t ratio
Age (years)	0.002	71.3 (n=10)	56.8 (n=53)	14.5	4.4	3.3
LDH (U/L)	0.024	484.2 (n=10)	338.2 (n=20)	145.9	61.2	2.4
P/F admission	0.043	138.1 (n=10)	204.6 (n=53)	-66.5	32.2	2.1
CD3+ (cells/µl)	0.028	337.4 (n=10)	585.9 (n=49)	-248.6	110.5	2.2
CD4+ (cells/µl)	0.042	232.3 (n=10)	381.1 (n=49)	-148.8	71.6	2.1
CD4/CD8 ratio	0.015	5.4 (n=10)	3.0 (n=49)	2.4	0.9	2.5
CD38+/HLA-DR+ on CD8+ cells (%)	0.026	13.5 (n=10)	7.6 (n=52)	5.8	2.5	2.3

[LDH, lactate dehydrogenase; P/F, partial pressure of oxygen [PaO_2]/fraction of inspired oxygen (FiO_2)] Multiple t-test with Holm-Sidak correction for multiple comparisons.

Regarding monocytes, Zhang and Zhou reported in their studies (22, 23) a relative increase in intermediate and non-classical monocytes. Instead, we detected normal value of classical and non-classical monocytes and a slightly reduction of intermediate monocytes. It should be underlined that our analysis was performed in samples collected early in the course of the disease, while it is unclear when the samples were collected in the studies conducted in China. Similarly to these studies, a fraction of monocytes observed in our cohort showed not only an increase in cell size but also in cell complexity, probably due to the presence of vacuoles in cytoplasm. We also noted an evident decrease of cell complexity in granulocyte population, that requires further investigation. These high-SC monocytes displayed a decrease of CD14 and HLA-DR antigen density. Notably, we also detected a reduced expression of HLA-DR, either as percentage or fluorescence intensity, on patients' monocytes with normal scatter properties. The same observation was made by Kuri-Cervantes et al. and by Giamarellos-Bourboulis et al. (10, 24). The latter also demonstrated an inverse correlation between HLA-DR molecules and serum levels of IL-6 (10). This dysregulated monocyte immune profile could be also related to weakened SARS-CoV-2 antigen presentation and defective crosstalk with T lymphocytes (25). It will be interesting to follow this population of atypical monocytes during the course of the disease.

Monocytes, considered the crucial players in the pathogenesis of lung damage caused by the cytokines storm, showed substantial phenotypical alterations already in the early phase of the disease. The mechanism leading to monocytes alteration is unclear, but a direct viral role can be suspected. Indeed, Zhang et al (22) showed how monocytes express ACE2, the entry receptor of SARS-CoV-2, and it has been reported how other viral infections (influenza A virus, vaccinia virus, vesicular stomatitis virus) can trigger rapid and substantial differentiation of monocyte profile toward dendritic cells (25).

Concerning lymphocyte compartment, Zhou and colleagues observed that CD4+ T lymphocytes were rapidly activated to become pathogenic Th1 cells, secreting proinflammatory cytokines. The resulting environment induced inflammatory CD14+CD16+ monocytes with high expression of IL-6 (26). In contrast, in our study the T helper ratio was oriented toward a Th2-like polarization. This could be due to IL-6 effect on the immune response towards Th2, by promoting early IL-4 secretion (27), even though in our cohort we did not detect any correlation between IL-6 values and Th-2 like cells. Likewise, Giamarellos-Bourboulis et al. did not detect an immune response oriented toward a Th1 phenotype in their cohort, with IFNγ values below the detection limit in all the patients (10). We acknowledge that in addition to the surface markers we have considered, employment of CCR4 would have helped better identify the Th2-enriched subset.

Finally, we observed some peculiar results stratifying the variables according to 28-day mortality. Indeed, in addition to the clinical variables which are expected to be linked with the disease outcome (age, P/F ratio, LDH), we observed lymphocytopenia and CTLs activation status more evident in those patients who died. It is hard with the current knowledges to understand the significance of these data. Both lymphocytopenia and lymphocyte activation could be the physiologic consequence of an ongoing viral infection, particularly evident in those with a severe outcome. However, it could be interesting to assess whether lymphocytes of these patients are able to control activated innate immunity and modulate inflammatory response. Indeed, in mice, Kim et al. demonstrated that after a viral infection, an unleashed innate immune response, due to the absence of residential T cells, can also be a direct cause of death (28).

It should be noted that our samples were collected at diagnosis, on average of 5 days from the appearance of symptoms. Considering that the mean incubation time of COVID-19 has been estimated to be 5 days, we can assume that our patients were on average of the 10th day after infection. For this reason, our results could be considered a faithful description of the immunological changes occurring in the early phases of the disease and could complement well the data provided by others. At the same time, we acknowledge that our research has limitations. First, it is a single center study, conducted on a small number of patients. Second, it involved only patients diagnosed in hospital, therefore with a severe disease. Third, we did not investigate the presence and the features of immune cells at alveolar level, where most of COVID-19's immunopathology occurs.

In conclusion, we present here the first description of immunologic features at diagnosis of a cohort of Italian COVID-19 patients that both confirm already available evidence and add novel elements to the COVID-19 jigsaw. Our data might suggest that, in the early phases of COVID-19, the virus can elicit an inflammatory response leading to a reduction in the number of both cytotoxic lymphocytes (CTL, NK cells, NKT cells) and helper lymphocytes. These changes, which are more pronounced in patients with adverse outcome, could represent the first steps in the cascade leading to the uncontrolled activation of monocytes in the context of cytokines storm. It is possible to speculate that an

early initiation of an immunomodulatory treatment might abrogate the immune system alterations leading to monocytes activation-dysfunction and to the consequent lung damage. Nevertheless, further prospective studies are needed to provide a precise picture of the immunologic profile during the course of the disease in order to define better therapeutic approaches and to improve the clinical management of COVID-19 patients.

"COVID-19 NETWORK" WORKING GROUP

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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 Comitato Etico Milano Area 2, Fondazione IRCCS Ca' Granda, Francesco Sforza 28, 20122 Milano. The patients/participants provided their written informed consent to participate in this study.

AUTHORS CONTRIBUTIONS

AL, LP, AB, and AG conceived the study. VC, EP, GL, GC, VS, GG, RG, and MM enrolled the patients. LP, AC, ET, MT, and FC performed the immunologic and biochemical analysis. AL, LP, and AB analyzed the data. AL and LP wrote the first draft. 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/fimmu.2020.560330/full#supplementary-material

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Improving Detection Efficiency of SARS-CoV-2 Nucleic Acid Testing

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Background: SARS-CoV-2 nucleic acid testing (NAT) has been routinely used for COVID-19 diagnosis during this pandemic; however, there have been concerns about its high false negative rate. We dissected its detection efficiency with a large COVID-19 cohort study.

Methods: We analyzed SARS-CoV-2 NAT positive rates of 4,275 specimens from 532 COVID-19 patients in Sichuan Province with different disease severities, statuses, and stages, as well as different types and numbers of specimens.

Results: The total positive rate of the 4,275 specimens was 37.5%. Among seven specimen types, BALF generated a 77.8% positive rate, followed by URT specimens (38.5%), sputum (39.8%), and feces/rectal swabs (34.1%). Specimens from critical cases generated a 43.4% positive rate, which was significantly higher than that of other severities. With specimens from patients at stable status, the SARS-CoV-2 positive rate was 40.6%, which was significantly higher than that of improved status (17.1%), but lower than that of aggravated status (61.5%). Notably, the positive rate of specimens from COVID-19 patients varied significantly from 85 to 95% during 3 days before and after symptom onset, to 20% at around 18 days after symptom onset. In addition, the detection rate increased from 72.1% after testing one throat swab, to 93.2% after testing three consecutive respiratory specimens from each patient.

Conclusions: SARS-CoV-2 NAT detection rates vary with patient disease severity and status, specimen type, number of specimens, and especially disease progression. Sampling as close to symptom onset as possible, and consecutively collecting more than one respiratory specimen could effectively improve SARS-CoV-2 NAT detection efficiency.

Keywords: SARS-CoV-2, COVID-19, nucleic acid testing, RT-PCR, detection rate, positive rate

HIGHLIGHTS

As SARS-CoV-2 NAT detection rates vary from 85 to 95% within 3 days before and after symptom onset, to 20% at 18 days after symptom onset, sampling as close to symptom onset as possible could effectively improve its detection efficiency.

INTRODUCTION

In late December 2019, an outbreak of coronavirus disease (COVID-19) caused by a novel coronavirus SARS-CoV-2, emerged in Wuhan, China and spread rapidly across China and further around the world (Chen et al., 2020; Huang et al., 2020; Zhu et al., 2020). As of May 1, 2020, there have been more than 3.18 million confirmed cases of COVID-19 with over 224 thousand deaths globally (Coronavirus disease (COVID-19) Situation Report, 2020). Accurate and rapid diagnosis of COVID-19 is crucial for disease treatment and transfection control. As the initial clinical manifestations of COVID-19 are non-specific, it is difficult to distinguish SARS-CoV-2 infection from other respiratory infections based only on clinical symptoms and signs or computed tomography (CT) imaging (Chen et al., 2020; Guan et al., 2020; Huang et al., 2020). SARS-CoV-2 nucleic acid testing (NAT), specifically real-time Reverse Transcription-Polymerase Chain Reaction (RT-qPCR) assay, as a confirmation test for COVID-19 diagnosis, has played a pivotal role in disease diagnosis, monitoring, surveillance, infection control, and prevention during the pandemic (Chu et al., 2020; Corman et al., 2020; Dennis Lo and Chiu, 2020; Guan et al., 2020; Lu et al., 2020).

However, the detection efficiency of SARS-CoV-2 NAT has been criticized due to a high false negative rate (60–70%) (Rainer et al., 2004; Fang et al., 2020; Wang P. et al., 2020; Wang Y. et al., 2020). Recent studies demonstrated that the positive rates of RT-qPCR tests for SARS-CoV-2 vary significantly between specimen types (Pan et al., 2020; Wang W. et al., 2020; Xie et al., 2020). Even among the most frequently collected specimen type, throat swabs, they only generated a 32% positive rate (Wang W. et al., 2020). In addition, four groups reported that the SARS-CoV-2 viral load in respiratory tract specimens peaked at 3, 5–6 days and 10 days soon after symptom onset in several COVID-19 cases, respectively (Peiris et al., 2003; Hung et al., 2004; Al-Tawfiq and Memish, 2020; Zou et al., 2020). Recently published data also showed that false negatives from one-time sampling were as high as 30–50% in real COVID-19 cases (Alfaraj et al., 2019). While, a large cohort study of MERS-CoV reported increasing number of specimens consecutively tested could effectively improve the positive rate of NAT (Hung et al., 2004).

Thus, to investigate whether sampling in different anatomic sites, at different disease progression stages, and with different number of samples from each patient, as well as whether patient disease severity and status contributes to the false-negative results of SARS-CoV-2 NAT, we performed a retrospective analysis of SARS-CoV-2 positive rates, based on a large cohort of 4,363 specimens from 532 laboratory-confirmed COVID-19 cases at 79 reference hospitals in Sichuan Province from January 10th, 2019 to March 1st, 2020.

MATERIALS AND METHODS

Cases and Specimens

A large cohort of 532 laboratory-confirmed COVID-19 patients presented in Sichuan Province, China, between January 10th, 2020 and March 1st, 2020 was included in the study. All patients in our study were diagnosed with COVID-19 based on the National Clinical Guidelines of COVID-19 (Edition 7) (The National Clinical Guidelines of COVID-19, 2020) and recorded in the Sichuan Provincial Health Commission Database. The study was approved by the Sichuan Provincial Health Commission and the ethics commissions of Sichuan Provincial People's Hospital and determined to be exempt from oversight given the use of pre-existing, de-identified data. As such, individual-level informed consent was not obtained.

All general information and clinical characteristics of the patients, as well as specimen types, sampling dates, and NAT results of 4,363 specimens were submitted to Sichuan Provincial Health Commission Database by 79 COVID-19 reference hospitals in in Sichuan Province. We extracted and reviewed the data to exclude cases and/or specimens with missing core data.

SARS-CoV-2 Nucleic Acid Testing

SARS-CoV-2 NAT specimens included upper respiratory tract (URT, including nasal swabs, pharynx swabs and nasopharyngeal swabs) specimens, lower respiratory tract (LRT, including sputum and BALF) specimens, feces, rectal swabs, and blood. Total RNA was extracted from the specimens with CFDA-certified manual or automatic RNA extraction methods following corresponding manufacturer instructions.

The real-time RT-PCR assays were conducted in qualified CDC and hospital clinical laboratories using CFDA-certified test kits, including DAAN gene Co., Ltd. (Guangzhou. China), Sansure biotech Co., Ltd. (Changsha, China), Shanghai zjbio-tech Co., Ltd. (Shanghai, China), BGI, Co., Ltd. (Shenzhen, China), and Geneodex, Co., Ltd. (Shanghai, China), which were accordant with WHO recommended assays targeting one, two, or three SARS-CoV-2 viral genes (RNA-dependent RNA polymerase gene, nucleocapsid protein gene and envelope protein gene) (Xu et al., 2020).

Statistical Analysis

Descriptive statistics were reported as mean ± standard deviation or median (interquartile range) for continuous variables and as frequency (percentage) for categorical variables. All COVID-19 cases were grouped into mild, moderate, severe, and critical based on symptom severity and into stable, improved, and aggravated based on disease status in the National Clinical Guidelines of COVID-19 (Edition 7) (Alfaraj et al., 2019). All specimens were grouped into nasal swabs, pharynx swabs, nasopharyngeal swabs, sputum, BALF, feces/rectal swabs,

blood, and unknown specimen groups based on specimen type, while into <0, 0–7, 8-14, 15–21, 22–28 and > 28 days after onset (d.a.o) of symptoms based on the difference in days between symptom onset and specimen collection. Missing specimen types were not excluded, but classified into other specimen type group, while missing the viral NAT results were excluded from analyses.

To compare the SARS-CoV-2 NAT positive rates of specimens between different specimen types, symptom severities, disease statuses and progression stages, we performed mixed-effects logistic regression models respectively, used an unstructured covariance structure to account for the correlations within laboratory and patient levels, and reported the odds ratios, 95% confidence intervals and *P* values. All models were adjusted for age and gender.

We considered p <0.05 as statistically significant for all analyses. All statistical tests were two-sided and analyzed using STATA version MP 15 (StataCorp LLC, College Station, TX, USA).

RESULTS

Patient and Specimen Profiles

A total of 532 laboratory-confirmed COVID-19 patients from 79 reference hospitals in Sichuan Province during January 10th, 2019 and March 1st, 2020 were included in our study. As of March 1st, 2020, 374 (70.3%) patients were discharged, three (0.6%) were dead, and 155 (29.1%) patients remain hospitalized. There were 52.6% male cases, and there were no differences in sex ratios among the four severity groups. Of the 532 patients, the majority were moderate cases (400, 75.2%), while 49 (9.2%), 51 (9.6%), and 32 (6.1%) were the mild, severe, and critical cases, respectively (**Table 1**). The median age was 45 years with a range of 0.1 to 87 years, and the ratio of the patients above 45 years increased from 26.5 to 49.7, 62.7, and 78.1% with increasing severity (**Table 1**).

A total of 4,363 specimens from the 532 patients were collected between 17 days before and 50 days after onset (d.a.o). Of the 4,363 specimens tested for SARS-CoV-2, 88 had recorded undetermined results, and were therefore excluded. Hence 4,275 specimens with SARS-CoV-2 results were eventually included in the study. Among them, the predominant specimens were 1,992 (46.6%) throat swabs, 1,009 (23.6%) sputum, and 574 (13.4%) feces/anal swabs. In addition, there were 319 (7.5%) nasopharyngeal swabs, 256 (6.0%) nasal swabs, 80 (1.9%) blood, nine (0.2%) BALF, and 36 (0.8%) others with unknown types (**Table 2**). The median number of specimens collected from each patient was six (ranging from one to 50) (**Table 2**).

Positive Rates of SARS-CoV-2 NAT in Different Specimen Types

Out of the 4,275 specimens, 1,605 (37.5%) were detected positive for SARS-CoV-2. Among seven specimen types, BALF (77.8%, 7/9), nasopharyngeal swabs (40.4%, 129/319), and sputum (39.8%,

TABLE 1 | Patient profile.

Characteristics	Severity, No. (%)				
	Total	Mild	Moderate	Severe	Critical
No. of Patients	**532**	**49 (9.2)**	**400 (75.2)**	**51 (9.6)**	**32 (6.1)**
Male	**280 (52.6)**	**26 (53.1)**	**203 (50.8)**	**30 (58.8)**	**21 (65.6)**
Age, median (range), year	**45 (0.1, 87)**	**31 (0.1, 71)**	**44 (0.25, 79)**	**48 (20, 84)**	**60 (32, 87)**
<44 year, No. (%)	263 (49.4)	36 (73.5)	201 (50.3)	19 (37.3)	7 (21.9)
≥45 year, No. (%)	269 (50.6)	13 (26.5)	199 (49.7)	32 (62.7)	25 (78.1)

TABLE 2 | Specimen profile.

Specimen type	Time course of disease, d.a.o, No. (%)						
	Total	<0	0-7	8-14	15-21	22-28	>28
[a]**URT specimen**	**2567 (60.0)**	**90 (3.5)**	**533 (20.8)**	**710 (27.7)**	**661 (25.8)**	**376 (14.7)**	**197 (7.7)**
Throat swabs	1992 (46.6)	75 (3.8)	441 (22.1)	573 (28.8)	512 (25.7)	254 (12.8)	137 (6.9)
Nasal swabs	256 (6.0)	5 (2.0)	33 (12.9)	55 (21.5)	63 (24.6)	64 (25.0)	36 (14.1)
[b]NP swabs	319 (7.5)	10 (3.1)	59 (18.5)	82 (25.7)	86 (27.0)	58 (18.2)	24 (7.5)
[c]**LRT specimen**	1018 (23.8)	11 (1.1)	108 (10.6)	222 (21.8)	279 (27.4)	2234 (22.0)	174 (17.1)
Sputum	**1009 (23.6)**	**11 (1.1)**	**105 (10.4)**	**220 (21.8)**	**276 (27.4)**	**223 (22.1)**	**174 (17.2)**
BLAF	9 (0.2)	0 (0.0)	3 (33.3)	2 (22.2)	3 (33.3)	1 (11.1)	0 (0.0)
Feces/rectal swabs	**574 (13.4)**	**1 (0.2)**	**23 (4.0)**	**111 (19.3)**	**178 (31.0)**	**151 (26.3)**	**110 (19.2)**
Blood	80 (1.9)	0 (0.0)	8 (10.0)	20 (25.0)	28 (35.0)	13 (16.3)	11 (13.8)
Others	36 (0.8)	0 (0.0)	1 (2.8)	2 (5.6)	16 (44.4)	11 (30.6)	6 (16.7)
Total	**4275**	**102 (2.4)**	**673 (15.7)**	**1065 (24.9)**	**1162 (27.2)**	**775 (18.1)**	**498 (11.6)**
No. of Specimens from each patient, median (range)	**6 (1, 50)**	**1 (1, 7)**	**1 (1, 8)**	**2 (1, 16)**	**2 (1, 16)**	**3 (1, 18)**	**4 (1, 27)**

[a]*URT, Upper Respiratory Tract.*
[b]*NP, Nasopharyngeal.*
[c]*LRT, Lower Respiratory Tract.*

402/1009) had the three highest positive rates. Throat swabs (38.5%, 767/1992), nasal swabs (35.9%, 92/256), and feces/rectal swabs (34.0%, 195/574) ranked fourth, fifth, and sixth, while blood only had a 5.0% (4/80) positive rate (**Table 3**). Notably, Compared to throat swabs, BALF [OR = 6.24, 95%CI(1.08, 35.92), $P = 0.04$] and sputum [OR = 1.24, 95%CI(1.01, 1.52), $P = 0.04$)] possessed significantly higher positive rates; in contrast, feces [OR = 0.74, 95%CI(0.58, 0.95), $P = 0.016$] and blood [OR = 0.06, 95%CI(0.02, 0.16), $P < 0.001$] showed significantly lower positive rates (**Table 3**).

There was no significant difference found in the positive rates among three URT specimens, including throat, nasal, and nasopharyngeal (NP) swabs. We then compared SARS-CoV-2 positive rates of URT specimens, sputum, and feces/rectal swabs, which were the most commonly accepted specimens for SARS-CoV-2 NAT, representing three different anatomic sites. The positive rate of URT specimens was significantly lower than that of sputum [OR = 1.24, 95%CI (1.02, 1.50), $P = 0.025$], but significantly higher than that of feces/rectal swabs [OR = 0.74, 95%CI (0.59, 0.93), $P = 0.011$] (**Table 3**).

Positive Rates of SARS-CoV-2 NAT Under Different Disease Severities and at Different Disease Statuses

To find whether the SARS-CoV-2 NAT positive rates vary with disease severity and status, we analyzed the positive rates of the 4,275 specimens among four disease severities and among three disease statuses. There were 34.1% (95/279), 37.3% (1237/3315), 38.1% (162/425), and 43.4% (111/256) positive SARS-CoV-2 NAT results in the mild, moderate, severe, and critical cases, respectively. Compared to the positive rate in the mild cases, only that in the critical cases was significantly increased [OR = 2.02, 95%CI (1.19, 3.41), $P = 0.009$] (**Table 4**).

TABLE 3 | SARS- CoV- 2 NAT positive rates in different specimens (n = 4,275).

Specimen type	Positive rate %(n/N)	Overall		URT vs. sputum vs. feces/rectal swabs	
		OR (95% CI)	*P* value	OR (95% CI)	*P* value
[a]URT specimens	**38.5 (989/2567)**			Reference	
Throat swabs	**38.5 (767/1992)**	Reference			
Nasal swabs	35.9 (92/256)	0.80 (0.57–1.12)	0.187		
[b]NP swabs	40.4 (129/319)	1.18 (0.86–1.60)	0.301		
[c]**LRT specimens**	40.2 (409/1018)				
Sputum	**39.8 (402/1009)**	1.24 (1.01–1.52)	**0.040**[d]	1.24 (1.02–1.50)	**0.027**[d]
BALF	77.8 (7/9)	6.24 (1.08–35.92)	**0.040**[d]		
Feces/rectal swabs	**34.0 (195/574)**	0.74 (0.58-0.95)	**0.016**[d]	0.74 (0.59–0.93)	**0.011**[d]
Blood	5.0 (4/80)	0.06 (0.02–0.16)	**<0.001**[d]		
Others	22.2 (8/36)	0.54 (0.21–1.37)	0.194		
Total	**37.5 (1605/4275)**				

[a]*URT, Upper Respiratory Tract.*
[b]*NP, Nasopharyngeal.*
[c]*LRT, Lower Respiratory Tract.*
[d]*P< 0.05 considered as significant.*

TABLE 4 | SARS-CoV-2 NAT positive rates under different disease severities, statuses and stages (n = 4275).

Patient classification	Positive rate % (n/N)	OR (95% CI)	*P* value
Disease severity			
Mild	34.1 (95/279)	Reference	
Moderate	37.3 (1237/3315)	1.22 (0.85–1.74)	0.279
Severe	38.1 (162/425)	1.32 (0.83–2.09)	0.241
Critical	43.4 (111/256)	2.02 (1.19–3.41)	**0.009**[b]
Disease status			
Stable	40.6 (1481/3650)	Reference	
Improved	17.1 (100/586)	0.23 (0.17–0.32)	**<0.001**[b]
Aggravated	61.5 (24/39)	2.81 (1.28–6.16)	**0.001**[b]
Disease stage			
<0[a] d.a.o	76.5 (78/102)	3.24 (1.78–5.92)	**<0.001**[b]
0–7[a] d.a.o	70.1 (472/673)	Reference	
8–14[a] d.a.o	33.8 (360/1065)	0.09 (0.06–0.12)	**<0.001**[b]
15–21[a] d.a.o	24.7 (287/1162)	0.03 (0.02–0.04)	**<0.001**[b]
22–28[a] d.a.o	31.4 (243/775)	0.03 (0.02–0.25)	**<0.001**[b]
>28[a] d.a.o	33.1 (165/498)	0.02 (0.01–0.04)	**<0.001**[b]

[a]*d.a.o days after onset.*
[b]*P < 0.05 considered as significant.*

Among three disease statuses, the SARS-CoV-2 NAT positive rates were 17.1% (100/586) under the improved status, 40.6% (1481/3650) under the stable status, and 61.5% (24/39) under the aggravated status. Compared to the stable status, the improved status had significantly lower SARS-CoV-2 positive rate with an adjusted odds ratio of 0.23 [95%CI (0.17–0.32), $P<0.001$], in contrast, the aggravated status had significantly higher positive rates with an adjusted odds ratio of 2.81 [95%CI (1.28–6.16), $P = 0.001$] (**Table 4**).

To reveal optimal specimen types in different severities and disease statuses, we analyzed the positive rates of URT specimens, sputum and feces/rectal swabs among four severities and three statuses. In the moderate cases, sputum [OR = 1.25, 95%CI (1.02–1.55), $P = 0.036$] showed a significantly higher positive rate, while feces/rectal swabs showed significantly lower positive rates in both the moderate [OR = 0.71, 95%CI (0.55–0.93), $P = 0.012$] and the critical [OR = 0.16, 95%CI (0.05–0.49), $P = 0.001$] cases (**Table 5**). At aggravated status, there was no significant difference in the positive rates among three specimen types (**Table 5**). Compared to URT specimens, sputum [OR=2.26, 95%CI (1.14–4.47), $P = 0.019$] had a significantly higher positive rate at improved status, and feces/rectal swabs [OR=0.71, 95%CI (0.55–0.91), $P = 0.007$] had a significant lower positive rate at stable status (**Table 5**).

Positive Rates of SARS-CoV-2 NAT During the COVID-19 Course

To analyze SARS-CoV-2 positive rates along the time course of COVID-19, we divided the 4,275 specimens into six disease stage groups, including <0 (102, 2.4%), 0–7 (673, 15.7%), 8–14 (1065, 24.9%), 15–21 (1162, 27.2%), 22–28 (775, 18.1%), >28 (498, 11.6%) d.a.o groups based on the different days between symptom onset and specimen collection (**Table 2**). The SARS-CoV-2 positive rate before symptom onset was 76.5% (**Table 4**). While after symptom onset, the viral positive rate continually declined from 70.1% (472/673) in the first week, to 33.8% (360/1065) in the second week and 24.7% (287/1162) in the third week, and subsequently increased to 31.4 and 33.1% in the fourth week and after (**Table 4**). Taking the positive rate in the first week as a reference, the weekly changes in the positive rates among six stages were statistically significant (**Table 5**). In addition, compared to URT specimens at corresponding stages, sputum showed significantly increased SARS-CoV-2 positive rates in the first week (82.9%) [OR = 2.88, 95%CI (1.18–7.02), $P = 0.020$] and the fourth week (37.7%) [OR = 2.29, 95%CI (1.34–3.92), $P = 0.003$] , but feces/rectal swabs had a significantly lower positive rate later than 28 d.a.o (27.3%) [OR = 0.42, 95%CI (0.21–0.84), $P = 0.013$] (**Table 5**).

To describe the trend of the SARS-CoV-2 positive rate varying across the time course of COVID-19 more specifically, we calculated the daily positive rates of URT specimens, sputum, feces/rectal swabs, and overall specimens between 5 days before and 40 days after symptom onset. As shown in **Figure 1**, the daily positive rates of overall specimens presented as a flat "U" shape through the disease course, with the highest rates of 85–95% during −3 and 3 d.a.o, the lowest rate of 20% at around 18 d.a.o (**Figure 1**). The second peak was a 52% positive rate at the late stage of 36 d.a.o (**Figure 1**). Both URT specimens and sputum had a similar varying trend of positive rate as overall specimens. The daily positive rates of sputum were higher than those of URT specimens across the entire disease course, except for an overlap later than 36 d.a.o. However, the daily positive rates of feces/rectal swabs continually decreased from about 50% at 2 days before onset, to 25% at 40 days after onset (**Figure 1**).

By investigating the number of daily tested specimens between −5 and 40 d.a.o in our study cohort, we revealed there was a normal distribution in overall specimens, as well as subtype

TABLE 5 | SARS-CoV-2 NAT positive rates of different specimens under different disease severities, statuses and stages.

Patient classification	[a]URTspecimen	Sputum			Feces/rectal swabs		
	Positive rate, %(n/N)	Positive rate, %(n/N)	OR (95%CI)	*P* value	Positive rate, %(n/N)	OR (95%CI)	*P* value
Disease stage	Reference						
Mild	32.6 (73/224)	21.7 (5/23)	0.54 (0.18–1.62)	0.270	63.0 (17/27)	2.52 (0.96–6.60)	0.059
Moderate	38.2 (759/1989)	39.6 (316/798)	1.25 (1.02–1.55)	**0.036**[d]	34.2 (153/448)	0.71 (0.55–0.93)	**0.012**[d]
Severe	40.8 (89/218)	41.4 (46/111)	1.40 (0.76–2.57)	0.275	31.8% (21/66)	0.92 (0.44–1.93)	0.821
Critical	49.3 (67/136)	45.5 (35/77)	0.77 (0.38–1.56)	0.463	15.2 (5/33)	0.16 (0.05–0.49)	**0.001**[d]
Progressive status	Reference						
Improved	12.0 (36/300)	23.8 (39/164)	2.26 (1.14–4.47)	**0.019**[d]	22.0 (20/91)	1.66 (0.75–3.67)	0.214
Stable	41.8 (937/2243)	42.7 (356/834)	1.15 (0.94–1.41)	0.184	36.5 (175/480)	0.71 (0.55–0.91)	**0.007**[d]
Aggravated	62.5 (15/24)	63.6 (7/11)	N/A[b]	0.287	33.3 (1/3)	N/A[b]	0.958
Disease stage	Reference						
<0[c] d.a.o	75.6 (68/90)	81.8 (9/11)	17.10 (0.76-193.34)	0.99	100.0 (1/1)	N/A[b]	N/A[b]
0–7[c] d.a.o	69.0 (368/533)	82.9 (87/105)	2.88 (1.18–7.02)	**0.020**[d]	52.2 (12/23)	0.49 (0.10–2.32)	0.370
8–14[c] d.a.o	31.7 (225/710)	41.4 (91/220)	1.55 (0.95–2.51)	0.076	36.9 (41/111)	0.85 (0.46–1.58)	0.614
15–21[c] d.a.o	23.1 (153/661)	23.9 (66/276)	1.35 (0.86–2.12)	0.195	33.7 (60/178)	1.26 (0.77–2.07)	0.353
22–28[c] d.a.o	27.9 (105/376)	37.7 (84/223)	2.29 (1.34–3.92)	**0.003**[d]	34.4 (52/151)	1.13 (0.63–2.03)	0.675
>28[c] d.a.o	35.0 (69/197)	37.4 (65/174)	1.11 (0.63–1.96)	0.721	27.3 (30/110)	0.42 (0.21–0.84)	**0.013**[d]

[aa] *URT, Upper Respiratory Tract.*

[b] *N/A, Not available.*

[c] *d.a.o days after onset.*

[d] *$P < 0.05$ considered as significant.*

groups, URT specimens, sputum, and feces/rectal swabs, across the entire disease course (**Figure 2**), excluding an inference of different specimen distributions.

Cumulative Positive Rates of SARS-CoV-2 NAT With Consecutive Specimens

The median d.a.o of the first specimen collected from the 532 patients was five with an interquartile range of one d.a.o to 10 d.a.o (**Table 6**). The median d.a.o of the second and third specimens sequentially collected were 13.5 and 15, respectively (**Table 6**). Thus, to find an optimal number of specimens for initial diagnosis of COVID-19 as sampling usually across early and middle stages during practically applying SARS-CoV-2 NAT in clinic, we investigated cumulative positive rates of consecutively collected URT specimens and sputum, which were predominant specimens in our study and also the most common used in clinic.

Among the 469 patients with throat swabs collected for SARS-CoV-2 NAT, 338 (72.1%) patients were detected positive after testing one swab, 364 (77.6%) after testing two consecutive swabs, and 378 (80.6%) after testing three consecutive swabs (**Figure 3**, **Table 6**). There were 508 patients with URT

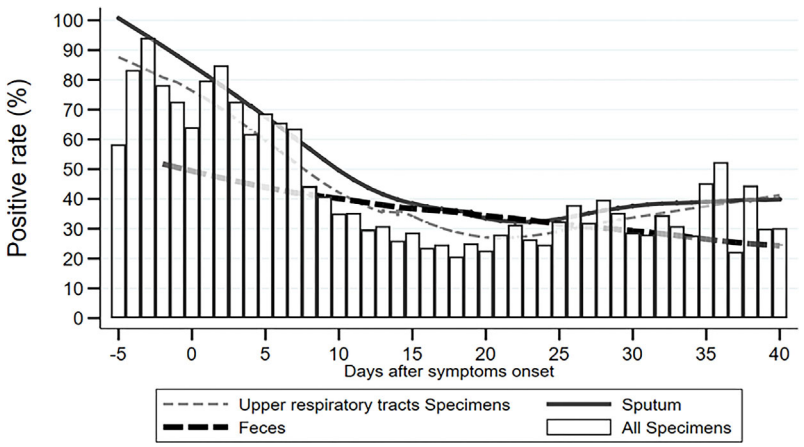

FIGURE 1 | Distribution of SARS-CoV-2 NAT daily positive rates across disease course.

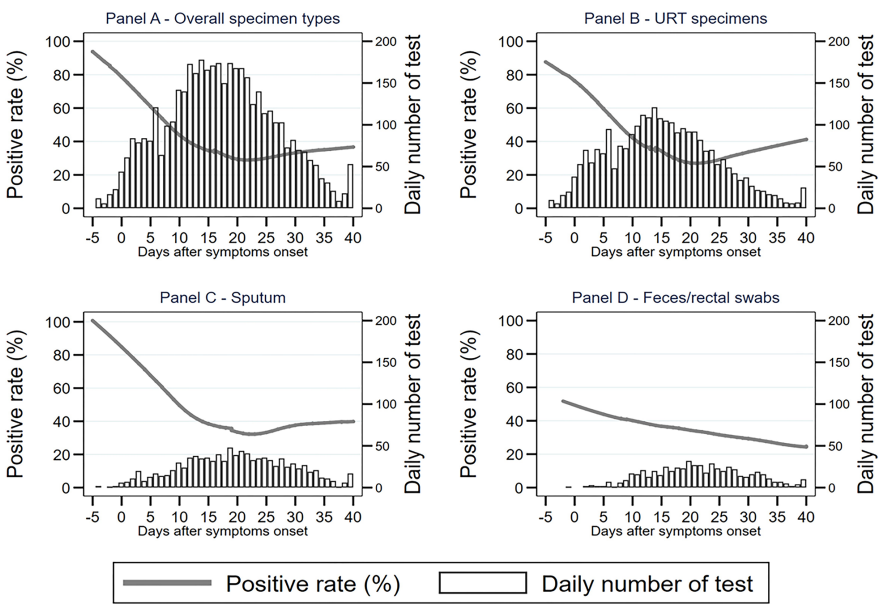

FIGURE 2 | The distribution of positive rates in different specimens across the disease course.

specimens collected for SARS-CoV-2 NAT. From those, 75.0, 81.7, and 85.2% were detected positive after one, two, and three consecutive specimens, respectively. While for 521 patients tested with either throat swabs or sputum specimens for SARS-CoV-2, the detection rate was 80.4% after one specimen tested, and increased to 86.4 and 89.6% after testing two and three specimens. The detection rate further increased to 82.7 and 89.7% after testing one and two specimens, respectively, for 532 patients with either URT specimens or sputum collection, and eventually reached 93.2% after testing three consecutive specimens (**Figure 3**, **Table 6**). The median d.a.o of the first, second, and third specimens collected in four specimen groups above were 5–7, 14, and 15–16, respectively. There was no significant difference observed among the four specimen groups above, thus excluding a probability of the difference in detection efficiency originated from sampling at different stages (**Table 6**).

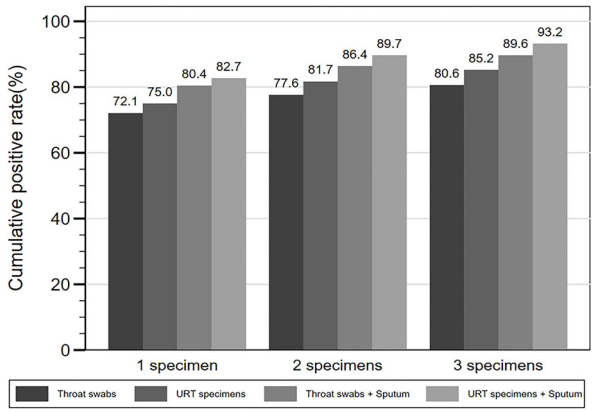

FIGURE 3 | Cumulative SARS-CoV-2 NAT positive rates of consecutive specimens.

DISCUSSION

The severity profile of COVID-19 cases included in our study is consistent with that across China reported by the WHO-China joint mission (WHO, 2020). The top three major specimens in the study were throat swabs, sputum, and feces/anal swabs, representing the most common collected specimen types and three different anatomic sites of SARS-CoV-2 viral load.

The overall SARS-CoV-2 positive rate of the 4,275 specimens was 37.5%, which is consistent with recent studies reporting positive rates ranging from 30 to 50% (Wang W. et al., 2020; Wang Y. et al., 2020; Xie et al., 2020). Among the seven specimen types, BALF had the highest positive rate, followed by nasopharyngeal swabs, sputum, throat swabs, nasal swabs, and feces/rectal swabs. The positive rates of three URT specimens, including throat, nasal, and nasopharyngeal swabs showed no significant difference, but were significantly lower than that of sputum, and higher than that of feces/rectal swabs. It suggests that the lower respiratory tracts have higher viral load compared to the upper respiratory tracts. LRT specimens, especially BALF had the highest detection rate for SARS-CoV-2 among all types of specimens, which is consistent with a recent study (Wang W. et al., 2020). However, considering the high infection risk and the low patient acceptance of the BALF collection procedure, it usually applies to critical cases only. Instead, sputum is an alternative LRT specimen type that is the best choice of SARS-CoV-2 NAT for COVID-19 patients with a productive cough. Otherwise, three URT swabs, including throat, nasal, and nasopharyngeal swabs are recommended for SARS-CoV-2 NAT. As the positive rate of feces/rectal swabs, especially blood specimens were significantly lower than that of respiratory specimens, they are not recommended for initial diagnosis of COVID-19.

The positive rate of SARS-CoV-2 NAT was not significantly different among patients with different COVID-19 disease severities, except for critical cases which had a significantly greater positive rate. However, the positive rate of SARS-CoV-2 NAT was significantly different among patients under the improved, stable and aggravated statuses, demonstrating that higher detection rates correspond to disease status. It suggests that SARS-CoV-2 viral load increases with disease status progression. For patients with

TABLE 6 | Cumulative SARS-CoV-2 NAT positive rates of consecutive specimens and time points of sampling.

No. of consecutive specimens	Total	Throat swabs		[a]URT specimen		Throat swabs and sputum		URT[a] specimens and Sputum	
	[b] d.a.o of sampling, median (c IQR)	Cumulative positive rate, %(n/N)	[b] d.a.o of sampling, median ([c] IQR)	Cumulative positive rate, %(n/N)	[b] d.a.o of sampling, median ([c] IQR)	Cumulative positive rate, %(n/N)	[b] d.a.o of sampling, median ([c] IQR)	Cumulative positive rate, %(n/N)	[b] d.a.o of sampling, median ([c] IQR)
one specimen	5 (1,10)	72.1 (338/469)	7 (2,13)	75.0 (381/508)	5 (1,12)	80.4 (419/521)	6 (2,12)	82.7 (440/532)	5 (1,10)
two specimens	13.5 (8, 20)	77.6 (364/469)	14 (8, 20)	81.7 (415/508)	14 (8, 19)	86.4 (450/521)	14 (8, 20)	89.7 (477/532)	14 (7, 20)
three specimens	15 (11, 21)	80.6 (378/469)	16 (10, 21)	85.2 (433/508)	16 (10, 21)	89.6 (467/521)	16 (10, 21)	93.2 (496/532)	15 (10, 21)

[a]*URT, Upper Respiratory Tract.*
[b]*d.a.o days after onset.*
[c]*IQR, interquartile range.*

moderate disease or under the improved status, the positive rate of sputum specimens was significantly higher than that of other specimen types. In contrast, the positive rates of feces/rectal swabs were significantly lower when patients had moderate and critical disease, or under improved status. It suggests that sputum, but not feces/rectal swabs has higher detection efficiency for SARS-CoV-2 NAT than URT specimens for patients in most of disease severities and at most of disease statuses.

Most importantly, our study revealed 85–95% positive rates at 3 days before and after onset of symptoms, but a 20% positive rate at 18 d.a.o. Statistical analysis revealed continually decreasing positive rates during the disease course of −3 to 24 d.a.o. It suggests that viral load peaks at very early stage of the disease, even a few days before symptom onset, but decreases rapidly one week after symptom onset (Pan et al., 2020; Young et al., 2020; Wolfel et al., 2020; Zou et al., 2020). Our results demonstrated that significantly reduced positive rates during the middle and late stages of the disease skew the overall detection rate of SARS-CoV-2 NAT to just 30–40%. However, when sampling as close to symptom onset as possible, the detection efficiency of SARS-CoV-2 NAT reached above 90%, which is high enough for initial diagnosis of COVID-19. Thus, the interval between sampling and symptom onset is the most important factor impacting detection efficiency of SARS-CoV-2 NAT compared to specimen type, disease severity and status. In addition, the second peak in the 5th week of the disease course probably represents a reversion of viral shedding during disease recovery, and could explain the reappearance of positive results in very few patients after two consecutive negative specimens. Interestingly, the positive rate of feces/rectal swabs continually decreased through the entire disease course, suggesting that it might correspond with disease progression as time.

Notably, our results show that testing a single throat swab generated only a 72.1% detection rate for COVID-19 patients. In contrast, three consecutive respiratory specimens, especially after including sputum, could significantly increase the detection rate to 93.2%. It suggests that consecutive respiratory specimens tested could effectively improve the detection efficiency of SARS-CoV-2 NAT, especially if missing the most optimal timepoint to collect specimens.

Some limitations should be known in the current study. First, our results were based on kits from at least five companies; we could not compare the difference between different kits, because we did have these data. Second, process of acquired sample would have an influence on the positive rate; most samples might have been got by nurses or physicians; we could not compare the difference between junior nurse/physicians or senior nurses/physicians. Third, the time span from sampling to testing might be an issue for the study.

Taken together, the study results revealed that the detection efficiency of SARS-CoV-2 NAT varies with specimen type, number of specimens, patient disease severity and status, and especially disease stages of specimen collection. Thus, to improve the detection efficiency of SARS-CoV-2 NAT, we highly recommend: 1) sampling as close to symptom onset as possible for initial diagnosis of COVID-19; 2) consecutively sampling 2–3 respiratory specimens with at least one LRT specimen if missing early stages of COVID-19.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

LJ, LW, and ZY designed the study. JieZ, KL, and LZ drafted the manuscript and analyzed the data. JianZ, ZR, TS, and HY helped to check the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Public Health

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Does Temperature Affect COVID-19 Transmission?

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This study utilizes the non-linear least squares method to estimate the impact of temperature on COVID-19 cases per million in forty-three countries, divided into three groups as follows: the first group is composed of thirteen countries that announced the first COVID-19 cases in January 2020, while the second and third groups contain thirteen and seventeen countries, respectively, that witnessed the pandemic for the first time in February and March of the same year. This relationship was measured after four time periods from the date of reporting the first case until April 1, April 15, May 15, and July 8, 2020. The results show an inverse relationship between COVID-19 cases per million and the temperature in the studies of the four-time periods for the three-country groups. These results were only significant statistically ($p < 0.1$) after 110.8, 164.8 days on average from the beginning of the pandemic in the case of "January" countries.

Keywords: COVID-19, SARS-CoV-2, infectious disease, COVID-19 transmission, temperature

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INTRODUCTION

There is no doubt that the containment of the COVID-19 pandemic, caused by the emerging coronavirus (SARS-CoV-2), is currently the primary concern worldwide. The characteristics of this pandemic made it difficult even for the most advanced health systems to control it after it started in Wuhan, China in late 2019.

COVID-19 has spread into most countries of the world due to its extremely high transmission rate of 2–2.5 (1). The number of COVID-19 cases globally as of July 8, 2020, at 08:24 GMT, was 11,965,661, of which 57.78% recovered, 4.57% died, and 37.65% were still active. According to official statistics, China, the source of the pandemic, managed to close 93.99% of cases with recovery, 5.54% with deaths (three cases per one million), and only 0.47% of cases were still active. While the US topped the list of the most affected countries, with a case number of about 3,097,417, representing 25.89% of global cases of which, 51.93% cases were still active. Furthermore, the combined COVID-19 cases in the US, Brazil, India and Russia amounted to 51.96% of the global cases during the study time. (https://www.worldometers.info/coronavirus/).

It is noteworthy that COVID-19 cases are mostly concentrated in the central and northern areas in the affected countries that represent capitals, densely populated cities, economic and financial centers, especially in the developed countries. For instance, New York, the US economic capital in the far northeast, was one of the most affected States. Likewise, the province of Lampodria in northern Italy, responsible for 40% of the industrial production (2), was the most affected area in Italy. Furthermore, COVID-19 has swept through Madrid, the Spanish capital, and the most important financial and economic center (3). In addition, the pandemic was

concentrated in Wuhan, the transportation and industry hub in central China. In Germany, the province of Bavaria, the second-largest German city, in terms of population and the producer of 18% of the gross German domestic production (4) was the most affected. As for France, the pandemic targeted the north-central region, Ile-de-France, the richest and the most important French and European region in terms of research, development, and innovation (5).

Coronaviruses are large, enveloped RNA viruses of both medical and veterinary importance (6). The envelope structures of SARS-CoV-2 are sensitive to physical and chemical conditions and can be destabilized or damaged by heat, ultraviolet (UV) light or extreme pH (7). The outermost structural protein of the SARS-CoV2 "Spike protein" showed active and inactive states at different temperatures (8). In such a way, regions that have low temperatures are more prone to infection than those with higher temperatures (9). The COVID-19 cases increased toward the Earth's poles with increasing latitude (7). Accordingly, coronavirus peaks occur in winter, taking the form of local epidemics that last a few weeks or months (10).

Several studies indicate that the transmission of COVID-19 is affected by temperature. An inverse correlation was found between temperature and the daily number of infections (11–17). Other studies determine the temperature range for this effect. For instance, the virus transmission is hindered by specific humidity above 6 g/kg and mean air temperature above 11°C (18). COVID-19 can be seasonal with the optimal temperature range of 5°C−14 and the peak of 10°C (19). Another study estimates that every 1°C increase in the minimum temperature leads to a decrease in the cumulative number of cases by 0.86 (20). In contrast, other studies deny or underestimate the effect of temperature on COVID-19 (21–25). It is indisputable that some of these results were affected by the methodology of analysis used, the countries chosen to carry out the study, and the other confounding factors that affect the phenomenon that may not have been neutralized in some of these studies.

Not only are climate and meteorological factors expected to affect the transmission of COVID-19 (17, 26), but there are also many other variables, i.e., social distancing, age, GDP per capita, ethnicities, health, poverty, diabetes, coronary heart disease, physical inactivity, alcohol consumption, tobacco abuse, and access to primary care (27). This paper investigates the impact of temperature on COVID-19 transmission, represented in cases per million.

METHODS

The transmission rate of COVID-19 is expressed as the daily number of infections (11, 25, 28), or the total number of confirmed cases (7, 9, 12, 16, 23). In other studies, the number of cases accumulated over a period of time (18, 20, 29), average daily cumulative rate of confirmed cases (13), or cases per 100000 (27) represent COVID-19 transmission. In addition, the virus spread is indicated as the growth rate of the confirmed cases (21, 24, 30), the effective reproductive number of infection (22), or the doubling time of the confirmed cases number (26). Others use cases per 1-km^2 (15).

In some studies, the temperature is expressed as the average daily temperature (13, 14, 20, 22, 25, 29), or the average temperature over a period of time (7, 9, 23). Others use the 14-day exponential moving averages (EMAs) of daily average temperature (28). For this study, the average temperature over a period of time is used. Countries are represented in terms of temperature by the most affected cities, or by capitals.

The non-linear least-squares method is employed to estimate the relationship between COVID-19 transmission and temperature using the STATA statistical software package (version 16.1; StataCorp LLC). The exponential function was suggested in Equation (11) to represent the relationship between the number of COVID-19 cases per million as a dependent variable (y), and the average temperature as an independent variable (x) (31).

$$y_{it} = \alpha e^{\beta Xit} \tag{1}$$

Where:

yit: is COVID-19 per million in country "i" at the end of the period "t"

α, β: is the model parameters.

xit: is the average temperature in the country "i" during the period "t."

Obtaining the natural logarithm of both sides of Equation (1), the following equivalent equation can be obtained:

$$ln\, y_{it} = \ln \alpha + \beta x_{it} \tag{2}$$

Where it was possible by converting to Equation (2) to obtain a formula for a linear regression model to which, the error component ε can be added to become as follows:

$$y'_{it} = \alpha' + \beta x_{it} + \varepsilon \tag{3}$$

This study assumes that the prevalence of COVID-19 increases as temperature decreases and vice versa. Hence, the main hypotheses are:

H0: there is no inverse relationship between COVID-19 per million and the temperature.

H1: there is an inverse relationship between COVID-19 per million and the temperature.

Given the low number of observations here, a level of significance ($p < 0.1$) has been adopted (32). Data on COVID-19 cases per million (**Supplementary Tables 3**, **7**, **11**) and temperature (**Supplementary Tables 4**, **8**, **12**) in forty-three countries were collected. These countries were divided into three groups, as follows: the first group consists of thirteen countries, eleven of which have experienced the pandemic for the first time in the last third of January 2020, namely: Australia, Finland, France, Germany, Italy, Malaysia, Russia, Spain, Sweden, UK, and the US, in addition to Japan and South Korea that witnessed the pandemic in the second third of the same month. The second group consists of thirteen countries, all of which have

reported the first case of COVID-19 in the last third of February 2020, namely: Armenia, Austria, Belarus, Croatia, Czechia, Denmark, Estonia, Ireland, Lithuania, New Zealand, Norway, Romania, and Switzerland. Finally, the last group consists of seventeen countries, all of which have experienced the COVID-19 pandemic during the first week of March 2020, namely: Albania, Bosnia and Herzegovina, Chile, Jordan, Moldova, Morocco, Paraguay, Peru, Poland, Portugal, Saudi Arabia, Serbia, Slovakia, Slovenia, Tunisia, Turkey, and Ukraine.

For more reliable results, comparable countries were intentionally chosen in the same group (**Supplementary Tables 1**, **5**, **9**). Furthermore, the primary comparison criterion was the extent to which the country succeeded in closing nearly half or more of the COVID-19 cases, accompanied by a decrease in the death rate attributed to the cases that were closed. This may indicate the status of the country's health system, as well as other sub-criterions considered, such as the number of tests per million as an indicator of spending on health in the country. It was also taken into account that there would not be a large disparity in the population, and that is why Brazil and Pakistan were excluded from the "February" group, for example. It was also taken into consideration that there was no disparity in the population density. Nevertheless, there were some necessary exceptions, such as the inclusion of France, Italy, and Spain in the "January" group, despite their high death rates, compared to the other

TABLE 1 | Results of the regression models.

Months	Days[a]	Actual No. of obs[b]	R^2_{adj}	F	Pro. > F	Intercept (S.E.)	*P*-value	Temp. (X) (S.E.)	*P*-value
January	68.8	10	−0.1197	0.04	0.8503	6.367 (0.489)	0.000	−0.010 (0.050)	0.85
	80.8	11	0.0005	1.01	0.3422	7.325 (0.491)	0.000	−0.037 (0.037)	0.342
	110.8	12	0.2261	4.21	0.0672	8.298 (0.574)	0.000	−0.089 (0.043)	0.067*
	164.8	12	0.2356	4.39	0.0626	9.318 (0.862)	0.000	−0.121 (0.058)	0.063*
February	35.2	11	0.0508	1.53	0.2472	6.882 (0.712)	0.000	−0.151 (0.122)	0.247
	49.2	11	−0.0941	0.14	0.7171	6.825 (0.666)	0.000	−0.034 (0.090)	0.717
	79.2	10	0.0647	1.62	0.2384	8.130 (0.706)	0.000	−0.094 (0.074)	0.238
	133.2	11	−0.0424	0.59	0.4608	8.611 (1.369s)	0.000	−0.083 (0.108)	0.461
March	28.35	14	0.1001	2.45	0.1438	4.994 (0.495)	0.000	−0.056 (0.036)	0.144
	42.35	15	−0.0453	0.39	0.5412	5.690 (0.647)	0.000	−0.028 (0.044)	0.541
	72.35	13	−0.0722	0.19	0.6701	7.043 (1.396)	0.000	−0.042 (0.103)	0.67
	126.35	13	−0.0908	0	0.9702	7.317 (2.086)	0.005	−0.005 (0.122)	0.97

[a]*Days from the 1st case reported.*
[b]*After excluding outliers, leverage and influencer observations.*
**Significant at 10% significance level.*

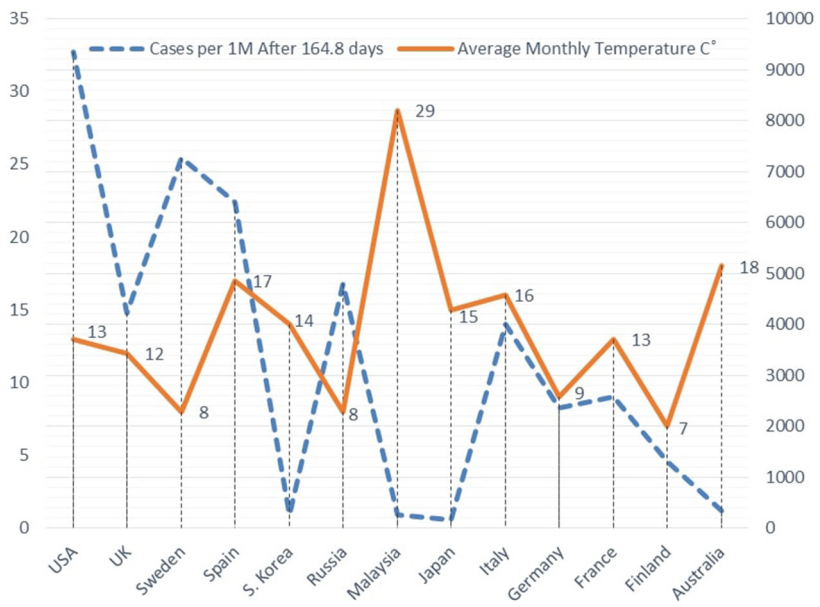

FIGURE 1 | COVID-19 cases per 1 M and temperature after 164.8 on average in January group countries.

group's members. However, they were included as a result of their similarity to other group's countries, in terms of their ability to close more than half of COVID-19 cases, as well as having high health spending, expressed in the number of tests per million. They also have a high median age as with the rest of the group - except Malaysia and Australia.

This relationship between the study's variables was measured for each group after four-time periods from the date of the first case reported until April 1, April 15, May 15, and July 8, 2020, respectively (**Supplementary Tables 2**, **6**, **10**).

For ensuring the validity of the results, Cook's distance and DFFITS tests were performed to show the influence of each observation on the fitted response values. The goodness of fit of the model parameters was checked by these two methods in which, outliers, leverage, and influential observations that affect the values of the fitted parameters were omitted (33).

Data on COVID-19, analyzed in this paper, was collected from one website (https://www.worldometers.info/), which provides global COVID-19 live statistics. The website is independent and is frequently cited as a source in journal articles. It was also voted as one of the best free reference websites by the American Library Association (http://www.ala.org/rusa/). As well, (https://www.timeanddate.com/) was utilized to obtain the monthly temperature in the most affected cities during the study's four-time periods. The site helps obtain the average monthly temperature directly without further calculations.

RESULTS

The relationship parameters between COVID-19 cases per million in the three studies' groups of countries and the average temperatures are estimated as follows (**Table 1**).

The results show an inverse relationship between the study's variables in the three groups of countries under study, in all the four-time periods since the first case was reported. The null hypothesis was rejected in favor of the alternative hypothesis at ($p < 0.1$) only after 110.8 and 164.8 days on average from the first case reported in the "January" group countries. **Figure 1** illustrates an example of the inverse relationship between cases per one million and weather temperature after 164.8 days in average, in the case of the "January" countries group.

By substituting the regression parameters for the "January" group countries "after 110.8 and 164.8 days" in Equation 3, Equations 4 and 5 can be obtained:

$$lny_{it} = 8.298 - 0.089\, x_{it} \quad (4)$$

$$lny_{it} = 9.318 - 0.121\, x_{it} \quad (5)$$

Applying e to both sides:

$$y_{it} = (e^{-0.089})^{x_{it}} + e^{8.298} = EXP\ (-0.089)^{x_{it}} + EXP\ (8.298) \quad (6)$$

$$y_{it} = (e^{-0.121})^{x_{it}} + e^{9.318} = EXP\ (-0.121)^{x_{it}} + EXP\ (9.318) \quad (7)$$

Equations 6 and 7 are used for predicting the development of COVID-19 cases per million, in terms of weather temperature (**Supplementary Tables 13**, **14**).

DISCUSSION

Although temperature is one of the factors that influence COVID-19 prevalence, there are other important factors that have worsened the situation in countries that were heavily invaded by the pandemic, such as the US, Spain, and Italy. Perhaps those countries were relatively late in imposing precautionary measures, unlike other similar countries, in terms of temperature at that time, such as China, South Korea, and Japan that managed to flatten the curve of new cases of COVID-19. In addition, the later countries utilized distinguished mechanisms of early mitigation measures well, including the big data techniques to contain the pandemic from its springs. Therefore, this was evidenced by the determination coefficient (R bar squared) in regression models (**Table 1**), which was 23.56% at most, indicating that 76.44% of the phenomenon is explained by other factors.

The preprint-results of this study (34) related the negative relationship between COVID-19 cases per million and temperature to the number of days since the first case was reported. Although temperature affects COVID-19 transmission in its early stages, cases per million reach a critical mass after the successive exponential increase, and temperature no longer has a significant influence on the pandemic transmission. As for this study, it coincides with the preprint's results, in terms of the direction of the relationship between the study variables.

TABLE 2 | Observed and estimated COVID-19 cases per million, in terms of temperature in the "January-group" countries.

Countries	After 110.8 days on average			After 164.8 days on average		
	Temp. (C°)	COVID-19 cases per 1 M		Temp. (C°)	COVID-19 cases per 1 M	
		Observed	Estimated in terms of temp.		Observed	Estimated in terms of temp.
Australia	20.4	276	654	18.29	348	1,213
Finland	3.6	1,124	2,915	7.43	1,311	4,526
France	10.2	2,175	1,620	12.57	2,586	2,427
Germany	6.4	2,092	2,272	9.14	2,367	3,678
Italy	13.4	3,702	1,219	16.14	4,002	1,575
Japan	11.6	127	1,430	15	158	1,808
Malaysia	29	212	304	28.71	268	343
Russia	4.2	1,801	2,763	8.43	4,802	4,009
S. Korea	10.2	215	1,620	14.29	272	1,971
Spain	13.4	5,868	1,219	16.86	6,400	1,443
Sweden	5	2,894	2,573	8.43	7,261	4,009
UK	10	3,489	1,649	12	4,218	2,601
USA	8.4	4,445	1,902	12.86	9,357	2,343

Observed cases per million are less than estimated
Observed cases per million are higher than estimated

In contrast, the effect of temperature on the prevalence of COVID-19 was not statistically confirmed here in ten out of twelve observations.

Perhaps, the preprint's results were affected by the crude comparison between incomparable countries, in terms of case ascertainment, connections between the country and the affected areas, population density, applied control measures to the country, and timing at which they were instituted. Regardless of the difficulties that concern the availability of a sufficient number of countries that would be compared, this study tries to include more comparable countries as much as possible. In addition, the preprint's results compared the relationship between the study variables after only two periods for two different groups of countries (72 days in the case of January countries, and after 44 days in the case of February countries), whereas this study intentionally deepens the analysis to estimate the relationship in four periods for each of the three groups of countries under study.

Referring to **Supplementary Table 13**, it turns out from **Table 2** that the observed COVID-19 cases per million after 110.8 days in average, from the first case, reported in France, Italy, Spain, Sweden, UK, and the US, were higher than its expected values, in respect to the average temperature. On the contrary, the observed COVID-19 cases per million, with regards to the temperature in Australia, Finland, Germany, Japan, Malaysia, Russia, and South Korea, were lower than expected after the same period. Likewise, the same group of countries showed almost identical behavior after 164.8 days on average except for Russia, where the numbers of observed COVID-19 cases were greater than expected (**Supplementary Table 14**, **Table 2**). The findings of this study assume that the data declared by countries are correct and accurate. But in the case of assumed underestimation or underreporting, actual cases per million in these countries can be expected as in **Table 2**. The same table show that the observed COVID-19 cases per million were less than their expected values in Japan by about 11 times, and by about 7–7.5 times in South Korea. Contrariwise, the observed COVID-19 cases per million were ∼4–5 times more than estimations in Spain, and three times in Italy, whereas, the COVID-19 cases per million observed in the US were twice to four times its estimated values.

One of the main criteria for selecting the "January" group countries was that they have advanced health systems and are expected to have a high degree of data reliability. Nevertheless, differences have emerged between the estimated and observed values. It is expected that these differences will be greater in the case of countries with less advanced health systems, and less reliable data recording. This was demonstrated when data about Togo and South Africa, instead of Turkey, was included in the "March" countries (**Supplementary Tables 15**–**19**). Through this data and using equations as in **Supplementary Table 19**, prediction tables of COVID-19 cases per million, in terms of temperature were obtained (**Supplementary Tables 20**, **21**). It is revealed from **Supplementary Table 22** that COVID-19 cases per million after 28.56 days in average, from the first case reported in Albania, Bosnia and Herzegovina, Chile, Moldova, Peru, Portugal, Saudi Arabia, Serbia, and Slovenia, were higher than its expected values, in terms of temperature. Far from this, the observed COVID-19 cases per million in Jordan, Morocco, Paraguay, Poland, Slovakia, South Africa, Togo, Tunisia, and Ukraine were lower than expected, in light of temperature. In a second period after 42.56 days on average, most of the above-mentioned courtiers showed a relatively large discrepancy between the observed and estimated COVID-19 cases per million (**Supplementary Table 22**). This large discrepancy may be due to underestimation or underreporting. In addition, this also may be associated with the number of tests per million, performed to detect COVID-19. For instance, it was noted that the estimated COVID-19 cases in Portugal that performed 124,698 tests per million were approximately twice the observed figures only. On the other hand, the estimated COVID-19 cases in Togo, which performed 4,025 tests per million at best, were 82 times the observed cases.

Finally, it is highly recommended that the relationship between COVID-19 cases per million and the temperature should be estimated at different time periods thereafter; in order to monitor the phenomenon, either in later stages or earlier than those observed in this study.

CONCLUSION

All the findings reached are presented in this study, including those introduced by the pre-print, or the attempts to replace some countries in the third group (**Supplementary Tables 19–22**). All the findings agree that the relationship between the temperature and the transmission of COVID-19 has an opposite direction, despite the variation in the level of significance, from being significant to insignificant. The differences in the criteria of selecting countries may lead to the variation in the statistical significance magnitude, but they have not affected the direction of the relationship. This study reports that the relationship between COVID-19 transmission and temperature is marginally and statistically confirmed ($p < 0.1$) in just two observations out of twelve. This may indicate that factors other than temperature are the most influential on the transmission of COVID-19.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

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

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This study has been released as a pre-print at (34).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.554964/full#supplementary-material

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Personality, Coping Strategies, and Mental Health in High-Performance Athletes During Confinement Derived From the COVID-19 Pandemic

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The COVID-19 outbreak has affected the sports field unprecedentedly. The emergency alert has deprived athletes of training in a suitable environment, as they are faced with cancellations of relevant events in their sports careers. This situation can cause stress levels and other emotional disorders similar to those experienced by athletes during periods of injury. Since the relationship between psychological factors and sports injuries is well-studied, the Global Psychological Model of Sports Injury (MGPLD) is applied to this historical situation for athletes. The purpose of this study was to analyze the relationships between perfectionism and trait anxiety with indicators of mental health (mood, depression, state anxiety, and stress) in high-performance athletes during confinement due to the COVID-19 pandemic, as well as to explore the coping strategies that athletes have applied and whether they are perceived as useful for managing negative emotional states. A cross-sectional study was conducted through online questionnaires during April 2020, adapting the Psychological Assessment Protocol of the High-Performance Sports Center of Murcia (Spain), to assess the psychological effects of confinement in a cross-cultural sample of 310 athletes (141 women and 169 men) from different countries in Europe, Asia, and America, and from diverse sports disciplines. The protocol comprised six instruments that test perfectionism, trait anxiety, mood states, stress, depression, coping strategies, and sleep. It was answered online via Google Forms. The results show that maladaptive perfectionism was related to all the indicators of athletes' mental health. However, athletes' levels of anxiety, stress, and depressive symptoms are relatively low, and the use of coping strategies such as cognitive restructuring and emotional calm was associated with lower levels of negative emotional states. Besides, the Iceberg Profile, a suitable fit for the mental health model, is observed in the mood of athletes, both in men and in women, although women showed higher levels of anxiety, stress, and depression than men. A strong relationship was observed between maladaptive perfectionism and martial arts sports discipline, superior to other sports. In short, it can be concluded that high-performance athletes in the

studied sample showed negative emotional state values below the expected average. Finally, the proposals for practical applications of the results collected are discussed.

Keywords: sports psychology, personality, high-performance athletes, coping, stress, COVID-19, confinement

INTRODUCTION

The emergence and expansion of the COVID-19 disease have caused a worldwide pandemic, affecting the political, economic, and social stage. To stop the spread of the disease, measures of confinement taken by most governments have interrupted the daily lives of the people, impeding athletes of training in a suitable environment, which can lead to negative consequences at emotional, cognitive, and behavioral levels. For most athletes, this sudden interruption in their training schedule will lead them to set new goals during the season when it resumes. For athletes at the end of their competitive stage, it can mean putting an early and abrupt closure to their careers, which can increase unpleasant emotions during confinement.

A recent review developed to study the existing evidence on the psychological impact of quarantine on people concluded, after analyzing 24 studies, that this situation can have adverse psychological effects such as post-traumatic stress symptoms, confusion, and anger (1). Other studies also show that confinement can affect well-being by inducing, promoting, or increasing substance abuse (2).

This situation of confinement is similar to the state of injury that most athletes suffer at some point throughout their sports careers. According to the literature, a series of psychological and emotional reactions may appear during the sports injury, as a response to both the injury itself (3–6) and the rehabilitation process (7). Moreover, the return to competition after a period of rest due to sports injury and the fear of relapse (8) are also the aims of this study.

The Global Psychological Model of Sports Injury [MGPLD; (9)] proposes that variables such as motivation, competitive anxiety, psychosocial stress, and coping mechanisms influence the different moments of the injury (before, during, and after). From this perspective, we consider that the confinement situation caused by the COVID-19 pandemic can have significant psychological effects on athletes, similar to those seen in people who carry out daily tasks in confinement also evidenced in studies about sports withdrawal (partial or total) because of sports injury.

Since this particular and sudden governmental response to COVID-19 was unexpected for the majority of the population, confinement measures might have entailed situations of unwanted isolation. For this reason, we explored the literature on the psychological consequences of confinement. From all the research conducted on human confinement, we highlight studies carried out on inmates in prisons and those related to performance tasks under a particular situation of confinement, as it is in study cases of astronauts and the crew of underwater vessels. A recent study in consequences of isolation shows how people who experience this isolation in prison present clinical or subclinical symptoms of mood disorders (depression and anxiety) and other added psychological symptoms such as loss of identity and stimulatory hypersensitivity (10, 11).

In other studies, such as Haney's research on prisoners in confinement (12), two different kinds of symptoms are illustrated. On the one hand, there are symptoms related to affect such as anxiety, migraines, tiredness and apathy, sleep problems, nightmares, palpitations, loss of appetite, and dizziness. On the other hand, there is a series of symptoms that Haney defines as the psychopathological effects of solitary confinement, such as irrational anger, hypersensitivity to stimuli, confused thinking process, perceptual distortions, hallucinations, and suicidal thoughts.

Because of the situation of confinement that convicts live in prisons, some studies have developed a series of measures to prevent the impact of pandemics in prisons. In the USA, the Centers for Disease Control and Prevention has developed a checklist to face epidemics (13), and similarly, the WHO has developed a recent and specific response guide to COVID-19 (14).

In the case of astronauts and crew of underwater vessels, besides social isolation (to a greater or lesser extent), there is also an adaptation to an extreme environment, which differs greatly from the usual habitat where people face their day-to-day tasks. These exceptional conditions are characterized by a reduced space, added to the absence of gravity, and the social isolation that produces changes in perceptive (15), cognitive, and sensorimotor levels (16). Also, the absence of light can cause changes in work and rest schedules, lack of sleep or difficulty falling asleep, changes in circadian rhythms, fatigue, etc. (17). Although there might be negative psychological consequences of confinement, there might also be protective factors, such as the resilience of individuals, that can minimize its effect and improve people's well-being (18).

Another set of consequences that have been observed is that low mood caused by isolation in crew members of submarines and space stations induces a series of dysfunctions in the immune system, such as hormonal alterations that increase the predisposition to get sick as a result (19, 20). Confinement, the same as in sports injury, can have adverse effects on certain psychological variables. These effects can take place during confinement itself, or, as in sports injuries, they can lead to psychological and social consequences once the period of confinement ends and the population is able to resume daily activities (21).

Psychological factors underlying the different stages of sports injury are growing in importance because it influences different processes related to a sports injury, and the potential for improvement in relapse intervention and prevention (22). Although the confinement situation derived from COVID-19 is a historically novel phenomenon, problems derived bear some similarities to those suffered by athletes during the different

stages of sports injury. Some of these implications are the interruption or limitation of sports activity, loss of autonomy, changes in the sports environment, loss of opportunities to improve sports records individually and collectively, interruption or limitation of non-related sports activities, and changes in personal and family life, including early retirement due to changes in competition schedules. Besides, larger problems such as substance abuse, social isolation, episodes of depression or anxiety, suicidal tendencies, self-esteem issues, and poorly perceived quality of sleep might be present. The latter factor is closely related to the athlete's quality of life, since poor perception of the quality of sleep can negatively influence athletes' well-being (23). In line with the conclusions of other authors regarding injuries, long periods of confinement could also stand an opportunity for personal growth and improvement of the psychological aspects of sports practice (24–27).

The current situation of confinement worldwide has led many athletes to adapt their sports training without the tools or suitable spaces to develop their training routines properly. This fact has led us to research how this unusual situation affects athletes and how they are experiencing it since all local, national, and international competitions have been canceled or postponed. It is in our particular interest to study the psychological effects, both negative and positive, that this situation can have on them.

Following the parallelism between injury and confinement, physiological and attentional changes as a consequence of stress caused by isolation can significantly influence athletes. If there is indeed a strong psychological relation with confinement and injury-perceived stress, those athletes with a higher level of stress and low self-control who do not put in place adequate coping resources would suffer a greater psychological impact once the competition resumes than those athletes with higher self-control and better competition stress management (28, 29).

The COVID-19 pandemic is unprecedented in terms of measures taken by countries and governments around the world; therefore, there are not many prior data to predict how this confinement will affect people in general and athletes in particular. For that reason, in the absence of a theoretical model of reference on which to base our hypotheses on the consequences that confinement by COVID-19 may have on athletes, we have advocated studying the parallelism of sports injuries and their psychological effects on the athletes.

Taking the Global Psychological Model of Sports Injury (9) as a starting point, we have selected those psychological variables that have been extensively studied in the field of sports injury and belong to the conceptual axis of sports injury. This axis forms a "galaxy" of factors, using the authors' metaphor, which influences the subjective experience of sports injury in particular ways.

Considering the aforementioned studies on this matter, the psychological variables selected for our study are the following: perfectionism (understood as concern over mistakes, personal standards, parental expectations, and organization), trait anxiety, state anxiety, stress and depression, mood state, coping strategies (understood as emotional calming, active planning/cognitive restructuring, mental withdrawal, seeking social support, and behavioral risk), and quality of sleep.

This study aims to analyze the relationships between perfectionism and trait anxiety with mental health indicators (mood, depression, state anxiety, and stress) in high-performance athletes during confinement due to the COVID-19 pandemic. In addition, the coping strategies that athletes have applied are explored and whether they are perceived as useful for managing negative emotional states.

MATERIALS AND METHODS

Participants

An incidental sampling was carried out to get the data sample. Athletes taking part in the study are formally enrolled in their respective sports federations. The inclusion criterion to be part of the sampled population was to be a high-level athlete or a high-performance athlete in their respective countries. The sample consists of 310 athletes (141 women) from 18 to 49 years of age ($M = 22.26$ years; $SD = 4.98$). The final sample answered all questions included in the protocol, except for 15 athletes who did not specify which sport discipline they belong to. For this matter, the analysis of psychological variables and sports modalities was set to 295. The most frequent sport practiced for this sample was football (14.9%), followed by athletics (12.2%), martial arts (11.9%), basketball (9.8%), and rugby (8.1%).

All subjects completed the *Psychological Assessment Protocol* online, voluntarily, and in accordance with the Helsinki Agreement protocol. Furthermore, this study has been approved ethically by the University of Trás-os-Montes e Alto Douro (UTAD, Portugal) Ethical Committee, code 23/DOC20/CE/UTAD (27/06/2018), and follows the Helsinki Protocol guidelines, including the informed consent from all participants.

Instruments and Materials

An *ad hoc* protocol was created, based on the injury protocol (30, 31) used in the Murcia High-Performance Center. This protocol assesses sociodemographic, personal, and sports data (name, age, sex, place of residence, household members, sport discipline, studies, course year, and online classes availability), and it consists of the following questionnaires.

The *Multidimensional Perfectionism Scale* [FMPS, (32)], Spanish version, adapted from the original FMPS (33), provides four subscales (instead of six, as previously suggested) for a multidimensional assessment of perfectionism: Concern over Mistakes (CM), Personal Standards (PE), Parental Expectations (PE), and Organization (O). The scale showed satisfactory reliability (Cronbach's α's $= 0.87$). The 35-item questionnaire is answered on a Likert-type scale from 1 (strongly disagree) to 5 (strongly agree); higher points show higher perfectionism while lower points indicate otherwise.

The *State-Trait Anxiety Inventory* [STAI-T; (34)], Spanish version, adapted from the original STAI-T (35), was used for the evaluation of competition anxiety. This scale has 20 items for assessing trait anxiety and 20 items for state anxiety. All items are rated on a four-point scale from 0 (hardly ever) to 3 (almost always). Higher scores indicate greater anxiety. The scale showed Cronbach's α's $= 0.93$.

The short version of *Depression, Anxiety, and Stress Scales* [DASS-21; (36)] Spanish version adapted (37) was used to measure common symptoms of depression, anxiety, and stress. This scale provides three subscales: Depression (DASS21-D), Anxiety (DAS21-A), and Stress (DASS21-S), each of them containing seven items. Each item comprises four response options scored from 0 (Does not apply to me at all) to 3 (Totally applies to me, or most of the time). The scale showed Cronbach's α's = 0.81.

The *Profile of Mood States* (38) was used in its Spanish adapted and validated version by Fuentes et al. (39). The short scale contains 29 items answered on a five-point scale from 0 (nothing) to 4 (very much). In this version, athletes reported on their mood state concerning each of the items on the instrument. The scale details five mood states: tension ($\alpha = 0.83$), anger ($\alpha = 0.85$), vigor ($\alpha = 0.83$), fatigue ($\alpha = 0.82$), and depression ($\alpha = 0.78$).

The *Approach to Coping in Sport Questionnaire* [ACSQ-1; (40, 41)] was used in its Spanish version (42). This scale contains 28 items answered in Likert-type form from 1 (never) to 5 (always). The questionnaire aims to find out how often athletes use certain coping strategies in competitive situations. The five dimensions of coping assessed are emotional calming (7 items; e.g., "I tried to block negative thoughts"), active planning/cognitive restructuring (6 items; e.g., "I tried to find something positive in what happened"), mental withdrawal (6 items; e.g., "I thought there was nothing left to do, and I accepted it"), seeking social support (4 items; e.g., "I talked to someone to figure out what I could specifically do to solve the problem"), and behavioral risk (5 items; e.g., "I was constantly changing strategies"). Internal consistency coefficient was acceptable ($\alpha > 0.7$).

A two-item questionnaire, created *ad hoc*, aims to assess the number of sleep hours and its quality, based on the *Sports Sleep Questionnaire* (CSD in Spanish) developed by Garcia-Mas et al. (23). The item of perceived quality of sleep is answered using a Likert-type scale from 1 (very bad) to 5 (very good). The second item, related to hours of sleep, was a numerical answer to indicate the number of hours that the subject usually sleeps.

Procedure

The online tool Google Forms was used via online messaging platforms and email, allowing a fast and efficient distribution of the *Psychological Assessment Protocol* to a wide spectrum of individuals who met the inclusion criteria. This selection approach benefitted the heterogeneity of the sample population. A cross-cultural sample was obtained in collaboration with several sport psychology working groups from China, Mexico, Portugal, Russia, and Spain. Initially, a total sample of 414 subjects was obtained, of which 103 were underage. These subjects and outlier values (a 68-year-old man) were excluded from the analysis, resulting in a final sample of 310 subjects. The athletes in our sample answered the psychological protocol throughout the month of April 2020. Several countries had been in alarm stage for approximately 2 weeks at that time, with the consequent suspension of sports training, competitions, and other sporting events. In addition, there were still no expectations regarding the end of the confinement measures or the resumption of trainings and sports competitions. The data obtained through Google Forms were exported to a Microsoft Excel spreadsheet for their adequate categorization. A descriptive and correlational analysis of the questionnaire responses was conducted using a software package for statistical analysis in social sciences, the statistical program SPSS v 20.

Data Analysis

The Kolgomorov–Smirnov normality test was applied in order to determine if parametric or non-parametric analysis was appropriate in this study. The results indicated that almost all variables present an abnormal distribution for this sample ($p < 0.05$), with the exception of STAI-T, POMSv, FMPSD, and ACSQ-U-CE ($p > 0.05$).

We applied a descriptive analysis, comparisons between groups and correlational analyses to the aforementioned sample. Given the data obtained from the results of the Kolgomorov–Smirnov test, the analysis of differences in means have been applied to several independent samples using the Kruskal–Wallis test, median difference analyses for two independent samples using the Mann–Whitney *U*-test, and correlational analysis using Spearman's Rho.

RESULTS

Table 1 shows the correlations between the ACSQ (Use/Efficacy) measurements and the STAI-T, DASS-21, POMS, FMPS, and Sleep Quality subscales. No significant correlation is shown between the different ACSQ factors (Use/Efficacy) and the hours of sleep, or between the ACSQ factors with the FMPSd factor except in ACSQUmw (*rho*=0.174; $p < 0.01$). The highest correlations are found in ACSQEcr and ACSQEem ($rho = 0.396$; $p < 0.01$), in a positive and negative direction.

There are significant, negative correlations between the emotional states perceived as negatives (anxiety, depression, stress, and fatigue) and the use of coping strategies in athletes, such as cognitive restructuring and emotional calming.

Table 1 shows a high number of correlations in all the psychological variables considered, especially in the variables of personality traits (anxiety and perfectionism), mood states (POMS), and mental health (DASS-21), and to a lesser extent in sleep variables (except for sleep quality).

The magnitude of these correlations is moderate (medium or relatively low), ranging from significant values of $rho = 0.119$ ($p < 0.05$) to values of $rho = 0.413$ ($p < 0.001$). In most of the significant correlations found, the probabilities are $p < 0.001$.

The greatest number of correlations found when considering coping strategies (use and efficacy) was realized when crossing them with the STAI-T, the three factors of the DASS-21, mood states, and, to a lesser extent, with those related to the quality of sleep.

Table 2 shows the correlations, through the Spearman correlation coefficient, between the sleep questionnaire STAI-T, DASS-21, POMS, and FMPS questionnaires. Sleep quality correlates significantly with most of the measured variables. Sleep

TABLE 1 | Spearman correlation coefficient between the ACSQ, STAI-Trait, DASS-21, POMS, FMPS, and Sport Sleep questionnaires.

	SQ	SH	STAI	DASSd	DASSa	DASSs	POMSt	POMSd	POMSa	POMSv	POMSf	FMPSA	FMPSD
ACSQUem	0.145*	0.011	−0.260***	−0.214***	−0.107	−0.138*	−0.086	−0.201***	−0.100	0.356***	−0.223***	0.332***	−0.060
ACSQUcr	0.124*	0.022	−0.288***	−0.365***	−0.159**	−0.219***	−0.138*	−0.271***	−0.131*	0.346***	−0.275***	0.218***	−0.143*
ACSQUmw	−0.207***	−0.024	−0.286***	0.291***	0.199***	0.264***	0.185***	0.253***	0.240***	−0.143*	0.186***	−0.083	0.187***
ACSQUbr	−0.046	−0.042	0.005	−0.011	−0.032	−0.036	0.049	0.074	0.060	0.228***	−0.025	0.207***	0.130*
ACSQUss	−0.009	−0.101	−0.023	−0.108	−0.039	0.011	0.088	0.004	0.041	0.076	0.044	0.144*	−0.048
ACSQEem	0.152**	0.072	−0.343***	−0.348***	−0.230***	−0.244***	−0.225***	−0.324***	−0.193***	0.372***	−0.299***	0.224***	−0.209***
ACSQEcr	0.199***	0.119*	−0.323***	−0.413***	−0.201***	−0.243***	−0.191***	−0.326***	−0.186***	0.386***	−0.277***	0.238***	−0.201***
ACSQEmw	−0.152**	−0.017	−0.197***	0.168**	0.194***	0.188***	0.177**	0.195***	0.232***	−0.059	0.109	−0.049	0.112*
ACSQEbr	0.054	0.076	−0.037	−0.111	−0.037	−0.055	0.041	−0.005	0.005	0.246***	−0.046	0.181***	0.130*
ACSQEss	0.097	−0.022	−0.090	−0.110	−0.056	−0.010	0.061	−0.066	−0.023	0.102	−0.014	0.093	−0.058

*Correlations are significant at the levels *p < 0.05; **p < 0.01; ***p < 0.001 (bilateral). ACSQ, Approach to Coping in Sport Questionnaire; ACSQUem, Use of emotional calm; ACSQUcr, Use of cognitive restructuring; ACSQUmw, Use of mental withdrawal; ACSQUbr, Use of behavioral risk; ACSQUss, Use of seeking social support; ACSQEem, Efficacy of emotional calm; ACSQEcr, Efficacy of cognitive restructuring; ACSQEmw, Efficacy of mental withdrawal; ACSQEbr, Efficacy of behavioral risk; ACSQEss, Efficacy of seeking social support; STAI-T, Trait Anxiety Inventory; DASS, Depression, Anxiety, and Stress Scales; DASSd, Depression; DASSa, Anxiety; DASSs, Stress; POMS, Profile of Mood States; POMSt, Tension; POMSd, Depression; POMSa, Anger; POMSv, Vigor; POMSf, Fatigue; FMPS, Multidimensional Perfectionism Scale; FMPSA, Adaptative; FMPSD, Dysfunctional.*

TABLE 2 | Spearman correlation coefficient between the Sport Sleep Questionnaire and STAI-T, DASS-21, POMS, and FMPS questionnaires.

	SH	STAI	DASSd	DASSa	DASSs	POMSt	POMSd	POMSa	POMSv	POMSf	FMPSA	FMPSD
SQ	0.476***	−0.348***	−0.272***	−0.370***	−0.374***	−0.302***	−0.350***	−0.295***	0.267***	−0.311***	0.077	−0.161**
SH		−0.152**	−0.101	−0.261***	−0.260***	−0.212***	−0.168**	−0.161**	0.076	−0.146**	−0.001	−0.044

*Correlations are significant at the levels **p < 0.01 and ***p < 0.001 (bilateral). Sport Sleep Questionnaire; SQ, Sleep quality; SH, Sleep hours; DASS, Depression, Anxiety, and Stress Scales; DASSd, Depression; DASSa, Anxiety; DASSs, Stress; POMS, Profile of Mood States; POMSt, Tension; POMSd, Depression; POMSa, Anger; POMSv, Vigor; POMSf, Fatigue; FMPS, Multidimensional Perfectionism Scale; FMPSA, Adaptative; FMPSD, Dysfunctional.*

hours correlates significantly with some variables, all correlations being negative.

With the exception of the correlations found between the sleep measures (quality and hours, $rho = -476$; $p < 0.001$), the correlational values found range from $rho = -0.134$ ($p < 0.05$) to $rho = 0.374$ ($p < 0.001$). Therefore, most of the significant correlations found show a moderate or relatively small magnitude, observing how most correlations are obtained at a significance level of $p < 0.001$.

Table 3 shows the correlations, through the Spearman correlation coefficient, between the FMPS factors and the POMS, DASS-21, and STAI-T questionnaires. The FMPSD correlated significantly with all the measures evaluated. FMPSA only correlated significantly and positively with POMSt and POMSv. As in previous tables, the correlations found are of medium or relatively low magnitude, ranging from values of $rho = 0.137$ ($p < 0.05$) to the highest values found with the STAI of $rho = 0.516$ ($p < 0.001$).

Table 4 presents the descriptive statistics and Kruskal–Wallis test for the STAI-T, POMS, DASS-21, and FMPS questionnaires, in relation to the variable sex. A *post-hoc* analysis has been performed using the Mann–Whitney *U*-test. Statistically significant differences were found in STAI-T ($p < 0.001$) and some factors of the POMS in relation to sex (POMSv, $p < 0.009$; POMSf, $p < 0.05$). DASS-21 scores reveal statistically significant differences in two of the three factors (DASS, $p < 0.004$; DASSs, $p < 0.000$). These results show how the sample of female athletes obtained the highest scores among all the variables considered, except for the POMS-VI, FMPSA, and FMPSD, where male athletes obtained the highest scores. From all these variables, only significant differences are shown with a $p < 0.001$ in the STAI, DASS-21 EST, and STAI-Total; with a $p < 0.01$ in DASS-21-AS and POMS-VI; and with a $p < 0.05$ in POMS-FA.

Table 5 shows the descriptive statistics and Kruskal–Wallis test for the ACSQ-Use/Efficacy questionnaire and the two items subtracted from the Sports Sleep Questionnaire, in relation to the variable sex. The results show no significant differences between male and female participants in their perception coping strategies, hours of sleep, and perception of quality of sleep.

Table 6 presents the descriptive statistics and analysis of the mean difference between the different sports modalities considering the responses of the STAI-T, POMS, DASS21, and the FMPS, applying the Kruskal–Wallis test for the multiple independent samples. *Post-hoc* analysis have been performed using the mean difference analysis for two independent Mann–Whitney *U*-samples. The mean scores obtained on STAI-T, POMS, and DASS21 do not show significant differences in relation to sports disciplines ($p < 0.05$). The relationship between FMPSD and MA presents a remarkable average score compared to the rest of sports. The results show statistically significant relationships in FMPS.

It can be seen how the highest scores on the STAI are obtained by Rugby players followed by basketball. In POMS-TE, DE, and CO, the highest scores have been obtained by soccer, basketball,

TABLE 3 | Pearson correlation coefficient between the FMPS, POMS, and DASS-21 questionnaires.

	POMSt	POMSd	POMSa	POMSv	POMSf	DASSd	DASSa	DASSs	STAI
FMPSA	0.137*	−0.076	0.108	0.256***	−0.056	−0.054	0.021	0.105	−0.004
FMPSD	0.294***	0.283***	0.270***	−0.126*	0.304***	0.323***	0.322***	0.334***	0.516***

*Correlations are significant at the levels *p < 0.05 and ***p < 0.001 (bilateral). STAI-State, Trait Anxiety Inventory; DASS, Depression, Anxiety, and Stress Scales; DASSd, Depression; DASSa, Anxiety; DASSs, Stress; POMS, Profile of Mood States; POMSt, Tension; POMSd, Depression; POMSa, Anger; POMSv, Vigor; POMSf, Fatigue; FMPS, Multidimensional Perfectionism Scale; FMPSA, Adaptative; FMPSD, Dysfunctional.*

TABLE 4 | Descriptive statistics and Mann–Whitney *U*-test of the STAI-T, POMS, DASS-21, and FMPS, for the sex variable.

	TOTAL X (SD) (*N* = 310)	Male X (SD) (*n* = 169)	Female X (SD) (*n* = 141)	*Z*	*Sig (p)*
STAI-T Factor					
STAI R(0–60)	20.80 (9.57)	19.21 (9.00)	22.68 (9.92)	−3.212	0.001***
POMS Factors					
POMSt R(0–24)	4.39 (4.52)	3.85 (3.97)	5.04 (5.03)	−1.860	0.063
POMSd R(0–20)	3.25 (3.24)	3.22 (3.06)	3.30 (3.46)	−0.259	0.796
POMSa R(0–32)	8.22 (5.23)	7.97 (4.76)	8.52 (5.75)	−0.320	0.749
POMSv R(0–20)	11.00 (3.86)	11.50 (3.73)	10.42 (3.95)	−2.612	0.009**
POMSf R(0–20)	4.40 (3.83)	3.94 (3.73)	4.95 (3.90)	−2.500	0.012*
DASS-21factors *R(0–21)					
DASSd	4.30 (3.55)	4.00 (3.27)	4.65 (3.83)	−1.229	0.219
DASSa	2.28 (2.77)	1.76 (2.45)	2.89 (2.99)	−2.877	0.004**
DASSs	4.89 (4.28)	4.20 (3.84)	5.72 (4.62)	−3.938	0.000***
FMPS factors					
FMPSA R(7–35)	46.80 (8.78)	46.99 (8.77)	46.57 (8.82)	−0.112	0.911
FMPSD R(6–30)	54.04 (14.23)	54.64 (14.48)	53.32 (13.94)	−0.783	0.434

*X (SD) = Average (Standard Deviation); R = Rank;*R = Full Scale Rank. STAI-T, Trait Anxiety Inventory; DASS, Depression, Anxiety, and Stress Scales; DASSd, Depression; DASSa, Anxiety; DASSs, Stress; POMS, Profile of Mood States; POMSt, Tension; POMSd, Depression; POMSa, Anger; POMSv, Vigor; POMSf, Fatigue; FMPS, Multidimensional Perfectionism Scale; FMPSA, Adaptative; FMPSD, Dysfunctional. Significant levels at *p < 0.05; **p < 0.01; ***p < 0.001 (bilateral).*

TABLE 5 | Descriptive statistics and Mann–Whitney *U*-test of the ACSQ and Sport Sleep Questionnaires, for the sex variable.

	TOTAL X (SD) (*N* = 310)	Male X (SD) (*n* = 169)	Female X (SD) (*n* = 141)	MW-*U*	*Z*	*Sig (p)*
ACSQ-Use/Efficacy						
ACSQUec R(7–35)	24.18 (5.21)	23.73 (5.62)	24.73 (4.66)	10,637.00	−1.645	0.100
ACSQUcr R(6–30)	22.00 (5.06)	21.63 (5.21)	22.43 (4.86)	11,003.50	−1.179	0.239
ACSQUmwR(6–30)	12.44 (5.06)	12.25 (5.16)	12.65 (4.94)	11,301.00	−0.800	0.424
ACSQUbr R(4–20)	9.16 (3.96)	9.32 (3.71)	8.98 (4.25)	11,164.00	−0.977	0.329
ACSQUss (5–25)	10.50 (4.94)	10.14 (4.57)	10.93 (5.34)	11,138.50	−1.009	0.313
ACSQEec R(7–35)	24.96 (6.074)	24.71 (6.27)	25.27 (5.84)	11,409.50	−0.661	0.509
ACSQEcr R(6–30)	21.77 (5.67)	21.52 (5.62)	22.06 (5.74)	11,144.00	−1.000	0.318
ACSQEmw R(6–30)	12.26 (5.65)	12.32 (5.94)	12.20 (5.32)	11,716.50	−0.271	0.787
ACSQEbr R(4–20)	10.08 (4.38)	10.23 (4.28)	9.91 (4.50)	11,316.00	−0.782	0.434
ACSQEss R(5–25)	13.38 (6.17)	12.91 (5.85)	13.94 (6.50)	10,832.50	−1.398	0.162
Sleep						
SQ R(1–5)	350 (1.21)	3.58 (1.21)	3.41 (1.21)	10,912.00	−1.331	0.183
SH	7.68 (1.58)	7.75 (1.56)	7.61 (1.61)	11,278.50	−0.760	0.447

*X (DS) = Average (Standard Deviation); R = Rank; *R = Full Scale Rank. ACSQ, Approach to Coping in Sport Questionnaire; ACSQUem, Use of emotional calm; ACSQUcr, Use of cognitive restructuring; ACSQUmw, Use of Mental withdrawal; ACSQUbr, Use of behavioral risk; ACSQUss, Use of seeking social support; ACSQEem, Efficacy of emotional calm; ACSQEcr, Efficacy of cognitive restructuring; ACSQEmw, Efficacy of mental withdrawal; ACSQEbr, Efficacy of behavioral risk; ACSQEss, Efficacy of seeking social support. Sport Sleep Questionnaire; SQ, Sleep Quality; SH, Sleep hours. Significant levels at *p < 0.05; **p < 0.01; ***p < 0.001 (bilateral).*

TABLE 6 | Descriptive statistics obtained from the FMPS and STAI questionnaires and Kruskal–Wallis test with *post-hoc* analysis with Mann–Whitney *U*-test (MW-U) carried out on the variable sport practiced.

		X (SD)	χ^2	Sig.	MW-U	Sig.
STAI	Athletics	18.89 (8.62)		0.142		
	Football	20.75 (9.17)	12.202			
	Rugby	23.83 (11.60)				
	Basketball	22.24 (11.41)				
	Cycling/triathlon	19.00 (5.80)				
	Martial arts	21.03 (8.94)				
	Climbing	12.82 (7.25)				
	Swimming/water polo	23.17 (8.54)				
	Other sports	20.65 (9.53)				
FMPSA	Athletics	46.42 (8.11)	24.681	0.001***	415.000	0.013*
	Football	43.84 (10.11)			404.500	0.000***
	Rugby	43.88 (7.76)			1497.500	0.006**
	Basketball	46.59 (7.33)			192.500	0.000***
	Cycling/triathlon	44.00 (15.22)			744.500	0.007**
	Martial arts	50.60 (7.65)			321.000	0.012*
	Climbing	44.27 (6.78)			85.500	0.006**
	Swimming/water polo	45.00 (7.69)			333.000	0.045*
	Other sports	48.44 (8.33)				
FMPSD	Athletics	51.61 (12.52)	27.691	0.002**	94.500	0.009**
	Football	48.61 (10.23)			536.000	0.021*
	Rugby	51.50 (12.13)			1360.500	0.001***
	Basketball	52.97 (11.10)			64.000	0.016*
	Cycling/triathlon	45.33 (16.14)			67.500	0.005**
	Martial arts	55.91 (15.64)			129.500	0.049*
	Climbing	41.91 (7.66)			338.500	0.020*
	Swimming/water polo	58.33 (16.45)			79.000	0.003**
	Other sports	58.10 (15.20)			12.000	0.035*
					179.500	0.000***

*X (DS) = Average (Standard Deviation); R = Rank; FMPS, Multidimensional Perfectionism Scale; FMPSA, Adaptative; FMPSD, Dysfunctional; STAI-State, Trait Anxiety Inventory. Level of significance: *p < 0.05; **p < 0.01; ***p < 0.001.*

and rugby players. At Vigor, the highest scores are obtained by swimmers/water polo players and MA wrestlers. The highest levels of Fatigue are observed in swimmers and fighters of MA.

Regarding the values of the DASS21, the highest scores in DE are obtained in MA fighters and basketball players. At ANS, the highest scores are obtained in swimming and basketball. In EST, the highest scores are obtained from swimmers, footballers, and cyclists/triathletes. The most adaptive perfectionist athletes are MA wrestlers, and the most maladaptive are MA swimmers/water polo players and wrestlers.

Despite the scores shown, only significant differences are shown by applying the Kruskal–Wallis test in Adaptive perfectionism ($p < 0.001$) and maladaptive perfectionism ($p < 0.01$). In both cases, there is a very high number of differences between groups of athletes, and many of them of $p < 0.01$. or $p < 0.001$.

Similar to the results obtained in **Table 6**, the application of *post-hoc* tests on the six variables using the Mann–Whitney *U*-test shows a very high number of significant differences between sports, finding in many of them a great variability in scores between sports ($p < 0.01$ or <0.001).

The descriptive statistics analysis and the Kruskal–Wallis test for the sport variable in relation to the results obtained in the ACSQ-U questionnaire are shown in **Table 7**. *Post-hoc* analysis have been performed using the mean difference analysis for two independent Mann–Whitney *U*-samples. Statistically significant differences were observed in emotional calming ($p < 0.01$) and seeking social support ($p < 0.04$).

In the descriptive table, it can be observed that MA athletes got the highest scores in ACSQ_U_CE, ACSQ_U_RC, ACSQ_U_CR, ACSQ_E_CE, and ACSQ_E_RC. In the ACSQ_U_RM variable, basketball players, followed closely by rugby players, are the ones that got the highest scores. The variable ACSQ_U_BAS highlights the highest scores for cycling and triathlon. In the variable ACSQ_E_RM, the highest scores for rugby players stand out. In the variable ACSQ_E_CR, the highest scores of the swimmers' group stand out.

The descriptive statistics analysis and the Kruskal–Wallis test for the sport variable in relation to the results obtained in the ACSQ-E questionnaire are shown in **Table 8**. *Post-hoc* analysis have been performed using the mean difference analysis for two independent Mann–Whitney *U*-samples. Statistically significant differences were observed in emotional calming ($p < 0.04$), mental withdrawal ($p < 0.01$), and seeking social support ($p < 0.00$). The martial arts sport discipline presents slightly higher average scores than the other sports disciplines in most factors.

Applying the Kruskal–Wallis test, statistically significant results are shown with a $p < 0.01$ in ACSQ_E_BAS and ACSQ_U_CE, and with a $p < 0.05$ in ACSQ_E_RM, ACSQ_U_CE, ACSQ_U_BAS, and ACSQ_E_CE. The application of the *post-hoc* test in the six variables using the Mann–Whitney *U*-test shows a high number of significant differences between sports, finding major score differences among sports in many of them ($p < 0.01$ or <0.001).

Figure 1 shows the profile corresponding to the average results of the POMS in the sample. As seen, both male and female athletes present an ideal mood profile, in line with the iceberg profile in which the vigor factor is at levels higher than the other factors.

Anxiety and depression scores collected through various instruments (STAI, DASS-21, and POMS) correlate significantly with each other, which indicates the reliability of the results obtained.

DISCUSSION

Due to the unexpected force of the COVID-19 outbreak's social impact, researchers from around the world and from a wide variety of disciplines responded promptly, applying accessible and previously applied models most of the time. In the case of sports psychology, the first model used was the well-known

TABLE 7 | Descriptive statistics and Kruskal–Wallis test with *post-hoc* analysis with Mann–Whitney *U*-test carried out on the sport variable in relation to the scores obtained in the ACSQ-U.

Test/Sport		X (SD)	χ^2	Sig.	Mann–Whitney	*Z*	Sig.
ACSQUec	Athletics	24.67 (5.060)	19.96	0.01**			
	Football	22.73 (5.11)			448.50	−2.09	0.036*
	Rugby	24.38 (4.48)			409.50	−3.56	0.000***
	Basketball	22.41 (4.62)			1653.50	−2.06	0.039*
	Cycling/triathlon	22.92 (6.44)			269.00	−2.33	0.019*
	Martial arts	26.89 (4.77)			243.00	−3.58	0.000***
	Climbing	23.18 (5.70)			964.50	−2.50	0.012*
	Swimming/water polo	24.00 (4.60)			112.50	−2.06	0.039*
	Other sports	24.85 (5.48)			1277.00	−2.10	0.036*
ACSQUcr	Athletics	22.75 (4.61)	7.15	0.52			
	Football	21.07 (4.83)					
	Rugby	22.71 (3.77)					
	Basketball	20.76 (5.90)					
	Cycling/triathlon	21.83 (6.39)					
	Martial arts	23.69 (3.84)					
	Climbing	22.18 (6.24)					
	Swimming/water polo	21.33 (4.84)					
	Other sports	21.96 (5.41)					
ACSQUmw	Athletics	10.72 (4.60)	9.19	0.32			
	Football	11.82 (4.42)					
	Rugby	13.42 (4.42)					
	Basketball	13.48 (5.63)					
	Cycling/triathlon	13.00 (3.27)					
	Martial arts	11.71 (3.98)					
	Climbing	12.09 (5.31)					
	Swimming/water polo	12.67 (5.50)					
	Other sports	13.11 (5.89)					
ACSQUbr	Athletics	9.03 (3.78)	12.39	0.13			
	Football	8.80 (3.56)					
	Rugby	8.42 (3.79)					
	Basketball	8.24 (3.48)					
	Cycling/triathlon	8.58 (4.54)					
	Martial arts	11.06 (3.75)					
	Climbing	8.73 (3.28)					
	Swimming/water polo	10.17 (3.54)					
	Other sports	9.31 (4.52)					
ACSQUss	Athletics	11.83 (5.54)	16.09	0.041*			
	Football	9.05 (4.00)			558.000	−2.274	0.023*
	Rugby	10.13 (4.079)			373.500	−1.968	0.049*
	Basketball	8.93 (3.34)			86.000	−3.573	0.000***
	Cycling/triathlon	14.33 (4.53)			78.500	−2.206	0.027*
	Martial arts	11.29 (4.16)			55.000	−3.424	0.001***
	Climbing	10.27 (5.85)			12.500	−2.212	0.027*
	Swimming/water polo	9.17 (4.35)			354.000	−2.181	0.029*
	Other sports	10.86 (5.42)					

*X (SD) = Average (Standard Deviation); ACSQ, Approach to Coping in Sport Questionnaire; ACSQUec, Use of emotional calm; ACSQUcr, Use of cognitive restructuring; ACSQUmw, Use of mental withdrawal; ACSQUbr, Use of behavioral risk; ACSQUss, Use of seeking social support. Significant levels at *p < 0.05; **p < 0.01; ***p < 0.001 (bilateral).*

Model of Sports Injuries [(43), MGLD: (9)] due to the similarity in the interruption of activity and the inherent uncertainty of the return to normal sports conditions, especially in long-term injuries. In the latest formulations, this model had added other variables such as self-efficacy (44) and environmental factors to the primary concepts of anxiety and stress. Likewise, unique

TABLE 8 | Descriptive statistics and Kruskal–Wallis test with *post-hoc* analysis with Mann–Whitney *U*-test carried out on the sport variable in relation to the scores obtained in the ACSQ-E.

Test/Sport		X (SD)	χ^2	Sig.	Mann–Whitney	*Z*	Sig.
ACSQEec	Athletics	24.97 (6.98)	17.072	0.049*			
	Football	23.16 (7.06)			514.500	−2.528	0.011*
	Rugby	25.96 (4.46)			217.000	−2.350	0.019*
	Basketball	22.48 (5.61)			257.000	−3.387	0.001***
	Cycling/triathlon	23.33 (7.39)			76.000	−2.537	0.011*
	Martial arts	27.09 (4.55)			942.000	−2.639	0.008**
	Climbing	27.00 (6.84)					
	Swimming/water polo	25.33 (4.80)					
	Other sports	25.75 (5.87)					
ACSQEcr	Athletics	22.67 (5.17)	13.202	0.105			
	Football	19.73 (5.91)					
	Rugby	21.79 (4.94)					
	Basketball	20.07 (6.19)					
	Cycling/triathlon	23.17 (5.70)					
	Martial arts	23.80 (4.50)					
	Climbing	22.18 (6.40)					
	Swimming/water polo	22.50 (4.03)					
	Other sports	21.95 (5.99)					
ACSQEmw	Athletics	11.83 (5.95)	19.642	0.012*	260.500	−2.607	0.009**
	Football	9.89 (4.30)			224.000	−3.933	0.000***
	Rugby	15.96 (6.04)			416.000	−2.527	0.012*
	Basketball	12.72 (5.16)			138.500	−2.532	0.011*
	Cycling/triathlon	13.67 (5.77)			555.500	−2.138	0.033*
	Martial arts	12.31 (5.55)			1637.000	−2.153	0.031*
	Climbing	13.27 (6.84)			238.000	−1.972	0.049*
	Swimming/water polo	12.33 (7.94)			262.500	−2.439	0.015*
	Other sports	12.21 (5.74)			738.500	−2.727	0.006**
ACSQEbr	Athletics	10.17 (4.53)	9,376	0.312			
	Football	9.32 (3.57)					
	Rugby	10.04 (4.41)					
	Basketball	8.72 (4.43)					
	Cycling/triathlon	9.50 (4.54)					
	Martial arts	10.86 (4.42)					
	Climbing	9.91 (5.06)					
	Swimming/water polo	12.50 (3.01)					
	Other sports	10.58 (4.73)					
ACSQEss	Athletics	16.81 (6.48)	20,276	0.009**			
	Football	24.97 (6.98)					
	Rugby	23.16 (7.06)			453,500	−3.285	0.001***
	Basketball	25.96 (4.46)			265,500	−2.521	0.012*
	Cycling/triathlon	22.48 (5.61)			267,000	−3.373	0.001***
	Martial arts	23.33 (7.39)			85,000	−1.988	0.047*
	Climbing	27.09 (4.55)			80,000	−2.701	0.007**
	Swimming/water polo	27.00 (6.84)					
	Other sports	25.33 (4.80)					

X (SD) = Average (Standard Deviation); ACSQ, Approach to Coping in Sport Questionnaire; ACSQEec, Efficacy of emotional calm; ACSQEcr, Efficacy of cognitive restructuring; ACSQEmw, Efficacy of mental withdrawal; ACSQEbr, Efficacy of behavioral risk; ACSQEss, Efficacy of seeking social support.

confinement experiences and their associated psychological aspects were studied, although it did not offer conclusive results (17, 18).

To mitigate this bias, the protocol designed for this particular study included several other variables, from coping strategies, anxiety, perfectionism, to behavioral ones, such as the perception

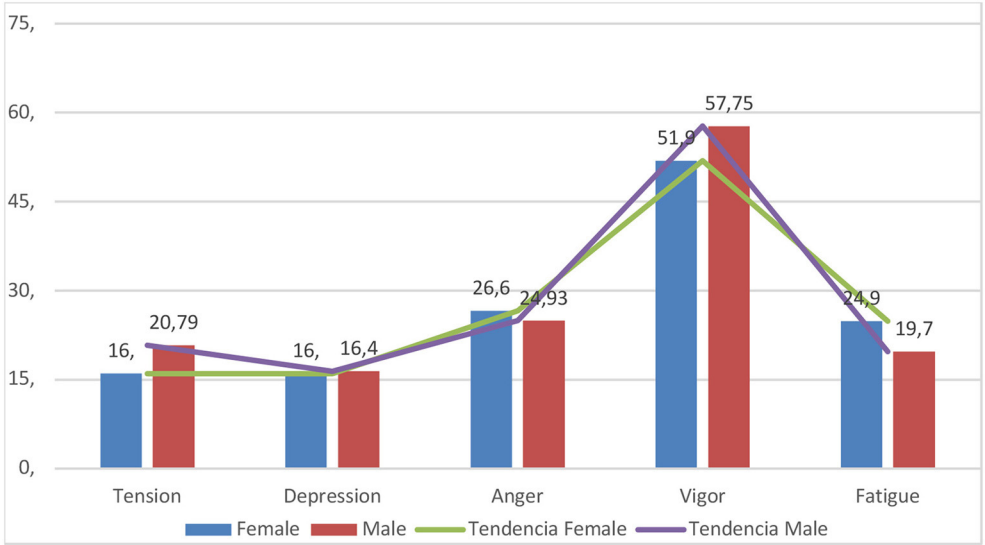

FIGURE 1 | Female and male profile in the POMS Scales.

of quality and quantity of sleep. In order to provide more control to the cross-sectional set of data, we collected it at least 15 days after the confinement began in each of the represented countries. At that time, there was no official plan to return to regular training sessions and hardly any expectation of resuming competitions at any sports level. In contrast, there was a continuous trickle of announcements informing cancellations and official postponements of major events, as was the worldwide emblematic case of the Tokyo Olympic Games (postponed to 2021).

Overall, the results of the present study show significant, negative correlations between the use of coping strategies in athletes, mainly on cognitive restructuring and emotional calming, and the emotional states commonly labeled as negative, such as depression, stress, anxiety, and fatigue. Although minor differences were found between males and females, the latter have shown higher scores in most psychological variables studied. The study of differences based on the sex variable is a relevant line of research where several studies have recently been carried out in this regard (45). However, it will be necessary to carry out specific investigations in this field of study. When it comes to sport disciplines, there are no significant differences among them except in the case of martial arts and the variable perfectionism, both subscales (adaptive perfectionism and maladaptive perfectionism). Implications of this last piece of data are discussed later in this section.

The results collected in this study have questioned in some way our expectations of the potential psychological impact on high-performing athletes, even when analyzed cross-culturally. According to our results, we can first observe that the main "clinical" values (a specific aspect of self-perception of mood), such as perceived stress, anxiety, or "depressive" symptoms, are relatively low. Within this triad, perception of stress was the highest value recorded, but without crossing the pathological range in any case. According to multiple previous studies, especially those related to anxiety and depression, a slight but significant difference can be observed toward higher values in female athletes than in male ones. The implication of this fact regarding performance is deeply debated by other authors and previous studies (46, 47).

In congruence with our results, Clemente-Suarez et al. (48) recently found little to no impact of confinement on the levels of anxiety of Olympic and Paralympic athletes. Authors attributed these phenomena to the larger experience of high-performance athletes in coping with competition-related anxiety and the higher cognitive resources professional athletes have. Similar results were observed in professional chess players by Fuentes-García et al. (49) despite the decrease of physical activity, which suggests that the cognitive resources of athletes potentially mitigate the negative effects of confinement. However, the same studies previously mentioned showed contradictory results in terms of academic levels and subjective perception of the confinement situation. On the one hand, chess players with university studies showed greater concern about the COVID-19 pandemic (49), while Olympic athletes with higher education showed more dissatisfaction with the confinement measures as a result of the COVID-19 pandemic (48).

In the second level of analysis related to mood states, it is interesting to observe that, using classic and contrasting measures with other studies (50) and, in the same line as findings discussed in the previous paragraph, the profiles match almost perfectly with the so-called "Iceberg profile" associated with high performance (38, 51, 52), despite a slight deviation corresponding to sex, a well-researched phenomenon reported in normal situations (53). It is highlighted that Vigor scores are the highest, while Depression scores are far below, clearly indicating that athletes have not experienced a decrease in their perception of energy in this situation of confinement (54).

Indeed, the results obtained regarding the athletes' perfectionism, as was indicated (21), supported all the results in the emotional spectrum. Nevertheless, the mean values of this trait never reached a considerable level among the athletes studied. However, inquiring into their possible connections, the values of the "good" and healthy perfectionism correlated positively with the mood state factors of Tension and Vigor. In a genuine sense, this fact adds no operative knowledge to our study, but there is no doubt that it enhances the facts found related to some authors advocating for more studies focused on the elite athletes' personality profiles, perhaps just one more brick on the wall. In another study carried out during confinement with a sample of Russian and Bulgarian university athletes, high values in adaptive perfectionism have been found, also significantly correlating with positive moods and adaptive coping strategies (55).

Complementarily and as expected theoretically, the "bad" perfectionism correlated negatively and significantly with Vigor and Tension POMS' factors, and positively with the negative ones, such as depression. This very same fact appears in all the aforementioned "clinical" factors considered (Depression, Anxiety, and Stress), and in trait anxiety personality scores. Overall, we must consider that lower values in our sample indicate that their skills or abilities were robust enough to overcome the constellation of black clouds surrounding this abnormal situation for elite athletes.

High-performance athletes follow strict training schedules to minimize the risk of making mistakes in competition; therefore, they train to perfect their technique and become meticulous with the "shape" of their execution. Although a certain degree of perfectionism is acceptable and expected in high-performance athletes, our results show interesting differences in perfectionism among sports disciplines. Martial arts (MA) is the sports discipline with the highest perfectionism (both adaptive and dysfunctional), followed by swimming. In practical terms, a single mistake in martial arts can lead to a knockout, which means losing the competition, and consequential physical risk (e.g., injury, death). Even if perfectionism may help reduce the chances of making mistakes, it also entails low tolerance for error, which, combined with inadequate stress-management mechanisms developed to avoid failure, could negatively impact performance. We are aware that this fact is beyond the scope of this paper; however, we find this evidence is worth mentioning.

Regarding the concept of perfectionism, the differences found according to sex are in line with the differences found in the variables anxiety and mood states already presented by other authors (56, 57). It is worth noting the importance of developing specific psychological tests to analyze relevant variables such as "Sports age category by sex" and "Sports age category" and may include other physical variables such as body mass index (BMI) in the study if needed, in line with previous research that refers to the existence of correlations between physical and psychological variables (44).

Therefore, we can assume that no indicator of psychological dysfunction has been observed in the sample of top-level athletes studied, supporting the findings of other confinement situations, in which only some signs of slowing cognitive processing appeared (58, 59) along with displacement of sleep phase, a typical syndrome of workers on night shifts, or the absence of perceived *zeitgebers* (17, 60).

Reinforcing this argument, another significant fact is related to the main behavioral indicator of this study: hours of sleep and the perceived quality of sleep. Sleep has been positively reported, meaning that athletes in the sample expressed a good quality of sleep and proper amounts of hours of sleep, allowing sleep to be perceived as restorative. However, we cannot conclude that alteration of circadian rhythms has not taken place since it remains plausible that athletes suffered changes in their bedtime routine and wake-up schedules because of the lack of objective measures regarding possible phase shifts.

In general terms, it can be observed that emotional state scores are low, interpreted as pleasant moods. Perhaps the "modulating" variable of these values is the use of coping strategies assessed with the ACSQ questionnaire, minimizing the negative psychological impact that the restrictive measures of this pandemic were expected to cause. Athletes mainly used coping strategies based on "emotional calming" and "search for social support," both perceived as effective strategies for emotional regulation (61). It deserves to be noted that these coping strategies are present in a cross-cultural sample of athletes, in comparison with the "textbook" behavioral approaches (62). Supporting this fact, it is also confirmed that the use of coping strategies correlates negatively and significantly with emotional states perceived as negative (anxiety, depression, stress) regardless of the low values of the latter.

Despite what is stated in the previous paragraph, and from the authors' point of view, these results, when critically analyzed, may "add fuel" to the already classic discussion between supporting the existence of a trait of hardiness (63), grit (64), or mental toughness (65), and supporting the relevance of training in coping strategies during the career of high-performance athletes. It is a very important discussion between modifiable and trainable variables or traits that can be otherwise detected and promoted (66, 67). From our data, a point appears in favor of the first of the assumptions, since the trait anxiety values have been remarkably low in the entire sample studied, with no screening being carried out before the inclusion of the practitioners. Certainly, this issue is more than susceptible to further research.

However, the authors acknowledge that even taking into account one of the most important limitations of the study, which we were not able to carry out because of technical and time constraints, the use of a mixed methodology (68) would allow for a more thorough analysis of the similarities of this confinement situation to some aspects of the psychological experiences of injured athletes. Perhaps the most relevant of these characteristics is the analysis of the psychological phases considered temporarily through which athletes with long-term injuries must face, especially those in which the time of return to practice is quite uncertain than more predictable forms of injury [very similar to those that appear in the well-known "psychological pain," for example, (69, 70)].

Although clearly anecdotal, we have a supporting statement by a well-known, high-level world athlete, Rafael Nadal, who, 3 weeks after confinement was declared in Spain, announced

through social media that he has decided to change his attitude toward the complicated situation caused by the coronavirus pandemic, confined as he is in Mallorca. "Good morning everyone, here we are. Difficult moments, however less time remains. As of today, I have tried to make a change, to be positive, and count the days that pass, because there is less time left. Greetings to you all and lots of encouragement, Vamos!" said the tennis player (71).

This line of thought leads us to agree with the reality that many of the previously studied confinement situations (58, 72) had very strict and elaborate work objectives, with previously established schedules that almost fully covered the subjects' time. Given this is definitely not the case (e.g., when athletes still do not have clear and reliable training plans to return to the practices and competitions), we must not forget the importance of self-established objectives (the data of the coping strategies of autonomy in decisions supports this suggestion), as it was highlighted by Glyn Roberts when arguing in favor of goal setting techniques in performance athletes (73). Speaking of which, this strategy has recently been confirmed to be effective in treating the psychological impact of medium- and long-term sports injuries (31).

Nevertheless, a specific conceptual model proposal has recently appeared ["Coronavirus Experience," CE, (74)] that is based on their previous Scheme of change for sport psychology practice model (75) that is not far from the model of response to sports injuries. CE is defined as an unpredictable, longitudinal, and multifaceted change event that consists of three phases: phase A, characterized by instability and confusion, emotional response, and appraisal; phase B, defined by coping and regression; and phase C, in which instability can increase or decrease depending on the previous sporting trajectory.

Continuing with the analogy of sports injuries, when most severe situations of confinement come to an end, it is likely that some athletes may develop post-traumatic stress symptoms (76), since, as in sports injuries, concerns over the injury process often arise, such as re-injury, when athletes resume training (29). Data analysis leads us to consider the possibility that fear of contagion or worry regarding another alarm state that involves confinement may be present in athletes once "normal" life is reestablished.

Therefore, the data collected does not corroborate the analogy with which this study began: to compare the possible psychological impact of the confinement due to the COVID-19 pandemic with the sports injury process. It is possible to establish that the athletes' experience of confinement did not suppose a significant, negative experience since they could compare their situation with the rest of the athletes from their respective sports, who were also confined, as proposed by the Social Comparison Theory (77). On the other hand, another possible explanation is that just as it happens when an athlete experiences an injury, confinement might have brought an opportunity to perceive a certain degree of control of the situation, including the implementation of strategies to improve psychological well-being that might promote the appearance of the phenomenon known as post-traumatic growth, consequently avoiding the impact of negative mood states on performance (78, 79).

Finally, it can be inferred from the results that sleep parameters, both objective and subjective, could constitute a light indicator to indirectly recognize the emotional state of athletes since it correlates negatively and significantly with emotional states perceived as negative (anxiety, depression, stress, fatigue, tension, and anger). As previously explained, sleep is a very sensitive factor to any sign of stress (80).

Limitations of the Study

This study has a series of evident limitations that we wish to indicate:

1. To ensure the quality of the data collected, we worked with those performance centers of our various research networks that (i) agreed to give a quick and supervised response from their athletes and (ii) had a sports psychology department that allowed us to homogenize the results obtained. This undoubtedly reduced the accessible sample, but we believe that it improved its reliability instead.
2. The models used determined the psychological variables used in the evaluation, which, in light of the results obtained, will require a major revision in the follow-ups of the sample that will be carried out in the phase of return to sports practice.
3. This study provides a relatively narrow time window, which may or may not be applicable to other, perhaps longer, lockdown situations.
4. Lastly, classical statistics have been used to analyze the collected data, but the experience of our group indicates that the use of Bayesian analysis, for example, to analyze the data probabilistically, may offer a complementary perspective that is more oriented to the possible prediction, than to a posteriori explanation.

Practical Applications

There seems to be a legitimate advantage in athletes who have seen their daily life altered and their future career affected, like the rest of mankind, but somehow managed to effectively cope with the psychological impact that was expected from an unprecedented crisis like this one. Since this research sample was accessed through the solidarity and joint efforts of an international network of psychology departments that are part of high-performance institutions, it could be assumed that most athletes in this sample have had some sort of contact with their respective psychology department throughout their professional careers. Also, the technical staff could have acted as an intermediator of psychological techniques and strategies that protect athletes from "giving up" in the face of challenges, even if some athletes in the sample might have not interacted directly with the sports psychology personnel in their institutions. If this interpretation proves accurate, high-school teachers and university professors, among other instructor figures, could be trained in coping strategies and crisis management to serve as role models for younger generations. Academic and institutional networks as the one that allowed this quick response to research obligations should be encouraged to provide high-speed examination of potential dangers.

Similar to the pathogenesis of COVID-19, psychological disorders are "invisible" to the general population until the symptomatology has reached a point where treatment is required. In the same way governments and institutions could have prevented the rate of dissemination of the coronavirus, psychological consequences of periods of crisis might be prevented as well. Protective factors against psychological disorders and promotion of well-being seem to have a strong relationship with the nature in which athletes have been trained to cope with stressful events. Our findings shed some light on the underlying psychological mechanisms that function as protective factors of negative mental states triggered by this emergency crisis. Ideally, preventive programs based on coping strategies and healthy habits could be transferred to the general public in organized sports and leisure activities. A more realistic approach could involve psychological training to those sectors of the community at risk of psychological dysfunction.

Also evident from the findings is the fact that, although the values of the "negative" psychological variables do not reach critical levels, their correlation with the athletes' sleep indicators is interesting and should not fall on deaf ears. The current facilities for collecting data in a mixed way (subjective and objective, such as by accelerometry) should be a standard monitoring practice—in the preventive line indicated in the previous point—within the protocols applied to high-performance athletes.

The results obtained in perfectionism follow the same line of other variables included in this research: there are differences in the sex variable. As already highlighted in previous research (56, 57), it is needed to develop future research examining certain variables of the athlete's personality and strictly sports-related, such as sports age category, sports category by sex, and category by weight, among other variables.

Future Lines of Research

Future lines of research should consider a longitudinal analysis of how these psychological variables develop from the imposition of confinement to the reestablishment of normality.

The fact that only psychological variables have been considered from a strictly quantitative perspective is a limitation, mainly because in those studies developed where there is limited bibliographic review on the subject studied, the most advisable thing is to carry out a mixed analysis, both quantitative and qualitative. In novel research topics, it is relevant to collect data from the subjects through non-standardized or open-response techniques. Therefore, the development of mixed models (qualitative–quantitative) is recommended, although the quantitative information included in this study is relevant from a theoretical and empirical perspective.

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 University of Trás-os-Montes e Alto Douro (UTAD, Portugal) Ethical Committee, code 23/DOC20/CE/UTAD (27/06/2018). The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

AG-M and AO designed the study as a whole. AO designed the questionnaires' protocol and FL adapted them in the online version. FL, AG-M, and AN prepared the draft of the introduction, with all the co-authors contributing to the revision and final version. FL carried out the data collection. FV, VG-E, RR-B, and AN were in charge of the statistical analyzes. FL, AO, and AG-M prepared the first draft of the discussion, with all the co-authors contributing to the final version and revisions. All authors contributed to the article and approved the submitted version.

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Clinical Characteristics and Diagnostic Challenges of COVID−19: An Update From the Global Perspective

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Clinical characteristics are essential for the correct diagnosis of diseases. The current review aimed to summarize the global clinical characteristics of the COVID-19 patients systematically and identify their diagnostic challenges to help the medical practitioners properly diagnose and for better management of COVID-19 patients. We conducted a systematic search in PubMed, Web of Science, Scopus, Science Direct, and Google Scholar databases for original articles containing clinical information of COVID-19 published up to 7th May 2020. Two researchers independently searched the databases to extract eligible articles. A total of 34 studies from 8 different countries with 10889 case-patients were included for clinical characteristics. The most common clinical symptoms were cough 59.6, fever 46.9, fatigue 27.8, and dyspnea 20.23%. The prominent laboratory findings were lymphocytopenia 55.9, elevated levels of CRP 61.9, aspartate aminotransferase 53.3, LDH 40.8, ESR 72.99, serum ferritin 63, IL-6 52, and prothrombin time 35.47%, and decreased levels of platelets 17.26, eosinophils 59.0, hemoglobin 29, and albumin 38.4%. CT scan of the chest showed an abnormality in 93.50% cases with bilateral lungs 71.1%, ground-glass opacity 48%, lesion in lungs 78.3%, and enlargement of lymph node 50.7%. Common comorbidities were hypertension, diabetes, obesity, and cardiovascular diseases. The estimated median incubation period was 5.36 days, and the overall case fatality rate was 16.9% (Global case fatality outside China was 22.24%: USA 21.24%, Italy 25.61%, and others 0%; whereas the case fatality inside the Hubei Province of China was found to be 11.71%). Global features on the clinical characteristics of COVID-19 obtained from laboratory tests and CT scan results will provide useful information to the physicians to diagnose the disease and for better management of the patients as well as to address the diagnostic challenges to control the infection.

Keywords: clinical characteristics, SARS coronavirus (SARS-CoV-2), novel coronavirus diseases (COVID-19), diagnostic challenges, systematic review

INTRODUCTION

In late December 2019, the Chinese government officially announced the novel coronavirus (2019-nCoV) outbreak in Wuhan. To contain the virus, Wuhan and eventually the whole Hubei province was put into massive lockdown. The virus, which was later named SARS-CoV-2, got out anyway in other countries of the world. Although limited to China initially, the novel strain of the coronavirus being super contagious compared to the previously known strains has quickly spread across the globe. WHO declared the virus as a global pandemic on March 11, 2020. By then the epicenter of the virus had been shifted from China to Eastern Europe followed by the USA. As updated by the World Health Organization (WHO) on May 28, 2020, the COVID-19 outbreak has reached 213 countries and territories worldwide with 5,593,631 confirmed cases and 353,334 deaths (1).

The virus is yet to be peaked in most of the countries that got infected, and on top of that public health experts have warned that the newest epicenter of the virus could be shifted to the densely populated south and south-east Asia (2). Despite the concerted efforts by every stakeholder, tens of thousands of new cases of COVID-19 have been arising every day. Suddenly the world is facing a severe crisis of ICU beds and artificial respirators. From frontline doctors and nurses to pharmacists, all healthcare professionals are now facing an intense workload. It would take years to develop a vaccine for this novel coronavirus considering the fact that we are yet to see any effective vaccines for the previous coronavirus outbreaks (e.g., SARS & MERS) (3). Besides no well-established and specific anti-viral drug for this virus is available right now. Under this context, it is essential to better elucidate the clinical characteristics of the virus for a clear understanding of the disease and proper management of the patients by the health care providers. Moreover, the diagnosis also coincides with many challenges that include false test results, sampling errors, asymptomatic cases, insufficient testing facilities and lack of consciousness among mass people. Due to higher cost for RT-PCR testing facilities and lack of skilled manpower, many countries could not make it available across every regions, specially remote areas. In addition, due to the unwillingness of the patients arising out of fear of contamination and tendency to avoid procedural complexities, many suspected cases remain undiagnosed. To get the actual scenario of the prevalence and incidences as well as for the proper management of COVID-19 addressing all the diagnostic challenges is of utmost importance. This systematic review presents the summary of published reports up to 7th May 2020 on the clinical characteristics of COVID-19 for a better understanding of the frontiers team (especially to the medical doctors) involved in the treatment and management of COVID-19 patients. Additionally, the challenges for the prevention, treatment and management of COVID-19 have been discussed in the review.

Abbreviations: COVID-19, Coronavirus diseases; Novel coronavirus 2019, 2019n-CoV; SARS-CoV-2, Severe Acute Respiratory Syndrome coronavirus 2; WHO, World Health Organization; MERS-CoV, Middle East Respiratory Syndrome Coronavirus; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; CRP, C-Reactive Protein; ICU, Intensive Care Unit; MINORS, Methodical Index for Non-randomized Studies; CDC, Center for Disease Control and Prevention; LDH, Lactate dehydrogenase; INF, Interferon; TNF, Tumor necrosis factor; ESR, Erythrocyte sedimentation rate; ARDS, Acute respiratory distress syndrome; IL, *Interleukins; RT-PCR,* Reverse transcription polymerase chain reaction; BUN, Blood urea nitrogen; CT Scan, computed tomography scan.

TABLE 1 | Search strategy.

Database	PubMed, Web of Science, Science Direct, Scopus, Wiley Online Library, Google Scholar
Articles included	Articles published up to 7th May 2020 were included in the study
Keywords used for the search	Clinical characteristics, Clinical features, Clinical symptoms, Findings, Diagnosis, Novel coronavirus 2019, COVID-19, SARS-CoV-2, Challenges
Language used	English
Inclusion criteria	i) Articles containing clinical characteristics of COVID-19 ii) Original articles iii) Case series iv) Published in English language
Exclusion criteria	i) Reviews ii) Meta-analysis iii) Case reports iv) Expert opinions v) Newspaper articles vi) Commentaries vii) Prospective viii) Correspondence

METHODOLOGY

Protocol

This systematic review protocol is designed following the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) Statement-2009 (4).

Literature Search Strategy

We conducted a systematic and comprehensive search of important online databases—PubMed, Web of Science, Science Direct, Scopus, Wiley Online Library, and Google Scholar. The articles published and available online up to 7th May 2020 were considered to include in this study. The keywords used for the searches are included in the **Table 1**. All the searches were done in the English language only.

Eligibility Criteria

The search results were subjected to a range of inclusion and exclusion criteria, as listed in **Table 1**. The inclusion criteria mainly include original peer-reviewed articles (articles based on direct clinical data of the COVID-19 patients in various clinical settings). We opted out case reports, meta-analyses, expert opinions, newspaper articles, and commentaries.

Screening and Study Selection

Following the eligibility criteria, two researchers were collaboratively involved in the screening and selection procedure of the articles of interest. The screening procedure was based on the PRISMA-2009 flow diagram, as presented in **Figure 1**. To

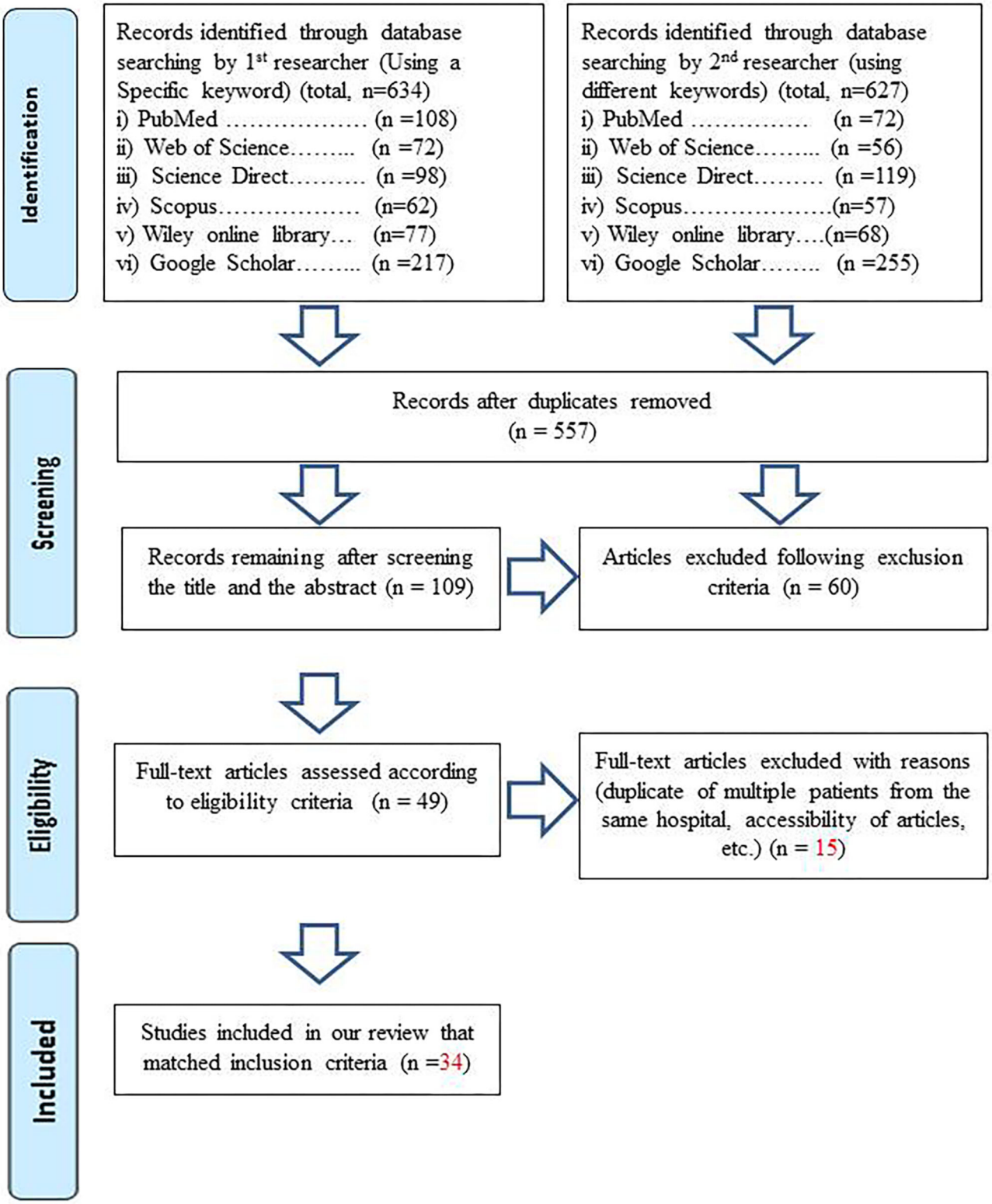

FIGURE 1 | PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) for the identification and screening of articles to include in the study according to eligible criteria.

present a global overview of the clinical trends of COVID-19, we have selected original studies from different hospitals across the world. The time period of each study and other related data were carefully screened to avoid any duplicates. Multiple studies from the same institutions were mainly analyzed and included avoiding data duplication. Observational studies

TABLE 2 | Studies included in the review.

Region	References	Journal	Hospital/Region	Total case patients *(n)*
Case studies in China (inside Hubei)	(5)	Allergy: European Journal of Allergy and Clinical Immunology	No.7 hospital of Wuhan	140
	(6)	SSRN Electronic Journal	Zhongnan Hospital of Wuhan University	221
	(7)	Clinical Infectious Diseases	Union hospital in Wuhan	69
	(8)	The Lancet	Outpatients of 30 hospitals in Wuhan designated for covid-19 treatment	124
	(9)	The Lancet Respiratory Medicine	Wuhan Jin Yin-tan hospital	52
	(10)	SSRN Electronic Journal	Renmin Hospital of Wuhan University	101
	(11)	Journal of the American Medical Association	Department of Critical Care Medicine, Zhongnan Hospital of Wuhan University	138
	(12)	The Lancet	Jin Yin-tan Hospital, Wuhan	41
	(13)	Chinese Medical Journal	9 tertiary hospitals of Hubei Province	137
	(14)	The Lancet	Jinyintan Hospital in Wuhan, China	99
	(14)	MedRxiv	Mobile Cabin Hospital of Optical Valley and Tongji Hospital of Huazhong University of Science and Technology in Wuhan	534
Case studies in China (outside Hubei)	(15)	Investigative Radiology	N/A	80
	(16)	Journal of Infection	57 hospitals across Beijing	262
	(17)	Journal of Infection	Wenzhou central hospital,Ruian people's hospital, Yueqing people's hospital	149
	(18)	China	Suzhou Fifth People's Hospital	69
	(19)	Clinical infectious diseases.	3 Grade IIIA hospitals of Jiangsu	80
	(20)	The BMJ	7 hospitals in Zhejiang	62
	(21)	Journal of Infection	Shanghai Public Health Clinical Center (SPHCC), Shanghai, China	249
	(22)	European Journal of Radiology	6 hospitals across Anhui province	73
	(23)	European journal of nuclear medicine and molecular imaging	Guangzhou Eighth People's Hospital	90
	(24)	European Respiratory Journal	First Affiliated Hospital of Zhengzhou University	18
	(14)	MedRxiv	5 hospitals across Zhejiang province	91
	(25)	European Review for Medical and Pharmacological Sciences	North Hospital of Changsha First Hospital	161
Global case studies outside China	(26)	MedRxiv	N/A	12
	(27)	Jama	Evergreen Hospital, USA	21
	(28)	Jama	12 hospitals in New York City, Long Island, and Westchester County, New York, within the Northwell Health system	5700
	(29)	Jama	72 hospitals of Lombardy ICU Network, Milan, Italy	1591
	(30)	The Lancet	N/A	17
	(31)	Journal of the American Medical Association	National Centre for Infectious Diseases, Singapore General Hospital, Changi General Hospital, and Sengkang General Hospital, Singapore	18
	(32)	International Journal of Biological Sciences	Centro Hospitalar Conde de São Januário, Macau	10
	(33)	Influenza and Other Respiratory Viruses.	Castle Hill Hospital, UK	68
	(34)	Travel Medicine and Infectious Disease	The Mediterranean Infection University Hospital Institute, France	280
	(35)	The Lancet	Self-Defense Forces Central Hospital, Japan	104
	(36)	Osong Public Health Res Perspect	N/A	28

reporting desired clinical parameters like symptoms, laboratory characteristics, and risk factors were included in the review. Case reports were discarded because they do not add significant value when stacked up against case studies. The whole procedure was overseen by two experienced researchers (an expert clinician and supervisor of this project).

Data Extraction and Entry

The full text of the selected articles was thoroughly examined for relevant data of this systematic review that included the author's name, name of the hospital where the research had been conducted, number of case-patients and their clinical features, etc. We divided the case studies into three categories, namely, (i) Case studies in Hubei province only (China) (ii) Case studies in China (outside Hubei) (iii) Global case studies outside China (**Table 2**). We recorded and summarized the extracted data in Microsoft Excel. The whole process was carefully monitored and adjusted, had there been any discrepancies, by two independent expert researchers, including a clinician.

TABLE 3 | Quality ratings of included studies according to MINORS*.

	References	①	②	③	④	⑤	⑥	⑦	⑧	Total score	Overall rating
Case studies in China (inside Hubei)	(5)	2	2	2	2	2	1	2	1	14	Good
	(6)	2	2	2	2	2	0	2	2	14	Good
	(7)	2	2	2	2	2	1	2	0	13	Good
	(8)	2	2	2	2	2	1	2	1	14	Good
	(9)	2	2	2	2	2	2	2	0	14	Good
	(10)	2	2	2	2	2	0	1	1	12	Satisfactory
	(11)	2	2	2	2	2	0	2	1	13	Good
	(12)	2	2	2	2	2	2	1	0	13	Good
	(13)	2	2	2	2	2	1	2	1	14	Good
	(14)	2	2	2	2	2	2	2	0	14	Good
	(14)	2	2	2	2	2	0	2	2	14	Good
Case studies in China (outside Hubei)	(15)	2	2	2	2	2	2	1	0	13	Good
	(16)	2	2	2	2	2	1	2	2	15	Excellent
	(17)	2	2	2	2	2	1	1	1	13	Good
	(18)	2	2	2	2	2	1	1	0	12	Satisfactory
	(19)	2	2	2	2	2	2	2	0	14	Good
	(20)	2	2	2	2	2	2	2	0	14	Good
	(21)	2	2	2	2	2	0	2	2	14	Good
	(22)	2	2	2	2	2	1	2	0	13	Good
	(23)	2	2	2	2	2	2	2	0	14	Good
	(24)	2	2	2	2	2	1	2	0	13	Good
	(14)	2	2	2	2	2	1	2	1	14	Good
	(25)	2	2	2	2	2	1	1	1	13	Good
Global case studies outside China	(26)	2	2	2	2	2	0	1	0	11	Satisfactory
	(27)	2	2	2	2	2	1	2	0	13	Good
	(28)	2	2	2	2	2	1	1	0	12	Satisfactory
	(29)	2	2	2	2	2	1	1	0	12	Satisfactory
	(30)	2	2	2	2	2	0	1	0	11	Satisfactory
	(31)	2	2	2	2	2	2	2	0	14	Good
	(32)	2	2	2	2	2	1	1	0	12	Satisfactory
	(33)	2	2	2	2	2	1	2	0	13	Good
	(5)	2	2	2	2	2	2	1	2	15	Excellent
	(6)	2	2	2	2	2	0	1	1	12	Satisfactory
	(7)	2	2	2	2	2	1	2	0	13	Good

**①A clearly stated aim; ②Inclusion of consecutive patients; ③Prospective collection of data; ④Endpoints appropriate to the aim of the study; ⑤Unbiased assessment of the study endpoint; ⑥Follow-up period appropriate to the aim of the study; ⑦Loss to follow up less than 5%; ⑧Prospective calculation of the study size.*

The items are scored 0 (not reported), 1 (reported but inadequate) or 2 (reported and adequate). The global ideal score being 16 for non-comparative studies. The scoring was characterized as follows: 15-16, Excellent, 13-14, good, 11-12, Satisfactory, 9-10, Weak, <9, Unacceptable.

TABLE 4 | Demographic and epidemiologic features.

Region	Global (Excluding China)	Only Hubei (China)	Outside Hubei (China)	Total/Mean	Percentage (%) on total
Number of studies	11	11	12	34	–
Number of case patients	7849	1656	1384	10889	–
Demographic features					
Median Age (in years) (Range)	51.3 (0.5–84)	55.6 (16–87)	45.1 (1–94)	49.8 (0.5–94)	–
Sex					
Male	5000/7849	867/ 1656	700/1384	6567/10889	60.3
Female	2849/7849	789/1656	684/1384	4422/10889	39.7
Smoking (current)	577/3683	38/767	26/80	641/4530	14.2
Severity on diagnosis/admission					
Severe/ ICU/ critical patient	2042/4640	212/592	154/1214	2408/6446	37.4
Non-severe/non-ICU patient	2598/4640	380/592	1060/1214	4038/6446	62.6
Epidemiological Data (Transmission Pattern)					
Travel/residence history in Wuhan	53/365	–	411/816	464/1181	39.2
Contact with people from Hubei Province/stay in Hubei	–	–	210/238	210/238	88.2
Contact with COVID-19 patient in family or community	10/292	395/1486	275/649	680/2427	28
No obvious contact history with COVID-19 patient	–	–	8/254	417/667	62.5
Resident of the infected area	280/347	–	137/320	19/308	6.2
Contact with person with fever	19/308	–	–	159/1486	10.70
Infection during hospitalization	–	159/1486	–	43/1486	2.89
Hospital staff	-	43/1486	-	43/1486	2.89
Huanan seafood wholesale market exposure	-	43/1486	-	118/1486	7.94
No obvious contact history with COVID-19 patient	-	118/1486	-	8/254	3.1

Quality Assessment

The validation instrument called "methodological index for non-randomized studies" (MINORS) was used to assess the methodological quality and bias risk of our data (**Table 3**). It is based on eight criteria for non-comparative clinical reviews, and the global acceptance score had been set to 16 (37).

RESULTS

Study Selection and Characterization

The result of our screening and selection protocol has been shown in **Figure 1**. We initially retrieved 557 articles after removing duplicates that were later shortened to 34 following the screening protocol for the selection of eligible articles. The relevant information of these finally selected articles along, with associated hospital or medical institution names, has been given in **Table 2**. The total number of case-patients in these studies is 10,889. We retrieved a total of 176 variables regarding these patients that included demographic features, laboratory findings, symptoms, comorbidities, etc. (**Tables 4**–**9**) from these articles for the review. Most of the studies were from China, particularly from the Hubei province. For convenience, the data were split into three groups as mentioned earlier. But we inferred our final results after combining them into a single dataset.

Demographic Characteristics

The mean age of the case population was 50.6 years (0.5–94). The percentage of the male population was 60.3% (6567/10889), whereas that of the female population was 39.7% (4322/10889). The case population includes Asian, European and North American patients. Around 14.2% (641/4530) of them were found to be current smokers (**Table 4**).

Clinical Symptoms

The most frequently observed clinical symptoms (**Table 5**) of the COVID-19 patients were cough/ dry cough 59.6 (2146/3598), fever 46.9 (4342/9242), fatigue 27.8 (1000/3598), dyspnea/shortness of breath 20.23 (728/3598), muscle ache/myalgia 12.64 (455/3598), diarrhea 11.95 (430/3598), headache 10.8 (389/3598), anorexia 9.9 (356/3598), sore throat 7.5 (270/3598), expectoration 7.48 (269/3598), upper airway congestion 6.67 (240/3598), and rhinitis 5.86 (211/3598). The lesser observed symptoms were pneumonia 2.89% (104/3598), abdominal Pain 2.31% (83/3598), nausea/vomiting 2.28 (82/3598), pharyngitis/pharyngalgia 1.83 (66/3598), chest pain 1.81 (65/3598), dizziness 1.56 (56/3598), chest tightness 1.25 (45/3598), malaise 0.61 (22/3598), chill 0.78 (28/3598), hemoptysis 0.42 (15/3598), heart palpitations 0.28 (10/3598), confusion 0.25 (9/3598), ARDS 0.22 (8/3598), belching 0.2 (7/3598), back discomfort 0.1 (3/3598), and arthralgia 0.03

TABLE 5 | Clinical symptoms of COVID-19 patients.

Symptoms	Global (Excluding China) (symptomaic patients/case patients)	Only Hubei (China) (symptomatic patients/case patients)	Outside Hubei (China) (symptomatic patients/case patients)	Patients with symptoms/Total no. of case patients	Percentage (%) on total
Cough/dry cough	338/558	1016/1656	792/1384	2146/3598	59.6
Fever	2020/6202	1200/1656	1122/1384	4342/9242	46.9
Highest temperature (°C)	37.34 (35.3–39.2)	38.5 (37.6–39.14)	39.0(36.99–41)	38.3 (35.3–41)	–
Fatigue	3/558	679/1656	318/1384	1000/3598	27.8
Dyspnea/shortness of breath	70/558	542/1656	116/1384	728/3598	20.23
Muscle ache/myalgia	75/558	287/1656	93/1384	455/3598	12.64
Diarrhea	29/558	284/1656	117/1384	430/3598	11.95
Headache	76/558	189/1656	124/1384	389/3598	10.8
Anorexia	–	305/1656	51/1384	356/3598	9.9
Sore throat	84/558	114/1656	72/1384	270/3598	7.5
Rhinitis/rhinorrhea	186/558	–	25/1384	211/3598	5.86
Upper airway congestion	22/558	218/1656	–	240/3598	6.67
Expectoration/sputum production	–	95/1656	174/1384	269/3598	7.48
Pneumonia	21/558	7/1656	76/1384	104/3598	2.89
Abdominal pain	2/558	74/1656	7/1384	83/3598	2.31
Nausea and vomiting	8/558	59/1656	15/1384	82/3598	2.28
Pharyngitis/pharyngalgia	–	57/1656	9/1384	66/3598	1.83
Chest pain	35/558	17/1656	13/1384	65/3598	1.81
Dizziness	2/558	18/1656	36/1384	56/3598	1.56
Chest tightness	–	–	45/1384	45/3598	1.25
Malaise	22/558	–	–	22/3598	0.61
Chill	1/558	–	27/1384	28/3598	0.78
Hemoptysis	–	9/1656	6/1384	15/3598	0.42
Heart palpitations	–	10/1656	–	10/3598	0.28
Confusion	–	9/1656	–	9/3598	0.25
ARDS	–	–	8/1384	8/3598	0.22
Belching	–	7/1656	–	7/3598	0.2
Back discomfort	–	–	3/1384	3/3598	0.1
Arthralgia	–	1/1656	–	1/3598	0.03
Others	3/558	–	–	3/3598	0.1
Asymptomatic	4/558	–	16/1384	20/3598	0.56

(1/3598). A minor portion, 0.56% (20/3598), of the patients of the case population was found to be asymptomatic as well.

Laboratory Findings

The laboratory findings (**Table 6**) included RT-PCR assay results, routine blood tests, and tests for various other blood-based biomarkers (e.g., coagulation factors, infection-related biomarkers, etc.). Among 8,650 patients, 8,253 (95.4%) were tested positive for SARS-CoV-2 in RT-PCR assay. Among the blood-based examinations, lymphocytopenia 55.9% (4177/7470) was most frequently observed. Other major lab findings include elevated levels of C-reactive protein 61.9% (830/1340), Aspartate aminotransferase 53.3% (3481/6537), Alanine aminotransferase 35.64% (2318/6503), lactate dehydrogenase 40.8% (392/973), ESR 72.99% (173/237), serum ferritin 63% (62/99), Interleukin-6 (IL-6) 52% (51/99), prothrombin time 35.47% (102/286), and D-dimer 28.06% (179/638). On the contrary, the levels of platelets 17.26% (160/927), eosinophils 59.0% (121/205), hemoglobin 29% (125/431), albumin 38.4% (187/487), and blood urea nitrogen (BUN) 20.9% (19/91) were reported to decrease.

Radiological Findings

The results of chest CT scans (**Table 7**) showed abnormality of at least one kind in 93.5% (1668/1785) of the case-patients. The predominant abnormality had been bilateral lungs found in 71.1% (1581/2223) of the patients. Other significant findings of the lung characteristics from CT scan result include Ground-glass opacity 48% (432/900), consolidation 21.88% (140/640), pleural effusion 20.6% (195/947), the lesion in lung 78.3% (180/230), enlargement of lymph node 50.7% (153/302), thickening of bronchial wall 30.3% (80/264), thickening of lung texture 84.9% (62/73), and thickening of Interlobular septal 47.1% (80/170).

TABLE 6 | Laboratory findings and physical examinations.

Laboratory findings	Global (Excluding China)	Only Hubei (China)	Outside Hubei (China)	Total/Mean (Range)	Total percentage (%)
SARS-CoV-2 RT-PCR assay + (%)	7147/7330	928/1139	178 / 181	8253/8650	95.4
Blood Routine					
Leukocytes ($\times 10^9$/L, normal range 3.5–9.5)	8.3 (1.7–16.9)	6.1 (3.2–13)	4.7 (2.48–6.95)	6.4 (1.7-16.9)	-
Increased–No./total No. (%)	5/68	103/702	38/637	146/1407	10.38
Decreased–No./total No. (%)	21/114	206/702	119/637	346/1453	23.81
Neutrophils ($\times 10^9$/L, normal range 1.8–6.3)	2.65 (0.7–4.5)	4.6 (1.62–9.13)	3.3 (2.0–5.9)	3.52 (0.7-9.13)	-
Increased–No. /total No. (%)	-	84/290	64/485	148/775	19.1
Decreased–No. /total No. (%)	1/10	-	52/ 389	53/399	13.28
Lymphocytes ($\times 10^9$/L, normal range 1.1–3.2)	1.1 (0.2–1.7)	0.85 (0.6–1.46)	1.1 (0.4–1.66)	1.02 (0.2–1.7)	–
Increased–No./total No. (%)	-	-	70/222	70/222	31.5
Decreased–No./total No. (%)	3407/5745	551/1017	219/708	4177/7470	55.9
Platelets ($\times 10^9$/L, normal range 125–350)	158 (116–217)	176 (127–263)	173.9 (78.25–238)	169.7 (78.25–263)	-
Increased–No./total No. (%)	-	14/223	14/382	28/605	4.63
Decreased–No./total No. (%)	12/121	37/263	111/543	160/927	17.26
Monocytes (%, normal range 3–8)	2.83 (0.5–12.22)	0.37 (0.23–0.55)	0.44 (0.27–0.7)	1.21 (0.23–12.22	–
Increased–No./total No. (%)	-	-	41/251	41/251	16.3
Decreased–No. /total No. (%)	-	-	1/171	1/171	0.6
Hemoglobin (g/L, normal range 130–175)	12.67 (8–17.2)	130 (118–146)	131.8 (120–152.3)	92.5 (8–152.3)	-
Decreased–No./total No. (%)	–	50/99	75/332	125/431	29
Eosinophils ($\times 10^9$/L, normal range 0.02–0.52)	–	0.011 (0.00–0.05)	–	0.011 (0.00–0.05)	–
Decreased–No./total No. (%)	–	121/205	–	121/205	59.00
Blood biochemistry					
Aspartate aminotransferase (U/L, normal range 15–40)	33.33 (9.0–71)	31.2 (22–48)	25.3 (15.75–39)	29.9 (9.0–71)	–
Increased–No. /total No. (%)	3283/5700	69/209	129/628	3481/6537	53.3
Decreased–No./total No. (%)	–	–	19/153	19/153	12.4
Alanine aminotransferase (U/L, normal range 9–50)	36.6 (19.3–55.0)	28.6 (16–53)	22.7 (12.0–39.5)	29.3 (12.0–55.0)	–
Increased–No. /total No. (%)	2197/5769	51/168	70/566	2318/6503	35.64
Decreased–No./total No. (%)	–	–	8/240	8/240	3.3
Albumin (g/L, normal range 35–57)	–	32.04	39.8(5.48–46.3)	35.92(5.48–46.3)	–
Increased–No./total No. (%)	–	–	3/240	3/240	1.3
Decreased–No./total No. (%)	–	97/98	90/389	187/487	38.4
Creatinine (μmol/L, normal range 64–104)	83.3 (7.6–343.1)	72.7 (56–87)	69.4(51.28–90)	75.13(7.6–343.1)	–
Increased–No./total No. (%)	–	7/140	116/451	123/591	20.8
Decreased–No./total No. (%)	–	21/99	81/371	102/470	21.7
Serum Creatinine Kinase (mmol/L, normal range 40–200)	117.67 (45–1290)	90.1 (51–219)	81.5(40.5–191)	96.42(40.5–1290	–
Increased–No./total No. (%)	1/10	30/199	35/291	66/500	13.2
Decreased–No./total No. (%)	–	23/99	76/211	99/310	31.9
Lactate dehydrogenase (U/L, range12–250)	381.3 (206.5–796)	266.8 (174–447)	246.02(94.5–554)	298.04(94.5–796)	–
Increased–No. /total No. (%)	33/114	184/338	179/521	392/973	40.8
Glucose (mmol/L; normal range 3·9–6·1)	–	8.2	6.3(1.97–7.7)	7.3(1.97–7.7)	–
Increased–No./total No. (%)	–	51/99	78/229	129/328	39.3
Decreased–No./total No. (%)	–	–	1/149	1/149	0.7
Total Bilirubin (μmol/L, normal value 4.0–17.1)	8.13 (4–18.81)	14.8 (8.4–31.7)	9.3 (5.4–15.43)	10.74 (4–31.7)	–
Increased–No. /total No. (%)	–	18/99	25/459	43/558	7.71
Decreased–No./total No. (%)	–	–	9/218	9/218	4.1

(Continued)

TABLE 6 | Continued

Laboratory findings	Global (Excluding China)	Only Hubei (China)	Outside Hubei (China)	Total/Mean (Range)	Total percentage (%)
Blood urea nitrogen (mmol/L; normal range 2.8–7.6)	–	6.6 (3.4–13.03)	4.4(3.3–5.9)	5.5(3.3–13.03	–
Increased–No. /total No. (%)	–	–	3/171	3/171	1.8
Decreased–No./total No. (%)	–	–	19/91	19/91	20.9
Lactate concentration (mmol/L normal range 0.5–1.6)	1.5 (1.1–2.1)	1.5 (0.7–3.2)	1.4(1.1–2.1)	1.47(0.7–3.2)	–
Hematocrit (%, normal range M: 40–50, F: 37–48)	12.67 (8–17.2)	40.3 (36.5–43.5)	–	40.3 (36.5–43.5)	–
Coagulation function	–	–	–	–	–
Activated partial thromboplastin time in seconds (normal range 21–37)	–	27.3	26(17.2–38.27)	26.65 (17.2–38.27)	–
Increased–No. /total No. (%)	–	6/99	40/149	46/248	18.55
Decreased–No./total No. (%)	–	16/99	2/80	18/179	10.05
Prothrombin time in seconds (9.4–12.5)	–	12.6 (10.1–17.39)	11.5(9.3–13.6)	12.05 (9.3–17.39)	–
Increased–No. (>1.00)/total No. (%)	–	85/137	17/149	102/286	35.47
Decreased–No./total No. (%)	–	30/99	7/229	37/328	11.28
D–dimer (ng/L; normal range 0–500)	438 (262–872)	712.2 (100–2800)	368 (106–2400)	506.1 (100–2800)	–
Increased–No./total No. (%)		113/249	56/389	179/638	28.06
Infection–related biomarkers					
Procalcitonin (PCT) (ng/ml, normal range 0–0.1)	0.7 (0.03–9.59)	0.24	0.35(0–2.6)	0.43(0.0–9.59)	–
Increased–No. (>1.00)/total No. (%)	–	125/677	58/329	183/1006	18.2
ESR (mm/h normal range 0–20)	–	20 (8–31)	32.95(9–90)	26.5(8–90	–
Increased–No. (≥20)/total No. (%)	–	114/157	59/80	173/237	72.99
Serum ferritin (ng/mL, normal range 21.0–274.7)	–	808.7	–	808.7	–
Increased–No. /total No. (%)	–	62/99	–	62/99	63.00
C–reactive protein (CRP) (mg/L, normal range <10)	30.94 (0.1–97.5)	37.8 (6.78–67.4)	10.3 (1.8–50.61)	26.35(0.1–97.5)	–
Increased–No./total No. (%)	17/96	379/508	434/736	830/1340	61.9
Interleukin−6 (pg/mL, normal range 0.0–7.0)	–	–	0/181	7.9 (6.1–10.6)	–
Increased–No. /total No. (%)	–	7.9 (6.1–10.6)	–	51/99	52.00
Decreased–No./total No. (%)	–	51/99	–	0/181	0
Physical Examinations					
Respiratory rate, breaths/min. (Normal range: 12–20)	16.33 (13.5–21)	20.5 (19–30)	22.5	19.8(13.5–30.0)	–
O_2 saturation,% (Normal range: 75–100)	96.9 (91–100)	–	91.78	94.34(91–100)	–
Mean Systolic pressure (mmHg) (Normal range−100)	128 (96–180)	91.5 (83–105)	114.5 (80–145.42)	111.3(80–180)	–
Heart rate /min (Normal range: 60–100)	92.4	–	88.44	90.42(52–125)	–

Physical Examinations

The results of the physical examinations (**Table 6**) that included respiratory rate 19.8 breaths/min (13.5–30.0), pulse oximeter O_2 saturation 94.34%, mean systolic pressure 111.3 mmHg (80–180), and heart rate 90.42/min (52–125) were found to be slightly higher than their respective normal range.

Comorbidities

Various underlying medical conditions (**Table 8**) were found in the case-patients, the most significant ones being hypertension 35.9% (3909/10889), diabetes 20.17% (2196/10889), obesity 15.95% (1735/10889), cardiovascular disease 13.92% (1516/10889), asthma 4.42% (481/10889), COPD 4.31% (469/10889) and malignancy 3.99% (435/10889). Several patients were also found to be co-infected with other viruses, 9.1% (244/2684), Bacteria 4.99% (24/481), and Fungus 3.43% (11/320).

Clinical Progression Data

The median incubation period of the patients is found to be 5.36 days (1.5–15) based on 12 studies involving 1080 patients. The median hospital stay of the patients is eight based on three studies. Median days from onset of illness to hospital admission is 4.83 (0–11) based on 13 studies. Global (outside China) case fatality rate was found to be 22.24% (969/4357), among which 21.24% (564/2655) was in the USA, 25.61% (405/1581) in Italy, and 0% (0/121) was in other countries (Singapore and Diamond Princess Cruise Ship while it was staying in Japan). In comparison

TABLE 7 | Radiological findings (Chest CT scan results).

Radiological findings	Global (Excluding China) (n)	Only Hubei (China) *(n)*	Outside Hubei (China) *(n)*	Total *(n)*	Percentage (%) in total
Chest CT					
Normal	49/164	22/1292	38/329	109/1785	6.11
Abnormality	115/164	1262/1292	291/329	1668/1785	93.5
Unilateral lungs		144/1292	137/306	281/1598	17.6
Bilateral lungs	57/122	1187/1529	337/572	1581/2223	71.1
Density and Inner Features					
Ground–glass opacity	53/112	69/236	310/552	432/900	48
Consolidation	32/112	25/137	83/391	140/640	21.88
Mixed	–	–	35.4/132	35.4/132	26.8
Other Features					
Pleural effusion	6/21	31/534	158/392	195/947	20.6
Lung lesion	–	–	180/230	180/230	78.3
Interlobular septal thickening	–	–	80/170	80/170	47.1
Crazy paving pattern	–	–	34/170	34/170	20
Spider web sign	–	–	20/80	20/80	25
Sub–pleural line	–	–	16/80	16/80	20
Bronchial wall thickening	5/21	–	75/243	80/264	30.3
Lymph node enlargement	–	–	153/302	153/302	50.7
Pericardial effusion	–	–	5/170	5/170	2.9
Paving stone sign	–	–	25/73	25/73	34.25
Thickening of lung texture	–	–	62/73	62/73	84.9
Pulmonary edema	2/21	–	–	2/21	9.5
Venous congestion	1/21	–	–	1/21	4.8
Atelectasis	1/22	–	–	1/22	4.55

the case fatality inside the Hubei Province of China was 11.71% (194/1656) (**Table 9**).

Exposure Pattern

A significant portion of the study cases has their exposure history linked to family members or their respective community 28% (680/2427) (**Table 4**) which is particularly the true for the study patients from China. Our review found that the exposure pattern in the global arena (outside China) has been largely centered on the imported cases 80.7% (280/347). This review also revealed that the hospital staff's exposure risk is 2.89% (43/1486) and the in-patients of the hospitals 2.89% (43/1486).

Age-Dependent Effects of COVID-19

It is assumed that, the age of the patients may influence the clinical features, disease progression, complexities, and fatality of the COVID-19. However, we have analyzed and included the available data on this aspect. The notable findings include faster disease progression, higher mortality rate, progressively lower lymphocyte count, higher ICU admission rate, higher mortality rate for those receiving mechanical ventilation, and higher risk of severe heart attack among the older patients (**Table 10**).

DISCUSSION

This systematic review is based on a random effect model. As such, it contains data from studies that took place in different countries, including China, the USA, Japan, South Korea, France, and Singapore. All the studies were published in peer-reviewed articles with patient data between late December 2019 and May 7, 2020.

We managed to retrieve data on clinical characteristics of COVID-19 from 10,889 infected patients. Various studies and reports worldwide showed that COVID-19 seems to infect the male population more frequently than it does to female population. Our review also corroborates this claim as 60.3% (6487/10889) of our case population are male and the rest 39.7% (4241/10889) are female. Previously older age had been predicted to be an essential factor for higher mortality in SARS and MERS patients (38). Several studies in our review have reported the same. During the initial transmission period, COVID-19 seemed to affect the elderly more, as reflected by the mean age of 49.8 years (0.5–94) of the case population included in the review attributing to the higher frequency of comorbidities observed among the elderly (39). But in the current situation, it seems that the virus can equally affect everyone irrespective of age. However, it is evident that the clinical characteristics and prognosis of the disease greatly vary among patients with different age groups. Patients over 60 years tend to show

TABLE 8 | Comorbidities.

Comorbidities	Global (Excluding China) ($n = 7849$)	Only Hubei (China) ($n = 1656$)	Outside Hubei (China) ($n = 384$)	Total ($n = 10889$)	Percentage (%) in total
Hypertension	3541/7849	296/1656	72/1384	3909/10889	35.9
Diabetes	2003/7849	160/1656	33/1384	2196/10889	20.17
Obesity (BMI ≥30)	1737/7849	–	–	1737/10889	15.95
Cardiovascular disease	1231/7849	164/1656	121/1384	1516/10889	13.92
Asthma	481/7849	–	–	481/10889	4.42
COPD	344/7849	110/1656	15/1384	469/10889	4.31
Malignancy/cancer	401/7849	25/1656	9/1384	435/10889	3.99
Chronic renal insufficiency	304/7849	26/1656	4/1384	334/10889	3.07
End-stage kidney disease	188/7849	–	–	188/10889	1.73
Hyperlipidemia	192/7849	–	–	192/10889	1.76
Cerebrovascular Disease	–	46/1656	116/1384	162/10889	1.49
Pneumonia	–	7/1656	76/1384	83/10889	0.76
Chronic liver disease	40/7849	31/1656	15/1384	86/10889	0.79
Chronic gastritis and gastric ulcer	0/7849	47/1656	11/1384	58/10889	0.53
History of solid organ transplant	57/7849	–	–	57/10889	0.52
Fatty liver and abnormal liver function	5/7849	43/1656	–	48/10889	0.44
Endocrine diseases	–	5/1656	39/1384	44/10889	0.40
HIV infection	43/7849	2/1656	–	45/10889	0.41
ARDS	8/7849	17/1656	2/1384	27/10889	0.25
Acute kidney injury	2/7849	22/1656	–	24/10889	0.22
Autoimmune disease	1/7849	10/1656	–	11/10889	0.1
Immunosuppression	3/7849	3/1656	3/1384	9/10889	0.08
Co-infection					
Other viruses	211/2364	33/320	–	244/2684	9.10
Bacteria	1/21	23/460	–	24/481	4.99
Fungus	–	11/320	–	11/320	3.43

TABLE 9 | Disease progression and other clinical data.

Disease progression and other clinical data	Global (Excluding China)	Only Hubei (China)	Outside Hubei (China)	Total/Mean (Range)	Percentage (%)
Median hospital stay of patients (days)	–	8	–	8	–
Median Incubation Period (days)	4.05 (2–9)	6 (5–13)	6.3 (1.5–15)	5.36 (1.5–15)	–
Days from onset of illness to dyspnea	4 (1–20)	–	–	4 (1–20)	–
Days from onset of illness to ICU	4.7 (1–14)	–	–	4.7 (1–14)	–
Days from Onset of symptoms to hospital admission	4 (0–11)	7 (4–11)	3.5 (0.8–8.2)	4.83 (0–11)	–
Days From hospital admission to death	–	4 (2–7)	–	4 (2–7)	–
Days from onset of illness to ARDS	–	8 (6–12)	–	8 (6–12)	–
Length of follow-up days	7.6 (1–15)	–	–	7.6 (1–15)	–
Total Death (Case Fatality Rate)*	Global: 969/4357 (22.24%) [USA: 564/2655 (21.24%), Italy: 405/1581 (25.61%), Other countries (Singapore, and Diamond Princess Cruise Ship while it was staying in Japan: 0/121(0%)]	194/1656 (11.71%)	5/893 (0.56%)	1168/6906	16.9

ARDS, Acute respiratory distress syndrome; ICU, intensive care unit.

**Based on the available follow-up data on patients from the selected case studies.*

TABLE 10 | Age dependent effects of COVID-19.

Parameter	Age related impact	References
Severity of disease	Median age of severe patients (62.0) were significantly older than non-severe ones (51.0)	Zhang et al. (6)
Disease progression	Disease progressed rapidly in older patients with ARDS and septic shock	Zhang et al. (6)
Survivors vs. non-survivors	Non-survivors were older (mean age: 64.5 years) compared to the survivors (mean age: 51.9 years) Mortality rate increases in older (<65 years) patients with comorbidities	Yang et al. (17)
Severe heart attack/death	Older patients (>70 years) with chronic medical conditions are likely to suffer a severe heart attack and death.	Shi et al. (10)
ICU admission	Patients treated in ICU were significantly older (median age: 66 years) than patients not treated in ICU (median age: 51 years)	Wang et al. (11)
Mortality rate of the patients who received mechanical ventilation	Mortality rate were found to be higher (97.2%) in older patients (>65 years) compared to 76.4% in case of younger patients (18–65 years).	Richardson et al. (28)
Lowest absolute lymphocyte count	Progressively lower for older patients	Grasselli et al. (29)
Median fraction of inspired oxygen (FiO2)	Lower (60%) in younger patient compared to the older patients (70%)	Grasselli et al. (29)

more severe clinical manifestations and relatively longer disease duration, meaning they would require more careful monitoring and more comprehensive medical interventions (40).

The Chinese Center for Disease Control and Prevention (CDC) reported that the vast majority (81%) of COVID-19 patients will develop only mild symptoms, and the rest develop severe illness i.e., they will require oxygen therapy (14%) or require ICU treatment (5%) (41). Usually, COVID-19 patients are diagnosed with signs of severe pneumonia. Our review found that the most commonly reported symptoms are dry cough (59.6%), fever (46.9%), fatigue (27.8%), and dyspnea/shortness of breath (20.23%). These symptoms, together with prior contact history with suspected patients, immediately would require medical attention. Other less frequent symptoms include myalgia, diarrhea, headache, anorexia, sore throat, rhinitis, upper airway congestion, expectoration, pneumonia, etc. The symptoms are similar to those of SARS and MERS (42, 43). In most cases, the symptoms are mild during the initial days of infection but can be very severe, particularly for the elderly and patients with underlying respiratory diseases. Unlike SARS and MERS, SARS-CoV-2 behaves mildly during the initial stage of infection, making it significantly more contagious than the previous coronaviruses since the condition can go unnoticed in some cases (44). New shreds of evidence emerging from across the globe suggest that the asymptomatic cases of COVID-19 infections are rising (45, 46). But our review found that only a small percentage (0.56%) (20/3598) of the patients were asymptomatic which may be attributed to the fact that the initial tests for COVID-19 were only being conducted for patients with a distinct illness. To screen out the asymptomatic cases, experts have emphasized on comprehensive epidemiological investigations of the suspects by disease control specialists. Besides, the asymptomatic patients have shown consistent abnormalities in their CT scan reports for which radiological investigations can be a useful diagnostic tool to find the asymptomatic variants (47).

The most notable laboratory finding of our review is lymphocytopenia found in 55.9% cases (4177/7470). It was also reasonably expected in the influenza virus (H5N1), SARS, and MERS (48). In H5N1 influenza the fall in lymphocyte count had been attributed to dendritic cell (DC) dysfunction suggesting that a similar mechanism related to dysfunctional adaptive immunity can cause the same in COVID-19 patients (49). Besides, another study has demonstrated the correlation between the lymphocytopenia and the clinical severity of SARS-CoV-2 infection. The study also infers that the lymphocytopenia might be an outcome of the death of lymphocytes or the damage of lymphatic organs like thymus or spleen directly by the virus itself (25).

Another significant finding of our review is the elevated level of CRP 61.9% (830/1340) which supports a study that demonstrated elevated CRP levels in COVID-19 patients even though they did not have any kind of coinfections (50). It is to be noted that the CRP level is usually increased in bacterial or viral infections. However, it does not demonstrate a significant elevation in the case of mild viral infections. Another study claimed the potentiality of CRP as a comprehensive predictive factor of COVID-19 prior to the changes in other inflammatory-related blood parameters e.g., leucocytes, lymphocytes, and neutrophils (5). Besides, CDC guidelines have also reported an elevated CRP level in COVID-19 patients with higher CRP levels indicative of the severity of the infection and poor prognosis (51). Previously it has been associated with Influenza H1N1 & H7N9 and the SARS epidemic (52, 53). In COVID-19 patients, this significantly elevated level of CRP can be explained by the excessive production of inflammatory cytokines due to immune response as well as the damage of the lung alveoli (50).

LDH level has been found to be increased by 40.8% (392/973) in COVID-19 patients. Although it is abundant in tissues, LDH level is low in blood circulation. Elevated LDH, as reported in SARS, reflects tissue necrosis corresponding to

hyperactive immunity (54). Besides, viral infections can cause lung tissue damage, which in turn raises the LDH level in blood circulation (55).

Our review revealed abnormal liver function tests in the case of patients with elevated ALT 36.65% (2318/6503) & AST 53.3% (3481/6537) level and lower albumin (38.4%) (187/487) and serum creatinine kinase level (31.9%) (99/310). One report has proposed that the hepatic dysfunction of COVID-19 patients can be linked to direct liver injury via viral hepatitis or due to abnormal levels of blood coagulative and infection-related functions, such as, elevated prothrombin time, D-dimer level, and serum ferritin, as found in our review (56).

We could not find sufficient data on the immunological parameters of the case patients. But one of the studies included in our review reported a higher IL-6 count in 51 (50%) of 99 patients (14). Besides, two recent studies have shown that IL-6 level was substantially increased in both severe and moderate patients. In addition to that, other pro-inflammatory parameters like IL10, IL2, IFN-γ, and TNF-α were found to be higher in more severe patients than in the moderate ones (21, 22). The magnitude of the cytokine storm or, more particularly, elevated IL-6 level has been associated with disease severity (13). It has been proposed as an essential parameter to predict respiratory failure risk based on a study that found a significantly higher IL-6 level in patients requiring ventilation (57).

The radiological findings showed that COVID-19 patients are accompanied by an abnormality in their CT scan report in 93.5% (1668/1785) cases. So CT scanning can provide an important base for early diagnosis of the virus. The typical CT scan features include bilateral lung 71.1% (1581/2223) and unilateral lung 17.6% (281/1598). The density characteristics of the lung lesions, found in 78.3% (180/230) cases, was mostly uneven, with ground-glass opacity 48% (432/900) as the primary presentation accompanied by consolidation in 21.88% (140/640) cases. CT scan reports have been demonstrated an association between disease progression and a higher rate of consolidative opacities (58). CT scan reports and the RT-PCR tests are generally found to be harmonious with a few exceptions. CT scan results can be a more specific diagnostic tool for the suspected COVID-19 cases since even the asymptomatic patients have shown abnormalities in their CT scan reports (46, 47). The less common CT findings include pleural effusion 20.6% (195/947), pericardial effusion 2.9% (5/170), bronchial wall thickening 30.3% (80/264), and interlobular septal thickening 47.1% (80/170) which have been reported as the disease progresses.

The median incubation period of the SARS-CoV-2 virus has been found to be 5.36 days (1.5–15). National Health Commission of China previously reported an incubation period of 1 to 14 days. The CDC has updated the mean incubation period to be between 2 to 14 days (51). However, various studies together with our findings suggest that the incubation period of SARS-CoV-2 is highly variable and requires more concrete statistically significant results. This high variability in the incubation period of SARS-CoV-2 has warranted a suitable quarantine period of at least 3 weeks to reduce the community transmission of the virus (59).

A significant portion of our case patients had one or more comorbidities indicating that a significant portion of our case patients is elderly. They are more likely to develop various chronic or acute clinical conditions on aging. Hypertension (35.9%) (3909/10889), diabetes (20.17%) (2196/10889), obesity (15.95%s) (1737/10889), cardiovascular diseases (13.92%) (1516/10889) and respiratory diseases (e.g., asthma-4.42%; COPD-4.31%) are the most frequent comorbidities found in the case patients of our review. The prevalence of comorbidities can be a major risk factor for severe patients compared to non-severe patients due to higher case fatality and poor prognosis (17). Thus, a proper medical history of the COVID-19 patients, particularly elderly patients, should be documented. Moreover, adequate clinical facilities should be made available for this group of patients.

Age-specific patient data was not sufficiently available in the case of most of our included studies. Still, for some crucial parameters, such as mortality rate and severity of the disease, a pattern was observed across the studies involving the older patients who, in general, possess a higher risk. **Table 10** has listed all of the available age-related effects that we were able to retrieve. One of the recent studies has indicated that the children's overall risk factors are not significantly affected by the age and sex (60). Unlike adults, the children are at a lower risk of developing severe symptoms or death.

Diagnostic Challenges of COVID-19

Article search strategy: For the collection of information on diagnostic challenges of COVID-19, we have extensively searched by using the keywords "COVID-19 and diagnostic challenges," "challenges for the diagnosis of novel coronavirus diseases," on Pubmed, Web of Science, Science Direct, Scopus, Wiley Online Library, Google Scholar databases and other online websites of reliable sources such as Gavi-The Vaccine Alliance (gavi.org), World Health Organization, US-FDA, etc.

Review Findings on the Diagnosis Challenges of COVID-19

Every day the novel coronavirus is presenting us with new clinical challenges. As countries worldwide are considering to curb the limits on social distancing measures, general testing and rapid diagnosis of the disease have become of utmost importance. Even though various efforts are undertaken both by the government agencies and international bodies, the diagnosis of COVID-19 is still challenging. For convenience, the information on the current challenges for the diagnosis of COVID-19 have been summarized as follows:

1) Although RT-PCR has been regarded as the gold standard for viral detection of SARS-CoV-2, it also comes with few challenges. The equipment and lab facilities required for this testing can be expensive. The testing environment requires a certain biosafety level e.g., BSL-2 cabinets for sample preparation or ideally a negative pressure room (61). Most of the traditional testing centers all around the globe do not have these facilities. On top of that, these facilities require technicians with enough expertise and experience to

perform the tests smoothly and flawlessly (62). Therefore, lack of testing centers and expert technicians are the primary diagnostic challenges faced by most of the countries all over the world. For this very reason, many countries failed to start coronavirus testing early in its transmission phase. Even when they had started testing, it was strictly restricted to suspected individuals with clear symptoms of the viral infection (63). Moreover, as countries all over the world are trying to increase their number of testing each day, this has put a serious pressure on the ready availability of reagents for the PCR reactions. Thus, shortage of testing kits is next big challenge to the rapid diagnosis of the disease.

2) Another crucial diagnostic challenge with RT-PCR testing is the risk of eliciting false-negative and false-positive results. It has been found that many patients with typical COVID-19 symptoms and CT features have shown a negative influence in RT-PCR testing (64). Therefore, a negative result should not preclude the patient as a COVID-19 suspect, and it should not be used as the only criterion of diagnosis. Besides, a false result due to human error cannot be overruled as under-trained technicians are performing many of these tests.
3) The collection of the patient sample (throat swab) presents another diagnostic challenge as this requires specific procedures to be followed for the sake of proper sample preparation for RT-PCR testing. Wrong sampling procedures can cause errors in RT-PCR testing (65). Moreover, a global shortage of personal protective equipment (PPE) has put additional challenges as adequate protective measures have to be made available for the staff involved in this sample collection procedure.
4) RT-PCR testing also faces another challenge that arises from the susceptibility of the viral mutations in the SARS-CoV-2 genome. Various studies have shown a rapid evolution of the virus (66, 67). Consequently, a false negative result may arise due to mutation in the primer or probe target regions (61). Although they are based on the most conserved regions of the viral genome, a slight variability can decrease the assay performance.
5) Asymptomatic patients have become a major challenge for both the clinicians and the administration. They are difficult to diagnose and isolate and they also pose a more significant threat of rapid and unchecked viral transmission. A recent study has found a similar viral load in asymptomatic patients compared to the symptomatic ones, indicative of their transmission potential (68). Another big concern is that most of these asymptomatic patients will not report to the hospitals or testing centers. Under such circumstances, their diagnosis solely depends on contact tracing or cluster screening by the disease control experts (68). Diagnosis of asymptomatic children is also challenging as their diagnosis is only limited to tracing their family history (69, 70). But this task is getting increasingly tricky both due to an overwhelming number of new cases arising each day and the limited workforce of the disease control centers.
6) Although COVID-19 patients have clear chest CT scan manifestations, several studies have reported usual CT scan reports despite being RT-PCR confirmed COVID-19 patients (9, 71). One study has found a false negative result of over 12% when attempted to predict the infection based on CT scan reports only (72). These reports clearly mean that clinicians have to be more vigilant in diagnosing the suspected cases of COVID-19 patients and consider multidimensional factors including laboratory parameters, CT scan reports, and other tests.
7) Additionally, most countries do not have a sufficient number of CT scan machines to support widespread testing of the rising number of COVID-19 patients (73). Besides, it takes a lot of effort to disinfect the machines after each test to prevent unwanted viral transmission.
8) Another challenge is that it requires multiple scans (at least 2, 6 days apart) for maximum accuracy in the diagnostic results meaning this would put additional pressure on the already scarce CT scan machines (74).
9) Sometimes suspected patients of the virus are showing unwillingness to come to test centers to confirm the presence of the virus. Some of the developing nations have reported these incidents, which were mostly attributed to the lack of concern, illiteracy, and the fear of social stigma (75). These patients remain undiagnosed for a considerable time till their symptoms get severe to the extent that they need hospitalization. So this group of patients presents us with a severe diagnostic challenge.
10) Serological detection tools utilize the detection of specific antibodies (IgM and IgG) against COVID-19 infections. These antibody tests have the advantage of low cost, fast detection and easy availability but suffer from low sensitivity as is seen with the antibody tests deployed for other coronavirus and influenza virus (76–78). However, IgM responses vary from person to person and require days to develop once they get infected. Considering that this may not be an useful tool for the accurate diagnosis of the viral infection except in confirming the late cases of COVID-19 and immunity of the recovered individuals (79). Due to these concerns, on April 1, 2020, the FDA granted Emergency Use Authorization (EUA). Still, they repeatedly expressed their doubts about the effectiveness of these tests in detecting the virus early during infection (80).

STRENGTH AND LIMITATIONS

To our knowledge, this is one of the first attempts to summarize the clinical characteristics and diagnostic challenges of COVID-19 taking into considerations of all the available data from global studies and variabilities so that the researchers, health care workers, policy makers and related stakeholders can get a contemporary overview of the COVID-19 situation.

The most noticeable limitation of our review is the lack of consistent data on every variable across the studies that we have included, which happened due to underreporting of symptoms, comorbidities, laboratory results, or exposure patterns of the case patients. These initially published studies have some issues related to lack of adequate laboratory

testing, which could be attributed to the fact that these studies had to be completed as fast as possible to keep people around the world up to date about the virus and to prepare them fast to manage the disease. Besides, we were able to fetch only a limited amount of data regarding the clinical progression and other epidemiologic characteristics that would otherwise come in handy in defining clinical characteristics. In addition, most of our studies were from China, as no data were available from other countries. It would be better to broaden the geographical scope of our review to get a more global scenario of the clinical characteristics of the outbreak.

CONCLUSION

This systematic review summarized the latest clinical findings of the novel coronavirus outbreak that has virtually affected the whole world. To the best of our knowledge, this is the first large scale review comprising of studies from across the globe focusing on the clinical features of the COVID-19 patients. Laboratory tests and CT scan reports, together with clinical symptoms will provide useful information for the correct diagnosis and better management of the patients. This review provides a comprehensive overview and clear features of the clinical characteristics of COVID-19 which will help the physicians to make proper clinical decisions and correct assessment regarding the patients. Additionally, we have reviewed the challenges being faced for the diagnosis of the disease across the world. Overcoming these obstacles to the fast and prompt diagnosis of COVID-19 will be crucial for the proper containment of the disease.

DATA AVAILABILITY STATEMENT

All data generated or analyzed in this study have been included in the article. The raw data will be provided by the corresponding and/or by the first author on reasonable request.

AUTHOR CONTRIBUTIONS

SMHI and MMRS: conceived and designed the study; SMHI and PTR: Collected data through article search in different online databases; KAK and MMRS: Checked and verified collected data; SMHI, PTR, MMRS, FK, and SA: Analysis and interpretation of data; SMHI and MMRS: prepared the manuscript draft; AAT, CLP, INM, and LCM critically reviewed the manuscript. LCM was also involved with the planning of the study. All authors read the manuscript, agreed to be accountable for all aspects of the work, and approved the final manuscript.

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Mathematical Modeling Predicts That Strict Social Distancing Measures Would Be Needed to Shorten the Duration of Waves of COVID-19 Infections in Vietnam

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Coronavirus disease 2019 (COVID-19) emerged in Wuhan, China in 2019, has spread throughout the world and has since then been declared a pandemic. As a result, COVID-19 has caused a major threat to global public health. In this paper, we use mathematical modeling to analyze the reported data of COVID-19 cases in Vietnam and study the impact of non-pharmaceutical interventions. To achieve this, two models are used to describe the transmission dynamics of COVID-19. The first model belongs to the susceptible-exposed-infectious-recovered (SEIR) type and is used to compute the basic reproduction number. The second model adopts a multi-scale approach which explicitly integrates the movement of each individual. Numerical simulations are conducted to quantify the effects of social distancing measures on the spread of COVID-19 in urban areas of Vietnam. Both models show that the adoption of relaxed social distancing measures reduces the number of infected cases but does not shorten the duration of the epidemic waves. Whereas, more strict measures would lead to the containment of each epidemic wave in one and a half months.

Keywords: COVID-19, SARS-CoV-2, epidemic model, multi-scale modeling, basic reproduction number

1. INTRODUCTION

The coronavirus disease 2019 (COVID-19) emerged in Wuhan, China and has caused a pandemic that has affected almost all countries around the globe. In the absence of effective vaccines or therapeutics against COVID-19, countries have resorted to non-pharmaceutical interventions (NPIs) to slow down the spread of the epidemic. These interventions have been proven to be effective against epidemics, such as SARS and 2009 swine flu, however, it is not clear what effects they have on the spread of COVID-19 as it is a novel disease. Mathematical modeling offers the opportunity to quantify the impact of these interventions and to design effective strategies that contain the spread of COVID-19.

Vietnam has been affected by the pandemic, but it has managed to control the epidemic in comparison with other countries. The first COVID-19 case in Vietnam was reported on Janruary 23, 2020. As of April 12, 2020, 260 positive SARS-CoV-2 tests were confirmed, and no deaths were reported. These results indicate that Vietnam adopted one of the most effective epidemic control strategies in the world. Indeed, the country was well-prepared to slow down the spread

of COVID-19 due to its experience in containing the SARS epidemic. The non-pharmaceutical interventions that have been adopted by Vietnam include preventing people from visiting areas with an elevated risk of infection, social distancing, school closures, shutting down borders with China, and isolating infected individuals. A nationwide lockdown was mandated from 1 to 15 April 2020. It consisted of limiting the movement of individuals to reduce the chances of disease transmission.

In this work, two mathematical models are used to analyze the dynamics of the spread of COVID-19 in Vietnam. The first is an SEIR model in which the population is divided into four compartments: susceptible, exposed, infectious, and recovered. We derive an analytical estimate for the basic reproduction number using this model and calculate it for the case of Vietnam. After model calibration using the reported data, we used it to study the impact of different levels of social distancing measures on the spread of the disease. The second model uses a multi-scale architecture to explicitly describe the transmission dynamics at the level of individuals (1, 2). The movement of individuals is simulated using a social-force model. This model is studied to investigate the impact of limited movement of the population on the spread of the epidemic.

The remainder of the paper is organized as follows: section 2 presents a summary of related works and how the paper contributes to the research field. Section 3 introduces the two models and the data that is used to calibrate them. Section 4 presents the results of numerical simulations quantifying the impact of relaxed and strict social distancing measures on the spread of the disease. The contributions and the limitations of the study are discussed in section 5.

2. RELATED WORKS

To gain a better understanding of the COVID-19 spread in Vietnam, it is urgent to build mathematical models that consider the impact of non-pharmaceutical interventions. Compartmental models that are widely used to describe the transmission dynamics of infectious diseases can be used to study the evolution of the COVID-19 epidemic. These models usually consist of a system of ordinary differential equations (ODEs). A compartmental model was used to evaluate the impact of non-pharmaceutical interventions in China (3). A susceptible-infected-recovered (SIR) model was previously formulated to study the effect of quarantine in containing the spread of COVID-19 (4). Susceptible-exposed-infectious-recovered (SEIR) models are used to simulate the transmission dynamics of infectious diseases with a relatively long incubation period (5). A sensitivity analysis of an SEIR model for COVID-19 was presented in a previous study (6). This analysis has shown that the early detection, early isolation, early treatment, and a comprehensive treatment strategy are necessary for slowing down the spread of the disease. SIR and SEIR models can be used to calculate the basic reproduction number ($\mathcal{R}_0$) (7, 8). The advantage of using the ODEs to implement compartmental models is the possibility to study the obtained systems analytically and numerically.

Individual-based modeling is another framework which can be used to implement compartmental models. Agent-based models explicitly describe the person-to-person transmission of the disease (9, 10). Some of these models describe the movement of individuals in space (11). Other individual-based models do not describe the movement of individuals but include several details on the spread of the disease under realistic conditions (12, 13). Multi-scale models integrate the processes regulating disease transmission at both the within-host and between-host levels (14). In this context, we have recently developed a multi-scale model to describe COVID-19 transmission dynamics in Italy, China, and Morocco (1, 2).

The main contribution of this work is that both continuous and agent-based approaches are used and compared to study the transmission dynamics of COVID-19 in Vietnam. We use two models to evaluate the impact of non-pharmaceutical interventions on the spread of the disease. The first belongs to the SEIR class and can be used to determine the basic reproduction number ($\mathcal{R}_0$) while the second is a multi-scale model which implicitly captures the movement of individuals. The aim of this study is not to provide an accurate forecast of the evolution of the pandemic, but rather to gain a deeper understanding of the impact of non-pharmaceutical interventions on the spread of the disease. Both models quantify the impact of lockdown policies on the propagation of the COVID-19 pandemic in Vietnam.

3. MATHEMATICAL MODELING OF COVID-19 TRANSMISSION DYNAMICS IN VIETNAM

3.1. Data Collection

The data on COVID-19 cases in Vietnam were collected from Ministry of Health (15). It shows that the number of daily cases has increased steadily over February and March. Then it started decreasing after the adoption of the social distancing measures on 31 March, 2020. Indeed, the Vietnamese government mandated a nationwide lockdown of 15 days from April 1, 2020 to April 15, 2020. During this lockdown, people are required to stay at home and not go out except for buying necessary goods or for going to work at factories. We have represented the collected data which include the daily and cumulative numbers of reported COVID-19 cases before and after the lockdown in **Tables 1**, **2**, respectively.

3.2. The SEIR Compartmental Model

We adapt the well-known SEIR model to describe the transmission dynamics of COVID-19 in Vietnam. The model splits the total population N in three classes: susceptible (S), exposed (E), and infected individuals (I). A schematic representation of the considered compartments and the interactions between them is represented in **Figure 1A**. It is an extension of the classical SIR Kermack and McKendrick model 1927 which includes a compartment of exposed individuals. We do not consider mortality in the model. This allows

TABLE 1 | Number of daily and cumulative reported number of COVID-19 in Vietnam from March 6, 2020 to March 30, 2020 before the nationwide lockdown.

Date (day/month)	Number of newly reported cases	Cumulative number
6 March	1	17
7 March	4	21
8 March	9	30
9 March	1	31
10 March	3	34
11 March	4	38
12 March	6	44
13 March	3	47
14 March	6	53
15 March	5	58
16 March	3	61
17 March	5	66
18 March	2	68
19 March	8	76
20 March	11	87
21 March	5	92
22 March	14	106
23 March	15	121
24 March	2	123
25 March	11	134
26 March	19	153
27 March	10	163
28 March	11	174
29 March	14	188
30 March	15	203

us to decouple R from the rest of the model. The model equations are, for $t > 0$,

$$\begin{cases} S'(t) = -\beta S(t)I(t), \\ E'(t) = \beta S(t)I(t) - \mu_E E(t), \\ I'(t) = -\mu_I I(t) + \mu_E E(t), \end{cases} \quad (1)$$

with the initial conditions

$$S(0) = S_0, \quad E(0) = E_0, \quad \text{and} \quad I(0) = I_0. \quad (2)$$

We consider that $E_0 = R_0 = 0$ (initially, there is no exposed and recovered individual). Moreover, we assume that $S(0) + E(0) + I(0)+R(0) = 1$ from which we have $S(t)+E(t)+I(t)+R(t) = 1$ for all $t > 0$. This means that we will work with the proportion to the total population. All the parameters of the model are described in **Supplementary Table 1**. Note that there is a slight difference in the used notations between our work and the study by Kuniya et al. (5). The identification parameter is denoted by ε in our paper, while it is denoted by p in the work by Kuniya et al. (5). Also, we refer to the onset rate by μ_e while in Kuniya et al. 2020, it is denoted by ε.

Inspired by a previous work (5), we consider an identification function

$$t \in \mathbb{R}^+ \mapsto X(t) = \varepsilon \times I(t) \times N.$$

TABLE 2 | Number of daily and cumulative reported number of COVID-19 in Vietnam from March 31, 2020 to May 3, 2020 following the application of the lockdown.

Date (day/month)	Number of newly reported cases	Cumulative number
31 March	4	207
1 April	11	218
2 April	9	227
3 April	10	237
4 April	3	240
5 April	1	241
6 April	4	245
7 April	4	249
8 April	2	251
9 April	4	255
10 April	2	257
11 April	1	258
12 April	2	260
13 April	5	265
14 April	1	266
15 April	1	267
16 April	1	268
24 April	2	270
3 May	1	271

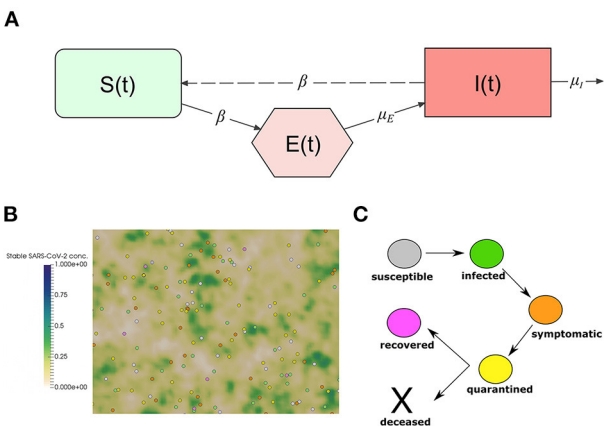

FIGURE 1 | (A) Interactions between the compartments of the epidemiological model (1). The continuous lines represent transition between compartments, and entrance and exit of individuals. The dashed line represents the transmission of the infection through the interaction between susceptible and infected individuals. **(B)** Snapshot of a numerical simulation of the model. Spheres represent individual people moving in a square section of 250 × 250 m. The color of each individual represents the class to which it belongs: white for susceptible, green for infected, orange for symptomatic, yellow for quarantined, and pink for recovered. The concentration of stable SARS-CoV-2 on surfaces is represented using the gradient of the green color. **(C)** The clinical course of COVID-19 patients in the model.

This quantity describes the number of infective individuals who are identified at time t, with N is the total population in Vietnam ($N = 97,338,579$) and ε is the identification rate. We can suppose that $\varepsilon \in [0.01, 0.1]$ (5). It allows to take in consideration the uncertainty due to the incomplete

identification of infective population. Then, we suppose that only a fraction of infectious individuals denoted by ε can be identified by diagnosis.

3.3. Multi-Scale Model of COVID-19 Transmission Dynamics

We adapt a previously developed model (1) to describe the transmission dynamics of COVID-19 in Vietnam. The model relies on a microscopic description to capture the movement of the individuals and the transmission of the disease. The model was used to gain insights into the impact of NPIs on the transmission dynamics of COVID-19 in Morocco (2). We study the transmission dynamics of COVID-19 in a population of 250 individual walking randomly in a square domain of 250 × 250 m. This corresponds to a population density of 1,000 inhabitant/km^2, which the minimal density in urban areas as defined by the U.S. Census Bureau (16, 17). The contact frequency of individuals increases as the population density grows (1, 18). We consider a closed system of individuals and we assume periodic boundary conditions on the movement of individuals to approximate larger systems. The agent-based aspect of the model makes it suitable to study the fine-grained aspects related to the impact of non-pharmaceutical measures. In particular, those which aim to reduce the movement of the population and reduce the chances of disease transmission. A snapshot of numerical simulation using the model is shown in **Figure 1B**. Details of the model implementation are provided in our previous study (1). In this section, we provide a summary of the main features of the model.

3.3.1. The Movement of Individuals

We use a social force model (19) to describe the movement of each individual agent. The model was used previously to describe the movement of pedestrian in crowded areas (20). We model each agent as a sphere particle which is subjected to several forces. We apply Newton's law to the center of each individual:

$$m_i \frac{dv_i}{dt} = f_i^{self} + \xi_i \tag{3}$$

$$\frac{dx_i}{dt} = v_i, \tag{4}$$

where x_i is the displacement of the individual, v_i is its velocity, m_i is its mass, ξ_i is a random perturbation and f^{self} is the self-driven force defined by:

$$f_i^{self} = m_i \frac{v_{d,i} - v_i}{\tau_i},$$

$v_{d,i}$ represents the desired velocity at which the i-th pedestrian wants to move. We consider that individuals tend to move to random directions, the amplitude of the desired speed is chosen to be following a normal distribution with an average of 1.34 m·s^{-1} ($\simeq$5 km/h) and a standard deviation of 0.26 m·s^{-1}, τ_i is a relaxation time.

3.3.2. Modes of Disease Transmission

Infectious individuals can transmit the disease by the mean of secreted droplets that bear the virus. These droplets can be inhaled by neighboring individuals. They also contaminate the neighboring surfaces. We consider both these modes of disease transmission in the model. First, we infectious individuals can infect susceptible individuals if the distance between them is <1 m. Direct transmission depends on the incidence of sneezing, coughing, breathing without mask, or handshaking. Therefore, we assume that the susceptible individual has a probability of p_d to contract the virus upon direct contact with infectious individual. We model the mode of indirect transmission by considering that infectious contaminate neighboring surfaces by secreting droplets which contain the virus. These surfaces can subsequently transmit the virus to susceptible individuals. We describe the concentration of stable SARS-CoV-2 on surfaces as follows:

$$\frac{\partial C}{\partial t} = W - \sigma C, \tag{5}$$

where W is the secretion rate of the virus by infected individuals and σ is the decay rate of stable SARS-CoV-2. The probability of viral infection by touching surfaces is estimated at:

$$p_{in} = \lambda \bar{C}(x_i), \tag{6}$$

where λ is a positive constant taken smaller than one and $\bar{C}(x_i)$ is the normalized concentration of the virus on local contaminated surfaces. Note that we only evaluate the possibility of indirect transmission once each day for each individual at a random moment of the day as the possibility of indirect transmission can be considered as a rare event.

3.3.3. Clinical Course of Infected Patients

Infected individuals do not develop symptoms until the end of the incubation period. However, they start transmitting the virus a day before the onset of symptoms. The median value for the incubation period is 5.1 days and can be sampled using a log-normal distribution (21, 22). After the onset of symptoms, infected individuals get isolated and go into quarantine at home or in a hospital. In this period of quarantine, the patient stops moving in the computational domain and interacting with other individuals. This phase can have two outcomes: the patient can either die or survive. If he or she survives, then they start moving and interacting with other individuals as before, but they become immunized to new infections. Recovered individuals refer to the state of individuals that already contracted the virus and no longer show symptoms. While immunity to re-infection by SARS-CoV-2 is still under investigation (23), we assume recovered individuals to be immune to new SARS-CoV-2 infections in the next few months. Median values for characteristic periods and the distribution used for their sampling them are given in **Supplementary Table 2**. The clinical evolution of infected individual is represented in **Figure 1C**.

3.3.4. Demographic Characteristics and Mortality Risks

We assume that the death probability for each patient depends on its characteristics and in particular age and pre-existing risk factors. The considered age-structure is introduced as a distribution function and used to sample the age of each patient. We restrict the population of individuals to the individuals older than 18. This is because individuals who are younger than 18 are less impacted by the disease and most of them are asymptomatic. We consider that the age of the individuals determines the risk of COVID-19-related mortality as represented in **Supplementary Table 3** (24).

Furthermore, we consider that individuals can also have one or many of the pre-existing risk factors which increase the mortality risk of COVID-19. These risk factors include chronic respiratory diseases, cardiovascular diseases, elevated blood pressure, diabetes, and cancer. We consider that the prevalence of these health conditions to be similar to the one observed in society. **Supplementary Table 4** provides the prevalence of these conditions and the corresponding death probability taken from a World Health Organization (WHO) report (25).

3.3.5. Computational Implementation

We used temporal integration to solve all the equations of the model. We took a very small-time step $dt = 10^{-4}$ h = 4.16×10^{-6} day to track all contacts between individuals. We use the Euler implicit scheme to solve the Newton's second law of dynamics and the finite different method to solve the equation for SARS-CoV-2 distribution (5). The model is implemented using the C++ language. To ensure code modularity, we have used an object-oriented programming (OOP) architecture. The post-processing of the results was done using the ParaView software and python scripts. The code can be accessed at: https://github.com/MPS7/SIM-CoV.

4. RESULTS

4.1. The SEIR Model Describes the Impact of NPIs on the Spread of the Disease

We begin by determining the parameters of the SEIR model that describe the evolution of the Covid-19 epidemic in Vietnam. Then we evaluate the impact of different prevention and control strategies on the spread of COVID-19. To achieve this, we use the least square method to fit the parameter β. Then, we reduce this parameter to model the impact of non-pharmaceutical interventions which aim to reduce the contact probability between individuals. Indeed, a lockdown aims to reduce the portion of mobile population which downregulates the contact rate. We estimate the basic reproduction number $\mathcal{R}_0$ for the epidemic COVID-19 in Vietnam. It is defined as the average number of new infections caused by an infectious individual in a susceptible population. This number describes the propagation speed of the epidemic. It is estimated using the following formula (26):

$$\mathcal{R}_0 = \frac{\beta S_0}{\mu_I} = \frac{\beta}{\mu_I}(1 - E_0 - I_0 - R_0) = \frac{\beta}{\mu_I}\left(1 - \frac{X(0)}{\varepsilon N}\right).$$

In the absence of any prevention or control strategy, the estimated basic reproduction number is $\mathcal{R}_0 = 4$. We can see that the value of the basic reproduction number is much higher than the same number at the beginning of the COVID-19 outbreak in Vietnam.

There exist other methods to estimate the basic reproduction number. For comparison purpose, we will use the method presented in a previous study (6). It defines the basic reproduction number as:

$$\mathcal{R}_0 = 1 + \gamma T_g + \rho(1 - \rho)(\lambda T_g^2),$$

where T_g is the generation time, ρ denotes the ratio of incubation period to generation time and we define γ as follows:

$$\gamma = \frac{\ln Y(t)}{t}.$$

Here $Y(t)$ denotes the number of symptomatic cases by time t. It can be defined as $Y(t) = iks$, where i is the number of confirmed cases, s is the number of susceptible cases, and k is the ratio of suspected to confirmed cases, equal taken equal to 0.695 (27). Applying this method to the results of numerical simulations, we estimate $\mathcal{R}_0 = 3.34$. Note that this method does not require the value of the contact rate (β) for the calculation of $\mathcal{R}_0$. As a result, it is possible to calculate the $\mathcal{R}_0$ using solely graphical data and the generation time of the disease.

Parameter values for numerical simulation are given in **Supplementary Table 1**. We estimate the contact rate to be $\beta = 0.4$. The identification rate ε does not affect much the basic reproduction number and the rate of infection. In numerical simulations, we set $\varepsilon = 0.08$ and we provide several predictions on the evolution of the epidemic. We show the model predictions for the evolution of the epidemic in the absence of NPIs in **Figure 2**.

We study the effect of non-pharmaceutical interventions with several cases of severity of the restrictions on the spread of COVID-19. We also explore the influence of the intervention duration. As stated before, the nationwide lockdown started on April 1, 2020. In **Figure 3**, we reduce the contact rate β to $0.1 \times \beta$ as soon as the intervention starts. This corresponds to a reduction of contacts by ninety percent of the normal value.

If the contact rate is below $0.08 \times \beta$, then the epidemic is contained in <2 months. More strict social distancing measures which reduces the contact rate by more than 95% would contain the epidemic in a period ranging from a month to a month and a half. We then look for the effect of the duration of the intervention on the evolution of the epidemic (**Figure 4**). The results suggest that the epidemic can persist and resurge again if the duration is not sufficient.

4.2. The Multi-Scale Model Quantifies the Effect of Lockdown on the Propagation of the Disease

We estimate the duration of the epidemic to be the difference between the moment of the onset where the number of actively infected individuals exceeds 5% of the population and the time

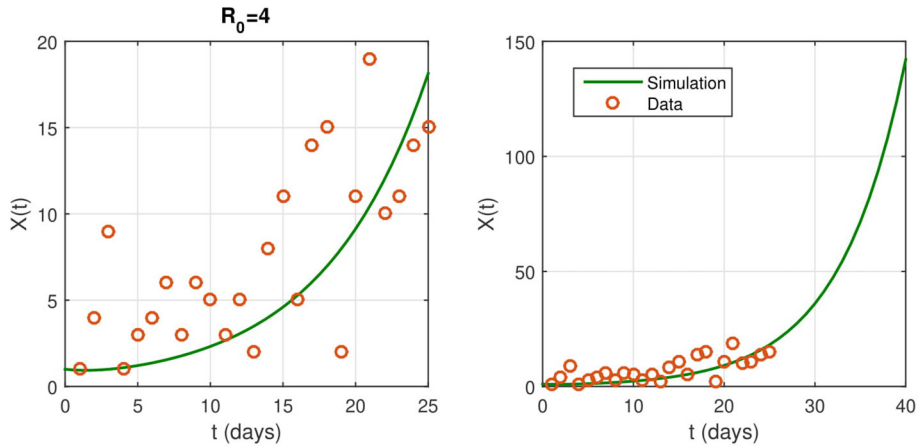

FIGURE 2 | (Left) The fitted curve for epidemic evolution, with $\varepsilon = 0.08$. The small red circles are the reported case data. **(Right)** an illustrations of the shape of $X(t)$ without any intervention. It predicts the spread of the epidemic.

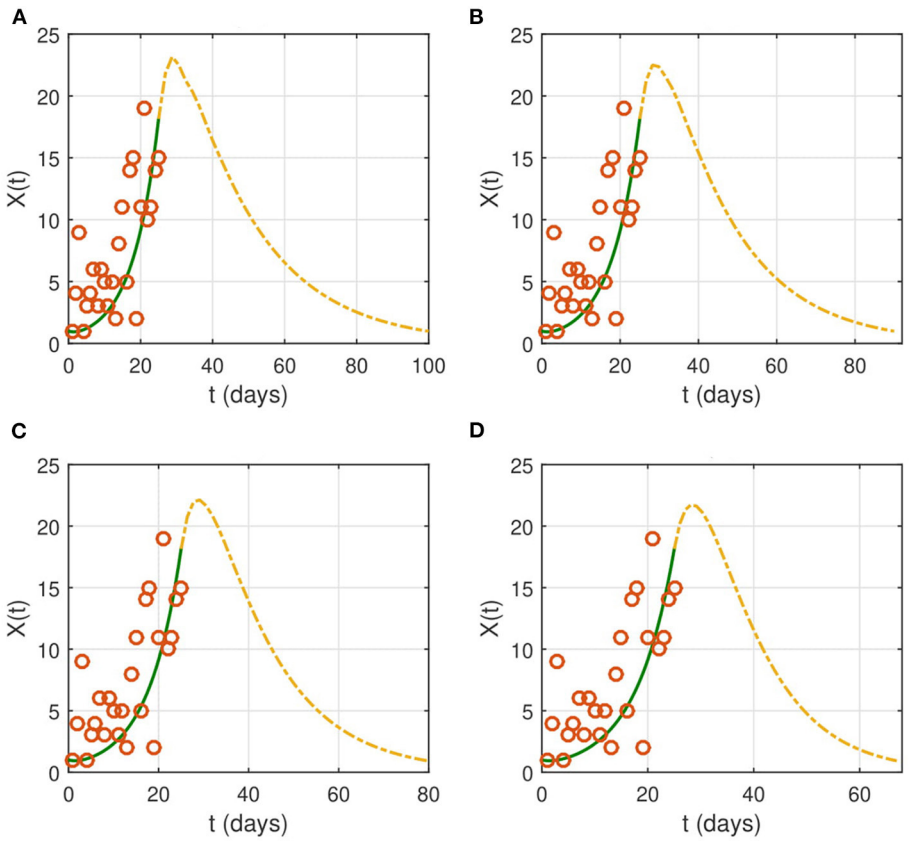

FIGURE 3 | The effect of NPIs on the evolution of the identification function $X(t)$. Lockdown starts on from April 1, 2020 ($t = 26$). We choose the following values for the contact rate: **(A)** $\beta_{new} = 0.1 \times \beta$, **(B)** $\beta_{new} = 0.08 \times \beta$, **(C)** $\beta_{new} = 0.05 \times \beta$, and **(D)** $\beta_{new} = 0.001 \times \beta$. The time interval of the X-axis represents the duration of the epidemic. The red circles represent the data for the number of reported cases.

when the same number drops below this value. We compute this duration using the following formula:

$$T_d = (N_2 - N_1)dt,$$

where N_2 is the number of iterations necessary to reach the end of the epidemic, N_1 is the number of iterations to reach the onset of disease spread, and dt is the time step. A lockdown is a social distancing measure which aims to reduce the movement

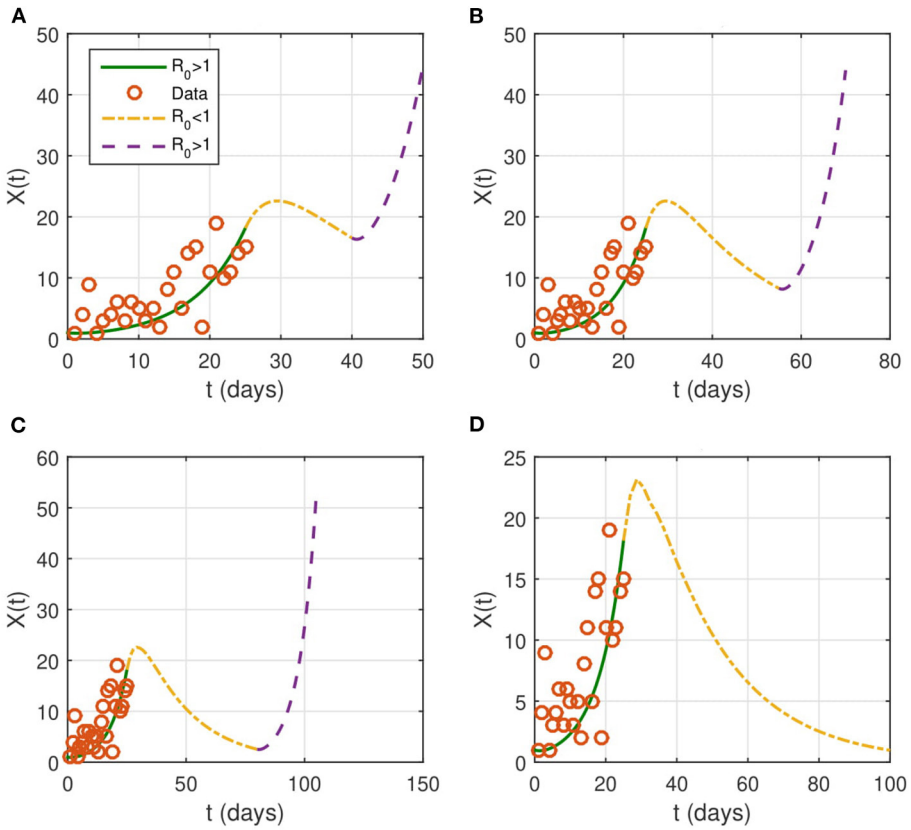

FIGURE 4 | Evolution of the identified infected population of $X(t)$. NPIs are considered from April 1 ($t = 26$), with $\beta_{new} = 0.1 \times \beta$ and last for a duration T. We take several intervention's duration: **(A)** $T = 15$ days, **(B)** $T = 30$ days, **(C)** $T = 45$ days, and **(D)** $T = 75$ days. The red circles represent the data for the number of reported cases.

of individuals to decrease to the transmission of the disease. However, full lockdown is an intervention that has a relatively high economic and social cost. Therefore, several countries have instead opted for a partial lockdown where only a proportion of the population is immobilized. In Vietnam, a lockdown was mandated on April 1, 2020. In this section, we use the multi-scale model to evaluate the effects of both full lockdown and partial lockdown on the evolution of the epidemic. In this model, we consider that the lockdown corresponds to the case where a portion of the population do not move which reduces the chances of contact between individuals. In the absence of control measures, numerical simulations show that the peak would be reached on May 1, 2020. At this moment, more than half of the population is infected and the virus concentration on the computational domain is high. By the end of the simulation time which corresponds to June 17, 2020, there are still a few cases of infected individuals who are not yet quarantined. Three stages of a numerical simulation showing the transmission dynamics in the absence of control measures are represented in **Figure 5A**.

We apply a partial lockdown, and we consider that only a half of the population move. In this case, the transmission dynamics remain the same, but the cumulative percent of infected cases is reduced to half. As before, the peak is reached on May 1, 2020, and the active number of infected individuals does not reach zero by the end of the simulation time. When we consider a stricter lockdown and immobilize 75% of the population, the peak is reached on April 18, 2020. The epidemic curve is flattened, and the cumulative percent of infected individuals falls to 29%. Still, the epidemic duration is not shortened and there a few infected cases remain on the computational domain by the end of the simulation time. We have represented three stages of a numerical simulation of the COVID-19 transmission dynamics in **Figure 5B**.

We study the transmission dynamics when a full lockdown is imposed from April 1, 2020. We consider that 90% of the population cannot move. The adoption of this intervention shortens the time of the epidemic. In this case, the peak is reached on the same day when the lockdown is imposed. The epidemic is resolved by May 15, 2020. The cumulative percent of infected individuals is reduced to 8%. We have represented the cumulative percent of infected individuals over time for the different strategies in **Figure 6**. The active percent of infected and symptomatic individuals are shown in **Figure 7**. It shows that a partial lockdown flattens the epidemic curve but does not shorten the duration of the epidemic. Whereas, a full lockdown stops the transmission of the disease and reduces the time of the epidemic.

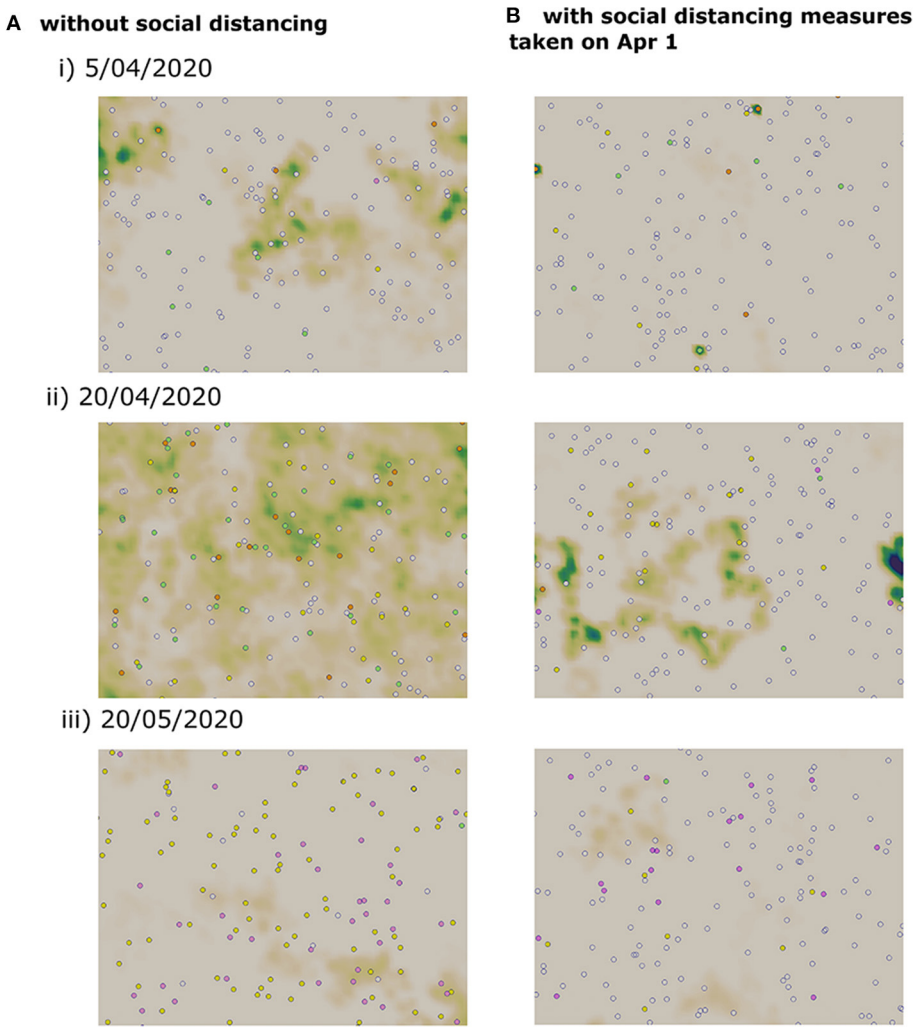

FIGURE 5 | Snapshots of numerical simulations showing the effect of social distancing on the transmission dynamics of COVID-19 in three different dates. **(A)** Without social distancing measures. **(B)** With a partial lockdown which targets 75% of the population.

In the absence of control measures, it is expected that 70% of the population would develop immunity against COVID-19. As a result, the population would be less susceptible to a new outbreak. However, this strategy of herd immunity is not guaranteed in the absence of evidence showing that recovered individuals develop immunity to COVID-19. The percent of recovered individuals decreases as the portion of immobilized population increases as shown in **Figure 8A**. In the absence of control measures, the case fatality rate (CFR) is equal to 6.4%. When a partial lockdown is applied and 50% of the population is immobilized, the CFR is 5.9%. When a full lockdown is imposed, the number of deceased patients reaches zero. We have represented the cumulative percent of deceased individuals for the different epidemic control strategies in **Figure 8B**. We represent the cumulative number of deceased patients in **Figure 8**.

5. DISCUSSION

This work aims to predict the evolution of the COVID-19 epidemic in Vietnam. To achieve this, we have used the available data on the early stages of the epidemic to calibrate two models. The first is an SEIR model which describes the transmission dynamics using four compartments: susceptible, exposed, infectious, and recovered. The second is a multi-scale model which describes the explicitly the movement of individuals, the transmission of the disease, and the clinical course of patients. The first approach is suitable for the study of the transmission dynamics of the disease at the population level. While the second is appropriate for the investigation of the fine-grained aspects that determine the spread of the disease at the individual level. Therefore, the two approaches complement each other and provide a deeper understanding of

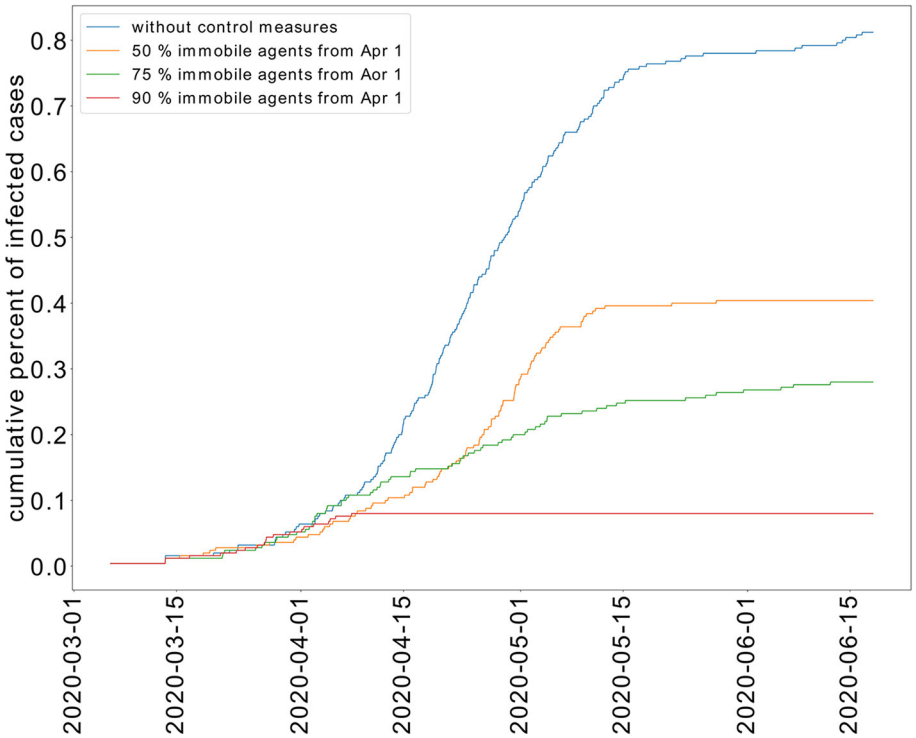

FIGURE 6 | Effect of limited movement of the population on the cumulative percent of infected individuals.

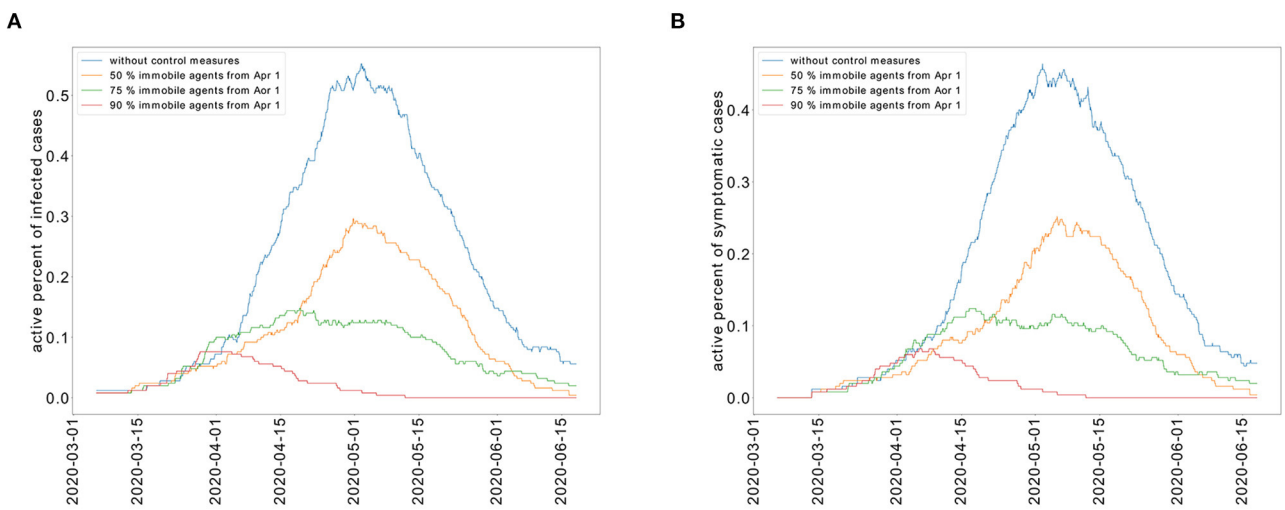

FIGURE 7 | Restricted population movement flattens the epidemic curve for the active percent of infected individuals **(A)** and symptomatic individuals **(B)**.

the transmission dynamics of COVID-19. We have previously applied these models to study COVID-19 transmission dynamics in Algeria (28), China, Italy (1), and Morocco (2).

Two models are used to predict the effects of a partial and full lockdown on the evolution of the epidemic in Vietnam. Both models show that a partial lockdown would reduce the cumulative number of infected individuals. However, it will take more than 2 months and a half after the lockdown beginning for the epidemic to resolve. Whereas, it takes only 1 month and a half for the epidemic to end the epidemic if a full lockdown is mandated. This last strategy was adopted in Vietnam and it shows the good agreement between the predictions of the two models and reported data as of May 3, 2020.

The two models rely on several assumptions. First, we consider closed systems in both models because international

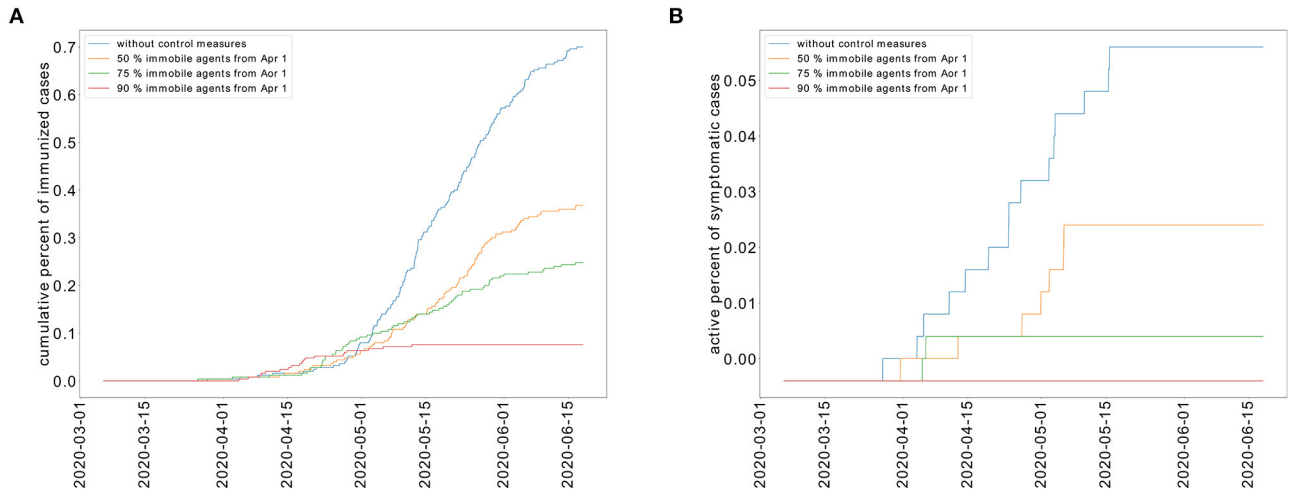

FIGURE 8 | Cumulative percent of recovered individuals **(A)** and deceased patients **(B)** for different levels of restricted movement.

flights are suspended. Second, we do not consider the changes in the population related to the birth of new individuals and deaths by other causes than COVID-19. Third, data related to COVID-19 mortality and infection dynamics are taken from clinical studies conducted in China. Finally, the effects of asymptomatic individuals and unreported cases are not taken into consideration because we do not have sufficient data on the transmission and infection dynamics of these individuals. In a forthcoming work, we plan to apply the model to investigate the transmission dynamics of COVID-19 in urban areas with realistic geometries (13).

DATA AVAILABILITY STATEMENT

The original contributions generated for the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors designed the study and planned the simulations. AC analyzed the data and conducted numerical simulations with the SEIR model. AB and AJ performed simulations with the multi-scale models. All authors contributed to the writing of the paper 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/fpubh.2020.559693/full#supplementary-material

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Going Viral: How Fear, Socio-Cognitive Polarization and Problem-Solving Influence Fake News Detection and Proliferation During COVID-19 Pandemic

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In times of uncertainty, people often seek out information to help alleviate fear, possibly leaving them vulnerable to false information. During the COVID-19 pandemic, we attended to a viral spread of incorrect and misleading information that compromised collective actions and public health measures to contain the spread of the disease. We investigated the influence of fear of COVID-19 on social and cognitive factors including believing in fake news, bullshit receptivity, overclaiming, and problem-solving—within two of the populations that have been severely hit by COVID-19: Italy and the United States of America. To gain a better understanding of the role of misinformation during the early height of the COVID-19 pandemic, we also investigated whether problem-solving ability and socio-cognitive polarization were associated with believing in fake news. Results showed that fear of COVID-19 is related to seeking out information about the virus and avoiding infection in the Italian and American samples, as well as a willingness to share real news (COVID and non-COVID-related) headlines in the American sample. However, fear positively correlated with bullshit receptivity, suggesting that the pandemic might have contributed to creating a situation where people were pushed toward pseudo-profound existential beliefs. Furthermore, problem-solving ability was associated with correctly discerning real or fake news, whereas socio-cognitive polarization was the strongest predictor of believing in fake news in both samples. From these results, we concluded that a construct reflecting cognitive rigidity, neglecting alternative information, and black-and-white thinking negatively predicts the ability to discern fake from real news. Such a construct extends also to reasoning processes based on thinking outside the box and considering alternative information such as problem-solving.

Keywords: COVID-19, fake news, problem-solving, fear, xenophobia, overclaiming, bullshit receptivity, socio-cognitive polarization

INTRODUCTION

"If it bleeds, it leads" cites a well-known mantra of journalism. When a story involves deaths or injury of some kind, it is more likely to be discussed on the media, to receive a higher number of clicks on the internet and be shared. News stories are often reported in a sensationalist form tailored to trigger an emotional response which can influence our ability to reason. Recent studies on the impact of misinformation during the COVID-19 pandemic highlight how unreliable and inflammatory information (Gallotti et al., 2020) may have threatened public health in several countries by altering individuals' perception of risk behaviors (Oh et al., 2020). For example, it appears that at the beginning of the pandemic the population was divided between those who were seriously concerned and reacted by seeking information and those who thought that COVID-19 was "no more than the flu" and thus resisted taking safety measures. Stanley et al. (2020) found that analytic thinking, measured via the Cognitive Reflection Test (CRT: Frederick, 2005), was a predictor of believing the pandemic was a hoax and resisting the adoption of safety measures like social-distancing and hand-washing to mitigate spread. A pandemic is a prototypical situation wherein collective behavior directly impacts the health and safety of every member of the group. Consequently, the belief in and proliferation of fake news during a global pandemic is a significant concern that can exacerbate a public health emergency. Indeed, misinformation has been instrumentalized by pushing divisive political ideologies with the consequence of preventing cooperation among individuals (Stella et al., 2018). In the present investigation, we attempted to gain a better understanding of specific social, cognitive, and emotional factors that contribute to individuals' tendency to believe in and share fake news during the early height of the COVID-19 pandemic in Italy and the United States.

Why does negative news echo faster than positive news? To answer this question, we highlight two components of news that contribute to its appeal: *alertness,* and *informativeness.* A first component that may explain why people are so attracted to negative news, whether it is fake or real, is that news tends to be *alerting* by inducing fear. From an evolutionary framework, fear makes people more sensitive to potential threats (Ohman and Mineka, 2001; Schaller et al., 2003; Balzarotti and Ciceri, 2014). Our cognitive system is particularly tuned toward potential sources of threat such as negative events, which, in most situations, are "more salient, and generally efficacious than positive events" (Rozin and Royzman, 2001, p. 297). Given its specific relevance to survival, human cognitive processing of negative stimuli is more elaborate, detailed, and complex compared to positive stimuli [i.e., negativity bias, see Rozin and Royzman (2001)]. Moreover, memories for negative information form more quickly and are more easily retrieved (Kensinger et al., 2006, 2007). A pandemic is a prototypical scenario that engenders a great deal of unresolved fear and anxiety that can leave people in a constant state of high alert. In parallel to the immune system, animal species have developed specific cognitive and behavioral responses that help us avoid infections (Schaller and Park, 2011). This disease-avoidance system drives both explicit and implicit safety measures against infection (e.g., increased hand-washing; Fleischman et al., 2011). For example, in response to fear people tend to be socially avoidant and less tolerant of foreigners, and are in general more xenophobic (Navarrete et al., 2007; Mortensen et al., 2010; Schaller and Park, 2011). Following this perspective, we predicted that fear of COVID-19 should relate to seeking out information about the disease, proactively taking actions to reduce the chances of being infected, and sharing as much information as possible.

A second factor involved in the proliferation of news is the need for *information*, which is especially pertinent during global crises such as the COVID-19 pandemic. Confirmed coronavirus (COVID-19) cases increased exponentially first in China, followed by Italy, Spain, central Europe, and then the United States, culminating in a worldwide public health emergency. From the earliest days of the outbreak, misinformation about COVID-19 circulated widely across social media, radio, talk shows, and national news media (Frenkel et al., 2020; Pennycook et al., 2020). Seeking out information can help resolve uncertainty during a time of heightened anxiety (e.g., Webster and Kruglanski, 1994; Kossowska and Bukowski, 2015). The limited amount of reliable scientific information during the beginning of the COVID-19 outbreak likely encouraged people to search for explanations that did not yet exist as the science underlying the biology and spread of the virus was still being investigated. This void of scientific consensus may have opened a wide avenue for the spread of pseudo-scientific and outright false information. In the context of threat, where feelings of uncertainty and fear make it difficult to anticipate or plan actions, people compulsively search for explanations and tend to base them on readily accessible pieces of information (Hogg and Adelman, 2013; Kossowska and Bukowski, 2015). Such a lack of reliable information, together with the fear of infection, might have compelled people toward pseudo-profound existential beliefs, as well as overclaiming confidence in unreliable information to make up for a lack of reliable information and overcome uncertainty-induced anxiety. Therefore we hypothesized that fear of COVID-19 should predict a greater likelihood of believing fake news, in particular COVID-related fake news; second we predict it would relate to individual's propensity to judge pseudo-profound statements as profound (measured by the Bullshit Receptivity Questionnaire; Pennycook et al., 2015) and the tendency for people to "self-enhance" when asked about their familiarity with general knowledge questions (Pennycook and Rand, 2017) assessed using the overclaiming scale (Paulhus et al., 2003). Moreover, we predicted that those with higher bullshit receptivity and worse overclaiming accuracy would be more likely to believe in fake news.

From the existing literature, we know that individual differences in thinking and reasoning modulate individuals' propensity to believe in fake news. Specifically, there is a positive correlation between solving the CRT and discerning fake from real news (e.g., Pennycook and Rand, 2017). Dispositionally analytical thinkers are, indeed, more resistant to believing fake news (e.g., Pennycook and Rand, 2017, 2019), but were also more likely to avoid safety measures at the beginning of the COVID-19 pandemic (Stanley et al., 2020). Stupple et al. (2017)

examined the role of "cognitive miserliness" as a determinant of poor performance on the CRT. According to the cognitive miserliness perspective, people often respond incorrectly on CRT items because they are "unwilling to go beyond the default, heuristic processing and invest time and effort in analytic, reflective processing" (p. 1). Additionally, reduced inhibitory control is found to be associated with lower CRT performances (Oldrati et al., 2016). Solving a problem entails being able to go beyond the first interpretation of a problem, accrue information, and incubate with potential solutions until the most appropriate solution is reached. The ability to detect fake from real news might also rely on mechanisms involved in problem-solving. This perspective finds support in a recent demonstration that people share false information about COVID-19 because they fail to think sufficiently about the accuracy of news content (Pennycook et al., 2020). In light of previous studies showing that stress, high risk-taking and anxiety of running out of time deteriorate creativity, and problem-solving performance (Salvi et al., 2016a; Salvi and Bowden, 2019; Duan et al., 2020) we hypothesized that the problem-solving performance would relate to a greater likelihood of detecting fake news, whereas fear of COVID-19 would lead to worse problem-solving performance.

To date, research on fake news has focused predominately around the 2016 US presidential election. Liberals and conservatives differ in cognitive style and the latter appear to fail at discerning fake from real news within a political context (Pennycook and Rand, 2017; Pennycook et al., 2018). There is scientific literature showing that political ideology is associated with cognitive rigidity/flexibility and different problem-solving styles, where liberalism is associated with a problem-solving style oriented toward insight, and conservatism toward step-by-step processing (Salvi et al., 2016b). Interestingly, insight problem-solving appears to lead to higher accuracy on problem-solving tasks, rely on brain regions responsible for novel and original associations and may also be involved in fake news detection (e.g., Salvi et al., 2016a, 2020; Shen et al., 2017; Cristofori et al., 2018; Danek and Salvi, 2018; Laukkonen et al., 2020). Additionally, there is evidence that conservatives and liberals differ in creativity (Dollinger, 2007). Overall, conservatives appear to be more structured, rigid, and prefer more direct answers, whereas liberals have more tolerance for ambiguity and complexity and tend to show greater openness (Jost et al., 2003). Conservatives present higher perceptual rigidity for example and appear to be more influenced by figures' global shapes as well as contextual information than liberals (Caparos et al., 2015). This difference is also reflected in neurocognitive functioning: Liberalism is associated with stronger anterior cingulate activity suggesting that liberals have a higher sensitivity for monitoring response conflict, whereas right-wing orientation is associated with greater neural sensitivity to fear and larger amygdala volume (Amodio et al., 2007; Jost and Amodio, 2011). These differences in problem-solving accuracy and the capacity to handle complexity may help explain people's ability to assess conflicting information provided by media outlets.

Recent studies investigating social media content have demonstrated that accounts with a high "bot score" (indexing the likelihood of being a bot, or fake account) promulgate conspiratorial narratives charged with alt-right ideology and are specifically oriented toward hateful and polarizing political ideologies (Stella et al., 2018; Ferrara, 2020). During the COVID-19 pandemic, hateful bots have been found to be more successful in attracting followers compared to counter-hate bots. Hateful and counter-hate bots appeared to interact and engage extensively with one another, promoting a culture of racism against Asians (Ziems et al., 2020). Because of the association between conservatism, rigidity in overall reasoning, and previous evidence on believing in fake news, we hypothesized that conservatism would predict fake news beliefs also in our dataset.

Tolerance of ambiguity is a well-established trait of personality known to predict creativity and problem-solving (Merrotsy, 2013). The Multidimensional Attitude Toward Ambiguity Scale (Lauriola et al., 2016) detects three different dimensions of intolerance for ambiguity: the affective (Discomfort with Ambiguity), cognitive (Moral Absolutism/Splitting), and epistemic (Need for Complexity and Novelty) components. Budner (1962) defined ambiguous situations or contexts as those which "cannot be adequately structured or categorized by an individual because of the lack of sufficient cues" (Budner, 1962, p. 30). Ambiguous situations are those which could be unclear, confusing, or interpreted in more than one way. Those who are intolerant of ambiguity tend to resort to black-or-white solutions and are distinguished for their quick and overconfident judgment, even at the neglect of reality (Frenkel-Brunswik, 1949). By contrast, those who are tolerant of ambiguity are attracted to situations they find ambiguous, challenging, and interesting. They are also individuals who score highly on the openness to experience and sensation-seeking behavior scales (McLain, 1993, 2009; Caligiuri et al., 2000; Lauriola et al., 2007). Individuals with low tolerance of ambiguity present an aversive reaction to ambiguous situations because the lack of information makes it difficult to evaluate risk and thus make decisions. These scenarios are perceived as a source of discomfort and people react to a perceived threat with stress, avoidance, delay, suppression, and denial (Budner, 1962; MacDonald, 1970; McLain, 1993; Furnham and Ribchester, 1995; Iannello et al., 2017). The tolerance of ambiguity scale negatively correlates with authoritarianism (MacDonald, 1970) and ethnocentrism (O'Connor, 1952) and positively with openness (Bardi et al., 2009) extraversion, and "novelty-seeking" (Rajagopal and Hamouz, 2009). Therefore, we hypothesized that intolerance of ambiguity, specifically captured by absolutism, could be associated with believing in fake news.

Xenophobia, specifically toward patients and Chinese visitors, is a final factor linked to the recent spread of anti-Asian hate seen during the COVID-19 pandemic (Ziems et al., 2020). On January 24, 2020, the fake news that "Chinese passengers from Wuhan with fever escaped the quarantine at Kansai International Airport" (Kansai International Airport., 2020) was spread through multiple social media channels. Despite Kansai International Airport denying that took place, xenophobia against Chinese people rapidly spread in Japan and all around the world. *#ChineseDon'tComeToJapan* started trending on Twitter, while "Chinese visitors [were being] tagged as dirty, insensitive, and even bioterrorists" (Shimizu, 2020).

Xenophobia has never been studied concerning fake news. Considering its relation to fear (see above) and potentially cognitive rigidity, we hypothesized xenophobia may play a part in the discernment of fake from real news, particularly COVID-19 news.

The shared literature on emotional, social, and cognitive factors underlying, political conservatism, intolerance for ambiguity, and xenophobic reactions suggest that people who score higher in these measures may be less likely to handle complexity and thus fail to seek out alternative explanations when assessing news. As such, we believe these factors share a common theoretical ground and belong to the same construct which we define as *Socio-Cognitive Polarization* (SCP; a factor capturing absolutism/intolerance of ambiguity, xenophobia, and conservative political ideology). Therefore, we hypothesized that they would be highly correlated to each other and would negatively predict the ability to discern fake from real news.

In sum, in this study, we sought to understand the potential role of a range of emotional, social, and cognitive factors underlying the infodemic during the early height of the COVID-19 pandemic. To this end, we investigated how fear of the COVID-19 pandemic is related to information seeking and proactive health behavior, fake news detection and sharing, propensity toward pseudo-profound beliefs, overclaiming false information, and problem-solving. We also investigated how these factors uniquely predict participants' ability to discern fake from real news. In particular, we expected that fear of COVID-19, problem-solving, and Socio-Cognitive Polarization would uniquely predict fake news detection and sharing. We administered a survey to participants from two countries that registered the highest case and death counts associated with COVID-19 during the early peak of the pandemic: Italy and the United States. The participants included a set of news headlines split by news-type (COVID-19-related or neutral) and veracity (fake or real); a series of questions to assess COVID-related fear and information-seeking proactivity; problem-solving tasks including the CRT and a set of visual and semantic puzzles (i.e., Rebus puzzles: MacGregor and Cunningham, 2008; Salvi et al., 2015a,b); and a series of scales measuring bullshit receptivity, propensity toward overclaiming, political ideology, xenophobia, and absolutism (See **Figure 1** for a summary of the experimental hypotheses).

METHODS

Pre-registration

We report materials used, our target sample size, how we determined data exclusions, our primary hypotheses, and our plan for primary analyses in our OSF preregistration available online (https://osf.io/tsvg5). Our materials and datasets can be found at our OSF preregistered project online (https://osf.io/4pd2u). In this paper we discuss only the first hypothesis of the preregistered study. Results of hypothses number two and three will be published separately.

Participants

Five hundred and sixty-five Italian and American volunteers participated in the study and completed the news and problem-solving portions of the survey (excluding outliers). The complete demographic characteristics of the respondents are available in the **Supplementary Table 1**. The two samples did not differ for age ($t_{(560)} = 0.43$; $p = 0.669$) and gender ($X^2 = 0.03$; $p = 0.985$), yet differed significantly in marital status and level of education (**Supplementary Table 1**). Because these differences suggest our samples reflect different populations, we decided to analyze and report the results of the two samples separately.

Italian Sample

Out of three-hundred and twelve participants who completed at least 50% of the study, three-hundred native Italian speakers completed all news and problem-solving items. Eight outliers were removed for performing three standard deviations away from the mean on our fake news variables, leaving us with a final sample of two-hundred-and-ninety-two participants (210 females, average age = 37.79, SD = 16.06). Missing values for nineteen participants (who failed to complete at least one item of the remaining scales) were imputed using the mean values across subjects for the respective variable. Participants were distributed as follows: 51.7% from Milan; 18.8% from Bergamo city[1]; 14.8% from other cities in the Lombardy region; 10.2% from other northern and central regions (Emilia Romagna, Liguria, Piemonte, Toscana, Trentino Alto Adige, Veneto); 2.8% from southern regions (Calabria, Campania, Sicilia).

American Sample

Out of three-hundred and forty-three participants who completed at least 50% of the study, two-hundred and seventy-five native American English speakers completed the news and problem-solving items. Two outliers were removed for performing at least three standard deviations away from the mean on our fake news variables, leaving us with a final sample of two-hundred and seventy-three participants (198 females, average age = 38.8, SD = 16.38). Missing values for twenty-one participants (who failed to complete at least one item of the remaining scales) were imputed using the mean values across subjects for the respective variable. Participants were distributed as follows: 35.2% from the state of Texas; 12.8% from the state of New York; 15.5% from Midwestern states (Illinois, Indiana, Iowa, Kansas, Michigan, Minnesota, Missouri, Nebraska, Ohio, Wisconsin); 13.6% from Western states (Arizona, California, Colorado, Hawaii, Idaho, New Mexico, Oregon, Utah, Washington), 13.2% from other Southern states (Arkansas, Florida, Georgia, Kentucky, Louisiana, Maryland, Mississippi, North Carolina, Oklahoma, South Carolina, Virginia, West Virginia); 9.9% from other Northeastern states (Connecticut, Maine, Massachusetts, New Jersey, Pennsylvania, Rhode Island).

[1]Among all the Italian cities Bergamo counted the highest number of deads for COVID-19.

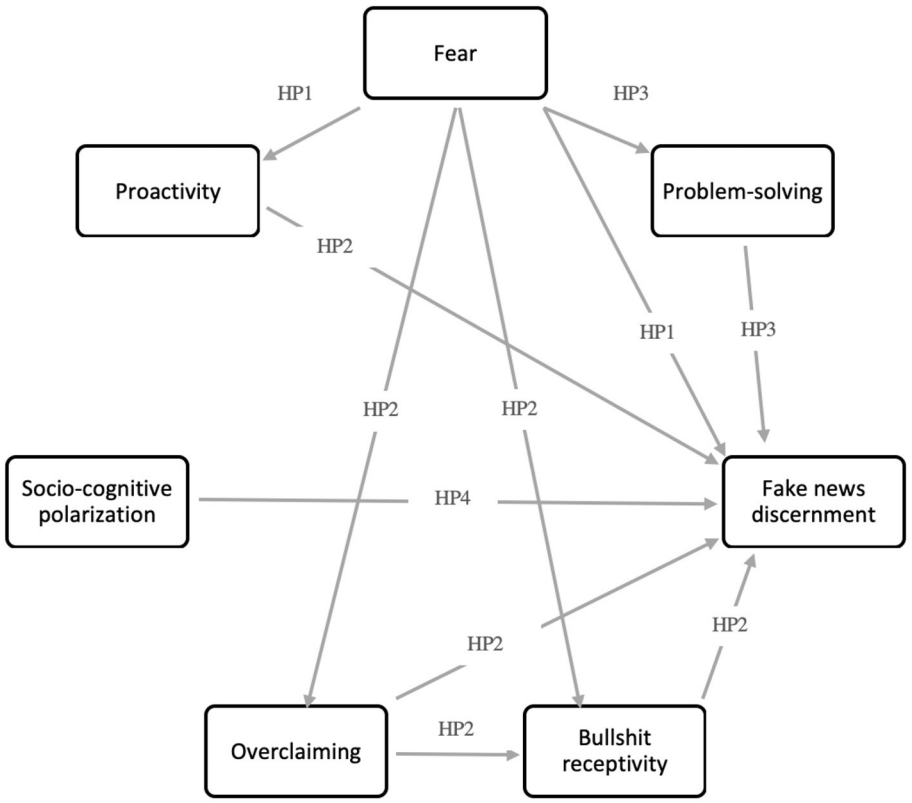

FIGURE 1 | Summary of our hypotheses and results. **significance at $p < 0.01$; ***significance at $p < 0.001$.

Sample Size Estimation

Based on data from previous studies investigating the relationship between fake news discernment and social and cognitive variables (Pennycook and Rand, 2019), we performed statistical power analyses for sample size estimation. Power analysis (at an alpha = 0.05 and power = 0.90) revealed that our final sample of participants (273 Americans; 292 Italians) is higher than the projected sample size ($N = 255$) needed to obtain the meaningful effect size for weak correlations ($r = 0.20$; GPower 3.1 software), as well as the projected sample size ($N = 247$) needed to obtain the meaningful effect size for weak to moderate increase in R^2 for fixed multiple linear regression with 8 tested predictors ($f^2 = 0.08$; G Power 3.1 software).

Procedure

Data were collected in Italy and the US during the COVID-19 pandemic peaks (Italy: 3–24 April 2020; US: 14–28 April 2020). Qualtrics online survey platform (www.qualtrics.com), hosted on the University of Texas at Austin and Università Cattolica del Sacro Cuore of Milan servers, was used to distribute the survey. Participants were recruited via email invitations, advertisements on social media platforms, as well as psychology and creativity associations' websites. Participation was voluntary. All participants gave written informed consent. Each session lasted ∼40 min.

Measures

After providing information about demographics (age, gender), marital status, level of education, type of news sources they consulted to seek information about COVID-19 (**Supplementary Table 1**), and political orientation, participants completed the online survey, which assessed fake news discernment, COVID-19-related fear and proactivity, bullshit receptivity, overclaiming tendencies, problem-solving, and socio-cognitive polarization. Clusters of individual sub-measures, which were theoretically similar and moderately to highly correlated ($r \geq 0.2$), were collapsed into higher-order factors by z-scoring individual measures and averaging across a specific factor. Our factor reduction procedure left us with a total of 10 predictor variables for our primary analyses: 3 demographic factors (age, sex, and level of education); 3 COVID-19 factors (COVID-19 fear, proactivity, and city case count); and 4 socio-cognitive factors (bullshit receptivity, overclaiming, problem-solving and socio-cognitive polarization). Correlation matrices for each collapsed factor can be found in the (**Supplementary Table 2**).

Fake-News Discernment

To measure the ability to judge the accuracy of COVID-19-related and neutral news headlines, 12 legitimate-looking news articles, inspired by news and titles found online, were created from scratch following the methodology of Pennycook and Rand (2017, 2019) (see **Figure 2**). The news articles were presented in a dedicated section of the survey. Six news headlines were factually accurate (real news) and six were false (fake news). The articles covered COVID-19-related news 50% of the time, whereas in the other 50% they covered more general topics, namely technology, nature, and employment (COVID-19 *vs.* neutral content). The news articles were balanced for credibility, plausibility, and sensationalism, through preliminary testing on a sample of 24 participants. Each headline was presented in the format of online newspaper headlines and it included a sensationalist headline, a thumbnail image, and a preview text from the article, while the sources were identical for each headline to control for source-bias.

For each news article, participants were asked: (1) if they were familiar with the article (response options: "No"; "Unsure"; "Yes"); (2) how accurate they believed the article was (a 5 points scale ranging from "Not at all accurate" to "Very accurate" was used); and (3) if they would share that article on social media (response options: "I would never share it online" (these data were removed from the analyses); "No"; "Maybe"; "Yes"). To make sure participants would not use any internet source to search for the answers, the news articles appeared on the screen for a maximum of 45 s. A discernment index, representing the ability to discern fake from real news, was calculated, following Pennycook and Rand's (2019) procedure, by subtracting the perceived accuracy of fake news from perceived accuracy of real news and dividing by 4. The discernment index ranges from −1 (i.e., complete belief in fake news and disbelief in real news) to 1 (i.e., complete belief in real news and disbelief in fake news), with 0 indicating no discernment between fake and real news. Similarly, a social-media sharing discernment index was calculated by subtracting willingness to share fake news from the willingness to share real news and dividing by 3. Individual discernment indices were computed for COVID-19-related news and neutral news.

COVID-19 Variables

Information about the type of news sources participants normally consulted to learn about COVID-19 was collected using a multiple answer question (options: daily or online newspapers, TV news, social media or news aggregates, government websites, radio, or podcasts).

Participants answered 6 questions about their perception, emotions, and behaviors toward COVID-19. More precisely, they were asked to rate, on a scale from 0 (low) to 100 (high), the perceived severity of COVID-19, the negative arousal associated with the possibility of being infected, and the perceived likelihood to be infected. Participants were also asked to evaluate the frequency of their proactive information-seeking behaviors to face the pandemic (i.e., search for more information and take actions to reduce their chances of infection) on a 7-point Likert scale. Individual Perceived Severity and Arousal scores were collapsed into one "COVID-19 fear" factor in the analysis, whereas proactivity scores were treated as a separate "proactivity" factor in the analysis.

Data about the spread of COVID-19 during the studied period (March 27–April 28, 2020), and more precisely country, state, and city-specific confirmed case and death counts, were retrieved from the Italian Civil defense COVID-19 database (GitHub, 2020b) and the New York Times COVID-19 database (GitHub, 2020a). On the last day of data collection, death counts reached 25,969 for Italy and 53,034 for the U.S. Daily counts of positive cases in each city were used as the "cases" factor in the analyses since we expected a local index of COVID-19 would be the best metric of environmental severity (see **Supplementary Table 1**).

Bullshit Receptivity Score (BRS)

The propensity to judge pseudo-profound statements as profound was measured using the Bullshit Receptivity Questionnaire by Pennycook et al. (2015). Pseudo-profound bullshit is defined by the authors as seemingly impressive assertions, which are presented as true and meaningful but are actually vacuous sentences with no discernible meaning (e.g., "Interdependence is rooted in ephemeral actions"). The scale includes actual profound statements (e.g., "All endings are also beginnings. We just don't know it at the time") and non-profound, mundane statements, which reported simple facts (e.g., "Some things have distinct smells"). An Italian version of the scale was created by translating the original statements and then verifying them with a back translation. Participants were asked to rate each statement on their profundity on a 5-point Likert scale (1 = Not at all profound; 5 = Very profound). Individual scores for bullshit, profound and mundane statements were computed (For the Italian translation see **Appendix 1**).

Overclaiming

Overclaiming is considered the tendency for people to "self-enhance" when asked about their familiarity with general knowledge questions (Pennycook and Rand, 2017). A shortened version of the Paulhus et al. (2003) overclaiming questionnaire was included in the survey. We administered a list of 13 different items with which participants had to rate their familiarity on a 7-point Likert scale (1 = Never heard of it; 7 = Very familiar). While 11 items indicated factual physical sciences topics, historical events, or historical figures, 2 foils were designed to detect if participants lied about their knowledge or overclaim. The items of the original scale were translated into Italian. To avoid participants researching the items on the Internet, the 13 questions were timed 60 s. A general knowledge score, consisting of the number of real items that received a score $\geq$ 4 (i.e., hits) and an overclaiming score, consisting of the number of foils which received a score $\geq$4 (i.e., false alarms), were computed. Finally, an accuracy score was calculated by subtracting the number of false alarms from the number of hits (Paulhus et al., 2003). A higher accuracy score indicates a lower tendency to overclaim.

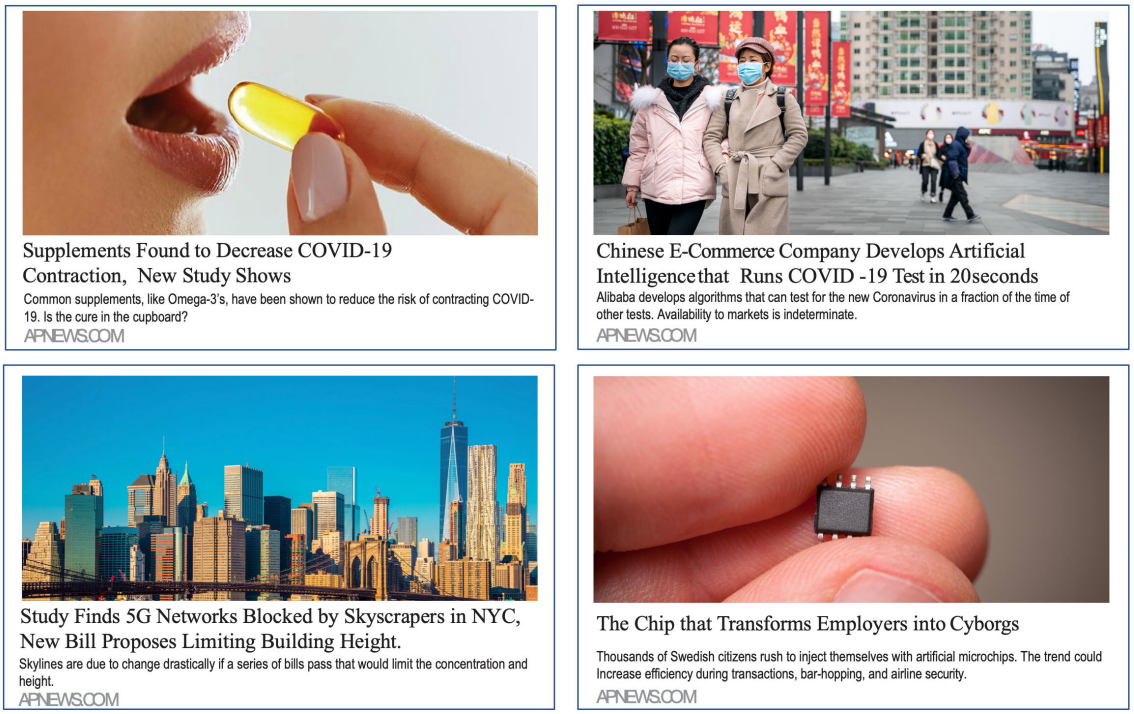

FIGURE 2 | Examples of fake news headlines presented to participants. We report one example for each type: Top left, COVID-19-related fake news; top right, COVID-19-related real news; bottom left, neutral fake news; bottom right, neutral real news. ©Images purchased from shutterstock.com. **significance at $p < 0.01$; ***significance at $p < 0.001$.

Problem-Solving

The problem-solving measures the performance of two cognitive tasks: a rebus puzzle-solving task (MacGregor and Cunningham, 2008; Salvi et al., 2015b) and four problems from the CRT (Frederick, 2005; Thomson and Oppenheimer, 2016).

Rebus Puzzles

Participants were administered 20 rebus puzzles taken from MacGregor and Cunningham (2008) and Salvi et al. (2015b). To solve each rebus puzzle, subjects had to merge verbal and visual clues to make a common phrase, such as: "Cycle, Cycle, Cycle"; solution: "Tricycle." These problems are solved through either insight or a step-by-step process. Subjects were asked to produce a text string response for each rebus and to self-report the problem-solving method they used to solve each rebus. The results on insight problem-solving during the COVID-19 pandemic will be reported separately from the present report.

CRT

Participants were administered four Cognitive Reflection Test problems (CRT; Frederick, 2005). CRTs are deceiving problems that are designed to elicit an immediate, yet incorrect, response. After further consideration, the correct solution becomes more apparent. The four problems were taken from Frederick (2005) and Thomson and Oppenheimer (2016), and more precisely the "bat and ball," "machines," "lily pads," and "Emily's" problem were selected. The Italian version of the problems was taken from Baldi et al. (2013). Each participant's percentage of correctly answered CRT items was calculated.

Socio-Cognitive Polarization (SCP)

The SCP factor included measures of absolutism (Lauriola et al., 2016), xenophobia (van der Veer et al., 2013), and conservatism (Robinson et al., 1999; Salvi et al., 2016b).

Absolutism

The Multidimensional Attitude Toward Ambiguity Scale (MAAS; Lauriola et al., 2016), which measures individual differences in tolerance *vs.* intolerance of perceived ambiguous stimuli, was administered to the participants. The 30-item version of the scale, which had both an Italian and American adaptation (Lauriola et al., 2016), was used. Responses were provided on a 7-point Likert scale (1 = Strongly disagree; 7 = Strongly agree). The Moral Absolutism/Splitting subscale, a measure of rigid and stereotyped "black-and-white" thinking (e.g., "There's a right way and a wrong way to do almost everything"), was of primary interest in the present investigation.

Xenophobia

Hostility and fear toward immigrants were assessed using the 14-item Xenophobia Scale created by van der Veer et al. (2013). Participants indicated their level of agreement with statements such as "Interacting with immigrants makes me uneasy" on a 7-point Likert scale (1 = Strongly disagree; 7 = Strongly agree). The

items of the scale were translated in Italian and then verified by a back-translation.

Conservatism

Political ideology was measured by two 7-point Likert scales (Robinson et al., 1999; Salvi et al., 2016b). Participants were asked to indicate their level of agreement with the following statements: "I endorse many aspects of conservative political ideology" and "I endorse many aspects of liberal political ideology." The conservatism score was calculated by subtracting the score for liberalism from the score for conservatism.

STATISTICAL ANALYSES

Overview

Our primary analyses investigate the relationship across our COVID-19-related factors (fear of COVID, COVID-19 information proactivity, city case count at the time of taking the survey), demographic factors (age, sex, education), cognitive factors (BRS, overclaiming accuracy, and problem-solving), and fake news factors (discernment, familiarity, and sharing). Secondary analyses probe the relation between problem-solving and SCP on fake news discernment in the context of the COVID-19 pandemic. Because we had reason to suspect our samples represented different populations (i.e., large differences in the level of education and in the type of news sources where they sought information about COVID-19; see **Supplementary Table 1**), separate correlations and regressions were performed for each sample.

COVID-19 and Socio-Cognitive Analyses

To test our series of hypotheses relating fear of COVID-19 to fake news discernment, sharing, proactive behaviors, BRS, and overclaiming, we initially performed correlations across COVID-19 factors (fear, proactivity, and city case count), fake news factors (discernment, familiarity, and sharing), and BRS and overclaiming factors. We tested our hypotheses that fear of COVID-19 would predict worse performance on problem-solving tasks by conducting a univariate regression between COVID-19 fear and problem-solving (with age, sex, and education as covariates) in each sample. The effect of type of news on discernment was analyzed through planned pairwise *t*-tests in each sample.

Fake News Discernment Regressions

To test our hypotheses that fear of COVID-19, problem-solving, and SCP would uniquely predict fake news discernment in each sample, we broadened our analyses with a series of planned univariate and multivariate regressions. Our reasoning for implementing multivariate regressions is to capture the strongest and most unique predictors of fake news discernment among the variables of interest by taking into account covariance and collinearity across all factors. We first performed bivariate correlations between each predictor and type of news discernment before fitting linear regressions using the generalized linear model glm() function in R3.6.3 (R Development Core Team., 2008) to determine how well each factor predicts fake news discernment. Our rationale for presenting correlations and univariate results is to illustrate the strength of each predictor when other factors are not taken into account. This was followed by a multiple stepwise regression in which fake and neutral discernment were separately regressed onto all COVID-19-related and cognitive factors, again using the glm() function with forward and backward selection in R3.6.3 (R Development Core Team., 2008). All regressions included sex, age, and education as covariates.

Given the widely recognized limitations of stepwise regression (Whittingham et al., 2006), we sought to isolate the most unique predictors of fake news discernment by fitting a cross-validated LASSO, or penalized regression, using the cv.glmnet() function (Friedman et al., 2010) in R3.6.3 (R Development Core Team., 2008). This method works by adding a L1-penalization term to the regression equation (Tibshirani, 1996), where larger λ-values correspond to the shrinking of more regression coefficients to zero. LASSO regression penalizes collinear coefficients and retains variables that most uniquely predict the outcome variable, thereby avoiding overfitting and multicollinearity [for a broader overview, see Gillespie et al. (2018), Yankov et al. (2019)]. We bootstrapped the cv.glmnet() function 1,000 times and extracted the λ with the smallest error deviance between predicted and actual observations. We applied each unique λ to final penalized regressions to identify the subset of factors most strongly predictive of fake news discernment. We executed this procedure four times, once for each unique fake news discernment variable (news type x country).

RESULTS

In support of our hypothesis that COVID-19 fear would predict likelihood to share news, we found that COVID-19 fear positively correlates with willingness to share both COVID-19 [$r = 0.15$, 95% CI = (−0.05, 0.35), $p = 0.01$] and neutral [$r = 0.17$, 95% CI = (0.07, 0.45), $p = 0.01$] news in the American sample (see **Table 1**). Importantly, the positive correlation indicates that COVID-19 fear relates specifically to willingness to share real news above and beyond fake news, contrary to our hypothesis that fear would make people more likely to share fake news. Additionally, in support of our hypothesis that proactivity (i.e., seeking information and taking safety measures) would relate to better discernment of fake news, we find that proactivity positively correlates with fake news discernment for COVID-19 [$r = 0.15$, 95% CI = (– 0.05, 0.34), $p < 0.05$) and neutral [$r = 0.15$, 95% CI = (–.06, 0.34), $p < 0.05$] news, as well as fear of COVID-19 [$r = 0.31$, 95% CI = (0.16, 0.42), $p < 0.01$] in the American sample. As predicted, BRS negatively correlates with both fake news discernment for COVID-19 [$r = -0.13$, 95% CI = (– 0.32, 0.07), $p < 0.05$) and neutral [$r = -0.14$, 95% CI = (−0.34, 0.06), $p < 0.05$] news, as well as positively correlates with COVID-19 fear [$r = 0.16$, 95% CI = (−0.04, 0.36), $p < 0.05$] in the American sample. This finding is in support of our hypothesis that those who score higher on BRS would in turn be less likely to detect fake news and that fear would be positively related to bullshit receptivity. BRS negatively correlates with overclaiming accuracy

[$r = -0.18$, 95% CI = (−0.37, 0.02), $p < 0.05$] in the American sample, suggesting those who are more likely to appraise pseudo-profound beliefs as profound are also more likely to overclaim the accuracy of unreliable information. Finally, overclaiming accuracy positively correlates with fake news discernment for COVID-19 [$r = 0.20$, 95% CI = (0.0, 0.39), $p < 0.05$] in the American sample, suggesting a relationship between two variables that rely on scrutizing potentially false information.

In the Italian sample, proactivity positively correlates with fake news discernment [$r = 0.18$, 95% CI = (−0.03, 0.36), $p < 0.01$] and sharing [$r = 0.18$, 95% CI = (0.07, 0.44), $p < 0.01$] of COVID-19 news. Interestingly, COVID-19 fear positively correlates with proactivity in both the American sample [$r = 0.31$, 95% CI = (0.19, 0.41), $p < 0.001$] and the Italian sample [$r = 0.36$, 95% CI = (0.18, 0.52), $p < 0.001$], highlighting a potentially beneficial role of COVID-19 fear on COVID-19-related information seeking. Similarly to the American sample, BRS positively correlates with COVID-19 fear [$r = 0.17$, 95% CI = (−0.03, 0.36), $p < 0.05$] in the Italian sample, supporting our hypothesis that fear would be positively related to receptivity of pseudo-profound statements. In the Italian sample BRS also correlates with proactivity [$r = 0.17$, 95% CI = (−0.07, 0.33), $p < 0.05$). From our results fear and fake news discernment did not significantly correlate in either sample. Contrary to our hypothesis that COVID-19 fear would be related to worse fake news discernment, there was no significant correlation in either sample between COVID-19 fear and fake news discernment variables. Notably, participants were more likely to discern COVID-19 fake news than neutral fake news in both samples (USA: $t_{(269)} = 10.524$, $p = 0.001$; Italy: $t_{(299)} = 18.554$, $p < 0.001$), suggesting a greater awareness of COVID-19 vs. neutral content during the pandemic.

COVID-19 and Problem-Solving

In support of our hypothesis COVID-19 fear negatively predicted performance on problem-solving tasks in both the American [$\beta = -0.20$, 95% CI = (−0.3, −0.1), $p < 0.01$] and Italian [$\beta = -0.24$, 95% CI = (−0.33, −0.14), $p < 0.001$] samples, while other predictors (age, education, number of cases) were insignificant (see **Figure 3**).

Fake News Discernment Regressions

In the American sample, COVID-19 fake news discernment significantly correlated with all the cognitive factors in the hypothesized directions (**Table 2**). In both the univariate and multiple stepwise regressions, the most unique cognitive predictors of COVID-19 discernment were SCP [$\beta = -0.22$, 95% CI = (−0.3, −0.1), $p < 0.001$] and overclaiming [$\beta = 0.16$, 95% CI = (0.06, 0.26), $p = 0.01$] and the most unique COVID-19 predictor was proactivity [$\beta = 0.13$, 95% CI = (0.03, 0.23), $p < 0.01$]. The LASSO identified SCP as the most unique (negative) predictor of COVID-19 discernment. In both univariate and multiple stepwise regressions, the most unique cognitive predictors of neutral discernment were SCP [$\beta = -0.13$, 95% CI = (−0.22, 0.02), $p < 0.05$] and problem-solving accuracy [$\beta = 0.24$, 95% CI = (0.14, 0.35), $p < 0.001$], the most unique COVID-19 predictor was proactivity [$\beta = 0.14$, 95% CI = (0.05, 0.26), $p < 0.05$], and the most unique demographic predictor was age [$\beta = 0.18$, 95% CI = (0.08, 0.28), $p < 0.01$]. The LASSO identified problem-solving as the most unique (positive) predictor of neutral discernment. In support of our hypotheses, these results suggest that while BRS and overclaiming accuracy are both related to how likely participants were to detect fake news, SCP and problem-solving ability are the strongest predictors of participants' ability to detect fake news. Specifically, SCP predicts believing in fake news headlines, whereas problem-solving predicts fake news detection in the American sample.

In the Italian sample, the factors that predict COVID-19 discernment were proactivity [$\beta = 0.19$, 95% CI = (0.1, 0.29), $p < 0.01$] and age [$\beta = 0.14$, 95% CI = (0.04, 0.23), $p < 0.05$], with the LASSO identifying proactivity as the most unique (positive) predictor (see **Table 3**). The sole factor that predicted neutral discernment is SCP [$\beta = -0.21$, 95% CI = (−0.31, −0.11), $p < 0.001$], which the LASSO identified as the most unique (negative) predictor. These result suggest that, similarly to the American sample, SCP predicts believing in fake news headlines (specifically neutral fake news) in the Italian sample.

DISCUSSION

The COVID-19 pandemic provided a natural context to study the critical impact of fear on how people seek, believe, and share information. Several aspects of human interaction with the media exacerbated fear during the COVID-19 pandemic. When the infection started to spread in Europe, especially in Italy, media reports adopted sensationalistic titles that tend to attract the most attention [e.g., on February 26, 2020 CNN titles "CDC official warns Americans it's not a question of if coronavirus will spread, but when" (McLaughlin and Almasy, 2020); or "Like a wartime curfew: Inside Italy's coronavirus quarantine zone," The Telegraph, on February 24 (Oliphant et al., 2020); "There is no truce," La Repubblica, on March 13, 2020 (Bocci et al., 2020)]. Even if little was known about the virus, such titles may have contributed to a state of alertness that drove people's behavior. The circulation of fake news and misleading information accelerated right after the beginning of the coronavirus outbreak (Chakravorti et al., 2020; Cinelli et al., 2020; Taylor, 2020). This spread of false narratives (e.g., implausible cures; Sommer, 2020), conspiracy theories (Ellis, 2020), and hate (Ferrara, 2020) favored attitudes and behaviors that undermine the governments' efforts to implement prevention measures (Abd-Alrazaq et al., 2020). The rapid and massive spread of misinformation has grown to such an extent that it has been referred to it as an "infodemic" ("We're not just fighting an epidemic; we're fighting an infodemic" -Word Health Organization., 2020), underlining the serious consequences of misinformation during the management of the viral outbreak. Information reliability becomes crucial when events that threaten many human lives take place (such as a pandemic or a natural catastrophe) since they impact the effectiveness of adopted safety measures (Zarocostas, 2020). The case of the COVID-19 pandemic is a clear example of such an event. Our theoretical background frames the belief in and sharing of fake news in the

TABLE 1 | Absolute correlation coefficients (Pearson's *r*) for fake news and covid factors for the USA (lower diagonal) and ITA (upper diagonal) samples.

	1	2	3	4	5	6	7	8	9	10	11
1. discernment COVID-19	–	−0.04	**0.23**	0.04	**0.26**	0.06	0.08	**0.18**	0.05	−0.02	0.05
2. discernment neutral	**0.29**	–	−0.01	**0.34**	−0.08	0.09	−0.12	−0.01	0.02	−0.03	0.07
3. familiar covid	**0.37**	**0.13**	–	0.02	0.02	0.07	−0.03	0.03	0.03	0.06	−0.02
4. familiar neutral	0.06	**0.4**	0.03	–	−0.03	**0.26**	−0.02	−0.04	−0.02	−0.04	0.03
5. share covid	**0.15**	**0.13**	**0.24**	0.06	–	0.02	0.08	**0.18**	0.04	0.03	0.01
6. share neutral	0.06	**0.14**	0.04	0.05	**0.27**	–	−0.01	0.01	−0.01	−0.07	−0.05
7. covid fear	0.09	0.04	0.07	0.06	**0.15**	**0.17**	–	**0.36**	0.04	**0.17**	0.02
8. proactivity	**0.15**	**0.15**	**0.16**	0.05	0.05	−0.02	**0.31**	–	0.07	**0.13**	0.09
9. cases	−0.09	−0.11	0.02	−0.06	−0.04	−0.05	0.06	0.01	–	0.15	0.03
10. BRS	**−0.13**	**−0.14**	−0.01	−0.09	0.03	0.02	**0.16**	0.03	0.1	–	0.08
11. overclaiming	**0.2**	0.12	0.09	0.11	−0.01	0.07	−0.03	0	0	**−0.18**	–

Bonferroni corrected for multiple correlations. Significant correlations are in bold. Significant correlations are in bold. r > |0.23| are statistically significant at p < 0.001, r > |0.18| are statistically significant at p < 0.01, r > 0.13| are statistically significant at p < 0.05.

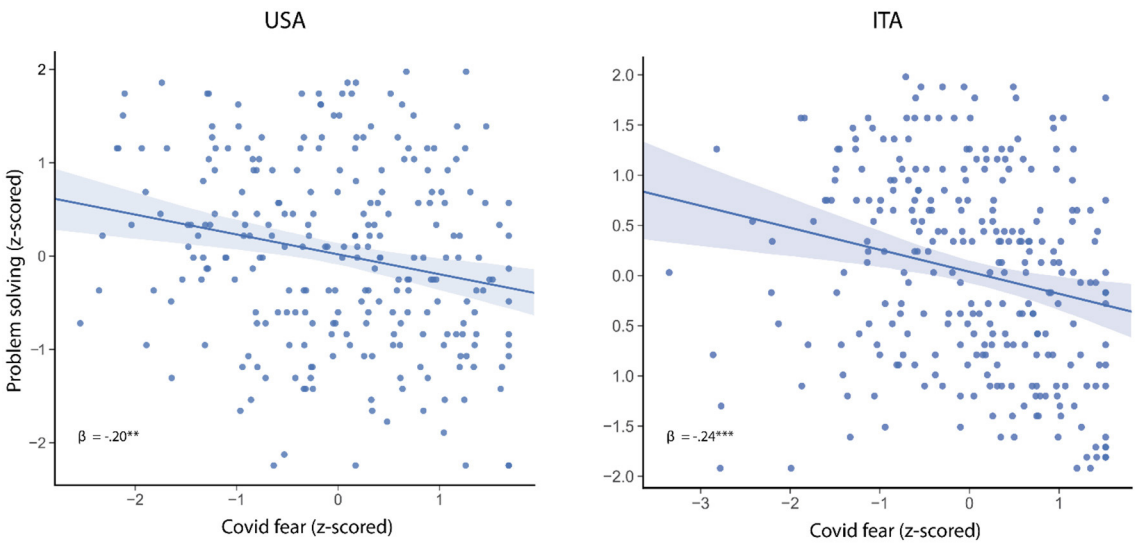

FIGURE 3 | Fear of getting COVID-19 negatively predicts problem-solving in the USA and ITA samples. Bands indicate 95% CI. **significance at $p < 0.01$; ***significance at $p < 0.001$.

context of individuals' need to resolve uncertainty during a time of heightened anxiety where people may be more vulnerable to fake news. This need has been exacerbated in a global pandemic, where scientific consensus and certainty was particularly elusive in the early days of the outbreak. During events like pandemics, fear-driven and instinctive behaviors activate promptly and people may develop more sensitivity to negative and overall novel information to help resolve uncertainty. This natural inclination toward information seeking may become problematic when sources of information contain misleading or outright false news stories.

Within this context, we investigated emotional, social, and cognitive factors that may influence fake news discernment and sharing, specifically the roles of COVID-19-related fear, seeking out information, BRS, overclaiming accuracy, problem-solving accuracy, and socio-cognitive polarization.

Our results showed that fear of COVID-19 is associated with proactive behaviors oriented toward seeking out information about the disease, taking actions to reduce the chances of being infected, and sharing real above and beyond fake news (despite the news sources consulted to gather information about the pandemic). As suggested by the literature, fear alters decision-making processes, and pushes people toward seeking information (Allen et al., 2014; Lin et al., 2014). We believe that the circumstantial lack of knowledge at the beginning of the COVID-19 pandemic did not just push people to seek information, but may have also increased their willingness to share this information over social media. We speculate that people share information because they believe that specific information would be interesting or useful to others. Thus, we conclude that people who took the risk of infection and severity of COVID-19 more seriously felt the urgency to seek out information related to

TABLE 2 | Correlation, standardized univariate regression, multiple stepwise, and Lease Absolute Shrinkage and Selection Operator (LASSO) coefficients for the American sample.

	USA Covid-19 news discernment				USA neutral news discernment			
	Univariate		Multiple regs		Univariate		Multiple regs	
Coefficient	***r***	***β***	***β***	**LASSO**	***r***	***β***	***β***	**LASSO**
Demographics								
Age	0.08				0.12		**0.18**	
Sex	0.10				−0.02			
Education	0.03				0.00			
COVID-19 factors								
Fear	−0.06	0.00			−0.05	−0.05	–	
Proactivity	**0.13**	**0.13**	**0.13**	–	**0.15**	**0.16**	**0.14**	–
Cases (city)	−0.08	−0.09			−0.11	−0.11		
Cognitive factors								
BRS	**−0.15**	**−0.15**	–	–	**−0.15**	**−0.17**	–	–
Overclaiming	**0.21**	**0.21**	**0.16**	–	**0.12**	0.08	–	–
Problem-solving	**0.18**	**0.18**	–	–	**0.21**	**0.27**	**0.24**	**0.065**
SCP	**−0.26**	**−0.26**	**−0.22**	**−2.6e-06**	**−0.16**	**−0.20**	**−0.13**	–

Predictors in the multiple stepwise linear regression are based on the best fitting solution with forward and backward selection. Significant coefficients are in bold. $r > |0.23|$ are statistically significant at $p < 0.001$, $r > |0.18|$ are statistically significant at $p < 0.01$, $r > |0.12|$ are statistically significant at $p < 0.05$. $\beta > |0.22|$ are statistically significant at $p < 0.001$, $\beta > |0.13|$ are statistically significant at $p < 0.01$, and $\beta > |0.12|$ are statistically significant at $p < 0.05$. All coefficients are standardized. $\lambda_{covid} = 0.26$; $\lambda_{neutral} = 0.16$.

TABLE 3 | Correlation, univariate regression, multiple stepwise regression, and Lease Absolute Shrinkage and Selection Operator (LASSO) coefficients for the Italian sample.

	ITA Covid-19 news discernment				ITA neutral news discernment			
	Univariate		Multiple reg		Univariate		Multiple regs	
Coefficient	***r***	***β***	***β***	**LASSO**	***r***	***β***	***β***	**LASSO**
Demographics								
Age	**0.15**		**0.14**		0.00			
Sex	0.01				−0.06		−0.03	
Education	0.00				0.09		0.014	
COVID-19 factors								
Fear	0.11	0.09		–	−0.11	−0.08	–	–
Proactivity	**0.2**	**0.19**	**0.19**	**1.8e-6**	0.00	0.01	–	–
Cases (city)	0.05	0.01			0.03	0.03		
Cognitive factors								
BRS	0.01	0.00	–	–	−0.02	−0.01	–	–
Overclaiming	0.04	0.02	–	–	0.07	0.07	–	–
Problem-solving	0.00	0.02	–	–	0.1	0.08	–	–
SCP	0.02	0.02	–	–	**−0.21**	**−0.20**	**−0.21**	**−3e-5**

Predictors in the multiple stepwise linear regression are based on the best fitting solution with forward and backward selection. Significant coefficients are in bold. $r > |0.21|$ are statistically significant at $p < 0.001$, $r > |0.20|$ are statistically significant at $p < 0.01$, $r > |0.14|$ are statistically significant at $p < 0.05$. $\beta > |0.21|$ are statistically significant at $p < 0.001$, $\beta > |0.19|$ are statistically significant at $p < 0.01$, and $\beta > |0.14|$ are statistically significant at $p < 0.05$. All coefficients are standardized. $\lambda_{covid} = 0.20$; $\lambda_{neutral} = 0.21$.

COVID-19 and share real news above and beyond fake news, hoping that circulating information may be helpful to others. This might illustrate a protective benefit to those who appraise the gravity of the pandemic by leading individuals to search for reliable sources of information. This result is in line with intuitive model of prosociality, which suggests that prosocial and helping behaviors often arises from intuitive, yet impulsive system 1 preferences (Shi et al., 2020). However, our results also reveal that fear of COVID-19 positively correlates with believing in pseudo-profound bullshit and negatively predicts problem-solving ability, which may illustrate adverse effects of fear induced by the distressing context of a pandemic. Although we cannot speculate a causal relationship between the two in our results the link between fear and believing in pseudo-profound statements may suggest that sensitivity to fear is paralleled with believing in meaningless claims, which extends to forms of

misinformation outside of the context of our fake news sample. Contrary to our prediction, the relation between fear and BRS seemed to be unique and independent from variables that have been hypothesized to be related to fear such as fake news discernment and overclaiming.

While we know that people acquire information to reduce fear and anxiety, our study provides evidence that fear leads also to sharing information and overestimating pseudo-profound statements. The relationship between fear, sharing, and bullshit receptivity may be explained by a desire to control the destabilizing lack of meaningful information. This effect might reflect the attitude toward creating spiritual meanings to explain, predict, and have an impression of control during unpredictable catastrophic events. The downside of these circumstances is that such a state reduces analytical thinking and our ability to solve problems. This result is in line with previous studies showing that stress, high risk-taking and anxiety of running out of time deteriorate creativity and problem-solving performance (Salvi et al., 2016a; Shen et al., 2018; Salvi and Bowden, 2019; Duan et al., 2020). Our results showed that problem-solving accuracy (as measured by both CRT and Rebus puzzles) correlates positively with a discerning fake from real news, indicating that an individual's willingness to engage in analytic and reflective thinking is associated with a reduced belief in fake news. In line with other studies, we found that individuals who perform better on the CRT (Bronstein et al., 2019; Pennycook and Rand, 2019), and visual-semantic puzzles (Sindermann et al., 2020) are better able to discern fake from real news. Tackling complicated problems requires continuous reframing and changing the initial representation of a problem to see it under a new light. We speculate that such mental exercise impacts other information processing skills. Thus, the relationship between being a good problem solver and detecting fake news may be explained by the willingness to invest time and effort in going beyond the default information. Problem-solving capacity may engender a greater tendency to question the information in news by investigating its accuracy further.

Our results replicate Pennycook and Rand (2020) findings that overclaiming accuracy and bullshit receptivity positively and negatively predict fake news discernment, respectively—but only in the American sample. This replication underscores a potential underlying feature of analytic thinking across our primary predictors (problem-solving and SCP) and supports our interpretation that those who are more willing to question default narratives, critically appraise a problem, and seek for new information are better suited to discern fake from real news. Finally, while problem-solving positively predicted fake news in the American sample, problem-solving was not a unique predictor of either neutral or COVID-19-related fake news in the Italian sample. Moreover, COVID-19-related fear only correlated with sharing real above and beyond fake news in the American sample. We have reason to believe our two samples represented populations with wide differences in their level of education (with the American sample scoring higher), which may explain the null relationship between problem-solving and fake news discernment in the Italian sample. Interestingly, the Italian sample scored much higher than the American sample on fear of COVID-19 (see **Supplementary Table 1**), which may have resulted in a ceiling effect, therefore preventing the detection of a relationship between fear and fake news variables in the Italian sample.

Our data indicate that higher levels of SCP (absolutism, conservatism, xenophobia) are associated with reduced fake news discernment. Absolutism refers to an individual's preference for rigid dichotomizations into fixed categories, which results in black-and-white thinking. People who score high in absolutism tend to have a polarized way of thinking by splitting representations of reality into opposite concepts that cannot coexist as distinct features of the same object (i.e., good-bad/right-wrong with no middle ground) (Frenkel-Brunswik, 1951; Budner, 1962; Lauriola et al., 2016). Verifying the news' reliability requires the willingness to go beyond a readily available piece of information, the motivation to search for alternative views on the same issue, and the conviction that beliefs should change according to evidence (Bronstein et al., 2019). Thus, when there are incongruencies within the news they are reading, it behooves individuals to seek more information in external resources for assessment (Edgerly et al., 2020). Following this logic, people who are high on absolutism tend to stick to a single view (Lewandowsky et al., 2012), maintain pre-existing established beliefs when presented with new information (Kruglanski et al., 1993), and are less likely search for alternative information (Ford and Kruglanski, 1995), which is likely to hinder the detection of fake news. Previous research suggests that intolerance for ambiguity is positively related to conservatism (Jost et al., 2003; Jost, 2017). Conservative ideology tends to correlate with preferences toward certainty, simplicity, and closure, and avoidance of uncertainty, novelty, and complexity. Other studies also suggest that conservatives may engage in heuristic/automatic thinking more often than liberals (Jost, 2017), and conservatism is positively related to lower mental effort (Eidelman et al., 2012; Van Berkel et al., 2015). Our results indicate that people who subscribe to conservative viewpoints were more likely to believe fake news, which replicates prior research suggesting that belief in incorrect information is prevalent among conservatives (Kull et al., 2003; Travis, 2010).

Xenophobia is also associated with the tendency to believe in fake news. To the best of our knowledge, there are no studies that demonstrate a relationship between xenophobic attitudes and fake news discernment. However, dogmatism, ethnocentrism, and intolerance for ambiguity are positively correlated [see Furnham and Marks (2013)]. Thus, it is plausible to assume that people reporting a high level of xenophobia are those who tend to be more dogmatic, rigid, and who are less open to considering alternative views, and thus are worse at fake news discernment (Bronstein et al., 2019).

We can conclude that the construct we named socio-cognitive polarization, which reflects cognitive rigidity, neglecting alternative information, and black-and-white thinking negatively predicts the ability to discern fake from real news. Such a construct extends also to reasoning processes, such as problem-solving, where thinking outside the box and consider alternative information is fundamental.

LIMITS OF THE STUDY

A major strength of this study is that data were collected during the critical early stages of the COVID-19 outbreak in two countries with the highest reported cases and deaths from the disease. However, the samples might not fully representative of the demographics of Italian and US populations. Nevertheless, it is worth noting that the two subsamples were well-balanced in terms of gender and age. The differences between the two subsamples are limited to education level, which, in any case, turned out to be a non-significant predictor of fake news discernment and information sharing, and to the type of news sources most frequently consulted to gather information about COVID-19. On the one hand, it can be argued that different media platforms would vary in terms of effort and actions directed to warn the readers about the potential threat of misinformation. On the other hand, even though Italian participants trusted TV news, whereas American participants preferred social media, predictions about fake news discernment were similar between samples, suggesting different media's responses to the threat of fake news during the pandemic was not a confounding variable in our study. Another limitation is that by administering the study online, we necessarily lose tight experimental control, which can introduce potentially confounding variables (e.g., impossible to know if each subject is fully attending to the experiment while in their home environment). Such caveats are of course true for online studies in general, and not unique to the present study. Another important issue is the current socio-cultural environment independent of the pandemic that could affect people's behavior, such as the impending 2020 US political election. Finally, as every subject is living through the same global pandemic, we do not have a putatively "pandemic free" sample to compare our results. Furthermore, in the present study, we did not measure trait and state levels of non-specific fear, and therefore our analyses regarding fear and fake news discernment, sharing, and problem-solving are limited to COVID-19 specific appraisals. To better understand how fear (or lack thereof) influences peoples' capacity to discriminate fake and real news stories, further research should also compare individuals with high vs. low levels of state or trait fear.

DATA AVAILABILITY STATEMENT

All data and analysis code are available on our OSF project page (https://osf.io/4pd2u/).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB at The University of Texas at Austin. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

This study is the result of 2.5 months of uninterrupted work of all the researchers involved, while they were quarantined for COVID-19. All the researchers contributed with continuous daily work to finish the study in time and publish the results as soon as possible. This work was possible thanks to the collaborative effort of everyone who was involved in the project and understood the importance of supporting science during the pandemic. CS contributed in work coordination, conceptualization, methodology, and writing. PI contributed in conceptualization, methodology, and writing. AC contributed in conceptualization, methodology, data curation, and writing. MM contributed in conceptualization, methodology, data analysis, data interpretation, and writing. SR contributed with creating and translating the questionnaires, data cleaning, formatting, and bibliography. JD and AA contributed in supervision, writing, reviewing, and final editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcomm.2020.562588/full#supplementary-material

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COVID-19 Dynamics: A Heterogeneous Model

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The mathematical model reported here describes the dynamics of the ongoing coronavirus disease 2019 (COVID-19) epidemic, which is different in many aspects from the previous severe acute respiratory syndrome (SARS) epidemic. We developed this model when the COVID-19 epidemic was at its early phase. We reasoned that, with our model, the effects of different measures could be assessed for infection control. Unlike the homogeneous models, our model accounts for human population heterogeneity, where subpopulations (e.g., age groups) have different infection risks. The heterogeneous model estimates several characteristics of the epidemic more accurately compared to the homogeneous models. According to our analysis, the total number of infections and their peak number are lower compared to the assessment with the homogeneous models. Furthermore, the early-stage infection increase is little changed when population heterogeneity is considered, whereas the late-stage infection decrease slows. The model predicts that the anti-epidemic measures, like the ones undertaken in China and the rest of the world, decrease the basic reproductive number but do not result in the development of a sufficient collective immunity, which poses a risk of a second wave. More recent developments confirmed our conclusion that the epidemic has a high likelihood to restart after the quarantine measures are lifted.

Keywords: COVID 19, dynamical model, epidemic, quarantine, antiepidemic measures, population immunity

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INTRODUCTION

We mathematically modeled the COVID-19 epidemic, as opposed to conducting a statistical analysis of the available data, because over the past 50 years no infectious disease has emerged that could be the basis for testing our model. Thus, SARS and Middle East respiratory syndrome (MERS) did not cause global epidemics. Acquired immunodeficiency syndrome (AIDS) is a disease which lasts for a long time and from which there is no recovery. Influenza epidemics also cannot be the correct basis for an analysis since these are either repeated epidemics in a partially immune population or epidemics where there is some cross-immunity. Therefore, in connection with the novelty of COVID-19, we focused on the development of a mathematical model.

We developed the mathematical model and submitted it for publication when COVID-19 was at its early phase. This disease was first identified in the city of Wuhan. The initial cases of COVID-19 were reported in late November 2019 (1). A month and a half after the first reports, on January 15, there were only 41 cases on record. Then, the number of cases grew rapidly (2–5). The number of cases increased by more than 1,000 from January 15 to February 15. Starting from January 2020, China took extreme quarantine measures. In mainland China, incidences of

the disease started to decline, but both the number of countries with an infected population and the incidence rate kept increasing (6, 7).

COVID-19 is caused by the virus SARS-CoV-2 (8–10). Clinical manifestations of the disease resemble those of SARS (11, 12). The mortality rate is lower than in SARS but the incidence rate and the total death toll are significantly higher (5, 13–15). The current COVID-19 epidemic differs in several aspects from the previous one caused by SARS, which was finally extinguished (16–21). First, COVID-19 has a higher basic reproductive number, R_0, than SARS (22–24). Second, in contrast to SARS, causative pathogen transmission in COVID-19 starts before the end of the incubation stage of the disease (25, 26). Third, unlike SARS, many cases of COVID-19 are asymptomatic, but they are accompanied by a spread of causative pathogens (27–29). These features of COVID-19 lower optimism over the belief that the current epidemic could be successfully controlled.

At the time of writing, several issues remain unclear regarding the spread of this pathogen around the globe, the ways to avoid mass morbidity, estimation of the total incidence rate, and the risk that the incidence rate could start growing after the emergency anti-epidemic measures are partially canceled. As noted above, the large number of unknowns regarding the disease motivated mathematical modeling of the disease progression.

To analyze COVID-19 dynamics, we developed a model that accounted for the heterogeneous composition of the human population (30–33), with subgroups affected differently by the disease. Our model explained the data that were available when the model was developed and predicted the epidemic progression in the case that the anti-epidemic restrictions were lifted. The subsequent developments matched the predictions of our model.

METHODS

The proposed dynamical model accounts for the heterogeneity of infection risk across different age groups. This feature of the model is important because the risk of developing COVID-19 strongly depends on patient age (34–36) and because measures against the disease spread include isolation of elderly individuals. Given these factors, it is important that infection risk, α, for different groups is incorporated in the dynamical model.

In our model, $I(\alpha,t)$ and $S(\alpha,t)$ are the proportions of infected and susceptible people, respectively, α is infection risk, t is time, and $dF(\alpha)$ is statistical distribution of infection risk across the population. ($\int dF(\alpha) = 1$). For an infinite isolated population, epidemic dynamics is defined by the set of differential equations (37):

$$\begin{array}{l} \frac{dI(\alpha,t)}{dt} = \alpha S(\alpha,t) \int I(\alpha,t)\, dF(\alpha) - \beta I(\alpha,t) \\ \frac{dS(\alpha,t)}{dt} = -\alpha S(\alpha,t) \int I(\alpha,t)\, dF(\alpha) + \gamma\,(1 - S(\alpha,t)) \end{array} \quad (1)$$

where $1/\beta$ is average disease duration from the time of infection till the end of pathogen transmission, and $1/\gamma$ is average lifespan for the people with lifelong immunity or average duration of sustained immunity for the people with transient immunity. The relationship between infection risk, α, and the basic reproductive number, R_0, is given by the equation:

$$R_0 = \frac{\int \alpha dF(\alpha)}{\beta} \quad (2)$$

For an epidemic that continues for several months, we can neglect the term $\gamma\,(1 - S(\alpha,t))$ that defines population renewal. In this case, the dynamical equations can be rewritten as:

$$\begin{array}{l} \frac{dI(\alpha,t)}{dt} = \alpha S(\alpha,t) \int I(\alpha,t)\, dF(\alpha) - \beta I(\alpha,t) \\ \frac{dS(\alpha,t)}{dt} = -\alpha S(\alpha,t) \int I(\alpha,t)\, dF(\alpha) \end{array} \quad (3)$$

The disease progression is usually described using discrete daily samples, where the variations of people's activities throughout the day are averaged out. Accordingly, if $J(\alpha,k)$ is the portion of infected people on day k then Equation (4) can be rewritten to have discrete steps:

$$\begin{array}{l} J(\alpha,k+1) = \alpha S(\alpha,k) \int_{\alpha} \sum_{n=0}^{N-1} J(\alpha,k-n) dF(\alpha) \\ S(\alpha,k+1) = S(\alpha,k) - \alpha S(\alpha,k) \int_{\alpha} \sum_{n=0}^{N-1} J(\alpha,k-n) dF(\alpha) \end{array} \quad (4)$$

where N is disease duration in days from the infection onset till the cessation of pathogen transmission, and $R_0 = N \int \alpha dF(\alpha)$.

Note that J cannot be greater than 1. Indeed, $J(\alpha,k+1) \geq 0$ if $J \geq 0$, $S \geq 0$ for any value of k or α. This follows from the first Equation in (5) because the right part of the equation is an integral of the numbers that are greater or equal to zero. Then, the sum of both Equations in (5) yields $S(\alpha,k+1) + J(\alpha,k+1) = S(\alpha,k) \leq S(\alpha,k) + J(\alpha,k) \leq 1$. The fact that S≥0 follows from the equation:

$$\begin{array}{l} S(\alpha,k+1) = S(\alpha,k) - \alpha S(\alpha,k) \int_{\alpha} \sum_{n=K}^{N-1} J(\alpha,k-n) \\ dF(\alpha) \leq S(\alpha,k) - \alpha S(\alpha,k)\left(1 - S(\alpha,k)\right). \end{array} \quad (5)$$

Indeed, in our case, $\alpha \leq 1$, so S does not exceed zero. For the cases where α is >1, the sampling rate could be increased.

The Equations 1, 3, and 4 belong to the susceptible–infected–recovered (SIR) class of models of an epidemic process (38). As followers from the model name, population members can be in one of three states: susceptible, infected, and immune. The susceptible-exposed-infectious-recovered (SEIR) models describe the initial period of an epidemic more accurately (39, 40). In these models, an additional state is added, called exposed, that corresponds to the very start of an infection. This state corresponds to the sterile period when, after being infected, a

person does not infect others. The following equation describe the SEIR dynamics:

$$J(\alpha, k+1) = \alpha S(\alpha, k) \int_{\alpha} \sum_{n=K}^{N-1} J(\alpha, k-n) dF(\alpha)$$
$$S(\alpha, k+1) = S(\alpha, k) - \alpha S(\alpha, k) \int_{\alpha} \sum_{n=K}^{N-1} J(\alpha, k-n) dF(\alpha) \quad (6)$$

where K is the duration of sterile period in days, and $R_0 = (N-K)\int \alpha dF(\alpha)$.

Our model of COVID-19 contains several additional assumptions. First, we assume that, during the initial stage of the disease, its incidence increases exponentially in the non-immune population. COVID-19's growth rate is significantly higher than that of SARS. At the initial stage of the SARS epidemic in 2003, the number of cases tripled during the month of April from 2,000 to 6,000 (41, 42). By contrast, in the second half of January 2020, the number of cases in Wuhan tripled in 3–4 days (i.e., a 40% increase per day). The rate of infection growth depends on R_0 and the disease duration—the factors that affect the distribution of time intervals between sequential infections. The lower limit for the time interval between infections is the time from infection onset till the beginning of virus shedding, and the upper limit is the sum of the interval from infection onset till the end of shedding and the duration of pathogen preservation in the external environment.

Quantifying the time interval between infections is difficult even for well-studied infectious diseases. This is because the beginning of the causative pathogen shedding does not always coincide with the end of the incubation period. Additionally, the time interval between infections is affected by factors such as changes in the intensity of the causative pathogen shedding at different stages of the disease, changes in patient behavior, and person-to-person variability. Because of these unknowns, we based our model on a simplified assumption that during the entire infectious period the infection rate remains constant, and the duration of infectious period, t_1, is equal to the duration of sterile period, t_2: $t = t_1 = t_2$. We performed modeling for different values of t.

RESULTS

We used our dynamical model to assess two factors that affect the epidemic progression: (1) the anti-epidemic measures designed to decrease the disease spread, and (2) the accumulation of collective immunity, especially in the high-risk groups.

Table 1 shows how the daily growth in the number of infection cases depends on R_0 and t. The estimation of R_0 is only an approximate of the daily growth because of the imprecise values of the sterile and infectious periods and because of the changes in time of virus shedding by an infected person and his/her interactions with other people. Additionally, infection control measures result in a decrease in the number of people interacting with the infected person. For example, the daily growth was 25% in Moscow at the beginning of the COVID-19 epidemic, and it decreased to 15% after the introduction of quarantine measures.

TABLE 1 | Daily increase in the number of infection cases at the epidemic's initial stage as the function of reproduction number, R_0, and the duration of sterile/infectious period, t.

R_0	t, days			
	3	4	5	6
2	19.2%	17.1%	15.3%	13.9%
3	32.5%	28.8%	25.9%	23.5%
4	42.9%	38.0%	34.2%	31.1%
5	51.7%	45.8%	41.2%	37.5%
6	59.3%	52.6%	47.3%	43.1%

Figure 1 shows the number of infected people as a function of time for a city with 10 million inhabitants; t is set to 5 days, and R_0 is set to 2 or 4. Here the results of a homogeneous model (solid lines) are compared with the results of a heterogeneous model (dashed lines). In the heterogeneous model, infection risk has a uniform distribution between 0 and $2R_0$. It is evident from this analysis that the overall incidence rate is lower when the heterogeneity factor is incorporated in the model. A noticeable slowdown in the incidence rate, however, is manifested only when the overall incidence rate has reached a sufficiently high value.

Anti-epidemic measures strive to reduce the COVID-19 infection rate even before it starts to naturally decrease because a substantial portion of the population (including hidden cases) are affected. We modeled the effect of anti-epidemic measures by decreasing R_0 from 4 to 2 (**Figure 1**). With these settings, anti-epidemic measures of moderate intensity shift the peak in incidence rate forward in time and reduce the peak amplitude. The total incidence does not change appreciably, as evident from the widening of the curve.

Ideally, the selection of appropriate anti-epidemic measures should be based on the quantification of R_0 early in the epidemic. One can estimate R_0 based on the disease duration and the growth of incidence rate in the beginning of an epidemic, when the growth is exponential (**Table 1**). During the exponential-growth stage, daily increase in the total number of cases is constant when expressed as the ratio of the number of cases on a given day to the cumulative sum of cases for the preceding day. **Figure 2** shows the dynamics of this ratio for several regions, including China's provinces and other countries. Points are median values, and error bars on the curve for China's provinces (red line) are quartiles. The value of 100% corresponds to the number of infected people doubling on a given day.

For Wuhan's data, the early 90% peak in growth rate is unreliable and can be disregarded because it corresponds to the very beginning of the disease diagnostics with very low samples. For subsequent data with more reliable measurements, the growth rate peaked at 40%, which corresponds to R_0 of 4 (see **Table 1**), and then decreased to 20% (i.e., R_0 of 2.5) in early February and clearly terminated in mid-February. Given the relatively low overall number of infections, this marked slowdown of infection progression occurred because of the anti-epidemic measures, not because of an accumulation of collective

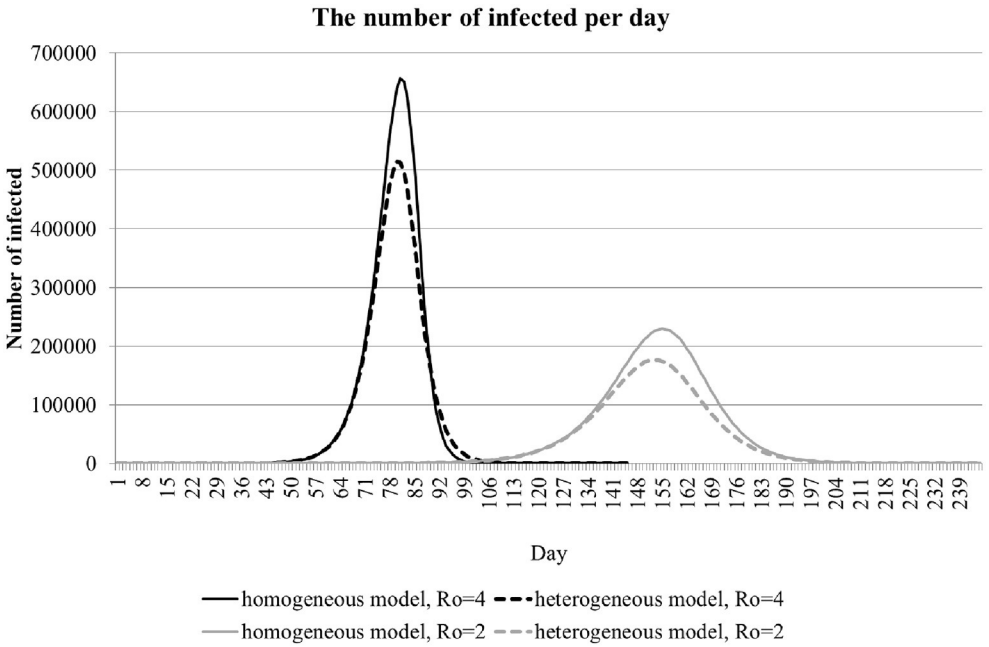

FIGURE 1 | The dynamics of the number of infections per day in a population of 10 million. The curves for homogeneous (solid lines) and heterogeneous (dashed lines) are shown for R_0 equal to 2 and 4.

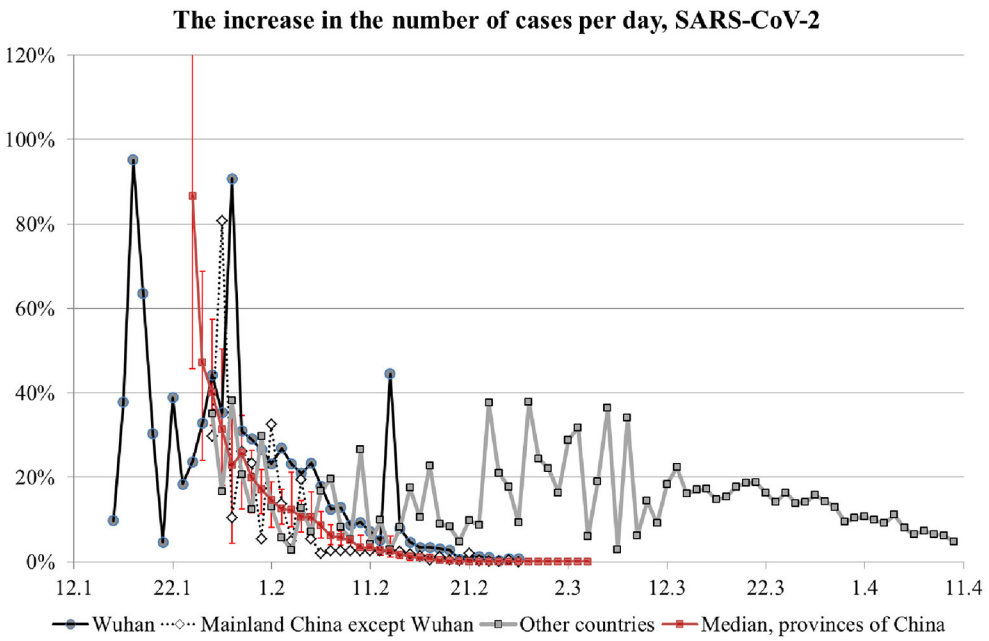

FIGURE 2 | Incidence rate growth rate in different regions.

immunity. A note should be made about the 45% surge in growth rate in Wuhan on February 13. It is related to a change in the methodology for calculating the number of cases. On that day, the cases previously considered as questionable were added to the report. Thus, the graph for Wuhan matches the prediction of our model where R_0 and the number of infections decrease because of anti-epidemic measures. A similar dynamic is seen for the rest of the world, where anti-epidemic measures were also undertaken and resulted in the growth rate decrease after March 13.

DISCUSSION

In this study, conducted during an early stage of the COVID-19 epidemic, we used a heterogeneous model to simulate the epidemic dynamic. With the heterogeneous model, we obtained more accurate results compared to the simpler, homogeneous models. Heterogeneity is an important factor for most infectious diseases. For example, for COVID-19, there is a population of individuals who are infected but do not show noticeable symptoms (20, 25–29). Asymptomatic individuals could be omitted from the medical reports. These people would transmit the infection to others and obtain specific immunity at the end of their infection period. These cases could be underreported because polymerase chain reaction (PCR), the existing methodology for diagnostics, cannot detect the individuals that recovered from the disease. Additionally, there is a bias toward testing mostly the patients with clinical symptoms. Furthermore, there is an age-related heterogeneity as the disease incidence increases with age (35, 43–45). The average age of patients with clinical symptoms is over 50 years old, whereas there are virtually no reported cases of infected children—a distribution that is at odds with the typical risk of infection for airborne infections, which is typically high for all age groups.

Our model accounts for the heterogeneity of infection risk and provides an estimate of the number of infections needed to accumulate for the epidemic to slow down. The model also allows us to assess the effect of anti-epidemic measures. We looked at two factors that reduce the epidemic's growth: (1) anti-epidemic measures, and (2) accumulation of a sufficiently large number of recovery cases from the illness in any form, including the recovery from a mild form without pronounced clinical symptoms. We modeled the first factor by decreasing R_0 and found that the epidemic progression slowed. The total number of eventually infected people, however, remained unchanged. This result brings importance to the second factor, which can guarantee that an infection has ended and would not restart.

The comparison of our model results with the data for Wuhan and the rest of the world indicates an R_0 of 4 at the start of the epidemic, followed by a decrease to 2.5 after the introduction of anti-epidemic measures, and finally a cessation of epidemic growth when the measures become strict. However, the total number of infected people is relatively low at this point, which could be insufficient for the second factor to guarantee that the epidemic has ended. Note that the decrease in incidence growth is almost the same for all Chinese provinces regardless of the huge discrepancies in morbidity levels among the provinces. For example, by March 4 the number of recorded cases in Hubei Province reached 67,466 while the median number of cases in the other 35 provinces amounted to just 245 cases. Such a dynamic is consistent with anti-epidemic measures taking their effect. Indeed, if the decrease in the growth rate was due to an accumulation of unreceptive cases, the decrease rate in the other provinces would have varied greatly. Moreover, a similar dynamic occurred for the rest of the world.

Based on these results, we concluded that:

1. The characteristics of COVID-19 differ markedly from SARS, which makes it hard to contain the disease spread to an affected territory unless the anti-epidemic measures are strict.

2. In the absence of effective anti-epidemic measures, more than 1% of the population could get infected. Should this happen, most cases will occur over a period of several months, which will cause great problems for the treatment of patients.

3. After lifting the emergency quarantine measures, the epidemic could restart because of an insufficient collective immunity level. This course of events should be seriously considered when "reopening" provinces and countries. The same conclusion was also reached by others (46–54).

During the time this manuscript was under review, the COVID-19 epidemic continued to develop, and the predictions of our model were confirmed. A spatial spread of the epidemic was observed in Asia (55, 56), Europe (57, 58), Africa (59, 60), South America (61), and North America (62–65). Moreover, as predicted by the model, lifting anti-epidemic measures resulted in a second wave of the epidemic across the world, which we are currently witnessing (66–69).

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://github.com/nytimes/covid-19-data.

AUTHOR CONTRIBUTIONS

AG, GL, and IS developed the model. AG, GL, ML, and IS interpreted the results and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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An earlier version of this manuscript has been released as a pre-print at biorxiv.org (70).

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BRIEF RESEARCH REPORT
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COVID-19–Related Fatalities and Intensive-Care-Unit Admissions by Age Groups in Europe: A Meta-Analysis

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Objectives: Precise international estimates of the age breakdown of COVID-19–related deaths and intensive-care-unit (ICU) admissions are lacking. We evaluated the distribution of COVID-19–related fatalities and ICU admissions by age groups in Europe.

Materials and methods: On April 6, 2020, we systematically reviewed official COVID-19–related data from 32 European countries. We included countries that provided data regarding more than 10 COVID-19–related deaths stratified by age according to pre-specified age groups (i.e., <40, 40–69, ≥70 years). We used random-effects meta-analysis to summarize the data.

Results: Thirteen European countries were included in the review, for a total of 31,864 COVID-19–related deaths (range: 27–14,381 per country). In the main meta-analysis (including data from Germany, Hungary, Italy, The Netherlands, Portugal, Spain, Switzerland; 21,522 COVID-19–related fatalities), the summary proportions of individuals <40, 40–69, and ≥70 years old among all COVID-19–related deaths were 0.1% (0.0–0.2; I^2 28.6%), 13.0% (10.8–15.4; I^2 91.5%), and 86.6% (84.2–88.9; I^2 91.5%), respectively. ICU data were available for four countries (France, Greece, Spain, Sweden). The summary proportions of individuals around <40–50, around 40–69, and around ≥60–70 years old among all COVID-19–related ICU admissions were 5.4% (3.4–7.8; I^2 89.0%), 52.6% (41.8–63.3; I^2 98.1%), and 41.8% (32.0–51.9; I^2 99%), respectively.

Conclusions: People under 40 years old represent a small fraction of most severe COVID-19 cases in Europe. These results may help health authorities respond to public concerns and guide future physical distancing and mitigation strategies. Specific measures to protect older people should be considered.

Keywords: coronavirus, SARS-CoV-2, COVID-19, mortality, epidemiology, age, meta-analysis, intensive care unit

INTRODUCTION

As of April 6, 2020, more than 1,000,000 confirmed cases and 65,000 deaths due to coronavirus disease 2019 (COVID-19) have been reported globally (1). Data from China and Italy have indicated that older adults are at higher risk of dying from COVID-19 than are younger people (2–4). However, most studies have emphasized the case-fatality rate (4–6) (i.e., the conditional probability of death among classified COVID-19 cases). This indicator is likely to be biased in the early phase of an outbreak, mostly because of preferential testing of people with more severe disease (e.g., hospitalized patients) and delays between the time of death and its official registration (7).

Despite a growing sense that SARS-CoV-2 can result in severe disease regardless of age (8), precise estimates of the age breakdown of COVID-19–related deaths and intensive-care-unit (ICU) admissions are lacking. Here we evaluated the distribution of COVID-19–related fatalities and ICU admissions by age in Europe. Such an analysis is critical to direct any potential relaxation of physical distancing and mitigation measures, which have been highly effective in reducing the number of cases but have severe economic and social consequences (9). We hypothesized that people < 40 years old represent a small fraction of most severe COVID-19 cases, as suggested by early reports from the Chinese Centers for Disease Control and Prevention (2).

MATERIALS AND METHODS

On April 6, 2020, one of the authors (JFC) systematically reviewed and extracted COVID-19–related mortality data from all 32 European countries participating in the European Center for Disease Prevention and Control surveillance network (i.e., European Union/European Economic Area and the United Kingdom), by collating official reports provided by local Public Health or Ministry of Health websites (**Supplementary Table 1**). We included countries if they provided data for more than 10 COVID-19–related deaths stratified by age according to pre-specified groups (i.e., <40, 40–69, ≥70 years old). For the included countries, we also extracted data regarding ICU admissions by age groups. A second author (DAK) verified study selection and data extraction, and disagreements were discussed to achieve consensus. We applied no language restrictions.

We used random-effects meta-analysis with Freeman-Tukey double arcsine transformation and exact confidence intervals (CIs) to estimate the proportion of age groups (<40, 40–69, ≥70 years old) among all COVID-19–related fatalities. We excluded participants with missing data for age. For mortality data, we conducted a sensitivity analysis that also included countries reporting data using slightly different age groups, with the following inclusion criteria: (1) the cutoff to define the "younger" age group was within 40–50 years and (2) the "intermediate" age group covered at least 20 years (e.g., United Kingdom, <40, 40–59, ≥60 years). We used the same age criteria to evaluate the distribution of age groups among COVID-related ICU admissions. Heterogeneity was quantified with the I^2 statistic.

TABLE 1 | COVID-19–related fatalities and intensive-care-unit (ICU) admissions by age groups in Europe (as of April 6, 2020).

	Belgium*	Finland**	France*	Germany	Greece***	Hungary	Italy	The Netherlands	Portugal	Spain	Sweden****	Switzerland	UK**
Date of report publication	April 6, 2020	April 6, 2020	April 2, 2020	March 29, 2020	April 5, 2020	April 6, 2020	April 5, 2020	April 6, 2020	April 6, 2020	April 3, 2020	April 3, 2020	April 6, 2020	April 6, 2020
Data up to	April 6	March 31	March 29	April 5	Unclear	April 5	April 6	April 5	April 3	March 29	April 6	April 5	April 5
Number of deaths by age (years), *N* (%)													
<40	9 (0.6)	0 (0.0)	29 (0.8)	1 (0.3)	1 (1.4)	1 (2.6)	42 (0.3)	2 (0.1)	0 (0.0)	13 (0.3)	1 (0.5)	3 (0.5)	43 (0.9)
40–69	112 (6.9)	2 (7.4)	319 (9.1)	46 (11.8)	18 (24.7)	9 (23.7)	2,359 (16.4)	221 (11.8)	41 (13.2)	441 (11.2)	50 (26.3)	61 (10.5)	353 (7.2)
≥70	1,489 (91.2)	25 (92.6)	3,128 (88.8)	341 (87.7)	54 (74.0)	28 (73.7)	11,979 (83.3)	1,643 (88.0)	270 (86.8)	3,050 (77.2)	139 (73.2)	519 (89.0)	4,501 (91.9)
Missing	22 (1.3)	n/a	47 (1.3)	1 (0.3)	n/a	n/a	1 (0.0)	1 (0.1)	n/a	449 (11.4)	n/a	n/a	n/a
Total	1,632 (100)	27 (100)	3,523 (100)	389 (100)	73 (100)	38 (100)	14,381 (100)	1,867 (100)	311 (100)	3,953 (100)	190 (100)	583 (100)	4,897 (100)
Number of ICU admissions by age (years), *N* (%)													
<40	n/a	n/a	417 (7.6)	n/a	0 (0.0)	n/a	n/a	n/a	n/a	97 (4.5)	26 (7.7)	n/a	n/a
40–69	n/a	n/a	2,327 (42.4)	n/a	43 (46.2)	n/a	n/a	n/a	n/a	998 (46.0)	215 (63.6)	n/a	n/a
≥70	n/a	n/a	2,744 (50.0)	n/a	50 (53.8)	n/a	n/a	n/a	n/a	664 (30.6)	93 (27.5)	n/a	n/a
Missing	n/a	n/a	0 (0.0)	n/a	0 (0.0)	n/a	n/a	n/a	n/a	430 (19.8)	4 (1.2)	n/a	n/a
Total	n/a	n/a	5,488 (100)	n/a	93 (100)	n/a	n/a	n/a	n/a	2,189 (100)	338 (100)	n/a	n/a

*Age groups: * <45, 45–64, ≥65 years; ** < 40, 40–59, ≥60 years; *** <40, 40–64, ≥65 years; **** <50, 50–69, ≥70 years. In Greece, intensive care unit admissions are patients under mechanical ventilation (intubated). n/a, not available.*

Statistical analyses involved use of Stata 15/SE (Stata Corp., College Station, TX, USA).

RESULTS

Descriptive Data

We identified official reports of COVID-19–related mortality data for all European countries except Cyprus. Eighteen countries (accounting for 1,113 deaths) were excluded because of unusable data: 11 were excluded because of lack of age breakdown of COVID-19 deaths, 5 because of fewer than 10 fatalities, and 2 because of age cutoffs not matching our pre-defined groups (**Supplementary Table 1**). Thirteen European countries were included in the review, for a total of 31,864 COVID-19–related deaths (range 27–14,381 per country; **Table 1**) (10–22).

COVID-19–Related Fatalities by Age Groups

In the main meta-analysis (7 countries; 21,522 COVID-19–related fatalities), the summary proportions of individuals <40, 40–69, and ≥70 years old among all COVID-19–related deaths were 0.1% (0.0–0.2; I^2 28.6%), 13.0% (10.8–15.4; I^2 91.5%), and 86.6% (84.2–88.9; I^2 91.5%), respectively (**Figure 1**). In a sensitivity analysis also including countries that reported slightly different age groups (13 countries; 31,864 COVID-19–related deaths), the summary proportions of individuals around <40–50, around 40–69, and around ≥60–70 years old among all COVID-19–related deaths were 0.2% (0.1–0.4; I^2 74.6%), 12.7% (10.0–15.7; I^2 97.4%), and 86.8% (84.0–89.4; I^2 97.1%), respectively (**Supplementary Figure 2**).

COVID-19–Related ICU Admissions by Age Groups

The meta-analysis of ICU admissions included data from 4 countries (8,088 COVID-19–related ICU admissions). The summary proportions of individuals around <40–50, around 40–69, and around ≥60–70 years old among all COVID-19–related ICU admissions were 5.4% (3.4–7.8; I^2 89.0%), 52.6% (41.8–63.3; I^2 98.1%), and 41.8% (32.0–51.9; I^2 99%), respectively (**Table 1** and **Supplementary Figure 3**).

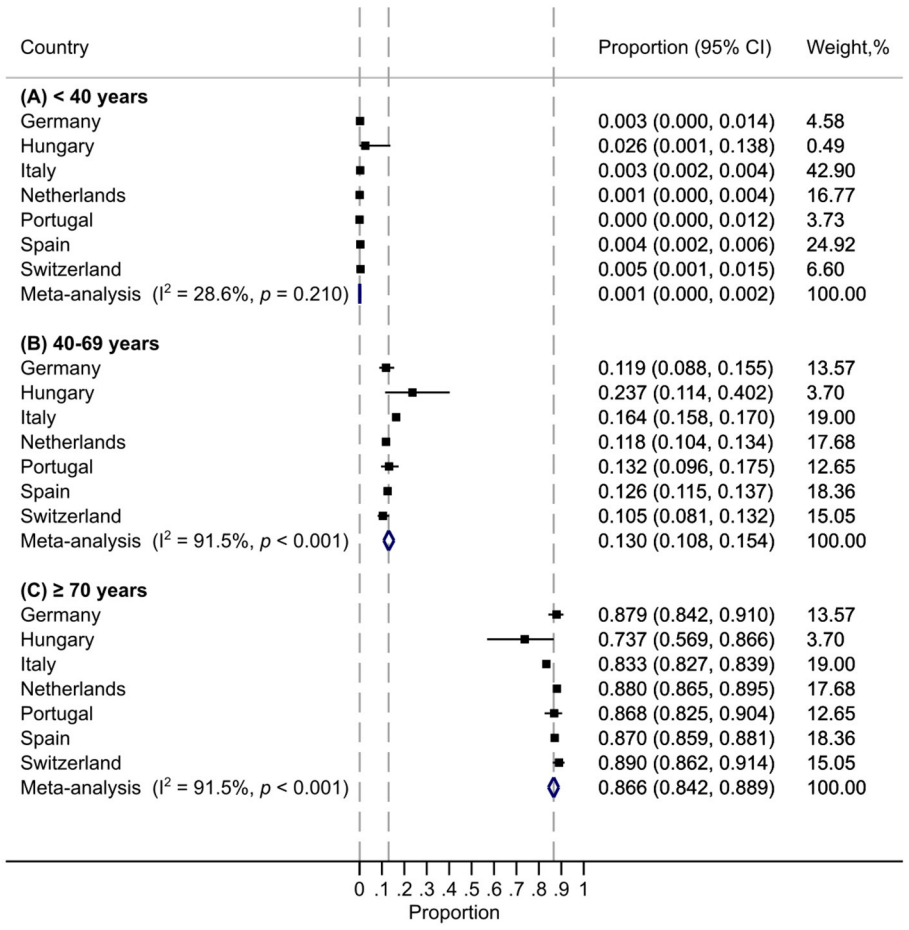

FIGURE 1 | Distribution of age groups for all COVID-19–related deaths in Europe: meta-analysis. Each vertical dashed line is aligned with the meta-analysis summary estimate of the corresponding age group.

DISCUSSION

This report describes the current distribution of COVID-19–related deaths and ICU admissions in Europe across age groups, using official reports from 13 countries. These data represent about half of the total of COVID-19–related deaths reported worldwide as of April 6, 2020 (1). Individuals <40 years old represented about 0.1 and 5% of COVID-19–related deaths and ICU admissions, respectively, whereas those >70 years old represented about 85 and 40%, respectively.

The distribution of COVID-19–related deaths by age in Europe differs from what was observed in the early phase of the pandemic in mainland China (as of February 11, 2020: <40, 40–69, ≥70 years old, representing 2.5, 46.6, and 50.8% of 1,023 COVID-19–related deaths, respectively) (4) and what is currently observed in the United States (as of April 7: <45, 45–64, ≥65 years old, representing 3.3, 17.6, and 79.0% of 2,214 COVID-19–related deaths, respectively) (23). This observation could reflect different patterns of patient characteristics, underlying risk factors, and management across settings as well as variability in the organization of healthcare and identification of causes of death across countries (24, 25). The age structure of each population might also be key: in 2019, Italy had the highest proportion of people aged ≥80 years in Europe (7.2%), whereas in the United States, for example, this proportion was as low as 3.6% in 2010 (26). However, the overall prevalence of obesity (body mass index ≥ 30 kg/m^2), reported as a risk factor for severe COVID-19, is much higher in the United States than Italy (42.4 vs. 10.5%) (27).

Our study has limitations. First, we could not include all European countries, mainly because official reports on COVID-19–related mortality data by age group were not always available. Second, we could not investigate the potential burden of underlying health conditions and other risk factors such as obesity and diabetes because this information was rarely reported. Third, we investigated COVID-19–related mortality data, but we acknowledge that methods for case ascertainment, case definitions, and SARS-CoV-2 RT-PCR testing strategies varied across countries. Some countries, such as France, provided detailed information for only in-hospital fatalities at the time of the study, which does not account for COVID-related deaths that occurred in other settings such as nursing homes (28), for a potential overrepresentation of younger patients. The definition of ICU cases also varied across countries, and Greece only reported the number of intubated patients.

Physical distancing is currently recommended in many countries for all age groups to slow the spread of COVID-19 and protect older people and, more broadly, the healthcare system. People < 40 years old represent a small fraction of the total number of most severe COVID-19 cases in Europe. These results, together with evaluations of the impact of comorbidities and risk factors in the course of COVID-19, may help health authorities respond to public concerns and guide future physical distancing and mitigation strategies. Relaxed physical distancing measures could be considered in people under 40 years of age, but specific measures aiming at protecting older people should be developed (29).

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.

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

JC, MC, and JT: conceptualization. JC and JT: methodology. JC: statistical analysis. JC and DK: data extraction and management. JC and JT: writing—original draft preparation. JC, DK, SM, SA, MC, and JT: writing—review and editing. MC and JT: 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.2020.560685/full#supplementary-material

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Clinical Symptom Differences Between Mild and Severe COVID-19 Patients in China: A Meta-Analysis

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Objective: The prognosis of mild and severe patients has prominent differences during the prevalence of COVID-19, and it will be significant to identify patients' potential risk of progressing to severe cases according to their first clinical presentations. Therefore, we aim to review the clinical symptoms of the COVID-19 epidemic systematically.

Methods: We searched PubMed, Embase, Web of Science, and CNKI (Chinese Database) for studies about the clinical features of COVID-19 in China from March 18 to April 18. Then we used REVMAN to conduct a meta-analysis.

Results: After screening, 20 articles including 3,326 COVID-19 confirmed cases were selected from 142 articles we retrieved at the beginning of our research. We divided all the cases into a severe group (including severe and critically severe patients) and a mild group according to the "Diagnosis and Treatment Protocol for Novel Coronavirus Infection-Induced Pneumonia" version 4 (trial). Of all the initial symptoms (including fever, cough, abdominal pain, anorexia, chest tightness, diarrhea, dyspnea, expectoration, fatigue, headache, hemoptysis, myalgia, nausea or vomiting, and pharyngalgia) we studied, we found that cough (odds ratio [OR] = 1.4, 95% confidence interval [CI]: 1.2–1.7; $p < 0.001$), fever (OR = 1.5, 95% CI: 1.2–1.9; $p < 0.001$), dyspnea (OR = 6.2, 95% CI: 3.6–10.6; $p < 0.001$), diarrhea (OR = 2.6, 95% CI: 1.3–4.9; $p < 0.001$), fatigue (OR = 2.1, 95% CI: 1.3–3.3; $p < 0.01$), expectoration (OR = 1.7, 95% CI: 1.2–2.6; $p < 0.01$), myalgia (OR = 1.6, 95% CI: 0.8–3.1; $p < 0.001$), hemoptysis (OR = 4.0, 95% CI: 1.5–11.3; $p < 0.001$), abdominal pain (OR = 7.5, 95% CI: 2.4–23.4; $p < 0.001$), and anorexia (OR = 2.8, 95% CI: 1.5–5.1; $p < 0.001$) had a different distribution in two groups and were statistically significant ($p < 0.05$).

Conclusion: COVID-19 patients whose initial manifestation is dyspnea, hemoptysis, anorexia, diarrhea, or fatigue, especially abdominal pain should be closely monitored to prevent disease deterioration.

Keywords: COVID-19, clinical features, differences, risk factor, meta-analysis

INTRODUCTION

An outbreak of a novel coronavirus-induced pneumonia occurred in Wuhan in December 2019, with the characteristics of being occult and infectious, with an incubation period of 3–7 days, usually no more than 14 days (1). On February 11, 2020, the World Health Organization (WHO) officially named the epidemic disease caused by the new coronavirus as coronavirus 2019 (coronavirus disease 2019) or COVID-19 (2). According to a study, COVID-19 can be transmitted between humans, and it has a significantly high RO (basic reproduction number, an essential index to evaluate the ability of a virus to spread), which was 3.77 times higher than that of MERS-CoV (3). An official report announced that the virus might have the feature of aerosol transmission. The virus can infect a human in a relatively closed space exposed to a high concentration of aerosols for a long time (4, 5). In the early stage of COVID-19, major symptoms were fever, dry cough, fatigue, and then breath difficulties, and severe cases can deteriorate to acute respiratory distress syndrome (ARDS) or septic shock, even death. On April 20, 2020, cumulative confirmed cases have reached 84,278 in China according to a report of WHO (6).

It is now universally acknowledged that severe COVID-19 cases have higher mortality than mild cases because severe cases are more likely to suffer ARDS, septic shock, or metabolic acidosis (7). So it is necessary to distinguish between severe and mild patients at an early stage. According to a report by Yong Gao (8), the levels of IL-6 and D-dimer can be measured to estimate the severity of COVID-19 and help to diagnose severe COVID-19 patients earlier. Also, Fei Zhou et al. (9) found that risk factors for death of adult patients with COVID-19 were a higher Sequential Organ Failure Assessment score, older age, and elevated d-dimer at admission. However, for countries or regions with poor health conditions, these methods may not be applicable. Our research aims to distinguish between severe and mild cases with COVID-19 at an early stage by analyzing initial clinical symptoms at admission, so it is easier to manipulate. We focused on 14 initial clinical presentations that most commonly occurred in COVID-19 patients and tried to determine the differences between mild and severe COVID-19 patients. It may help enable the implementation of effective interventions and likely lower the mortality of COVID-19 patients.

At present, over 200 countries are involved in this epidemic, and pneumonia induced by COVID-19 has become an enormous threat to global public health. Many cases emerged inside and outside China over the past month (2, 10–13). Although there have been many studies on clinical case analysis, the limited number of cases in each study may lead to different results and more significant bias. Therefore, our study involved over 3,000 confirmed cases to reveal the clinical symptoms of COVID-19 patients and provide help for clinical prevention and control of the epidemic disease.

TABLE 1 | Bias risk assessment.

Study	①	②	③	④	⑤	⑥	⑦	⑧	⑨	⑩	⑪	⑫	Score
Cai Q.	2	2	2	2	2	1	2	0	2	2	2	2	21
Dong L.	2	2	2	2	2	1	1	0	2	2	2	2	20
Fang X.	2	2	2	2	2	0	0	0	2	2	2	2	18
Guan W.	2	2	2	2	2	0	1	0	2	2	2	2	19
Huang C.	2	2	2	2	2	0	1	0	2	2	2	2	19
Li D.	2	2	2	2	2	1	1	0	2	2	2	2	20
Li K.	2	2	2	2	2	1	2	0	2	2	2	2	21
Li Y.	2	2	2	2	2	1	1	0	2	2	2	2	20
Liu K.	2	2	2	2	2	1	2	0	2	2	2	2	21
Liu W.	2	2	2	2	2	0	2	0	2	2	2	2	20
Lu Z.	2	2	2	2	2	0	0	0	2	2	2	2	18
Wan Q.	2	2	2	2	2	0	1	0	2	2	2	2	19
Wan S.	2	2	2	2	2	1	2	0	2	2	2	2	21
Wang D.	2	2	2	2	2	0	1	0	2	2	2	2	19
Wu C.	2	2	2	2	2	1	1	0	2	2	2	2	20
Xiang T.	2	2	2	2	2	1	0	0	2	2	2	2	19
Yuan J.	2	2	2	2	2	1	1	0	2	2	2	2	20
Zhang J.	2	2	2	2	2	0	1	0	2	2	2	2	19
Zhang W.	2	2	2	2	2	1	2	0	2	2	2	2	21
Zheng F.	2	2	2	2	2	0	2	0	2	2	2	2	20

① A clearly stated aim. ② Inclusion of consecutive patients. ③ Prospective collection of data. ④ Endpoints appropriate to the aim of the study. ⑤ Unbiased assessment of the study endpoint. ⑥ Follow-up period appropriate to the aim of the study. ⑦ Loss to follow up less than 5%. ⑧ Prospective calculation of the study size. ⑨ Appropriate selection of control group. ⑩ Synchronization of control group. ⑪ Baseline comparable between groups. ⑫ Appropriately statistical analysis. The global ideal score being 24 for comparative studies.

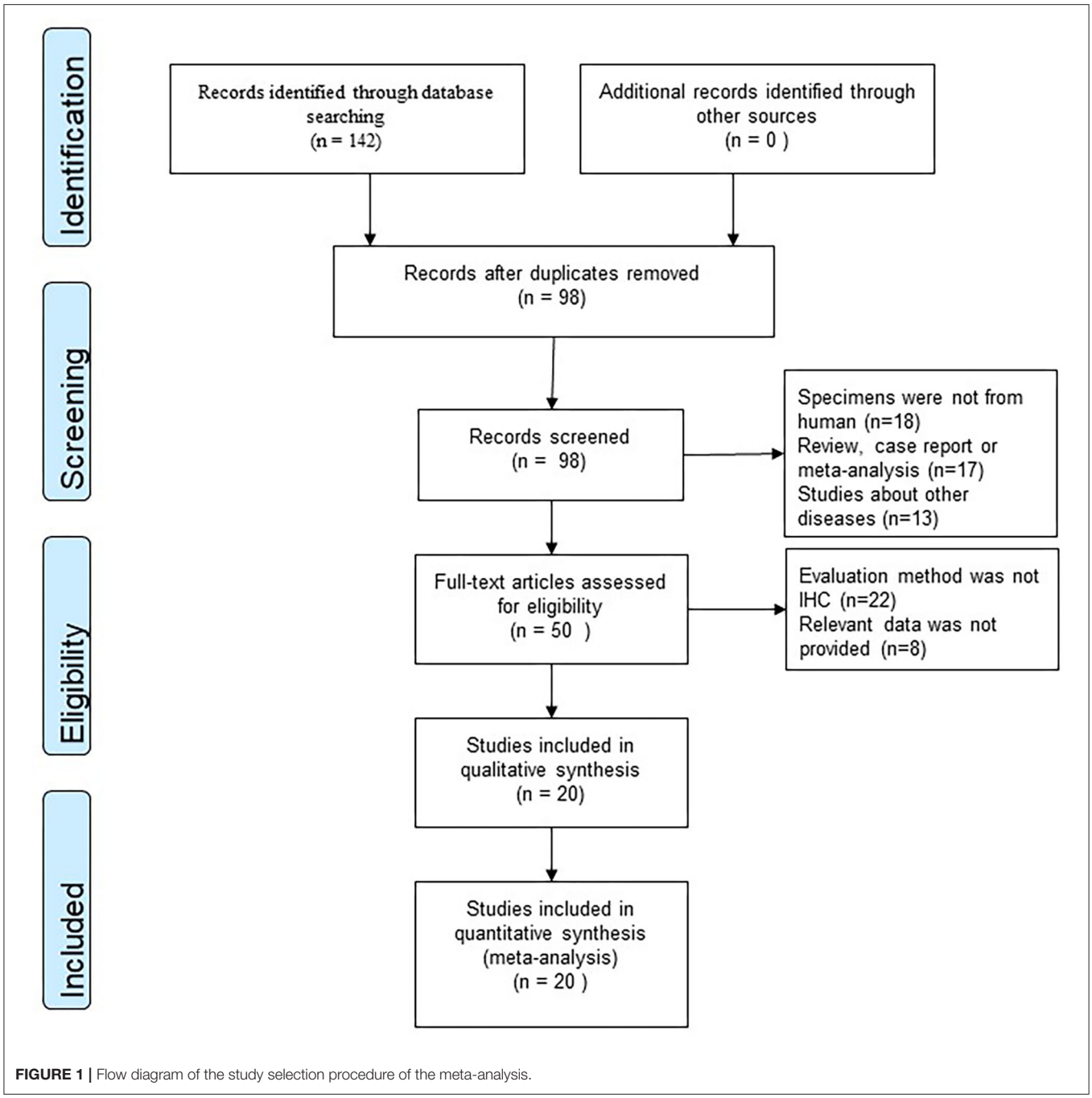

FIGURE 1 | Flow diagram of the study selection procedure of the meta-analysis.

METHODS

Search Strategy and Inclusion Criteria

We retrieved four databases, PubMed (https://pubmed.ncbi.nlm.nih.gov/), Embase (https://www.embase.com/), Web of Science (http://isiknowledge.com/), and CNKI (https://www.cnki.net/), to acquire case analysis studies on coronavirus disease 2019. Articles published between March 18, 2020, and April 18, 2020 were included. The search terms we used were as follows: ("SARS-CoV-2" or "nCoV" or "COVID-19," or "coronavirus") AND ("clinical feature" or "clinical characteristic" or "clinical symptom"). We evaluated all the search results according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement. Original articles related to COVID-19 patients in China without restriction on study design or study type were included. Studies on pregnant and infant patients, without reliable clinical data, and outside China were excluded.

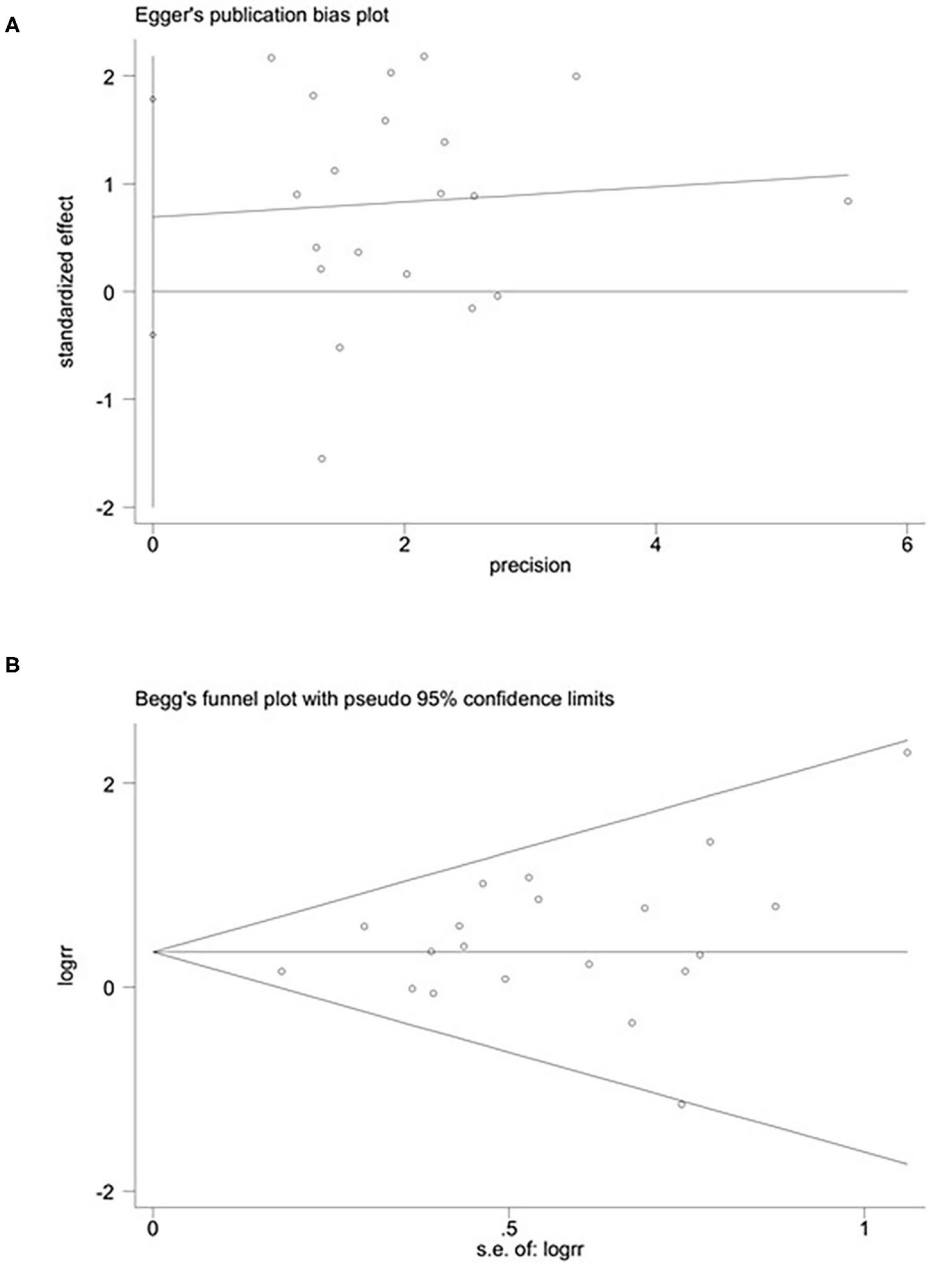

FIGURE 2 | Publication bias: Begg's **(A)** ($p > 0.05$) and Egger's **(B)** ($p > 0.05$) funnel plots for possible publication bias in the current study. No publication bias was found, indicating credible results.

Data Extraction and Quality Assessment

Information, including the first author, initial symptoms researched, and a sample size of severe and mild groups, was extracted from each study, and a Microsoft Excel database was used to record the details. Two independent reviewers (Xiaobo He and Xudong Feng) conducted the selection and assessment of article quality. Any disagreement was solved by consulting a professional investigator (Maoming Xiong).

Bias Risk Assessment

We used MINORS (**Table 1**) to assess the bias risk of included studies.

Data Analysis

We performed statistical analysis with REVMAN (Review Manager version 5.3, The Nordic Cochrane Center, The Cochrane Collaboration, Copenhagen, 2014). Crude odds ratios (ORs) and 95% confidence intervals (CIs) were used to estimate

TABLE 2 | Clinical characteristics of the include studies on COVID-19.

Study	Abdominal pain		Anorexia		Chest tightness		Cough		Diarrhea		Dyspnea		Expectoration		Fatigue		Headache		Hemoptysis		Myalgia		Nausea or vomiting		Pharyngalgia	
	S/T	N/T	S/T	N/T	S/T	N/T	S/T	N/T	S/T	N/T	S/T	N/T	S/T	N/T	S/T	N/T	S/T	N/T	S/T	N/T	S/T	N/T	S/T	N/T	S/T	N/T
Cai Q.	–	–	–	–	–	–	27/58	78/240	4/58	5/240		–	–	–	3/58	10/240	0/58	5/240	–	–	–	–	–	–	0/58	2/240
Dong L.	–	–	–	–	–	–	7/14	20/45	–	–	–	–	2/14	10/45	6/14	2/45	2/14	3/45	–	–	4/14	4/45	–	–	2/14	3/45
Fang X.	–	–	–	–	–	–	14/24	31/55	3/24	1/55	4/24	5/55	3/24	7/55	–	–	–	–	–	–	–	–	–	–	2/24	1/55
Guan W.	–	–	–	–	–	–	122/173	623/926	10/173	32/926	65/173	140/926	61/173	309/926	69/173	350/926	26/173	124/926	4/173	6/926	30/173	134/926	12/173	43/926	23/173	130/926
Huang C.	–	–	–	–	–	–	11/13	20/28	0/13	1/25	12/13	10/27	5/13	6/26	–	–	0/13	3/25	1/13	1/26	–	–	–	–	–	–
Li D.	–	–	–	–	–	–	14/17	43/63	4/17	9/63	15/17	4/63	14/17	43/63	10/17	24/63	3/17	9/63	–	–	3/17	19/63	–	–	–	–
Li K.	–	–	–	–	–	–	24/25	41/58	–	–	7/25	2/58	9/25	6/58	–	–	3/25	6/58	–	–	5/25	10/58	–	–	2/25	4/58
Li Y.	–	–	–	–	–	–	18/27	19/22	27/27	4/22	–	–	–	–	24/27	8/22	–	–	–	–	–	–	–	–	–	–
Liu K.	–	–	–	–	–	–	18/21	36/43	–	–	–	–	14/21	22/43	17/21	31/43	–	–	–	–	–	–	–	–	–	–
Liu W.	–	–	–	–	–	–	4/11	30/67	–	–	6/11	14/67	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Lu Z.	–	–	–	–	–	–	16/34	22/67	–	–	–	–	7/34	20/67	–	–	2/34	7/67	–	–	–	–	–	–	–	–
Wan Q.	–	–	–	–	–	–	16/21	76/132	–	–	8/21	23/132	13/21	39/132	12/21	25/132	–	–	–	–	9/21	9/132	–	–	–	–
Wan S.	–	–	–	–	3/40	9/95	35/40	67/96	13/40	5/95	18/40	0/95	7/40	5/95	–	–	11/40	23/95	3/40	1/95	–	–	–	–	0/40	24/95
Wang D.	3/36	0/102	24/36	31/102	–	–	21/36	61/102	6/36	8/102	23/36	20/102	8/36	29/102	29/36	67/102	3/36	6/102	–	–	12/36	36/102	4/36	10/102	12/36	12/102
Wu C.	–	–	–	–	–	–	68/84	95/117	–	–	50/84	30/117	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Xiang T.	–	–	–	–	0/9	4/40	6/9	13/40	0/9	2/40	–	–	4/9	3/40	3/9	6/40	–	–	–	–	–	–	–	–	0/9	7/40
Yuan J.	–	–	8/57	9/82	–	–	18/31	95/192	0/31	12/192	15/31	0/192	7/31	20/192	–	–	1/31	10/192	–	–	–	–	–	–	–	–
Zhang J.	6/57	2/82	–	–	–	–	45/53	45/67	9/57	9/82	24/53	20/67	–	–	39/53	51/67	–	–	–	–	–	–	5/57	19/82	–	–
Zhang W.	3/9	3/56	0/9	1/56	–	–	3/9	15/56	3/9	3/56	–	–	1/9	5/56	0/9	8/56	0/9	2/56	–	–	–	–	0/9	1/56	0/9	3/56
Zheng F.	–	–	–	–	–	–	21/30	80/131	1/30	16/131	9/30	14/131	–	–	15/30	49/131	4/30	8/131	–	–	4/30	14/131	0/30	6/131	–	–

S/T, severe cases/total cases; N/T, non-severe cases/total cases.

TABLE 3 | Meta-analysis outcomes of clinical features.

Clinical features	Number of studies	Number of patients	Heterogeneity		OR (95%CI)	*P*-value
			I^2(%)	*p*-value		
Abdominal pain	3	342	0	0.67	7.5 (2.4, 23.4)	0.0005
Anorexia	3	342	43	0.17	2.8 (1.5, 5.1)	0.0009
Chest tightness	2	184	0	0.72	0.7 (0.2, 2.4)	0.55
Cough	20	3,267	8	0.36	1.4 (1.2, 1.7)	0.0002
Diarrhea	13	2,552	56	0.007	2.3 (1.3, 4.9)	0.005
Dyspnea	13	2,590	72	<0.00001	6.2 (3.6, 10.6)	<0.00001
Expectoration	14	2,367	51	0.01	1.7 (1.2, 2.6)	0.009
Fatigue	13	2,436	62	0.002	2.1 (1.3, 3.3)	0.002
Fever	–	–	45	0.02	1.5 (1.2, 1.9)	0.0005
Headache	12	2,480	0	0.94	1.1 (0.8, 1.6)	0.48
Hemoptysis	3	1,273	0	0.77	4.0 (1.5, 11.3)	0.008
Myalgia	8	1,773	67	0.006	1.6 (0.8, 3.1)	0.15
Nausea or vomiting	5	1,602	44	0.9	0.9 (0.6, 1.5)	0.66
Pharyngalgia	9	2,005	54	0.03	1.2 (0.5, 2.7)	0.69

the strength of the association between clinical features and COVID-19 ($p < 0.05$ was considered statistically significant). The I^2 index, which indicates the percentage of the total variation across studies, was used to assess statistical heterogeneity (14). When $I^2 < 50\%$, a fixed-effects model was used to determine OR, while when $I^2 > 50\%$, a random-effects model was selected. We used Begg's rank correlation test and Egger's weighted regression method to assess potential publication bias, with $p < 0.05$ indicating statistically significant publication bias (15).

RESULTS

Study Characteristics

According to our research method, we collected 142 studies from four databases (PubMed, EMBASE, Web of Science, and CNKI). **Figure 1** shows the flow chart of selection, and no publication bias existed (**Figure 2**). Details were as follows: 98 articles were further browsed after duplicates were removed. Of these studies, those which were reviews, case reports, or meta-analyses (17 articles); related to other diseases (13 articles); or not about humans (18 articles) were excluded. Then we conducted a qualification assessment of the remaining 50 articles. Twenty-two articles were excluded for not using IHC as an evaluation method, and eight articles were excluded for lacking relevant data. Finally, 20 studies were analyzed. We extracted and analyzed 14 symptoms (including fever, cough, dyspnea, expectoration, hemoptysis, abdominal pain, diarrhea, chest tightness, headache, myalgia, nausea or vomiting, pharyngalgia, anorexia, and fatigue) with REVMAN. For research purposes, we divided all the initial symptoms into four types: fever, respiratory symptoms (including cough, dyspnea, expectoration, hemoptysis, chest tightness, and pharyngalgia), digestive symptoms (including abdominal pain, diarrhea, and nausea or vomiting), and neurological symptoms (including anorexia, fatigue, myalgia, and headache). Details of patients' clinical features and meta-analysis outcomes are shown in **Tables 2**, **3**, respectively. The initial symptoms we studied in this research are shown in **Figure 3**, and the indexes marked with a symbol ($\surd$) are statistically significant.

Associations Between Fever and Severe Cases

As shown in **Figure 4**, meta-analysis results revealed that fever (OR = 1.5, 95% CI: 1.2–1.9; $p < 0.001$) frequently occurred in patients with severe COVID-19 pneumonia compared with the mild cases.

Associations Between Respiratory Symptoms and Severe Cases

As shown in **Figures 5, 6**, meta-analysis results revealed that initial respiratory symptoms including cough (OR = 1.4, 95% CI: 1.2–1.7; $p < 0.001$), dyspnea (OR = 6.2, 95% CI: 3.6–10.6; $p < 0.001$), expectoration (OR = 1.7, 95% CI: 1.2–2.6; $p < 0.01$), and hemoptysis (OR = 4.0, 95% CI: 1.5–11.3; $p < 0.001$) occurred frequently in patients with severe COVID-19 pneumonia compared with the mild cases, while chest tightness (OR = 0.7, 95% CI: 0.2–2.4; $p > 0.05$) and pharyngalgia (OR = 1.2, 95% CI: 0.5–2.7; $p > 0.05$) did not show this characteristic.

Associations Between Digestive Symptoms and Severe Cases

As shown in **Figure 7**, meta-analysis results revealed that initial digestive symptoms including abdominal pain (OR = 7.5, 95% confidence interval [CI]: 2.4–23.4; $p < 0.001$) and diarrhea (OR

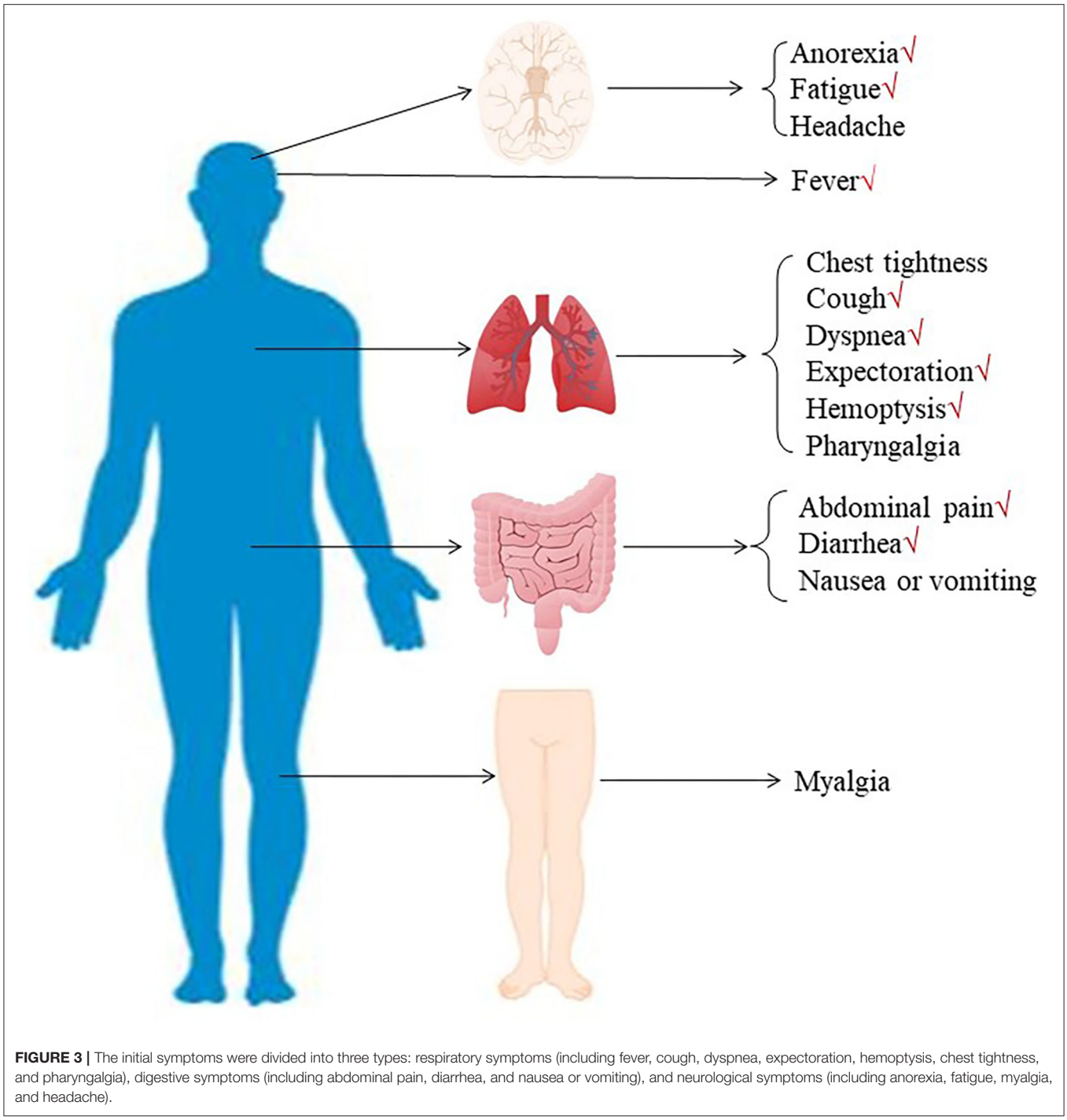

FIGURE 3 | The initial symptoms were divided into three types: respiratory symptoms (including fever, cough, dyspnea, expectoration, hemoptysis, chest tightness, and pharyngalgia), digestive symptoms (including abdominal pain, diarrhea, and nausea or vomiting), and neurological symptoms (including anorexia, fatigue, myalgia, and headache).

= 2.6, 95% CI: 1.3–4.9; $p < 0.001$) occurred frequently in patients with severe COVID-19 pneumonia compared with the mild cases, while nausea or vomiting (OR = 0.9, 95% CI: 0.6–1.5; $p > 0.05$) did not show this characteristic.

Associations Between Neurological Symptoms and Severe Cases

As shown in **Figure 8**, meta-analysis results revealed that initial neurological symptoms including anorexia (OR = 2.8, 95% CI: 1.5–5.1; $p < 0.001$) and fatigue (OR = 2.1, 95% CI: 1.3–3.3; $p < 0.01$) occurred frequently in patients with severe COVID-19 pneumonia compared with the mild cases, while headache (OR = 1.1, 95% CI: 0.8–1.6; $p > 0.05$) and myalgia (OR = 1.6, 95% CI: 0.8–3.1; $p > 0.05$) did not show this characteristic.

Publication Bias

We used Begg's rank correlation test and Egger's weighted regression method to assess publication bias statistically. As

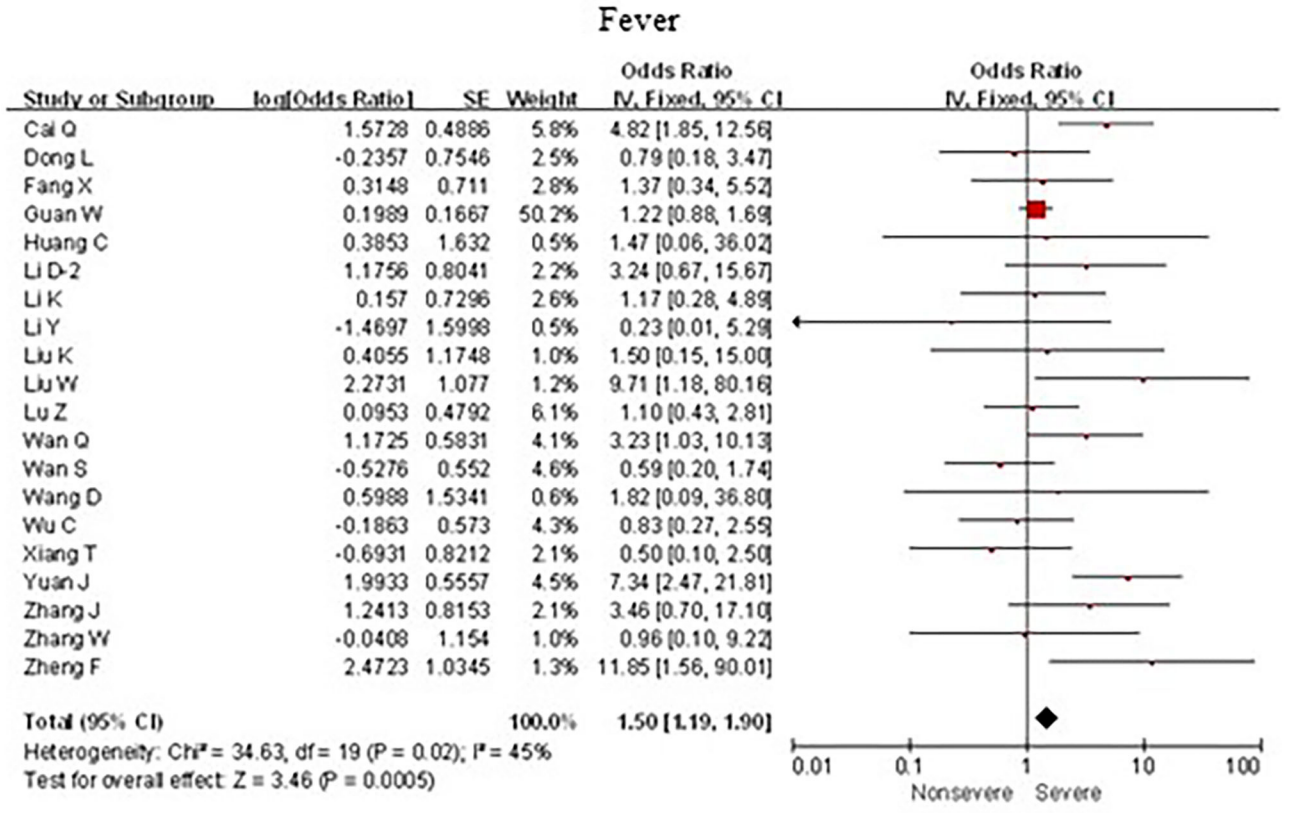

FIGURE 4 | Forest plots of odds ratios (ORs) showed the associations between fever and disease progression of COVID-19 pneumonia.

shown in **Figures 2A,B**, neither Begg's ($p = 0.453$) nor Egger's ($p = 0.246$) test provided clear evidence of publication bias. These results showed the credibility of the findings reported in this meta-analysis.

DISCUSSION

Over the past month, nearly one million cases of coronavirus disease 2019 were confirmed in China and other countries in Asia, Africa, Europe, and America. COVID-19 has become an enormous threat to human health around the world and seriously hindered the development of the world economy and the progress of humanity. As a member of the Coronaviridae family, which is distributed widely in humans and other mammals such as bats, masked palm civets, and pangolins (16, 17), the novel coronavirus 2019 has a lower mortality but higher morbidity than MERS-CoV and SARS-CoV (18, 19). The novel coronavirus 2019 has been designated a Public Health Emergency of International Concern by the WHO because of its high infectivity (20). According to clinical symptoms, laboratory indicators, and imaging findings, COVID-19 is classified as mild, normal, severe, and critical (7). The diagnostic criteria for severe are any of the following: shortness of breath, respiratory rate (RR) > 30 times/min; oxygen saturation < 93% at rest; arterial blood oxygen partial pressure (PaO_2)/oxygen concentration (FiO_2) < 300 mmHg (1 mmHg = 0.133 kPa) (when the altitude is above 1,000 m, PaO_2/FiO_2 should be corrected according to the following formula: $PaO_2/FiO_2 \times$ [atmospheric pressure (mmHg)/760]). Pulmonary imaging showed that the lesions significantly progressed within 24–48 h, and >50% were managed according to severity (21). According to the analysis of existing clinical characteristics, severe patients tend to have dyspnea after 1 week in some cases, moderate to low fever, or even no obvious fever. Severe cases were more likely to rapidly develop to ARDS, septic shock, metabolic acidosis, and coagulopathy that are difficult to correct compared with non-severe patients. Besides, kidney, heart, and other organ damage, and even multiple organ failures were more likely to occur in severe patients (22, 23). It is necessary to predict and diagnose severe COVID-19 correctly as early as possible. Especially for countries and regions with poor medical conditions, we can make a preliminary classification of the severity according to the patient's signs, thereby reducing unnecessary medical waste and making the best use of medicines.

According to a statistical description of 656 and 1,994 patients with new coronary pneumonia counted by Alfonso J. Rodriguez-Morales and Long-Quan Li, the most common clinical symptoms

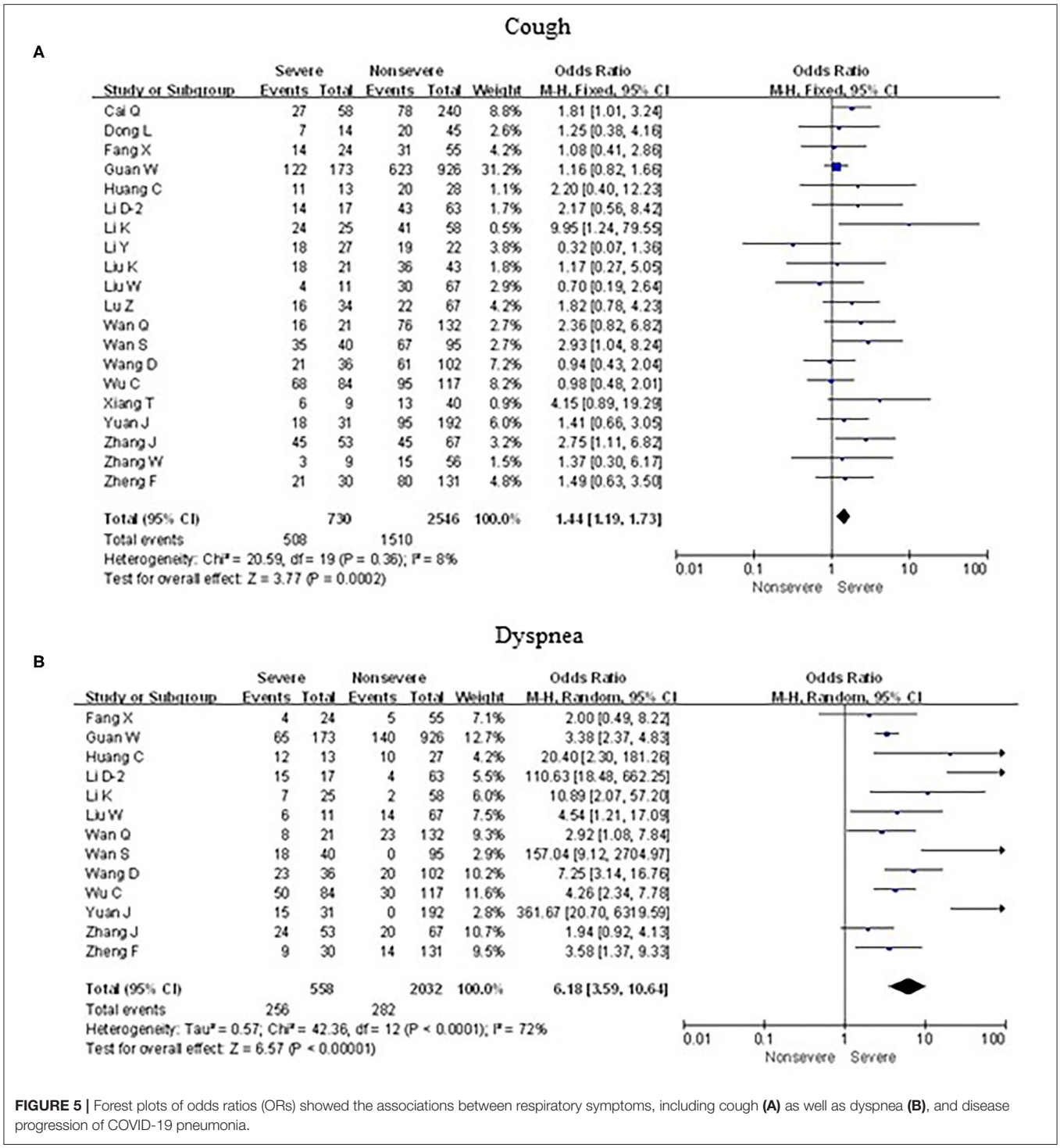

FIGURE 5 | Forest plots of odds ratios (ORs) showed the associations between respiratory symptoms, including cough **(A)** as well as dyspnea **(B)**, and disease progression of COVID-19 pneumonia.

of COVID-19 were fever, cough, and dyspnea (24, 25). Many similar studies have proved this conclusion. However, few studies have discussed how to initially assess the severity of patients with new coronary pneumonia in a short period, to facilitate earlier treatments and reduce complications and mortality. This meta-analysis involved the latest studies from March 2019 to April 2019 to analyze the clinical characteristics of the novel coronavirus 2019. Our study, which included 3,326 confirmed cases, provided more credible results of clinical features of the novel coronavirus 2019. Although all the studies were case studies, there was no publication bias. In this study, the dominant clinical features of COVID-19 patients were cough

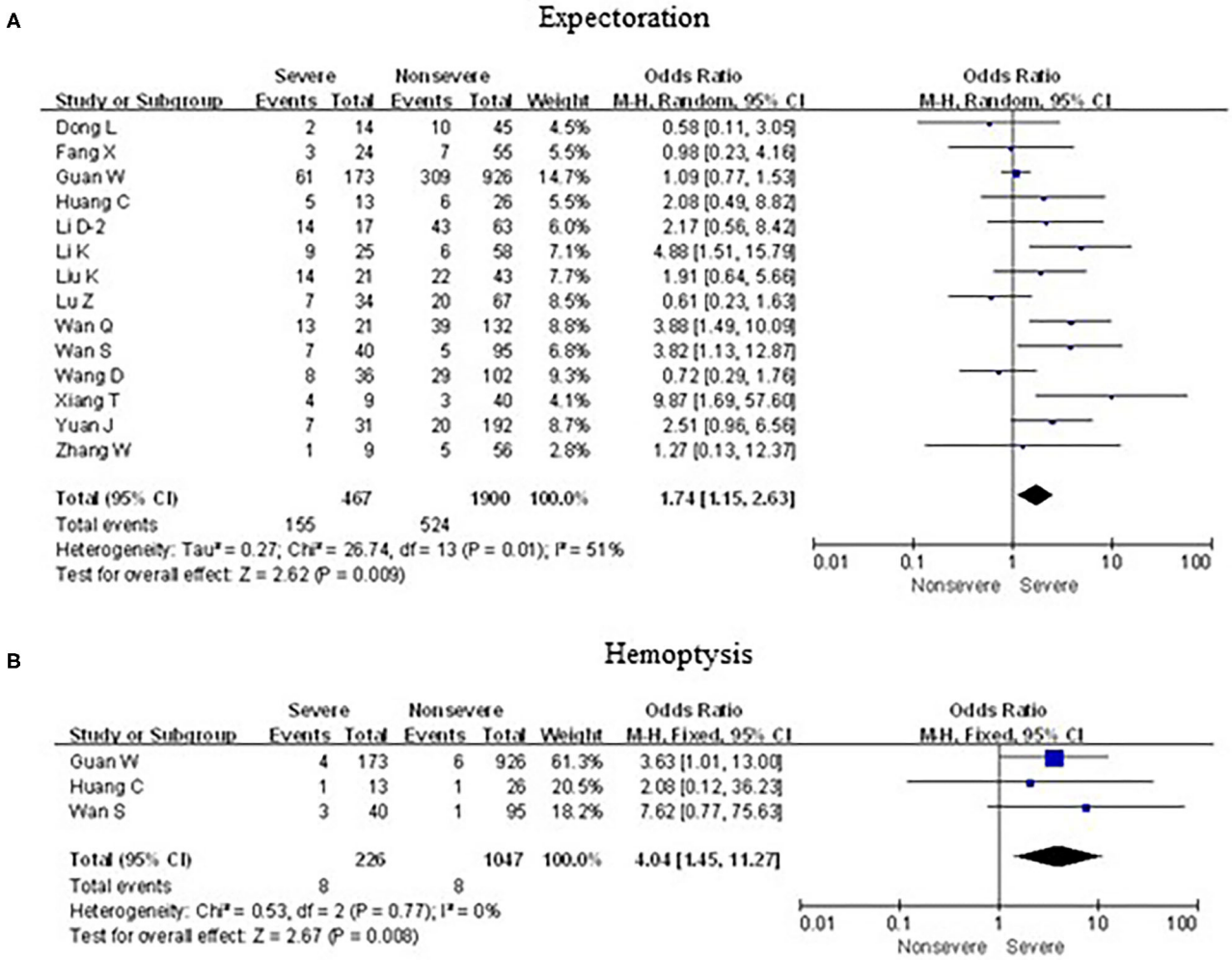

FIGURE 6 | Forest plots of odds ratios (ORs) showed the associations between respiratory symptoms, including expectoration **(A)** as well as hemoptysis **(B)**, and disease progression of COVID-19 pneumonia.

(98.5%) and fever (93.4%), which were similar to other studies on initial symptoms of COVID-19 patients in China (22, 26, 27). By analyzing the distribution differences of initial symptoms between severe cases and mild cases, we found that some initial symptoms including abdominal pain (OR = 7.5, 95% CI: 2.4–23.4), dyspnea (OR = 6.2, 95% CI: 3.6–10.6), hemoptysis (OR = 4.0, 95% CI: 1.5–11.3), anorexia (OR = 2.8, 95% CI: 1.5–5.1), diarrhea (OR = 2.6, 95% CI: 1.3–4.9), fatigue (OR = 2.1, 95% CI: 1.3–3.3), expectoration (OR = 1.7, 95% CI: 1.2–2.6), fever (OR = 1.5, 95% CI: 1.2–1.9), and cough (OR = 1.4, 95% CI: 1.2–1.7) occurred more frequently in severe COVID-19 patients than in mild COVID-19 patients. However, chest tightness (p = 0.55), headache (p = 0.48), myalgia (p = 0.15), nausea or vomiting (p = 0.66), and pharyngalgia (p = 0.69) did not show a significant difference between patients with severe and mild COVID-19. In other words, the symptoms, including abdominal pain, anorexia, diarrhea, dyspnea, expectoration, fatigue, cough, fever, and hemoptysis, had a different distribution in severe cases and mild cases of coronavirus disease 2019, especially abdominal pain, dyspnea, and hemoptysis. The outcomes of our study were of great significance in predicting and diagnosing severe 2019 coronavirus disease, reducing the mortality and preventing its further spread. Some countries with severe epidemics do not have sufficient medical resources such as Italy, Spain, and India. They should pay more attention to the COVID-19 patients whose initial symptoms are abdominal pain, dyspnea, hemoptysis, anorexia, diarrhea, or fatigue because these patients are more likely to deteriorate to a severe type of 2019 coronavirus disease. Moreover, to further reduce the mortality of patients, medical workers should monitor their vital signs closely.

Severe acute respiratory syndrome (SARS) is an infectious disease belonging to the coronavirus family that occurred in 2002, and it has very similar initial symptoms and complications as the new coronavirus (1). According to research published

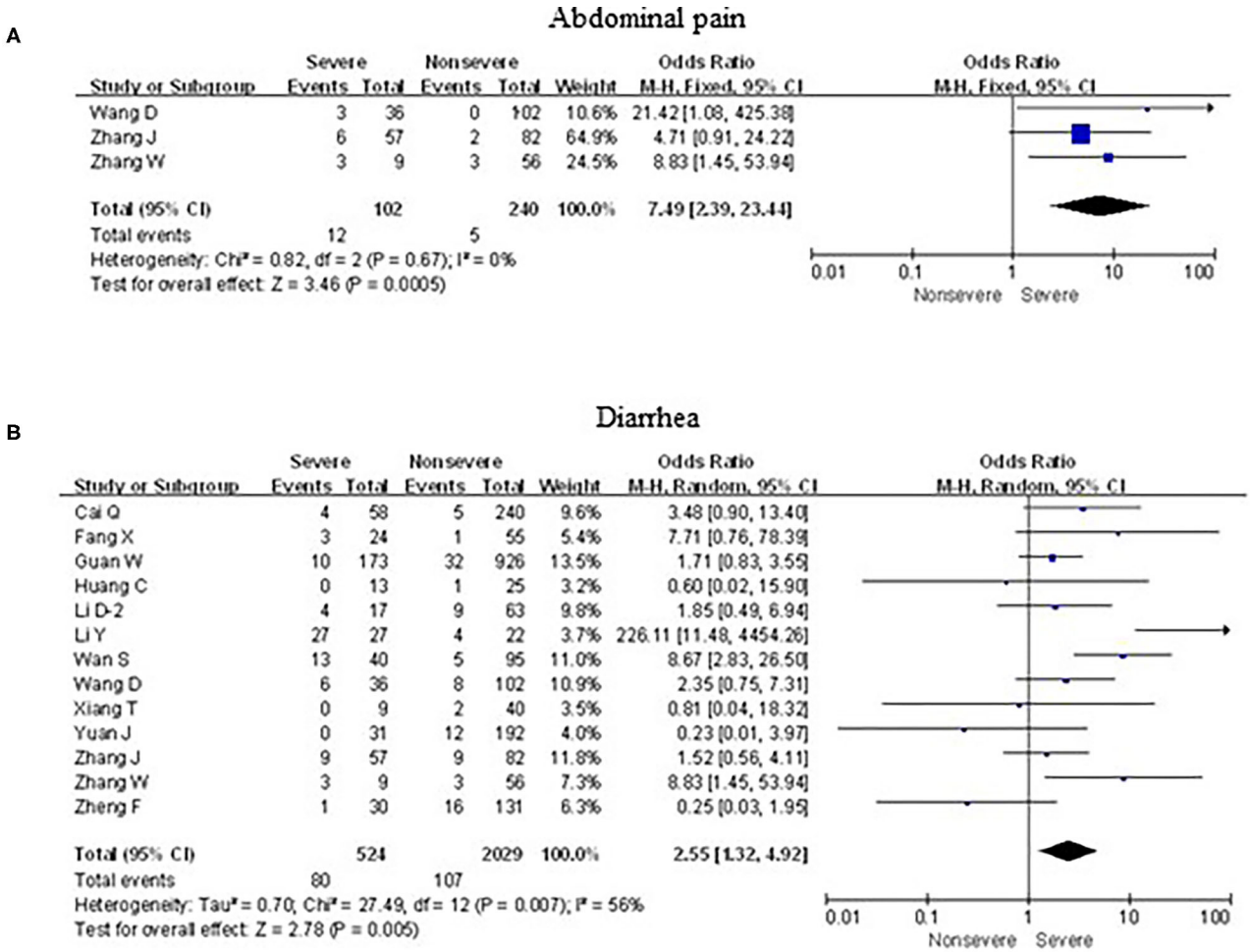

FIGURE 7 | Forest plots of odds ratios (ORs) showed the associations between digestive symptoms, including abdominal pain **(A)** as well as diarrhea **(B)**, and disease progression of COVID-19 pneumonia.

in *The Lancet Infectious Diseases* in 2004, symptoms such as fever, cough, and respiratory distress could serve as prediction and monitoring indicators for critical SARS (28). The result is consistent with our research. Moreover, Daozheng Huang et al. summarized 4,972 patients with COVID-19 from the end of 2019 to February 12, 2020 (29). They found that some clinical symptoms such as dyspnea, vomiting, and diarrhea had distinct differences between severe and non-severe patients, but no apparent differences were found in the symptoms of headache, fever, myalgia, and arthralgia. Also, calcitonin $\geq$ 0.05 ng/mL, creatinine $>$ 104 μmol/L, lymphocyte count $<1.5 \times 10^9$/L, and bilateral involvement of chest CT were significant risk factors for severe COVID-19. The above findings are highly consistent with our results, while vomiting and fever are contrary to our results. Therefore, it is necessary to expand the sample size of the research subjects for further exploration. What is certain is that when patients show abdominal pain, anorexia, diarrhea, dyspnea, fatigue, cough, or hemoptysis, they are more likely to be diagnosed as severe cases in the later development of the disease.

Our study still has some limitations. First, all the studies included were from China, so it would be better to involve studies in other countries to get a more comprehensive result of COVID-19, and the outcomes might be affected by geographical and ethnic differences. Second, more detailed patient information such as laboratory findings and ages were not involved in our meta-analysis for lack of relevant data. Third, some outcomes (including hemoptysis, abdominal pain, and anorexia) were defined with only three studies since many articles have not been published yet, which might increase the risk of bias. Finally, we performed this meta-analysis during the ongoing outbreak of COVID-19, and many regions affected by COVID-19 have not yet presented clinical data, which might skew the results of the analysis.

In conclusion, our study analyzed 3,326 cases and found that some initial symptoms especially abdominal pain, dyspnea,

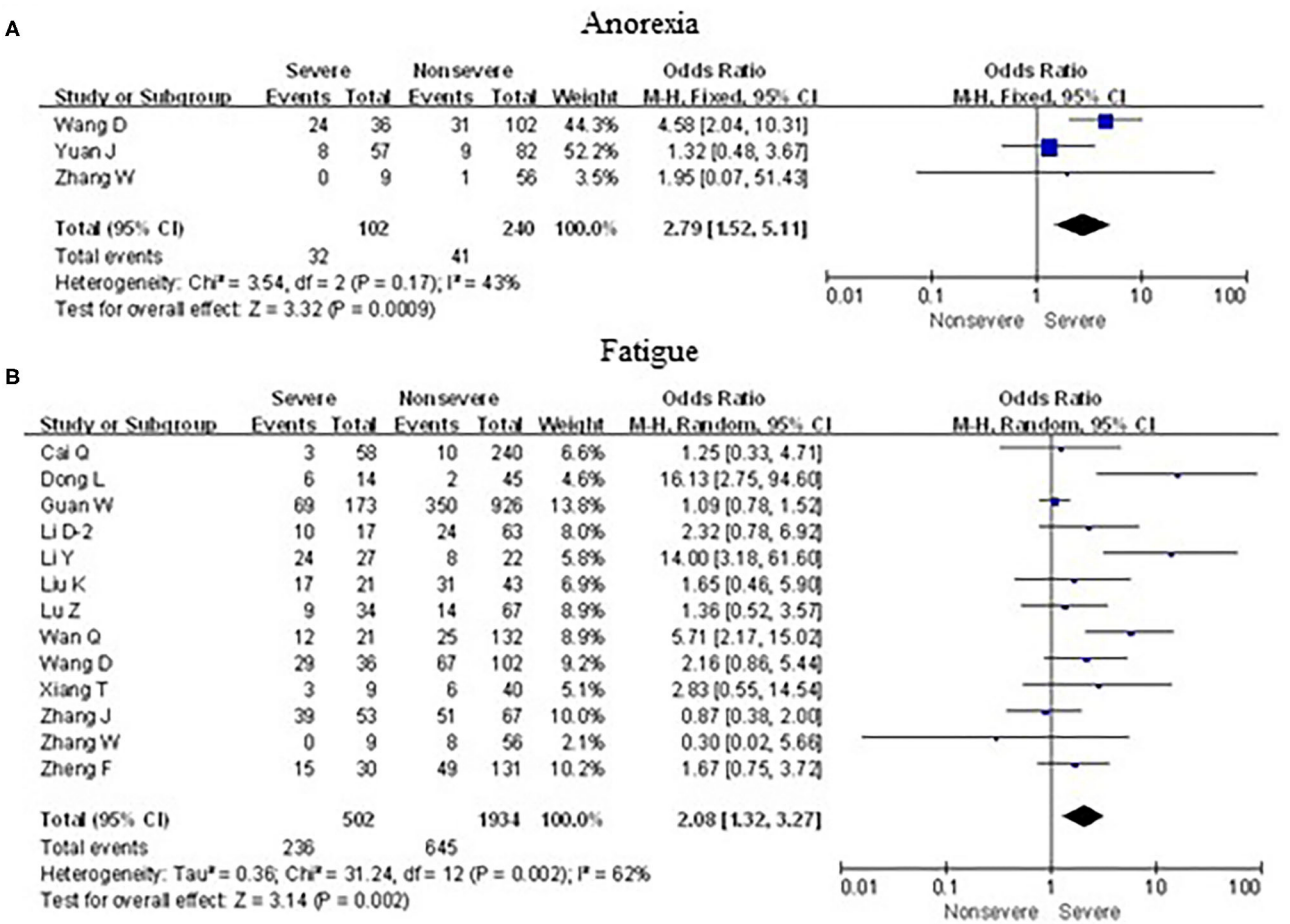

FIGURE 8 | Forest plots of odds ratios (ORs) showed the associations between neurological symptoms, including anorexia **(A)** as well as fatigue **(B)**, and disease progression of COVID-19 pneumonia.

hemoptysis, anorexia, diarrhea, and fatigue were significantly correlated with a severe type of 2019 coronavirus disease, suggesting that these symptoms could predict severe novel coronary pneumonia. Hospitalized COVID-19 patients with these initial symptoms should be paid more attention to keep a stable vital sign.

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

XH: data statistics and analysis. XC: draft writing. XF: literature screening. SC: language modification. HW and MX: paper modification. All authors contributed to the article and approved the submitted version.

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PERSPECTIVE
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Vitamin C and COVID-19

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In numerous animal studies, vitamin C has prevented and alleviated viral and bacterial infections. In a few dozen placebo-controlled trials with humans, vitamin C has shortened infections caused by respiratory viruses, which indicates that the vitamin can also influence viral infections in humans. In critically ill patients, plasma vitamin C levels are commonly very low. Gram doses of vitamin C are needed to increase the plasma vitamin C levels of critically ill patients to the levels of ordinary healthy people. A meta-analysis of 12 trials with 1,766 patients calculated that vitamin C reduced the length of ICU stay on average by 8%. Another meta-analysis found that vitamin C shortened the duration of mechanical ventilation in ICU patients. Two randomized placebo-controlled trials found statistically significant reduction in the mortality of sepsis patients. The effects of vitamin C on acute respiratory distress syndrome (ARDS) frequently complicating COVID-19 pneumonia should be considered. Vitamin C is a safe and inexpensive essential nutrient.

Keywords: artificial respiration, ascorbic acid, coronavirus, critical care, pneumonia, respiratory distress syndrome, respiratory tract infections

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INTRODUCTION

About 100 animal studies have shown that vitamin C can prevent and alleviate many kinds of viral and bacterial infections (1, 2). Because of the great diversity in the infectious agents in those studies, it is evident that the effect of vitamin C is not restricted to any specific virus or bacterium. Furthermore, in the early literature, pneumonia was reported to be common in patients suffering from vitamin C deficiency (3, 4), which also indicates that the vitamin can have clinically relevant effects in the protection against infections in humans. Vitamin C may improve the immune response to viral infections through the stimulation of the proliferation and function of T-lymphocytes and NK-lymphocytes, and the production of interferon (2, 5, 6).

EVIDENCE INDICATING THAT VITAMIN C MIGHT INFLUENCE COVID-19

Several of the numerous animal studies on vitamin C and infections (1, 2) are relevant when considering the potential role of the vitamin against the new SARS-CoV-2 coronavirus. Vitamin C increased the resistance of chick embryo tracheal organ cultures to infection caused by an avian coronavirus (7), and protected broiler chicks against an avian coronavirus (8). In addition, in septic mice with acute respiratory distress syndrome (ARDS), vitamin C administration downregulated proinflammatory genes, enhanced epithelial barrier function, and improved alveolar fluid clearance (9, 10). In addition, the deficiency of vitamin C increased lung pathology caused by influenza A in mice (11).

A number of controlled vitamin C trials in humans are also important when considering the new coronavirus. A few dozen placebo-controlled trials with humans showed that regularly

administered ≥1 g/day vitamin C shortened infections caused by respiratory viruses in adults by 8%, and in children by 18% (12). Respiratory viruses form a heterogeneous group and their distribution varies over time and location. Therefore, types of viruses have varied between the trials and it is unlikely that the benefit of vitamin C is explained by effects on just a certain respiratory virus or virus group. Because the effect of vitamin C on the diverse group of respiratory viruses seems non-specific, it seems plausible that vitamin C may also have effects on the new coronavirus.

In the placebo-controlled trials on the common cold (12), the magnitude of effect of regularly administered vitamin C has not been very large and does not justify regular vitamin C supplementation in normal situations. However, the new coronavirus causes an illness that is much more severe than ordinary respiratory virus infections and frequently causes pneumonia complicated by ARDS. Therefore, even moderate benefits of an 8–18% decrease in the duration of respiratory virus infections would justify consideration of vitamin C supplementation. Moreover, in the early literature, pneumonia was described as a common complication of frank vitamin C deficiency, scurvy, and two small controlled trials indicated that vitamin C might have therapeutic benefits against pneumonia (3, 4).

The particular concern with COVID-19, the disease caused by the novel coronavirus, is that ICU treatment is needed for a rather high proportion of patients. There is much evidence that critically ill patients have reduced plasma levels of vitamin C, which is explained by the increased depletion of the vitamin in their body so that one third of ICU patients may have as low vitamin C levels as vitamin C deficient patients (13, 14). In particular, a recent survey found that out of 18 COVID-19 patients, 17 had undetectable vitamin C levels and one patient had a very low level (15). Another recent study also reported low vitamin C plasma levels in COVID-19 patients, and non-survivors had half the plasma level of survivors (16). Although 0.1 g/day of vitamin C can maintain ordinary plasma levels in healthy persons (17), critically ill patients need much higher doses (2–3 g/day) to increase the plasma vitamin C levels to the ordinary range (13, 18). It would therefore seem reasonable to screen plasma vitamin C levels in ICU patients and administer vitamin C to those with low levels. Unfortunately, vitamin C assay with HPLC is quite expensive and therefore not usually available in daily practice, and the cheaper tests are less accurate.

A meta-analysis of 12 controlled trials with 1,766 patients found that vitamin C had shortened ICU stay on average by 8% (13). Another meta-analysis of eight trials found that vitamin C shortened the duration of mechanical ventilation in patients who needed the longest ventilation (19). Furthermore, Zabet et al. (20) reported that vitamin C reduced mortality in 28 sepsis patients by 78% ($P = 0.01$; based on 2/14 vs. 9/14) and Fowler et al. (21) reported that vitamin C reduced mortality in 167 patients with sepsis and ARDS by 35% ($P = 0.01$; based on 25/84 vs. 38/83). A reanalysis of the latter trial showed that during the 4-day vitamin C administration, mortality was decreased in the vitamin C group with RR = 0.19 (95% CI 0.06–0.55) (22). During the 4-day intervention, the number needed to treat was 5.5 (95% CI 3.5–12.5), which means that one death was prevented in five to six patients by vitamin C (22).

DISCUSSION

Although there is as yet no direct evidence indicating that vitamin C is beneficial specifically against COVID-19, the reported benefits of vitamin C in the ICU context suggest that it could be considered for patients. Based on the dose vs. plasma level analyses, it is unlikely that a healthy person would benefit from daily vitamin C doses over 0.5 g/day (17). However, for patients suffering from a respiratory virus infection, 6–8 g/day of oral vitamin C was significantly more effective than 3–4 g/day (1). In the recent studies with patients with sepsis (20) and with sepsis and ARDS (21–23), the doses of intravenous vitamin C were 7–16 g/day for 3–4 days.

Vitamin C is an essential, inexpensive nutrient. Due to the severe clinical course of COVID-19 pneumonia, even moderate benefits may be worthwhile. However, the excellent safety profile of vitamin C and the necessity of ICU treatment for a high proportion of COVID-19 patients may justify consideration of clinical application of vitamin C, even before the results of large clinical trials are available (24). Vitamin C has been proposed for COVID patients also by other authors (25–27).

AUTHOR CONTRIBUTIONS

HH and AM participated in the revision of the manuscript.

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Nowcasting and Forecasting the Spread of COVID-19 and Healthcare Demand in Turkey, a Modeling Study

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Background: This study aims to estimate the total number of infected people, evaluate the effects of NPIs on the healthcare system, and predict the expected number of cases, deaths, hospitalizations due to COVID-19 in Turkey.

Methods: This study was carried out according to three dimensions. In the first, the actual number of infected people was estimated. In the second, the expected total numbers of infected people, deaths, hospitalizations have been predicted in the case of no intervention. In the third, the distribution of the expected number of infected people and deaths, and ICU and non-ICU bed needs over time has been predicted *via* a SEIR-based simulator (TURKSAS) in four scenarios.

Results: According to the number of deaths, the estimated number of infected people in Turkey on March 21 was 123,030. In the case of no intervention the expected number of infected people is 72,091,595 and deaths is 445,956, the attack rate is 88.1%, and the mortality ratio is 0.54%. The ICU bed capacity in Turkey is expected to be exceeded by 4.4-fold and non-ICU bed capacity by 3.21-fold. In the second and third scenarios compliance with NPIs makes a difference of 94,303 expected deaths. In both scenarios, the predicted peak value of occupied ICU and non-ICU beds remains below Turkey's capacity.

Discussion: Predictions show that around 16 million people can be prevented from being infected and 94,000 deaths can be prevented by full compliance with the measures taken. Modeling epidemics and establishing decision support systems is an important requirement.

Keywords: mathematical modeling, COVID-19 modeling, covid-19 simulation, TURKSAS, epidemic forecasting

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INTRODUCTION

Infectious diseases can persist in certain populations (endemic), spread at a sudden rate and affect wider populations (epidemic), or turn into a global threat (pandemic) as in the 1918 Spanish flu (1). Coronaviruses, which were first detected in 1960, have been observed in humans to date and have seven subtypes, and are responsible for the SARS outbreaks in 2003 and MERS in 2012 (2).

A new type of coronavirus (later named Sars-Cov-2) first drew attention on 31 December 2019 after 27 pneumonia cases with unknown etiology were detected in Wuhan, China and reported to

the World Health Organization (WHO) (3, 4). The epidemic caused by the virus, called COVID-19, spread rapidly between countries and continents and was ultimately considered pandemic by the WHO on 11 March 2020 (5).

The rapid progression of the COVID-19 pandemic and its devastating effects in many countries has revealed the vital nature of epidemic modeling studies to evaluate the course of the epidemic and its burden on countries' health systems properly. Stochastic, deterministic and agent-based models have been used in the scientific literature to model the spread of COVID-19 (6, 7).

Turkey has also taken precautions due to the COVID-19 pandemic and many additional measures were implemented after the identification of the first national case on March 11, 2020 (8). These measures include Non-Pharmaceutical Interventions (NPIs) such as school closures, cancellation of arts and sports events, mandatory quarantine for people who have traveled to the country from abroad, the general closure of public places such as cafes/cinemas/wedding halls, making mask usage in grocery stores obligatory, curfew for the citizens over the age of 65 and under 20 and those with chronic illnesses (9–11).

This study aims to estimate the total number of infected people, evaluate the consequences of social interventions on the Turkish healthcare system, and predict the expected number of cases, intensive care needs, hospitalizations and mortality rates in Turkey according to possible scenarios *via* a modified version of the SEIR-based outbreak modeling method. Thus, it aims to contribute to the pandemic response policies adopted in Turkey by providing an epidemiological framework.

MATERIALS AND METHODS

Study Design

This study was carried out according to three different dimensions. In the first dimension, the actual number of people infected in the community was estimated using the number of deaths in Turkey. In the second dimension, the expected total numbers of infected people, deaths, hospitalizations, and intensive care unit (ICU) bed needs were predicted in the case of no intervention.

The predictions in the second dimension include cumulative numbers only. Thus, additional calculations were required to predict the distribution of healthcare needs, patients, and deaths over time. Therefore, a third dimension was added to the study to model the distribution of the expected numbers to determine the health resources required based on this model, and to predict the impact of social interventions on the progression of the epidemic.

In this third dimension, the SEIR model was used for estimations and predictions. This model divides society into four main compartments during the epidemic: those who are not yet infected (Susceptible), those who have been exposed to the agent but show no signs of infection (Exposed), those who have had symptoms of the disease (Infectious), those whose who have either recovered or died from the disease (Removed) (12).

First Dimension Assumptions and Forecasting Algorithm

The ratio of deaths in the total infected population is identified in the literature as the Infection Fatality Ratio (IFR) (13). There may be a time shift bias in the estimations based on the number of deaths. For more accurate estimates, the number of deaths observed on a given day should not be compared to the number of infectious people for the same day; instead, it should be compared to the day the infection started (14). Thus, in this dimension of the study, the number of infected people was estimated by using the number of deaths based on IFR. According to the studies, the time that elapses from initial symptoms to death is about 18 days (13). The number of infected people was estimated according to a delay of 18 days, and the remaining days were projected with a quadratic growth curve which has the highest R^2 value (0.9936). This study used the average IFR [0.66% (0.39–1.33)] and age-specific IFR values which were adjusted for the United Kingdom and the United States in Imperial College London (ICL) modeling based on calculations by Verity et al. (13).

Second Dimension Assumptions and Forecasting Algorithm

The COVID-19 overall infection rate for Turkey was considered to be 81% (15). 2018 TurkStat census data was used for age stratification. Using the expected age-specific hospitalization and intensive care ratios, total hospitalization numbers and ICU needs are estimated for each age group. First dimension values were used for IFR values. By applying age-specific IFR values to the expected number of infected people in the relevant age group, the highest number of expected deaths was determined (13, 15). In this dimension, it was assumed that no measures were taken, and the pandemic is free to spread throughout society.

Third Dimension Assumptions and Forecasting Algorithm

In this dimension of the study, a SEIR-based model was created, and a simulator called TURKSAS was developed by adding transmission dynamics as well as clinical dynamics and NPIs dynamics. The TURKSAS model structure is as presented in **Figure 1**.

Mathematical Equation of the Model

1*st Group*, *Asymptomatic cases*;
2nd Group, *Symptomatic cases*
***(p:** proportion|**ICU:** Intensive Care Unit);*
p_1, *Asymptomatic case proportion*;
p_2, *Symptomatic case proportion*;
py, *Symptomatic and will apply to the hospital*;
ph, *Symptomatic and will have mild disease*;
pi, *will recover from the hospital*;
pk, *will need ICU Bed*;
pt, *will recover from ICU*;
pö, *Fatality rate among ICUs accordingto IFR*;
R0, *Number of people contaminated by an infected*;
T_{inc}, *Incubation period*;
T_{inf}, *Infectious period*;
S, *Susceptible*;

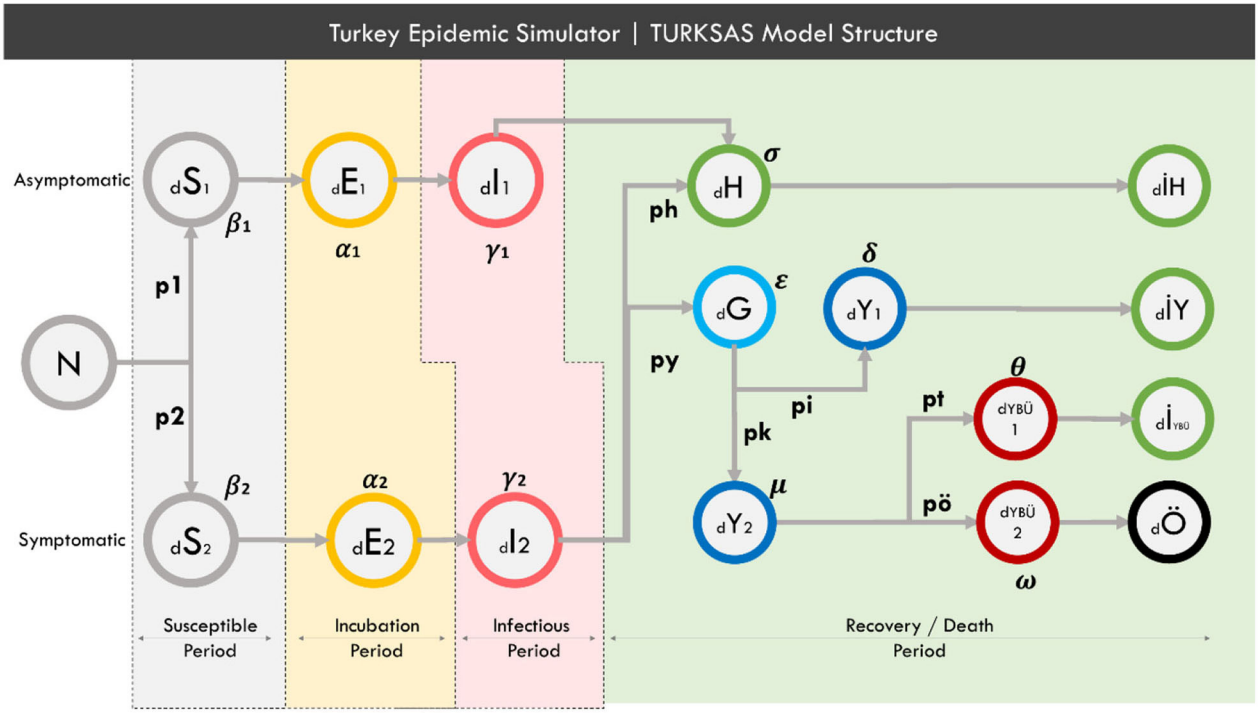

FIGURE 1 | TURKSAS model structure. In a time section; N, Total population; d, delta (expressing the change of the related cluster over time). S- E- I, The number of Susceptible-Exposed-Infected people, respectively, in the relevant time section. H, Infected who have mild symptoms. IH, Those who have recovered with mild symptoms. G, Infected and have not yet applied to the hospital. Y, Infected who apply to the hospital and have occupied non-ICU beds. Y, Those who have recovered in hospital and been discharged. Iybu, Those who have recovered from ICU. YBÜ1, Those who will recover in ICU. YBÜ2, Those who will die in ICU. Ö, Those who have died. For other parameters, see section Mathematical Equation of the Model.

E, *Exposed*;
I,*Infectious*;
H, *Mild cases*;
İH, *Recovered with mild symptoms.*
G, *Infected but have not yet applied to the hospital*;
$\boldsymbol{Y_1}$, *Applied to the hospital and will recover*;
$\boldsymbol{Y_2}$, *Applied to the hospital and need ICU*;
İY, *Recovered from the hospital without ICU need*;
***YB*Ü1** : *Still in ICU and will be recovered*;
***İyb*ü**, *Recovered from ICU*;
YB*Ü*2 : *Still in ICU and will die*;
Ö:*Died*

$$\frac{dS_1(t)}{dt} = -\frac{S_1(t)}{N_1}.I_1(t).\beta_1$$
$$\frac{dS_2(t)}{dt} = -\frac{S_2(t)}{N_2}.I_2(t).\beta_2$$
$$\frac{dE_1(t)}{dt} = \frac{S_1(t)}{N_1}.I_1(t).\beta_1 - \alpha_1.E_1$$
$$\frac{dE_2(t)}{dt} = \frac{S_2(t)}{N_2}.I_2(t).\beta_2 - \alpha_2.E_2$$
$$\frac{dI_1(t)}{dt} = \alpha_1.E_1 - \gamma_1.I_1$$
$$\frac{dI_2(t)}{dt} = \alpha_2.E_2 - \gamma_2.I_2$$

$$\frac{dH(t)}{dt} = \gamma_1.I_1 + ph.\gamma_2.I_2 - \sigma.H$$
$$\frac{d\dot{I}H(t)}{dt} = \sigma.H$$
$$\frac{dG(t)}{dt} = py.\gamma_2.I_2 - \varepsilon.G$$
$$\frac{dY_1(t)}{dt} = pi.\varepsilon.G - \delta.Y_1$$
$$\frac{d\dot{I}Y(t)}{dt} = \delta.Y_1$$
$$\frac{dY_2(t)}{dt} = pk.\varepsilon.G - \mu.Y_2$$
$$\frac{dYBU_1(t)}{dt} = pt.\mu.Y_2 - \theta.YBU_2$$
$$\frac{dYBU_2(t)}{dt} = p\ddot{o}.\mu.Y_2 - \omega.YBU_2$$
$$\frac{d\ddot{O}(t)}{dt} = \omega.YBU_2$$

$$p_2 = 1 - p_1 \qquad ph = 1 - py \qquad pi = 1 - pk$$
$$pt = 1 - p\ddot{o} \qquad p\ddot{o} = ((IFR/p2)/py)/pk$$
$$N_1 = p_1.N \qquad N_2 = N - N_1$$
$$\alpha_1 = \frac{1}{T_{inc1}} \qquad \alpha_2 = \frac{1}{T_{inc2}}$$

$$\beta_1 = \frac{R0_1}{T_{\inf 1}} \qquad \beta_2 = \frac{R0_2}{T_{\inf 2}}$$

$$\gamma_1 = \frac{1}{T_{\inf 1}} \qquad \gamma_2 = \frac{1}{T_{\inf 2}}$$

$$\sigma = \frac{1}{Mild\ to\ recovery\ duration}$$

$$\varepsilon = \frac{1}{Hospitalization\ lag}$$

$$\delta = \frac{1}{Hospitalized\ to\ recovery\ without\ ICU}$$

$$\mu = \frac{1}{Hospitalization\ to\ ICU\ duration}$$

$$\theta = \frac{1}{ICU\ to\ recovery\ duration}$$

$$\omega = \frac{1}{ICU\ to\ death\ duration}$$

Because the incubation period, infectious period and basic reproduction number (R0) variables differ between symptomatic and asymptomatic cases, these two groups are considered to be separate community layers within this model. Also, it is assumed that asymptomatic cases will not apply to the hospital and die. The R compartment was also restructured to predict the need for health care. Some infected people will recover with mild symptoms without requiring hospital admission (H). Some will be late to apply to the hospital even though they show symptoms (G). After the delay, these people will apply to the hospital (Y). It is assumed that all positive cases admitted to the hospital will initially be transferred to the non-ICU beds. Some of these patients will recover directly from the service (İY) and some will recover and be discharged from ICU (YBU1). Others will go to ICU (YBU2) and then die (Ö).

Due to a lack of studies that estimate the local clinical care dynamics and durations in Turkey, we used coefficients and assumptions from various scientific studies.

Transmission Dynamics

The average incubation period was accepted to be 4.6 days for asymptomatic cases and 5.1 days for symptomatic cases, and the infectiousness period was accepted to be 6.5 days for both groups (15, 16). Symptomatic cases were considered to be two times more infectious than asymptomatic (15). It is assumed that R0 values are between 2 and 3 for Turkey (17, 18). Considering that the study on the Diamond Princess (cruise ship) was close to a prospective cohort design, the rate of asymptomatic cases was accepted to be 17.8% in our study (19).

Clinical Dynamics

It has been assumed that people with mild symptoms will not apply to the hospital and their recovery will take 22 days (20). The delay time for hospital admissions is considered to be 5 days, and the period from hospitalization to recovery is considered to be 10 days (21). The duration of recovery from ICU to discharge is considered to be 15 days, and the duration from ICU to death is considered to be 7 days (22, 23). Duration for referral to ICU after hospitalization was assumed to be 5 days from expert opinion. The duration from the onset of symptoms of the disease to death is considered to be 17.8 days (13). The total ICU bed and non-ICU bed capacity of Turkey is considered to be 38,098 and 193,095, respectively (24).

TABLE 1 | Effect of NPIs on Rt value (8) and assumptions regarding social compliance with policies.

NPIs	Date	Relative percentage reduction in Rt	Social compliance (%)
School Closure	12 March 2020	20%	100%
Self İsolation	13 March 2020	10%	80%
Public Events Ban	16 March 2020	12%	80%
Social Distancing	18 March 2020	11%	80%
Curfew > 65*	27 March 2020	14.3%	90%
Curfew, <20*	5 April 2020	14.3%	90%

NPIs, Non-pharmaceutical Interventions.

**In the ICL 30 March report, the total effect of lockdown was measured to be 50%. Turkey has applied curfew for over 65 s and under 20 s to date. We assumed this effect for three different age groups consulting expert opinion as C65: 14.3% C20: 14.3% and C21–64: 21.4%.*

NPIs Dynamics

NPIs decrease the number of contacts, which accordingly decreases the value of time-varying reproduction number (Rt) directly. This decrease affects all outputs over the β-value in the equation. The impact of social interventions on the Rt value in European countries is presented in detail in the ICL March 30 report (25). In TURKSAS, the impact values from the ICL report were used and simulations were made specific to the dates when each intervention was activated. It was also calculated the extent to which the social interventions applied in Turkey reduced the default Rt value in the model over time. The dates the NPIs were applied, relative percentage reduction in Rt, and assumptions about social compliance to NPIs in Turkey are presented in **Table 1**.

RESULTS

First Dimension

According to the estimates based on the number of deaths (announced daily), the number of infected people on March 17 was 75,909. The number of infected people in society according to IFR and the future projection are presented in **Figure 2**.

Second Dimension

In the case of the free spread of the pandemic without any interventions, the expected age-stratified distribution of the maximum total number of cases, total need for ICU and non-ICU beds and deaths are presented in **Figure 3**. The maximum total number of hospitalizations was estimated to be 3,418,398, intensive care hospitalizations to be 856,422 and deaths to be 414,203.

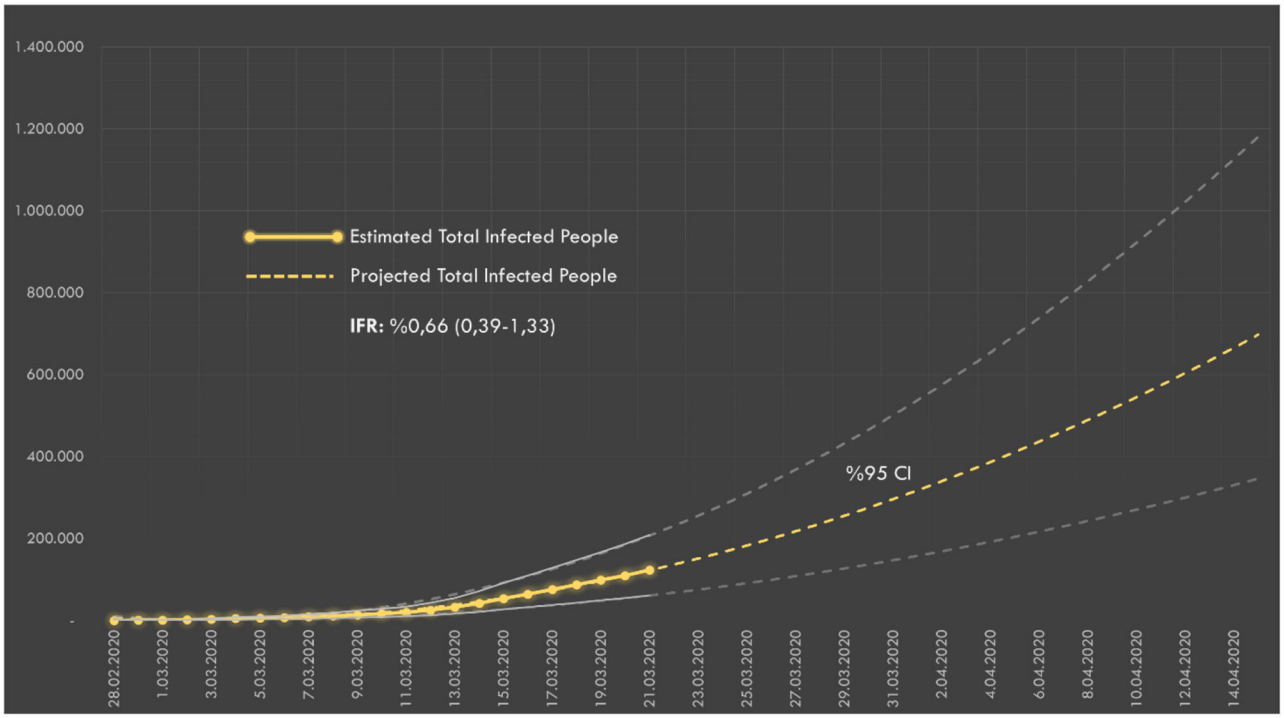

FIGURE 2 | The estimated number of infected people over the number of deaths in Turkey. IFR, Infection Fatality Rate.

Age Groups	Turkey Population	k1	Max. Expected Cases	k2	Max. Expected Hospitalisations	k3	Max. Expected ICU Cases	IFR	Max. Expected Deaths
0-9	12.881.568	81%	10.434.070	0,1%	10.434	5,0%	522	0,002%	209
10-19	12.725.029	81%	10.307.273	0,3%	30.922	5,0%	1.546	0,006%	618
20-29	12.780.455	81%	10.352.169	1,2%	124.226	5,0%	6.211	0,030%	3.106
30-39	12.882.447	81%	10.434.782	3,2%	333.913	5,0%	16.696	0,080%	8.348
40-49	11.139.044	81%	9.022.626	4,9%	442.109	6,3%	27.853	0,150%	13.534
50-59	8.857.551	81%	7.174.616	10,2%	731.811	12,2%	89.281	0,600%	43.048
60-69	6.042.751	81%	4.894.628	16,6%	812.508	27,4%	222.627	2,200%	107.682
70-79	3.107.727	81%	2.517.259	24,3%	611.694	43,2%	264.252	5,100%	128.380
80-89	1.275.636	81%	1.033.265	27,3%	282.081	70,9%	199.996	9,300%	96.094
90-99	170.023	81%	137.719	27,3%	37.597	70,9%	26.656	9,300%	12.808
100+	4.990	81%	4.042	27,3%	1.103	70,90%	782	9,300%	376
Total	81.867.221		66.312.449		3.418.398		856.422		414.203

FIGURE 3 | In the case of no interventions, the expected age-stratified distribution of the maximum total case, hospitalization, ICU cases and deaths. k1, Attack rate. k2, age-specific proportions of hospitalization among symptomatic cases. k3, age-specific proportions of ICU need among hospitalized people. IFR, Infection Fatality Rate.

Third Dimension

Scenario 1: No Intervention

The estimations in the second dimension were also simulated in SEIR-based TURKSAS simulator (**Table 2**). The expected total number of infected people was 72,091,595 and the total number of deaths was 445,956. The attack rate was 88.1% for a pandemic period as the entire society is considered to be the population at risk. The expected mortality ratio was 0.54%.

It is predicted that all ICU beds and non-ICU beds will reach 100% occupancy rate in May, while the need for ICU and non-ICU beds would reach its peak in June. At the peak point, the ICU bed capacity would be exceeded by 4.4-fold and the non-ICU bed capacity by 3.21-fold (**Figure 4**).

Scenarios 2 and 3: Social Compliance to NPIs (<100% Compliance and 100% Compliance)

The effects of the NPIs applied in Turkey on Rt are presented in **Figure 5**. According to the calculations made by taking into account the compliance rates with the interventions, the value of Rt is estimated to decrease from 3 to 1.38.

Predictions in first scenario (<100% compliance) and the second scenario (100% compliance) are presented in **Table 3**. Compliance with social interventions makes a 94,303 difference in the expected number of deaths. In both scenarios, the predicted peak value of occupied ICU and non-ICU beds remains below Turkey's healthcare capacity.

For the second and third scenarios, the predicted numbers of total daily deaths and required ICU and non-ICU beds are presented in **Figure 6**.

TABLE 2 | Predictions for the first scenario (in the case of no intervention).

	Value	Unit
Expected total cases	72,091,595	Cases
Attack rate	88.1	%
Expected total deaths	445,956	Deaths
Mortality	0.54	%
Daily occupied ICU beds peak	168,790	Beds
Date of peak	June 2020	date
ICU bed capacity exceeded	4.44	Fold
Date ICU beds are 100% full	May 2020	Date
Daily occupied non-ICU bed peak	618,928	Beds
Date of peak	June 2020	Date
Non-ICU bed capacity exceeded	3.21	Fold
Date non-ICU beds are 100% full	May 2020	Date

ICU, Intensive Care Unit.

Scenario 4: General Curfew Intervention

We predicted that if a curfew were declared for the 21-64 age group, Rt would drop to just below 1 (0.98) and the pandemic would tend toward an end (i.e., non-exponential increase in infection rate). The predicted situation if such a curfew was applied for the 21–64 age group on April 15 is presented in **Table 4** and **Figure 7**. According to these predictions, the expected number of deaths would be 14,230 and the peak daily values for ICU and non-ICU bed demand would be well below the country's healthcare capacity.

DISCUSSION

Estimating and predicting the burden of epidemic diseases on society and the healthcare system in the most accurate way possible is important to ensure the efficient use of the health resources. Although expert opinions are valuable for the

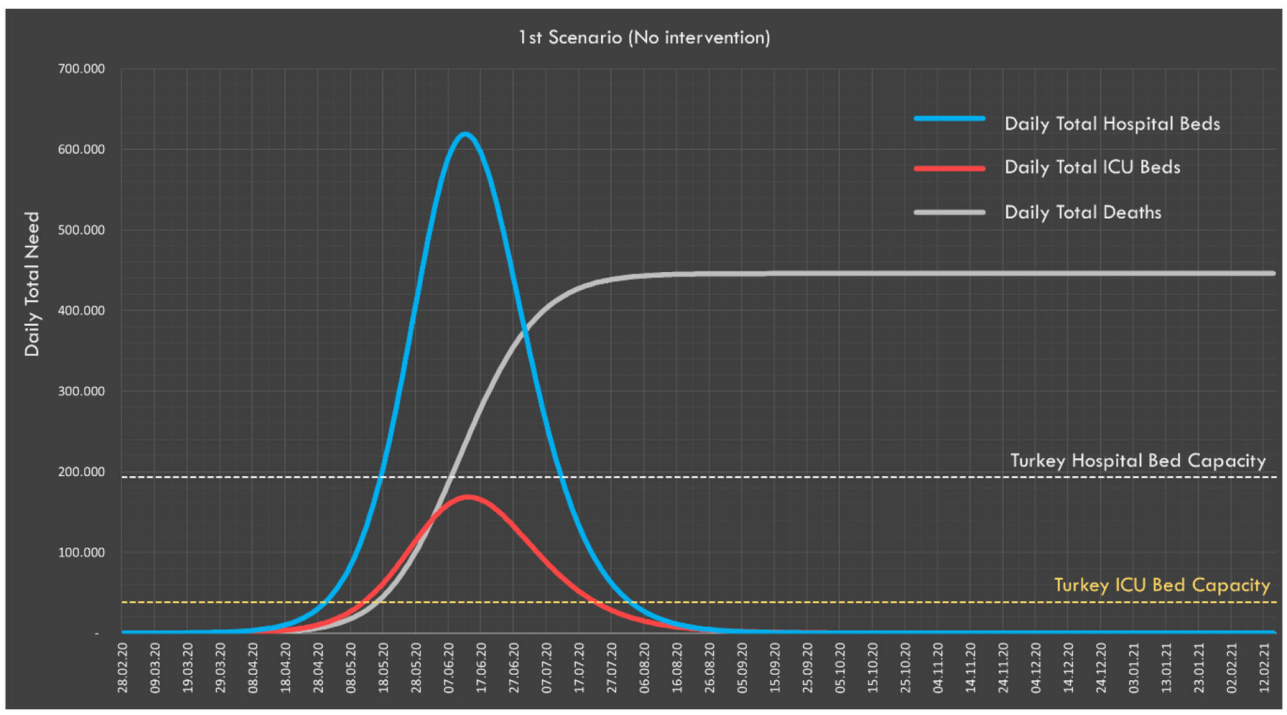

FIGURE 4 | In the worst-case scenario, the need for ICU and non-ICU beds and daily distribution of expected deaths.

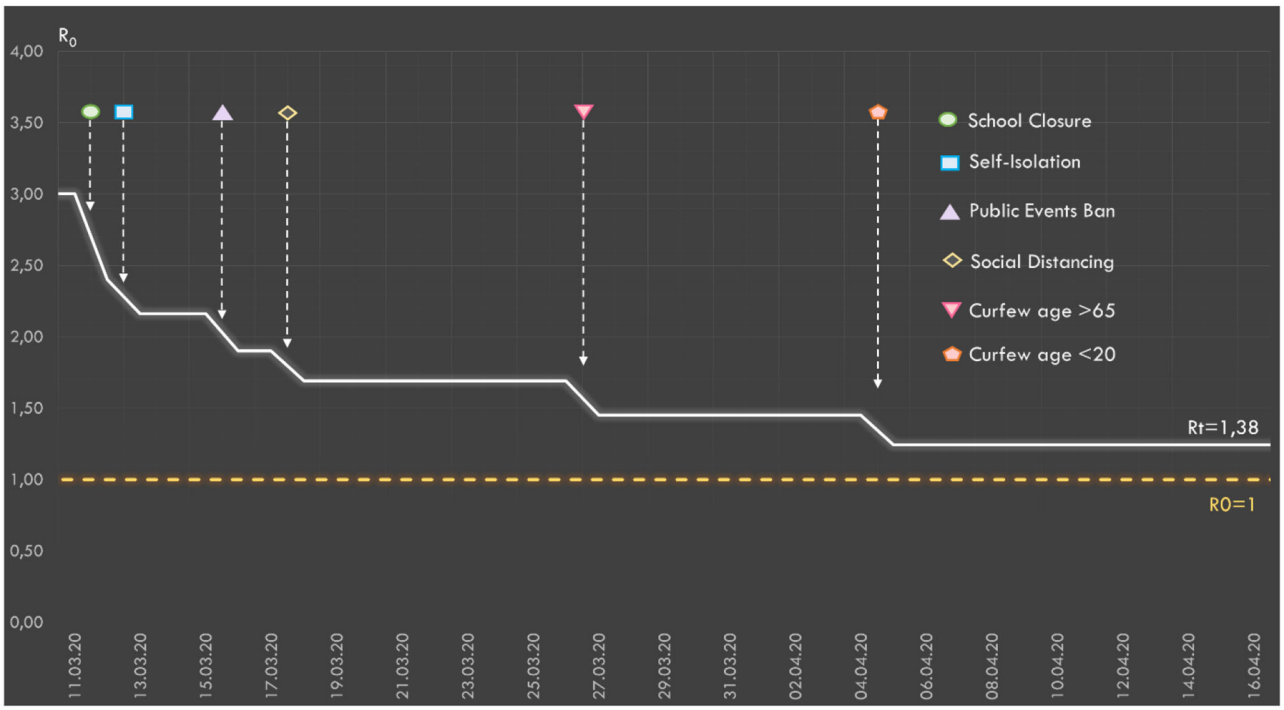

FIGURE 5 | The relative effect of the social interventions applied in Turkey on Rt values.

predictions relating to the pandemic, it is difficult to find up-to-date evidence to support expert opinions in pandemics that are not frequently experienced. Due to the devastating social effects of epidemics, there is no possibility of experimenting for most interventions, and there are also, of course, ethical limitations involved. For this reason, modeling outbreaks using assumptions supported by the scientific literature and establishing decision support systems based on objective criteria is an important, if not vital, requirement (26).

First Dimension

The first dimension of the study is to nowcast the actual number of infected people using the IFR. In the estimation of the actual number of cases, the case fatality rate (CFR) and IFR concepts are often confused. The CFR refers to the ratio of the number of deaths in a given time segment to diagnosed cases. However, this rate includes only those who are admitted to hospital and who have been identified, not the proportion of infected people in the community. If perfect conditions were observed and all patients could be followed, how many infected people would die is expressed by the IFR (13). For this reason, it is more appropriate to use IFR in the estimation of the final number of deaths and CFR to estimate the number of deaths in a given time period (14).

We estimated the number of cases in Turkey as 120,000 on 21 March. According to the ICL report, this number was 7 million for Spain as of 28 March 2020; 5.9 million for Italy and 600,000 for Germany (15).

TABLE 3 | Predictions for the second scenario (<100% social compliance) and the third scenario (100% social compliance).

	2nd scenario	3rd scenario	Difference	Unit
Expected total cases	32,528,665	16,502,277	16,026,388	Case
Attack rate	39.7	20.2	19.58	%
Expected total deaths	229,415	135,113	94,303	Case
Mortality	0.28	0.17	0.12	%
Daily occupied ICU beds peak	28,821	14,220	14,601	Bed
ICU bed capacity exceeded	0.76	0.37		Fold
Daily occupied non-ICU bed peak	100,402	49,127	51,275	Bed
Non-ICU bed capacity exceeded	0.52	0.25		Fold
Total recovered	30,174,033	12,678,861	17,495,172	Case

ICU, Intensive Care Unit.

Second Dimension

In this dimension, the maximum number of infected people was estimated to be 66 million, the number of deaths 414,000 with a consequent mortality rate of 0.54%. According to scientific data for the population of Turkey, this would not be expected to be worse than these numbers.

Third Dimension

In SEIR-based studies, generally, asymptomatic and symptomatic cases have not previously been differentiated according to incubation time, infectivity time and Rt variables. In this study, these two groups were included in the model separately. The

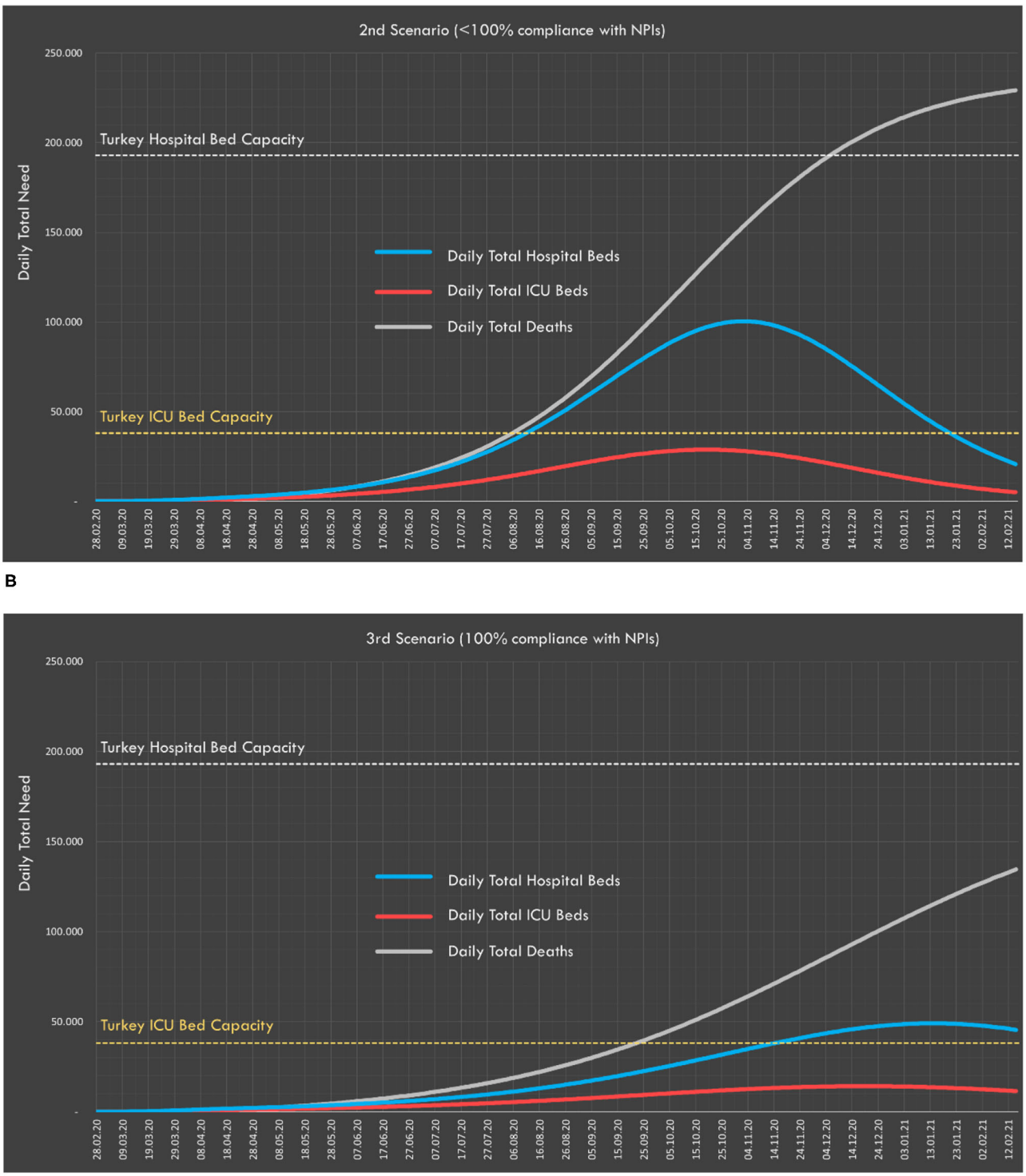

FIGURE 6 | Daily distribution of total ICU and non-ICU beds and expected deaths for the **(A)** second and **(B)** third scenarios.

proportion of asymptomatic cases can be up to 78% in the studies performed according to the symptoms on the day the PCR sample was taken (27, 28). However, the WHO stated that 75% of cases that were asymptomatic developed some symptoms at a later stage and the wholly asymptomatic proportion of the population is actually quite low, and therefore not a major

determinant of the pandemic (29). In the study conducted on the Diamond Princess, 17.9% of all cases were stated to be asymptomatic (19). In our study, it was accepted that the closest study to the cohort design was the Diamond Princess and this same percentage was accordingly used in our calculations. Unlike previous studies, the R compartment was structured with the addition of clinical dynamics in order to evaluate the associated healthcare needs.

In the third dimension of the study, according to this worst-case scenario, a total of 72 million people would be infected in Turkey, and 446,000 people would be estimated to die. According to the ICL report, if there is no intervention, 510,000 deaths would be expected in the UK and 2.2 million in the United States.

TABLE 4 | Predictions for the fourth scenario (general curfew intervention).

	Value	Unit
Expected total cases	594,924	Case
Attack rate	0.7	%
Expected total deaths	14,230	Deaths
Mortality	0.02	%
Daily occupied ICU beds peak	1,355	Beds
Date of peak	May 2020	Date
ICU bed capacity exceeded	0.04	Fold
Daily occupied non-ICU bed peak	2,146	Beds
Date of peak	May 2020	Date

Also, it is calculated that the ICU bed capacity would be exceeded by 30-fold for the UK (15). In our study, the ICU bed capacity in Turkey would be expected to be exceeded by 4.4-fold.

In the second and third scenarios, the expected number of cases and deaths were also calculated according to whether society is partially (second scenario) or fully (third scenario) compliant with the social interventions applied. Predictions show that around 16 million people can be prevented from being infected and 94,000 deaths can be prevented by full compliance with the measures taken. With the measures that Turkey has taken so far, the highest expected need for ICU beds would be under the existing capacity, and indeed ICU bed capacity would not be exceeded were either of these scenarios to be realized. In the fourth scenario, with the implementation of a general curfew that covered all age groups, it was predicted that the total number of cases will be 600,000 and the number of deaths would be <15,000.

In our study, we estimate that Rt has decreased to 1.38 as a result of the existing measures in Turkey. This decreases the rate of spread and attack rate of the pandemic. However, in the case of no intervention the attack rate would be 88.1%, while in the case of a general curfew and other NPIs, this value would decrease to 0.7% and overall mortality rates would decline from 0.54 to 0.02%. Complete control of the pandemic is possible by keeping Rt below 1. For this, additional measures would clearly be needed.

In our study, deaths due to exceeding the number of ICU and non-ICU beds were not considered. Also, in case of exceeding intensive care and

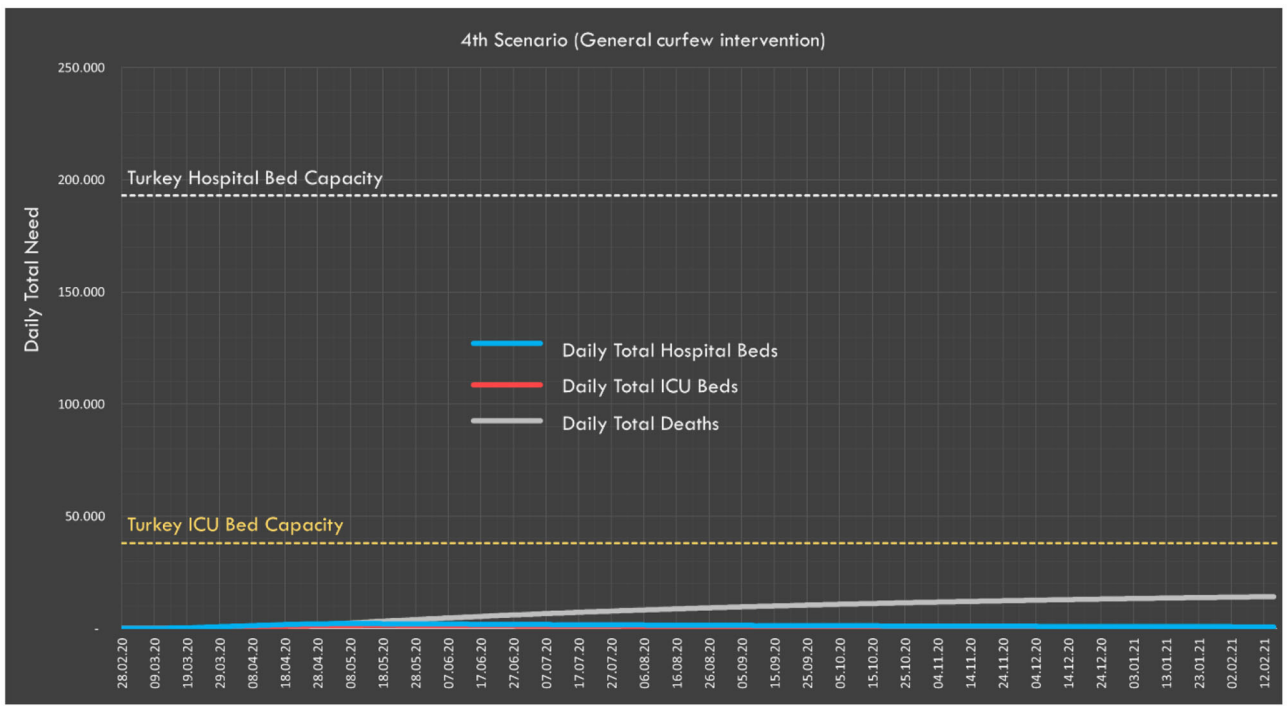

FIGURE 7 | In the fourth scenario, expected daily hospital and ICU bed demand, and distribution of deaths.

healthcare capacity, deaths that may result from disruption of healthcare services are not included in the calculations.

Considering that many global and local parameters affect the results, it is quite difficult to draw definitive conclusions or to make clear statements about the natural course of the disease. Mathematical models are important tools in this period where rapid and evidence-based political decisions should be made under the already devastating effects—and potential future effects—of the epidemic. The estimates in this study show that the progressive stages of the pandemic should be carefully projected, and intervention strategies should be evidence-based. The ultimate goal of all NPIs is to maintain the number of cases within the limits that the relevant healthcare system(s) can intervene with until any vaccine or medical treatment method is available, thereby minimizing deaths and disabilities by providing healthcare to as many patients as possible.

We have conducted our modeling at the early stages of the COVID-19 pandemic with the best possible use of limited epidemiological parameters. Hence, ethical, legal, and economic dimensions were ignored in the suggestions presented in this study. Ethical, economic, and social aspects of interventions assessed above need to be considered in future research. The applicability of widespread interventions, which concern not only health but also the economy and social life, should be evaluated through studies in this field.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: (1) TurkStat Data (http://tuik.gov.tr/PreIstatistikTablo.do?istab_id=1632) (2) Turkey Health Statistics (https://www.saglik.gov.tr/TR,62400/saglik-istatistikleri-yilligi-2018-yayinlanmistir.html).

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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This manuscript has been released as a pre-print at MedRxiv Arslan et al. (30).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.575145/full#supplementary-material

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Pressure Injury Prevention in COVID-19 Patients With Acute Respiratory Distress Syndrome

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Coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was identified in China in December 2019 and became a pandemic in a short period of time. While most infected people might have mild symptoms, older people and people with chronic illnesses may develop acute respiratory distress syndrome (ARDS). Patients with ARDS with worsening hypoxemia require prone positioning to improve the respiratory mechanics and oxygenation. Intubated patients may stay in a prone position up to 12–16 h, increasing the risk of pressure injury (PI). Frequent skin inspections and PI risk assessment in COVID-19 patients will be challenging due to hospital infection control measures aimed to reduce the risk for health professionals. In this perspective article, we summarize the best practice recommendations for prevention of PI in SARS-CoV-2-infected ARDS patients in prone positioning. Prior to positioning patients in prone position, the main recommendations are to (1) conduct a skin assessment, (2) use pressure redistribution devices, (3) select an appropriate mattress or an overlay, (4) ensure that the endotracheal tube securing device is removed and the endotracheal tube is secured with tapes, (5) use a liquid film-forming protective dressing, and (6) lubricate the eyes and tape them closed. Once a patient is in prone position, it is recommended to (1) use the swimmer's position, (2) reposition the patient every 2 h, and (3) keep the skin clean. When the patient is repositioned to supine position, healthcare professionals are advised to (1) assess the pressure points and (2) promote early mobilization.

Keywords: acute respiratory distress syndrome, COVID-19, guidelines, intensive care, pressure injury, pressure points, prone positioning, ventilation

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INTRODUCTION

Coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was identified in China in December 2019 and rapidly became a pandemic (1, 2). While most infected people have mild symptoms, older people and people with chronic illnesses may become critically ill and develop viral pneumonia and acute respiratory distress syndrome (ARDS) (3–5), requiring admission to an intensive care unit (ICU) (6). The pathophysiology of SARS-CoV-2 ARDS differs from that of the typical ARDS (7). The pathophysiological mechanism of COVID-19-related ARDS is pulmonary micro-thrombosis (8).

The results of lung and skin biopsy of critically ill SARS-CoV-2-infected patients demonstrated generalized thrombotic microvascular injury (7). SARS-CoV-2 infection results in cytokine storm and a local and systemic inflammatory response syndrome leading to macro- and microthrombosis (8, 9). The three factors of Virchow's triad—reduced blood flow, endothelial injury, and hypercoagulability—increase the risk of thrombosis in severe COVID-19 patients (8).

Most ICU-admitted COVID-19 patients need non-invasive ventilation, a non-rebreathing mask, and prone positioning to increase oxygen delivery (10) as well as high-flow nasal oxygen through specialized nasal cannula in negative-pressure rooms (11). Up to 5% of COVID-19 patients with ARDS may require endotracheal intubation (12, 13). In general, patients with ARDS of any etiology with worsening hypoxemia (PaO_2:FiO_2 < 100–150 mmHg, $FiO_2 \geq 0.6$, PEEP $\geq$ 10 cm of water, and tidal volume of 6 ml/kg of predicted body weight) require prone positioning to improve the respiratory mechanics, improve oxygenation, and offload the weight of the heart (14, 15). When applied early (usually 12 to 24 h after the initiation of mechanical ventilation) with other lung-protective strategies and adopted for a prolonged period, the prone position is associated with reduced mortality, particularly in patients with severe hypoxemia (16–18).

Prone positioning was reported in the management of ARDS in critically ill patients with severe acute respiratory syndrome (SARS) (19) and Middle East respiratory syndrome (MERS) (20) coronavirus infections and is used to manage ARDS in COVID-19 patients (12). Initially, it was found to be effective in one small-scale study of 52 critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China (21), with further anecdotal evidence from day-to-day clinical practice in ICU. A recent small-scale study (22) on the use of prone positioning in non-intubated patients with COVID-19 and hypoxemic acute respiratory failure in Turkey, managed outside the ICU, reported that oxygenation increased in only a quarter of patients and was not sustained in half of those after resupination. Self-proning of COVID-19 patients is increasingly used in several countries and has become a standard treatment in the management of ARDS patients with hypoxia (23). With some precautions, prone positioning is used in the management of COVID-19-related ARDS in pregnant women (24). A case report from Japan suggests that, although prone positioning may mitigate hypoxemia, its role in reducing mortality in COVID-19 patients with ARDS is unclear, particularly in patients with a secondary superinfection (25), which is often associated with sepsis, shock, and multiple organ failure (26).

The intubated patients may remain in a prone position up to 16 h per day, alternating with 8 h in supine position (27). Prone positioning increases the risk of developing hospital-acquired pressure injury (HAPI) (16, 28), and this risk is higher when compared to the supine position (29). A Wuhan study reported that the mean hospital stay of COVID-19 patients with pneumonia was 22 days (5); prolonged ICU admission (30) and increased hospital stay (31) are independent risk factors for the development of HAPI.

While the studies reporting HAPI incidence in COVID-19 ARDS patients have not yet been published, individual case reports have reported that patients cared in prone position are at risk of developing multiple severe device-related PI on their face, requiring a consultation and an intervention by plastic surgeons (32). Furthermore, diarrhea is a common gastrointestinal feature in COVID-19 patients (33, 34), and in addition to other risk factors, such as immobility and reduced perfusion, diarrhea may contribute to the development of incontinence-associated dermatitis and a pressure injury in the sacral area (35). This risk is even higher in older patients with COVID-19 ARDS and requires immediate attention (36).

Repositioning and pressure relief are important strategies to reduce the risk (37). However, clinical experience reports the need to involve up to seven people to reposition the intubated patient. Frequent skin inspections and risk assessment in COVID-19 patients could be challenging due to hospital infection control measures aimed to reduce the risk for health professionals working in ICU (11, 38).

In general, there is a global health professional knowledge deficit on PI prevention, with early detection (39–45) and standard preventive interventions recommended in clinical practice guidelines not fully implemented (46) in the context of COVID-19 clinical care. Health professionals may lack awareness of pressure points typical for patients in prone position and may have misconceptions related to the specific equipment required for prone positioning (47). In this perspective article, we summarize the best recommendations for the prevention of PI in SARS-CoV-2-infected ARDS patients in prone position.

PRONE POSITION: PRESSURE INJURY PREVENTION

The latest version of *Prevention and Treatment of Pressure Ulcers/Injuries: Clinical Practice Guideline*, *International Version* (37) acknowledges evidence derived from one low-quality study (29), indicating that prone positioning is associated with a higher incidence of HAPI compared with supine positioning. The reported incidence of HAPI is said to be 5 to 15% as derived from low- and moderate-quality studies (48–50). The main recommendation is to avoid the extended use of prone positioning unless required for the management of a medical condition ((37), p. 126). However, COVID-19 ARDS management requires prone positioning for extended periods of time, and therefore, using appropriate support surfaces and pillows and patient repositioning as soon as feasible are key preventive strategies recommended by the guidelines (37). Facial pillows and chest padding can be used to redistribute pressure. The main pressure points in the prone position are the forehead, chin, cheeks, shoulder (anterior), elbow, chest (breasts), genitalia (particularly male), anterior pelvic bones (iliac crests and ischium), knees (patella), dorsal feet and toes, and nose (if positioned incorrectly), which should be inspected as soon as feasible ((37), p. 139), especially if supplies of personal protective equipment are limited.

According to the guidelines ((37), p. 126), the implementation strategies for HAPI prevention in the prone position include the following:

COVID-19

- caused by SARS-CoV-2
- most infected people have mild symptoms
- some are severely affected

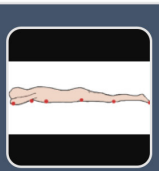
Intensive Care

- some with COVID-19 develop viral pneumonia and Acute Respiratory Distress Syndrome (ARDS) and require intensive care
- ICU admitted COVID-19 patients with ARDS may need non-invasive or invasive ventilation

Prone Position

- indicated for patients with ARDS of any aetiology with worsening hypoxaemia
- improves respiratory mechanics and oxygenation

however this increases risk of pressure injury



Pressure Points in Prone Position

- forehead, chin, cheeks, shoulder (anterior), elbow, chest (breasts), genitalia (particularly male), anterior pelvic bones (iliac crests and ischium), knees (patella) dorsal feet and toes, nose (if positioned incorrectly)

Pressure injury prevention prior to proning

- conduct skin assessment
- use pressure redistribution devices to offload pressure from the bony prominences
- select an appropriate mattress or an overlay
- ensure the endotracheal tube securing devise is removed
- use a liquid film-forming protective dressing and
- lubricate the eyes and tape them closed

Pressure injury prevention once proned

- use the swimmer's position
- where feasible reposition patient every two hours
- keep the skin clean and conduct regular skin cheks
- ensure patients have adequate nutrition and hydration

When repositioned back to supine position

- assess the pressure points
- document a comprehensive skin assessment at all stages
- promote early mobilization

Summary of risk mitigation strategies for health professionals

- follow recommended intensive contact and droplet precautions
- follow recommended airborne precautions for aerosol-generating procedures
- provide adequate training and monitor health professionals' compliance

FIGURE 1 | COVID-19. Pressure Injury prevention in ARDS patients in prone position. Infographic.

TABLE 1 | COVID-19 patient care: Empiric additional precautions*.

Contact and droplet precautions	
Personal protective measures	• use a medical mask • wear eye or facial protection (goggles or face shield) • wear a clean, non-sterile, long-sleeved gown • use gloves • remove and dispose of all personal protective equipment appropriately • maintain hand hygiene • use a new set of personal protective equipment when care is given to a different patient • refrain from touching face with potentially contaminated gloved or bare hands
Equipment-related precautions	• use either single-use and disposable or dedicated equipment (e.g., thermometers, stethoscopes, and blood pressure cuffs) • if shared among patients, clean and disinfect equipment between use for each individual patient (e.g., ethyl alcohol 70%) • use designated portable X-ray or other diagnostic equipment
Engineering and environment-related precautions	• place patients in adequately ventilated single rooms (60 L/s per patient) • group patients together when single rooms are not available • ensure patients' beds are placed at least 1 meter apart regardless of the suspected COVID-19 diagnosis • clean and disinfect surfaces with which the patient is in contact
Patient transporting precautions	• avoid moving and transporting patients unless medically necessary • use predetermined transport routes to minimize exposure for staff, other patients and visitors, and have the patient wear a medical mask • ensure that health care workers transporting patients wear appropriate personal protective equipment and perform hand hygiene • notify the area receiving the patient of any necessary precautions as early as possible prior to their arrival
Administrative measures	• where possible, designate a team of health care workers to care exclusively for suspected/confirmed cases to reduce the risk of COVID-19 transmission • limit the number of staff, family members, and visitors who are in contact with suspected/confirmed COVID-19 patients • maintain a record of all people entering a patient's room
Airborne precautions for aerosol-generating procedures	
Personal protective measures	• use a particulate respirator approved by country-specific occupational safety and health standard • perform the seal check, if you use a disposable particulate respirator • note that facial hair may prevent a proper respirator fit • use eye protection (i.e., goggles or a face shield) • wear a clean, non-sterile, long-sleeved gown and gloves • wear a waterproof apron, if a gown is not fluid-resistant
Engineering and environment-related precautions	• perform procedures in an adequately ventilated room ("natural ventilation with air flow of minimum 160 L/s per patient or in negative pressure rooms with at least 12 air changes per hour and controlled direction of airflow when using mechanical ventilation")
Administrative measures	• limit the number of health professionals present in the room to the absolute minimum required for the patient's care and support

**These precautions were adapted from the World Health Organization Infection prevention and control during health care when COVID-19 is suspected. Interim guidance. 19 March 2020.*

1) Use of pressure redistribution support surface or positioning devices to offload pressure points on the face and the body,
2) Checking for uneven pressure redistribution, focusing on main pressure points unique to prone position, and positioning of medical devices,
3) Use of additional PI preventive strategies, including prophylactic silicone dressings over the bony prominences and under medical devices,
4) Assessing the face and body areas in the main pressure points at each rotation.

A recent review (51) of PI prevention in non-COVID-19 patients, placed in prone position, reported that the main preventive strategies include (1) conducting a skin assessment before proning and following repositioning to the supine position, (2) keeping the skin clean and moisturized, (3) repositioning to offload pressure points on the face and the body, (4) use of positioning devices, and (5) application of dressings, such as hydrocolloids, transparent film, and silicone, to decrease facial skin breakdown (51). Practical suggestions provided by Wounds International (52) include the need to (1) protect bony prominences on the front of the body prior to prone positioning, (2) lubricate the eyes and tape them closed, (3) select an appropriate mattress or an overlay, (4) ensure that the endotracheal tube securing devices are removed—the endotracheal tube should be secured with tapes with the help of a respiratory therapist, (5) ensure the use of liquid film-forming dressing such as SKIN-PREP to decrease trauma on removal, and (6) place the patient's face in swimmer's position when prone, i.e., turn the face to the side toward a flexed arm and put the other arm behind the patient. The swimmer's position allows movement of the head and the endotracheal tube (and a nasogastric tube) at the same time, which should be done every 2 h.

We have summarized the main points of PI prevention in patients in prone position in the infographic (**Figure 1**).

Finally, considering the pathophysiology of SARS-CoV-2 in relation to severe thrombosis (7, 8), patient repositioning as well as early mobilization should be prioritized. Specific guidelines with detailed instructions on how to prepare patients in prone position for care and how to reposition in the supine position are available for intensive care units (15, 53).

The patient's position and the duration in prone position need to be well-documented. Repositioning of unconscious patients into a prone position should be conducted by a team of at least four health professionals, following individual hospital policies, standard safety practices (37), and COVID-19 occupational safety guidelines. PI management relies on team work (37), and the assistance of other team members may be required (54), which should be arranged according to the risk mitigation strategies for health professionals (55).

The main routes of occupational SARS-Cov-2 transmission for health professionals include droplets, airborne transmission, especially during invasive respiratory procedures, and contact transmission (26). In addition to standard measures, health professionals should apply contact and droplet precautions and airborne precautions for aerosol-generating procedures to mitigate the risk of SARS-Cov-2 transmission (56). The main risk mitigation strategies for health professionals (**Table 1**) include the need to be trained in fastidiously applying, wearing, and removing personal protective equipment, which prevents droplets, contact, and airborne transmission; to perform aerosol-generating procedures in a well-ventilated environment, preferably in a negative-pressure room; to allocate a team of healthcare workers to care exclusively for suspected/confirmed cases; and to limit the number of healthcare workers present in the room to the absolute minimum required for the patient's care and support (56).

DISCUSSION

Prone positioning may be effective in the management of SARS-CoV-2 ARDS (10), although this position is associated with an increased risk of HAPI (28). HAPI is a well-known indicator of the quality of care in acute settings (57). Patient influx coupled with a shortage of nursing staff and related caregiver fatigue may influence the quality of care. Preventable PI in acute care can interfere with the patients' recovery, can increase hospital stay, and may contribute to death from PI complications, such us osteomyelitis and sepsis (58). Stages III and IV PI are frequently colonized with methicillin-resistant *Staphylococcus aureus* (59) and multi-resistant Gram-negative bacilli (60), which increase the risk of bacteremia (58) and associated mortality (61). We have discussed the main recommendations for PI prevention in COVID-related ARDS patients in prone position from the latest version of *Prevention and Treatment of Pressure Ulcers/Injuries: Clinical Practice Guideline*, *International Version* (37) and included practical suggestions from the field. In summary, they include specific recommendations for the preparatory stage, care in prone position, and care after repositioning in supine position.

Prior to positioning patients in prone position, the main recommendations are to (1) conduct a skin assessment, (2) use pressure redistribution devices to offload pressure from bony prominences, (3) select an appropriate mattress or an overlay, (4) ensure that the endotracheal tube securing device is removed and that the endotracheal tube is secured with tapes, (5) use a liquid film-forming protective dressing, and (6) lubricate the eyes and tape them closed.

Once the patient is prone, it is recommended to (1) use the "swimmer's position," i.e., turn the face on the side toward a flexed arm and put the other arm behind the patient, (2) reposition the patient every 2 h, i.e., turn the patient's face to the left and lift the left arm if their face was positioned to the right and their right hand was extended, and (3) keep the skin clean.

When the patient is repositioned to supine position, health care professionals are advised to (1) assess the pressure points and (2) promote early mobilization.

The management of PIs is costly to health systems (62–67). Studies show that PI prevention is more cost-effective than treatment (68). Prevention of HAPI in COVID-19 patients would help to avoid additional financial burden to an increasingly drained health system (69–71), particularly in countries significantly impacted by the COVID-19 outbreak (72). In order to preserve healthcare resources and to ensure adequate hospital capacity for the management of COVID-19 patients, many countries have deferred elective surgeries (73–77) and extended elective surgery waiting time. When the restrictions on elective surgeries are lifted, a sizable proportion of hospital beds might be occupied by COVID-19 patients requiring HAPI care if the preventive practices were suboptimal, given that patients with HAPIs have longer adjusted length of hospital stay (63). In addition to health system costs, there are extreme human costs associated with PI development (66), which further strengthens the importance of prevention of HAPIs in COVID-19 patients. Finally, the predicted second wave of COVID-19 cases (78), the lack of evidence on acquired immunity after COVID-19, and the risk of potential re-infection (79) in the absence of a COVID-19 vaccine (80) may result in increased hospital admissions, highlighting the need to speed up quality improvement in this field.

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

VT and LT conducted the literature search and drafted the manuscript with support and guidance from CW, AJ, and HT. VT designed the infographic. All the authors critically reviewed and contributed to the individual parts of the manuscript and approved the final version.

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Artificial Intelligence and Telehealth may Provide Early Warning of Epidemics

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The COVID-19 pandemic produced a very sudden and serious impact on public health around the world, greatly adding to the burden of overloaded professionals and national medical systems. Recent medical research has demonstrated the value of using online systems to predict emerging spatial distributions of transmittable diseases. Concerned internet users often resort to online sources in an effort to explain their medical symptoms. This raises the prospect that incidence of COVID-19 may be tracked online by search queries and social media posts analyzed by advanced methods in data science, such as Artificial Intelligence. Online queries can provide early warning of an impending epidemic, which is valuable information needed to support planning timely interventions. Identification of the location of clusters geographically helps to support containment measures by providing information for decision-making and modeling.

Keywords: artificial intelligence, COVID-19, telehealth, virus, digital disease detection, epidemiology, pattern recognition

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INTRODUCTION

The current pandemic involving the COVID-19 virus appears to have overloaded medical systems worldwide, which are nearly at the breaking point due to insufficient resources in the face of the exponential growth of the virus. Thousands of retired medical staff are returning to volunteer their services, but the effort remains concentrated in the urban areas while regional areas are suffering even more from an acute shortage of medical staff. A pre-print from the Peter Doherty Institute for Infection and Immunity recently discussed the rapid production of a national COVID-19 plan using existing influenza pandemic preparedness techniques that have been developed over the course of many years (Moss et al., 2020). Their clinical care modeling study confirmed that the COVID-19 could rapidly overwhelm Australia's health sector capacity, and re-iterated case targeted measures including isolation of infected individuals and the quarantining of their close contacts. Furthermore, social and physical distancing measures will reduce the number of potential contacts, and in doing so, ease the public health workload (Moss et al., 2020). Preparedness in combination with predictive measures could slow and eventually help to arrest the spread of the virus.

Reviews on internet-based surveillance techniques have been described in the literature (see, for example, Brownstein et al., 2009; Aiello et al., 2020). The techniques used are often based on tracking disease outbreaks by gathering information from the internet, such as from news aggregators, blogs, crowd sourcing platforms and expert-curated discussions. An advantage of using these techniques is that they do not rely on traditional sources of disease surveillance, which are sourced from hospitals and laboratory-based systems from patients who seek care for an illness (Aiello et al., 2020). Rather,

these emerging surveillance techniques use real-time data readily available from virtual platforms. Digital disease detection, as it is aptly called, is considered to be an extension of traditional systems but not a replacement (Wilson and Brownstein, 2009; Milinovich et al., 2014). These emerging technologies have thus far proven to be very useful when used alongside traditional methods, but can still be improved to reflect advances in online technologies.

In the last decade, with the rise of machine learning, Artificial Intelligence (AI) applications have grown rapidly and there is increasing interest in analyzing online queries from members of the public, as well as data mining of electronic health records for disease detection. There is untapped potential for using online queries, information discovery and predictive analytics. There is also potential for the combination of internet software tools with AI and Telehealth in the developing world, including Africa, Middle East and South-East Asia. For example, the COVID-19 crisis has been discussed recently in the context of different geographical regions with respect to such issues as AI, big data, blockchain and internet of things (IoT) (Ting et al., 2020). Another recent review highlighted studies using AI to support identification of disease outbreaks to support policy development in low income and medium income countries, noting such methods as expert systems, machine learning, and natural language processing (Schwalbe and Wahl, 2020).

POTENTIAL OF ARTIFICIAL INTELLIGENCE AND TELEHEALTH

To help mitigate the social and economic impacts of the virus, the use of AI has many attractions. Although humans contribute empathy and intuition to medicine and public health practice, they are biological creatures prone to fatigue, stress, depression, and an assortment of physical and emotional concerns that would affect online search performance. These human qualities can lead to errors in judgment. AI can help to automate detection and localization of virus clusters online and free scarce professional staff for other duties. The high throughput of automated systems could provide valuable information very early in an epidemic that could lead to timely interventions.

Recent reports have revealed that AI algorithms may be able to augment or replace some human-based processes. For example, the United States Center for Disease Control and Prevention warned about the virus on January 6th, 2020. Three days later, the World Health Organization (WHO) made a similar announcement to the public. However, a Canadian AI health monitoring platform known as *BlueDot*–an AI algorithm that sifts through foreign language news, airline ticketing, animal and plant disease networks, and official proclamations–forewarned of the impending pandemic on December 31st, 2019 (Niiler, 2020).

There have been other AI developments in response to COVID-19. For example, an online AI-based diagnostic tool has been developed by the Sydney start-up DetectED-X. The online tool analyses computed tomography (CT) sectional scans of the human torso and rapidly identifies the presence of COVID-19 in the lungs (Detected-X, 2020). Another example is the portable AI device *FluSense*–which was modified for COVID-19 tracking. This was originally designed to analyze influenza trends by detecting and collecting real-time coughing data, along with crowd sizes, noting that coughing is a primary symptom of infection (University of Massachusetts Amherst, 2020). Thermal-imaging is now being used routinely to identify individuals with a fever at various public screening points (Ting et al., 2020).

In addition to diagnostic imaging tools and portable devices, clues on the spread could be obtained by using a number of online queries relating to the combination of targeted search keywords, such as fever, coughing, and recent international travel (using an expert system based on Boolean logic). A regional map of the number of queries could provide information discovery, such as the location of local clusters. There are several influenza-based applications which could be used as a launching pad for COVID-19 AI developments. For example, Deiner and co-workers detected the spatial spread of a conjunctivitis epidemic by tracking online queries on disease symptoms by users of social media such as Google Trends and Twitter (Deiner et al., 2016). A simple example of AI was used in the form of Boolean logic to process the queries. The survey was highly specific to tweets associated with eye disease relating to conjunctivitis symptoms and involved filtering of word content. A further illustration of using internet-based trends and AI-based modeling can be found in Teng et al. (Teng et al., 2017). The authors used Google Trends for the surveillance of the Zika virus. Their study found strong correlations between online searches related to the virus and actual number of reported cases using a machine learning and autoregressive integrated moving average (ARIMA) model.

In the preceding examples, we can identify online platforms, such as Google Trends, as being useful data mining tools for providing data analytics. There are different schools of thought with respect to the application of virtual data for disease detection. On one hand, *BlueDot's* CEO believes social media data is "too messy" to be used in digital disease detection (Niiler, 2020). However, social media networks are providing first-hand information, and are valuable data sources, even if the data are not being collected with a health objective (Denecke, 2017). Thus, researchers, are using social media posts (together with news reports and data from official public health channels) along with machine learning and natural language processing to parse through available data sources and detect mentions of specific COVID-19-related symptoms (Knight, 2020).

The advantage of collating statistics for online queries and social media postings include early warning and the localization of biosecurity threats and epidemics to enable timely intervention by medical authorities. A conceptual process to serve as a baseline for development of an AI-based approach for early warning of an emerging epidemic is depicted in **Figure 1**.

Following initial screening by online AI, suspicious cases could be referred to medical staff for further investigation. The AI algorithm has the computational power to meet and even exceed the performance of a human doctor in diagnostics, but is missing intuition and soft skills, which are desirable in clinical management, patient guidance and support.

As an example, a preliminary analysis carried out by the authors using Google Trends and the search terms "Corona Virus Australia" reveals an increasing trajectory of COVID-19 concerns in Australia from late December 2019 to March 2020

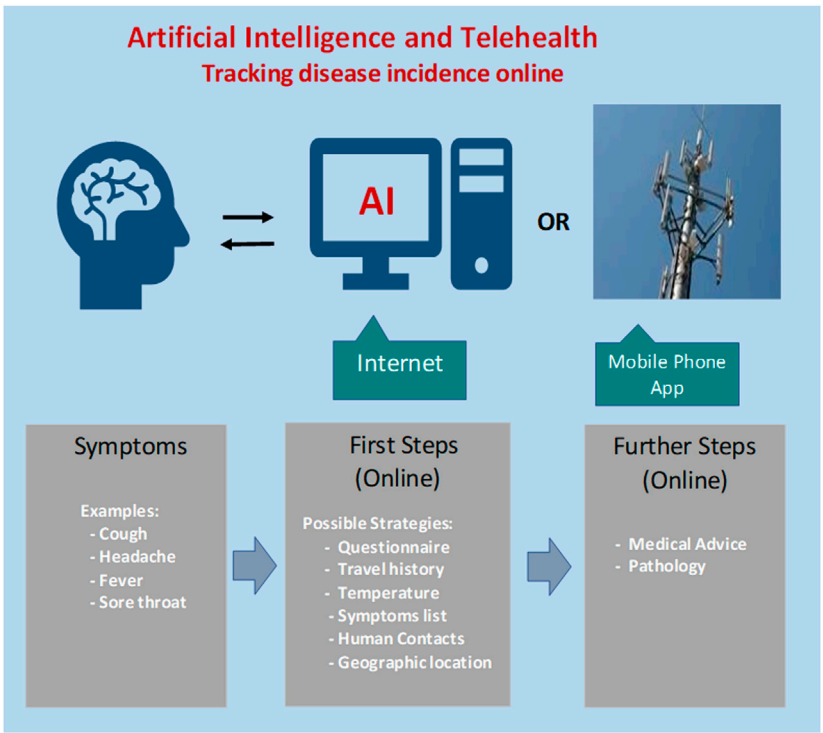

FIGURE 1 | Steps for designing an artificial intelligence-based tracking procedure for regional incidence of COVID-19 virus activity. Concerned citizens with possible symptoms could login and answer a series of questions. Feedback would indicate whether further follow-up is warranted.

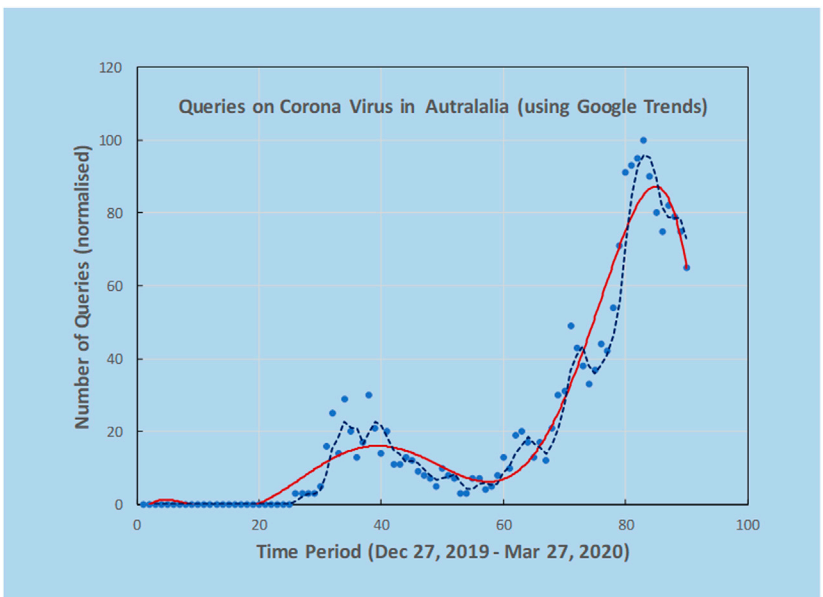

FIGURE 2 | Using Google Trends, it was possible to investigate online search activity over a period of three months using the keywords "Corona Virus Australia". Solid line is an estimate of trend by fitting a polynomial curve to the scatterplot data from Google Trends and the dashed line is 3-point moving average. Peaks indicate high interest at certain dates that could be correlated with news reports. Note the double peak in query dates.

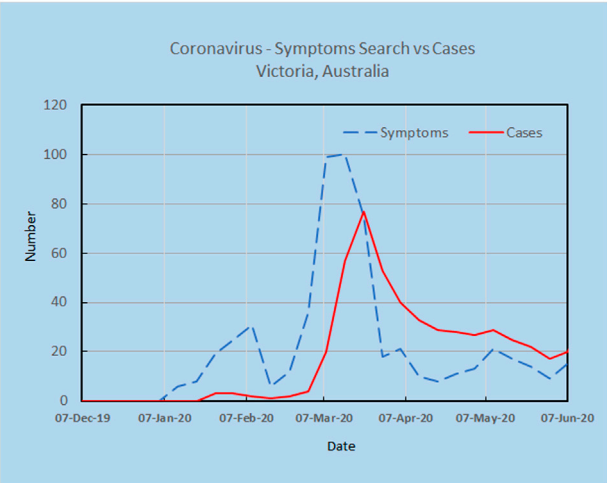

FIGURE 3 | Online search for symptoms reached a maximum during 8–15 March, while the peak for cases was 22 March, providing 1–2 weeks warning.

(**Figure 2**). Using AI techniques alongside these preliminary trends, the potential spread and direction of COVID-19 may be estimated before actual confirmed cases begin to surface. This is shown in **Figure 3**, where the search for symptoms over a six-month period reached a maximum during 8–15 March, while the peak of cases occurred at 22 March, thus providing 1–2 weeks prior warning. The search terms employed were subject to analysis by Boolean logic and were "Victoria AND coronavirus AND symptoms" (Source: Google Trends, Accessed 4 July 20). The results are indicative that monitoring online queries on symptoms from the public may reveal new outbreaks, with prior warnings of 1–2 weeks, which is significant given the exponential growth rate of the virus. These results are from a pilot study only and need to be confirmed with larger trials. There is a need to study the design and structure of search queries to remove ambiguities and to ascertain whether the search performance changes as the epidemic evolves.

Apart from Google Trends, there are also other social media platforms, such as Twitter, that could be used to track COVID-19 or other epidemics. For example, Paul and co-workers describe influenza forecasting using data collected from the Twitter community (Paul et al., 2014). The authors compared this form of forecasting to the more traditional and gold-standard of reporting (i.e., historical influenza-like illness (ILI) data from the CDC). The ILI data has the disadvantage that there is a lag of 1–2 weeks (i.e., time between a patient being diagnosed to when their data appears in the ILI report). The initial reports are often riddled with inaccuracies which are corrected by the CDC over time. Using a basic linear autoregressive model, the authors found that a model incorporating Twitter data outperformed an equivalent model relying solely on ILI data. They found that incorporating Twitter data can reduce influenza forecasting error by 17–30% over a baseline that uses only historical data. Additionally, Twitter can forecast 2–4 weeks ahead of models incorporating historical data. Such findings were mirrored in several other publications, of which we present two for brevity. Achrekar et al. (Achrekar et al., 2011), who found a correlation between the number of flu-related Tweets and reported ILI cases using a autoregressive model with exogenous inputs. Also, Signorini et al. (Signorini et al., 2011) used support-vector regression to make quantitative estimates of ILI values using Twitter feeds, and demonstrated that influenza data gathered from Twitter could accurately track reported disease levels.

Despite the promise shown for mining information from postings on social media, there are also uncertainties in data quality worth consideration, which may include accuracy of data extraction, updating periods of search engines, data authenticity, user motivation, and extent of media coverage. There may also be subconscious bias in search terminology by users due to age, gender and ethnicity, together with under-representation by the elderly with limited access to the internet (Benke, 2017). Failure to adequately address some of these issues may have contributed to the spectacular failure of the Google Flu Trends algorithm to detect the non-seasonal A/H1N1 pandemic in 2009 (Ginsberg et al., 2009; Cook et al., 2011). The Google Flu Trends model–a linear regression model with 45 unique search queries - was updated, but later resulted in overestimation for the 2012–2013 influenza season. As a result, Google removed the Google Flu Trends application from the public domain and it is now accessible only to researchers (Aiello et al., 2020).

To improve confidence of online searches, replication of online Google Trends searches together with spatial stratification of sampling may help to improve results. Results may also be improved by considering different AI approaches, or their combinations, including machine learning, neuro-linguistic programming, and sentiment analysis. AI can also use a *continual learning* approach–where the model continually and autonomously learns from a stream of data (Zenke et al., 2017). For example, machine learning may be used to train on the time-series data documenting the number and location of search queries as a means to estimate weights for a predictive model.

MOBILE PHONES

Within the context of COVID-19, a team at the University of Oxford (Bourne, 2015) suggested epidemic control through mobile phone tracing (Ferretti et al., 2020). Similar systems have been deployed in Asia, in which a phone app allows a central database to collect data on user movement and coronavirus diagnosis. According to the authors, "By keeping a temporary record of proximity events between individuals, it can immediately alert recent close contacts of diagnosed cases and prompt them to self-isolate." The Australian Government (2020) has also released an official Coronavirus App. The app allows users to check their symptoms via a questionnaire (e.g., checking whether patients experience breathlessness or drowsiness), and which contains many more features, such as current status of COVID-19 cases in Australia and registering (Australian Government–Department of Health, 2020).

There are now many countries around the world, including those with very high infection rates, using various methods such as mobile phone apps to track locations of mobile phone users. By providing aggregated data, trends of interests are captured. It would be interesting to conduct further research on how the number of queries on symptoms may change during the actual course of an epidemic.

CONCLUSION

There are very real and effective benefits arising from using AI and big data, together with online resources, such as Google and Twitter. The contribution of AI is that it can detect patterns and possible clusters from analyzing online queries of symptoms, which is much faster than physical testing of citizens. The ability to provide early warning of a new epidemic even by a few days is critical when there is exponential growth involved and this may lead to faster interventions that may save lives. Although online platforms cannot replace physical testing, they can provide timely information to support modeling the spread and potential trajectory of epidemics.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

JA and KKB developed the idea and wrote the manuscript. KKB designed the figures.

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OPINION
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A Care Delivery Model of Temporary Transfer of Medical Workers and Equipment to Confine a Pandemic

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1. INTRODUCTION

Since December 2019, the coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome-coronavirus-2 (SARS-COV-2) virus reached pandemic levels (1). As of March 1, 2020, there are 79,968 confirmed cases in China, and among these confirmed cases, 2,873 deaths occurred (2). Once the COVID-19 develops to critical illness, the mortality rate will rise drastically (3, 4). Among critically ill COVID-19 patients, 63.5% were treated with high-flow nasal cannula though controversial, 71% with mechanical ventilation, 11.5% with prone position ventilation, 11.5% with extracorporeal membrane oxygenation (ECMO), and 17% with continuous Renal Replacement Therapy (CRRT) at Wuhan Jin Yin-tan hospital (Wuhan, China) resulting in an unprecedented surge in critical care resource utilization (4). Similarly, most countries encountered this challenge since the outbreak of COVID-19 throughout the world, resulting in an urgent need of sharing the successful anti-plague strategies between different countries.

2. IMBALANCE OF MEDICAL RESOURCE IN HUNAN

As one of the provinces closest to the Hubei, the epicenter of China, Hunan province has adopted bundled care delivery model strategies to prevent the spread of the COVID-19, including a model of medical team delivery to the sites with increased disease burden due to the regional difference in economic development, number of beds, physicians, nurses, respiratory therapists, and other medical personnel (5, 6). The Chinese hospital network is built on a multi-layered hub-and-spoke model (7, 8). According to the number of beds, departments, medical personnel, equipment, and expertise, hospitals in China are classified as primary, secondary, and tertiary hospitals (9). A primary hospital is a typical township hospital with <100 beds focusing on primary care. Secondary hospitals are located within medium sized cities, counties, or districts and contain between 100 and 500 beds providing comprehensive health services. Subsequently, tertiary hospitals with more than 500 beds provide specialist health services and they serve as medical hubs providing care to multiple regions.

In Hunan, the province next to the COVID-19 epicenter Wuhan, most tertiary hospitals are located in the capital city Changsha. In Changsha, there are nine tertiary hospitals and except Hengyang city, all other cities have one or no tertiary hospitals. The data are showing distribution inequality, especially the access to tertiary care hospitals within the Hunan province (**Table 1**) (10, 11). As a result, many hospitals located in small towns or prefecture-level cities do not have the

TABLE 1 | The number of tertiary hospital, beds, medical workers, physicians, and nurses in each city of Hunan province.

City	Population (10 K)	Confirmed cases	Tertiary hospital	Beds	Medical workers	Physician	nurse	Tertiary hospital/100 k	Bed/100 k
Changsha	815.47	242	9	77,253	81,548	30,793	38,727	0.110	947.3
Xiangtan	286.48	36	1	19,336	19,508	7,530	8,765	0.035	674.9
Zhuzhou	402.08	80	1	26,811	26,616	10,534	12,003	0.025	666.8
Yueyang	579.71	156	1	35,172	31,595	15,187	11,324	0.017	606.7
Changde	582.72	82	1	39,679	35,750	17,387	13,198	0.017	680.9
Chenzhou	474.45	39	1	33,485	29,669	11,693	13,051	0.021	705.7
Hengyang	724.34	48	4	46,537	42,260	17,936	17,352	0.055	642.4
Yiyang	441.38	60	1	28,141	24,964	11,167	9,876	0.023	637.5
Loudi	393.18	76	1	20,138	21,127	9,484	8,133	0.025	512.1
Zhangjiajie	153.79	5	0	10,838	9,140	3,303	3,949	0	704.7
Yongzhou	545.21	44	1	40,794	32,165	13,656	12,908	0.018	748.2
Huaihua	497.96	40	1	35,033	31,258	12,005	13,355	0.020	703.5
Shaoyang	737.05	102	1	43,760	35,224	14,478	14,536	0.014	593.7
Xiangxizhou	264.75	8	0	14,457	16,741	5,817	7,207	0	546.1

infrastructure and staffing conditions to provide high-quality treatment for COVID-19 patients. As such, facing the problem of the regional imbalances of medical resources, most severe patients in primary and secondary hospitals could be transferred to higher-level tertiary hospitals. Along the lines of hub-and-spoke system of any healthcare enterprise, centralized care leads to more efficient utilization of scarce resources such as intensive care and improved clinical outcomes (12). However, the benefits provided by a centralized care model needs to be balanced against the risk of infection transmission due to inter-hospital transfer, delay in access to intensive care, and loss of expertise in peripheral hospitals (12). Especially due to its high transmissibility, inter-hospital transfer strategies are challenging during the COVID-19 pandemic (13, 14). Thus, it would be reasonable to reduce the number of transfer processes once a COVID-19 is identified.

3. ANTI-COVID STRATEGY ADOPTED IN HUNAN

Aligning with the strategy of reducing the transfer of COVID-19 patients, Hunan Health Commission formulated a strategy to facilitate the treatment of COVID-19 patients at the prefecture-level cities instead of transfers. This is learned from the valuable experience of the fight against SARS virus in 2003. At that time, China also set up dedicated doctors and nurses led by experienced respiratory and infectious disease experts in various provinces to provide special care for all patients admitted to designated wards (15). Hunan Province has carried out very strict quarantine inspection (16) and isolation measures. Through timely identification, isolation, and active and effective treatment, the epidemic situation can be controlled. Until the end of the SARS epidemic, there were only seven SARS patients in Hunan Province, and all of them were cured. During the current pandemic, improvements were made on the basis of the previous experiences. The core idea of this strategy was to assign a medical "anti-COVID team" to each city of Hunan, resulting in a total of 14 teams being deployed. Each "anti-COVID team" consisted of experts from the critical care medicine, pulmonary and critical care medicine, infectious diseases, and emergency medicine departments from the tertiary hospitals of Changsha, the capital city of Hunan. Besides the "anti-COVID team" for each city, there are two additional teams "airway management team" and "ECMO team" shared by all cities in Hunan. The "ECMO team" experts are in charge of ECMO management. The "airway management team" was established with four respiratory therapists (RT) experienced in the management of emergency airways. Once there is an urgent need for RT or ECMO operations in a city, members of these two teams will be mobilized and transferred to the city to aid the treatment of COVID-19 patients. The urgent need is notified by the "anti-COVID" team dispatched to the designated hospital and then transferred to Hunan Health Commission that is responsible for deploying the members of "airway management team" and "ECMO team." For example, Loudi is a prefecture-level city of Hunan and has a population of 3.87 million. As of March 11, 2020, the Center Hospital of Loudi, the only designated hospital to treat COVID-19 patients in Loudi, have received 76 confirmed cases, including five severe illness and two critically ill patients (17). Those two critically ill patients needed invasive mechanical ventilation, prone position and ECMO. However, in Hunan province, only four tertiary hospitals, namely the Xiangya hospital of Central South University, the second Xiangya hospital of Central South University, the third Xiangya hospital of Central South University, and Hunan Provincial People's Hospital, have professional RTs. As a result, physicians or nurses mainly perform mechanical ventilation in the Center Hospital of Loudi without training in respiratory care or mechanical ventilation, which brings many challenges to the treatment of the patients. Even worse, the Center Hospital of Loudi does not have completed isolated rooms. Patients of Covid-19 are separately isolated in temporary rooms of 10 m^2. This hospital is also confronted with problems of lacking medical equipment (e.g., high-flow

nasal oxygen and ECMO). Thus, in order to provide high-quality treatment for two critically ill patients in Loudi, besides the "anti-COVID team," the Hunan Health Commission further mobilized an RT from the "airway management team" and two ECMO experts from the "ECMO team" to Loudi. In addition, the transferred medical personnel are also responsible for training the physicians and nurses, who work in the designated hospital with diagnosis, treatment, respiratory care, and ECMO. With the efforts of transferred and local medical personnel, there was no death case in Loudi city caused by COVID-19 disease as of December 18, 2020. The mortality rate of COVID-19 cases in Hunan Province was <0.4%, while the overall mortality rate of COVID-19 patients in all provinces except Hubei was about 0.6%. In Hubei Province, as the first place of the epidemic in China, the total mortality rate of COVID-19 confirmed patients in Hubei Province reached 6.6% due to the darkest time in the early days of the epidemic.

The case of assigning Loudi with "anti-COVID team" from provincial capital was not specific to Hunan, but part of China's anti-epidemic strategy. This was a different approach compared to other countries with an emphasis on models, where patients were transferred to expert centers (18). The "peripheralized care deliver model" can help underdeveloped regions to access the rich medical resources of developed regions within a country.

4. DISCUSSION

While the care delivery model mentioned above is exciting and now tested during a pandemic, there were also many problems. First of all, this model would not have worked if the tertiary hospitals were overwhelmed with a surge of patients preventing sharing the experts to peripheral hospitals. This model also unmasked the scarcity of limited qualified personnel. Interestingly, there are approximately 140 RTs participating in the "anti-COVID teams" across the China, which highlights the need to train personnel, who can manage ventilators. Respiratory therapy has become a formal career in China only in 2019 and there are no official certification or license for RTs at this moment (19). In addition, only one university has provided degree in respiratory therapy. There are a total of 259 students in respiratory therapy, who graduated from the university since 2004. Unexpectedly, 31% of these graduates engaged in other jobs rather than respiratory therapy (20, 21). Besides this university, there are only two other colleges providing short-term training program for physicians or nurses. As a result, almost all primary and secondary hospitals and even some tertiary hospitals do not have professional RT. The tasks of RTs in these hospitals are mainly conducted by physicians and nurses highlighting the need for increased efforts devoted to the education of respiratory therapy in China. In addition, the success of the care delivery model presented in this paper benefits a lot from the medical equipment transferring between tertiary hospitals and peripheral hospitals, which could lead to a threat of running out of medical equipment when there are many critical cases. Moreover, the transferring of medical equipment is also harmful to the efficiency of the care delivery model due to the time costs on the disassembling, transporting, installation, and testing. Thus, it is better to add equipment infrastructure in the various peripheral locations, allowing the deployed teams to be immediately operational when another pandemic happened in future.

In summary, the care delivery model in China during the COVID-19 pandemic was creative and successful within that country and its regulations. The transfer of healthcare personnel between cities and even hospital systems would require regulatory adjustments for such a system to be adapted in other countries.

AUTHOR CONTRIBUTIONS

HZ and DY prepared the draft of the commentary under supervision of HL and AK. HL and AK revised the manuscript. MY and LL collected the data used in the paper. All authors contributed to the article and approved the submitted version.

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Organizational Compliance During COVID-19: Investigating the Effects of Anxiety, Productivity, and Individual Risk Factors Among Iranian Healthcare Employees

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This study investigates the impact of anxiety, productivity, and individual characteristics on employee compliance in an Iranian medical science university during the COVID-19 outbreak. The data of 160 healthcare employees of various professions were collected with reliability and validity on the measurements performed. Two regression tests revealed that higher anxiety reduces and higher productivity increased compliance. Participants with higher education and non-medical professions were found to have higher compliance. Productivity was also found to be positively associated with tenure and having a medical position. Implication and limitation are discussed.

Keywords: organizational compliance, anxiety, productivity, COVID-19, regression, Iran

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INTRODUCTION

Coronavirus has already had enormous effects on almost all aspects of human life. The widespread of this virus since the beginning of 2020 has faced many healthcare organizations and systems worldwide with unprecedented pressure to the point of collapse. Therefore, organizational integrity and the elements related to healthcare organizational culture have become even more crucial than before in the global fight against the Coronavirus. Organizational compliance, the level of adherence to the organizational regulations, procedure, and standards (Gershon et al., 1995), is considered an essential organizational culture element (Gershon et al., 1999) with a significant role in maintaining organizational integrity.

Organizational compliance could be affected and hindered by social and organizational crises, which, in turn, can influence organizations' output and functionality. For example, as evident in the case of recent infectious diseases such as SARS and MERS, previous studies showed that medical staff's lack of adherence to treatment protocols contributed to the failure of the fight against pandemics (McCarthy et al., 2016; Smith et al., 2016; Adeniyi et al., 2018). Thus, to develop the current understanding of organizational compliance during crises, this study looks at the effect of two significant constructs of anxiety and productivity on compliance.

Anxiety creates affective and cognitive deterrence and interferes with employee judgment (Barlow, 1991), and causes uncertainty, helplessness, and physiological arousal (Grupe and Nitschke, 2013). Various forms of anxiety are known for their behavioral impacts (Rahmani, 2017). Productivity, perceived as the employees' efficiency (Drewnowski, 2019), affects employee

self-efficacy, the perception of being able to accomplish a specific task (Bandura, 1994). Thus, both anxiety and organizational productivity could affect compliance in organizations. Furthermore, similar to other organizational factors, compliance could be affected by individual characteristics such as gender, tenure, education, and individual characteristics such as smoking, overweighing, and stress (O'Reilly and Chatman, 1986; Gershon et al., 1995).

Contextually, this study focuses on a medical science university in the Markazi province of Iran. As one of the worse hit countries by the pandemic, Iran is a significant case study that could contribute to our understanding of the global challenges induced by the Covid-19. Previous studies showed that responding to the pandemic, Iranians showed a higher level of anxiety than the Chinese (Jahanshahi et al., 2020). Furthermore, the level of anxiety among the Iranian general public is directly associated with infection levels in the regions and provinces (Moghanibashi-Mansourieh, 2020). While almost the entire country was heavily affected by the disease, this study investigates a healthcare organization in Markazi province, one of the first epicenters of the diseases in Iran (Arab-Mazar et al., 2020).

Furthermore, as the healthcare staff's efficiency proved to be distinctive in reducing the disease toll across the globe, an organizational approach to the pandemic's consequences is of eminent significance. According to the World Health Organization, about 20–60% of staff and medical staff are infected with the virus; for example, in Italy, 20% of responding healthcare employees were infected (Lancet, 2020). Recent research showed that a sizeable portion of healthcare staff in Iran needs emotional and psychological support due to anxiety and distress resulting from Covid-19 (Zhang et al., 2020). Thus, it is crucial to investigate the impact of anxiety on various aspects of organizational culture and performance in an Iranian healthcare organization.

As one of the most widely used and cited health promotion models, Precede-Proceed provides concrete steps to facilitate individual health behavior changes. Essentially, the Precede-Proceed model posits that health behavior not only is driven by knowledge, beliefs, and attitudes but also needs to be enabled and reinforced (Green and Kreuter, 1991). The Precede component refers to the diagnostic and assessment phases that aim to identify health problems, existing resources, and risk factors. In contrast, Proceed stages involve intervention implementation and evaluation of the process and health outcomes and their long-term ramifications in the community (Ransdell, 2001). Highlighting people's interactive nature and their environment, the Precede-Proceed model allows for a comprehensive approach to decipher the complexity in individual health behavior and design the most effective intervention programs.

In the past, the Precede-Proceed model has been adopted as a framework to improve heart-healthy behaviors among low-income citizens (Paradis et al., 1995), decrease children injuries (Gielen and McDonald, 1997), increase youth physical activity (Welk, 1999), improve asthma educational programs (Chiang et al., 2004), and so on. In the current study, the Precede-Proceed model serves as the theocratical basis for exploring how risk factors at the individual level influence one's compliance in Iran's unique cultural setting during the COVID-19 pandemic. The study's findings will help advance our knowledge of health professionals' organizational behaviors and provide insight into how we can better protect frontline workers during the time of significant uncertainties.

LITERATURE REVIEW

Organizational Compliance

Within the broader organizational context, compliance is defined as "a state of accordance between an actor's behaviour or products on the one side, and predefined explicit rules, procedures, conventions, standards, guidelines, principles, legislation or other norms on the other" (Foorthuis and Bos, 2011, p. 261). Organizational compliance can be reflective, i.e. self-conscious, or non-reflective, i.e. non-self-conscious (Paulsen, 2016). Compliance, or interchangeably adherence, within the healthcare context, is defined as doing or avoiding behaviors as recommended by healthcare professionals in individual (e.g. doctors) or general (e.g. public service announcement) levels to improve the quality and status of one's health condition (Martin, 2014). Information, motivation, and strategy are the necessary elements of successful compliance (DiMatteo et al., 2012).

Employees' compliance with organizational rules and regulations is perceived to be impacted by and, at the same time, an indicator of organizational culture (Hu et al., 2012). Investigation of compliance within healthcare organizations showed that strong and effective leadership is an essential element in healthcare professionals' hygiene compliance during the spread of infectious diseases (De Bono et al., 2014). Emphasizing and monitoring compliance via ethical programs lead to the lower cases of unethical conduct, increased ethical advice, seeking and ethical awareness in the organizations (Weaver and Treviño, 1999).

Previous studies of compliance have studied patients' compliance and adherence. Such studies, for example, showed that poor compliance to medical care increases the risk of health outcomes (Karvinen et al., 2013), and compliance among cancer patients could be disrupted due to the psychological or behavioral effects of the treatment procedure (Andersen et al., 1994).

Previous research on healthcare employees' compliance investigated the precedents and outcomes of compliance in the healthcare facilities. Carthey et al. (2011) indicated that information overload causes non-compliance in the United Kingdom's National Health Service (NHS). Flodgren et al. (2019) concluded that the local opinion leaders' interventions, alone or in combination with other interventions, can increase compliance among healthcare professionals. Another study showed that organizational feedback and personalized action plan could increase hand-hygiene compliance (Fuller et al., 2012). Increased compliance with antidepressant therapy is correlated with reduced absenteeism costs among healthcare employees (Birnbaum et al., 2010).

Anxiety

Anxiety occurs when a person's stressful life conditions become too long or frequent. If the body's nervous system fails to endure the stress resistance phase and the body remains under pressure for a long time, it will be worn out and become vulnerable to physical and mental illness, such as anxiety (Barlow, 1991). The causes of anxiety are divided into four categories (Barlow, 1991): first, biological and physical factors such as the levels of some hormones in the blood; second, genetic and hereditary factors; third, morbid anxiety, the tendency of the nervous system to decrease the amount of excitement of condensed instincts; and fourth, environmental and social factors such as family problems, feelings of separation and rejection, or sudden and unexpected changes, such as earthquakes, illness, and death of a loved one. Anxiety and worrying can cause physical symptoms such as palpitations, shortness of breath, tremors, sweating, feeling tightness and muscle tension, decreased concentration, and insomnia.

Previous studies of anxiety and stress concerning organizational compliance showed that the higher levels of compliance with medical care indicated less stress measured by heart rate variability (Karvinen et al., 2013). Gershon et al. (1999) indicated that anxiety at the workplace could reduce organizational compliance. Healthcare employees are high-risk groups during the prevalence of infectious diseases and are more prone to mental disorders such as anxiety due to high awareness, changing working hours, insufficient rest, and other factors (Ciorlia and Zanetta, 2005; Huttunen and Syrjänen, 2014). Covid-19 is a pandemic with an unprecedented impact on healthcare organizations, which has increased anxiety and distress among healthcare staff (Zhang et al., 2020). The following hypothesis is proposed to investigate the possible effect of anxiety on healthcare staff's compliance:

H1. The higher level of anxiety reduces organizational compliance of healthcare employees

Productivity

Workplace productivity is defined in terms of absenteeism i.e. taking workdays off, and presenteeism i.e. reduced performance, measured using work disability, work loss, and work limitation (Drewnowski, 2019). Lofland et al. (2004) added compensation to absenteeism and presenteeism to measure productivity. Two approaches to human capital and friction cost are used to measure the components of productivity. In the first method, monetary value is assigned to lost productivity, while the second approach associates frequency, length, and cost of friction with the friction period (Lofland et al., 2004). Moon et al. (2012) proposed that organizational productivity, synonymous with efficiency, could be measured by the overall output of a specific number of employees based on a fixed amount of input.

Previous organizational healthcare research has generally approached productivity as an outcome of the research (Riedel et al., 2001). For example, employees with a higher Body Mass Index (BMI) and employees with sleep disturbance were less productive (Gates et al., 2008; Rosekind et al., 2010). Riedel et al. (2001) proposed a healthcare organizational productivity model to increase productivity and reduce costs. In this model, disease prevention, health promotion, acute and chronic illness management, environmental health and safety, and health corporate culture are the model's antecedents. The antecedents' impact is mediated by reduced absenteeism, improved performance, creativity and motivation, reduced accident and saving costs, and reduced healthcare cost.

While the possible effect of productivity on compliance with workplace precautions is understudied, some previous research included productivity and compliance as study variables. A study of medical adherence to workplace productivity among Asthma patients did not reveal a significant impact on the total health-related quality of life (Joshi et al., 2006). A study of the association between preventive service compliance, productivity, and employee dental claim showed that those with preventive dental claims showed higher compliance and productivity; however, the study did not directly link productivity and compliance (Burton et al., 2017).

Previous organizational compliance studies confirmed the impact of cognitive processes on employees' compliance with organizational regulation (Carthey et al., 2011; Flodgren et al., 2019). Perception of self-productivity in the organization could impact one's role in the organization and how one is competent in the organizational culture. As compliance is a manifestation of organizational culture (Hu et al., 2012), we propose the following hypothesis to investigate the impact of productivity on organizational compliance:

H2: Productivity increases organizational compliance.

Individual (Risk) Factors

Previous studies showed that individual factors such as gender, tenure, profession, and education could be correlated with compliance (Gershon et al., 1995). Gershon et al. (1995) found a difference in the compliance level based on profession and education, but they did not find a difference based on gender and tenure in their United States healthcare employee sample. As culturally, Iran is considered to be higher in masculinity and power difference than the United States (Farzianpour et al., 2016), it is possible that gender and tenure also affect compliance among the Iranian healthcare employees. Masculine cultures strive to distinguish between how men and women are expected to think and behave, and power difference refers to the accepted and expected inequality in power distribution (Liu et al., 2019). Moreover, in another study, tenure was found to negatively correlate with compliance, which shows that while newer employees develop their relationship with their organization based on compliance, over time, internalization of organization goals and values and pride in affiliation may develop instead (O'Reilly and Chatman, 1986). Along with personal characteristics, individual risk factors such as smoking, experiencing a high level of stress, overweighing, and being at risk at the workplace, (e.g. having direct contact with

COVID-19 patients) can affect compliance with organizational regulations in healthcare services (Gershon et al., 1995; Gershon et al., 1999). The following hypotheses are proposed to investigate these possibilities:

H3: Organizational compliance level differs based on personal characteristics (including gender, tenure, profession, and education).

H4: Organizational compliance level differs based on individual risk factors (including smoking, taking stress medicine, weight, and direct contact with COVID-19 patients).

In addition to compliance, productivity could also be associated with the individual (risk) factors. Previous studies showed personal risk factors, such as smoking and overweight, cause illnesses, increase absenteeism, and reduce productivity (Riedel et al., 2001). Regarding tenure, previous research showed contradictory findings. While more tenured staff are more likely to control their negative emotions and engage in less counterproductive behaviors, it is also possible that the power and influence related to their long tenure lessen their conformity at work (Ng and Feldman, 2010). Thus, the following research questions are presented to understand the possible impact of personal factors on organizational productivity:

RQ3: Do personal characteristics (including gender, tenure, profession, and education) impact organizational productivity?

RQ4: Do individual risk factors (including smoking, taking stress medicine, weight, and direct contact with COVID-19 patients) impact organizational productivity?

Figure 1 illustrates the relational study model.

METHODS

Analytic Strategy

Firstly, the scales' construct validity was established by examining their factor structure using confirmatory factor analysis (CFA). We relied on several fit indices to ascertain the model fit, including the comparative fit index (CFI), Tucker Lewis index (TLI), the root mean square error of approximation (RMSEA), and the standardized root mean square residual (SRMR). "Good fit," in terms of CFI and TLI values means greater than 0.90, while RMSEA and SRMR should ideally be less than 0.08 (Hu and Bentler, 1999). Convergent validity of the constructs was measured using Average Variance Extracted (AVE), which should be higher than 0.5 (Hair et al., 2014). Also, two hierarchical multiple regressions were conducted with compliance and productivity as the dependent variables. For compliance, demographic variables, including income, gender, tenure, and education, were entered at stage one of the regression to control for background characteristics. Dummy variables are used to measure the categorical variables' effect with more than two categories, and different sets of dummy variables should be added to the regression models at different stages (Field, 2009). Thus, the dummy variables related to profession were added at the second level. The individual risk factors, including taking stress medication, direct contact with COVID-19 patients, BMI, and smoking habits, were added to the third model. Finally, model four was formed using anxiety and productivity. The first three models were replicated for productivity. Data management and statistical analysis were performed using SPSS version 25.0 (SPSS Inc., Chicago, Illinois).

Participants

Participants were 160 employees of a medical university in the city of Arak in central Iran, one of the country's initial epicenters of COVID-19. The university consists of different medical and

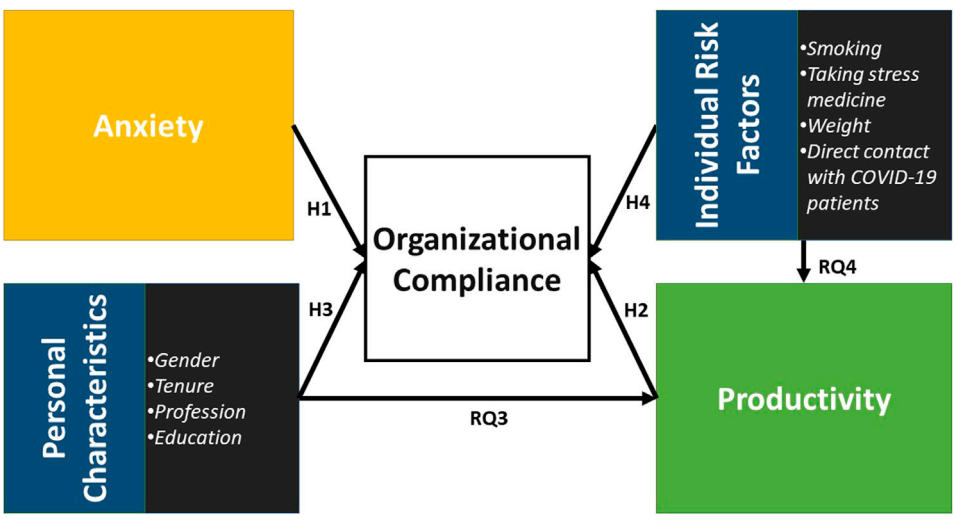

FIGURE 1 | The study model.

TABLE 1 | Participant demographics.

Variable	*n*	%
Gender		
Female	88	55
Male	72	45
Age		
20–29	19	11.9
30–39	76	47.5
40–49	54	33.8
50–59	11	6.9
Profession		
University teaching staff	22	13.8
Healthcare professionals	101	63.1
Laboratory professionals	11	6.9
Administrators	26	16.3
Education		
No university degree	19	11.9
Undergraduate university degree	74	46.3
Postgraduate university degree	67	41.9
Smoking habit		
Smoker	41	25.6
Quitter	25	15.6
Non-smoker	94	58.8
Monthly income		
10 M[a] thru 19.9 M	1	0.6
20 M thru 29.9 M	13	8.1
30 M thru 39.9 M	17	10.6
40 M thru 49.9 M	35	21.9
50 M thru 99.9 M	43	26.9
100 M thru 199.9 M	18	11.3
200 M thru higher	33	20.6
Taking stress medication		
Yes	20	12.5
No	140	87.5
Direct contact with COVID-19 patients		
Yes	79	49.4
No	81	50.6

Note: [a]million iranian rials.

educational sections and is responsible for planning and implementing medical services in the region. Following the ethic committee approval by the university (research ethic code: IR.ARAKMU.REC.1398.330), this study used self-report questionnaires to collect participants' gender (male or female), age, profession (the type of occupation at the university), education (the last achieved educational degree), financial condition (monthly income in Iranian rial) and individual risk factors (i.e. smoking habit, Body Mass Index (BMI), taking stress medication and direct contact with the COVID-19 patients). The questionnaire was uploaded to Google Forms, and the link to the survey was distributed using organizational email lists. To ensure the validity of translation, the Farsi draft of the questionnaire items was translated back to English by two different translators and compared with the original English items. The information on the participants is presented in **Table 1**.

Measures

Anxiety

Anxiety was measured by six descriptive symptoms of anxiety (e.g., "unable to relax") derived from the scale developed and validated by Beck et al. (1988). All items were scored on a seven-point scale, ranging from '1 = have not had at all' to '7 = Have had too much to bear'. The Cronbach's alphas for this scale was 0.89. Also, CFA results revealed that despite the slightly high RMSEA, the six-item model showed a satisfactory model fit (chi^2 *(9)* = 21.2; CFI = 0.97; TLI = 0.96; RMSEA = 0.09; SRMR = 0.02), indicating the acceptable construct validity of the scale. AVE for anxiety was 0.6, which reflects the convergent validity of this construct.

Compliance

Compliance was measured by eight items (e.g., "wear disposable gloves whenever there is a possibility of exposure to blood or other body fluids") derived from the scale developed and validated by (Gershon et al., 1999). All items were scored on a range of 0–100, where 0 means never and 100 means always. The Cronbach's alphas for this scale was 0.96. Also, CFA results revealed that despite the slightly high RMSEA, the eight-item model showed a satisfactory model fit (chi^2 (20) = 33.36; CFI = 0.99; TLI = 0.99; RMSEA = 0.07; SRMR = 0.02), indicating the acceptable construct validity of the scale. AVE for anxiety was 0.77, which reflects the convergent validity of this construct.

Productivity

Productivity was measured using a question asking for the perceived change in the level of efficiency due to the COVID-19 based on a scale ranging from 1 = has become much less to 7 = has become much more. Means, SD, reliability, and correlations of the constructs are presented in **Table 2**.

RESULTS

The hierarchical multiple regression with compliance as the dependent variable was formed (**Table 3**) and revealed that at stage one, income, gender, tenure, and dummy variables contributed significantly to the regression model, [F (5, 154) = 7.52, $p < 0.001$] and accounted for 17% of the variation in "compliance". Introducing the dummy variable of profession to the second model improved it significantly: [F (5, 154) = 6.40, $p < 0.001$] and accounted for 25% of the variation in "compliance". This change in R^2 was significant, [F (3, 151) = 3.83, $p < 0.05$]. Introducing the BMI, smoking habit, dummy variables for stress medication and COVID-contact variables explained an additional 4.3% of the variation in compliance, but this change in R^2 was not significant, [F (5, 146) = 1.80, p = 0.117]. Adding stress and productivity to the regression model explained an additional 18.9% of the variation in compliance, and

TABLE 2 | Means, standard deviation, reliability coefficients, and correlations.

Variable	M	SD	α	(1)	(2)	(3)	(4)
(1) body mass index	25.47	3.29	—	—			
(2) productivity	3.56	1.64	—	–0.14	—		
(3) anxiety	20.24	8.18	0.90	0.11	–0.01	—	
(4) compliance	0.50	0.28	0.96	–0.09	0.17*	–0.43**	—

*Note: *p < 0.05, **p < 0.01.*

TABLE 3 | Regression Model for compliance.

Regressor	Model 1	Model 2	Model 3	Model 4
Intercept	0.22	0.51	0.89	0.82
Income	–0.30**	–0.14	–0.18	–0.16
Gender	–0.10	–0.10	–0.04	–0.14
Tenure	0.08	0.06	0.09	–0.08
Undergraduate university vs no university degree	0.48***	0.43***	0.42***	0.41***
Undergraduate university vs postgraduate university	–0.21*	–0.06	–0.04	–0.03
Healthcare professionals vs teaching staff		–0.23**	–0.29***	–0.28***
Healthcare professionals vs lab technicians		0.05	–0.04	0.03
Healthcare professionals vs administrators		–0.16*	–0.22*	–0.21**
Taking stress medication			0.14	0.08
Direct contact with Covid patients			–0.17*	–0.07
Body Mass index			–0.01	0.05
Smokers vs quitters			0.02	–0.02
Smokers vs non-smokers			-0.15	–0.03
Anxiety				–0.41***
Productivity				0.26***
F	7.52***	6.40***	4.73***	9.05***
ΔF		0.06*	0.04	0.19***
R^2	0.2	0.25	0.3	0.49
R^2_{adj}	0.17	0.21	0.23	0.43

Note: *p < *0.05*, **p < *.01*, ***p = < *0.001*.

TABLE 4 | Regression model for productivity.

Regressor	Model 1	Model 2	Model 3
Intercept	0.54	–2.35	7.23**
Income	0.26*	0.05	–0.07
Gender	–0.01	0.00	0.07
Tenure	0.09	0.12	0.16*
Undergraduate university vs high school	0.01	0.09	0.06
Undergraduate university vs postgraduate university	0.25*	0.03	0.01
Healthcare professionals vs teaching staff		0.36***	0.24***
Healthcare professionals vs lab technicians		–0.03	–0.26***
Healthcare professionals vs administration		0.18*	-0.01
Taking stress medication			0.06
Direct contact with Covid patients			0.55***
Body mass index			0.01
Smokers vs quitters			–0.04
Smokers vs non-smokers			–0.24*
F	1.65	3.67***	6.89***
ΔF		6.71***	10.26***
R^2	0.05	0.16	0.38
R^2_{adj}	0.02	0.19	0.33

Note: *p < *0.05*, **p < *0.01*, ***p =< *0.001*.

this change in R^2 was significant [F (2, 144) = 26.39, p < 0.001]. Thus, this model was retained for analysis purposes. When all 15 independent variables were included in stage three of the regression model, only stress, performance, education, and job were significant predictors of compliance. Together, the 15 independent variables accounted for 49% of the variance in compliance.

Furthermore, a hierarchical multiple regression with productivity as the dependent variable was formed (**Table 4**), and showed that at stage one, income, gender, tenure, and dummy variables for education, did not contribute significantly to the regression model, [F (5, 154) = 1.65, p = 0.149]. Introducing the dummy variable of profession to the second model improved it significantly: [F (8, 151) = 3.67, p < 0.001] and accounted for 11.8% of the variation in productivity. This change in R^2 was significant, [F (3, 151) = 6.71, p < 0.001]. Introducing the BMI, dummy variables for smoking condition, taking stress medication, and COVID-contact variables explained an additional 21.8% of the variation in normal compliance, and this change in R^2 was significant [F (5, 146) = 10.25, <0.001]. Thus, this model was retained for analysis purposes. When all 13 independent variables were included in stage three of the regression model, only tenure, profession, performance, education, and job were significant predictors of compliance. Together, the 13 independent variables accounted for 38% of the variance in compliance.

DISCUSSION

This study investigated the effect of anxiety and productivity on organizational compliance of a medical science university in Iran during the outbreak of the COVID-19 infectious disease. Anxiety and stress are often experienced simultaneously, especially when facing significant uncertainties in life. When exposed to chronic stressors, individuals tend to engage in detrimental health behaviors such as drinking, smoking, binge eating, etc. (Rosenbaum and White, 2015). While previous studies have mostly focused on the impacts of anxiety and stress on individual health outcomes, the current study explored how anxiety and stress are linked to compliance behaviors. The findings of the study suggest that increased stress and anxiety led to fewer compliance behaviors. During COVID19, health compliance behaviors are unprecedentedly important as affected individuals can appear asymptomatic and still transmit the virus to others (Holshue et al., 2020). Whether or not to comply with

the medical instructions is rooted in one's moral reasoning and psychological state (Harper et al., 2020). Our study has confirmed that a poor psychological state can result in less health compliance behaviors, which in turn leads to decreased health outcomes in the community. This finding is in line with previous studies showing stress and anxiety deteriorate compliance (Gershon et al., 1999; Karvinen et al., 2013). The fear of COVID19 is unique as the virus can swiftly transmit across large populations. While fear may encourage a range of risk-reducing behaviors, it may also instill various negative emotions in people that lead to impaired health decisions (Harper et al., 2020). As evidenced in this study that stress leads to compromising compliance, government and health organizations are warranted to engage in effective communication that helps mitigate uncertainty and stress amidst the general population. The findings of this study contribute to our understanding of the Precede-Proceed model by focusing on health compliance, an individual health behavior that also has fatal consequences on others. As the world is becoming increasingly interdependent, future health studies/models need to pay more attention to behaviors and decision-making at a community-level.

This study also found that people with higher education backgrounds are more likely to comply with instructions. This positive relationship between education and health compliance is consistent with previous findings. Education is positively associated with the perception of self-efficacy and perceived benefits of health-promoting behavior, and those with higher education are more likely to comply with medical instructions (Garcia-Pena et al., 2001; Hacihasanoğlu and Gözüm, 2011). Future studies are warranted to continue control for education when studying individual health behaviors.

Furthermore, this study showed a significant difference in compliance among the employees of the various organizational sections. Medical science university teachers and administrators showed a higher level of compliance. Previous studies also showed that profession could affect compliance (Gershon et al., 1999). The effect of profession could be related to the more stressful nature of tasks among the healthcare professionals and lab technicians who are more involved in diagnosing and treating the patients compared to the policymaking, educational, and supporting role of the other employees in the healthcare organizations.

The positive effect of productivity on compliance is also a significant finding of this study. Majorly, the previous organizational studies have approached productivity as an outcome, while this research showed that once employees perceive themselves as more efficient at work, they are more likely to comply with the organizational regulations. Previous research showed that productivity is positively associated with participative activity in organization (Rosenberg and Rosenstein, 1980). Also, productivity is positively associated with different forms of psychological and mental abilities such as organizational involvement, organizational commitment, and perceived self-efficacy (Bandura, 2000; Eastin and LaRose, 2000; Wolf and Zwick, 2008; Phipps et al., 2013). Organizational commitment could be related to the psychological attachment with the organization to be more productive and reduce the intention to leave. Previous studies also indicated the association of compliance and increasing organizational commitment (Fritz, Arnett, and Conkel, 1999). The impact of productivity on compliance is related to the satisfaction employees perceive due to their work efficiency, increasing their attachment to the organization, and boosting their compliance. Especially, being efficient during the Covid-19 crisis entails both organizational and social satisfaction. During the crisis, healthcare employees worldwide were greatly appreciated for their extraordinary commitment and service. The feeling of being useful in battling the COVID-19 could have resulted in a higher self-efficacy and commitment, and compliance. This finding is in line with the previous study that indicated motivation as a necessary element of compliance (DiMatteo et al., 2012).

Further related to productivity, the study showed that healthcare professionals and lab technicians are more productive than the university teaching staff and administrators. Furthermore, the study showed that direct contact with COVID-19 patients increases workplace productivity. Both healthcare professionals (i.e. doctors and nurses) and lab technicians are highly involved with the COVID-19 patients, and their task entails an excessive level of care and commitment, associated with higher productivity, as mentioned before. Interestingly, the healthcare professional showed higher productivity and lowered compliance, while the total sample showed that productivity increased compliance. One explanation is that although working in a crisis circumstance has entailed higher workplace efficiency, healthcare professionals used more non-complying ways to go around the organizational regulations and accomplish their tasks more efficiently. Future studies should investigate the relationship between compliance and creativity during crises.

Furthermore, the study showed that tenure increases productivity among employees. The longer-tenured employees may have a better perception of their tasks to deal with the crisis. However, it is also essential to consider the effect of age. Further research is needed to investigate the independent effect of tenure on productivity. The study's finding concerning tenure is in line with previous research showing tenure increased creativity and in-role performance and decreased self-rated counterproductive behavior in the organizations (Ng and Feldman, 2010).

Limitation

The results of this study should be interpreted in light of some considerations. First, the possibility of self-bias in the report of compliance is a common limitation in the self-report measurement of compliance studies (De Bono et al., 2014). The same consideration is relevant to how people assess their productivity in the workplace. Second, despite the efforts, the respondent participation and small sample size impose restrictions on the generalizability of the finding. Further studies with higher sample sizes are warranted to measure the effect of various constructs on organizational compliance in

healthcare organizations. Especially due to the cultural aspects of compliance, investigating this construct in diverse cultural contexts is crucial. Third, the disproportional sizes of some variables could have possibly affected some results.

CONCLUSION

The purpose of this study was to investigate the effect of anxiety, productivity, and various individual factors on healthcare organization employee compliance with organizational regulations during the COVID-19 outbreak. A significant theoretical contribution of this study is the sizable impact of anxiety on organizational compliance. This finding indicates the significance of caring for the employees' mental health, especially during crises. The study also contributed to the current knowledge by showing that working under pressure and higher perceived risks can increase organizational productivity, which could lead to higher compliance, but the deteriorating effect of anxiety on compliance is much higher. The study also showed that education has a crucial role in increasing organizational compliance, and investing in employee education can benefit organizations by increasing employee compliance.

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 Dr Mohammad Jamalian, director of research ethics committee, Arak University of Medical Science . The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FV: Data Collection, Introduction (Anxiety) CZ: Introduction, Data analysis, Discussion, Theoretical framework AG: Method, Data analysis. proof-reading DR: Data collection, Introduction, compliance, productivity, individual risk factors, analysis, discussion, theoretical and method design.

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REVIEW
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Immunopathological Changes in SARS-CoV-2 Critical and Non-critical Pneumonia Patients: A Systematic Review to Determine the Cause of Co-infection

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The ongoing COVID-19 pandemic originating from Wuhan, China is causing major fatalities across the world. Viral pneumonia is commonly observed in COVID-19 pandemic. The number of deaths caused by viral pneumonia is mainly due to secondary bacterial or fungal infection. The immunopathology of SARS-CoV-2 viral pneumonia is poorly understood with reference to human clinical data collected from patients infected by virus and secondary bacterial or fungal infection occurring simultaneously. The co-infection inside the lungs caused by pneumonia has direct impact on the changing lymphocyte and neutrophil counts. Understanding the attribution of these two immunological cells triggered by cytokines level change is of great importance to identify the progression of pneumonia from non-severe to severe state in hospitalized patients. This review elaborates the cytokines imbalance observed in SARS-CoV-1 (2003 epidemic), SARS-CoV-2 (2019 pandemic) viral pneumonia and community acquired pneumonia (CAP), respectively, in patients to determine the potential reason of co-infection. In this review the epidemiology, virology, clinical symptoms, and immunopathology of SARS-CoV-2 pneumonia are narrated. The immune activation during SARS-CoV-1 pneumonia, bacterial, and fungal pneumonia is discussed. Here it is further analyzed with the available literatures to predict the potential internal medicines, prognosis and monitoring suggesting better treatment strategy for SARS-CoV-2 pneumonia patients.

Keywords: SARS-CoV-2, COVID-19, cytokines, lymphocyte, neutrophil, viral pneumonia, interleukins, interferon

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INTRODUCTION

The pandemic of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is currently causing a huge number of deaths globally, identified on 1st December 2019 in Wuhan, China (1). The SARS-CoV-2 emerges from Riboviria realm belonging to the coronaviridae family under the genus betacoronavirus responsible for global deaths causing SARI (severe acute respiratory infection) in humans (2). The SARS-CoV-2 infection can rapidly infect immunosuppressed patients and aging individuals were considered to be at higher risk of acquiring coronavirus disease-19 (COVID-19) (3). The entry of this virus is responsible for causing secondary bacterial or

fungal co-infection increasing the criticality in patients (4). On 11th February 2020 World Health Organization (WHO) named the disease caused by coronavirus as COVID-19 and changed the virus name from 2019-nCoV to SARS-CoV-2 due to its high similarity with epidemic SARS (SARS-CoV-1) coronavirus (5). Presently COVID-19 has spread across all the continents except Antarctica (6).

The transmission of coronavirus across the species is possible through antigenic shift (7). Where possibly two viral antigenic genes recombined within the host cell gives rise to a new epidemic virus, previously observed during influenza virus pandemic (8). A study conducted in India in collaboration with China (Wuhan institute of virology), USA and Singapore (published in August 2019) suggested that bats are natural reservoir for several viral species including Ebola and Marburg virus (9). Coronavirus similar to influenza virus causes upper and lower respiratory tract infection (LRTI), it gradually in association with bacterial superinfection results in severe lower respiratory tract infection giving rise to fatal condition (10). SARS-CoV-2 is responsible for upper and LRTI causing severe acute respiratory infection (SARI) (3). Similar to SARS-CoV-1, it leads to acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) further progresses to sepsis or myocardial injury resulting in death of the patient (11, 12). Apart from respiratory failure and sepsis as major cause of raising death toll, myocarditis, acute myocardial injury, ischemic stroke related deaths and multiple organ failure like acute kidney injury are being seen in COVID-19 patients (13–15).

SCOPE AND FOCUS

The immune activation during pneumonia caused by fungi or bacteria or co-infection seen in viral pneumonia patients is highlighted here. The main purpose of this review article is to identify the possible cause of secondary pneumonia infection observed in COVID-19 patients. In this review the overall immunopathological changes and immune imbalance observed previously during SARS-CoV-1 pneumonia epidemic, presently in SARS-CoV-2 pneumonia pandemic, moderate and severe infection, respectively, are discussed in detailed manner to reveal the potential cause of superinfection. Beside that immune activation during community acquired pneumonia (CAP) caused by fungi or bacteria or co-infection seen in SARS-CoV-2 virally challenged patients are highlighted. Although there is no major difference in terms of cytokine storm observed in SARS-CoV-1 and SARS-CoV-2 patients however to understand if dysbiosis causes superinfection is reviewed. This review would be helpful in distinguishing the role of anti-inflammatory drugs during the progression of moderate to severe form of SARS-CoV-2 pneumonia caused by cytokine storm.

At present there are several scientific questions arising from the existing pandemic situation. The reason behind children and women are less fatally challenged by COVID-19 than the aged males. The potential reason why majority of patients survive COVID-19 disease without any complications while others struggle or die irrespective of known comorbidities is reviewed here. This article would further help to design a better clinical investigational plan for studying and monitoring viral pneumonia in patients. Here the significance of reporting the basic cytokines level in serum or bronchoalveolar lavage (BAL) fluids of SARS-CoV-2 pneumonia patients is briefly discussed. Apart from that epidemiology, virology, clinical symptoms, prognosis, therapeutic interventions and potential limitations are discussed in this detail review article along with the immunopathology and cytokine imbalance observed in SARS-CoV-2 severe and non-severe pneumonia patients.

METHODOLOGY

To find the potential cause of pneumonia in SARS-CoV-2 infected patients, systematic review of literature was performed and the procedure of Ahmed et al. was followed (16). Using the terms "cytokine level in SARS patients" and "2019-nCoV" pubmed was searched on or before 14th February 2020, results obtained as 56 and 1,257 published articles respectively. All the research articles reported on SARS-CoV-1 and SARS-CoV-2 patients published between 1st January 2003 till 14th February 2020 and 1st December 2019 till 14th February 2020, respectively, were reviewed. Duplicate articles and literatures other than English language were excluded. The articles reported on virology immunopathological changes, symptoms and cytokine profiles in COVID-19 patients were included. After following the inclusion and exclusion criteria 22 studies on SARS-CoV-1 and 45 research articles on SARS-CoV-2 were selected and saved for reading. The studies emphasizing on cytokines levels of either interleukin-1beta (IL-1β) or interferon-gamma (IFN-γ) or both in SARS-CoV-1 and SARS-CoV-2 infected Chinese patients was the inclusion criteria and selected in **Table 2**. The reason for choosing this criterion is discussed in paragraph 12 & 15. Upon implementation of the inclusion criteria, 9 papers on SARS-CoV-1 were selected for preparation of **Table 2**. Similarly, 12 research articles on immunopathological changes and cytokine profiles of COVID-19 patients were presented in **Table 1**. Among those two articles were utilized in **Table 2** to describe cytokine imbalance of IL-1β/IFN-γ. One research article related to cytokine storm in COVID-19 patients published in April 2020 was added in **Tables 1**, **2** as its findings demonstrated cytokine imbalance of IL-1β and IFN-γ (23). To ensure that updated information on CAP, SARS-CoV-1, and COVID-19 are reviewed here google authors, pubmed, WHO, and center for disease control and prevention (CDC), respectively, were searched with appropriate terms or questions during manuscript preparation. Methodology is described with a flow chart in **Figure 1**.

EPIDEMIOLOGY

Particularly while discussing about viral pneumonia neither the death rate of pneumonia nor the viral infections resulting in LRTI could be underestimated. As per WHO, the overall deaths resulted due to LRTI was reported to be three million,

TABLE 1 | Represents number of patients included in each studies immunopathological changes reported in COVID-19 (2019-nCoV) infected patients.

Study	Number of patients	Immunopathological changes (Lymphocytes and neutophils counts)	Cytokine and chemokine levels in COVID-19 patients	References
1	1	Mild changes in blood like leukopenia and thrombocytopenia.	NA	(17)
2	2	Lymphocyte, Neutophil, WBC counts were in normal range for patient 1, in Patient 2 lymphocyte count reduced.	NA	(18)
3	4	Neutophil count reduced in discharged adult patients upon treatment but patients severely affected had high neutrophil and low lymphocyte count.	NA	(19)
4	6	Adult patient displayed high neutophil level and an elderly patient depleted in neutophils count. Lymphocyte count decreased in two other elderly patients.	NA	(20)
5	9	Average lymphocyte count increased and neutrophil count remained within range in children.	IL-6, IL-17F, IL-22 levels were higher than normal range.	(21)
6	13	Reduced lymphocyte count and neutrophil count in medium range.	NA	(22)
7	41	Highly Significant difference noted in neutophil count in ICU patient compared to Non-ICU, significantly low lymphocyte count in ICU patients.	IL2, IL7, IL10, GSCF, IP10, MCP1, MIP-1α, and TNF-α were significantly high in ICU patients.	(1)
8	50	Significant decrease in lymphocyte count and increase in neutrophil count observed in critically ill patients compared to moderate group.	IP-10, MCP-3, HGF, MIG and MIP-1α levels were high in critical patients (ICU).	(23)
9	99	In 35% patients lymphocyte count decreased and in 38% patients neutrophil count increased during SARS-CoV-2 pneumonia.	IL-6 level was elevated in 52% patients.	(24)
10	137	Lymphocyte count below 1×10^9/L was found in 27% patients.	NA	(25)
11	138	Neutophil count significantly increased in fatal cases than non-fatal cases. Lymphocyte count reduced among fatal cases than non-fatal patients.	NA	(12)
12	191	Lymphocyte count was significantly lower in non-survivor than survivors.	IL-6 level was elevated substantially in non-survivors.	(26)
13	710	No significant changes observed in survivor or non-survivor groups.	NA	(27)

NA, not available; IL, Interleukins; TNF-α, Tumor necrosis factor-α; IFN-γ, Interferon-γ; CXCL10/IP-10, IFN-γ induced protein 10; MCP-3, monocyte chemotactic protein-3; HGF, hepatocyte growth factor; MIG/CXCL9, monokine induced IFN gamma; MIP-1α, macrophage inflammatory protein 1 alpha.

excluding tuberculosis related deaths (41). As of 1st Dec 2020 1 year after its outbreak COVID-19 has infected more than 61.8 million individuals which caused above 1.4 million deaths globally while these numbers are still rising steadily. SARS-CoV-2 has spread to several territories more than 200 nations have already reported several cases of COVID-19. As per prediction of WHO 2–5% crude mortality rate would prevail for COVID-19 in world population, at present approximately it is 2.2% after 1 year (6). The ongoing pandemic situation raised by COVID-19 will put additional burden on overall global death rate caused by infectious diseases. Unlike Influenza or SARS-CoV-1, the current pandemic COVID-19 is causing lesser number of deaths in children (26). Men are at higher risk than female exposed to SARS-CoV-2 the exact reason is unknown. At the same time smoking was not found to be a predisposing factor for the patient's complications (42). Due to presence of co-morbidities like age, gender, diabetes, anemia and immunosuppressed or immunocompromised conditions including HIV or cancer, respectively, increases the chance of fatality (24).

As of 1st December 2020, countries like USA, India, Brazil, Italy, France, and Russia are most affected by this pandemic. Currently SARS-CoV-2 is more infectious than previously known epidemic SARS virus (6). The current pandemic imposed by COVID-19 has caused more deaths in shorter period than epidemic SARS-CoV-1 lasted for 6 months causing <800 deaths (43). COVID-19 has a crude death rate of below 5% in global population whereas MERS and SARS had ∼10 and 35% of mortality rate, respectively, (11). At present COVID-19 has higher mortality than influenza and seasonal influenza, however the accurate death rate would be available after 1 year. As the viral shredding takes place at 1–2 days post infection of SARS-CoV-2 hence the recovery rate is collectively high. The reproductive rate (R0) for COVID-19 was estimated by WHO was 2–2.5 (44).

TABLE 2 | The imbalance of IL-1β and INF-γ/IP-10 in CAP (severe), SARS-CoV-1 (severe and non-severe), and SARS-CoV-2 (severe and non-severe) are shown in this table.

Diseases and Severity	CAP (Severe)	SARS-CoV-1 (non-severe)	SARS-CoV-1 (severe)	SARS-CoV-2 (severe)	SARS-CoV-2 (non-severe)
CYTOKINES/CHEMOKINE					
IL-1β	• IL-1β level high in BALF than in serum of fatal cases (28). • Low IL-1β level high in Seven (35%) out of 20 total CAP patients (29). • IL-1β level increased notably in fatal case (30) • IL-1β increased in severe CAP group (31).	• IL-1β level Significantly high in all the patients except three patients during hospitalization compared to normal (32). • IL-1β level was significantly high for 13 days monitored for 20 days of hospitalization (33). • IL-1β did not increase in recovering patients compared to normal (34).	• IL-1β level was (25–49%) higher in fatal cases than normal in lungs demonstrated using IHC (35). • IL-1β level no change in infected patients but increased in a fatal case detected by mRNA expression (31). • 50% increased IL-1β level compared with normal patients (36).	• The IL-1β levels in ICU patients were significant higher than normal but no major difference found in ICU and non-ICU groups (1). • IL-1β level in critically ill patients were significantly low compared with severely ill patients (23).	• Highest statistical significance was observed in IL-1β level of non-ICU patients compared with normal (1). • IL-1β level was in normal range for pediatric patients (21).
IFN-γ/IP-10	• IFN-γ level was significantly higher in Severe CAP patients than non-severe CAP and healthy (29). • IFN-γ level increased in two patients during acute phase but decreased with time (30). • IFN-γ level increased, IP-10 level did not change in pneumonia group (31). • Significantly higher than the SARS-CoV-1 patients 42% patient had CAP (37). • No major difference found in IFN-γ level of CAP and normal patients (38).	• IFN-γ levels not measured (32). • Significantly high for 3 days monitored for 18 days, IP-10 was not monitored (33). • IFN-γ level did not vary in recovering patients compared to normal (34).	• IFN-γ levels in severe and non-severegroups did not vary significantly compared to control group. IP-10 level was high in SARS-CoV-1 patients (31). • IFN-γ level increased by 50% compared to normal (36). • The level of IFN-γ was lower than CAP but the IP-10 level was significantly higher than CAP group (37). • Significantly high in fatal than non fatal patients (39) • No Significant change in serum INF-γ level of severe and non-severepatients (40).	• IFN-γ was significantly higher in the ICU patients compared with the healthy individuals (1). • IP-10 level varies with time monitored in critical, severe and moderate pneumonia. Major statistical significance was observed in the critically ill patients and in moderate pneumonia patients after 15 days of infection (23).	• No difference observed in IFN-γ levels of ICU and non-ICU patients but IP-10 was high in ICU but not in non-ICU patients (1). • IFN-γ level was in normal range except for two pediatric patients (21).

The non-severe group contains hospitalized patients, the severe group patients required frequent oxygen therapy and critical group patients were admitted in ICU under invasive mechanical ventilator.

VIROLOGY OF SARS-CoV-2 VIRUS

To date seven species of Coronaviruses has been identified and was found to be threatful to human respiratory system. Among them four species like hCoV-229E, OC43, NL63, and HKU1 are considered as less infectious whereas viruses like MERS, SARS, and SARS-CoV-2 are considered highly infectious and fatal (20). The SARS-CoV-2 is very closely similar to the SARS virus which was previously well known for causing epidemic of 2002–2003. The SARS-CoV-2 antigen or spike (S) protein is capable of binding to human angiotensin converting enzyme II (ACE2) receptor as it has structural similarity to SARS-CoV-1. This virus was found to be 79.6% similar to the SARS-CoV-1 (45). It was found that SARS-CoV-2 has 98.7% nucleotide homology to horseshoe bat coronavirus (46). The isolated strains of SARS-CoV-2 from infected patients had 99.98% similarity with each other, suggesting no antigenic drift noted during early phase of outbreak (18, 46). The SARS-CoV-2 viral genome is similar to coronavirus (CoV) strains indentified in pangoline and bats (47).

The SARS-CoV-2 virus failed to infect laboratory mice by gaining entry through the ACE2. However, this viral infection in mice could be achieved by cloning human-ACE2 (hACE2) in mice to obtain pathogenecity (48). ACE2 are widely expressed on T-lymphocyte cells of mammalian lungs (49, 50). Using Immunohistochemistry (IHC) a study demonstrated the expression pattern of ACE2 which is widely expressed in alveolar epithelial cells, bronchial epithelial cells, bronchial serous glandepithelial cells, monocytes/macrophages, gastric parietal cells, myocardial cells, distal convoluted renal cells, adrenal cortical cells, sweat gland of epithelial cells, and acidophilic cells of pituitary. The ACE2 were not expressed in bronchial mucous gland epithelial cells, follicular epithelial cells of thyroid and gastric chief cells (35). A study analyzed RBD (receptor binding domain) gene of 103 strains of SARS-CoV-2 found two types of molecular divergence in its genomes S (Serine) type and L (Leucine) type. The L-type strains was dominant and aggressive in nature compared to S-type was seen during the early phase of Wuhan outbreak (51).

The SARS-CoV-2 genome is differentiated into two basic types of non-structural proteins and structural proteins encoding genes. There are overall 16 non-structural proteins in orf1a/b encoded by this gene sequence necessary for suppressing host

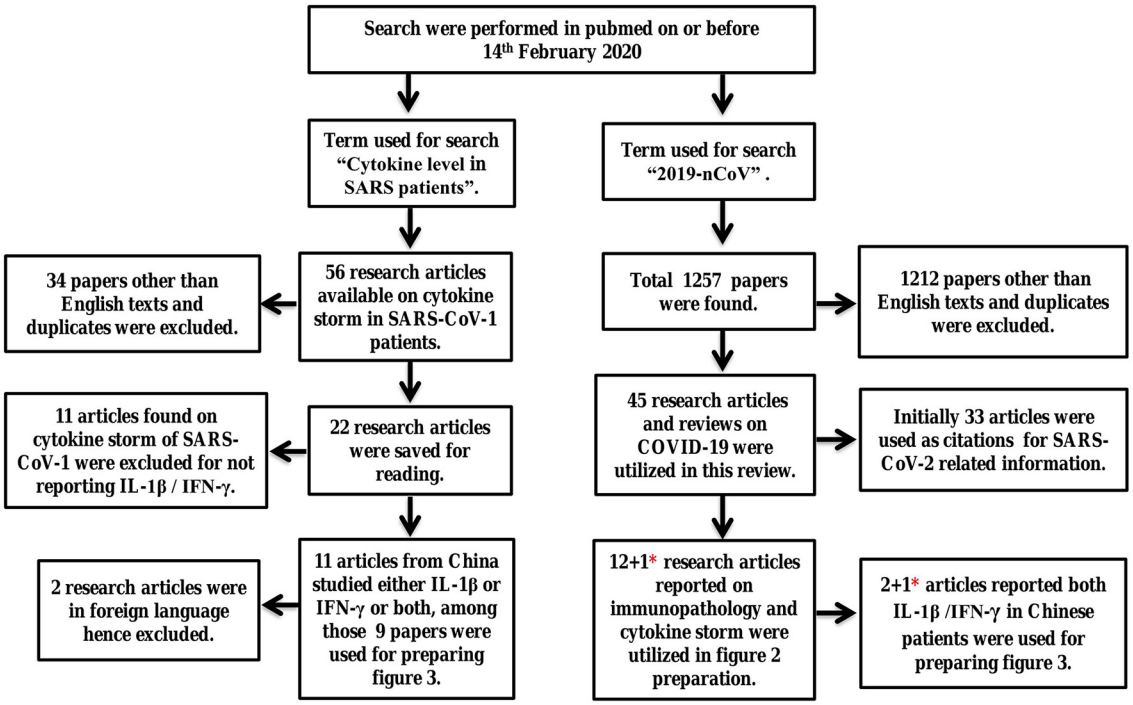

FIGURE 1 | Systematic review of literature to study the immunopathology and immune imbalance resulting in pneumonia. Here the term SARS refers to SARS-CoV-1 and 2019-nCoV refers to SARS-CoV-2 virus. *One research article on SARS-CoV-2 published in April 2020 was added later to both **Tables 1**, **2**.

defense mechanism, replication, reverse transcription, helicase, host specific binding elements etc. The structural proteins are of four types spike (S), envelop (E), membrane (M), and nucleocapsid (N) proteins these proteins are synthesized by four known genes ORF2 (S), ORF4 (E), ORF5 (M), and ORF9 (F), respectively (20, 52). Additionally there are eight open reading frame genes named as ORF1ab, ORF1a, ORF3a, ORF6, ORF7a, ORF7b, ORF8, and ORF10 responsible for respective protein generation. The viral genome is responsible for producing sixteen (16) non-structural proteins these functions in inducing host mRNA cleavage (NSP1), binding to prohibitins (NSP2), proteinase activity (NSP3), membrane formation (NSP4), NSP polypeptide generation (NSP5), autophagosome generation (NSP6), dimerizing (NSP7), helicase (NSP9), stimulates NSP16 (NSP10), unknown (NSP11), RNA polymerase (NSP12), RNA caping (NSP14), endoribonuclease activity (NSP15), methylation, binding, and stimulation of NSP's (NSP16) (52). Among all these NSP1 is known for suppressing antiviral host response by inhibiting interferon (IFN) response genes and degrades host cellular mRNA (53).

PROGNOSIS

The levels of different cytokines in COVID-19 patients were closely monitored in hospitalized patients. The Interferon gamma-induced protein 10 (IP-10) levels could be potentially distinguished in the severe and non-severe hospitalized patients. Gradual rise or decrease in its level would suggest if patient would require prolong hospitalization or early discharge (23). The use of computerized tomography (CT) scan or magnetic resonance imaging (MRI) to confirm the bilateral opacities of patchy shadows present in patient's lungs caused due to viral infection followed by pneumonia could be confirmed, ARDS associated changes could be observed during the severe cases of COVID-19 (17, 54). Early identification of fungal or bacterial co-infection in COVID-19 cases would potentially increase the survival chances of the subjects.

CLINICAL SYMPTOMS OF SARS-CoV-2

As per WHO, symptoms like fever, cough, sore throat, nasal congestion, malaise, headache, and muscle pain were observed in uncomplicated COVID-19 patients (3). The non-ICU patients presented high fever than the patients in ICU (1, 12). Possibly due to early and regulated secretion of pro-inflammatory cytokine like IL-1β in non-ICU patients reduced the probability of ICU admission. Symptoms like constipation and abdominal pain was rare in SARS-CoV-2 patients (12, 19). While symptoms like rhinorrhoea, diarrhea, and vomiting was commonly seen in hospitalized patients (12, 24, 25, 27). Liver dysfunction in 2–11% of patients was a frequent comorbidity, elevated hepatic enzymes was seen in 14–53% of infected cases (55).

IMMUNOPATHOLOGY OF SARS-CoV-2 INFECTION

Activation of lymphocytes and neutrophils during superinfection process can directly modify the diseased state. Hence monitoring these two cell types would reveal the disease progression in patients (56). However, the change in lymphocyte and neutrophils counts observed during acute respiratory infection severe and non-severe conditions are mainly triggered by the acute phase cytokine storm (57). In 2012 a cohort study conducted involving patients suffering from pneumonia infection or influenza. In the H1N1 infected patients it was observed that neutrophil-lymphocyte count ratio (NLCR) below 10 tend to get less hospitalized than the patients with NLCR above 10 (58). The lymphocyte and neutrophil count is the primary criteria under SIRS score to be monitored in hospitalized viral pneumonia patients (3).

In the current pandemic condition serological studies suggested that the patients requiring intensive care had significantly high level of neutrophil counts and low level of lymphocytes entirely opposite was seen in the discharged individuals (19, 22, 25, 27). In COVID-19 adult patients the lymphocyte counts remained average whereas in elderly patients found to be below the lower range of 1.2×10^9/L (20). Fei Zhou et al. reported lymphocytes count was below 1.0×10^9/L in 38 out of 137 patients (26). Studies have confirmed neutrophil count in ICU patients to be significantly higher than Non-ICU ones, while the lymphocyte count was not much different in these two groups of patients (1, 12). The lymphocyte count decreased in both critically ill survivor and non-survivor groups of COVID-19 (23, 27). The average lymphocyte count increased while the average neutrophil count in pediatric patients with non-severe SARS-CoV-2 pneumonia remained within normal range. The age of the patients ranged from 2 months to 15.6 years all the patients survived. The new born (>2 months old) and children <4 years among these nine children showed early recovery had highest level of lymphocyte and moderate level of neutrophils beside that all were positive for viral shredding post recovery from disease (21). The lymphocyte count and cytokine/chemokine levels in COVID-19 patients are illustrated along with the immunopathology in a chart (**Table 1**).

IMMUNOPATHOLOGY OF SARS-CoV-1 AND CAP INFECTION

The progression of pneumonia in severe cases of SARS-CoV-1 or SARS-CoV-2 could be confirmed by evaluating the neutrophil count it could be high in serum but drastically increases in BALF samples (39). Isolation of BALF from patient lungs could be difficult mainly while an epidemic outbreak has already occurred as the patient intake in hospital could be unusually high. Due to the ability of CoV to infect and suppress the IFN induced defense mechanism in patients, this possibly resulted in depletion of T-cells leading to lymphocytopenia and T-cell exhaustion in adults (50, 53, 59).

It was reported that decrease in lymphocytes and increase in neutrophils could be visible in patients exposed to endotoxemia (60). In CAP and SARS-CoV-1 pneumonia patient's neutrophil count were higher than the average lymphocyte counts, similar to the SARS-CoV-2 pneumonia patients (1). Previously in SARS-CoV-1 infected patients leukopenia and lymphocytopenia were noted, SARS-CoV-1 affected individuals showed lower counts of overall T-cells, CD4+ T-cells, CD8+ T-cells, natural killer (NK) cells, and B-cells in serum samples (56). Also a study conducted in 2014 where they focused on both viral pneumonia and CAP patients showed minor difference between these two groups identified in the lymphocytes counts but not in the neutrophil counts. The neutrophil count remained high and equal in both viral and non-viral bacterial pneumonia groups (61). Later in a study's effort to distinguish SARS-CoV-1 pneumonia and CAP mediated changes found the neutrophil counts were substantially high in CAP cases than SARS-COV-1 pneumonia (severe and non-severe) cases but the lymphocyte count was reduced in SARS-CoV-1 but not in CAP group (37). Lymphocyte cells are responsible for producing significant amount of IFN-γ and tumor necrosis factor (TNF-α) in the SARS-CoV-1 infected patients (62). The lymphocyte count plays a decisive role during viral infection; patients with initial high level had lower risk of acquiring and dying due to pneumonia (24). The neutrophil and lymphocyte count shows similar trend in both SARS-CoV-1 and SARS-CoV-2 pneumonia patients (1).

IMMUNE ACTIVATION DURING BACTERIAL PNEUMONIA

Pneumonia can be caused by bacteria like *Streptococcus pneumoniae*, *Mycoplasma pneumonia*, *Klebsiella pneumoniae*, *Chlamydia pneumoniae*, *Staphylococcus aureus*, *Legionella pneumophila*, *Pseudomonas aeruginosa,* and *Haemophilus* influenzae type b (Hib) (58). *Streptococcus pneumoniae* is the most virulent pathogen capable of causing most fatal and common form of pneumococcal infection. Upon binding to the respiratory tract pneumococcal species further colonizes, multiplies, and spreads the infection in multiple regions of human body like ear (otitis), blood (bacteraemia), brain (meningitis) (63). In the existing pandemic viral pneumonia the most commonly identified bacterial species includes *Acinetobacter baumannii, Klebsiella pneumoniae,* and methicillin resistant *Staphylococcus aureus* (24).

The binding of the bacteria to the epithelial cells of bronchus or alveoli is the primary step toward infection. Upon the entry of bacteria in the bronchus through the nasopharyngeal space into the trachea moves further to alveoli, it infects the alveolar epithelial cells (AEC) this activates immune cells like macrophage and dendritic cells through cytokine release (64). Gram negative bacterium, lipopolysaccharides (LPS) induces IL-1β release from AEC it activates macrophage via NFkB mediated signaling pathway resulting in pro-IL-1β secretion (65). The proinflammatory cytokines activates the dendritic cells which in turn facilitates proliferation of the macrophage and neutrophils

through the release of pro-inflammatory cytokines like tumor necrosis factor alpha (TNF-α), interleukin IL-1β, IL-6, IL-8, and IFN-γ (66).

The cytokines like IL-1RA, IL-6, IL-8, and IL-10 were found in initial phase of invasion, also known as acute phase cytokines (67). The initial titers of pro-inflammatory cytokine release leads to the activation of naive T-cells, it produces GM-CSF, G-CSF, IFN-γ, TNF-α, IL-1α/β, and IL-12 it enhances the ability to fight bacterial infection (38). The recruited T-cells play a significant role in bacterial clearance during the course of infection (68). T-cells are prominently known for IFN-γ production during respiratory viral infection but not during pneumonia (62). T-helper 17 cells play a vital role during bacterial infection by releasing IL-1β and IL-6; it activates and recruits neutrophils to the site of infection (69). Due to these above facts IFN-γ and IL-1β levels should be observed in patients with CAP. The levels of both pro-inflammatory and anti-inflammatory cytokines are important to be monitored in patients suffering from pneumonia.

Alveolar macrophage mediated inflammatory responses includes the cytokines release like IFN-α/β/γ, TNF-α, IL-1β, IL-6, and IL-8 these cytokines release causes fever, pain, headache and cough formation (70). The anti-inflammatory cytokines like IL-1 receptor antagonist (IL-1ra), transforming growth factor (TGF)-α/β and IL-10 plays a crucial role in suppressing the destructive function of inflammatory cytokines during critical condition (71, 72). The major role of pro-inflammatory cytokines IL-1β is to continuously recruit polymorphonuclear (PMN) neutrophils at the site of infection to effectively reduce the bacterial load this causes ALI and ARDS (73). IL-1β mediated chemokine (CXCL1/2) release is necessary for suppressing infection via neutrophil activation, similarly IL-8 mediated CXCL2 release simultaneously triggers neutrophil activation and proliferation (74, 75). Thus, increased IL-1β level has negative impact on patient's survival during severe CAP, IL-1ra, and IL-8 release downregulates the negative impact of IL-1β (69, 76).

The disrupted epithelial cells and macrophages signals the proliferation of neutrophils causing the accumulation of cellular debris along with blood, neutrophils and macrophage forms fluid in the alveoli giving arise to pulmonary edema (70, 77). Failure of neutrophils to degrade the bacteria within it via phagocytosis mediated degradation could increase the bacterial load causing neutropenia increasing the criticality in patients often observed during multi drug resistant (MDR) pneumonia (78). The up-regulation of granulysin and perforin mediated bacterial cell lysis beside that the neutrophil extracellular traps (NET), ROS/NOS, anti-microbial peptides and protease enzyme produced by the alveolar macrophages also by PMN neutrophils all together terminates the bacteria (70, 75).

IMMUNE ACTIVATION DURING FUNGAL INFECTION

Presently fungal pneumonia is seen in patient confirmed to be *Candida glabrata, Candida albicans,* and *Aspergillus flavus* (24). During the course of fungal pneumonia dendritic cells and macrophages activates the NK cells through release of IL-12 (79). Sequentially NK cells IFN-γ release leads to maturation of Th1 cells. On the other side dendritic cells causes proliferation of Th17 cells by secreting TGF-β, IL-6, IL-1β, IL-21, and IL-23. Both the activated T-helper cells mediated neutrophil maturation and proliferation at the fungal infection site. The cytokines level of IFN-γ, IL-12, IL-17A/F, IL-23, and IL-22 has protective role in neutralizing the fungal invasion (80). Activation of neutrophil leads to similar complications like bacterial pneumonia, however early and balanced proliferation of neutrophils is important for patient's survival. Fungal pneumonia has differential observations from bacterial pneumonia in ground glass opacities (GGO) obtained from the CT-scan report (81).

CYTOKINES IMBALANCE LEADS TO VIRAL PNEUMONIA

The viral pneumonia is a co-infection caused initially by any virus like influenza A/B viruses, respiratory syncytial virus, enterovirus, adenovirus, rhinovirus, metapneumovirus, parainfluenza virus, bocavirus, MERS and SARS coronaviruses, in association with secondary bacterial or fungal infection results in fatality, it is also known as superinfection. This type of co-infection was commonly seen in the previous influenza virus pandemic of 2009 (10, 82). The viral entry causes disruption of alveolar epithelial cells (AEC), it cascades the signal for production of cytokines like granulocyte/macrophage colony-stimulating factor GM-CSF it signals maturation and recruitment of monocyte and macrophage cells (83). The production of TNF-α by macrophage cells present at very low level during acute infection phase within the alveoli, signals AEC to produce GM-CSF (84). Later the activated and matured macrophages play a vital role in proliferation of dendritic cells and recruitment of macrophages which all together leads to chemotactic recruitment of neutrophils (85). GM-CSF is produced by AEC has a prominent role in recruitment and activation of dendritic cells important for adaptive immune response, it promotes the raising level of alveolar macrophages mandatory for innate immune response to tackle bacterial invasion (68). All the viruses mainly targets lymphocytes for its growth and replication.

Acute respiratory infection caused by viruses sub-sequentially engages the immune cells at the site of infection and changes the composition of the lungs and gastro intestinal tract known as dysbiosis. While suppressing the viral load the opportunistic bacteria or fungi residing in the nasopharyngeal space invades easily the alveoli of the lungs (86). The predepository factors attributed by influenza virus or coronavirus infection is mainly mitigated by the release of IFN-γ, it downregulates IL-1β level necessary to fight against bacterial or fungal infection demonstrated in mice (87). In another study shown in mice model the derogatory role of IFN-γ initiates after it binds with macrophage cells and impairs neutrophil recruitment during bacterial co-infection (88). Similarly using a murine model it was revealed that IL-1β production is inhibited by IFN-γ during influenza infection (89). It is further reviewed

here if the mechanism of dysbiosis exists in SARS-CoV-2 mediated cytokine storm leading to cytokine imbalance and pneumonia progression. The IFN-γ level shoots high during pneumococcal pneumonia but not in the case of staphylococcal pneumonia, so alternatively IP-10 level would be suitable to observe IFN-γ mediated changes in clinical studies (90, 91). In severe/fatal scenario of SARS-CoV-2 infection causes imbalance of IFN-γ and IL-1β cytokine levels and related molecular pathways resulting in dysbiosis is yet to be studied.

CYTOKINES LEVELS IN PNEUMONIA PATIENTS

During the severe pneumonia it was observed that level of IL-1β was at higher concentration during fatal condition than non-fatal patients. The proinflammatory cytokine level tends to double in the BAL fluid than in the blood of viral pneumonia challenged individuals (60). The patient's BAL fluid had elevated IL-1β than in serum due to presence of bacterial infection, IFN-γ level was not confirmed (28). The IFN-γ level was found to be similar compared to healthy pediatric patients infected with *Mycoplasma pneumoniae* pneumonia found in a meta-analysis (92). IFN-γ levels were found to be normal in a study involving children suffering from severe pneumonia (38). In a study focusing on patients infected by *Legionella* pneumonia found low level of IL-1β except the fatal case, the level of IFN-γ elevated during acute phase but it diminished with time (30). The CD8+ T-Cells are responsible for producing and maintaining high levels of IFN-γ required for clearing the viral load at the same time IFN-γ plays a major role in recruitment of T-cells, but its level remains insignificant during non-severe CAP (93).

In severe CAP (where three out of 10 patients suffered from Haemophilus influenzae and H1N1) both the BAL fluid and serum cytokine level showed high IFN-γ and low IL-1β levels in CAP patients. Severe CAP group compared to non-severe CAP shows high level of IFN-γ but not IL-1β suggests possible role of dysbiosis causing this imbalance during co-infection. Cytokines like IL-8 and IL-1β are responsible for the activation of neutrophil, during severe pneumonia infection these were found to be elevated in BALF of fatal cases (29). The IL-1β levels were elevated in severe CAP patient's BAL fluid samples, possibly because this study enrolled both pneumococcal pneumonia and viral pneumonia patients. Thereby, it is possible that due to lymphocytopenia or leukopenia caused by any of the other comorbid conditions may raise the chances of dysbiosis resulting in pneumonia.

CYTOKINES IMBALANCE IN SARS-CoV-1 PNEUMONIA PATIENTS

The cytokines level often changes depending on the type of bacterial and fungal species. Difference in cytokine level could be observed when groups are sorted as per secondary infections (90, 92). The IL-1β level was found to be ∼25–49% higher in dead patient's AEC upon SARS-CoV-1 infection, whereas TNF-α level was reported to be around 50–75% higher in same fatal cases, IFN-γ level was not measured in fatal cases by IHC (35). In a study conducted in Hong Kong included 20 patients infected with SARS-CoV-1, under non-severe scenario the serum IL-1β level was significantly higher (above 1.3 ng/L considered significant) for 13 days post infection until recovery, whereas it was not observed in IFN-γ level (above 15.6 ng/L considered significant) in same patients while monitored for 18 days, it is possibly because both severe and non-severe patients were included in the same group (33). The IL-1 gene expression pattern measured in SARS-CoV-1 severe and non-severe patients did not vary with results for IL-1β measured by enzyme immuno assay (EIA) (94, 95).

The IFN- γ level was not found to be high in SARS-CoV-1 group (one patient had ARDS), but substantially elevated in CAP group, however the IP-10 level was higher in SARS-CoV-1 infected individuals than CAP group (37). The level of IFN-γ in end stage of severely infected SARS-CoV-1 patients was not substantially different from normal patients, but IP-10 level increased in fatal cases than in normal or convalescent groups. There is a clear indication from above clinical data that presence of high viral load caused prolonged activity of IFN-γ and viral replication resulted in lymphocytopenia. IL-1β level did not changed in hospitalized SARS-CoV-1 infected patients but was increased in fatal cases detected by mRNA expression (31). IL-1β leads to neutrophil activation necessary during acute phase of pneumonia, slower secretion of IL-1β increases bacterial growth resulting in faster disease progression. Viral load never declines in severely infected viral pneumonia fatal cases. The failure in up-regulation of signature level of IL-1β is necessary for suppressing the bacterial load causes dysbiosis it increases complications or results death (86).

The serum level of IL-1α and IFN-γ were found at non-significant level among severe and non-severe SARS-CoV-1 patient groups. Anti-inflammatory cytokines like TGF-β and IL-10 level increased in convalescent group (40). IL-1β level in non-severe SARS-CoV-1 infected children were substantially high before and after the treatment with anti-inflammatory drugs. The IL-1β level was reported to be induced by the activation of caspase-1 dependent pathway triggered by viral entry in macrophage cells (32). The lymphocytes count trends to decline during SARS-CoV-1 and SARS-CoV-2 viral pneumonia suggesting IFN-γ release from alternative cellular sources. IFN-γ is produced by NK cells, T-cells and antigen presenting Cells (APC) during viral infection, suggesting NK cells and APC are alternative source of IFN-γ during coronavirus infection (88).

Kao-Jean et al. reported that IFN-γ level was not substantially different in the SARS-CoV-1 fatal and non-fatal cases, but the level of IFN-γ was several fold higher in acute stage of viral infection in patients than normal individuals (39). IL-1β increased with severity of SARS-CoV-1 in critical patients while its levels remained normal in non-severe patients (34, 36). The mechanism of dysbiosis could be seen in these clinical studies with SARS-CoV-1 pneumonia critical patients. The imbalance of

IL-1β and INF-γ/IP-10 in CAP (severe), SARS-CoV-1 (severe and non-severe) and SARS-CoV-2 (severe and non-severe) are shown in **Table 2**.

CYTOKINES IMBALANCE IN SARS-CoV-2 PNEUMONIA PATIENTS

The cytokine storm taking place in SARS-CoV-2 infected patients suggested that pro-inflammatory cytokine IL-6 and anti-inflammatory IL-10 levels to be substantially elevated in both mild and severe cases (24). Cholin Huang reported high level of IL-2, IL-7, IL-10, G-CSF, IP-10, TNF-α, MCP-1, and MCP-1A increased in ICU patients with SARS-CoV-2 pneumonia (1). Previously in middle east respiratory syndrome coronavirus (MERS-CoV) severely infected patients the levels of IFN-γ, TNF-α, IL-15, and IL-17 were reported to be high (96).

The immediate recruitment of neutrophil is required during pneumonia, delayed activation are due to ongoing fight against viral invasion delays the GM-CSF production (83). The TNF-α and IL-1 mediated GM-CSF production induces controlled neutrophil recruitment and vice versa (97, 98). Additionally GM-CSF enhances survival, proliferation and phagocytosis of immune cells, GM-CSF plays an important role during the life threatening cases of sepsis (68). GM-CSF was not significantly high in ICU admitted patients of SARS-CoV-2 compared to the non-ICU patients; this suggests the importance of GM-CSF during the early phase of infection in severely affected patients (1). Beside that the T-cell decreases the viral load during CoV invasion, but the T-cells are the prime target of this virus (99).

Elevated level of IFN-γ was a usual marker for SARS-CoV-2 infected severe as well as in non-severe groups, but no major difference between severe or non-severe groups. The level of IL-1β was significantly high in ICU patients compared with healthy individuals however the IL-β level was below 2 pg/ml. Increased IP-10 level was related to high IFN-γ activity which was high in ICU patients. IL-1β level remained in normal range for healthy individuals but increased in both ICU and non-ICU (low statistical significance) held patients the patient's serum which were collected soon after admission and was not regularly monitored (1). A study sorting SARS-CoV-2 pneumonia groups as per the requirement of MV considered critical, while non-invasive MV considered as severely ill and least MV requiring patients grouped as moderate. Upon prolong monitoring of IL-1β for 24 days post infection in these 3 focus groups where statistical significance in critical and severe patients was seen on 15th day. The IL-1β level decreased in critical group suggesting disease progression while IP-10 level remained high even after prolonged treatment with immunosuppressant observed 15 days after onset. The increasing level of IP-10 and MIG seen in these groups modulates the disease and signals toward severe condition of lungs, while decreased level was positively correlated with healing when IP-10 was below 1,000 pg/ml in serum. The IP-10 and MIG genes are induced by IFN-γ activity during viral pneumonia. It was reported that reduced IL-1β level in critically ill ICU patients compared to less critical ones, in that study G-CSF, M-CSF, CTACK, IL-18, IL-13, MIP-1α, MIP-1β, MCP-3, MIG, HGF, IL-1ra, IL-1β, and IP-10 were monitored regularly in patients (23). Similarly observed in influenza-pneumonia and CAP there was clear indication that IFN-γ downregulates the IL-1β level similar trend is observed in COVID-19 pandemic but further study is required at molecular level in future to draw this conclusion. This calls for further study to reveal the underlying intercellular mechanism causing down regulation of IL-1β by IFN-γ in critically ill patients.

During severe instance of sepsis the IFN-γ level rises and it has detrimental effect on individual life expectancy. In the same study it was reported that IFN-γ single nucleotide polymorphism gives rise to increased susceptibility to pneumonia in Chinese population (100). Similarly IL-1β gene polymorphism was observed in Iraqi children found increased susceptibility to pneumonia infection. It was observed in the study that IL-1β level was not substantially increased in CAP patients compared to healthy individuals (101). Further it was noted that IL-10 encoding SNP genes were found responsible for exacerbating systemic inflammatory response syndrome (SIRS) score during CAP infection but not the TNF-α and IL-6 cytokines (102). Any of these SNP gene mutations in COVID-19 patients play an important role in progression of pneumonia. Immunosuppressive corticosteroids could be effectively used to reduce the burden of SIRS. The levels of the cytokines, interferons and chemokines were GM-CSF, G-CSF, TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, IL-17A/F, IL-22, and IP-10 were monitored in SARS-CoV-2 pneumonia patients (1). Excess production of IL-1β has negative effect on patient's survival and disease recovery but early induction of this pro-inflammatory cytokine possibly leads to early or quick recovery.

CYTOKINE LEVELS IN SEVERE SARS-CoV-2 CHILDREN AND AGED (FEMALES AND MALES)

In most recent study supporting the concept of dysbiosis could be observed where pediatric patients suffered from SARS-CoV-2 pneumonia on an average the level of IFN-γ increased while IL-1β level remained normal during the course of treatment in hospital with antivirals and interferon-alpha (IFN-α). The SARS-CoV-2 infected children <4 years demonstrated early recovery with increased viral shredding for prolonged time where lymphocyte and IFN-γ level elevated although neutrophil and IL-1β level remained within normal range (21). This evidence collectively suggested children <4 years accommodate virus possibly due to higher lymphocyte count promoting constant viral replication for several days post recovery and required prolong monitoring of viral titer. The lymphocytes level in children within 1 month to 3 months remains high due to enhanced activity of thymus but it trends to decline with old age, it could be a possible reason for reduced deaths of children by COVID-19 pneumonia (103). Similarly IL-17F and IL-22 were found significantly higher in children, these two cytokines protects during fungal invasion however fungal pneumonia was not reported in the pediatric patients (21). The Th2 cells

are well known for secreting IL-17F and IL-22 during viral infection (80).

In mice model it was demonstrated upon *Acinetobacter baumannii* infection of lungs, resulted in death of aged mice but not young mice, even vaccination against this species did not protected the older mice (104). This specific superinfection was common in COVID-19 patients of Wuhan; its presence exacerbates to irreversible condition and leads to confirmed death. In the current pandemic, women are less fatally affected by the virus than men. Possibly due to the fact that in females of age group above 50 years has higher lymphocyte counts compared to males of same age group is responsible for protection against infection. In this study to further demonstrate gender difference of withstanding infection found higher leukocyte count in Chinese males than its female counterpart (105). Sexual dimorphism in immunity was seen in female mice with enhanced disease fighting abilities against Staphylococcus induced peritonitis than the male mice (106).

TREATMENT STRATEGY

The treatment strategy involves the use of antiviral medicines to tackle the viral loads in coronavirus infected patients. However, certain antivirals are ineffective in treating the COVID-19 patients, drugs like ganciclovir, acyclovir and ribavirin may not prove effective in patients. Drugs like neuraminidase inhibitors and protease inhibitor lopinavir/ritonavir along with IFN-α in combination were reported to be effective in SARS-CoV-2 challenged patients (19). The antiviral oseltamivir effective against influenza was found effective in reducing SARS-CoV-2 viral load upto a greater extent (1). Antibiotics against pneumococcal infection could induce effective suppression of bacterial growth in the patients with pneumonia. Antibiotics like amoxicillin, azithromycin, and fluoroquinolones were in use for reducing bacterial burden in patients (19, 107). Antiviral agents like umifenovir, remdesivir, and chloroquine were found to be effective against SARS-CoV-2 in patients (108). Drugs like cephalosporins, quinolones, carbapenems, and tigecycline were successfully used against methicillin resistant *Staphylococcus aureus* along with linezolid and other antifungal drugs showed inhibitory activity against pneumonia in severely infected patients (24). Corticosteroid like methylprednisolone, dexamethasone, and methylprednisolone sodium succinate along with intravenous immunoglobulin therapy were reported to be in use for patients suffering from MDR pneumonia (19). Heparin was recommended after successfully demonstrated clinically to reduce coagulopathy in patients with sepsis (108). In a non-randomized study the serum transfusion from COVID-19 recovered or convalescent patient to SARS-COV-2 infected critically ill patients recovered within 11–13 days post transfusion (109). Cytokine absorption devices are currently in use for managing critical conditions; it eliminates the excess amount of circulating cytokines in the blood of SARS-CoV-2 pneumonia patients (110).

Interferon-γ level has negative effect on patient's susceptibility to withstand severe condition like bacterial sepsis and not recommended by WHO for the existing COVID-19 treatment (111). Cellular therapy involving the cytokines like GM-CSF was clinically proven and suggested (112). IFN-α is more suitable for SARS-CoV-1, SARS-CoV-2, and MERS virally challenged individuals as demonstrated in mice (113, 114). Early inhalation of GM-CSF and treatment using IL-6 inhibitor (tocilzumab) for patients suffering from viral pneumonia has repressive role in sepsis progression (112, 115). The treatment strategy, essential cytokine markers, disease progression and prognosis are described in brief with a flow chart (**Figure 2**).

The IL-1 inhibitor (anakinra) showed patients treated with it recovered from the COVID-19-pneumonia. Two different clinical studies demonstrated anti-IL-1 receptor has positive impact on patient's survival. In a non-randomized study set up patients treated with anti-IL-1 receptor antagonist saved significant number of lives. All the eight patients were comorbid with hemophagocytic lymphohistiocytosis (HLH) a rare immune disorder responsible for high morbidity. Increased number of lymphocyte and macrophage cells is commonly seen in patients with HLH. By treating with anakinra 300 mg/day for seven days, four patients required mechanical ventilator, three were treated with corticosteroids and one attended quick recovery among them three patients died (116). This study suggested that hyperactivated immune system may delay the recovery process in patients. A clinical study could be conducted further to confirm the efficacy of IL-1 inhibitor beside corticosteroid treatment group in severely ill patients. Similarly, a cohort study involving COVID-19 patients comparing with a historical placebo group with the anti-IL-1 treated group found reduced number of deaths in treatment group. In this study none of the patients suffered from cancer but suffered from other comorbidities and were treated with 200 mg/day for 3 days than 100 mg/day for seven days (117). Both studies involving SARS-CoV-2 pneumonia patients suggested further studies should be conducted to conclude the efficacy of anti-IL-1 antibodies. In future randomized clinical study including patients suffering from multidrug resistant SARS-CoV-2-pneumonia should be enrolled and treated with anti-IL-1 to identify its efficacy.

LIMITATIONS

The studies discussed in this review mostly focused on studies of Chinese population and published during the early phase of pandemic. The initial review of literature search was limited to pubmed, other repository or databases were not searched. The cytokine storm seen during epidemic SARS-CoV-1 and initial wave of SARS-CoV-2 was mainly highlighted to review the cause of dysbiosis. The cytokine levels could be diverse in populations of different global regions affected by COVID-19 due to varying secondary infections is not reviewed here. The immunopathological trends should remain the same and may not vary significantly. The side effects attributed by treatment regimen in COVID-19 patients are not discussed here. Few of the COVID-19 literatures discussed here measured the cytokine level upon admission to the hospital (1). Few authors reported patients were monitored for

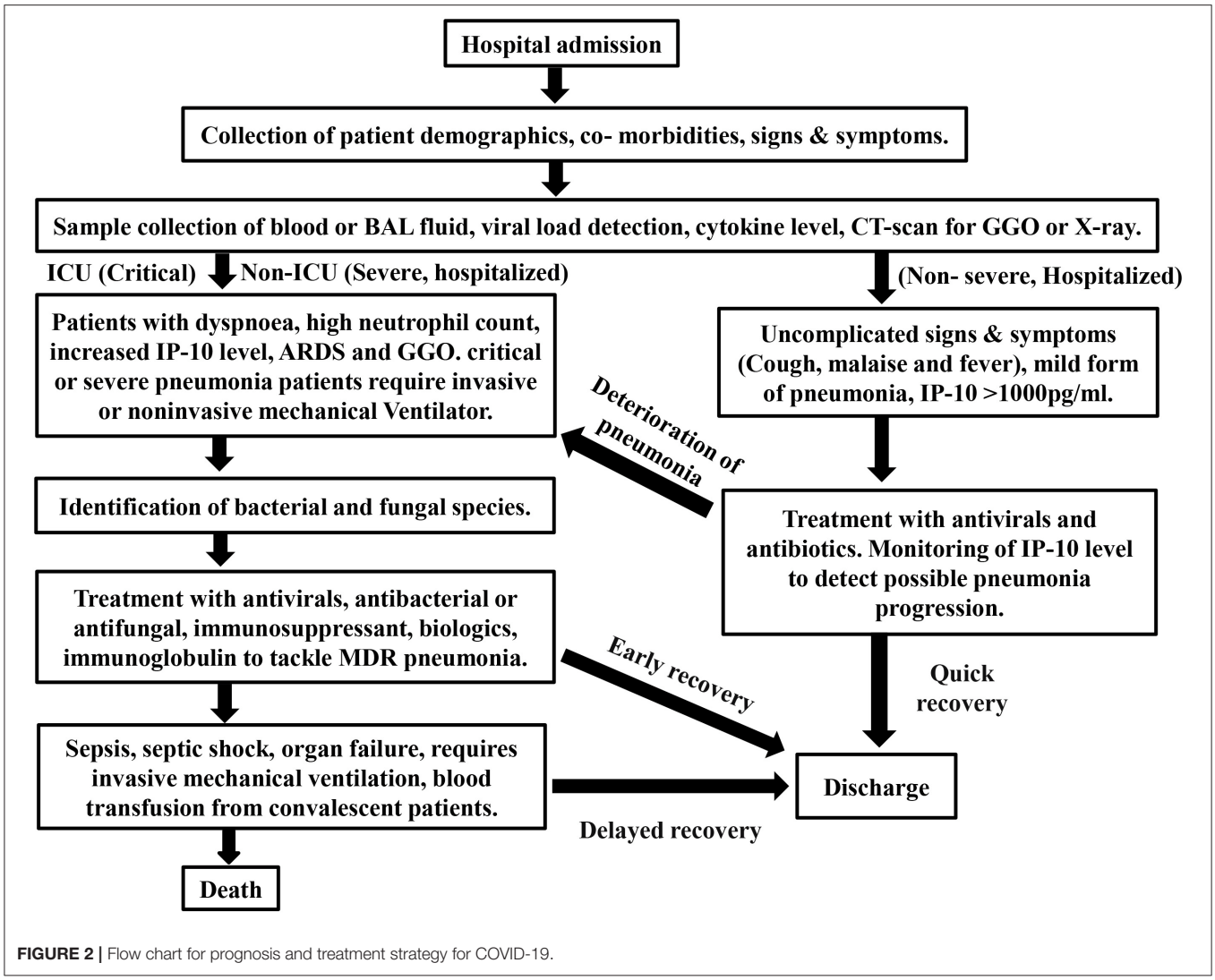

FIGURE 2 | Flow chart for prognosis and treatment strategy for COVID-19.

cytokines levels throughout the hospitalization phase and was treated with antivirals, immunosuppressant and interferon-alpha (IFN-α) (23). Accurate mortality rate of COVID-19 and basic reproduction number of SARS-CoV-2 are yet to be estimated. The level of cytokine in COVID-19 non-hospitalized patients is not discussed here.

CONCLUSION

Signature cytokine levels of IL-1β is an important factor activated during acute phase of viral pneumonia, as its level may not be significant obtained from large number of patients infected by varying bacterial or fungal species (1, 92). It is presently seen in fatal cases that homeostasis of immune system is not maintained resulting in imbalance of immunological cells and cytokine levels mainly IL-1β and IFN-γ during dual mode of infection such as viral pneumonia increases the complications in COVID-19 patients (87, 118). Low level of lymphocytes mainly Th2 cells during the acute phase of viral infection causes failure in recruitment of anti-bacterial neutrophils required to tackle the secondary infection (69). In the severe cases of viral pneumonia patients died due to bacterial burden but not due to primary viral infection (12). Observed in MERS, SARS-CoV-1, and COVID-19 patients it is understood that initially delayed but gradual increase of IL-1β complicates patient's condition (23). Increase in cytokines levels of IL-1β, IFN-γ, IL-6, IL-8, and IL-10 are the main cause and sign of disease progression (11). Pre-acquired gene polymorphism of IL-1β or IFN-γ genes in COVID-19 patients and superinfection by *Acinetobacter baumannii* in elderly COVID-19 patients may raise the chances of death irrespective of known co-morbidities.

Hereby it is suggested that similar to MuLBSTA score and SIRS score, the chemokine (IP-10) fold change, along with lymphocyte and neutrophil counts should be closely monitored for early detection and progression of SARS-CoV-2 pneumonia

(3, 119). The initial level of white blood cells (WBC) types, cytokines profiles along with the GGO lesions detected in CT-scan report, beside the rectal swab test done to detect viral shredding are useful for prolong monitoring in patients (25, 120). The isolation and identification of specific fungal or bacterial species would help in precise treatment with antibiotics (54). During viral pneumonia pandemic it was recommended to collect the BAL fluid for immunological studies rather than collection of blood samples (60). As observed in MERS, SARS-CoV-1, and COVID-19 patients it is understood that initially delayed but gradual increase in cytokines like IL-1β, IFN-γ, IL-6, IL-8, and IL-10 is the main cause of rapid sepsis progression (11). Immuno-compromised patients suffering from viral pneumonia are subjected to prolonged mechanical ventilation either invasive or non-invasive it may prove to be fatal or worsen the disease condition prolonging the recovery time (77). Targeting the GM-CSF-IL-1β axis in humans could be an effective method to attain therapeutic benefit during early phase of viral pneumonia which is explored clinically (NCT04569877).

AUTHOR CONTRIBUTIONS

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

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Severe Acute Respiratory Syndrome Coronavirus 2 Viral RNA Load Status and Antibody Distribution Among Patients and Asymptomatic Carriers in Central China

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This study aimed to monitor severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viral loads and specific serum-antibodies (immunoglobulin [Ig] G and M) among confirmed patients and asymptomatic carriers from returning healthy travelers. The throat swabs, sputum, and stool samples from 57 hospitalized coronavirus disease (COVID-19) patients and 8 asymptomatic carriers, among 170 returning healthy travelers were tested using reverse-transcription real-time polymerase chain reaction. SARS-CoV-2 IgM/IgG antibodies were detected *via* serum chemiluminescence assay. Sequential results showed higher viral RNA loads in the throat, sputum, and stool samples at 3–12 and 6–21 days after symptom onset among severely ill COVID-19 patients. Shorter viral habitation time (1–8 days) was observed in the oropharyngeal site and intestinal tract of asymptomatic carriers. The IgG and IgM response rates were 19/37 (51.4%) and 23/37 (62.6%) among the 29 confirmed patients and 8 asymptomatic carriers, respectively, within 66 days from symptom or detection onset. The median duration between symptom onset and positive IgG and IgM results was 30 (n=23; interquartile range [IQR]=20–66) and 23 (n=19; IQR=12–28) days, respectively. Of 170 returning healthy-travelers to China, 4.7% were asymptomatic carriers (8/170) within 2 weeks, and the IgG and IgM positivity rate was 12.8% (12/94). IgM/IgG-positivity confirmed 3 suspected SARS-CoV-2 cases, despite negative results for SARS-CoV-2 RNA. Compared with other respiratory viral

infectious diseases, COVID-19 has fewer asymptomatic carriers, lower antibody response rates, and a longer antibody production duration in recovered patients and the contacted healthy population. This is an indication of the complexity of COVID-19 transmission.

Keywords: severe acute respiratory syndrome coronavirus-2, coronavirus disease, viral RNA load, asymptomatic carriers, IgM, IgG

INTRODUCTION

Coronavirus disease 2019 (COVID-19) (Zhou et al., 2020) was officially declared a pandemic and public health emergency of international concern by the World Health Organization, indicating that it may result in substantial morbidity and mortality (Jones, 2020). As of February 16, 2021, more than 109,820,928 confirmed cases of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections have been reported from 209 countries, including 101,569 cases in China. In total, 2,417,402 patients have died because of COVID-19 (Peeri et al., 2020). However, data on viral load kinetics in confirmed cases and carriers and the production time and distribution of antibodies among patients and the contacted healthy population remain scarce to date (Wu et al., 2020). Currently, there are no data on the proportion of asymptomatic carriers and antibody distribution among the returning healthy travelers from endemic areas. Thus, this study aimed to monitor SARS-CoV-2 viral RNA loads and specific serum-antibodies (immunoglobulin [Ig] G and M) in confirmed patients and asymptomatic carriers among returning healthy travelers.

MATERIALS AND METHODS

Study Subjects and Design

This was a retrospective case–control study on the viral RNA load and antibodies in confirmed COVID-19 patients and asymptomatic carriers. We evaluated all hospitalized COVID-19 patients (n=57) and asymptomatic carriers (n=8) who were admitted to Henan Provincial People's Hospital between January 2, 2020, and April 29, 2020, and were diagnosed with SARS-CoV-2 infection by positive nucleic acid and antibody tests. In addition, 170 healthy travelers who were returning to China were also evaluated. Patients with negative real-time reverse-transcription polymerase chain reaction (rRT-PCR) results or no SARS-CoV-2-specific IgM and IgG, with a definite diagnosis of other diseases, and with a negative SARS-CoV-2 nucleic acid or antibody test were excluded from this study. Case definitions of confirmed human infection and asymptomatic carriers with SARS-CoV-2 were in accordance with the latest diagnostic criteria (version 7) issued by the Chinese National Health Committee (5). This included patients that had a contact history with confirmed or suspected COVID-19 patients and those that tested positive for SARS-CoV-2 nucleic acid in throat swabs, sputum, or stool samples, or tested positive for IgM/IgG in serum. Further, the patients who had abnormal chest computed tomography (CT) findings and with fever and respiratory symptoms were defined as confirmed cases. Among the patients with a contact history who tested positive for SARS-CoV-2 RNA, those who had normal chest CT findings and no fever or other respiratory symptoms within 14 days of isolation were defined as asymptomatic carriers (Jin et al., 2020).

In total, 65 individuals were evaluated in this study, including 57 confirmed COVID-19 patients and 8 asymptomatic carriers. Among the 57 confirmed COVID-19 patients, 54 patients were local residents and 3 patients who had returned to China from Iran and France were confirmed to have COVID-19 based on IgM or IgG positivity despite negative nucleic acid test results. Notably, 3 patients and 8 asymptomatic carriers were among the 170 returning travelers.

This study was approved by the Institutional Ethics Board of Henan Provincial People's Hospital (20190050) and was conducted in accordance with the Declaration of Helsinki. The ethics committee waived the requirement for written informed consent for the patients' participation in this study.

Diagnosis and Data Collection

Throat swab, sputum, and stool samples for suspected cases were collected for SARS-CoV-2 testing using rRT-PCR assays (Shanghai Zhijiang Biotechnology Ltd., China). Cycle threshold (C_t) values of ≤44 from the rRT-PCR were considered positive. The duration of the continuous positive C_t value was the number of days of viral habitation time in each patient (Padoan et al., 2020). Viral RNA load was presented as RNA copy number of SARS-CoV-2. Ct values of rRT-PCR were converted into RNA copy number of SARS-CoV-2. The RNA copy number was calculated using a reference method as follows. C_t values were inversely related to the viral RNA copy number, with C_t values of 30.86, 28.68, 24.56, and 21.48, corresponding to 1.5×10^4, 1.5×10^5, 1.5×10^6, and 1.5×10^8 copies/mL, respectively (Zou et al., 2020). Negative samples were denoted with a C_t value of 45, which was under the limit of detection. Additionally, blood specimens for IgM and IgG detection using chemiluminescence assay (Beijing Beier Biotechnology Ltd., China) were considered positive at a cut-off value if the number of antibodies were ≥8 U/mL (Zou et al., 2020). Among the 65 patients, only 37 patients underwent IgM/IgG testing (23 of whom tested positive) because the antibody test was not available in our hospital until 24 February 2020. Information regarding the dates of illness onset, visits to clinical facilities, and hospital admissions were collected from clinical records. The incubation period was defined as the time from exposure to illness onset and was estimated among patients who could provide the exact dates of close contact with individuals who had confirmed or suspected SARS-CoV-2 infection. Throat swab, sputum, and stool samples

were collected and tested as the standard point of care diagnostic workup. However, these samples were not collected and tested at the same time. According to the latest Chinese COVID-19 diagnosis and treatment guidelines (version 7, released on March 4, 2020), in suspected patients, whose diagnosis is confirmed by nucleic acid testing, a throat swab is the first sample to be collected, followed by sputum and stool. Additionally, during the patient's treatment, physicians generally decide on the number and interval of sample collections based on the patient's response to treatment and recovery status. However, patients are required to have two consecutive negative nucleic acid test results with an interval of >24 h before hospital discharge. Available sequential C_t values of rRT-PCR assays and IgM and IgG test results for those included were obtained from the Henan Provincial People's Hospital laboratory information system. Dates of disease onset, hospitalization and classification of COVID-19 severity were recorded. The date of illness onset was defined as the day when any symptoms were noticed by the patient and later confirmed by a physician. Severity classification was defined using the diagnostic and treatment guidelines for SARS-CoV-2 issued by the Chinese National Health Committee (version 7) (Jin et al., 2020). Patients were defined to have severe COVID-19 if they met one of the following criteria: 1) respiratory distress with respiratory frequency ≥30/min; 2) pulse oximeter oxygen saturation ≤93% at rest; and 3) oxygenation indexes (artery partial pressure of oxygen/inspired oxygen fraction) ≤300 mmHg.

Statistical Analyses

Continuous variables are summarized as either means and standard deviations if they were normally distributed or as medians with interquartile ranges (IQRs) if they were non-normally distributed. Meanwhile, categorical variables are presented as the percentages of patients in each category. Categorical variables were compared using chi-square or Fisher's exact tests, and continuous variables were compared using Student's *t* tests or Mann-Whitney *U* tests, according to their distribution. The Pearson correlation coefficient (*r* value) was used to describe the correlation between continuous variables, including Ct value, and the IgG/M response of patients and asymptomatic carriers. All statistical analyses and graphs were generated and plotted using GraphPad Prism version 8.00 software (GraphPad Software Inc.) or SPSS software version 20.0 (IBM Corp., Armonk, NY, USA). P-values <0.05 were considered statistically significant.

RESULTS

Characteristics of the Study Subjects

Of the 57 assessed patients and 8 asymptomatic carriers among 170 healthy traveling returnees, 7 patients required intensive care unit (ICU) admission. Of them, 6 died of severe disease. The remaining 58 patients had mild-to-severe illness or were asymptomatic carriers. Among the 36 severe patients, the mean (standard deviation) age was 60.4 years (16.5), 55.4% were men and 54 (83.1%) patients had at least one coexisting condition. The mean time from admission to symptom onset in severe patients was 11.6 (8.4) days. The mean length of hospitalization of severe patients was 11 (6.6) days, and the average time taken to obtain a positive PCR result was 13.0 (8.3) days. Furthermore, 29 patients had a history of travel to Hubei Province or contact history with confirmed patients, while 3 patients and 8 asymptomatic carriers were among the 170 overseas returnees during the COVID-19 outbreak. **Table 1** shows this information in more detail.

Viral RNA Loads in Throat Swab, Sputum, and Stool Samples

The C_t values were analyzed in a total of 297 samples, including 185 throat swabs, 56 sputum, and 9 stool samples, collected from the 65 patients. The viral RNA loads (inversely related to C_t values) detected 3–12 days after symptom onset were higher than those detected after 12 days from symptom onset. The viral RNA loads detected in the sputum, throat, and stool were higher among severely ill patients than among those with mild disease and asymptomatic carriers. The viral RNA loads in the throat swab and sputum samples peaked approximately 3–12 days after symptom onset. The C_t values ranged from 34 to 36 (10^5–10^8 copies/mL; **Figures 1A, B**) (Zou et al., 2020). Notably, viral RNA was detected in stool samples from 4 symptomatic patients and 1 asymptomatic carrier (**Figure 1C**). The viral RNA loads in stool samples peaked at approximately 6–21 days after symptom onset, with C_t values ranging from 23 to 33 (10^5–10^8 copies/mL). Among the severely ill patients, the viral RNA loads were higher in the throat swab and sputum samples, and this lasted 33–66 days (**Figure 1D**). Unexpectedly, we found that 8 asymptomatic carriers, on different return flights from Iran, France, Cambodia, and Thailand between March 8, 2020 and April 29, 2020, had a positive nucleic acid result in their oropharyngeal site and intestine tract that lasted 1–8 days, with C_t values ranging from 33 to 34 (10^5–10^8 copies/mL) (**Figure 1A**).

Correlation and Comparison of Ct Values Among Sputum, Throat Swab, and Stool Samples

Available sequential C_t values of every 1-day interval were used to determine the correlation of the viral RNA loads among the throat swab, sputum, and stool samples from the 65 confirmed patients and carriers. The viral RNA loads were significantly correlated between the throat swab and sputum samples (n=28 pairs, R=0.8018, p=0.0088; **Figure 2A**). Similarly, the viral RNA loads were also significantly correlated between the stool and sputum samples (n=8 pairs, R=0.9621, p=0.0389; **Figure 2B**) and between stool and throat swab samples (R=0.98, p=0.0156; **Figure 2C**). Meanwhile, the viral RNA loads and positive PCR duration differed among the sputum, throat swabs, and stool samples. Stool samples had higher viral RNA loads and shorter positive PCR duration than did sputum and throat swab samples (**Figure 1D**). However, there were no significant differences in

TABLE 1 | Demographics and clinical characteristics in confirmed patients as well as carriers of COVID-19[a].

Characteristics	Patients with severe disease (n = 36) n (%)	Patients with mild disease and carriers (n = 29) n (%)
Age, years, mean (*SD*)	60.4 (16.5)	38.8 (14.4)
Male	16 (45.7)	20 (69.0)
Time of admission from symptom onset, days, mean (SD)	11.6 (8.4)	6.4 (2.2)
Length of hospitalization, days, mean (*SD*)	11 (6.6)	8.8 (4.0)
Time of positive PCR result, days, mean (*SD*)	13.0 (8.3)	4.2 (3.6)
Time of symptom onset, days, mean (*SD*)	27.1 (9.8)	13.3 (2.3)
30-day mortality	6 (17.1)	0 (0)
Hospitalization in ICU	7 (20)	0 (0)
History of travel to Hubei Province during COVID-19 outbreak	15 (41.7)	14 (48.3)
Contact with confirmed patients	17 (47.2)	6 (20.7)
Fever	28 (80)	15 (51.7)
Dry cough	33 (94.3)	18 (62.1)
Fatigue	13 (37.1)	6 (20.7)
Diarrhea	6 (17.1)	10 (34.5)
Shortness of breath	12 (34.3)	1 (3.4)
Expectoration	10 (28.6)	1 (3.4)
COPD	2 (5.7)	1 (3.4)
Diabetes	16 (45.7)	1 (3.4)
Hypertension	10 (28.6)	3 (10.3)
Cardiovascular disease	6 (17.1)	0 (0)
Hepatitis B	1 (2.9)	2 (6.9)
Hypoalbuminemia	8 (22.9)	1 (3.4)

COPD, chronic obstructive pulmonary disease; CT, computed tomography; ICU, intensive care unit; SD, standard deviation.
[a]*Total number of patients with available data.*

the viral RNA loads or positive PCR duration between the sputum and throat swab samples from 27 patients that had paired sputum and throat swab samples (**Figures 1D** and **2D**).

Correlation and Comparison of Viral Duration for Patients by Ward and Severity Classification

To examine viral presence, we compared the duration in days for positive C_t values according to the type of ward and severity classification of the patient. The duration for available positive C_t values (47 patients and 8 asymptomatic carriers) in the sputum, throat swab, and stool samples was longer for patients in the ICU (n=7) than for those in the general ward (n=46) (median: 13 days [IQR=9.5–25.25 days] vs. median: 6.5 days, [IQR=2–14] p=0.0396, **Figure 3A**). In addition, the duration until available positive C_t values were acquired was longer among severe cases (n=30) than those among mild cases and asymptomatic carriers (n=23) (median=12 [IQR=8–18.85 days] vs. median=2 [IQR=1–4.25 days], p<0.001, **Figure 3B**). There was a mutual linear positive relationship among the number of days until a positive C_t value, hospitalization days, and days from symptom onset to SARS-CoV-2 detection (**Figures 3C, D**).

Characteristics of Asymptomatic Carriers and Confirmed COVID-19 Cases Based on Antibodies

Among the 170 returning healthy travelers, 12 individuals had serum samples that tested positive for SARS-CoV-2 based on specific IgM or IgG antibodies. Further, eight throat swab samples from these individuals were positive for SARS-CoV-2 on rRT-PCR. The positive antibody and nucleic acid results were taken from different individuals among the 170 returning cases. Eight individuals with positive rRT-PCR results had no typical signs or symptoms of SARS-CoV-2, such as fever or cough, within the 14-day mandated quarantine period. Their chest CT scans showed normal imaging features (**Figure 4A**), and their laboratory test results were within normal range (**Table 2**). These 8 (4.7%) individuals were diagnosed as asymptomatic carriers. The IgM and IgG positivity rates were 12.8% (12/94). Among the 56 patients, 3 suspected patients with both positive IgM and IgG results but negative rRT-PCR results were diagnosed with COVID-19 according to the latest diagnostic criteria (version 7) (Jin et al., 2020). These 3 individuals had signs of infection, abnormal laboratory test results (**Table 1**), and abnormal findings on CT (**Figure 4B**).

Distribution of IgM and IgG Antibodies Among Confirmed Patients and Carriers

Among the 29 antibody-positive patients, only 55.2% (16/29) and 58.6% (17/29) were IgG and IgM positive, respectively, within 66 days from symptom onset. Meanwhile, 3 of the 8 asymptomatic carriers had positive serum IgM and IgG results. The sensitivity of rRT-PCR for COVID-19 diagnosis in the 66 days following symptom onset was 94.6% (53/56), which was significantly greater than the 62.2% (23/37) sensitivity of the antibody test ($\chi 2$ = 16.95, p<0.001). Of the 29 confirmed patients and 8 asymptomatic carriers among the 170 healthy traveling returnees, 20 patients had positive antibody test results during the 66 days from symptom onset and 3 asymptomatic carriers showed IgM antibody response within 14 days. The median duration from symptom onset to IgG response was 30 days (IQR = 20–66 days), while it was only 23 days (IQR=12–28 days) for IgM-positive patients and asymptomatic carriers.

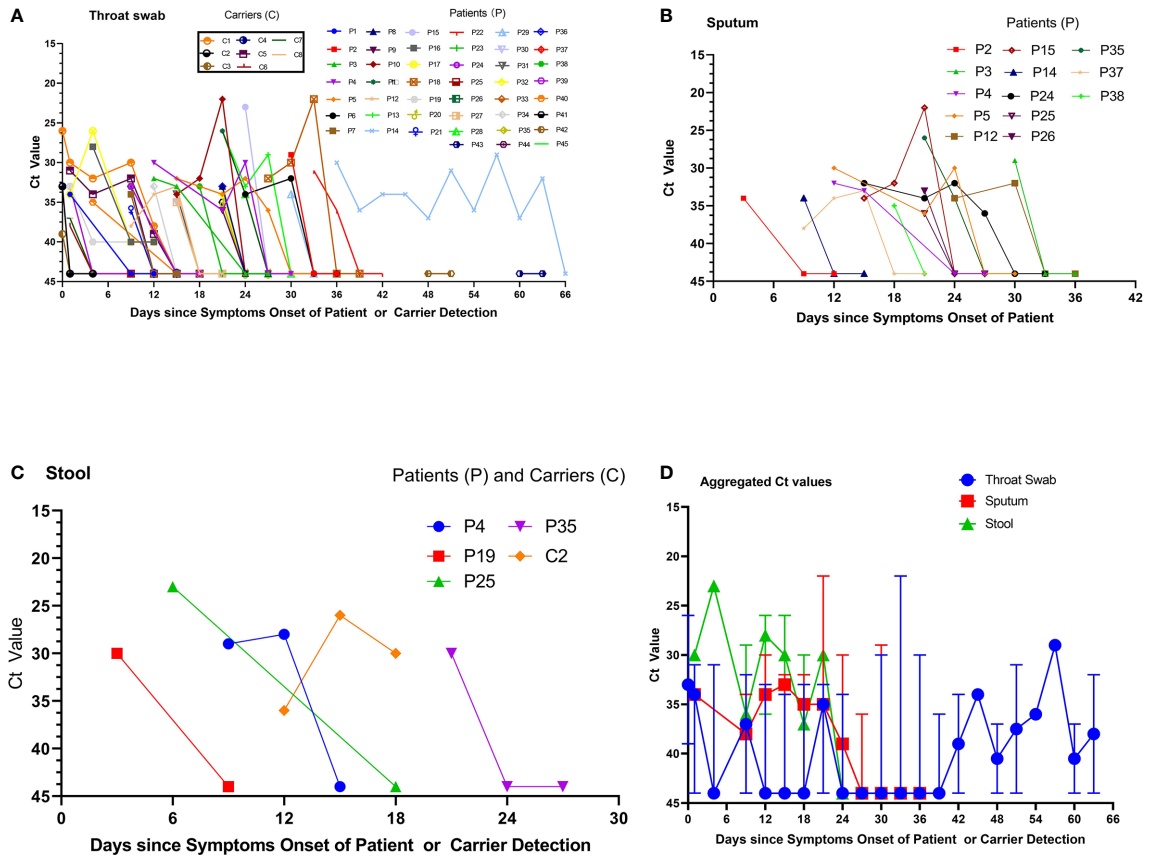

FIGURE 1 | Viral RNA loads detected in throat swabs, sputum samples, and stool specimens obtained from patients infected with severe acute respiratory syndrome coronavirus 2. **(A)** The cycle threshold (C_t) values of Orf1b in the reverse-transcription polymerase chain reaction (rRT-PCR) assay using throat swab specimens were obtained from the available 45 patients. The labels for the 8 returning asymptomatic carriers are provided in the separate text boxes. **(B)** The C_t values of sputum samples and **(C)** the C_t values of stool samples from four patients and one carrier. **(D)** The aggregated C_t values of Orf1b on the rRT-PCR assay for the 56 cases and 8 carriers, based on the duration from symptom onset to nucleic acid detection.

Comparison of the Duration to Positive IgG and IgM Results and Positive Ct Value

We compared the duration until positive IgM and IgG results were detected among the available 29 patients and 8 asymptomatic carriers and found a significant difference in the duration (p=0.004, **Figure 5A**). However, there was no significant difference in the duration until a positive IgM result and a positive C_t value were obtained (**Figures 5B, C**). Moreover, the duration until a positive C_t value was obtained was correlated with the duration to IgG response onset (R=0.6495, p=0.0163; **Figure 5D**). The IgM or IgG positivity rate was higher among severely ill patients than among mild cases and asymptomatic carriers: 71.4% (20/28) vs. 50% (5/10) (χ^2 = 8.828, p=0.002).

DISCUSSION

There are currently limited data on the proportion of asymptomatic carriers and antibody distribution among the returning healthy travelers from endemic areas. In this study, asymptomatic infections accounted for 4.7% (8/170) of the returning cases among the healthy population, and the antibody production rate was 12.8% (12/94). This study presents the viral RNA load kinetics and distribution of antibodies in patients and asymptomatic COVID-19 carriers in central China. We speculated that during the 2 weeks following disease onset, disease transmissibility was higher. With recovery onset, the production rate for the antibody response increased to 62.2% (23/37) at 66 days after symptom onset. These data indicate that compared with other known respiratory viral infectious diseases such as hand-foot-and-mouth disease, mumps, and rubella, this emerging infectious disease caused by SARS-CoV-2 has fewer asymptomatic carriers and generates a lower antibody response rate among healthy contacts. Moreover, a longer duration is required for antibody production among both recovered patients and healthy contacts. Further, it is unclear whether the IgG antibody is protective, indicating the complexity of COVID-19 transmission. Therefore, we believe that this new virus may have just started spreading from animals to humans and it may persist in humans for a long time. To the best of our best knowledge, this is the first report of 3 confirmed COVID-19 cases detected using specific IgM or IgG

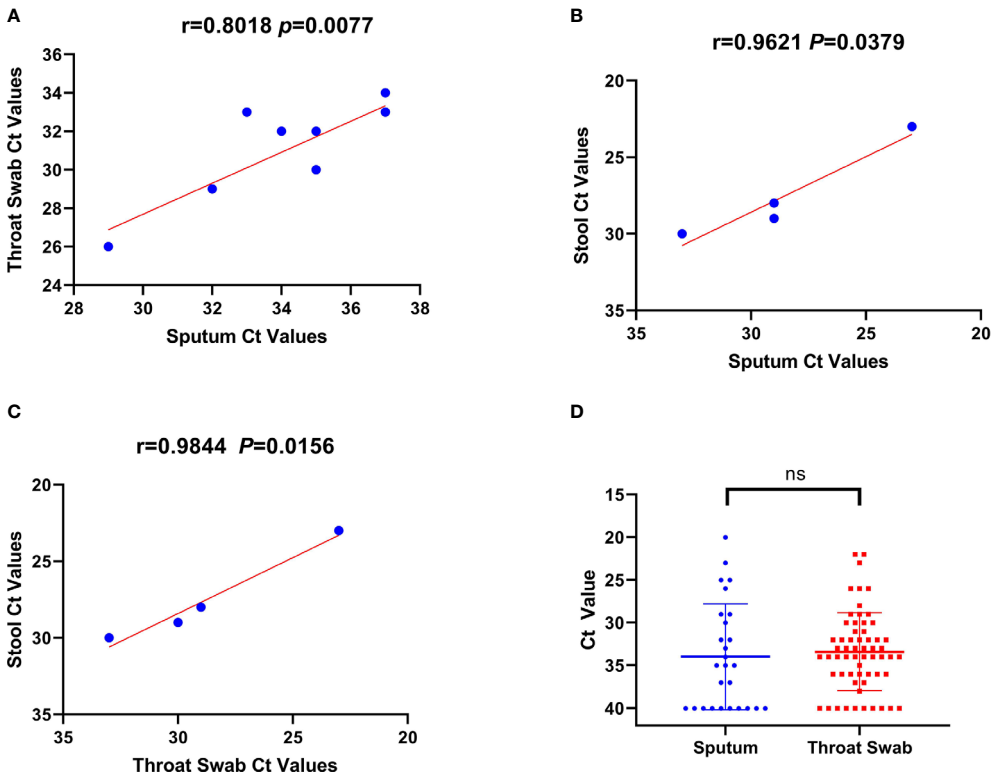

FIGURE 2 | Correlations and comparisons among viral RNA loads detected in throat swabs, sputum samples, and stool samples obtained from patients infected with severe acute respiratory syndrome coronavirus 2. **(A)** The correlation of viral RNA loads between the sputum and throat swab samples. This shows the cycle threshold (C_t) values of Orf1b in the reverse-transcription polymerase chain reaction assay that were detected from throat swab samples obtained from 56 patients and sputum samples from 13 patients. **(B, C)** The correlation of C_t values among throat swab, sputum, and stool samples. There was a positive linear relationship between C_t values for the viral RNA loads among the sputum, throat swab, and stool samples obtained from patients. **(D)** The comparison of C_t values for viral RNA loads between the throat swab and sputum samples. There was no significant difference in the C_t values between sputum and throat swab samples.

antibody tests from serum specimens with negative rRT-PCR results from throat swab samples among Chinese nationals who returned from endemic countries. Moreover, this is the first report of 8 (4.7%) asymptomatic carriers diagnosed 2 weeks after returning to China among the same population.

A positive IgM result indicates a recent infection with SARS-CoV-2. Additionally, IgG-positive results indicate that the body has begun to establish an immune defense. By testing patients and carriers for IgM and IgG antibodies and identifying time points at which they start producing antibodies, it is possible to monitor the extent to which COVID-19 spreads and the infection duration (Erensoy, 2020). A previous report showed that the rRT-PCR assays could be used to test throat swab samples to detect asymptomatic carriers with a travel history who later transmitted the infection to their contacts (Kannan et al., 2020; Lai et al., 2020). To better manage asymptomatic infections, the Chinese government has stipulated that from April 1, 2020, health authorities should report the daily number of new cases and outcomes of asymptomatic carriers nationwide. On February 18, 2021, there were 338 cases of asymptomatic carriers in China. Among them, 282 were returning asymptomatic carriers. However, the proportion of asymptomatic carriers and rate of antibody production among healthy contacts are unknown. Our findings reinforce that for identification and contact screening of individuals traveling from epidemic countries, it is important to conduct joint detection for SARS-CoV-2 using respiratory samples for nucleic acid testing and blood samples for IgM or IgG antibody testing (Bai et al., 2020).

Consistent with the findings in previous studies, the virus was detected in stool specimens in this study in addition to samples from the upper respiratory tract (Chen et al., 2020; Won et al., 2020). Diagnostic and treatment guidelines recommend detection of SARS-CoV-2 *via* throat swabs using nucleic acid testing (Jin et al., 2020). A stool sample for nucleic acid testing or a blood sample for specific IgM or IgG antibody detection should be obtained from patients highly suspected of COVID-19 but with continuously negative nucleic acid test results from throat swabs.

Unlike SARS-CoV and Middle East respiratory syndrome coronavirus infection (Chafekar and Fielding, 2018; Xu K. et al., 2020), the SARS-CoV-2 viral RNA load is highest during the early phase of the illness then continues to decrease until the end of the second week. In severe cases, the high viral RNA load can last up to 2 months. The duration of the virus infection is positively correlated with the disease severity and symptom duration, suggesting that we should detect, diagnose, and isolate the patients as early as possible to prevent community transmission and mortality.

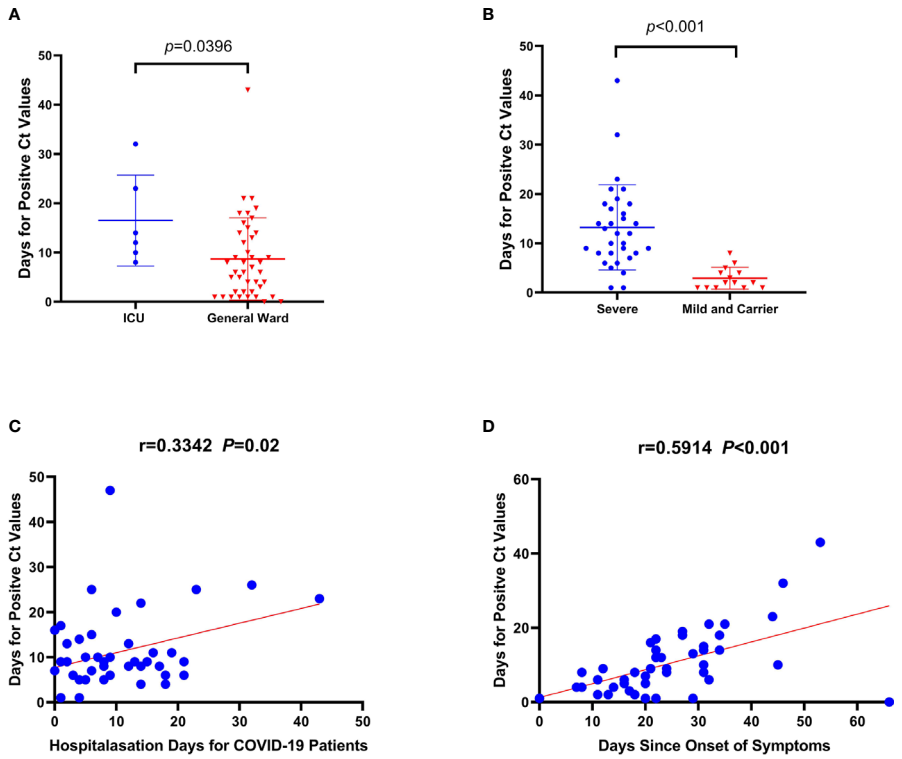

FIGURE 3 | Correlations and comparisons for viral RNA loads detected among coronavirus disease patients admitted to the intensive care unit (ICU) and the general ward, and between severely ill and mildly ill patients and returning carriers with severe acute respiratory syndrome coronavirus 2 infection. **(A)** Comparison of the duration (days) for obtaining positive cycle threshold (C_t) values since symptom onset between ICU and general ward patients. **(B)** Comparison of the duration (days) for positive C_t values between severe and mild patients, and among asymptomatic carriers. **(C, D)** The linear correlations between days for positive C_t values and hospitalization days, and days since symptom onset to nucleic acid detection, respectively. There was a significant difference in duration until positive C_t values between severe and mild patients, and for asymptomatic carriers. Similarly, the same significant difference in duration was observed for positive C_t values between patients from the ICU and the general ward. The duration until a positive C_t value in patients was related to hospitalization duration and the duration of symptoms in patients.

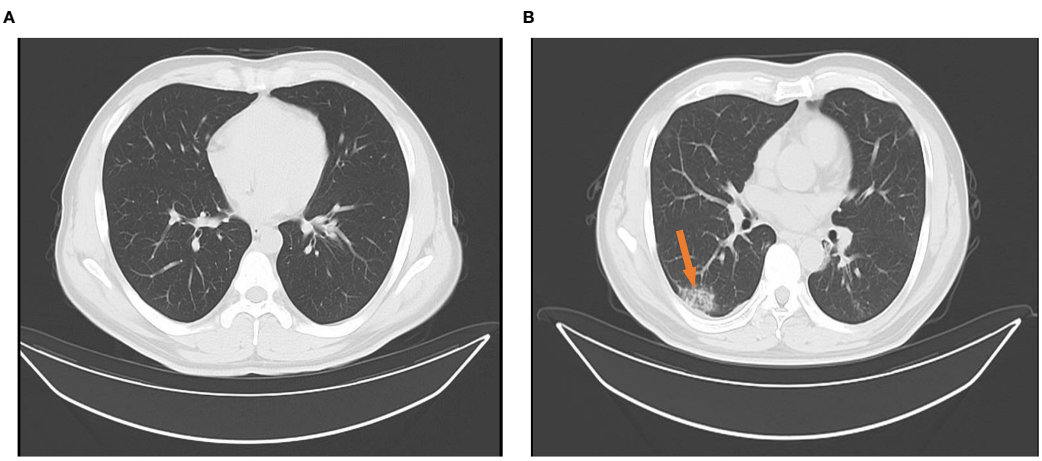

FIGURE 4 | Chest computed tomography findings for confirmed cases and returning carriers with severe acute respiratory syndrome coronavirus-2 infection. **(A)** Normal chest computed tomography characteristics among returning asymptomatic carriers with only positive reverse-transcription polymerase chain reaction (rRT-PCR) results and no signs or symptoms of coronavirus disease (COVID-19) within 14 days. The images were taken the day after hospital admission and after 14 days. **(B)** A solitary rounded ground-glass opacity in the upper lobe (arrow) of the left lung in a confirmed COVID-19 patient with only a positive specific IgM or IgG without sequential positive rRT-PCR assay results. The images were taken when patients were hospitalized or on the next day.

TABLE 2 | Laboratory and physical examination results for the eight asymptomatic carriers and three confirmed cases with severe acute respiratory syndrome coronavirus 2 diagnosed according to a positive antibody test.

Parameter	Reference Range	Carrier 1	Carrier 2	Carrier 3	Carrier 4	Carrier 5	Carrier 6	Carrier 7	Carrier 8	Patient 1*	Patient 2*	Patient 3*
PCR	–	+	+	+	+	+	+	+	+	–	–	–
IgM	–	–	–	–	–	–	+	+	+	+	+	+
IgG	–	–	–	–	–	–	–	–	–	+	+	–
C-reactive protein (mg/L)	0.0–10	0.6	0	0.3	8.8	2.5	2	0.5	1.5	35.4	48.6	3.3
Eosinophils (109/L)	0.02–0.52	0.05	0.02	0.1	0.06	0.38	0.13	0.04	0.12	0.02	0.01	0
Lymphocytes (109/L)	1.1–3.2	1.8	1.55	1.2	2.22	2.48	2.55	0.91	1.55	1.38	3.38	1.55
Neutrophils (109/L)	1.8–6.3	3.48	5.21	2.26	2.2	2.25	5.93	2.03	1.86	3.01	4.11	4.35
White blood cell count, (109/L)	3.9–9.9	5.5	6.8	4.1	3.4	5.5	9.1	3.4	3.9	5	8.1	6.2
D-dimer (mg/L)	0–0.5	0.19	0.19	<0.19	0.21	0.19	0.19	0.19	0.19	0.2	0.83	0.28
Procalcitonin (μg/L)	0–0.25	0.04	0.08	0.05	0.03	0.06	0.05	0.09	0.14	0.12	0.28	0.03
Chest CT	–	–	–	–	–	–	–	–	–	Abnormality	Abnormality	Abnormality
Signs and symptoms	–	–	–	–	–		–	–	–	Fever, Cough	Fever, Cough	Fever, Cough

**Two sequential negative PCR results for severe acute respiratory syndrome coronavirus 2 with an interval of more than 24 hours. +: positive; -: negative. PCR, polymerase chain reaction; CT, computed tomography; Ig, immunoglobulin.*

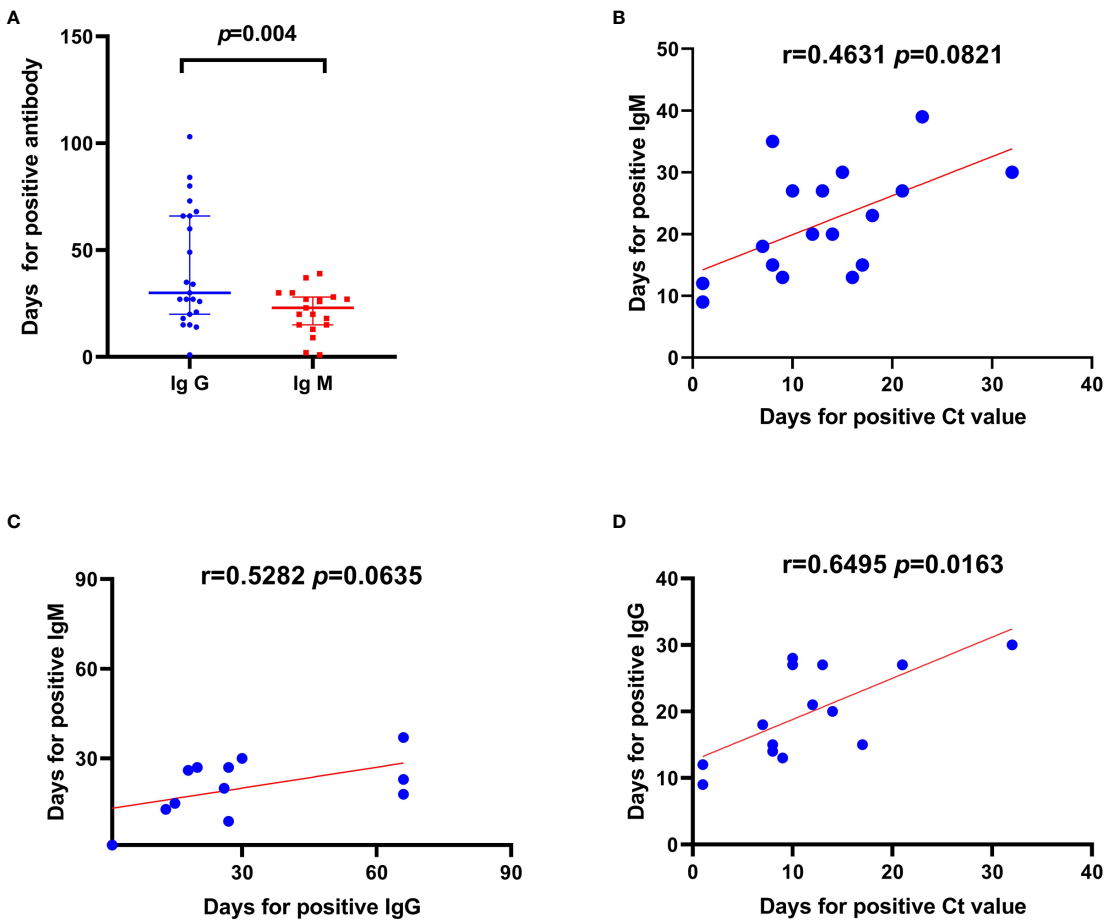

FIGURE 5 | Correlations and comparisons in duration to positive IgG and IgM result and duration until a positive cycle threshold (C_t) value was detected from patients with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection. **(A)** Comparison in duration (days) until a positive antibody test since symptom onset between IgG and IgM. **(B)** Linear correlations between duration until positive C_t values and duration until positive IgM. Linear correlations between duration until C_t value positivity and IgM **(C)** and IgG **(D)** positivity. There was a significant difference in the duration until C_t value positivity between IgM and IgG positivity. Moreover, the duration until C_t value positivity was correlated to the duration until IgG positivity in patients with SARS-CoV-2 infection.

This study has some limitations. First, the patients may not be representative of the general population of COVID-19 patients in China (Fan et al., 2020; Wu and McGoogan, 2020). Second, we cannot estimate the time point that these patients were exposed to the virus and when viral shedding *via* respiratory secretions and stool started. Third, the virus was not cultured from respiratory secretions and stool specimens because we do not have a professional Bio-safety Level 3 laboratory in our hospital (Xu X. et al., 2020). Finally, there were no sequential IgM or IgG antibody distribution results available for the SARS-CoV-2 infected patients throughout the duration of their illness. The IgG and IgM positivity rates only accounted for 51.4% and 62.6% of the 29 confirmed patients and 8 carriers within 66 days following symptom onset, respectively. The IgM antibody is known to be produced in the early stages of an infectious disease, whereas the IgG antibody is produced during the recovery period (Kam et al., 2020; To et al., 2020). We found that the median duration from symptom onset to IgG and IgM positivity was 30 and 23 days, respectively. This indicated that in COVID-19, initiation of IgG antibody production is longer than that for IgM production at 30 days versus earlier than 23 days. More samples should further be observed to confirm the phenomenon (Amanat et al., 2020).

In conclusion, we found that there are fewer asymptomatic COVID-19 carriers among the returning healthy travelers. Additionally, there are lower rates of antibodies produced among recovered patients and the contacted healthy population. These findings indicate the complexity of COVID-19 transmission and suggest that this infectious disease is likely to occur in humans for a long time.

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.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Ethics Board of Henan Provincial People's Hospital (20190050). The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

YY and HW designed the study, analyzed the data and prepared the manuscript. JinZ and WL contributed to the collection and interpretation of the laboratory and clinical data. NJ and JX analyzed the antibody data of patients and carriers. GL, YL, SW, YW, and LL were involved in the project management and organizational work. BM and JiaZ collected data and EF reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Clinical Significance of an IgM and IgG Test for Diagnosis of Highly Suspected COVID-19

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Background: Nucleic acid detection and CT scanning have been reported in COVID-19 diagnosis. Here, we aimed to investigate the clinical significance of IgM and IgG testing for the diagnosis of highly suspected COVID-19.

Methods: A total of 63 patients with suspected COVID-19 were observed, 57 of whom were enrolled (24 males and 33 females). The selection was based on the diagnosis and treatment protocol for COVID-19 (trial Sixth Edition) released by the National Health Commission of the People's Republic of China. Patients were divided into positive and negative groups according to the first nucleic acid results from pharyngeal swab tests. Routine blood tests were detected on the second day after each patient was hospitalized. The remaining serum samples were used for detection of novel coronavirus-specific IgM/IgG antibodies.

Results: The rate of COVID-19 nucleic acid positivity was 42.10%. The positive detection rates with a combination of IgM and IgG testing for patients with COVID-19 negative and positive nucleic acid test results were 72.73 and 87.50%, respectively.

Conclusions: We report a rapid, simple, and accurate detection method for patients with suspected COVID-19 and for on-site screening for close contacts within the population. IgM and IgG antibody detection can identify COVID-19 after a negative nucleic acid test. Diagnostic accuracy of COVID-19 might be improved by nucleic acid testing in patients with a history of epidemic disease or with clinical symptoms, as well as CT scans when necessary, and serum-specific IgM and IgG antibody testing after the window period.

Keywords: COVID-19, nucleic acid test, IgM, IgG, CT scan

INTRODUCTION

COVID-19 was named by the World Health Organization on January 12, 2020. Coronaviruses are a large family of viruses that cause colds and more serious diseases (1). COVID-19 is caused by a novel coronavirus strain that had not previously been found in humans. Common signs of infection include respiratory symptoms, fever, shortness of breath, and dyspnea. In severe cases, infection can cause pneumonia, acute respiratory syndrome, kidney failure, and even death. There is currently no specific treatment for COVID-19 (2). However, many symptoms can be managed and must be treated according to the clinical situation of each patient. The main routes of transmission of COVID-19 are respiratory droplets and contact transmission. Aerosol and fecal-oral routes of transmission must be further clarified. Epidemiological investigations have shown that cases can be traced to close contact with individuals with confirmed infection (3, 4).

According to the sixth edition of the diagnostic criteria, COVID-19 cases are divided into two categories: suspected cases and confirmed cases. As of midnight on February 28, 2021, a total of 89,912 confirmed cases had been reported in China; 85,066 cases had been cured, and 4,636 deaths had occurred. COVID-19 exhibited a sudden outbreak worldwide (5). Timely and accurate diagnosis is crucial for detection and patient therapy. However, in clinical practice, detection standards have varied with the rapidly growing awareness of COVID-19. Nucleic acid detection, chest CT, epidemiological history, and clinical manifestations are recognized as the diagnostic basis (6, 7). However, nucleic acid detection has the limitations of operator errors, time consumption, and proneness to contamination. The specificity of CT results is also limited. IgM/IgG antibody detection has the advantage of being simple and easy to perform, and has high sensitivity.

In our study, we aimed to provide a quick, simple, and accurate diagnostic method by evaluating the clinical significance of IgM and IgG for the diagnosis of highly suspected COVID-19 cases.

TABLE 1 | Characteristics and clinical features of patients with suspected COVID-19.

Characteristic/clinical features	*n*	%
Sex		
Male	24	42.11
Female	33	57.89
Age in years		
<18	4	7.02
18–45	43	75.44
46–65	10	17.54
Clinical symptoms		
Fever	35	61.4
Cough	27	47.37
Fatigue	5	8.77
Shortness of breath	1	1.75
Asymptomatic	4	7.02
Others (headache, sore throat, diarrhea, and so on)	8	14.04
Imaging findings		
Characteristic changes	40	70.18
Normal	17	29.82

MATERIALS AND METHODS

A total of 63 patients with suspected COVID-19 were observed, 57 of whom were finally enrolled, including 24 males and 33 females who were 2–63 years of age (8). Six patients were excluded because of a lack of serum samples. The characteristic features of the patients are described in **Table 1**. Selection was performed according to the diagnosis and treatment protocol for COVID-19 (trial sixth edition) released by the National Health Commission of the People's Republic of China. The patients who met the standards for suspected COVID-19 were enrolled, and those who did not were excluded. Suspected cases of COVID-19 were defined according to the presence of at least one of the following clear epidemiological history criteria: (1) a history of travel or residency in Wuhan or the surrounding area, or in communities with COVID-19 cases within 14 days before

TABLE 2 | Exposure times for NAAT and serological tests.

Groups	Sample number	Exposure time, day			
		Mean ± SD	Range	Median (25%, 75%)	*S-W*-test
NAAT					
All	43	12.86 ± 9.94	1–34	12 (3, 20)	$P = 0.0041$
Positive	18	10.28 ± 7.15	1–34	9 (3, 17.5)	$P = 0.0560$*
Negative	25	14.72 ± 12.31	1–21	14 (3.5, 24.5)	$P = 0.0301$
Serological test					
All	43	23.21 ± 8.48	6–39	24 (17, 29)	$P = 0.4585$*
Positive	34	22.82 ± 7.93	10–39	24 (17.75, 29)	$P = 0.4670$*
Negative	9	24.67 ± 10.70	6–39	26 (14.5, 35.5)	$P = 0.5789$*

$P > 0.05$ means the data are normally distributed. Nucleic acid amplification tests, NAAT.

onset; (2) a history of contact with people with COVID-19 (positive nucleic acid test) within 14 days before onset; (3) a history of contact with patients infected with COVID-19 from Wuhan and surrounding areas, or a history of contact with people with fever or respiratory symptoms from communities with COVID-19; and (4) cluster onset. In addition, patients were required to have the following clinical manifestations: (1) fever and/or respiratory symptoms; (2) imaging features of COVID-19; and (3) normal or diminished white blood cells in early stages of disease and a diminished lymphocyte count. If there was no clear epidemiological history, the above three clinical manifestations were necessary for inclusion. This was a retrospective study approved by the Ethics Committee of Shenzhen Hospital, Southern Medical University (NYSZYYEC20200009). The requirement for informed consent was waived because the data were anonymous. Study participants shared the results in strict accordance with the rules of the Ethics Committee of Shenzhen Hospital, Southern Medical University.

Laboratory Examination

The routine blood parameters were detected on the second day of hospitalization. The remaining serum samples were used for detection of IgM and IgG. Primary screening through nucleic acid amplification from pharyngeal swabs was performed with two kits from six companies (DAAN, Sansure Biotech, BGI, ShangHai ZJ Biotech, Geneodx, and Biogerm) in ∼20 hospitals in ShenZhen. The time after SARS-CoV-2 exposure to nucleic acid amplification tests (NAAT) and serological tests are described in **Table 2**.

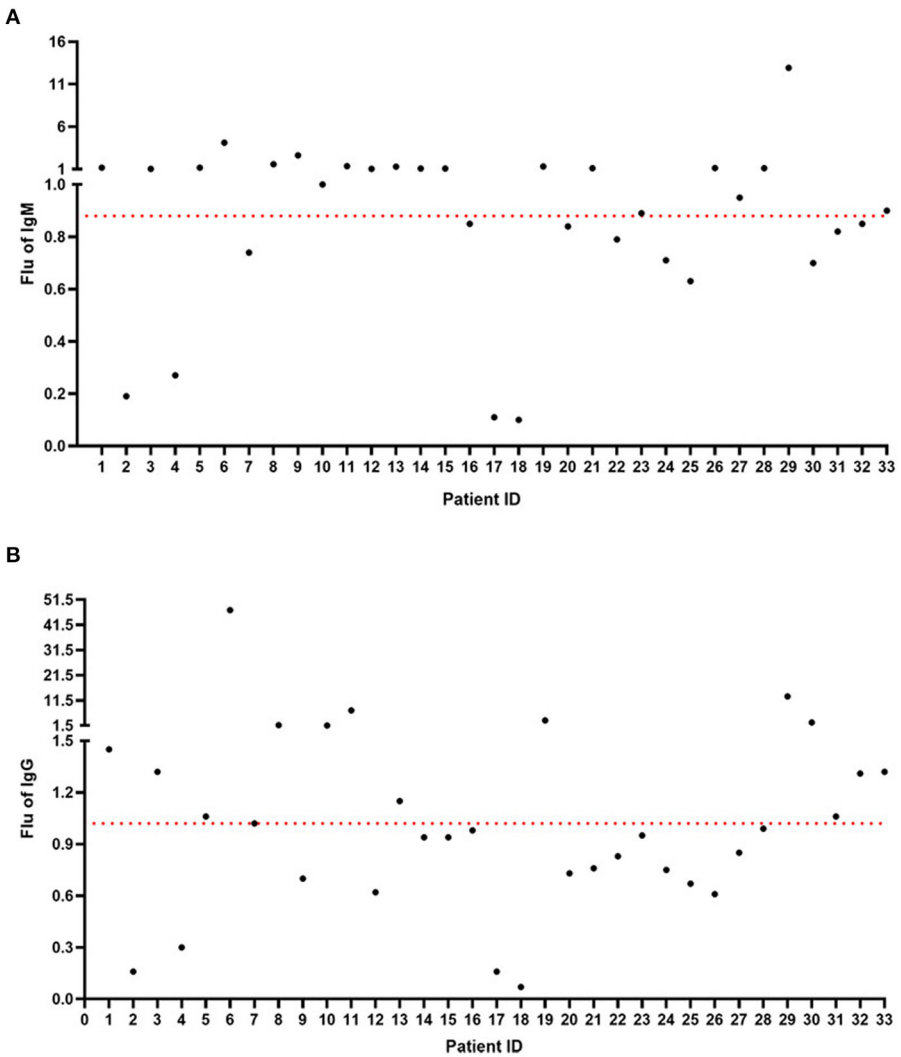

FIGURE 1 | IgM and IgG detection among the 33 patients with negative COVID-19 nucleic acid results. **(A)** The IgM-value of 20 patients was more than 0.88 U/L. **(B)** The IgG-value of 15 patients was more than 1.02 U/L.

Most serum samples were obtained 2 weeks after virus exposure; there was only 1 in 6 days and 4 within 2 weeks. The rest of the serum samples were obtained between 14 and 39 days, which is a good detection window period for IgM/IgG (9, 10). A COVID IgM/IgG antibody kit, which was sent to BIMT (Beijing Institute of Medical Device Testing) for product verification, was used with a Time-Resolved Immunofluorescence Analyzer to perform fluorescence immunochromatographic assays (Beijing Diagreat Biotechnologies Co., Ltd., Lot: 20200214, Beijing, China). The procedures of nucleic acid, IgM, and IgG detection were performed strictly according to the manufacturer's manual. A total of 242 healthy people without related diseases were tested, and the values were measured. The 95% confidence intervals for IgM and IgG were 0.44–0.88 U/L and 0.50–1.02 U/L, respectively. These results provided by Beijing Diagreat Biotechnologies Co., Ltd. suggested that the cutoffs for IgM and IgG were 0.88 and 1.02 U/L.

Data Analysis

Statistical analyses were performed in statistical analysis system software SPSS 19.0. Count data are expressed as percentages. The Shapiro–Wilk normality test was used to evaluate whether the data were normally distributed. Normally and non-normally distributed data are presented as mean ± SD and medians (25th percentile and 75th percentile). Non-parametric tests and two-sided χ^2-tests were used to compare the differences between groups, and a P-value < 0.05 was considered statistically significant.

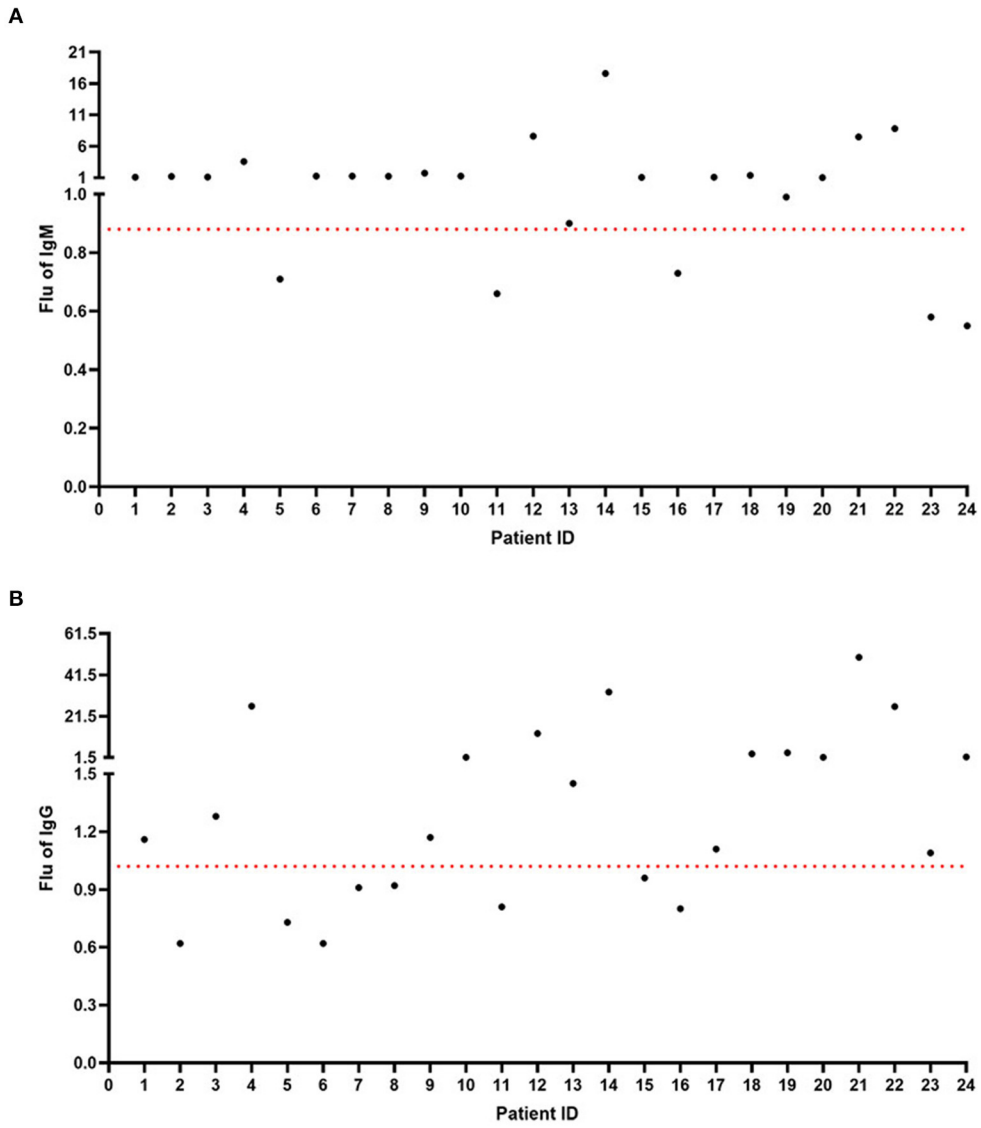

FIGURE 2 | IgM and IgG detection among the 24 patients with positive COVID-19 nucleic acid results. **(A)** The IgM-value of 19 patients was more than 0.88 U/L. **(B)** The IgG-value of 16 patients was more than 1.02 U/L.

RESULTS

Clinical Characteristics and COVID-19 Nucleic Acid Testing

According to the diagnostic standards for suspected COVID-19, 57 patients were enrolled in our study. All 57 patients underwent three nucleic acid tests, and each time, the results were confirmed with two COVID-19 nucleic acid test kits. Among the 57 patients, 24 patients had a positive nucleic acid test, and 33 patients had a negative nucleic acid test the first time, and all 57 patients had a negative nucleic acid test the second and third times. The positivity rate of COVID-19 nucleic acid testing in the 57 suspected COVID-19 cases was 42.10%.

IgM and IgG Single Detection of COVID-19

According to the nucleic acid test results, we performed IgM and IgG detection through the Diagreat company. As shown in **Figure 1A**, among the 33 patients with negative COVID-19 nucleic acid results, the IgM value of 20 patients was more than 0.88 U/L, and the positivity rate was 60.61%. As shown in **Figure 1B**, the IgG-value of 15 patients was more than 1.02 U/L, and the positivity rate was 45.45%. As shown in **Figure 2A**, among the 24 patients with positive COVID-19 nucleic acid results, the IgM value of 19 patients was more than 0.88 U/L, and the positivity rate was 79.17%. As shown in **Figure 2B**, the IgG-value of 16 patients was more than 1.02 U/L, and the positivity rate was 66.67%.

Comparison of Exposure Times for NAAT and Serological Tests

The time from the first exposure to infection to nucleic acid testing ranged from 1 to 34 days. One patient had a negative nucleic acid result 34 days after exposure; however, the IgM detection result was positive. The results were partly different from the current understanding that the median incubation period for COVID-19 is 3 days, with a minimum of 0 days and a maximum of 24 days.

A comparison of exposure times for NAAT and serological tests (**Figure 3**) revealed no statistically significant differences in SARS-CoV-2 exposure times between the NAAT positive group and the NAAT negative group. The results were the same as those of the serological tests.

Combination of IgM and IgG Detection of COVID-19

Figure 4A shows the combination of IgM and IgG detection of COVID-19. Among the 33 patients who had a negative nucleic acid test, the percentages of IgM(+)IgG(+), IgM(−)IgG(+), IgM(+)IgG(−), and IgM(−)IgG(−) were 36.37, 12.12, 24.24, and 27.27%, respectively. The positive diagnostic rate with a combination of IgM and IgG detection for 33 patients with negative COVID-19 nucleic acid test results was 72.73%. Compared with a negative nucleic acid test and IgM and IgG single detection, the combination of IgM and IgG detection had a significantly higher positivity rate ($P < 0.01$). As shown in **Figure 4B**, in the 24 patients with a positive nucleic acid test, the combination of IgM and IgG detection of COVID-19 resulted in percentages of IgM(+)IgG(+), IgM(−)IgG(+), IgM(+)IgG(−), and IgM(−)IgG(−) of 62.50, 8.33, 16.67, and 12.50%, respectively. The positive diagnostic rate of a combination of IgM and IgG detection for 24 patients with COVID-19 negative nucleic acid test results was 87.50%. Compared with a nucleic acid positive test and IgM and IgG single detection, the combination of IgM and IgG also had a significantly higher positivity rate ($P < 0.01$).

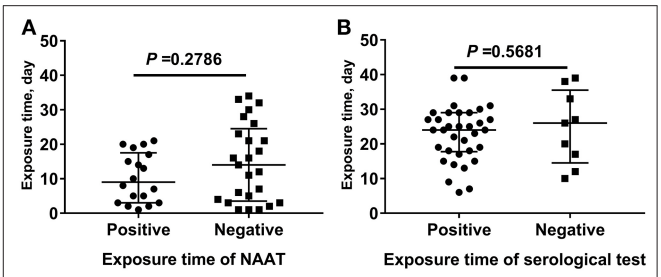

FIGURE 3 | Comparison of exposure times for NAAT and serological tests. **(A)** The SARS-CoV-2 exposure time between the nucleic acid amplification test (NAAT) positive group and the NAAT negative group. **(B)** The SARS-CoV-2 exposure time between the serological test positive group and the serological test negative group.

CT Scan of Two Special Patients

A patient (number 55, female, 62 years old) presented with fatigue and fever on February 19, 2020. The nucleic acid detection results of pharyngeal swabs were negative on February 19 and 20. Serum IgM and IgG results were positive, and the values were 7.49 and 50.03 U/L. Chest computed tomography (CT scan) was performed on February 20, 2020, and the results are shown in **Figure 5A**. Characteristic changes in positive imaging findings were observed. In the lower region of both lungs in this patient, the CT scan showed large fuzzy shadows and ground-glass opacity (GGO), and a slightly fan-shaped distribution. Clinical symptoms, serological tests, and characteristic changes in the CT imaging of this patient were consistent.

Another patient (number 39, female, 35 years old) presented with cough and diarrhea on February 3, 2020. The nucleic acid detection results of pharyngeal swabs were positive on January 28, 2020. Serum IgM and IgG results were negative, and the values were 0.71 and 0.73 U/L. A CT scan was performed on February 8, 2020, and the results are shown in **Figure 5B**. The CT results showed no clear lesions in both lungs. Serological tests and characteristic changes in CT imaging of this patient were consistent.

Association of CT Results With PCR Results and With Serological Results

Chest computed tomography (CT) scans of patients were assessed in the hospital. Characteristic changes of positive imaging findings included the following: multiple small patches and ground-glass opacity in both lungs, and infiltration and consolidation of opacity. Associations of CT results with PCR results and with serological results are shown in **Figure 6**. We found no statistically significant difference in the proportion

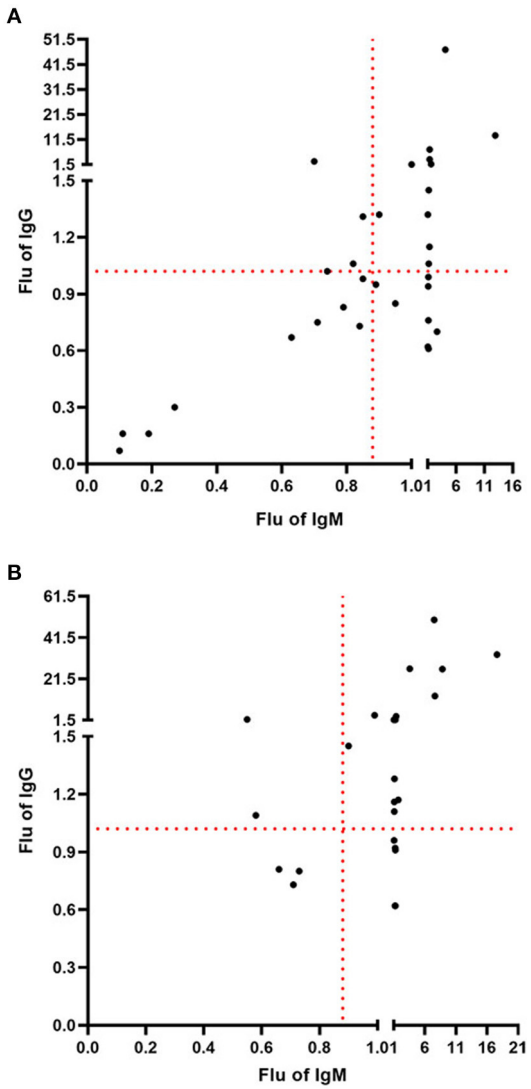

FIGURE 4 | Combination of IgM and IgG detection of COVID-19. **(A)** The positive diagnostic rate with a combination of IgM and IgG detection for 33 patients with negative COVID-19 nucleic acid test results was 72.73%. **(B)** The positive diagnostic rate with a combination of IgM and IgG detection for 24 patients with positive COVID-19 nucleic acid test results was 87.50%.

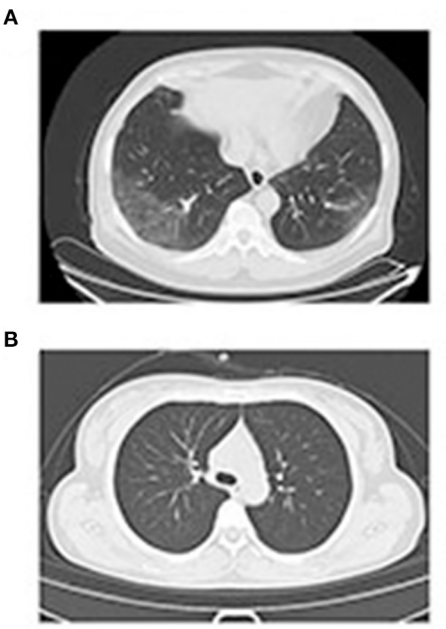

FIGURE 5 | Patient case CT scan. **(A)** For patient 55, the nucleic acid detection result was negative, but the IgM and IgG results were positive. In the lower regions of both lungs, large fuzzy shadows, GGO, and a slightly fan-shaped distribution were observed. **(B)** In patient 19, the nucleic acid detection result was positive, but the IgM and IgG results were negative, and no clear lesions were found in the lungs.

of positive NAAT between the positive and negative CT imaging groups. The results were the same as those for the serological tests.

DISCUSSION

In our study, the COVID-19 nucleic acid positivity rate in the 57 cases of suspected COVID-19 was 42.10%. The nucleic acid test results may be false negative or false positive. Throat swab samples were collected in our study, and prior studies have demonstrated that sample location is a very important factor in nucleic acid detection. In one study, researchers analyzed a total of 72 nasal swabs and 72 throat swabs and obtained 9 consecutive samples from each patient; they detected a higher viral load shortly after the onset of symptoms and found that the viral load in the nose was higher than that in the throat (11). Studies have confirmed that bronchoalveolar lavage fluid specimens show the highest positive rates (14 of 15; 93%), followed by sputum (72 of 104; 72%), nasal swabs (5 of 8; 63%), fibrobronchoscope brush biopsies (6 of 13; 46%), pharyngeal swabs (126 of 398; 32%), feces (44 of 153; 29%), and blood (3 of 307; 1%) (12, 13). A systematic review and meta-analysis of SARS-CoV-2 using RT-PCR in different types of clinical specimens also revealed that the lower respiratory tract had the highest positive rate followed by rectal swab then sputum (14). In order to increase the accuracy of detection, one nasopharyngeal swab and one oropharyngeal swab are suggested to be collected into a single collection tube at the same time (15). Moreover, in clinical practice, when collecting throat swab specimens, medical staff must wear protective clothing. In this study, different medical staff performed the sample collection, and this procedure might have affected the nucleic acid detection results. Specimen collection operators should be trained (15) and an amplification reagent with "internal standard" was suggested to check whether the sample is qualified (16). As shown in **Figure 5A**, the nucleic acid detection results for patient 55 were negative, but the IgM and IgG results were positive. Characteristic changes in positive CT imaging findings were

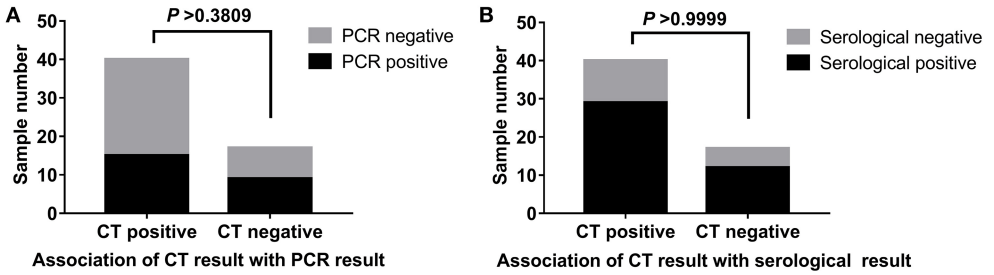

FIGURE 6 | Association of CT results with PCR results and with serological results. **(A)** The proportion of positive NAAT between the positive and negative CT imaging groups. **(B)** The serological results between the positive and negative CT imaging groups.

found. Finally, because of potential laboratory contamination, the positive results might have been false positive. As shown in **Figure 5B**, the nucleic acid detection result for patient 19 was positive, but the IgM and IgG results were negative, and the CT results showed no clear lesions in both lungs. One study has analyzed 126 German citizens who left Wuhan and were required to pass screening for clinical signs of infection: two passengers' nucleic acid tests were positive after quarantine for 14 days, but the two patients did not develop symptoms. The researchers reconfirmed the results through other methods. The results suggested that people with no fever, no symptoms, or only mild symptoms of infection may ignore their potential infectivity (17). Laboratory results for a COVID-19 nucleic acid positive group of 31 patients and a negative group of 23 patients have been found to be mainly characterized by diminished lymphocyte counts and elevated C-reactive protein levels; except for dyspnea, significant differences were observed in the clinical characteristics of the COVID-19 nucleic acid negative and positive groups (18). Studies have demonstrated that the clinical features of COVID-19 nucleic acid positive and negative patients are similar (19). We also studied the associations among CT results, PCR results, and serological results (**Figure 6**), and found no statistically significant difference in the proportion of positive NAAT between the positive and negative CT imaging groups. The results were the same as those for the serological tests. CT scans for the diagnosis of COVID-19 lack specificity (20). Faster and more accurate methods are urgently needed for the diagnosis of COVID-19. The positive diagnosis rates with a combination of IgM and IgG detection for patients with COVID-19 negative and positive nucleic acid tests were 72.73 and 87.50%, respectively. For 1 year, the SARS-CoV-2 antibody method including an enzyme-linked immunosorbent assay, a rapid immunochromatographic assay, and a chemiluminescent immunoassay has been applied and its clinical value is being evaluated (21). A study revealed that IgM/IgG-based detections can also result in false positive/false negative outcomes (22).

Some limitations are present in our study. First, the relatively small sample size, differences in IgM and IgG antigen binding sites, and differences in COVID-19 nucleic acid test design may have resulted in bias of the results. Second, the positivity rate of IgM and IgG tests may have been affected by the different times between viral exposure and detection. Earlier and different times should be examined, and the detection values of IgM and IgG should be further validated. Third, the detection value of IgM and IgG should be followed up in a future study.

CONCLUSION

Compared with nucleic acid detection, IgM and IgG detection may provide a quick, simple, and accurate detection method for suspected COVID-19 cases. IgM and IgG antibody detection can identify suspected cases with negative nucleic acid tests. Diagnostic accuracy of COVID-19 might be improved by nucleic acid testing in patients with a history of epidemic disease or with clinical symptoms, as well as CT scans when necessary, and serum-specific IgM and IgG antibody testing after the window 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

The studies involving human participants were reviewed and approved by the Ethics Committee of Shenzhen Hospital, Southern Medical University (NYSZYYEC20200009). Written informed consent for participation was not provided by the participants' legal guardians/next of kin because: This is a retrospective study and the data were anonymous, so the requirement for informed consent was therefore waived.

AUTHOR CONTRIBUTIONS

XJ, YT, JunW, KH, and YL contributed to the study design. JunlW and HZ contributed to data collection. JL and HH contributed to the collection of clinical specimens. XJ, ZC, and LZ contributed to experiments and data collection. PZ contributed to the data analysis. XJ, PZ, and YT contributed to the manuscript

preparation. All authors contributed to the article and approved the submitted version.

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COVID-19 Epidemic in Malaysia: Epidemic Progression, Challenges, and Response

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COVID-19 pandemic is the greatest communicable disease outbreak to have hit Malaysia since the 1918 Spanish Flu which killed 34,644 people or 1% of the population of the then British Malaya. In 1999, the Nipah virus outbreak killed 105 Malaysians, while the SARS outbreak of 2003 claimed only 2 lives. The ongoing COVID-19 pandemic has so far claimed over 100 Malaysian lives. There were two waves of the COVID-19 cases in Malaysia. First wave of 22 cases occurred from January 25 to February 15 with no death and full recovery of all cases. The ongoing second wave, which commenced on February 27, presented cases in several clusters, the biggest of which was the Sri Petaling Tabligh cluster with an infection rate of 6.5%, and making up 47% of all cases in Malaysia. Subsequently, other clusters appeared from local mass gatherings and imported cases of Malaysians returning from overseas. Healthcare workers carry high risks of infection due to the daily exposure and management of COVID-19 in the hospitals. However, 70% of them were infected through community transmission and not while handling patients. In vulnerable groups, the incidence of COVID-19 cases was highest among the age group 55 to 64 years. In terms of fatalities, 63% were reported to be aged above 60 years, and 81% had chronic comorbidities such as diabetes, hypertension, and heart diseases. The predominant COVID-19 strain in Malaysia is strain B, which is found exclusively in East Asia. However, strain A, which is mostly found in the USA and Australia, and strain C in Europe were also present. To contain the epidemic, Malaysia implemented a Movement Control Order (MCO) beginning on March 18 in 4 phases over 2 months, ending on May 12. In terms of economic impacts, Malaysia lost RM2.4 billion a day during the MCO period, with an accumulated loss of RM63 billion up to the end of April. Since May 4, Malaysia has relaxed the MCO and opened up its economic sector to relieve its economic burden. Currently, the best approach to achieving herd immunity to COVID-19 is through vaccination rather than by acquiring it naturally. There are at least two candidate vaccines which have reached the final stage of human clinical trials. Malaysia's COVID-19 case fatality rate is lower than what it is globally; this is due to the successful implementation of early preparedness and planning, the public health and hospital system, comprehensive contact tracing, active case detection, and a strict enhanced MCO.

Keywords: coronavirus, pandemic, movement control order, Malaysia, COVID-19

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INTRODUCTION

Pandemics of the 20–21st century

Throughout the 20th century, three influenza pandemics occurred over several decades; the most severe was the "Spanish Flu" (caused by an A(H1N1) virus), estimated to have resulted in 20–50 million deaths in 1918–1919. Milder pandemics occurred subsequently in 1957–1958 (the "Asian Flu" caused by an A(H2N2) virus) and in 1968 (the "Hong Kong Flu" caused by an A(H3N2) virus), which were estimated to have caused 1–4 million deaths each.

An influenza pandemic caused by the A(H1N1) virus erupted in the 21st century (2009–2010). For the first time, a pandemic vaccine was developed, produced, and deployed in multiple countries during the first year of the pandemic. The H1N1 pandemic was however milder than the ones before, estimated to cause between 100,000 and 400,000 deaths globally in its first year (1).

History of Epidemics in Malaysia

Newspapers in Malaya had as early as September 1918 carried reports of the raging influenza pandemic in South Asia. The only details of the spread of the epidemic were substantially documented from the medical report of the British North Borneo from June to November. The account indicates most possibly the transmission of the influenza virus from the maritime and land routes ferrying passengers and migrant workers from the South China Sea to the rest of the hinterland. This was the Spanish Flu brought in from Europe which resulted in 34,644 deaths among the 3,584,761 population then, giving a fatality rate of almost 1% (2).

Over a period of 8 months in 1999, the Nipah virus infected 265 Malaysians and killed 105. Malaysia's response was delayed because it was initially misidentified as Japanese encephalitis. The SARS outbreak of 2003, which infected 8,098 and killed 774 people globally, claimed only two lives in Malaysia. The present COVID-19 was brought into Malaysia by Chinese tourists from Wuhan *via* Singapore and Malaysian citizens who traveled to high COVID-19–infected countries such as Italy and Indonesia.

Origin of COVID-19

Pneumonia of unknown etiology was detected in Wuhan City, Hubei Province of China on December 31, 2019, whereby, the WHO China Country Office was informed. From December 31, 2019 through January 3, 2020, a total of 44 cases of pneumonia of unknown etiology were reported in China, of which the causal agent was not identified.

On January 7, 2020, the Chinese authorities identified a new type of coronavirus. China shared the genetic sequence of the novel coronavirus for countries to use in developing specific diagnostic kits on January 12, 2020. WHO later received further detailed information from the National Health Commission China on January 11–12, 2020 that the outbreak was associated with exposures in a seafood market in Wuhan City.

On January 13, 2020, the Ministry of Public Health, Thailand reported the first imported case of laboratory-confirmed novel coronavirus case (2019-nCoV) from Wuhan, Hubei Province, China. On January 15, 2020, the Ministry of Health, Labor and Welfare, Japan reported an imported 2019-nCoV from Wuhan.

As of January 20, 2020, a total of 282 confirmed cases of 2019-nCoV have been reported in China (278 cases), Thailand (2 cases), Japan (1 case), and Korea (1 case). Cases in Thailand, Japan, and Korea were exported from Wuhan City, China. Among the 278 confirmed cases in China, 258 cases were reported from Hubei Province, 14 from Guangdong Province, 5 from Beijing Municipality, and 1 from Shanghai Municipality.

On January 30, 2020, WHO declared the outbreak of COVID-19 a public health emergency of international concern. WHO's greatest concern was the potential for the virus to spread to countries with weaker health systems which will be ill-prepared to deal with the outbreak (3).

A modeling study published in *The Lancet* on January 31 estimated that, on average, every infected individual is infecting 2.68 additional individuals (4). The specifics of how the virus is transmitted from person to person have also yet to be defined. It is still unknown whether the virus can be spread by the fecal–oral route, for example. The disease pathogenesis is shrouded in mystery. How does the virus replicate in different sites, and how does that relate to the severity of disease?

It was also uncertain how long patients remain infectious. Thus, it is difficult to decide on the period of isolation. There is also the possibility that the virus is mutating into transmissible forms. Older patients with comorbidities seem to be most at risk of developing severe disease as a result of infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Information from China stated that case fatality is around 2%.

Transmission of COVID-19

COVID-19 is largely spread *via* droplets in the air and is a respiratory illness. These droplets are typically expelled when an infected person coughs or sneezes. They become increasingly less infectious once symptoms develop, so that a person's viral load declines steadily. However, infected persons keep shedding the virus after they recover from COVID-19 for around 2 weeks in both their saliva and stools. Infected persons with mild or no symptom can have a very high viral load in their upper respiratory tracts. They can shed the virus through spitting, touching their mouths, or noses or possibly through talking. SARS-CoV-2 has also been found to persist for days on surfaces (5).

Fever, dry cough, and tiredness are the most commonly reported symptoms, and in mild cases people may get just a runny nose or a sore throat. In the most severe cases, infected persons experience breathing difficulty, and ultimately organ failure may develop. Some cases are fatal. The authorities in China have placed the Wuhan population under quarantine or lockdown, and stopped trains and flights out of the city. They have suspended certain long-distance bus routes, including

those that depart or arrive in Beijing. On March 11, WHO announced the outbreak to be a pandemic, which means that multiple countries are seeing sustained transmission between people, causing disease or death (**Figure 1**) (6).

COVID-19 EPIDEMIC PROGRESSION IN MALAYSIA

First Wave of Outbreak (January 25 to February 15, 22 Cases)

The first three cases of COVID-19 in Malaysia were imported cases, confirmed on January 25, 2020. Imported cases are defined as infection acquired from outside Malaysia with reference to the travel history of the individual case. The three cases were detected on tracing and screening after communication from the Singapore Ministry of Health that eight close contacts of a confirmed case of a Chinese nationality in Singapore had traveled into Johor, Malaysia. By February 15, the number of cases in Malaysia increased to 22, consisting of 12 persons under investigation (PUI), eight close contacts of confirmed cases, and two Malaysian evacuees of humanitarian aid mission from Wuhan, China. Most of the cases in the first wave were imported cases or of Chinese nationality (15 out of 22 cases) and close contacts, while only two cases were of local transmission (7). After the 22nd case, no new case was reported for 11 days, which formed the first wave of COVID-19 outbreak in the country. All cases of the first wave recovered from the infection.

The *Guidelines for COVID-19 Management in Malaysia (No. 05/2020)* was developed by the Ministry of Health Malaysia in response to the novel virus (8). The guidelines define PUI as those who "developed acute respiratory infection, and had traveled/resided in foreign countries, or being in close contact with confirmed cases within 14 days before onset, or attended an event associated with a known outbreak." Close contacts are described as those who "worked, traveled or lived together with a COVID-19 patient."

Second Wave of Outbreak (February 27 Onwards, 5,945 Cases by April 29)

The second wave of the outbreak started on February 27, and lasting until the present work was undertaken. From February 27, new cases began to appear as people who had international travel history to countries such as China, Japan, Italy, and Australia started to manifest symptoms. Clusters of cases began to form from the close contacts of confirmed cases who attended meetings and events together, which generated several generations of infections. The number of cases reached a total of 129 on March 10 from among the PUIs, close contacts, and evacuees of humanitarian aid missions.

The Sri Petaling Tabligh Cluster

On March 11, a sporadic COVID-19–positive case was detected among 600 surveillance sampling of patients with influenza-like illness (ILI) and severe acute respiratory infection (SARI). At the same time, the International Health Regulations (IHR) Focal Point (FP) in Brunei informed the IHR FP Malaysia that a COVID-19 case in Brunei had attended a tabligh (Islamic missionary) convention held at Masjid Jamek in Sri Petaling, Kuala Lumpur from February 27 to March 3, 2020. The event was purportedly attended by 14,500 Malaysians who had since gone back to their respective states throughout Malaysia (9). There were also 1,500 oversea attendees who had returned to their countries across Asia. The initially sporadic case was also linked to the tabligh convention in Sri Petaling.

Sri Petaling Tabligh became the largest cluster of COVID-19 infection that triggered local transmission across all states in Malaysia (**Figure 2**) (10). The Ministry of Health (MOH) Malaysia immediately urged all tabligh attendees to contact the local district health offices for screening and risk assessment (11). On March 15, the number of daily new cases surged from 41 to 190 cases from across all states, with most of them linked to the Sri Petaling Tabligh cluster. As the number of new cases continued to exceed 100, and totaling 553 cases on the next day, MOH Malaysia announced the country to be in the late containment phase of COVID-19 (**Figure 3**) (11), where prompt responses were necessary to stop the disease spread. Later, Malaysia announced the Movement Control Order (MCO), commencing on March 18 to contain the virus through social distancing strategy. On March 19, Malaysia recorded a total of 900 cases, which ranked Malaysia as the country with the fourth highest number of cases in Asia, and the first in Southeast Asia (12).

The Sri Petaling Tabligh cluster generated a number of subclusters especially at Islamic educational institutions (*madrasah*) in several states. Some of the significant subclusters (cases as of April 29) were at Sungai Lui in Hulu Langat, Selangor (157 cases); Sendayan, Negeri Sembilan (81 cases, 1 death); Jerantut, Pahang (81 cases); Rembau, Negeri Sembilan (53 cases); and Jasin, Melaka (40 cases) (13). A number of other tabligh clusters were also identified, namely, the Makasar Tabligh Johor (27 cases, 1 death), Makasar Tabligh Sabah (9 cases), and Pakistan Tabligh Sabah (6 cases) (13). Screening activity from targeted approach among madrasah and tahfiz schools reported 342 cases (5.5%) out of 6,229 students, teachers, and staff screened (14). A wedding reception in Bangi, Selangor was also connected with the cluster, reporting 96 cases which included a number of healthcare workers. By March 19, MOH managed to trace 10,650 of the Sri Petaling Tabligh attendees and detected 513 positive cases in the cluster (15).

As of April 29, a total of 33,577 people had been examined, and 32,590 samples had been taken from the Sri Petaling Tabligh cluster. The cluster reported 2,167 cases which represented 6.5% of total samples taken from the cluster, and 36% of the total 5,945 cases in Malaysia. The number of index cases from the cluster was 767 cases, and up to 5 generations chain of transmission among contacts (family members and friends) were traced in the cluster (16). On May 21, the percentage of cases from the cluster increased to 47% (3,347 cases) out of the total cases (7,059 cases) (17).

The Sri Petaling Cluster came to an end on July 8 (zero active case from the cluster) with the last case being reported on June 11 (18). This cluster represented 38.9% of the total positive

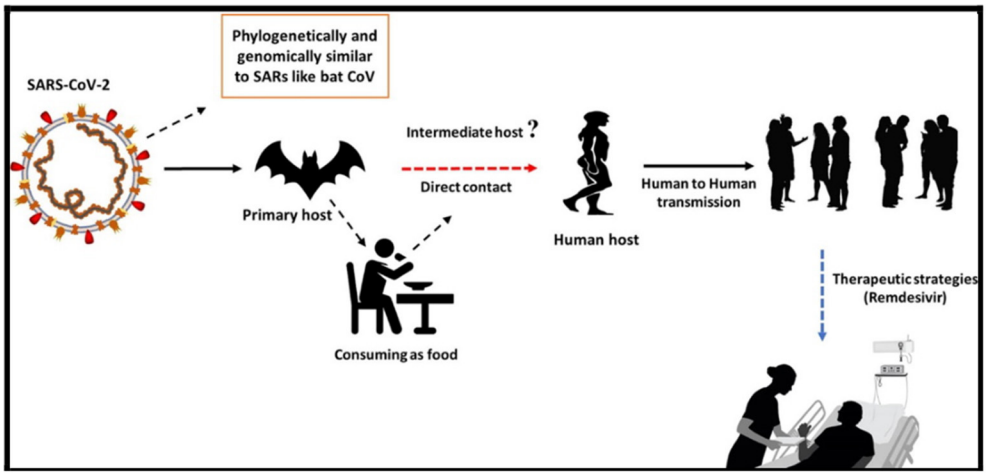

FIGURE 1 | The transmission of COVID-19 (6). License: CC BY-NC-ND 4.0.

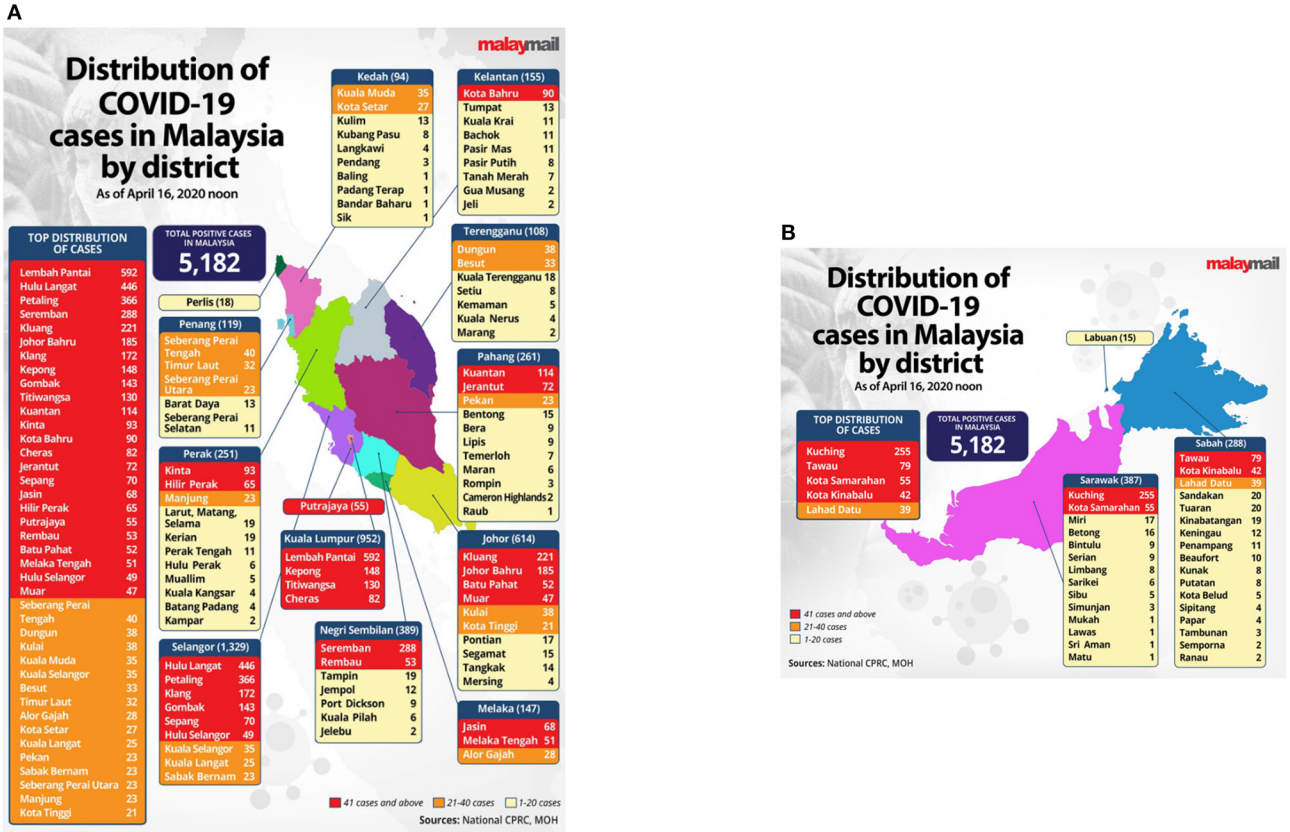

FIGURE 2 | Distribution of COVID-19 cases by district in Malaysia as of April 16, 2020. **(A)** Peninsular Malaysia, **(B)** island of Borneo, Malaysia (10). Source: Malay Mail. Permission has been obtained from the copyright holders.

cases in Malaysia at the time, with 2,550 (75.6%) Malaysian cases from seven states and 825 (24.4%) non-Malaysian cases from 28 countries. During the 4-month ordeal, a total of 42,023 samples were taken with a positive case rate of 8.03% (3,375 cases), case recovery rate of 98.99% (3,341 cases), case fatality rate of 1.01% (34 cases), and intensive care unit (ICU) treated case rate of 2.58% (87 cases) with 29 cases on ventilators. The number of deaths from this cluster constituted 28.1% of all deaths in Malaysia at

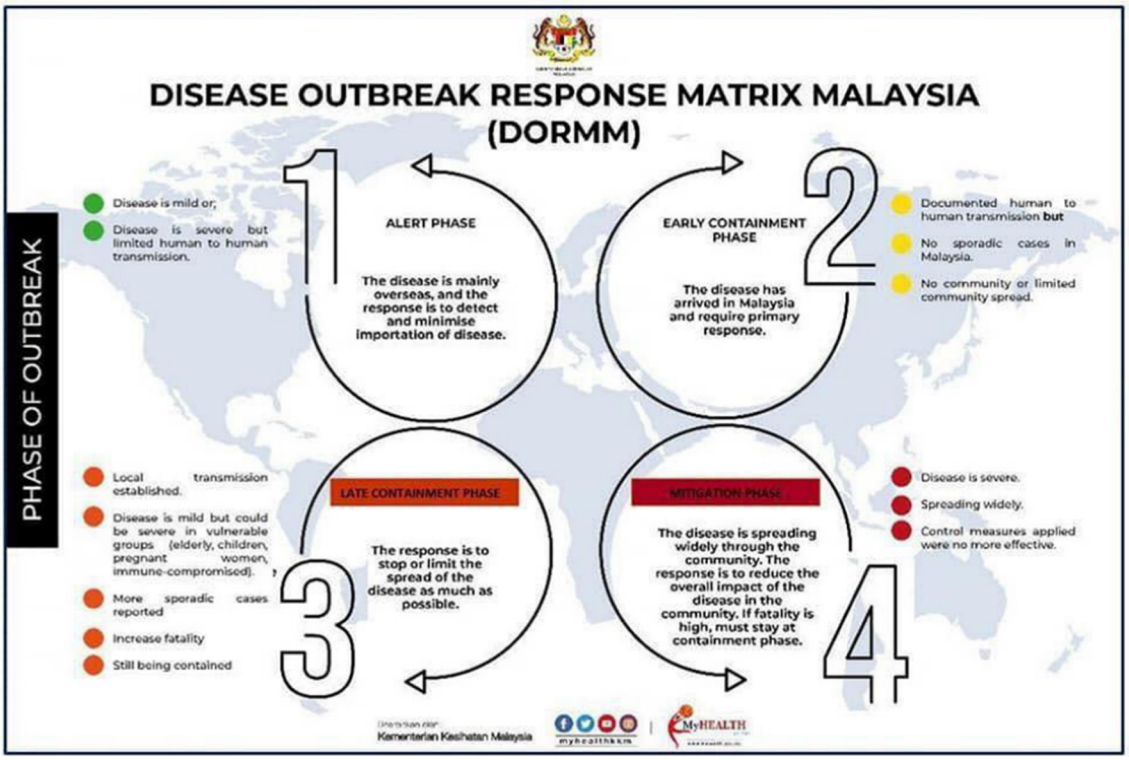

FIGURE 3 | Disease outbreak response matrix Malaysia (11). Source: Ministry of Health, Malaysia. Permission has been obtained from the copyright holders.

the time, where those aged 60–79 recorded the most number of deaths (65%). On the other hand, a total of 2,187 (64.8%) positive cases were asymptomatic.

Other Clusters and Case Concentrations

Other clusters of positive cases in Malaysia were formed from local mass gatherings and imported cases of PUIs traveling from oversea countries. In Sarawak, three major clusters were registered: a 3-day church gathering in Kuching recorded 176 cases and three deaths; a PUI from Italy recorded infections among 63 cases with five deaths; and a hospital cluster recorded 56 cases, of which 29 cases (52%) were linked to the church gathering (19). In Kuantan, Pahang, a cluster from a PUI with travel history to Bali, Indonesia recorded 43 cases with three deaths, including 10 healthcare workers at a medical center (20). Besides that, a total of 164 imported cases were reported among Malaysians who returned from Indonesia including students (21). From April 3 to 26, a total of 139 cases (1.1%) were detected among 12,672 Malaysians who returned from overseas (14). Among non-Malaysians such as travelers, immigrant workers, and asylum seekers, the MOH detected 601 cases (22), of which most were nationalities from Indonesia and the Philippines.

In relation to clusters, several areas that reported highly concentrated number of cases had been placed under enhanced Movement Control Order (EMCO) to contain the local transmission rates (23). These areas are the Sungai Lui village (156 cases) connected to Sri Petaling Tabligh; Bandar Baru Ibrahim Majid in Kluang, Johor (193 cases, 4 deaths) (24); and Masjid India Road where many foreign immigrants were staying (180 cases) (25). The number of cases at several local wholesale markets in Selayang, Selangor and northern Kuala Lumpur also reported an increase (79 cases), and had been linked to the Sri Petaling Tabligh cluster (26). As wholesale markets are normally visited by large number of people including traders from other markets, the MOH also prompted visitors who believed to have been exposed to come forward for screening, to avoid another huge outbreak such as the Sri Petaling Tabligh event (27).

On the other hand, as healthcare workers (HCWs) carry high risks of infection in direct contact with patients, MOH Malaysia reported that most HCWs got infected outside the healthcare settings and not when handling patients (19). Out of 325 cases (5.8% of total) among HCWs, 70% were infected through community transmission (28).

Third Wave of Outbreak (October 8–Present)

After lowering to a record of between single- and double-digit number of cases from July to September 2020, Malaysia entered the third wave of outbreak in early October with the highest number of cases coming from Sabah (8,082), Selangor (3,357), Kuala Lumpur (2,853), and Kedah (1,940) from September 1 to October 19 (The Star, 2020) (29). The most significant number of

cases was recorded in Sabah, where several large clusters of cases have been identified. These clusters are mainly concentrating in the east of Sabah including Lahad Datu, Semporna, Tawau, and Sandakan area. The largest cluster is the Benteng Lahad Datu cluster at the Lahad Datu District Police Headquarters, which led to several subclusters including the Tawau prison subcluster. The number of cases in Sabah worsened after the Sabah state election on September 26, where persons who returned from high-risk areas in Sabah to peninsular Malaysia were also tested positive (MOH, 2020) (30). At the start of the third wave, the total number of confirmed cases was 14,368 on October 8. In just 2 months by December 3, the number of cases has shot by 381% to 69,095. This also means that 21% of the cases occurred over slightly <10 months during the first and second waves, while 79% of the cases occurred in just 2 months during the third wave (31).

Infection of Vulnerable Population

Similar to the global situation, the elderly and those with chronic diseases in Malaysia were more vulnerable to the COVID-19 infection (32). It was found that the incidence per population rate of COVID-19 cases was the highest among age group 55 to 64 years in the population (33). The first two deaths in Malaysia occurred on March 17 (**Figure 4**). Among the case fatalities, 63% were reported to be aged above 60 years, and 81% had chronic diseases such as diabetes, hypertension, and heart diseases (34). To protect the vulnerable groups, and at the same time ensure adequate supply of medicine for them without being exposed to high-risk environments such as hospitals, MOH encouraged the use of PharMarchy Value Added Services (VAS) available at MOH healthcare facilities such as medicines through post, drive-through, locker, and appointment system. Besides, a guideline on "Recommendations for the COVID-19 Pandemic for Private, Public and NGO Residential Aged Care Facilities" was also developed especially to manage the elderly care institutions. As of May 1, Malaysia recorded 103 deaths, with a case fatality rate of 1.7% which was lower than the reported rate of 3–4% by WHO (35).

Detected Mutation of SARS-CoV-2 in Malaysia

One of the notable clusters in the early second wave was the cluster involving the 26th case who had travel history to Shanghai but developed symptoms after a month. As the case had attended several meetings before being aware of the infection, the cluster recorded a total of 121 cases, which was considered as exceptionally infectious compared with the other cases. The Institute for Medical Research later discovered a possible mutation in the virus strain isolated from the 26th case, which might have caused the virus to be more aggressive and contagious (36).

The COVID-19 disease, which is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been suggested to be triggered by spike mutation of the SARS-CoV from bats, which enabled it to infect humans (37, 38). Phylogenetic network analysis of the SARS-CoV-2 found the virus in three major variants: strain A which closely resembles that of the bat coronavirus; strain B which is mutated from strain A by a synonymous and a non-synonymous mutations; strain C with a non-synonymous mutation from strain B (39). The main virus strain for the infection in Malaysia is of strain B, which is found exclusively in East Asia. However, it was reported that the other two strains—strain A mostly found in the USA and Australia, and strain C in Europe—were also present in Malaysia (40).

THREATS AND CHALLENGES

The origin of COVID-19 outbreak in Malaysia can be traced back to the first case arriving on Malaysian shores on January 25, 2020, when a passenger from China (en route via Singapore) was tested positive for the virus (41). Since then, a historical new chapter unfolded, shaking the pillars of the national public health system and ultimately testing the perseverance of the nation as a whole.

Disease Containment

The MOH spearheads the national outbreak management of this global pandemic as declared by WHO (42). During the early phase of COVID-19 worldwide spread, even before the first reported case in Malaysia, the MOH has come up with a comprehensive preparedness plan. This plan encompassed several key components including enhanced screening and inter-agency collaborations at entry points (airports, seaports etc.,); bolster sampling at health clinics and hospitals; designation of hospitals and laboratories nationwide as "treating" and "sampling" centers, respectively, empowering the public health surveillance system through active case detection and robust contact tracing; and the adequate stockpiling of personal protective equipment (PPE) and medications needed (43, 44).

The earliest response once the outbreak occurred in Malaysia followed a common standard outbreak management framework emphasizing on thorough case definition formation and case identification process. The MOH distributed the national Guideline on COVID-19 Management aimed in assisting frontliners in every step of management involving COVID-19 cases, including early case detection with clear case definitions used from the outset. The guideline is easily accessible online and has since undergone dynamic editing and updating processes at various points in time as the virus spreads further globally (8). Initially, singular cases were reported almost daily, which in time doubled and later exponentially increased when several clusters mushroomed. As clusters expanded beyond the first generation of contacts, more vigilant contact tracing and testing were done. One notable cluster in Sri Petaling, Kuala Lumpur affiliated to a religious gathering began to surface in early March. Cases began to spread all over Malaysia after the conclusion of the assembly with attendees returning to their hometowns. Moreover, infected international participants later spread the virus to their native countries with cases emerging in neighboring countries like Brunei, Singapore, Cambodia, and Thailand (45). A nationwide call for approximately 16,000 local attendees to come forward for testing ensued (46). Active case detection

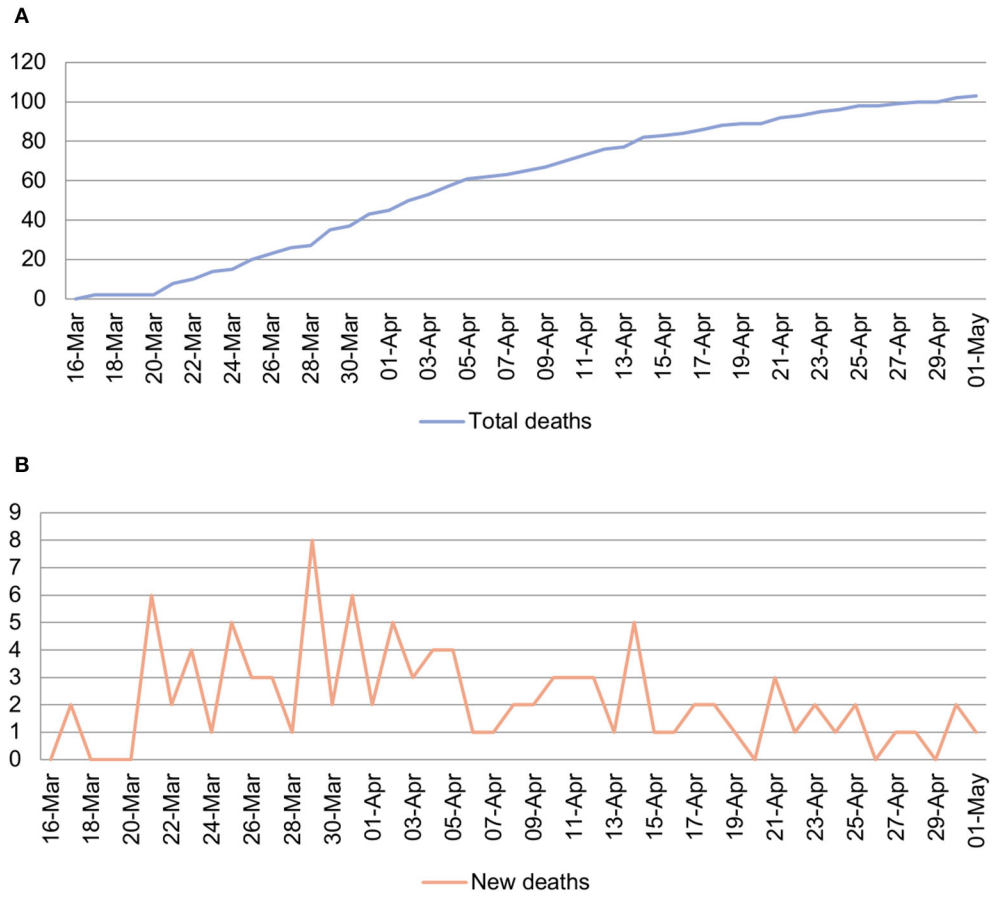

FIGURE 4 | Number of total deaths **(A)** and new deaths **(B)** from March 16 to May 1 in Malaysia. Originally created by the co-author S.C.K.

and mapping of participants led to several mass sampling areas nationwide. Everyone was included in these targeted samplings, from symptomatic to asymptomatic individuals, and both local and foreigners were also not spared; more importantly, tests were carried out by MOH for free. MOH policy clearly stated that no one should be left behind since the virus discriminates no nationality (47). This policy ensures that everyone was treated equally and equitably.

Another method of vigorous case identifications and contact tracing involves targeted active cluster identification. Several areas in Peninsular Malaysia for example recorded sharp case increments justifying localized EMCO to these particular areas. These EMCOs imposed strict no in–out movement, aimed ultimately at reducing and breaking disease transmission. Two such areas in Kuala Lumpur were locations swollen with foreign workers, immigrants, refugees, and asylum seekers thriving with their daily life and businesses, many among them considered marginalized and vulnerable (48, 49). Major samplings of all the residents living in these areas regardless of their nationality were indeed daunting, but consequently led to further detection of cases and their subsequent treatment. Contact tracing has become the mainstay and core activity to curb disease spread and not only confined to regular close family members or contacts, but expanded well beyond that to include shops or places visited by each case. The government also developed and urged the use of technology *via* mobile phone apps aimed to assist COVID-19 outbreak management, and to facilitate contact tracing of people who may be exposed to infected individuals, namely, MySejahtera and MyTrace apps (50). Soon, the three T's of "trace–test–treat" became a new mantra, and this triad was carried out vigorously early on and throughout this ongoing outbreak mainly by MOH staff on the ground (from district and state public health offices) with the help of other agencies, e.g., police, immigration, civil defense personnel, and so on.

Federally imposed isolation and quarantine may be deemed radical by some or even draconian by few, yet these measures (isolation and quarantine) have been two of the most successful tools available in fighting outbreaks since the dawn of epidemics (51). Experience from China showed that these two measures played significant roles in determining the course of COVID-19 and reducing the effective reproduction number (Ro) there (52). In Malaysia, isolation and quarantine of close contacts and travelers returning from abroad were carried out in 409

designated and gazetted quarantine centers nationwide (53). These centers include training centers, technical institutes, community colleges, hotels, and former National Service camps. Quarantine of close contacts and travelers in designated centers were crucial since the MOH estimated that up to 15% of those instructed to self-quarantine at home did not comply with the order (54). In the beginning, these centers were used to house close contacts of positive cases especially among those from the Sri Petaling religious gathering cluster and others from the EMCO areas. Subsequently, as the global spread of the virus outside China worsened, the government brought back Malaysians from all over the world through chartered flights. All of the returnees and close contacts were quarantined for 14 days during which they will undergo viral real-time reverse transcription-PCR (rRT-PCR) sampling twice before they are allowed to return home (if both results returned negative). This "quarantine and test" method later allowed for more case detection among these "travelers" cluster during the later stages of the MCO, subsequently exceeding the number of local infections.

In terms of treatment, the challenge obviously involves selecting a suitable range of evidence-based medications including repurposed drugs to be used in Malaysia. One specific drug that reached stardom and gained renewed popularity for use in COVID-19 treatment is chloroquine (or its more potent derivative hydroxychloroquine). In the COVID-19 management guideline distributed by the MOH, the use of chloroquine and hydroxychloroquine with or without various antiviral medications has been suggested at different clinical stages of the virus (55). These two antimalarial drugs have been suggested to influence the virus–receptor binding of COVID-19 and may impair virus attachment and entry (56). With time, these medications garnered hype worldwide leading to hoarding by several countries despite scanty evidence in regards to their effectiveness (57). However, in light of recent evidences, these repurposed drugs came under heavy scrutiny and was found to have no significant beneficial effect on the treatment and prophylaxis of COVID-19 (58). Malaysia, meanwhile, retracted the use of these off-label drugs in June after analysis showed no effect among 500 COVID-19 patients (59). In the meantime, Malaysia has pledged their commitment with WHO into the global "Solidarity Trial"—an international initiative testing several drugs for the treatment of COVID-19 (60). Nine COVID-19–treating hospitals nationwide were selected to test the efficacy of four treatment regimens using the combination of remdesivir, lopinavir/ritonavir, interferon beta, chloroquine, and hydroxychloroquine (61). WHO recently published their finding on the said trial, dubbed the world's largest randomized clinical trial on COVID-19 therapeutics, and indicated that the regimens had little or no effect on 28-day mortality or the in-hospital course of COVID-19 among hospitalized patients (62).

In the vaccination front, the government looked into several initiatives that may ensure Malaysians will adequately receive successfully developed COVID-19 vaccines from multiple sources worldwide. The government recently pledged their commitment to the global COVID-19 Vaccine Global Access (Covax) partnership, spearheaded by WHO, while also collaborating with the Chinese government, for the procurement of COVID-19 vaccines once they are available (63). A recent concern among many countries including Malaysia is the speed of vaccine procurement. Some Malaysian neighboring countries have started rolling out vaccines procured at rather premium prices because these countries entered into advanced purchase agreements much earlier (before the publication of interim trial data). Singapore, for example, allocated around S$1 billion for vaccine procurements and has since commenced the inoculation of their population (approximately one-fifth of Malaysia's population)—almost the same amount of budget allocated by the Malaysian government for vaccine procurement for our population (64). In a race against time, the government estimated that RM2.05 billion will be used, out of the initial allocated RM3 billion budget, for ongoing vaccine purchase agreements and procurements which involve several pharmaceutical firms worldwide as well as vaccines acquired via Covax. Most of these vaccines require the two-dose regime and will include the Pfizer-BioNTech vaccine, which has obtained conditional approval from the governing National Pharmaceutical Regulatory Agency (NPRA) of MOH, expected to supply 20% or 6.4 million of Malaysia's population (65, 66). It will be rolled out by end of February 2021, during the commencement of the MOH-announced "National Covid-19 Immunization Plan," outlining a framework of three phases to vaccinate over 80% or 26.5 million of the population to achieve herd immunity over a 1-year period (by February 2022), en route to becoming the largest vaccination program in this country (67). Priority will be given to frontliners in the first phase, followed by the inoculation of vulnerable and high-risk groups (elderly and those with comorbidities), before finally reaching healthy adults aged 18 and above. The Malaysian Prime Minister himself pledged that he will be among the earliest to receive the vaccine (68). The MOH-developed MySejahtera app mentioned earlier will serve as one of the platforms for vaccinees' selection, invitation, enrollment, side effects monitoring, and certification while also reminding vaccinees of their second jab appointments when due (69). All these efforts complement the ongoing research into the most appropriate medications to fight COVID-19 and efficacious vaccines that can provide immunity to this novel virus.

Threats to the Healthcare System

An important aspect into the ability of nations to combat any new outbreak and one as devastating as COVID-19 is the coping ability of its healthcare system. The whole healthcare system may be stretched thin with improper management and administration. The Malaysian MOH has since the outset prepared for the worst case scenarios and outlined the plan in clear and easily accessible guidelines (8). In times of crisis, the collective collaboration of both public and private healthcare sectors is needed and none should be allowed to work in silos. One such ongoing collaboration is the performance of COVID-19 rRT-PCR tests by certified public and private laboratories (stand-alone or hospital laboratories). As we know, WHO defines a confirmed COVID-19 case as "a person with laboratory confirmation of COVID-19 infection" and the recommended

routine testing is through detection of COVID-19 virus RNA by nucleic acid amplification testing (NAAT) such as rRT-PCR (70, 71). In the beginning, as with the government policy to indiscriminately test locals and foreigners during contact tracing, active case detection, and mass sampling, most tests were carried out in government laboratories and they were able to cope with the daily demand of testing. However, with the increasing number of testing per day and increased workload of these laboratories to cope with the turnaround time, private hospitals and laboratories opened their services with significantly reduced fees to share the burden and dependence on public laboratories. Uberization of COVID-tests *via* home sampling was also established with help from the private laboratories. At one point in time, the combined ability of all 43 laboratories (public and private) nationwide reached a maximum of 16,635 rRT-PCR tests per day (72).

By the end of April 2020, the total tests conducted in Malaysia were estimated to exceed 150,000 with the ratio of about 4,700 tests per million population (73). Although, this figure is far lower in comparison with South Korea (11,980 tests per million population) and Singapore (about 17,000 tests per million population), the tests conducted by Malaysia from early on is more of a targeted testing. Tests were carried out undiscriminatingly on symptomatic or asymptomatic local or foreign individuals who were either close contacts (family, workplace, marketplace, school), or those who live in red zone areas, tahfiz (religious) school students, homeless centers, old folks homes, wet markets, construction workers, healthcare workers, returning travelers, and many other risk groups. Meanwhile, the MOH has also approved and received the first batch of antigen rapid testing kit from South Korea which has a reported sensitivity level of 84.4% (74). This effort complements the use of antibody rapid testing kits that have been used to aid the investigation of this ongoing outbreak and to detect seroprevalence of the virus in the community. Surveillance testing has also been conducted nationwide in various sentinel clinics and hospitals for patients who presented with ILI and SARI where out of 6,100 clinical samples collected, 71 (1.16%) were positive for COVID-19 (75). These different testing modalities in the future are hoped to help boost the testing capability and ultimately improve the detection of COVID-19 cases in the country.

Once cases have been tested positive for the virus, the next challenge stems. The adequacy of designated hospital beds along with its ICU beds and ventilators will come into question. The MOH has prepared several contingency plans looking into different best to worst case scenarios. Early on, the government designated 34 public hospitals as the admitting and treating hospitals for COVID-19 nationwide (8). These hospitals were selected based on stringent criteria, among others the number of beds, healthcare staff (specialists, doctors, nurses, etc.,), and adequate infrastructural ability and support system. It later included two other university hospitals into the list. To put into perspective, there are about 150 public hospitals throughout Malaysia (76). In the meantime, large private hospitals have also pledged their readiness for COVID-19 patients if the situation worsens (77). The MOH also introduced "step down" centers where cases who are asymptomatic and clinically stable can be transferred to these centers. This may free up beds in the designated hospitals and reduces the risk of stretching the resources needed. The COVID-19 pandemic has truly challenged the ability of the healthcare system in many countries globally, and Malaysia to some degree experienced the same problem. ICU beds and ventilators are two critical commodities in times of crises. The MCO imposed by the government had generated positive consequences with the number of incidence dropping to two digits toward the end of the third phase of MCO (from April 15 to 28, 2020). Some 40 cases (from the total 1,758 active cases) were receiving treatments in ICU with 18 of them requiring ventilation support (75). As of early May 2020, the health system was able to cope with the demand, with utilization of ventilators standing at around 30% of the total capacity allocated for COVID-19 management (78). The government also allocated a special RM500 million budget to purchase equipment like ventilators and PPE (79).

Other inevitable threats to any healthcare system are the adequacy of manpower and sufficiency of PPE supply. These two must go hand in hand because proper management of COVID-19 requires that each healthcare staff involved must be provided with the appropriate level of PPE. In this time of disaster, PPE are valuable assets hoarded and "hijacked" by some leading to huge demand and inadequate supply to others (80, 81). The scarcity of PPE in certain parts of the world has led to infections and deaths of healthcare staff from COVID-19 infection (82, 83). In Malaysia, the MOH use every possible method of acquiring adequate supplies of PPE for every healthcare staff involved in COVID-19 management (84). Healthcare workers are advised to strictly adhere to guidelines given by the ministry in using the appropriate level of PPEs for different activities they perform during their daily involvement with COVID-19 management. Nevertheless, it is worth acknowledging that many individuals, local entrepreneurs, businesses, and private companies have donated PPEs and even provided monetary funding to buy PPEs. They contributed to ensure that all MOH frontliners are well-protected. Some even went to the extent of voluntarily sewing isolation gowns, head covers, and boot covers while other groups created DIY head covers and donated them to frontliners (85). Everybody is coming together to do their part to help the country battle the COVID-19 pandemic.

Healthcare staff is one of the integral parts in the fight against this virus. As an instrument of the public health system, the healthcare staff serve to protect the health and wellness of the general population. They are the frontliners risking their own safety and health and responsible in activities related to COVID-19. The Ministry mobilizes reinforcements of healthcare workers from states with less COVID-19 cases to states with multiple red zone districts (defined as having 40 active cases or more). MOH has also called on private and retired medical staff to contribute in COVID-19 management nationwide, with a special budget allocated to hire them on contract (79, 86). This is very important to avoid burnout and exhaustion among healthcare workers which could be detrimental to their physical and mental health. The MOH takes this seriously and provided regular tips in avoiding burnouts to all its healthcare staff (87). Many

healthcare staff risk being exposed to contracting the infection themselves. Some 325 ministry healthcare staff have been tested positive for COVID-19 so far, and investigations into these cases concluded that none of them contracted the disease in their line of work, with 70% attributable to social gatherings, oversea trips, and others (28). Nevertheless, the guideline provided by MOH clearly outlines the levels of PPE that staff should abide to base on available evidence, which is the responsibility of every staff involved in the COVID-19 management (8). The Health Director-General (DG) has been one of the most respected authorities in the country and his daily national live broadcast ensured a smooth and comprehensive risk communication to all (88). In times of crises, working hand in hand with other agencies ensures a more holistic approach toward achieving the common goal of containing the spread of the virus. These interagency collaborations were carried out in many instances during the mitigation phase when the MCO was initiated and continued throughout.

MITIGATION STRATEGY

Movement Control Order

Social quarantine or more popularly known as lockdowns coupled with social distancing has become an almost standard protocol in the control of COVID-19 spread across the world. Lockdown was first implemented in Wuhan, the epidemic epicenter in China, on January 23, 2020. This was soon followed by 15 other cities in Hubei Province, of which Wuhan is its capital, and later by several administrative areas in China. The Wuhan lockdown was only lifted some 2.5 months later on April 8, while in most of Hubei, it was lifted earlier on March 25. Many were concerned of this draconian measure which violated individual rights and were skeptical of its effectiveness. The move, even though commended by WHO, also said that it was beyond its guidelines in epidemic control and was an unprecedented public health measure (89). However, as the pandemic spread to the rest of the world, lockdown implemented in varying degrees became a household term. It is estimated that 1.7 billion or 20% of the world's population have been instructed by their governments to stay home (90).

Malaysia took a similar approach when the number of COVID-19 cases started to escalate during the second wave and implemented the MCO. A phase 1 MCO was first initiated for 2 weeks from March 18 to 31, 2020. This was extended another 2 weeks into the phase 2 MCO from April 1 to 14, and subsequently another 2 weeks into the phase 3 MCO from April 15 to 28 and a further 2 weeks into phase 4 MCO from April 29 to May 12. Thus, the total MCO or lockdown period was intended for 8 weeks. However, from May 4 onwards, the MCO was converted to a conditional MCO (CMCO), with respect to the partial opening of the economic sector as announced by the Prime Minister on his Special Labor Day Address on May 1, 2020. The CMCO continued until June 9, after which the recovery MCO (RMCO) was activated from June 10 to August 31. During RMCO, the economic, education, religious, hospitality, and tourism sectors were reopened, but with strict standard operating procedures (SOPs). These include meetings, conventions, exhibitions, and weddings. The international borders, however, remained closed except for approved travel. On August 28, it was announced that the RMCO was extended until December 31, 2020.

Following the Sabah state election on September 26, 2020, resulting in cases of COVID-19 escalating again with a third wave beginning on October 8, the government reinstated a second CMCO in the state of Sabah on October 13 and in Malaysia's most urbanized area, the Klang Valley comprising Kuala Lumpur, Putrajaya, and Selangor, from October 14 onward. The other states in Peninsular Malaysia, with the exception of Perlis, Kelantan, and Pahang, also joined the Klang Valley in the CMCO on November 9. Under the CMCO, cross-district and cross-state travel were again prohibited unless for work and with prior permission from the police; schools and educational institutions were closed; public events like concerts, conventions, and weddings were again prohibited; religious services were limited to a small group; and public premises like bars and theaters were closed.

The MCO in its various forms was enforced through the Prevention and Control of Infectious Diseases Act 1988, whereby, under Section 11(2) of the Act, the Minister of Health may, by regulations made under this Act, prescribe the measures to be taken to control or prevent the spread of any infectious disease within or from an infected local area (91). Under Section 11(3), an authorized officer may also direct any person or class or category of persons living in an infected local area or in any part thereof to subject himself or themselves to isolation, observation, or surveillance, the period of which is being specified according to circumstances, or to any other measures as the authorized officer considers necessary to control the disease. It is also supplemented by the Police Act 1967 (92).

Subsequently, the Prevention and Control of Infectious Diseases (Measures within the Infected Local Areas) Regulations 2020 was gazetted under the Act by the Minister of Health on March 18, 2020 to facilitate the enforcement of the MCO (91). The MCO incorporated three key measures, namely, implementation of border control, control of public movement, and prohibition of public gathering and promotion of social distancing. Malaysia closed its international border entry points except for foreigners leaving the country and for Malaysians returning from overseas. In terms of movement control, all non-essential work places, commercial establishments, and services were ordered to close down, so that the population nationwide will be confined to their homes and were only allowed to venture out either to perform any official duty, to make a journey to and from any premises providing essential services, to purchase, supply, or deliver food or daily necessities, to seek healthcare or medical services, or for any other special purposes as may be permitted by the Director General of Health. With respect to public gathering, all religious services, wedding receptions, sports events, conferences, cinemas, and public gatherings were disallowed during the MCO period. Malaysians returning from overseas were initially subjected to self-quarantine at home but subsequently they were subjected to mandatory quarantine at government-managed quarantine sites for 14 days. They are tested for COVID-19 on arrival and then again on the 13th day

of quarantine. If the result of the second test is negative, they will be allowed to leave quarantine.

There was a slight hitch in the initial implementation of the MCO as it was announced on March 16 to take effect after midnight on March 18. As expected, there were some panicked buying of foods and daily supplies by the population, even though they were told that supermarkets, convenient stores, and restaurants will remain open throughout the MCO. An unexpected event, however, was the exodus of people, especially students from the capital Kuala Lumpur and the surrounding Klang Valley to their homes. Some families also took the opportunity of the 2-week MCO to return to their hometowns across states. This caused congestions at train and bus stations and highways leaving the Klang Valley. Social distancing was ignored, and it is uncertain if the chaos created had resulted in COVID-19 transmission. The government was also worried that the exodus from the Klang Valley might have helped spread the disease to the other parts of Malaysia.

Enhanced Movement Control Order

From March 27, specific locations were subjected to a stricter order called the EMCO. Malaysia employed a targeted approach in tackling the COVID-19 epidemic by first identifying high-risk districts and localities. Districts with no active or cumulative case within a 14-day period are termed as green districts, those with 1 to 20 cumulative cases within 14 days are termed as yellow districts, those with 21 to 40 cumulative cases within 14 days are termed as orange districts, while those with more than 40 cumulative cases within 14 days are termed as red or high risk districts. Within each red district, potentially explosive localities are identified and an EMCO may be enforced in those localities. As of April 23, 2020, seven EMCOs were designated, whereby a total lockdown was enforced with the assistance of the police and armed forces. The EMCO can be enforced on a village, a housing area, a commercial area, or an apartment or a condominium (**Figure 5**) (93). In an EMCO, all residents are required to remain indoor at all times, a medical base is set up, door-to-door screening of all residents for COVID-19 using the RT-PCR method is conducted, and all business activities in the area are ceased except for essential services. Essential food supplies are provided for free to all residents by the Social Welfare Department, all entry and exit points in the area are guarded, and all food deliveries are allowed to deliver only to a designated area. This strategy proved to be very effective in controlling the spread of COVID-19 cases. In a number of these EMCO areas like the Menara City One, Selangor Mansion, Malayan Mansion, Selayang Baru, and residential areas around the Kuala Lumpur Wholesale Market, they are inhabited by foreigners and migrant workers, some of whom are illegals. A number of index cases from these EMCO areas were from the Sri Petaling Tabligh cluster.

Social Distancing

The Ministry of Health Malaysia defines close contact to a confirmed COVID-19 case as being in social presence of within 1 m from a confirmed case for a duration of not <15 min. Thus, the recommended social distancing for the public is to be apart from each other at a distance of not <1 m. In some countries like the USA and the UK, a social distancing of 2 m is recommended instead. Public Health England (PHE) describes social distancing as steps taken to reduce social interaction between people to reduce the transmission of COVID-19 (94). According to PHE, the objectives of social distancing are more than maintaining the physical distance between persons, which are to

1. Avoid contact with someone who is displaying symptoms of COVID-19 which include a high temperature and a continuous cough.
2. Avoid non-essential use of public transport when possible.
3. Work from home, where and when possible.
4. Avoid large and small gatherings in public spaces like restaurants, leisure centers, and in closed spaces.
5. Avoid gatherings with friends and family while keeping in touch using remote technology such as phone, internet, and social media.
6. Use telephone or online services to contact your general practitioner or other essential services.

Epidemic Progression

On March 23, 2020, the Malaysian Institute of Economic Research (MIER) predicted an epidemic peak of 5,070 active COVID-19 cases by April 12, 2020 (**Figure 6**) (95). This triggered concerns that our healthcare system might be overwhelmed. Thus, efforts were made to increase the number of available hospital beds, for example, the setting up of a temporary medical facility to house mild COVID-19 patients in Serdang, increasing the availability of ICU beds, ordering of medical ventilators, and ordering of personal protective equipment for medical staff.

Flattening the Epidemic Curve

The implementation of the MCO, especially phases 1, 2, and 3, has clearly managed to flatten the epidemic curve. **Figure 7** shows the number of daily new COVID-19 cases reported in Malaysia. The number of new cases peaked at 235 on March 26, 2020 (94). **Figure 8** gives the number of daily active cases reported in Malaysia (96). Active cases are cases that are still under treatment in hospitals, which is essentially the cumulative number of COVID-19 cases in Malaysia, minus the total number of recovered cases discharged and the total number of deaths. The highest number of active cases reported was 2,596 cases on April 5. This figure is only 51.2% of the 5,070 cases predicted to occur on April 12 by MIER (95). Therefore, the epidemic curve has been flattened by about half of what it should have been if proactive measures like the MCO were not taken by Malaysia. The epidemic peak also occurred a week earlier on April 5 instead of on April 12 as predicted by MIER.

EXIT STRATEGY

Many countries in Asia and Europe which have been under lockdowns are now strategizing for an exit strategy to reopen

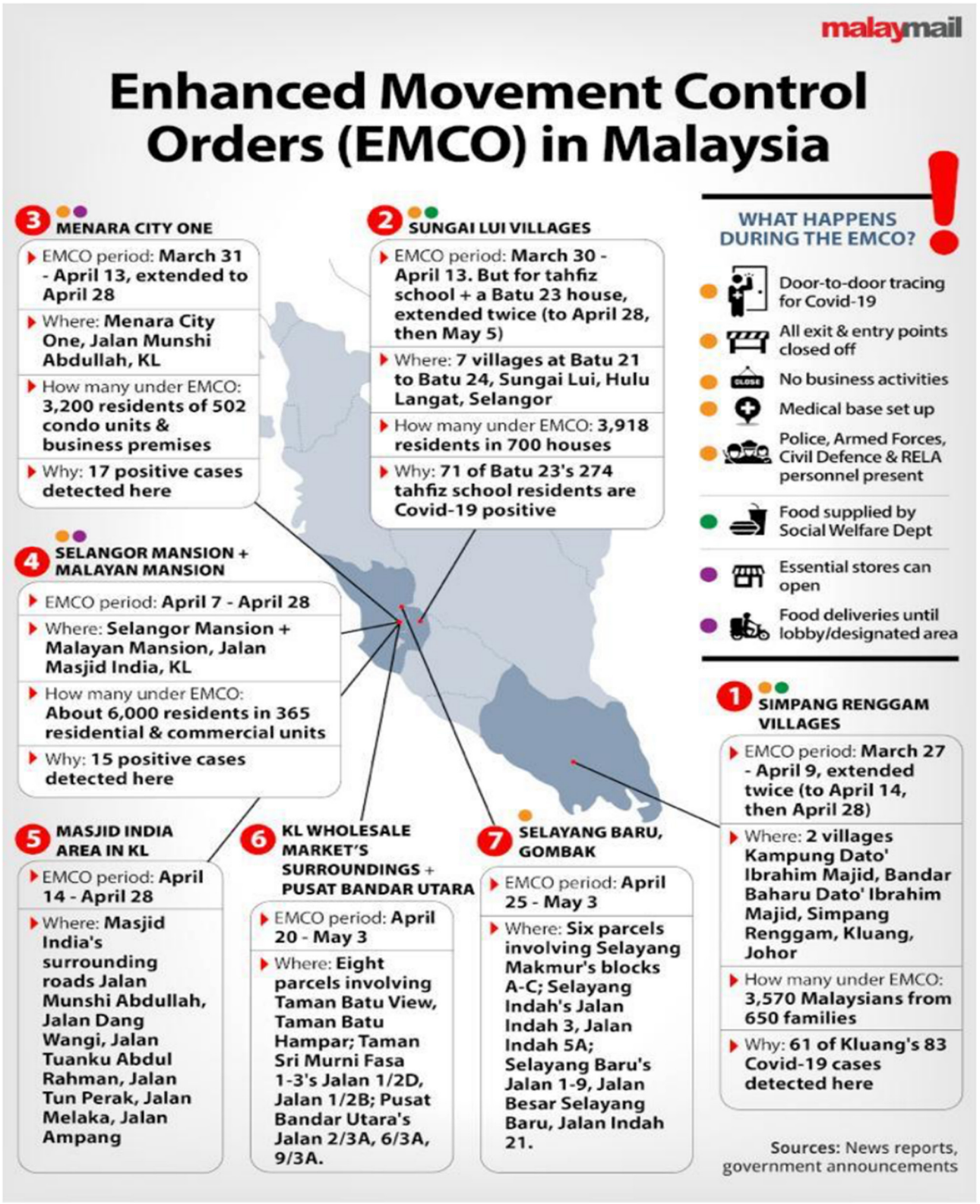

FIGURE 5 | Enhanced movement control order in Malaysia (93). Source: Malay Mail. Permission has been obtained from the copyright holders.

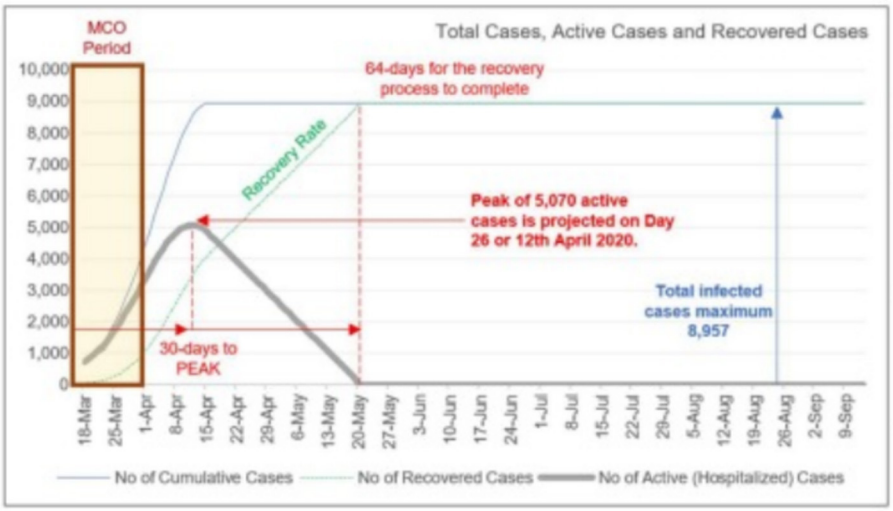

FIGURE 6 | Projection of epidemic peak by the Malaysian Institute of Economic Research (95). Source: Malaysian Institute of Economic Research. Permission has been obtained from the copyright holders.

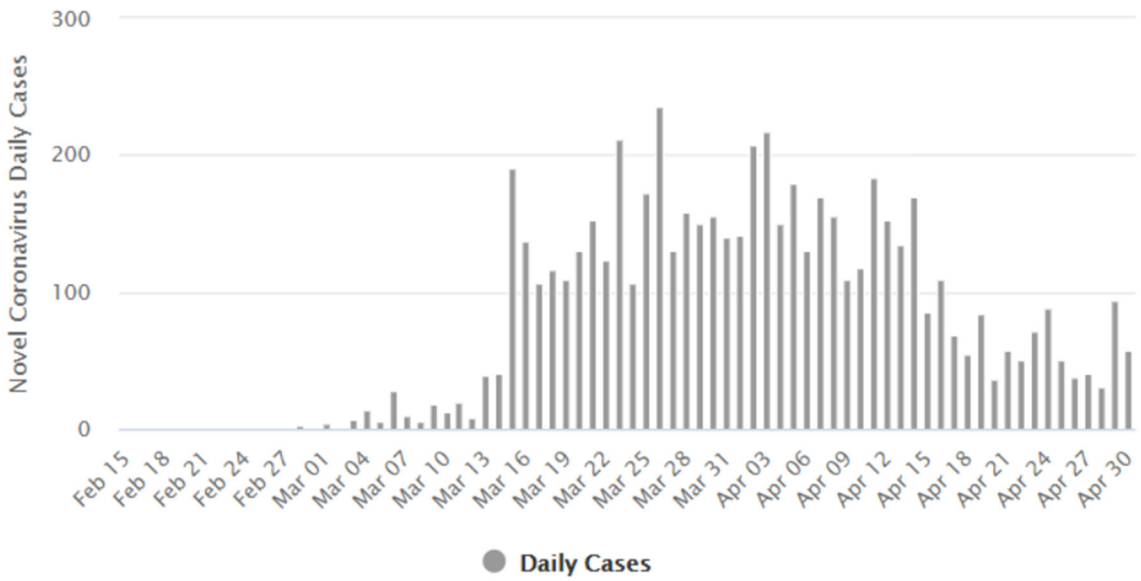

FIGURE 7 | Number of daily new COVID-19 cases reported in Malaysia (96). Source: Worldometers.info. Figure is an open source from the Worldometers.info website.

their countries in order to revitalize their economic sector and activities. Austria was one of the earlier European countries to impose a lockdown on March 16, 2020 which has strictly shut down its entire public system and businesses (97). It acted swiftly to close down bars, restaurants, schools, theaters, non-essential shops, and other places of gathering. Austria has since reopened on April 14, 2020 but urged its public to maintain social distancing when in public areas. Three weeks into reopening, it has not seen a new spike in infections (98).

Reopening the Country

WHO announced six conditions a country must acquire before lifting a lockdown (99). These conditions provide justifications for a country to make a decision to lift restrictions on their social and economic activities:

1. Disease transmission is controlled.
2. Health system capacities are in place to detect, test, isolate, and treat every case and trace every contact.
3. Outbreak risks are minimized in special settings like health facilities and nursing homes.

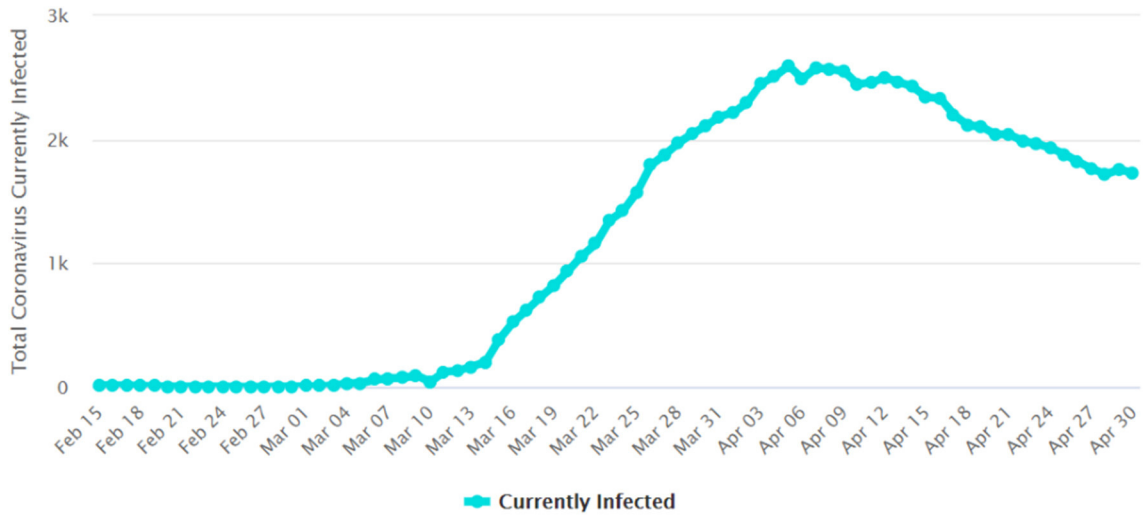

FIGURE 8 | Number of daily active COVID-19 cases reported in Malaysia (96). Source: Worldometers.info. Figure is an open source from the Worldometers.info website.

4. Schools, workplaces, and other essential places have established preventive measures.
5. The risk of importing new cases can be managed.
6. Communities are fully educated, engaged, and empowered to live under a new normal.

However, WHO warned that lifting the lockdown too soon and not carefully managing the opening of a country may lead to a resurgence in new COVID-19 cases and undo all the disease containment efforts that has been painstakingly achieved thus far under lockdown (100).

The MOH has envisaged a soft landing for an exit strategy, in which the social and education sectors may have to wait a longer period and the travel ban may continue after MCO is lifted. In line with WHO's recommendations the MOH felt that the following criteria has been met by Malaysia:

1. The implementation of public movement control to reduce the rate of infection among locals.
2. Upgraded capacity of healthcare facilities.
3. Improved ability to care for population at risk, such as the elderly, treated patients, as well as persons with disabilities.
4. Community empowerment in COVID-19 prevention.
5. Malaysia's border control has been tightened to prevent the importation of positive COVID-19 cases.
6. Adoption of the new-normal practices including social distancing and good personal hygiene.

The undesired impact of the MCO or lockdown is its damaging impacts on a country's economy, which may plunge a country into a recession with widespread unemployment. In a Labor Day address to the nation on May 1, 2020, the Malaysian Prime Minister announced that the country loses about RM2.4 billion a day during the MCO period. The total loss has been estimated at around RM63 billion up to the end of April. If the MCO was to continue for another month in May 2020, Malaysia stands to lose another RM35 billion in revenues, accumulating the loss to RM98 billion (101). This is indeed a significant economic impact on a small developing country like Malaysia with limited economic resources. Thus, Malaysia has to carefully plan an exit strategy that will both help to contain the spread of COVID-19 at a manageable level, but at the same time allow its economic sector to restart.

In facing the current economic crisis amid the COVID-19 pandemic, the Malaysian government adopted a strategy that includes six approaches:

1. A firm action and resolve in controlling the spread of the COVID-19 pandemic by implementing public movement control.
2. Build an economic resilience through a stimulus package known as PRIHATIN to improve people's economy.
3. Regenerate or restart the economy on a structured and controlled basis.
4. Implement economic recovery strategies in facing the new normal.
5. Strengthen or revitalize the economy for future sustainability.
6. Restructure or reform the economic foundation to allow the country and its people to migrate into an era of living with the new normal.

Thus, the Prime Minister also announced a reopening of the country's economic and public sectors on May 4. This essentially converted the fourth MCO into what is called a conditional MCO (CMCO). Under CMCO, there are several categories of industries and businesses that are still not permitted to operate. These businesses or activities involve public gatherings and body contact, whereby social distancing will be difficult to maintain.

Businesses and activities that are still not permitted are movie theaters, karaoke centers, reflexology centers, entertainment centers, nightclubs, theme parks, Ramadhan bazaars, public Ramadhan iftar, Aidil Fitri bazaars, carnival sales, all forms of conferences, and exhibitions, social and cultural events (e.g., weddings, concerts, cultural performances), feasts, open houses, public iftar, monthly gatherings at government and private departments, all forms of council inaugurations and assemblies, religious activities (religious parades, Friday prayers, all activities of worshiping or assembling in mosques, prayer houses, and houses of worship), cross-border travel (except for purpose of attending work and returning home after stranded in villages or elsewhere), and cross-state travel to return to villages for Aidil Fitri holiday. Meanwhile, educational activities in schools, colleges, and institutes of higher learning will continue to be conducted remotely. EMCO in designated areas continued to be enforced. Local public movement are no longer restricted to a 10-km distance but must be within a state. Malaysia's international borders remained closed to entering foreigners and exiting Malaysians.

The MOH has set SOPs for the reopening of the economic sector and businesses starting May 4, 2020 (101). These SOPs will emphasize the following considerations:

1. Social distancing.
2. Personal hygiene.
3. Appropriate use of face mask.
4. Immediate reporting of COVID-19 case to the MOH.
5. Priority in protecting vulnerable population (infants, children, elderly, and handicapped person).
6. Sick persons with symptoms to undergo health screening.
7. Social distancing in public transport.
8. Promotion of online transactions.

Mass Antibody Testing

Antibody or immunoglobin (IgM and IgG) that are produced in our body's immune system will help to stop foreign viruses from harming our body. Hypothetically, those people who have been exposed to COVID-19 and recovered are expected to develop some level of immunity against the SARS-CoV-2 virus. Therefore, mass antibody testing can be a strategy for countries to detect their population immune response to COVID-19 by specifically looking for antibodies developed against the virus (102).

Several countries mostly in Europe and the USA have started collecting mass antibody data (103). These data would be used as one of the decision-making tools of risk assessment and management for a country to lift its restrictions and open its market. However, WHO has warned countries that there is currently no evidence that people who have recovered from COVID-19 and have antibodies are protected from a second infection. Therefore, this perception of an "immunity passport" should be adopted with precaution (104).

Malaysia has also considered conducting random antibody testing in the red zones areas to know the prevalence of infection in the community, especially among infected persons who have not been detected. This antibody testing helps the government to contain the number of sporadic cases in the country (105).

Achieving Herd Immunity

Globally, COVID-19 serological datasets from the patients admitted into the hospital with severe symptoms, whereby most of them develop immunoglobulin G (IgG) antibodies of symptomatic infection, correlate with the virus disappearance (106). However, currently there are not enough serological datasets from the non-hospitalized people with positive COVID-19 but without symptoms. A country needs to have mass serological dataset of its community to gauge the status of population immunity toward COVID-19. However, with the current level of natural population exposure to this pandemic, the required level of herd immunity is unlikely to be achieved (107). Meanwhile, experts at Johns Hopkins University warned that mass exposure to the virus in the hope of achieving herd immunity could result in increased mortality which could overwhelm the capacity of a country's healthcare system (108). Malaysia does not want to take the risk of allowing for herd immunity as it was unclear and there is lack of evidence that recovered patients may develop immunity (109). Nevertheless, currently Malaysia has no case of COVID-19 reinfection by recovered patients (110).

Vaccination

In fighting the global spread of COVID-19, vaccination would be the best approach to acquire herd immunity against the virus. However, in reality, the world's medical experts still have much to learn about this novel virus, and vaccine would only be available within 1 to 1.5 years. While waiting for the arrival of the vaccine, the MOH urge the public to keep practicing social distancing and maintain good personal hygiene.

Malaysia is also calling for cooperation with other vaccine-producing countries to work together in developing the COVID-19 vaccine, and is willing to share her facilities, data, and resources toward this effort. Malaysia is willing to participate in the clinical trials once the vaccine is made available. Malaysia is considered highly suitable for the human vaccine trials, as Malaysia is a multi-racial country (111).

CONCLUSION

Even though COVID-19 is a global pandemic, expression of the epidemic may differ from one country to another. This may relate to the virus genetics, vulnerable population characteristics, population's behavior, and the country's response to the crisis. We presented here Malaysia's challenges and response to the epidemic in the hope of sharing our experiences that can be lessons learned for others. Malaysia as a developing country with limited resources faces mounting challenges in responding to the crisis. However, with good cooperation between government agencies and the public, the country managed to overcome the first two waves of the epidemic. Nevertheless, the third wave proved to be more daunting, but is kept under control at the time of writing of this article. Hopefully, Malaysia will be able to keep the epidemic at bay, at least until the arrival of the vaccine.

AUTHOR CONTRIBUTIONS

ZH has contributed in writing for part 1 - introduction. SK has contributed in writing for part 2 - COVID-19 epidemic progression in Malaysia. MM has contributed in writing for part 3 - threats and challenges. JH has contributed in writing for the abstract and part 4 - mitigation strategy. MA has contributed in writing for part 5 - exit strategy. All authors contributed to the article and approved the submitted version.

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Outcomes of COVID-19 Admissions in the New York City Public Health System and Variations by Hospitals and Boroughs During the Initial Pandemic Response

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Introduction: In the initial pandemic regional differences may have existed in COVID-19 hospitalizations and patient outcomes in New York City. Whether these patterns were present in public hospitals is unknown. The aim of this brief study was to investigate COVID-19 hospitalizations and outcomes in the public health system during the initial pandemic response.

Methods: A retrospective review was conducted on COVID-19 admissions in New York City public hospitals during the exponential phase of the pandemic. All data were collected from an integrated electronic medical records system (Epic Health Systems, Verona, WI). Overall, 5,422 patients with at least one admission each for COVID-19 were reviewed, with a study of demographic characteristics (including age, gender, race, BMI), pregnancy status, comorbidities, facility activity, and outcomes. Data related to hospitalization and mortality trends were also collected from City of New York website. These data often involved more than one facility and/or service line resulting in more location or treatment facility counts than patients due to utilization of services at more than one location and transfers between locations and facilities.

Results: Higher mortality was associated with increasing age with the highest death rate (51.9%) noted in the age group >75 years (OR 7.88, 95%CI 6.32–10.08). Comorbidities with higher mortality included diabetes (OR 1.5, 95% CI 1.33–1.70), hypertension (OR 1.62, 95% CI 1.44–1.83), cardiovascular conditions (OR 1.66, 95% CI 1.47–1.87), COPD (OR 1.86, 95% CI 1.39–2.50). It was deduced that 20% of all New York City COVID-19 positive admissions were in public health system during this timeframe. A high proportion of admissions (21.26%) and deaths (19.93%) were at Elmhurst Hospital in Queens. Bellevue and Metropolitan Hospitals had the lowest number of deaths, both in borough of Manhattan. Mortality in public hospitals in Brooklyn was 29.9%, Queens 28.1%, Manhattan 20.4%.

Conclusion: Significant variations existed in COVID-19 hospitalizations and outcomes in the public health system in New York City during the initial pandemic. Although

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outcomes are worse with older age and those with comorbidities, variations in hospitals and boroughs outside of Manhattan are targets to investigate and strategize efforts.

Keywords: SARS-CoV-2, COVID-19, mortality, outcomes, variations, public hospitals, health systems, initial pandemic

INTRODUCTION

It has emerged that regional differences may have been present across COVID-19 hospitalizations and patient outcomes in New York City. Whether such patterns are also present in public health hospitals is largely unknown. Such information could be useful to public health infrastructure and to help understand the initial pandemic response to COVID-19 as well as to strategize future approaches should outbreaks occur. As prior studies have presented observations in private hospitals in the New York area (1), the aim of this brief study was to investigate COVID-19 hospitalizations and patient outcomes in the public health system during the initial pandemic response.

METHODS

A retrospective study was conducted on COVID-19 patients admitted to New York City public hospitals during the exponential phase of the pandemic. This public health system encompasses 11 hospitals in four different boroughs of New York City with two hospitals in Queens (Elmhurst Hospital and Queens Hospital), three in Manhattan (Bellevue Hospital, Metropolitan Hospital, and Harlem Hospital), three in Brooklyn (Kings County Hospital, Coney Island Hospital and Woodhull Hospital), and three in the Bronx (Jacobi Medical Center, Lincoln Hospital and North Central Bronx Hospital). The study was approved by Biomedical Research Alliance of New York (BRANY) and the Institutional Review Board. All data were collected from an integrated electronic medical records system (Epic Health Systems, Verona, WI). Demographics and outcomes including mortality on COVID-19 confirmed cases by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay of nasopharyngeal swabs were analyzed. The data were collected for the period of 5 weeks from March 6th 2020 to April 9th 2020 comprising of the initial exponential phase, and completion of analysis was done by April 16th 2020. Variables were obtained including age, sex, race, body mass index, and pregnancy status, discharges, and mortality as well as discharge disposition.

TABLE 1 | Demographics of patients admitted in the initial pandemic with COVID-19 positive results to New York City public hospitals.

	Admissions		**Deceased**	
Characteristic	***n* = 5,422**	**%**	***n* = 1,663**	**%**
<17 years old	25	0.46	1	0.06
18–44 years old	885	16.26	117	7.04
45–64 years old	2,146	39.43	450	27.06
65–74 years old	1,165	21.41	410	24.65
>75 years old	1,201	22.07	685	41.19
Female	2,045	37.58	604	36.32
Male	3,376	62.04	1,059	63.68
Unknown	1	0.02	0	0.00
Race				
White	495	9.10	201	12.09
Black	1,725	31.70	500	30.07
Asian	295	5.42	101	6.07
Others*	2,555	46.95	762	45.82
Unknown	382	7.02	107	6.43
Pregnant	85	1.56	0	0.00
BMI (kg/m^2)^	*n* = 3,235^		*n* = 1,078^	
≤18.4	79	2.44	29	2.69
18.5–24.9	697	21.55	244	22.63
25.0–29.9	1,135	35.09	372	34.51
30.0–39.9	1,039	32.12	324	30.06
>40.0	285	8.81	109	10.11

**Includes Hispanic and non-Hispanic population.*
^BMI only known for 4,313.

TABLE 2 | Deaths of admitted in the initial pandemic COVID-19 patients in New York City public hospitals.

NYC Public Hospital	Deceased Percent (%)	Orig N	Expected, Eq Distrib	Readmissions[#]	Adj pctgs*	OR	95% CI	*p*-value
Bellevue	16.2	708	585	123	0.250	0.45	0.37–0.56	<0.001
Harlem	28.9	397	328	69	0.085	0.96	0.76–1.20	0.7
Metropolitan	17.7	264	218	46	0.115	0.51	0.37–0.70	<0.001
Elmhurst	23.9	1,396	1,153	243	0.050	0.74	0.64–0.85	<0.001
Queens	35.7	610	504	106	0.050	1.31	1.09–1.56	<0.001
Lincoln	31.1	680	562	118	0.100	1.06	0.89–1.26	0.06
Jacobi	26	640	528	112	0.115	0.83	0.69–1.0	0.22
North Central Bronx	20.1	250	206	44	0.030	0.59	0.43–0.81	<0.001
Kings County	23.8	686	566	120	0.150	0.73	0.61–0.89	0.01
Coney Island	33.7	564	466	98	0.005	1.20	0.99–1.44	0.00
Woodhull	35	370	306	64	0.050	1.27	1.01–1.58	0.00
Sums	100	6,565	5,421	1,144	1.000	–	–	–
NYC Borough								
Manhattan	20.4	1,317	1,125	192	0.445	reference	reference	reference
Queens	28.1	1,931	1,650	281	0.105	1.52	1.29–1.80	<0.001
Bronx	27.4	1,497	1,279	218	0.245	1.47	1.24–1.76	<0.001
Brooklyn	29.9	1,602	1,369	233	0.205	1.66	1.40–1.98	<0.001
Sums	100	6,347	5,422	925	1.000	–	–	–
Comorbidity								
Diabetes	33.6	1,828				1.50	1.33–1.70	<0.001
Hypertension	33.2	2,561				1.62	1.44–1.83	<0.001
End Stage Renal	29.3	140				1.06	0.74–1.54	0.74
cardiovascular disease	33.1	2,723				1.66	1.47–1.87	<0.001
Pulmonary disease	30.9	731				1.17	0.98–1.39	0.07
COPD	41.5	193				1.86	1.39–2.50	<0.001
Sums	100	8,176						

**Adjusted percentages are for original population base rather than admission/readmission counts.*

[#]Readmissions is the estimated number of patients entered more than once due to change in service location.

Deceased patients included patients who died during admission, also including mortalities in the emergency room at the time of presentation. We used descriptive statistics to characterize our study population. Continuous variables were calculated as mean, median, and ranges. Categorical variables were expressed as counts and percentages. We used Microsoft Excel 365 with add-in program Analysis ToolPak for statistical analysis. Univariate analysis was done using contingency tables and odds ratio with confidence interval was calculated to study the effect of different variables on mortality trends. Chi square independent tests (df = 2–11) were used to calculate *p*-value to determine the significance. A $P < 0.05$ was considered statistically significant, with significant Chi squared values dependent upon numbers of groups tested. Data related to hospitalization and mortality trends of New York City were collected from City of New York website COVID-19 data link[1], which was compared with our data. City data on daily deaths and hospitalizations were used and compared with corresponding data generated for the public hospitals in this study. Data related to bed count including critical care beds for each hospital were obtained from New York State Department of Health website. These bed counts are the official approved beds for each hospital before the start of the pandemic, any increase in bed counts during the pandemic were not accounted for. Bed counts were used as a surrogate for resource and staff allocations. Due to overlapping services and change of service type between facilities, which impacted 21% of patients, amounts of overlap and re-admission had to be taken into account as part of the evaluation of boroughs and facilities evaluations, resulting in adjustments of services data based upon patients-visits activities data for H+H for the year 11/01/2019-10/31/2020 were evaluated for percent distribution of services per facility.

RESULTS

A total of 18,147 patients were tested for COVID-19 during our study period and 11,599 (63.9%) were reported positive. Of these 11,599 patients, 5,422 patients (46.7%) were admitted to one of the eleven hospitals. Of the 5,422 patients admitted, 1,663 patients (30.6%) died and 2,158 (39.8%) were discharged, 1,601

[1]https://www1.nyc.gov/site/doh/covid/covid-19-data.page

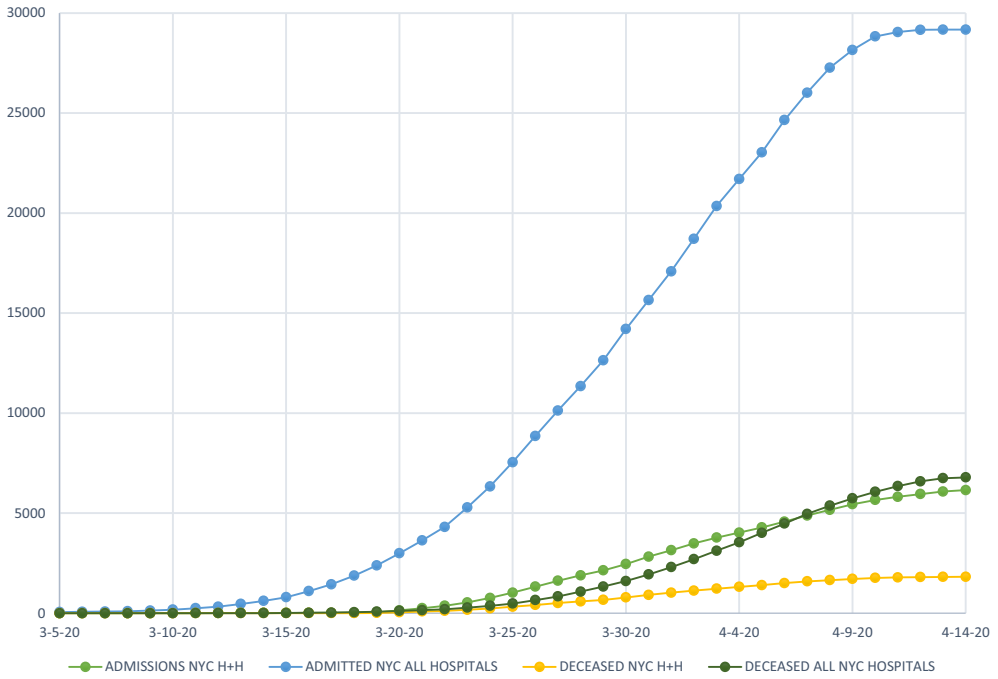

FIGURE 1 | Comparison of admission and death trends of New York City public hospitals with all New York City hospitals.

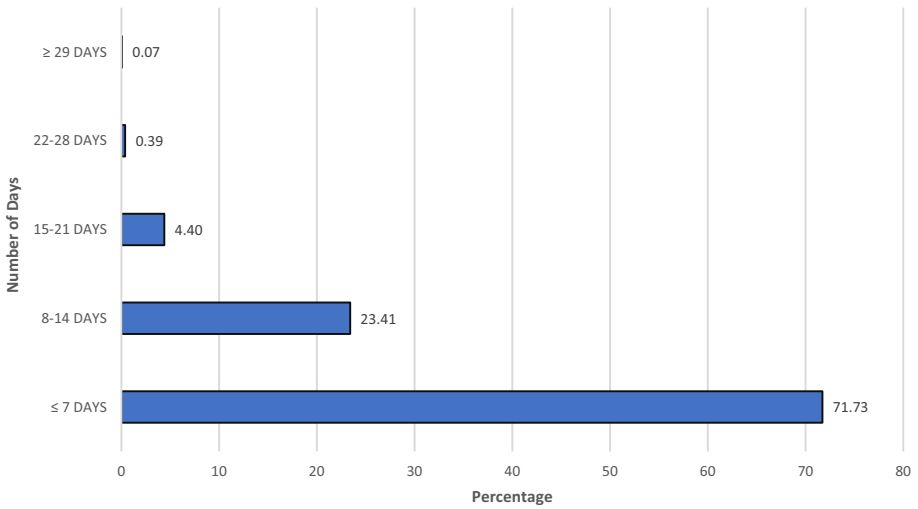

FIGURE 2 | Length of stay in hospital in the initial pandemic of COVID-19 in positive patients in New York City public hospitals.

patients were still admitted at the conclusion of the study. There were overall 1,521 (28.1%) inpatient deaths while 142 (2.5%) died in the emergency room at the time of presentation. The median age of the patients in our study population was 62 years (IQR 50–73 years). Ages 39–64 years contributed to 39% of the total admissions. Only 25 patients (0.46%) were <18 years of age. The majority of the admissions (78.7%) were noted as non-white including black or listed as other (which includes Hispanic and non-Hispanic populations). BMI was available for 3,235 of the admissions with the majority (67.2%) in the range of 25–39 kg/m^2 (**Table 1**). Mortality was more prevalent in age group >75 years (**Table 1**). On univariate analysis of age groups, higher mortality was associated with increasing age and age group >75 years was associated with the highest mortality rate (OR 7.88, 95% CI 6.32–10.08). In the group <18 years of age just one death was seen (0.06% of all deaths). Hospitalization was more common in males (62.04%) than females (37.58%) with a slightly higher mortality rate noted in males (**Tables 1**, **2**). Eighty-five patients (1.56%)

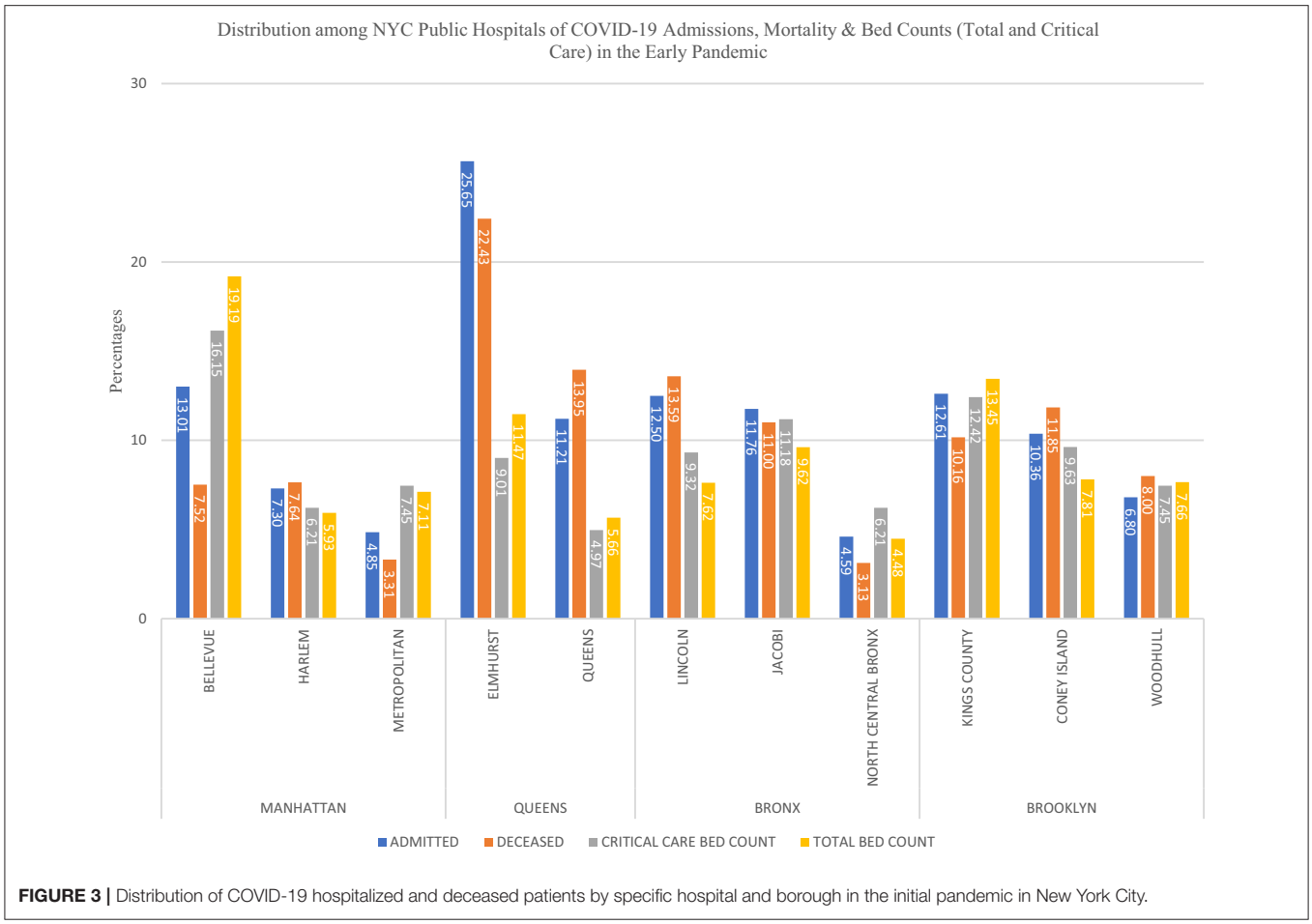

FIGURE 3 | Distribution of COVID-19 hospitalized and deceased patients by specific hospital and borough in the initial pandemic in New York City.

were pregnant with no reported death at the time of analysis of this study. Comorbidities associated with higher mortality include diabetes (OR 1.5, 95% CI 1.33–1.70), hypertension (OR 1.62, 95% CI 1.44–1.83), and cardiovascular conditions (OR 1.66, 95%CI 1.47–1.87) and COPD (OR 1.86, 95% CI 1.39–2.50) (**Table 2**). From data available on City of New York website[1], 20% of all COVID-19 positive admissions were in public health system during this timeframe (**Figure 1**). Median length of stay was calculated to be 5 days (IQR 2–9 days) among patients discharged or deceased and 72% of the study population had a hospital stay of <7 days (**Figure 2**). The distribution of the admissions during the COVID-19 pandemic and patient outcomes across the public hospitals were correlated with bed counts and ICU bed counts for each hospital are shown in **Figure 3**. Statistical differences for outcomes are shown in **Table 2**. Bed count information was from reference[2].

The first part of **Table 2** depicts data for patient-facility encounters, 1,144 of the 6,565 events (21%) were patients whose care was linked to more than one institution related to COVID-19 admission, such as a readmission, treatment provided by a second location, and/or transfer with readmission to a new facility. The most common reasons for these events could not be defined based upon the data provided by the EMR review. However, transfers included additional care to a new or special facility, i.e., patients transferred out of ICU to another location for a special care facility such as Bellevue for managing severely ill patients or facilities that provide a unique care for COVID-19 patients related to pregnancy or psychiatric care. The percent of admissions in the entire health system were noted to be highest in Elmhurst Hospital and Bellevue Hospital and similarly discharge rates were higher in these two hospitals, 52.8% (OR 1.84, 95% CI, 1.57–2.15, $p < 0.001$) and 51.9% (OR 1.77, 95% CI 1.57–1.99, $p < 0.001$), respectively. A high proportion of admissions (21.26%) and deaths (19.93%) were at Elmhurst Hospital. Differences in bed counts at two locations are noted, Bellevue in Manhattan and Elmhurst in Queens, with Bellevue having higher bed counts among the two. Examining variations in hospitals showed that Bellevue (16.2%, OR 0.45, 95% CI 0.37–0.56) and Metropolitan Hospitals (17.7%, OR 0.51, 95% CI 0.37–0.70) had the lowest number of deaths during this timeframe, both are in borough of Manhattan. Mortality in public hospitals in Brooklyn was 29.9% and Queens 28.1% and hospitals located in Manhattan 20.4%. The proportion of admissions was highest in Queens (30.4%) followed by Brooklyn (25.2%). Manhattan (20.8%) had the lowest proportion of admitted patients among public hospitals. While comparing data across different boroughs of New York City,

[2]https://profiles.health.ny.gov/hospital/view/103016#inspections

Brooklyn had the lowest discharge rate (36.6%, OR 0.72, 95% CI 0.62–0.83, $p < 0.001$).

DISCUSSION

This study included 5,422 confirmed COVID-19 patients admitted in New York City public hospitals during the early phase of the pandemic. There are limited studies analyzing the public health system response during this timeframe. From data available from City of New York website[1], we deduced that 20% of all COVID-19 positive admissions were in NYC public health system. Our study demonstrates the evidence of variation in patient outcomes among the 11 hospitals in NYC public health system, located in four different boroughs. In public hospitals in New York City, the overall mortality in admitted patients was demonstrated to be 28.1% during this time in the pandemic, which is slightly higher than other studies that have evaluated patient outcome in areas around the City (1) and in Manhattan alone (2) with mortality from 10–21%. Our data is in accordance with others (3, 4) with co-morbidities such as diabetes, hypertension, cardiovascular and pulmonary diseases contributing toward higher mortality. In a study conducted by the Northwell Health system (1) in New York involving 5,700 patients, mortality rate was seen to increase with age with much higher rates above 70 years and much lower rates in those below 30 years. Also noted in this study was a greater gender difference in mortality rate than that noted in our study with higher rates noted among males. Similar findings are noted in a study from Wuhan, China (3) which showed a statistically significant increase in mortality with age as well as a trend toward higher mortality in male patients. A study focusing on boroughs in this timeframe and hospitalization and death rates (5) due to COVID-19 found highest rates in the Bronx and lowest in Manhattan which may indicate an inverse correlation with affluence and socioeconomic status and a direct correlation with the proportion of ethnic minorities in different boroughs. Variability between different boroughs was also noted in our study with highest mortality rates noted in Brooklyn and Queens and lowest in Manhattan. Limitations of this study are the ones that are typical of the analysis of any initial pandemic. The COVID-19 status of other admitted patients in NYC public health system that were not included in the study was not known which might have led to underestimation of total number of cases. As we are still in an ongoing epidemic, we were not able to investigate a control population that may account for all the differences seen. In addition, as this study sole focus was on the initial pandemic response in the New York City health system, the outcomes of 1,601 patients who remained admitted during the study period were not incorporated into outcomes analysis. It is likely that disproportionate burden of COVID-19 has been borne by lower income and minority communities in New York City in the pandemic (5), such variations in the hospitals based on locations in different boroughs remain an important focus. Additional efforts and investigation of regional differences can likely help to strategize efforts that will help to improve outcomes of patients in public health system in the ongoing 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 Biomedical Research Alliance of New York (BRANY). 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

AD contributed to study design, data and analysis, and writing. TA and TM data collection, editing, and analysis. BA and RE study design, organization, analysis, and writing. All authors contributed to the article and approved the submitted version.

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The Coronavirus Intervention in Ethiopia and the Challenges for Implementation

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The coronavirus has been rapidly spreading across different parts of the globe. The pandemic will have a severe impact unless coordinated preventive measures are undertaken. This paper examines the COVID-19 intervention and the challenges for implementation in Mekelle, Tigray, northern Ethiopia. Telephone interviews, personal observation and document reviews were used as data collection techniques. Results showed that the majority of the population in Mekelle are aware of the pandemic. Most people practiced handwashing with soap and water. But there is limited physical distancing in religious institutions, market places, and coffee houses where many people convene. With this, staying at home remains a challenge among the majority. Still, there is a belief that the pandemic is a "punishment from God", while others believe that it is an illness of the old and does not affect the young. Generally, applying the recommended COVID-19 prevention measures, including the physical distancing, seem unattainable as the majority continue to overlook government advice. As related studies such as the fight against the Ebola outbreak in West African countries showed, this emanates from the lack of trust in media messages. In Tigray, religious leaders and community elders have a significant influence on their respective community members. Messages from these individuals remain trusted and can easily reach the majority due to social networks. This highlights that besides enforcing strict and prolonged measures from the government, reliable two-way communication can help achieve the desired behavior changes towards complying with the COVID-19 prevention measures among the different segments of the population in Mekelle.

Keywords: COVID-19, physical distancing, communication, behavior change, mekelle, Ethiopia

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INTRODUCTION

The COVID-19 pandemic continues as a significant worldwide health threat. The World Health Organization has declared it a public health emergency of global concern calling for concerted efforts from across all nations to stop the spread of the virus (World Health Organization, 2020). Most African countries face an enormous mission to contain the rapidly spreading pandemic due to poor healthcare and limited resources. However, the Mo Ibrahim Foundation (2020) debates that the continent appears to have comparative advantages to contain the pandemic. The foundation's claims are based on two viewpoints. First, it is related to the demography as most of its population is in the age of lower than 20 years. The assumption is that younger populations appear to suffer milder symptoms compared to older people. Second, it is associated with a warm climate in that influenza

and respiratory viruses mainly transmit during cold rainy seasons. This is related to earlier studies that "high temperature and high relative humidity significantly reduce the transmission of COVID-19" (Wang et al., 2020, p.1). Nevertheless, this was immediately overruled, affirming that the virus could be transmitted to all areas, hot and humid climates (World Heath Organization, 2020b). Based on this, there is no concrete evidence to recognize the foundation's claims as the COVID-19 is a new pandemic with no explicitly recommended antiviral treatment or vaccine to date. However, these claims represent the rapidly circulating myths and misconceptions in most African countries, including Ethiopia.

Although most low-income countries are in the early stages of the pandemic, there are considerable uncertainties regarding the trajectory of the disease. Higher transmissibility is expected in such settings due to larger household sizes, overcrowding and inadequate water and sanitation, which will affect the adoption of recommended preventive measures (Dahab et al., 2020). Ethiopia is exceptionally vulnerable to the pandemic due to its relatively tenuous health systems, inadequate infrastructure, population mobility, and susceptibility to social and political unrest. Thus, it becomes a priority for the nation to implement effective intervention strategies to contain the rapidly transmitting virus. After the first COVID-19 case on 13 March 2020, the Ethiopian government has adopted different measures endorsed by the World Health Organization. These measures include informing the public about regular handwashing with water and soap, physical distancing, contact tracing, self-isolation and quarantine measures. Also, the government has announced school and workplace closures, limited public gatherings and establishing COVID-19 task forces at different levels. Similar measures proved effective in reducing the spread of the virus in different countries, including New Zealand, China, Singapore and South Korea.

On 26 March 2020, in addition to the measures mentioned above, the Tigray state government has declared a region-wide state of emergency to prevent the spread of the virus. Within the region, all travels have been banned for 15 days (extended until June 2020). A ban was put on all social activities, mass gatherings such as in market places, religious institutions, cafes and restaurants (with some amendments at later stages); to ensure physical distancing. Despite these measures, the number of COVID-19 cases is increasing in Ethiopia, with 54,409 cases reported as of 2 September 2020 (Ethiopian Public Health Institute, 2020). As of 5 October 2020, a total of 6070 confirmed COVID-19 cases and 37 deaths in Tigray. The most significant proportion, 2,270 (37.4%), of the cases were in the age group of 25–34 years, followed by 1,872 (30.84%) in the age group 15–24 years (Tigray Regional Health Bureau Report, 2020).

The numbers mentioned above do not distinctly show the exact level of the pandemic in the country and Mekelle. This is because the number of COVID-19 cases depends on the testing capacity of nations. For instance, from March 13 to mid-April 2020, Ethiopia did not have its COVID-19 testing laboratory and sent samples to South Africa. Later, there was only one testing center in the capital Addis Ababa, and samples from different parts of the country were sent to the capital. Although there are testing centers in most cities, including Mekelle, they are inadequate compared to a large number of populations in the country. Therefore, low testing capacity affects population-based testing rates and data about the overall status of the pandemic in the country. With the rapidly transmitting virus, it is clear that the number of cases could significantly increase in Mekelle. However, due to the genocide in Tigray by the Ethiopian PM Abiy Ahmed[1], there is a blackout since the end of October 2020. Most of the public health service centers and media houses have been partially working or destroyed. Hence, it is challenging to obtain the exact figure about the status of COVID-19 cases in Mekelle.

Social Media and the COVID-19 Pandemic

Social media became significant sources of information for the public, often covering different themes. Studies have shown that social media have become the primary sources of health information and public engagement (Moorhead et al., 2013; Fernández-Luque and Bau, 2015; Freeman et al., 2015). They are highly cost-effective for information exchange among individuals supporting public health education (Obar et al., 2012; Scott and Maryman, 2016). Social media contributed to fundraizing within China and outside its borders during pandemics, enabling scientists to collaborate, brainstorm and identify solutions (Sokolov, 2020), become useful in tracking diseases (Yasinski, 2020). Social media were also effective in disease mapping (Signorini et al., 2011). However, different factors are affecting the use of social media for health communication. One of them is a growing level of misinformation and fake news through these platforms. Misinformation refers to false information disclosed without the intention of harming others (Jack, 2017). But certain practices are taken as dangerous because of their disruptive effect rather than their deceiving content (Bayer et al., 2019). The spread of false information can have severe consequences for public health (Scheufele and Krause, 2019). Misinformation is associated with severe public health consequences, such as increased public fear and loss of vaccine confidence (Larson et al., 2014; Larson, 2018). Different studies have documented that social media are used to spread harmful health messages, including anti-vaccine rhetoric (Dunn et al., 2015; Tomeny et al., 2017), misinformation about the Zika virus (Sharma et al., 2017) and Lyme disease (Basch et al., 2017); and the Ebola-related prevention and treatment strategies (Oyeyemi et al., 2014).

With the spread of COVID-19, Tedros Adhanom, the Director-General of the World Health Organization, claimed that "We're not just fighting an epidemic; we are fighting an infodemic" (World Heath Organization, 2020c). This shows that misinformation is common, and it is equally affecting the COVID-19 prevention campaigns. Misinformation about the COVID-19 is rapidly spreading (Chakravorti, 2020; Taylor, 2020). The BBC's Reality Check (2020) has listed down several

[1]Abiy Ahmed is the 2019 Noble Prize winner for Peace. Yet, in November 2020, he declared genocide in Tigray. He used rape and hunger as weapons of war. Jointly, the Ethiopian military forces, the Amhara Militia, and the Eritrean military forces have committed unspeakable civilian atrocities in Tigray.

COVID-19-related misleading information that has widely spread throughout the African continent. These include consuming alcohol as throat sanitizer (a governor of Nairobi, Kenya); urging Africans not to wear blue face masks claiming they are contaminated with toxins (Amazon); wearing a mask warrants protection from the virus and that if wearing them, social distancing is needless (a regional governor, Nigeria); and inhaling hot steam can kill the virus (Tanzanian president) among others.

Another misinformation comes from the government of Madagascar who declared that the country had produced herbal tea as a remedy for the COVID-19. The nation's president Rajoelina claims the validity of the herbal tea asserting that "Tests have been carried out and two people have now been cured by this treatment" (Times of Israel, 2020). Despite these claims, the World Health Organization warned that, to date, there is no specific medicine recommended to prevent or treat the coronavirus. The African Center for disease Control (CDC) has requested further details from Madagascar about the reliability of the proposed herbal medicine. Along with the widely disseminated misinformation, myths and cultural barriers are significantly affecting the pandemic intervention in Africa. Padayachee and du Toit (2020) compiled several myths that have been extensively disseminated in African countries. Some of the myths include that the COVID-19 does not affect Africans; the coronavirus cannot survive in Africa's warm climate; spray alcohol and chlorine all over your body to protect the coronavirus. WHO declared that besides the pandemic threat, an infodemic had been generated by a large amount of information available on the matter (World Heath Organization, 2020a). However, most nations, including Ethiopia, have limited techniques to block the spread of misinformation.

Public Health Emergency and Social Behavior

The social behavior in a given setting determines the level of transmission of a pandemic (Del Valle et al., 2005; Xiang et al., 2016). Accordingly, employing behavioral and social interventions have become indispensable to alleviate the impacts of outbreaks. Interventions should be based on "community engagement, participation and ownership and on intersectoral coordination and collaboration for prevention, control and mitigation strategies to work" (World Health Organization, 2012, p.vi). During public health emergency, mass media play significant roles in disseminating messages to educate the public about the pandemic and its preventive measures. However, most of them do not recognize the context of intervention. Understanding the context becomes indispensable as the nature and scope of public health education may differ based on the intervention setting and the level of risk perception among the public. This is whether to focus on individual behavior or collective behaviors of the target population. It is related to Hofstede (2001) concepts of collectivism and individualism, which suggest that while individual-based decisions are appreciated in developed western countries, collective decisions are valued in some developing countries.

In western countries, individual behavior would be decisive to control the spread of COVID-19 rather than government action (Anderson et al., 2020). However, in Ethiopia, where family attitudes hold a significant part of the community than individual roles (Ethiopian Public Health Institute, 2005), and individually referenced health interventions may not sufficiently achieve the desired behavior changes. But most of the behavior change intervention strategies, as Singhal (2003, p. 21) asserts, "focus on the tree and not enough on the forest of which the tree is a part." Related studies about HIV/AIDS in Africa have shown that intervention programs focusing on individuals have failed as they lacked community orientation (Panos, 2003; Singhal, 2003; Singhal and Rogers, 2003; Airhihenbuwa and Obregon, 2006). One of the challenges of interventions of these type is that they lack a precise analysis of the target behavior as they mostly focus on implicit common-sense models of behavior (Michie et al., 2009).

Public Health Intervention in Ethiopia

The Ethiopian public health intervention focuses on prevention-orientated public health policy. The Ministry of Health (MOH) implements the intervention through the Health Extension Program using Health Extension Workers (HEWs). This program is a platform for delivering primary health care (Assefa et al., 2019) through regular public health education. This pivotal role in achieving the Millennium Development Goals in Ethiopia. The country has got global recognition and remarkable success in achieving most of the health MDGs, including "67% reduction in under-five mortality, a 71% decline in maternal mortality ratio, a 90% decline in new HIV infections, a decrease in malaria-related deaths by 73% and a more than 50% decline in mortality due to tuberculosis" (Assefa et al., 2017, p.5). A recent study about the Health Extension Program in Ethiopia has concluded the central role of health extension workers in ensuring a well-functioning primary health care system (MERQ, 2019).

Despite encouraging trends in public health intervention in the country, there are limitations. One of the limitations is that most public health intervention programs are top-down in format limiting community engagement. Communities have defined roles in problem identification and strategy implementation to promote holistic response to achieve the desired health intervention. There are no clearly recognized health communication strategies for rural and urban settings. This shows that a limited understanding, practice and research about the role of communication in public health. World Heath Organization (2011) argues that the instrumentality of effective health communication strategies appears to have been ignored in most parts of Sub-Saharan African countries, and apparently, Ethiopia joins this category.

Several studies examined health communication in Ethiopia. Most of them focused on family planning and HIV/AIDS prevention campaigns (see Cho and Witte, 2005; EPHI, 2005; Farr et al., 2005; Getachew, 2005; Gulilat, 2006; Tibebe, 2006; Hiwot, 2007; Zelalem, 2010; Bekalu and Eggermont, 2013;

Nigussie, 2019). These studies indicated that health communication positively contributed to raising awareness of target populations about respective health issues. However, their roles in promoting behavior changes were minimal. Different factors affected behavior changes of populations towards risky behaviors. First, most of the campaigns have focused on urban areas with extensive use of media. Media has played a significant part in informing the public about the epidemic (Farr et al., 2005; Zelalem, 2010). However, programs that broadcast from urban centers may fail to improve the HIV/AIDS knowledge of rural people and disadvantage them relative to their urban counterparts (Bekalu and Eggermont, 2013). Consequently, the country has shown some of the greatest differences in AIDS awareness across its regions. For instance, striking lack of knowledge in the Somalia, Gambela, and Benishangul-Gumuz regions; the Somalia region had the lowest levels of awareness as only 50% of women, and 64% of men had heard of AIDS-and this lack of knowledge was evident across all HIV/AIDS knowledge domains (CSA, 2016). Second, the behavior change communication strategies primarily focused on individuals (Ethiopian Public Health Issue, 2005; Nigussie, 2019). Nevertheless, a health system that is individually referenced to lifestyle risk factors and underpinned by a biomedical model will be doomed to failure as it applies a "one size fits all" policy ignoring cultural differences (Farmer et al., 2012). One size fits all rural health package limitations because it affects the diversity and choice of experiences that can be offered for all cultural groups (Sypek et al., 2008; McBain-Rigg and Veitch, 2011). This shows that the health communication field has been "missing the message" because it has concentrated on "putting out messages [rather than] fostering an environment where the voices of those most affected . . . can be heard" (Panos, 2003, p.22). Third, the epidemic intervention communication strategies are not context-specific, as there are no clearly stated communication strategies for rural HIV/AIDS prevention campaigns (Getachew, 2005; Nigussie, 2019).

In Ethiopia, where people are conservative to culture and religion, it requires employing coordinated and inclusive public health education rather than uniquely relying on media messages. Religious organizations and community elders can play vital roles in raising the awareness of their respective community members about the pandemic. Therefore, it is essential to design health communication strategies that fit the specific setting to promote community engagement and collective action. Particularly during outbreaks, communicating health messages requires special attention. During an emergency, all affected people take information differently, process it in their way and act differently (Reynolds et al., 2002). One of the essential measures for pandemic prevention is implementing national risk communication and community engagement plans using existing pandemic influenza or other public health communication procedures (World Health Organization, 2020d). Risk communication helps people develop a sense of control over their health and safety, which allows them to react to risk factors with more reasoned responses (World Health Organization, 2005). Also, it is helpful to build trust in the response and increases the probability that health advice is followed. It minimizes and manages false rumors and misinformation that undermine the response and may lead to further disease spread (World Heath Organization, 2018). However, Ethiopia does not have a full-fledged Risk Communication and Community Engagement (RCCE) readiness nor the experience in handling pandemics the world has faced previously, such as SARS or Ebola. In Ethiopia, culture, religion and social ties determine the way people respond to public health interventions. Thus, achieving the COVID-19 interventions in Mekelle relies on understanding the broader context of the intervention setting rather than simply focusing on disseminating the pandemic-related messages. This study examines the levels of applying the recommended COVID-19 prevention measures in Mekelle and the challenges for implementation. It focuses on the following questions:

1. What is the level of awareness of people about the COVID-19 in Mekelle?
2. What is the level of applying the recommended COVID-19 prevention measures in Mekelle?
3. What are the factors (if any) affecting the implementation of the COVID-19 prevention measures?

The Study Context

Ethiopia is located in the horn of Africa and is known as one of the oldest nations on the continent. It occupies 1.1 million square kilometers and is bordered by Eritrea to the North, Djibouti and Somalia to the East, Sudan to the West and Kenya to the South. Based on Worldometers (2021), Ethiopia has a population of 116,690,885. Nevertheless, it is challenging to get the exact official figure as the country did not undertake Population and Housing Census after May 2007. With the current population, the country becomes one of the most populated nations in east Africa and the second most populated nation in the continent, next to Nigeria. Ethiopia is a diverse country with more than 80 ethnicities, varied geographic and climatic conditions, rich traditions and multifaceted history.

The major languages in Ethiopia are Oromifa, Amharic and Tigrigna. However, due to the federalism system, each region has its respective office language. Tigray is one of the states within the Federal Democratic Republic of Ethiopia, with total area coverage of 80,000 km2. It is located in the northern part of the country and its capital Mekelle is 762 km away from the country's capital, Addis Ababa. Tigray borders with Eritrea from the north, Sudan in the west, Afar Regional State in the east and Amhara Regional State in the southwest. Mekelle is 783 km away from Addis Ababa, the Ethiopian capital, with a population of 524,000 people (Population Stat., 2020). High levels of poverty characterize most parts of Ethiopia. Still, most people rely on food aid for their survival. More than 8 million people are currently estimated to require food assistance in Ethiopia, 4.5 million acutely malnourished residents, and 9.5 million need non-food emergency assistance (Mercy Corps, 2020). In Tigray, there is a high level of poverty. The poverty levels in Tigray were found 61.4, 48.5 and 31.8% in 1999/00, 2004/05 and 2010/11, respectively, while the 2015/16 survey result shows that the poverty rate of Tigray has declined to

27% (Central Statistics Agnecy, 2016). However, this makes the region's situation more dangerous compared to the national average of 23.4% (World Bank 2017; cited in Dejene and Cochrane, 2019). The COVID-19 has posed additional challenges to alleviate poverty. Based on the Economic Commission for Africa (2020) report, it is estimated that between 5 million and 29 million people will be pushed below the extreme poverty line of $1.90 per day due to the impact of COVID-19. This will have a tremendous impact on low-income earners, including daily laborers in the construction sector, informal businesses, and street vendors.

MATERIALS AND METHODS

This study employed a case study. The case study has been espoused in social science and health care research for many years (Stake, 2008; Creswell, 2012; Yin, 2012). Swanborn (2010) indicated that cases could be located at the micro (persons and interpersonal relations), meso (organization, institution), or macro levels (communities, democracies, societies) and involve one actor or multiple actors. The case study becomes relevant as it is useful for discovering new behaviors, processes, or anything we have little knowledge of it, responding to 'how and why questions about a contemporary set of events (Meyer, 2001, p. 330).

This study was undertaken in Mekelle, the Tigray capital, in northern Ethiopia. There are different reasons to select Mekelle. First, as the capital, it is one of the most densely populated cities in Tigray. The pandemic seems mostly critical in dense urban settings, often with a fast transmission (Florida, 2020). Second, Mekelle is a gateway for people travelling to and from Addis Ababa, where the majority of the COVID-19 cases are reported. Third, there is a high level of population mobility from rural to urban, including to Mekelle and vice versa. This study was undertaken purely on qualitative research principles, and telephone interviews, personal observation and document reviews were used as data collection techniques. The study subjects were journalists (private and government), health experts (private clinics and government hospitals) working in the frontlines, religious leaders, and the general public.

Telephone interviews: Due to travel restrictions in Mekelle, it was challenging to undertake face-to-face interviews. I could not use video-calling (Skype/Zoom) due to a limited internet connection in the city. Thus, a telephone interview was used to contact interviewees. Telephone interviews have several advantages. It is cost-effective, allowing interpersonal communication without a face-face interaction (Carr and Worth, 2001). Telephone interviews provide higher levels of anonymity and privacy (Carr and Worth, 2001; Holt, 2010; Vogl, 2013); as many people are shy and are not comfortable with face-to-face interactions (Tucker and Parker, 2014). This study used a semi-structured interview format; and adopted a conversation style approach (Kvale and Brinkmann, 2008; Rowley, 2012). Interviewees were purposefully selected based on their roles in the COVID-19 intervention campaigns. Interviewees comprised journalists (both private and government), health experts, and religious leaders.

Thirty-five individual interviews were held, each lasting 15–20 minutes. Interviewees comprised 6 religious leaders (Catholic (1), Orthodox (2), Muslim (1), and protestant 2) participants; 6 health experts (3 from private clinics and 3 from government hospitals); and 23 journalists (4 from FM 104.4; 4 from Dimtsi Weyane radio; 8 from DW TV; and 7 from Tig TV).Telephone interviewing has limitations. One of the limitations was creating a good interview ambience, including ensuring participants are comfortable and interview distractions are kept to a minimum. Also, there were instances of mobile network failures and mobile phones getting switched off during interviews. With this, some interviewees were reluctant to answer calls despite prior consent for the interview.

Personal observation: A total of 15 hours of personal observation was undertaken in selected sites in three rounds.The personal observation was used to observe, recognize and record the levels of practicing the recommended COVID-19 prevention measures by the general public in Mekelle city. In relation to what to observe and how to observe, Gobo (2008, p.162) outlines three issues, including the "social structures, common-sense interpretations/explanations given by individual participants and the context of action." This can be attained using a focused observation by recording actions using a checklist of questions to "tick off pre-established actions" (Marshall and Rossman, 2014, p.139).

For personal observation, sites were purposefully selected, including market places, churches/mosques, and coffee houses where a large number of people congregate. Observation focused on to what levels people practice handwashing with water and soap; the extent of applying physical distancing; whether the public used face masks; avoided visiting crowds, and the home-stay subsequent government measures. Personal observations were undertaken in three rounds (at early stages of the pandemic, soon after the lockdown and stay-at-home measures, and after the government lifted the lockdown measures) to evaluate the continuity or disparities in complying with the recommended prevention measures.

Document reviews: Document reviews for this study focused on four editorial policy documents from private and government broadcast media houses (radio and TV) in Mekelle. The media houses were the Tigray television station, the Dimtsi Weyane radio, FM Mekelle 104.4 and the Dimtsi Weyane television stations. These media houses were purposefully selected based on their relevance for health education-related programs and to examine the extent of coverage of the COVID-19-related news and programs in each of them.

DATA ANALYSIS AND DISCUSSION OF RESULTS

Data Analysis

Data analysis was undertaken qualitatively using thematic analysis. Audio outputs in the local language (Tigrigna) were transcribed into English, and responses were coded in a matrix that contains different thematic categories. Then, tabulated responses were thematically ranked from the most frequently

mentioned to the least frequent ones. Observation-based field notes were coded into themes based on the matrix of observation to identify and formulate all ideas, themes or issues they suggest (Emerson et al., 2011). Document analysis was made based on the procedures described by Startt and Sloan (2003). The procedures focused on the evidence, the context, and constructing generalizations (ibid, pp. 201–202). Finally, observation-based field notes and tabulated responses from the interview and extracts and texts were carefully analyzed so that sensible meanings emerge from the recurring themes.

Ethics Statements

For this study, ethical clearance was reviewed and obtained from the Ethical Review Committee at the College of Health Sciences, Mekelle University. The participants provided their written, informed consent to participate in the study. Choices of participants are adhered to in the research process, including the choice to withdraw from the study at any stage. The identity of the research participants was kept confidential. All the quotations from the interviewees have been attributed to pseudonyms.

DISCUSSION

Media Coverage of the COVID-19 in Mekelle

As a new public health emergency, the COVID-19 has received extensive media coverage globally. It has been much more prominent in the media than the recent pandemics, including Ebola (Wahl-Jorgensen, 2020). In Mekelle, both private and government media houses have produced several news and programs about the pandemic. Editorial policy documents from the Tigray television, the Dimtsi Weyane television, FM Mekelle 104.4, and the Dimtsi Weyane radio stations showed that each media house has extensively covered the pandemic-related news and programs. The COVID-19-related programs in these media houses focused on different topics, including COVID-19 and gender, psychology, food security, agriculture, and investment. But there are differences in the levels of news and program coverages within these media houses. The Tigray television had the most coverage (138 news and 45 programs); the Dimtsi Weyane television (45 news and 36 programs); FM Mekelle 104.4 (52 news and 39 programs); and the Dimtsi Weyane radio (43 news and 38 programs). Despite these differences in the news and programs, these media houses have broadly covered the pandemic to educate the public. Nevertheless, extensive media coverage of the pandemic may not necessarily pledge to realize the contents of the message. Instead, it is a one-directional information dissemination mechanism and does not mean individuals are effectively communicated to; they are simply exposed to "information dumping" (Moemeka, 2000, p. 9). The basic reason for this is that people have different levels of understanding and interpreting media messages, which determines their response towards the COVID-19 messages. This highlights the need to broadcast consistent messages to different audiences as each may have varying levels of perception and response to a crisis.

Responses of audiences to a crisis can be categorized into three groups: freeze, flight and fight (Bracha, 2004, p.679); each pattern involving different communication forms. The freeze cluster entails clear incentives to make people more alert to the risks they are facing, and the flight cluster needs to be informed that even within isolation, there are risks that will need to be addressed (Collins et al., 2020, p.5). But the fight cluster involves good advice on how to channel their need for action into behavioral responses that reduce the risk to themselves and others while avoiding blaming others who are alleged to be causing or amplifying the risk (Renn, 2015). Yet, there are no programs specific to different segments of the population, including the marginalized and vulnerable groups, who constitute a significant number in Mekelle. In relation to this, a radio journalist argues that:

> We know that there are listeners with diverse needs and priorities. Unfortunately, our media does not have programs explicitly focusing on these individuals. But designing programs specific to their needs become helpful in promoting inclusive and coherent media messages.

Besides the lack of classifying audiences, there are limited local sources to design context-based pandemic-related news and programs. Affirming this, a TV journalist contends that:

> . . .there are limited sources locally that can provide detailed information about the COVID-19. Mostly, we rely on international media to produce news and programs. However, it is challenging to translate and contextualize the information we obtain from foreign sources.

These views highlight that media houses produce the COVID-19 news and programs without realizing these factors and the context of intervention, which may restrain the awareness levels of different segments of the public in Mekelle.

The Level of People's Awareness About the COVID-19

Communicating relevant, accurate, and timely health information to at-risk populations is a critical factor for promoting public health (Kreps and Maibach, 2008). Media plays a significant role in informing the public about pandemics (Farr et al., 2005; Rubin et al., 2010). An essential component of media is messaging relevant information, which educates the public with what is known about the virus and information about health behaviors that can reduce individual-level risk (Frieden and Lee, 2020). Results showed that extensive media coverage about the COVID-19 has helped to raise the awareness of people in Mekelle. A TV journalist describes this as:

> At the early stages of the pandemic, there were confusions. But currently, there are great levels of awareness among the people in Mekelle. Each time

> we ask respondents in different parts of the city, we get a good level of awareness about the COVID-19 and its prevention methods.

A health expert from a public hospital also adds that:

> With the rising levels of misinformation, getting the right information about the COVID-19 is challenging. But most people seem that they are well informed about it. Each time you ask them about it, they give you details. This shows increasing levels of public awareness about it.

The above assertions are consistent with other studies about the roles of media in creating awareness about public health, leading to positive behaviors and prevention practices (Wakefield et al., 2010; Wakefield et al., 2011; Jung et al., 2013; Collinson et al., 2015; Majumder et al., 2015). Notwithstanding the levels of media coverage, several factors determine positive behavior changes and collective action toward pandemic prevention. Notably, designing context-based COVID-19 news and programs becomes vital to educate the majority of the public. In Mekelle and other parts of Tigray, people are highly interconnected through religion, kinship and cultural ties. Understanding these factors helps to design messages consistent with the values and cultural positions of people. Also, it promotes community engagement in the production and dissemination of the COVID-19 messages. However, a lack of understanding of the context would neither "take the people into confidence; nor attempt to learn from them" (Moemeka, 2000, p. 4). Community engagement promotes trust in the media messages as community members become key actors in information generation and raise their awareness levels leading to behavior change.

The Level of Applying the Recommended COVID-19 Prevention Measures

With no explicitly recommended antiviral treatment or vaccine for the COVID-19 to date, it requires realizing the recommended prevention measures. Containing the rapidly spreading virus relies on the public having accurate perceptions of personal and societal risk factors. This becomes vital as people's behavior can fundamentally influence and alter the spread of a pandemic (Funk et al., 2009; van Bavel et al., 2020). Observation results showed that most people in Mekelle had demonstrated the readiness to apply the recommended COVID-19 prevention measures during the early stages of the pandemic. Most of them practiced regular handwashing with soap and water, wore face masks, utilized sanitizers, and stayed at home due to closures to schools and government offices. However, there were limited practices in physical distancing, notably in churches and mosques, market places, coffee houses and restaurants where a large number of people congregate. Based on this, it can be argued that the majority of the public has continued to neglect government advice about physical distancing.

One of the reasons for this is that, still, there is a belief that the pandemic is an illness of the old and does not affect the young. Therefore, despite diverse COVID-19 education programs through different media, most people, particularly the young generation, ignore these messages. The reason to overlook them can be the lack of preparedness from the public to cooperate and implement the recommended prevention measures. In Mekelle, it seems business as usual for most people despite the mounting numbers of the COVID-19 cases. Hence, it requires identifying the factors preventing these individuals from applying the COVID-19 prevention measures.

Factors Affecting the Implementation of the COVID-19 Prevention Measures

Most nations have shown the readiness to realize the COVID-19 prevention measures. But others, notably the developing countries, are struggling to deal with it. There are different reasons for the differences in implementing the recommended pandemic prevention measures. One of the reasons is related to the degree of preparedness and resource mobilization of nations. Ethiopia has a flawed healthcare system and limited resources to control the pandemic. Hence, educating the public becomes a priority to contain the rapidly spreading virus. Journalists and health experts were given the responsibility to educate the public. However, it was challenging for both professionals to handle their duties effectively. Results showed that journalists experienced different challenges in covering the pandemic. A TV journalist describes it as:

> Covering the COVID-19 is a challenging job for journalists in our context. Some of the challenges posed to journalists include the lack of adequate knowledge about the pandemic, lack of personal protective equipment and the widely disseminating misinformation among the public.

Similarly, a radio journalist also adds that:

> Producing news and programs about the COVID-19 is demanding. As the pandemic is new, you do not get as many details as required. The sources you contact may not provide you with detailed information. With this, each time you reach sources, there is a high degree of uncertainty about personal and family safety issues.

Health experts working in the frontlines also face several challenges that affected their pandemic prevention campaigns. About this, a health expert from a government hospital contends that:

> We have been working a lot to educate our people about the COVID-19 and its prevention measures. However, the pandemic has caused fear and confusion among the public, which affected our intervention efforts. Similarly, as professionals, we face insecurity in our lives.

The issue of fear and confusion about the pandemic from the professionals and the public can affect the pandemic intervention efforts. Also, shortages of personal protective equipment have significantly affected the interest of professionals to engage in the COVID-19 prevention campaigns. Thus, the challenges mentioned above require special attention both from the media houses and respective government offices. Other challenges affecting the COVID-19 prevention in Mekelle are the widely spreading myths and misconceptions, religious proclivity, and the poverty and livelihood concerns of the public.

Myths and Misconceptions About the COVID-19

Myths and misconceptions have tremendously affected the COVID-19 prevention efforts in most African countries (Frenkel et al., 2020; Russonello, 2020). Since the first COVID-19 case in Ethiopia, several myths and misconceptions have circulated among the public. One of the commonly mentioned myths in Mekelle was eating spicy food to protect the virus. Others advocated that washing your body with "Holy water" protects from the virus. Consequently, several people traveled to "Holy water" centers even though the government introduced restrictions against it at later stages. The other most widely disseminated myth among the general public was consuming lemon juice, garlic and ginger to prevent the virus. Observation results showed unanticipated inflation in prices of these items in the markets. Social media has exacerbated the dissemination of these myths that further intensified the confusion among the public. Moreover, several COVID-19-related misconceptions were circulating among the public in Mekelle. One of the commonly mentioned misconceptions was that the pandemic is an imported disease manufactured in the US laboratory to reduce the rapidly growing populations in Africa. The persistence of myths and misconceptions forced people to rely on traditional medicine and cultural practices to prevent the pandemic. This can be related to the lack of trust in the government's preparedness and ability to respond to the pandemic. This is consistent with other studies in Sierra Leone about the government's incompetence to prevent the Ebola pandemic (Prati and Pietrantoni, 2016; Yamanis et al., 2016).

Ethiopia is in an uncertain political environment where there is mistrust between the federal government and regional governments. Likewise, there is confusion among the public that the COVID-19 prevention campaigns might have been compromised for political gains. For instance, one of the confusions that led to the mistrust toward the government was Ethiopian Prime Minister Abiy Ahmed, who publicly declared that the nation is extracting a herbal medicine that cures the virus. Although this claim is against the guidelines and procedures outlined by WHO, the PM insisted that the nation is extracting the herbal. This is unrealistic and shows interest in political gains rather than a coordinated effort to fight against the pandemic. These messages, along with the rapidly disseminating misinformation, have significantly affected the pandemic prevention efforts. However, there are no systems in place to regulate the COVID-19-related misinformation in Ethiopia, nor are there timely media responses about the misleading information.

Globally, there is limited research about disseminating false information during health emergencies (Pulido et al., 2020). Therefore, it requires debunking the myths and ending the traditional medicine hype. Experience from the HIV/AIDS pandemic shows similar perceptions and limited behavior changes, which claimed many lives in Mekelle and other parts of the country. Hence, it should be an excellent lesson in realizing the COVID-19 prevention campaigns through holistic response. This can be attained through providing accurate and appropriate information from the media, health experts and relevant government offices. Equally, it is essential to realize the peculiarities of the intervention setting and utilize risk communication and community engagement strategies accordingly.

Religious Proclivity Among the General Public

Culture and religion are an integral part of the social and moral fabric of the people in Mekelle. Accordingly, most people favor participating in cultural and religious activities. Despite the measures to avoid crowds and public gatherings, most people continued to participate in the cultural and religious ceremonies in Mekelle. Observation results showed that a large crowd attends funeral rite, and the majority of the Christian religious followers attend the "Sunday Mass" and other religious events. The same applies to Muslims attending the "Friday Prayers" and funeral rites. Therefore, irrespective of the awareness levels about the pandemic, many people are failing to comply with the recommended COVID-19 prevention measures. Some of the factors leading to negligence to prevention measures include cultural and religious misunderstandings and misinformation about the virus. Results showed that the majority of the public in Mekelle still believe that the COVID-19 is a "punishment from God" or "Allah" due to people's immoral acts. In relation to this, a religious leader argues that

> The coronavirus is one of the most worrying pandemics we have experienced. I believe that it is a result of our sinful acts. . . .we need to cleanse ourselves and become closer to Him. We need to unite and pray for His forgiveness.

Another religious leader also adds that:

> . . .I believe that voracity and individualism are dominating our world. This is not what we have learned from Him. Thus, we need to pray and come back to the basics of our religious teachings. That is the only way to fight the virus.

Others accentuated the power of praying to prevent the severe impacts of the pandemic. Emphasizing this, a religious leader argues that:

> Most nations, including those with quality healthcare, are struggling to cope with the dreadful impact of the pandemic. We do not have the resources to prevent it. We pray for His absolution. Had it not been for the power of God, the impact of the pandemic would have been worse than anyone can imagine.

Acknowledging the power of praying to prevent the anticipated impact of COVID-19, the Ethiopian Inter-Religious Council has declared a nation-wide one month of prayers earlier in March 2020. Religious leaders believed that this program incites family solidarity, inspiring people to practice the recommended measures through family influence. Religious leaders and community elders are the most trusted and accepted individuals by their respective community members. Engaging these individuals in the COVID-19 prevention programs can bridge the gap as people are more inclined to cultural and religious practices. This is consistent with related practice about the Ebola in West Africa and the vital roles of community engagement in preventing the virus. In relation to this, Osoro (2017) indicated that one of the valuable lessons learnt from the Ebola outbreak in Sierra Leone was engaging the communities in response to the outbreak. Community engagement in pandemic prevention ensures trust and confidence in the prevention efforts. It contributes to reporting suspected COVID-19 cases and minimizing stigma and discrimination to those affected. It is vital as cultural insiders and leaders can help address people's misperceptions and enhance trust to encourage health system use (Abramowitz et al., 2015; Kennedy and Nisbett, 2015).

Poverty and the Livelihood Concern

Poverty and livelihood concerns are the significant challenges in the COVID-19 prevention campaigns in developing countries. This is because the COVID-19 continues to impact people's livelihoods and income opportunities, leading to food insecurity (World Food Program, 2020). Most people in Mekelle are low-income earners that participate in the informal sector, often relying on daily wage to feed their family and cover related expenses. The COVID-19 has posed additional challenges to their livelihoods efforts as most of them are struggling to earn income. Consequently, they are confronting the harsh reality of contracting the virus while working for survival. Observation results have shown that daily laborers in the construction sector, informal businesses, and street vendors have continued their daily activities despite lockdown measures.

People working in the informal sectors have been most affected by COVID-19 response measures such as stay at home orders, closure of open markets and shutdowns in many sectors (Demeke and Kariuki, 2020). The lockdown measures became a predicament for these individuals as the measures threaten their efforts for survival. The challenge is not only for food items but also to cover expenses for personal protection equipment such as face masks, sanitizers and medication. There were several media reports in Mekelle asserting that donors have been supporting people in need in the form of food and sanitation items. But with high levels of need for assistance, these contributions cannot sufficiently satisfy their needs. Overall, the lockdown measures that proved useful in the pandemic prevention campaigns in most of the developed nations seem impractical in Mekelle. One of the reasons for the inapplicability of lockdown measures is that most people rely on daily income to survive and not refrain from attending their workplaces or informal businesses. This, in turn, places individuals at risk and immensely affects the pandemic prevention campaigns.

CONCLUSION

To contain the rapidly spreading virus, the Tigray State government has adopted different measures endorsed by the World Health Organization. Broadcast media houses have extensively covered the pandemic-related news and programs to educate the public. These programs have positively contributed to raising the awareness of the public about the pandemic. However, there are limited results in realizing behavioral changes among the public in applying the recommended COVID-19 prevention measures. Notably, a limited practice in wearing face masks, a lack of avoiding crowds and no physical distancing in market places, religious institutions, coffee houses, and restaurants. Different factors are affecting the implementation of the recommended prevention measures in Mekelle. These include an increasing level of myths and misconceptions about the pandemic, religious factors associating the COVID-19 with a "punishment from God" or "Allah", a perception that the pandemic affects only the "old", and persistent poverty levels of most people, who ignored the lockdown and stay-at-home measures.

The number of COVID-19 cases has been rapidly increasing in Mekelle. Thus, it obliges introducing the following measures to develop behavior changes among the public. First, it requires understanding the overall context of the intervention setting to identify the nature and preferences, media literacy and related factors of the target population. This helps to design inclusive and relevant messages targeting diversified segments of the public. Second, along with the media messages, it requires promoting interpersonal and group communication to maintain an effective flow of pandemic-related messages among the population. This enhances two-way communication and fosters dialogue at the household and community levels to comply with the preventive measures.

Third, several myths and misconceptions resulting from cultural beliefs and religious practices, seem to distract holistic response toward the pandemic prevention campaigns. Therefore, it necessitates debunking the myths and misconceptions. Lastly, as most people in Mekelle are interconnected through culture and religion, it requires empowering community elders and religious leaders to achieve the desired behavior change among the people. As Gould and Marsh (2004, p.16) argued, "positive health behaviors are more likely to be attained and sustained when the people within a cultural setting are involved in a contextual transformation process." In general, inclusive pandemic prevention communication for a sustained behavior change

necessitates employing consistent and coordinated messages from different groups, including the media, community elders and religious leaders.

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 Ethical review Committee, Mekelle University, College of Health Sciences. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

This paper is conceptualized and solely written by HN.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcomm.2021.562512/full#supplementary-material

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Vaccine Hesitancy and Rejection of a Vaccine for the Novel Coronavirus in the United States

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The arrival of the COVID-19 vaccine has been accompanied by increased discussion of vaccine hesitancy. However, it is unclear if there are shared patterns between general vaccine hesitancy and COVID-19 vaccine rejection, or if these are two different concepts. This study characterized rejection of a hypothetical COVID-19 vaccine, and compared patterns of association between general vaccine hesitancy and COVID-19 vaccine rejection. The survey was conducted online March 20-22, 2020. Participants answered questions on vaccine hesitancy and responded if they would accept the vaccine given different safety and effectiveness profiles. We assessed differences in COVID-19 rejection and general vaccine hesitancy through logistic regressions. Among 713 participants, 33.0% were vaccine hesitant, and 18.4% would reject a COVID-19 vaccine. Acceptance varied by effectiveness profile: 10.2% would reject a 95% effective COVID-19 vaccine, but 32.4% would reject a 50% effective vaccine. Those vaccine hesitant were significantly more likely to reject COVID-19 vaccination [odds ratio (OR): 5.56, 95% confidence interval (CI): 3.39, 9.11]. In multivariable logistic regression models, there were similar patterns for vaccine hesitancy and COVID-19 vaccine rejection by gender, race/ethnicity, family income, and political affiliation. But the direction of association flipped by urbanicity (P=0.0146, with rural dwellers less likely to be COVID-19 vaccine rejecters but more likely to be vaccine hesitant in general), and age (P=0.0037, with fewer pronounced differences across age for COVID-19 vaccine rejection, but a gradient of stronger vaccine hesitancy in general among younger ages). During the COVID-19 epidemic's early phase, patterns of vaccine hesitancy and COVID-19 vaccine rejection were relatively similar. A significant minority would reject a COVID-19 vaccine, especially one with less-than-ideal effectiveness. Preparations for introducing the COVID-19 vaccine should anticipate substantial hesitation and target concerns, especially among younger adults.

Keywords: COVID-19, demography, disease outbreaks, vaccines, surveys and questionnaires

INTRODUCTION

The pandemic of novel coronavirus disease (COVID-19) (1) has caused huge disruptions to life in the United States, which on March 26, 2020, became the country with the most cases globally. By late March 2020, researchers understood the disease to be more severe in older age groups (2), although reports of cases in children and young adults also circulated widely in the news (3).

Widespread uptake of the COVID-19 vaccine could control spread of the disease, but high uptake of vaccine is not guaranteed. Studies during the H1N1 pandemic in 2009 found that many individuals did not want to get vaccinated at the later points during the epidemic (4, 5), which could be due to apathy, desensitization, or a belief that there is a lower probability of illness. Individuals also may be less accepting of a pandemic vaccine if they perceive it to be less safe or effective (6). Because newly developed vaccines have not been on the market long, the general population may perceive these vaccines to be less safe and want more information on the safety profile of the vaccine (7, 8). Additionally, given the proclivity of RNA viruses like SARS-CoV-2 to mutate rapidly, it is not entirely clear how effective any potential vaccine will be. While all vaccines go through rigorous clinical trials (9), members of the general public may not understand this process well. For these reasons, assessing how perceived effectiveness and safety could influence acceptance of a potential COVID vaccine over the course of an outbreak is important. Moreover, the currently available COVID-19 vaccines all have varying attributes in terms of efficacy and risk of adverse events (10).

Vaccine hesitancy, an increasingly recognized global phenomenon (11), could also play a role in limiting people's desires for a COVID-19 vaccine (12), or could itself be impacted by the epidemic (13). Vaccine hesitancy is defined by the WHO as the "delay in acceptance or refusal of vaccines despite availability of vaccine services. Vaccine hesitancy is complex and context specific, varying across time, place and vaccines. It is influenced by factors such as complacency, convenience and confidence" (14). Over the course of the 2009 H1N1 outbreak, negative attitudes towards vaccination in general in France increased dramatically from 9.6% to 38.2% (15). This could be correlated with decreases in risk perceptions, but more information is needed on how risk perceptions, vaccine hesitancy, and vaccine acceptance interrelate for an emerging outbreak of an infectious disease. Given the rapid development of a COVID-19 vaccine, and its deployment among adults, who have fewer vaccination recommendations than children, it will be important to document how vaccine hesitancy in general differs from the specifics of COVID-19 vaccine rejection.

Another question remains about whether acceptance of a vaccine would vary by age of the individual or safety/effectiveness profile of the vaccine. Anecdotally, it is thought that younger adults are not taking the virus seriously, with frequent news stories about young adults taking spring break trips (16), and news in the early phase of the pandemic focused on risks in older adults. The aims of this study are to estimate differences in vaccine hesitancy and COVID-19 vaccine acceptance by generation, and to characterize if acceptance is affected by how safe or effective the vaccine is.

Understanding vaccination attitudes at the beginning of the epidemic is uniquely important because research from previous epidemics has shown that acceptance of vaccines and compliance towards public health recommendations decline over time (4, 15, 17). Additionally, understanding to what extent US adults would accept a new vaccine for COVID-19 would help the government to design risk communication messages regarding the deployment of new vaccines for COVID-19.

METHODS

Study Population

US adults who were part of the sampling frame of the survey research firm, Dynata, were eligible for inclusion into this study. Dynata recruits participants through social media and other advertisements, and notifies them of their eligibility to participate in surveys. We built an age-gender nested quota system into the model, whereby a set number of individuals were sought across female/male gender and six age groups (18-24 years old, 25-34 years old, 35-44 years old, 45-54 years old, 55-64 years old, and 65-99 years old), with numbers roughly equivalent to their distribution in the US population. This cross sectional survey was implemented March 20-22, 2020.

We sought a sample size of 800. At this size, with an alpha of 0.05 and a power of 80%, and a proportion of 50% (a statistically conservative estimate of what proportion of the population supports a given public health action) the margin of error is 4%, which we judged to be sufficiently precise.

Questionnaire

Participants responded to a similar set of questions, but participants who mentioned that they had a parent over the age of 60 or a child under the age of 18 were asked additional questions. The questionnaire is publicly available: https://doi.org/10.6084/m9.figshare.13303121. The questionnaire was pre-tested in 16 individuals ranging in age from early 20s to late 60s.

Outcome Variables

The study had two outcomes: potential COVID-19 vaccine rejection and vaccine hesitancy. We asked all participants whether they would accept a hypothetical COVID-19 vaccine. Individuals were randomized into four conditions, where the safety and effectiveness attributes of the COVID-19 vaccine changed. Across the four categories, participants read that the vaccine was either (1): 95% effective with a 5% risk of fever, (2) 50% effective with a 5% risk of fever, (3) 95% effective with a 20% risk of fever, or (4) 50% effective with a 20% risk of fever.

Vaccine hesitancy came from a 10-item scale developed by the World Health Organization (WHO) Strategic Advisory Group of Experts on Immunization (SAGE) Vaccine Hesitancy Working Group (18). Because the original scale's developers' original purpose was to assess parental attitudes towards pediatric vaccination, we modified the scale to ask about the

individual's own vaccinations, not their child's. Participants responded about their agreement on 10 different statements on a 5-point Likert scale. In the analysis, we reordered the responses for certain questions (L1-L4, L6-L8) so that for all items, an increase represented greater vaccine hesitancy. Overall this scale had good internal reliability, the standardized Cronbach alpha was 0.89. The psychometric properties of the original pediatric scale have been previously studied (19). We summed this scale (possible range from 10-50), and then dichotomized the scale at 25, based on a validated measure (20).

Independent Variables

The primary independent variable was respondent age, which we categorized by generation. Due to a limited number of responses among individuals of the "Silent Generation" (individuals ≥75 years old) they were collapsed in with Baby Boomers (56-74 years old) for analysis. GenX included individuals 40-55 years old, Millennials 24-39 years old, and GenZ 18-23 years old (21).

For demographics, we used similar wording to previous questionnaires. Participants responded to the same race/ethnicity questions that are on the US Census and the 2019 Behavioral Risk Factor Surveillance System (BRFSS) (22). Due to participant sample sizes, we collapsed the race/ethnicity categories into non-Hispanic White, non-Hispanic Black, Hispanic, and other. We asked about gender identity using guidelines from the American Association of Public Opinion Researchers (23), although no one selected an "other" gender in this survey. A question on urbanicity came from the National Health Interview Survey (24).

We also asked about perceived risk of being infected within the next month using a scale from 0% to 100%. A previous study of H1N1 influenza included a similar question (5). We considered this variable to be continuous in the analysis.

Statistical Analysis

We ran multivariable logistic regression models, corresponding to the two different outcomes: COVID-19 vaccine rejection and general vaccine hesitancy. We used the same set of demographic predictors (participant gender, urbanicity, generation, race/ethnicity, family income, and political affiliation) based on *a priori* considerations. For vaccine rejection, we also included general vaccine hesitancy, perceived risk of infection, and the safety and effectiveness characteristics as additional independent variables in a "full model". To assess the interaction of generation and perceived risk, we included a cross-product term between these variables. We calculated the least squares marginal means for each outcome by generation to account for confounding by covariates in the multivariable regression models. We display parameter estimates and 95% confidence intervals (CI).

We compared the strength of odds ratios in the vaccine hesitancy and COVID-19 vaccine rejection by creating two observations per person, with the outcome of one of these observations being for vaccine hesitancy and the other for vaccine rejection. We then specified an interaction term between every predictor variable and a dummy variable for whether this was the hesitancy or vaccine rejection outcome. The model included Generalized Estimating Equation (GEE) methods with an independent correlation matrix to account for two data points per individual. A similar approach was used in a previous study (25). We display the P-value from the interaction terms.

All data were analyzed in SAS version 9.4 (SAS Institute, Cary, NC), and plots were generated in R version 3.6.0 (R Foundation for Statistical Computing, Vienna, Austria).

Ethical Approval

This study was deemed exempt by the University of Michigan Health Sciences and Behavioral Sciences Institutional Review Board (#HUM00179335). Participants read an information sheet which explained the risks and benefits of the study, which they had to agree to prior to starting the questionnaire. Participants were not given a direct research incentive but were given reward points through Dynata which they could use to exchange for gift cards.

RESULTS

In total,1,068 individuals clicked on the link to start the online survey and responded to at least one question: 271 (25.4%) did not respond to any questions beyond the screening questions (age and gender) on the start screen, and 50 (4.7%) did not consent, leaving 747 participants (70.0%). We excluded 34 individuals (4.6%) who spent less than 3 minutes on the survey, leaving a total sample size of 713.

Table 1 shows demographic characteristics of the study population, and the proportion who are vaccine hesitant or who would reject a COVID-19 vaccine by group. The sample was demographically diverse. Study participants were 54.3% female and 32.5% said they lived in a rural area. A plurality, about one-third (34.1%), were ≥56 years old, a majority (74.5%) were non-Hispanic White, and most participants reported family income either between $2,000-$4,999 (28.5%) or $5,000-$9,999 (30.5%).

COVID-19 Vaccine Rejection

Overall, 8.4% of individuals would reject a hypothetical COVID-19 vaccine that was 95% effective with a 5% risk of fever, whereas 12.2% would for a vaccine that was 95% effective and had a 20% risk of fever, 22.2% would for a vaccine 50% effective with a 5% risk of fever, and 29.5% would for a vaccine 50% effective with a 20% risk of fever (**Figure 1**). In the multivariable model for vaccine rejection accounting for vaccine attributes, vaccine hesitancy, risk perceptions, and the interaction between generation and risk perceptions (**Table 2**), we found that all these variables were significant. A vaccine with a 20% risk of fever had 1.63 times greater odds of being rejected compared to a vaccine with only a 5% risk (95% CI: 1.03, 2.57), and a vaccine 50% effective had 4.08 times greater odds of being rejected compared to a vaccine with a 95% effectiveness (95% CI: 2.44, 6.83). These differences translate to 95% effective vaccines being rejected by 12.8% of the population (95% CI: 8.6%, 18.7%), whereas 50% effective vaccines were rejected by 33.0% (95% CI: 25.6%, 41.4%). There was a smaller disparity by safety: a vaccine with a 5% risk of fever would be rejected by 17.5% (95%

TABLE 1 | Demographics of online survey panel, United States, March 2020.

		Count (column %)	Vaccine hesitant (row %)	Reject COVID-19 vaccine (row %)
Overall		713 (100%)	230 (33.0%)	131 (18.4%)
Participant's gender	Male	326 (45.7%)	98 (31.0%)	51 (15.6%)
	Female	387 (54.3%)	132 (34.8%)	80 (20.7%)
Participant's residence	Rural	227 (32.5%)	88 (40.2%)	37 (16.3%)
	Urban	471 (67.5%)	139 (29.9%)	93 (19.7%)
Participant's generation	Baby boomer and silent generation	242 (34.1%)	48 (20.5%)	40 (16.5%)
	GenX	222 (31.3%)	60 (27.6%)	41 (18.5%)
	Millennial	176 (24.8%)	80 (46.2%)	32 (18.2%)
	GenZ	70 (9.9%)	41 (59.4%)	17 (24.3%)
Participant's race/ethnicity	Non-Hispanic White	531 (74.5%)	146 (28.0%)	86 (16.2%)
	Non-Hispanic Black	50 (7.0%)	33 (70.2%)	17 (34.0%)
	Hispanic	53 (7.4%)	24 (47.1%)	12 (22.6%)
	Other	79 (11.1%)	27 (36.0%)	16 (20.3%)
Monthly family income	<$2,000	140 (20.2%)	70 (51.1%)	39 (27.9%)
	$2,000-$4,999	198 (28.5%)	70 (36.3%)	43 (21.7%)
	$5,000-$9,999	212 (30.5%)	60 (28.7%)	30 (14.2%)
	≥$10,000	144 (20.7%)	27 (19.1%)	18 (12.5%)
Political affiliation	Republican	216 (31.8%)	71 (33.3%)	37 (17.1%)
	Democrat	262 (38.5%)	76 (29.7%)	41 (15.6%)
	Independent	202 (29.7%)	74 (37.6%)	51 (25.2%)
Perceived risk of infection within next month	*median (IQR)*	*32% (11%-51%)*	–	–

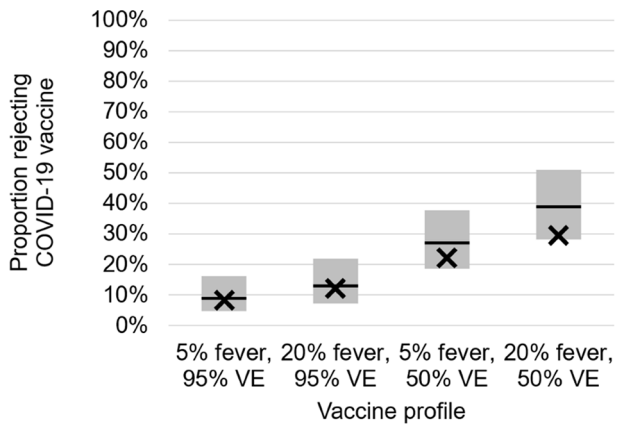

FIGURE 1 | Modeled (bars) and observed values (X) for vaccine rejection by vaccine effectiveness (VE) and risk of fever. Modeled estimates and 95% confidence intervals from least square means marginal proportions, accounting for age, urbanicity, race/ethnicity, income, and political affiliation.

CI: 12.5%, 23.9%) and this was 25.5% (95% CI: 18.8%, 33.7%) for a vaccine with a 20% risk of fever.

Vaccine hesitancy and perceived risk were significantly associated with COVID-19 vaccine rejection. Those vaccine hesitant were significantly more likely to reject COVID-19 vaccination (OR: 5.56, 95% CI: 3.39, 9.11). Increases in risk perceptions were associated with decreases in vaccine rejection (OR: 0.97, 95% CI: 0.95, 0.98). The association of risk perceptions and vaccine rejection varied by generation, with significant attenuation for Baby Boomers versus Millennials. **Figure 2** shows how the slope of the relationship between risk perceptions and vaccine acceptance is sharper for later generations: for Baby Boomers there is less of a relationship between risk perception and vaccine acceptance, whereas this is highly apparent for GenZ.

Comparison of COVID-19 Vaccine Rejection and General Vaccine Hesitancy

Table 2 shows results from multivariable models for COVID-19 vaccine rejection and vaccine hesitancy using the same set of predictors. There was no significant difference in COVID-19 vaccine rejection by generation, however there was a significant generational difference in vaccine hesitancy. Baby Boomers (OR: 0.40, 95% CI: 0.25, 0.65) and GenX (OR: 0.54, 95% CI: 0.34, 0.85) had lower odds of vaccine hesitancy compared to Millennials. The difference in the strength of association between generation and vaccine hesitancy and between generation and vaccine rejection was significant (P=0.0037).

Race/ethnicity was significantly related to both COVID-19 vaccine rejection and vaccine hesitancy, and the strengths of association between race/ethnicity and both outcomes were similar. COVID-19 vaccine rejection was higher in non-Hispanic Black individuals compared to non-Hispanic White individuals (OR: 2.86, 95% CI: 1.40, 5.87). And we found that participants who were non-Hispanic Black also had higher levels of hesitancy (OR: 4.07, 95% CI: 1.96, 8.42) than participants non-Hispanic White.

Higher levels of income were associated with less COVID-19 rejection and lower vaccine hesitancy scores. The association between income and COVID-19 rejection and between income and vaccine hesitancy was similar. For example, vaccine rejection was lower in those with higher income (>$10,000 *vs* $2,000-$4,999 OR: 0.53, 95% CI: 0.29, 1.00), and for this same comparison the odds of vaccine hesitancy was 0.44 (95% CI: 0.25, 0.77).

TABLE 2 | Impact of demographic factors on general vaccine hesitancy and COVID-19 vaccine rejection, online survey panel, US, March 2020.

	COVID-19 vaccine rejection (full model) OR (95% CI)	COVID-19 vaccine rejection (abbreviated model) OR (95% CI)	Vaccine hesitant OR (95% CI)	P-value[a]
Participant's gender				0.3494
Male	ref	ref	ref	
Female	1.34 (0.82, 2.18)	1.36 (0.90, 2.06)	1.09 (0.76, 1.56)	
Participant's residence				0.0146
Rural	0.61 (0.36, 1.03)	0.74 (0.48, 1.16)	1.36 (0.93, 1.97)	
Urban	ref	ref	ref	
Participant's generation				0.0037
Baby Boomer (≥56 years)	0.54 (0.19, 1.50)	1.11 (0.63, 1.94)	0.40 (0.25, 0.65)	
GenX (40-55 years)	0.81 (0.31, 2.10)	1.16 (0.67, 1.99)	0.54 (0.34, 0.85)	
Millennial (24-39 years)	ref	ref	ref	
GenZ (18-23 years)	1.20 (0.35, 4.16)	1.19 (0.58, 2.45)	1.34 (0.71, 2.51)	
Participant's race/ethnicity				0.7793
Non-Hispanic White	ref	ref	ref	
Non-Hispanic Black	1.87 (0.80, 4.39)	2.86 (1.40, 5.87)	4.07 (1.96, 8.42)	
Hispanic	1.29 (0.54, 3.07)	1.44 (0.69, 3.03)	1.56 (0.81, 2.99)	
Other	2.76 (1.25, 6.10)	1.76 (0.89, 3.49)	1.35 (0.72, 2.53)	
Monthly family income				0.5541
<$2,000	0.91 (0.49, 1.69)	1.25 (0.74, 2.11)	1.62 (1.00, 2.63)	
$2,000-$4,999	ref	ref	ref	
$5,000-$9,999	0.59 (0.32, 1.08)	0.60 (0.35, 1.03)	0.76 (0.48, 1.20)	
≥$10,000	0.68 (0.33, 1.39)	0.53 (0.29, 1.00)	0.44 (0.25, 0.77)	
Political affiliation				0.4363
Republican	0.78 (0.43, 1.41)	0.77 (0.47, 1.27)	1.10 (0.70, 1.71)	
Democrat	0.71 (0.41, 1.26)	0.48 (0.29, 0.78)	0.58 (0.37, 0.90)	
Independent	ref	ref	ref	
Vaccine hesitant				
No	ref	–	–	
Yes	5.56 (3.39, 9.11)	–	–	
Increase in 1 percentage point in perceived risk	0.97 (0.95, 0.98)	–	–	
Vaccine safety				
5% fever risk	ref	–	–	
20% fever risk	1.63 (1.03, 2.57)	–	–	
Vaccine effectiveness				
95% effective	ref	–	–	
50% effective	4.08 (2.44, 6.83)	–	–	
Generation * perceived risk interaction				
Risk * Baby Boomer	1.03 (1.01, 1.06)	–	–	
Risk * GenX	1.02 (1.00, 1.05)	–	–	
Risk * GenZ	0.99 (0.96, 1.03)	–	–	

[a]*Difference in estimates from COVID-19 vaccine rejection model and vaccine hesitancy model.*

Political affiliation was related to vaccine rejection and vaccine hesitancy. Those identifying as Democrats were less likely to reject the COVID-19 vaccine and less likely to be vaccine hesitant compared to Independents.

DISCUSSION

This study examines acceptance of a COVID-19 vaccine, and how it is affected by vaccine hesitancy in the early phase of the COVID-19 epidemic. We surveyed a demographically diverse group of U.S. adults between March 20 and 22, 2020. During this interval the estimated number of cases increased from 18,747 to 33,404. Our study found generational differences in vaccine hesitancy, with less hesitancy in older adults. However, this did not translate into reduced acceptance of the COVID-19 vaccine among younger adults.

In our study, a large majority of individuals would accept a COVID-19 vaccine, but a small and significant minority stated they would reject it. As expected, US adults were more accepting of a COVID-19 vaccines if they were safer or more effective. We do not know how safe or effective the COVID-19 vaccine will be, but if it mimics the influenza vaccine (26), it could be similar to our profile of 50% effectiveness and 5% risk of fever, which would be rejected by almost one-fourth of the population. Because we found differences in vaccine rejection by race/ethnicity and income, there could also be spatial differences in vaccine rejection, and therefore pockets of susceptibility within the country.

COVID-19 vaccine acceptance may also change over time. Two previous cross-sectional surveys this year found that between late January and late February 2020, acceptance of a COVID-19 vaccine increased from 48% to 65% (27). As the outbreak becomes more real to Americans, their acceptance of a

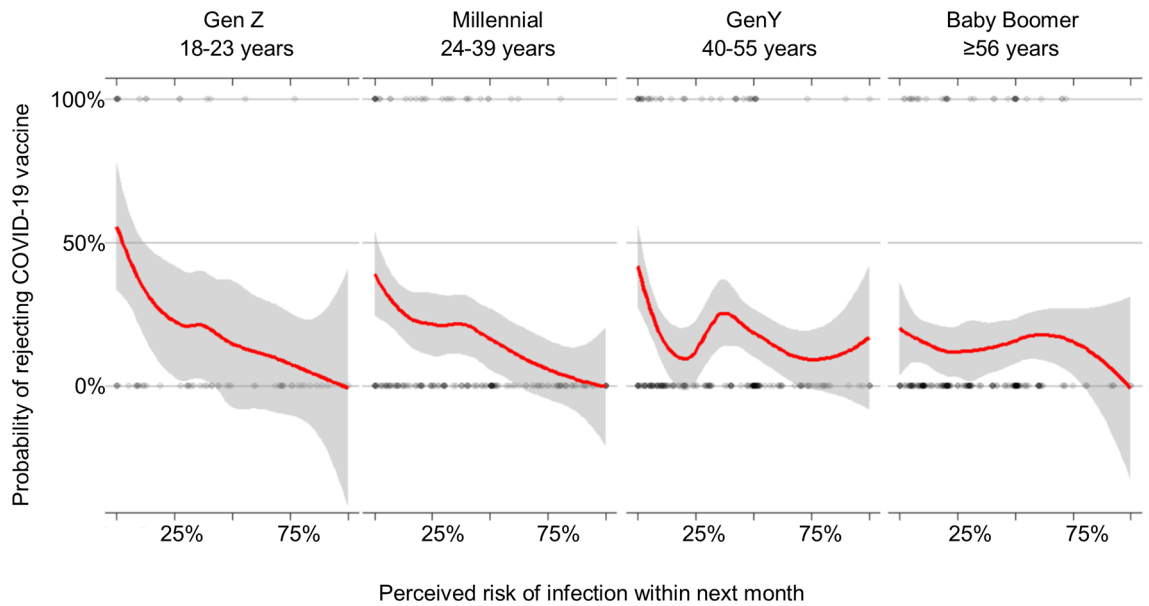

FIGURE 2 | Relation between risk perceptions and COVID-19 vaccine acceptance, by generation, US, March 2020.

vaccine may increase. This finding, in turn, would relate to the positive relationship we found between risk perceptions and vaccine acceptance, which has been echoed in other research (28). It is worthwhile for future research to observe the changes of vaccine acceptance and how it is related to the spread of disease and actions taken by the government.

Vaccine hesitancy may also increase over the course of the COVID-19 pandemic. In a study in France during the 2009 H1N1 influenza outbreak, negative attitudes towards vaccination increased rapidly, with the researchers speculating this was correlated both with concerns about the safety of a newly introduced H1N1 influenza vaccine and with heightened controversy over the perceived seriousness of the vaccine (15).

If vaccine hesitancy does increase, this could differentially impact younger generations and lead to lower uptake among younger adults. Therefore, how we deliver effective messages to the groups with high vaccine hesitancy to influence their behaviors is critical. A study of adult preferences for vaccines found that provider recommendations were just as important as effectiveness of the vaccine (8). Accordingly, strong promotion from health professionals could counter lower effectiveness of the vaccine.

We found that the relationship between risk perceptions and vaccine acceptancy varies by generation. One of the possible explanations could be that older generations are highly accepting of vaccines, regardless of their risk perceptions, whereas younger generations have higher intent when they perceive their personal risk to be higher. Future research could explain the reasons for this discrepancy, but it could be possibly tied to experience with previous outbreaks/pandemics, more appreciation for vaccines across the life-span, or more experience with vaccine-preventable diseases, such as measles, polio, or pertussis, which are now relatively rare. Regardless, vaccine education among younger generations should also focus on increasing risk perceptions. These promotions will be important for two reasons. One, if perceived risk decreases over time, as it has in previous outbreaks (4, 5), younger adults may become even more less likely to be vaccinated. Two, similar to the influenza vaccine (26), the COVID-19 could be even less effective in older adults compared to younger adults. Maintaining high vaccination coverage in younger adults could be key to creating adequate herd immunity that protects older adults.

General vaccine hesitancy itself was strongly related to rejection of the COVID-19 vaccine. There is already concern in some anti-vaccine groups that a COVID-19 vaccine could be compulsory (29). Our study found that vaccine hesitancy was higher in individuals among those with lower monthly incomes. This finding contrasts with previous research which has found that those with higher income tend to have higher vaccine hesitancy, lower vaccine coverage (30, 31), and higher incidence of vaccine-preventable disease (32). However, other studies have found no such relationship (33, 34). In contrast to many previous studies focusing on parents' hesitancy to pediatric vaccines, our study asked adult participants about their hesitancy to adult vaccination. It is likely that patterns of vaccine hesitancy differ when directed at an adult rather than at their children. For example, a previous study which presented participants with information about influenza vaccines with different attributes found that parents were more risk sensitive when considering vaccinating their child than considering the vaccines for themselves (35). And another study which looked separately at preferences among parents for childhood vaccines and adults for adult vaccines found that effectiveness was more important in the analysis of parents than in the analysis of adults (8).

Strengths and limitations

This survey used Internet-based samples to allow rapid data collection during the pandemic and to avoid person-to-person contact. However, Internet samples may have inherent biases. There is sampling bias in that individuals who participate need to have access to the internet, and so individuals of lower socioeconomic status will be less likely to participate. Additionally, individuals may answer rapidly with little thought, which is why we removed individuals from our analytical sample who completed the survey in a short period of time. We also note that constructs in our study, including items related to vaccine hesitancy or interpretations of effectiveness or fever, could differ across participants. Other factors, like education, could impact vaccination behaviors, but were not included in the survey.

CONCLUSIONS

In this survey of US adults in late March 2020, we found that a large majority of individuals would accept a COVID-19 vaccine. However, about one-third would reject the vaccine if it was only 50% effective – which is a reasonable estimate compared to the seasonal influenza vaccine. In general we found similar patterns for vaccine hesitancy and COVID-19 vaccine rejection, indicating that thoughts about vaccinations in general and for COVID-19, specifically, are highly correlated. Vaccine hesitancy may increase over the course of the outbreak, and if vaccine hesitancy increases and perceived risk of infection decreases, younger adults in particular may be less likely to become vaccinated. Acknowledging generational differences in risk perceptions could help the government tailor messages to promote vaccines. Additionally, stressing the safety of the vaccine will be important when rolling out the COVID-19 vaccine.

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 below: https://doi.org/10.3886/E130422V1.

ETHICS STATEMENT

This study was reviewed by the University of Michigan Institutional Review Board (#HUM00176454). Before starting the survey, participants read an informed consent form, which they could download as a PDF, and clicked a button to agree.

AUTHOR CONTRIBUTIONS

S-FS conceptualized the study and wrote the original draft. AW obtained funding, conceptualized the study, contributed to visualization, and wrote the first draft. NM wrote the original draft, and contributed to visualization. LP and BZ-F contributed to methodology, and contributed critically to reviewing the manuscript. YL conceptualized the study and contributed critically to reviewing the manuscript. All authors contributed to the article and approved the submitted version.

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From Plant Survival Under Severe Stress to Anti-Viral Human Defense – A Perspective That Calls for Common Efforts

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Reprogramming of primary virus-infected cells is the critical step that turns viral attacks harmful to humans by initiating super-spreading at cell, organism and population levels. To develop early anti-viral therapies and proactive administration, it is important to understand the very first steps of this process. Plant somatic embryogenesis (SE) is the earliest and most studied model for *de novo* programming upon severe stress that, in contrast to virus attacks, promotes individual cell and organism survival. We argued that transcript level profiles of target genes established from *in vitro* SE induction as reference compared to virus-induced profiles can identify differential virus traits that link to harmful reprogramming. To validate this hypothesis, we selected a standard set of genes named 'ReprogVirus'. This approach was recently applied and published. It resulted in identifying 'CoV-MAC-TED', a complex trait that is promising to support combating SARS-CoV-2-induced cell reprogramming in primary infected nose and mouth cells. In this perspective, we aim to explain the rationale of our scientific approach. We are highlighting relevant

background knowledge on SE, emphasize the role of alternative oxidase in plant reprogramming and resilience as a learning tool for designing human virus-defense strategies and, present the list of selected genes. As an outlook, we announce wider data collection in a 'ReprogVirus Platform' to support anti-viral strategy design through common efforts.

Keywords: viral diseases, early cell reprogramming, ReprogVirus, somatic embryogenesis, alternative oxidase (AOX), aerobic fermentation, stress tolerance, SARS-CoV-2

BACKGROUND

Effective immunologic protection contributes to resilient behavior of higher organisms. It is essentially based on the diversity of innate and adaptive cell responses and cell memory tools (1–4). Immunologic responses are energy consuming and require efficient metabolic reprogramming. However, metabolic reorganization is only recently recognized as an integrated part of immunology (5–8). It is increasingly understood that plants and animals have similar responses and cell memory mechanisms to manage immunology (1, 3). These insights enable science to profit from experimental systems across organisms and to apply a higher degree of abstraction for gaining relevant knowledge on early reprogramming events that link to overall resilience.

Somatic Embryogenesis (SE) – An Experimental Tool to Identify Markers for Early Reprogramming and Resilience

In plants, SE can be induced *in vitro* as a model for a resilient response upon severe stress of highly variable origins (9–18). SE induction depends essentially on the death of neighboring cells [(19); see also in (17)] and is defined as asexual regeneration of plants from single or few somatic cells, which can subsequently develop into an embryo in a similar process as it is known for zygotic embryogenesis in seeds [see reviews in (20)]. The discovery of SE in plants in 1958 revolutionized cell biology and stem cell research (9, 10). For the first time, it was revealed that totipotency could be acquired from differentiated somatic cells as it had been predicted by Haberlandt in 1902 (21, 22). SE is routinely used in plant biotechnology to massively propagate selected genotypes from individual plants. It can be utilized to help plants growing-out of virus threats, when propagation is induced from healthy parts of an infected plant (23). SE induction can be seen as an example of environment-inducible, molecular-physiological plasticity, a trait that is *per se* important marker for understanding resilient performance (17, 24–26).

It is common knowledge that energy-consuming reprogramming in eukaryotes is complex, individual- and context-dependent and integrates hormonal, epigenetic and metabolic actions regulated through a wide network of cell signaling factors, second messengers and transcription factors. Our group contributed to this knowledge with several research, perspective and reviewing papers [see e.g. in (11, 14, 26, 27)]. Typically, cell reprogramming covers dedifferentiation and *de novo* differentiation associated with autophagy and cell cycle regulation [see in (11, 17)]. Interaction within molecular networks relies upon cell origin, actual cell status, within cell distribution and structuration, cell communication and environmental signaling. Biochemical insights tell us that small variation at any level might have large consequences depending on thermodynamics, reactant and product concentrations, intermolecular forces, space organization and time. Consequently, relevant markers for reprogramming including those induced by viruses must be based on complex traits as confirmed by Costa et al. (Preprint 28).

Carbohydrate supply is essential for *in vitro* induction of SE (11, Preprint 28, Preprint 29). Sugars and sugar phosphates interact in plants and animals with hormone pathway networks and play central role in signaling to modulate energy metabolism and energy availability. Down-stream of sugars two important antagonistic protein kinases are involved in energy sensing and physiological adaptation (30–32). While sucrose non-fermenting-1-related protein kinase1 (SNRK1) is activated when energy is depleted (31, 33, 34), TOR (target of rapamycin, mTOR in mammals) is induced in situations of energy excess and stimulates cell cycle progression (G1/S and G2/M transitions) and cell proliferation (35). This stimulation involves transcription factors of the E2F family (36, 37). However, it was shown that a short six-hour pulse of one molar sucrose was sufficient to induce SE in hormone-free medium (16). This observation points to a more complex role of sucrose in cell reprogramming beyond energy supply. Sucrose is known to act as a signaling molecule (32, 38), in addition to acting as an osmotic stressor that can disrupt communication within and between cells (16).

Sucrose was also shown to trigger aerobic alcohol fermentation in support of respiration and synthesis of higher molecular weight compounds, such as, lipids (39). The phytohormone auxin and its distribution play critical roles for SE induction (40). However, sucrose could induce SE even in auxin-depleted medium (14). 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic herbicide that provides auxin activity, was shown to stimulate ethanol secretion in cultured carrot cells. Ethanol secretion was more dependent on sucrose availability than on oxygen availability, and linked to alcohol dehydrogenase (ADH) activity. Cell differentiation was shown to be critical for the amount of secreted ethanol (41, 42). Recently, Fan et al. (43) identified hormone and alcohol degradation pathways as the most activated during early stages of SE. Ethanol has been

demonstrated to reduce ROS levels in stress performance and led to high induction of alternative oxidase (*AOX*) and glutathione-S-transferase transcripts relative to several other tested genes (44). Aerobic alcohol fermentation was found to play a critical role in controlling tissue level concentration of pyruvate in plants and thereby, adapt respiration rates primarily to energy status rather than to oxygen availability (45).

2,4-D is frequently used in plant biotechnology, because it can induce SE with high efficiency. It seems to impose higher oxidative stress levels than seen for native auxins (46, 47). Reactive oxygen species (ROS) enforced by ROS-induced ROS release (RIRR) and reactive nitrogen species (RNS) can integrate outer and inner cell signals and coordinate together adaptive cell and organism responses (48). Slight variations in ROS and RNS levels can have strong effects on cell fates (49, 50). Excess of nitric oxide (NO) and ROS can lead to production of peroxynitrite ($ONOO^-$), which can cause nitration and subsequent inhibition of a broad range of cellular protein functioning and nitro-oxidative stress (51). ROS are known to interact with redox-sensitive protein cysteine thiol groups relevant for energy metabolism and metabolic channeling linked to cell differentiation and cell cycle regulation (51, 52, pre-print 53, 54). Downstream signaling pathways of NO constitute post-translational protein modifications by S-nitrosylation, including SUMOylation, phosphorylation, persulfidation and acetylation, which plays important role on altering protein functions either positively or negatively (55). Plant alcohol dehydrogenase 2 (ADH2) functions as nitroso-glutathione reductase (GSNOR) (56) and has high similarity to ADH5/GSNOR in human cells (Costa JH, not shown). GSNOR is involved in NO homeostasis and interferes with auxin signaling and polar auxin transport in higher plants (57). In animals, GSNOR was connected to mitochondria maintenance and cell longevity (58, 59). It can modulate redox signaling and, its overexpression in tomato could increase ROS and NO scavenging efficiency (60). Competence for SE induction was shown to be positively linked to the amount of anti-oxidant secondary plant compounds and enzymes (18, 26, 61–65). It is relevant to mention that high levels of NO can counteract SE induction, highly lightening the importance of balanced ROS/RNS homeodynamics in cells. Scavenging of NO by phytoglobins (66, 67) is suggested to integrate oxidative stress and auxin metabolism with the acquisition of SE competence. In plants, NO is produced mainly by the cytosolic nitrate reductase (NR) and mitochondrial electron transport-mediated nitrite to NO reduction (68).

AOX Integrates ROS/RNS Signaling, Aerobic Fermentation and Respiration During Reprogramming - A Learning Tool for Virus Defense?

We hypothesized that a better understanding of the role of AOX during SE induction can help to reveal mechanisms that could be used to confront harmful virus-induced reprogramming in human cells. This hypothesis had been explored through original research (Preprint 28) and confirmed our approach.

AOX functions universally in a vast variety of organisms across all kingdoms (69). Most probably, *AOX* gene got transferred into eukaryotes from prokaryotes *via* primary endosymbiosis (70, 71). However, *AOX* is not present in vertebrates and arthropods and the majority of bacteria lost *AOX* during the course of evolution (72). Nevertheless, in 2005 an Alternative Consortium was created to explore a beneficial role of AOX in mitochondrial oxidative phosphorylation that could alleviate phenotypic effects of widespread OXPHOS deficiencies in human diseases (73, 74). Currently, *AOX* is being explored in animals, which overexpress *AOX* ubiquitously [e.g. (75)] as a tool to understand respiratory control mechanisms (76–78). Studies on transgenic *AOX*-mice revealed differential effects of AOX on acute and chronic hypoxia, which helped to better understand pulmonary oxygen sensing mechanisms vital e.g. for respiratory distress syndromes (79). Recently, it has been shown that viral infection, particularly respiratory viral infections upregulate ROS production [e.g. (80, 81)]. Overexpression of *AOX* in mouse displayed substantially reduced ROS generation (82). Also, cigarette smoke-induced mitochondrial stress and ROS production was shown to be relieved in *AOX*-mice attenuating lung dysfunction and tissue damage linked to chronic obstructive pulmonary disease (known as COPD) (83).

Mitochondrial AOX was proposed as functional marker for plant cell reprogramming (27). It demonstrated significant role in homeostasis, reprogramming and plant growth adaptation in response to diverse abiotic and biotic stresses (26, 84–90). Short- and long-term fine-tuning of *AOX* at transcriptional level was shown to be important for positive effects on performance (85, 91). Recently, relevance of *AOX* for predicting plant robustness from early reprogramming has been substantiated (26). In plants, virus tolerance is essentially regulated by salicylic acid, a hormone that acts on ROS accumulation (92). It involves a highly complex regulatory network, where AOX plays a role by modulating mitochondrial redox/ROS signaling (93). Fu et al. (94) revealed that NO acted as inducer of AOX in response to *Tobacco mosaic virus* (TMV) infection. *AOX* transcript accumulation took place when cytochrome-c-oxidase (COX) was inhibited by TMV, or NO or KCN.

In several applied plant systems of reproducibly stimulated morpho-physiological reprogramming, it was shown that early up- and down-regulation of *AOX* transcript levels is typical and coincides with critical phases of *de novo* induced morpho-physiologic events (induction, initiation, and realization). This included carrot SE induction and seed germination (24, 26), olive root induction for propagation from shoots (95, 96), callus induction from quiescent root tissue (97, 98), and *Hypericum perforatum* germination (99). In carrot seedlings, chilling also induced oscillating *AOX* transcript levels. *AOX* transcripts peaked after 45 minutes and prior to high induction of a specific anti-freezing gene only after 24h (98). These results are in agreement with state-of-the art knowledge on the importance of flexible short- and long-term fine-tuning of

AOX at transcriptional level besides the protein level to enable known positive effects on plant performance (85, 91). To unravel the precise role of *AOX* and its isoforms during reprogramming integrated in complex signaling networks (100–102), it was suggested that measuring transient changes in respiration *in vivo* in seconds to minutes should be performed (103, 104).

The extraordinary role of AOX for reprogramming involves four major aspects for cell and tissue determination: (a) *AOX* is stress-induced and drives ROS level equilibration (105); *AOX* was shown to be involved in both scavenging and generation of NO (68). Cvetkovska and Vanlerberghe (106) demonstrated that overexpression of *AOX* led to lower NO production and *AOX* knockdown led to increasing NO. AOX scavenges electrons, thus it was expected to prevent in the mitochondrial electron transport chain electron leakage to nitrite and concomitant NO formation at the sites of complex III and complex IV. Later, Cvetkovska et al. (107) found that scavenging of NO could prevent NO inhibition of COX. Recently, Vishwakarma et al. (68) showed that bacterial elicitor flg22 treatment led to excess of NO, superoxide, peroxynitrite and tyrosine nitration. Moreover, *AOX* overexpression reduced peroxynitrite and tyrosine nitration suggesting that AOX-mediated NO removal can prevent downstream toxic products, (b) AOX is critical for mitochondrial ROS signal transduction towards mitochondria-nucleus retrograde communication (108–110), (c) AOX contributes to prevent excessive plant cell death by regulating ROS levels (17, 111, 112), and, (d) pyruvate is a major metabolic regulator of AOX (104, 113–117), which links to the role of sugar and the central branch point between respiration and fermentation (118). AOX activation can avoid energy and carbon shortage for anabolism by maintaining the tricarboxylic acid cycle active also when oxygen concentration is reduced (45). In *AOX*-overexpressing transgenic mice, presence of AOX enhanced mitochondrial respiratory rates through forward electron transport from succinate dehydrogenase (cII) both under phosphorylating (presence of ADP) and non-phosphorylating (absence of ADP) conditions (76). Lack of *AOX* in transgenic plants resulted in high ethanol production associated with injuries (118). Thus, AOX can help in decreasing fermentation and, thus can be expected to avoid harmful effects by excessively induced fermentation products (lactic acid, ethanol).

Standard Genes Profile 'ReprogVirus' for Exploring Virus-Induced Early Reprogramming in Relevant Primary Infected Human Cells - A 'Ready-to-Use' Approach

Viruses are known to 'abuse' host cell's competence and structures for reprogramming. Any virus infection provokes struggling for commanding coordination of the host cell program and this starts in the initially infected cells. Therefore, it is challenging to early stop virus-induced harmful reprogramming and avoiding at the same time suppressing the host's defense and survival strategy. As reviewed in Costa et al. (Preprint 28), viruses typically capture host cell signaling and metabolism. Changes in host cell redox homeostasis and central carbon metabolism are recognized as most critical events during viral infection and essential for virus replication. Viruses can influence host cell cycle to arrest or progress in favor of their own replication, where E2F1 of the E2F transcription factor family plays major role. In plants, TOR-suppression by silencing or inhibition resulted in impressively reduced virus replication, resistance or elimination of viral infection. Further, host microtubule (MT) assembly is critical for virus entry, replication and spread. Enzymes catalyzing posttranslational MT modifications were identified as suitable targets for drug development to combat viral infection (119).

Based on this knowledge and the characteristics of 'reprogramming for survival' during SE induction and supported by our validating results on the overall approach (Preprint 28) we selected a set of genes for a 'ready-to-use' standard profile to explore virus-induced early reprogramming. The standard profile consists of genes related to ROS/RNS equilibration, anti-oxidant activities, NO production, G6PDH, MDH1 and 2, lactic fermentation, structural cell organization, energy status-signaling, cell cycle regulation, and regulation of apoptosis/programmed cell death and includes IRF9 and IRF3 as markers for the immune system response plus transcription factors NF-KB1 and NF-KB-RELA. The complete list of genes is given in **Table 1**.

OUTLOOK

Recent advancements in virus research increasingly reveal good relevance of transcriptome data for cell and organism performance (120–123). It is also understood that it will be important to focus on gene sets (Preprint 124). The presented standard profile of selected genes is now available to be broadly applied. It can identify critical early traits of harmful virus-induced cell reprogramming by rapid *in vitro* - screening of a diversity of virus types and variants. It should be applied under commonly accepted standard conditions in relevant human cells or tissues of primary importance for defined diseases. Currently, the profile 'ReprogVirus' was used by our team to trace corona virus-related reprogramming (Preprint 28). Transcriptome profiles were explored by using the data available in public domain from transcriptomic experimental studies in Genbank (NCBI). It proved to be helpful in identifying a complex SARS-CoV-2-induced trait named 'CoV-MAC-TED' (Preprint 28), which covers early ROS/RNS balancing, aerobic fermentation regulation and cell cycle control. Potential impact from this trait is promising to support running and new initiatives of anti-SARS-CoV-2 therapy designs as broadly discussed (Preprint 28).

Here, we announce the initiation of the 'ReprogVirus Platform' to enable appropriate wide data collection under standardized conditions and data processing. The strategic flow diagram in **Figure 1** provides a straightforward instruction for data collection. In parallel, regulatory data of 'ReprogVirus' at

TABLE 1 | List of genes selected as 'ReprogVirus' for analyses in *Homo sapiens*.

Function	ReprogVirus	Gene members (accession numbers)
ROS/RNS equilibration	*ADH* (alcohol dehydrogenase)	*ADH5* (NM_000671.4)
Anti-oxidant activities	*SOD* (superoxide dismutase)	*SOD1* (NM_000454.5)
		SOD2 (M36693.1)
	Catalase	Catalase (NM_001752.4)
	GPX (glutathione peroxidase)	*GPX-1* (NM_000581.4)
		GPX-2 (NM_002083.4)
		GPX-3 (NM_002084.5)
		GPX-4 (NM_002085.5)
		GPX-5 (NM_001509.3)
		GPX-6 (NM_182701.1)
		GPX-7 (NM_015696.5)
		GPX-8 (NM_001008397.4)
	GSR (glutathione reductase)	*GSR* (NM_000637.5)
NO production	*NOS* (nitric oxide synthase)	*NOS1* (NM_000620.5),
		NOS2 (NM_000625.4)
		NOS3 (NM_000603.5)
Lactic fermentation	*LDH* (lactate dehydrogenase)	*LDH-A* (NM_005566.4)
		LDH-B (NM_002300.8)
		LDH-C (NM_002301.4)
		LDH-AL6A (NM_144972.5)
		LDH-AL6B (NM_033195.3)
Structural cell organization	*ACT* (Actin)	*ACT-A1* (NM_001100.4)
		ACT-B (NM_001101.5)
		ACT-G1 (NM_001199954.2)
	TUB (Tubulin)	*TUB-A1B* (NM_006082.3)
		TUB-A1C (NM_001303114.1)
		TUB-A4A (NM_006000.3)
Glycolysis	*ENO* (Enolase)	*Eno1* (NM_001428.5)
		Eno2 (NM_001975.3)
		Eno3 (NM_001976.5)
	HK (Hexokinase)	*HK1* (NM_000188.3)
		HK2 (NM_000189.5)
		HK3 (NM_002115.3)
	PFK-M (Phosphofructokinase)	*PFK-M* (NM_001166686.2)
	GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)	*GAPDH* (NM_002046.7)
	PK (Pyruvate kinase)	*PKLR* (XM_006711386.4)
		PKM (NM_002654.6)
Energy status-signaling	*SNRK* (sucrose non-fermenting-1-related kinase)	*SNRK* (NM_017719.5)
Cell cycle regulation	*mTOR* (target of rapamycin)	*mTOR* (NM_004958.4)
	E2F transcription factor	*E2F1* (NM_005225.3)
Regulation of apoptosis/cell death	*CASP* (Caspase)	Caspase in [*CASP8* (NM_001228.4); *CASP9* (NM_001229.5); *CASP10* (NM_032977.4)]
		Caspase ex [*CASP3* (NM_004346.4); *CASP6* (NM_001226.4); *CASP7* (NM_001227.5)]
	Bcl gene	*BCL-xL* (Z23115.1)
Markers for the immune system response	*IRF* (interferon regulatory factor)	*IRF9* (NM_006084.5), *IRF3* (NM_001571.6),
Viruses-activated transcription factors	*NF-KB1*	*NF-KB1* (NM_003998.4)
	NF-KB-RELA	*NF-KB-RELA* (NM_021975.4)
Other key genes	*G6PDH* (*Glucose-6-phosphate dehydrogenase)*	*G6PDH* (NM_000402.4)
	MDH (Malate dehydrogenase)	*MDH1* (NM_005917.4)
		MDH2 (NM_005918.4)

DNA/RNA and protein levels can be explored and collected. In case of choosing to analyze expression of individual genes (RT-qPCR), regulatory data regarding transcriptome could be obtained by exploring public databases.

The platform will provide integrative data analyses using Artificial Intelligence methodologies to identify final targets for designing specific and/or unspecific anti-viral strategies. More specifically, we intend to apply deep learning techniques to identify gene expression patterns from individual genes or from a combination of genes. These patterns will be automatically correlated with a virus or a set of viruses using a distinct deep neural network. As deep learning architecture we foresee the use of multi-head attention mechanisms in a transformer-based, variational auto-encoder network, allowing the identification of the most relevant parts of the input. Moreover, we will also apply and evaluate other CDNN

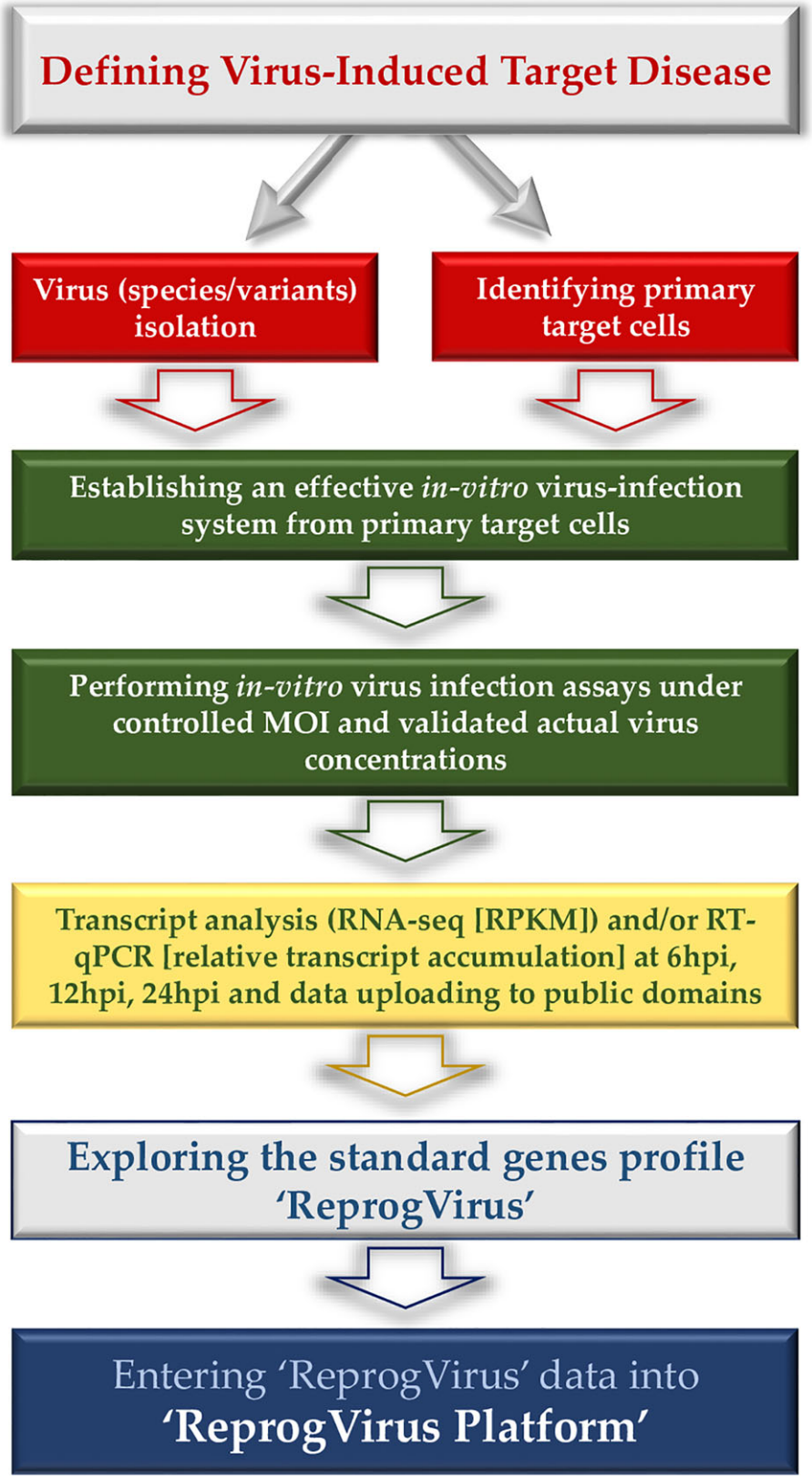

FIGURE 1 | Flow diagram - data collection for 'ReprogVirus Platform'.

(clustering deep neural networks), such as deep embedding clustering and GANs (Generative Adversarial Networks) (125).

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

BA-S initiated scientific approach and concepts in close collaboration with JHC and CN, coordinated their final development for the presented perspective through common discussions among all *FunCROP* net members and wrote the manuscript. RB contributed to manuscript writing and prepared overall ms for submission. PQ supports this initiative through his competence in Artificial Intelligence methodologies. SRK helped BA-S in overall *FunCROP* group coordination. KJG helped in writing manuscript parts related to NO metabolism. All authors contributed to the article and approved the submitted version.

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ROS/RNS Balancing, Aerobic Fermentation Regulation and Cell Cycle Control – a Complex Early Trait ('CoV-MAC-TED') for Combating SARS-CoV-2-Induced Cell Reprogramming

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In a perspective entitled 'From plant survival under severe stress to anti-viral human defense' we raised and justified the hypothesis that transcript level profiles of justified target genes established from *in vitro* somatic embryogenesis (SE) induction in plants as a reference compared to virus-induced profiles can identify differential virus signatures that link to harmful reprogramming. A standard profile of selected genes named 'ReprogVirus' was proposed for *in vitro*-scanning of early virus-induced reprogramming in critical primary infected cells/tissues as target trait. For data collection, the 'ReprogVirus platform' was initiated. This initiative aims to identify in a common effort across scientific boundaries critical virus footprints from diverse virus origins and variants as a basis for anti-viral strategy design. This approach is open for validation and extension. In the present study, we initiated validation by experimental transcriptome data available in public domain combined with advancing plant wet lab research. We compared plant-adapted transcriptomes according to 'RegroVirus' complemented by alternative oxidase (AOX) genes during *de novo* programming under SE-inducing conditions with *in vitro* corona virus-induced transcriptome profiles. This approach enabled identifying a *ma*jor complex *t*rait for *e*arly *de novo* programming during SARS-CoV-2 infection, called 'CoV-

MAC-TED'. It consists of unbalanced ROS/RNS levels, which are connected to increased aerobic fermentation that links to alpha-tubulin-based cell restructuration and progression of cell cycle. We conclude that anti-viral/anti-SARS-CoV-2 strategies need to rigorously target 'CoV-MAC-TED' in primary infected nose and mouth cells through prophylactic and very early therapeutic strategies. We also discuss potential strategies in the view of the beneficial role of AOX for resilient behavior in plants. Furthermore, following the general observation that ROS/RNS equilibration/redox homeostasis is of utmost importance at the very beginning of viral infection, we highlight that 'de-stressing' disease and social handling should be seen as essential part of anti-viral/anti-SARS-CoV-2 strategies.

Keywords: SARS-CoV-2, redox biology, alternative oxidase, tubulin, mTOR, melatonin, repurposing drugs

INTRODUCTION

Towards the tail end of 2019, a corona virus disease caused a pandemic outbreak. The virus is designated as 'severe acute respiratory syndrome corona virus 2' (SARS-CoV-2) and has apparently zoonotic origin. SARS-CoV-2 is a virion enveloping a single positive-stranded RNA genome, which is capable of entering into human cells by using the physiologically important, hormone-related receptor ACE2 (angiotensin converting enzyme, 2). The complex and diverse multi-systemic effects of the virus on individuals and its pandemic impact are widely discussed in comparison to two other similar viruses with endemic impacts from the *Coronaviridae* family, SARS-CoV and MERS-CoV (1–6). These similar viruses exhibit more drastic clinical impairment, but showed less human-to-human transmission rates and lower mortality (7). Also, SARS-CoV-2 has thermodynamic advantages than former Corona virus SARS-CoV through binding with higher affinity to ACE2 receptors (8).

The intense damage caused by viruses to human and the possibility of similar virus threats in the future triggered a worldwide debate and tremendous efforts have been initiated across all society levels. The global academic community aims to widen the knowledge dimensions for developing specific and wider antiviral concepts and strategies. In this context, we intend to contribute with relevant knowledge, which can be useful for developing intervention strategies well before virus spread can start to become an exponentially growing threat for the infected person and, thus, for the community.

Viruses are non-living structures. However, they are able to gain power over human cell programs and individual organism fitness due to their dominating presence. This is in conformity with knowledge on structuralism and biosemiotics in Theoretical Biology, which acknowledges the importance of relationships and signs rather than characteristics of individual components for the functioning of systems (9). In an abstract sense, virus reproduction depends on the virus-inherent 'structural power' in relation to other components in the system. This term is taken from peace research related to socio-economic systems, where inequality is recognized as the main driver for structural violence (10, 11). This view can also be applied for the relation of viruses to their hosts. In fact, viruses have comparatively low Gibbs energy due to their chemical compositions (12, 13). This makes their replication highly competitive and a driving force against the host cell metabolism. Virus-induced cell reprogramming favors virus replication, but demands at the same time cell reprogramming for host defense and survival. As a consequence of this conflict, any viral infection provokes struggling for commanding coordination of host cell program and this starts in the initially infected cells. Competing for bioenergy and for 'territories' is decisive for the success of virus reproduction and evolution.

Virus spread and its natural evolution escaped quite successfully human control. Currently, there is no awareness of any effective and simple broad anti-viral treatment available that could also be used to treat SARS-CoV-2 infections. When viral structures enter in contact with living cells, the latter cannot ignore their presence. Ignorance would be helpful and nothing harmful would happen to the host cells. However, this is way behind possible, because viral structures interact at the outer cell surface in a way that stimulates cell's program-commanding components, such as reactive oxygen species (ROS) (14) and growth factors (15), which in turn induce changes in intracellular signaling cascades. Successful virus propagation relies on manipulated cell programming that allows abusing the host's energy resources by help of the proper host. Since viruses are non-living structures, damage of the host is not a target but might happen as collateral circumstance (16). Therefore, virus docking to cell membranes and the subsequent membrane fusion event that enable entrance of the virus particles into living cells can be seen as 'structural violence'. It is a sort of 'hostile takeover' as it is known from economy. Correspondingly, virus affects host system management and makes it act in favor of the incoming structures and risks thereby the healthy status or even life of the host cell and, finally, the overall organism (16).

Primarily virus-infected cells act as super-spreaders. In case of corona viruses, cell-cell fusion between infected and adjacent, uninfected cells were reported to form giant, multinucleated cells that allow rapid spread within infected organisms obviously even without being detected and neutralized by virus-specific antibodies (17). Massive virus replication is energy-costly, weakens and endangers the host and can cause a pandemic threat. And there is a second danger too that comes 'secretly and as gratis' when virus replication is not stopped during the very

early phase of infection: virus coding sequences might undergo modifications due to the host's and/or virus-driven error-prone molecular machinery. Consequently, when the rate of virus replication progresses and reaches massive propagation, the probability of virus code evolution increases and, this might enhance in turn virulence (18, 19). However, a widened diversity of virus coding structures reduces the chance that the immune system of individual organisms and of present and future networking populations are prepared to keep virus threat at low level. And thirdly, in case of SARS-CoV-2, once acquired immunity might also not last for a prolonged time (20). For these three reasons, it is of utmost importance to understand the very first steps in virus-induced cell reprogramming. This is influential to develop efficient and sustainable anti-viral concepts for confronting viral infection by administrating early or even prophylactic therapies.

In a parallel perspective paper entitled 'From plant survival under severe stress to anti-viral human defense' we proposed using a standard profile of selected genes ('ReprogVirus') to trace early footprints of wide varieties of viruses under standardized *in-vitro* conditions (21). We raised and justified the hypothesis that transcript level profiles of justified target genes established from *in vitro* somatic embryogenesis (SE) induction in plants as a reference compared to virus-induced profiles can identify differential virus signatures that link to harmful reprogramming. This interdisciplinary approach was explained in details, including especially the use of SE induction in the two plant species *Arabidopsis thaliana* and carrot (*Daucus carota* L.) as efficient experimental tools to identify markers for early reprogramming and resilience and the focus on alternative oxidase (AOX). Here, we follow strictly that approach in order to initiate scientific validation of the underlying hypothesis and enable progressing in a common effort towards the wider perspective for anti-viral strategy-design.

BACKGROUND

Virus Captures Host Cell Signaling and Metabolism

Viral infections causing respiratory complications are known to change host cell redox homeostasis, which involves balancing ROS/RNS as a critical event (22). RNA viruses were suggested to utilize oxidative stress during infection to control genome RNA capping and genome replication (14, 23, 24). In airway epithelial cells, virus-induced ROS was found to originate from diverse oxidase activities, including NADPH oxidases, dual oxidase, and xanthine oxidase [reviewed in (22)]. Similar to plant superoxide dismutase (SOD), animal SOD plays a central role as an oxidative stress indicator and also as an anti-oxidative stress defender along with a set of other ROS scavenging enzymes, such as, catalase, GPX (glutathione peroxidase) and GSR (glutathione reductase) was found to be induced upon viral infection (25) and also highlighted in stem cell research as an indicator for cell reprogramming (26). Likewise, nitric oxide (NO) was found to be involved in virus replication (27, 28). In humans, NO production during viral infection depends on nitric oxide synthase (*NOS*). Inducible NOS (NOS2) produced much higher amounts of NO for a prolonged duration as compared to constitutively expressed neuronal (*NOS1*), endothelial (*eNOS* or *NOS3*) and mitochondrial *NOS* (29). Biogenesis of higher levels of NO can suppress type 1 helper T-cell-dependent immune responses, which can impair type 2 helper T-cell-biased immunological host responses (29). eNOS is mostly present in endothelial cells and its functionality can be restored with renin- and angiotensin-converting enzyme-inhibitors or angiotensin receptor blockers, both commonly used to regulate blood pressure in hypertensive patients (30).

Several metabolic changes have been reported in virus-infected animal and/or plant host cells that were related to central carbon metabolism: e.g. (a) increased rate of glycolysis linked to pools of nucleotides and amino acids essential for replication (31, 32), (b) differential mTOR (mammalian target of rapamycin) pathway regulation and intracellular calcium signaling linked to enhanced Krebs cycle, (c) down-regulation of glycolysis that severely affected viral infection (33–35), (d) encoding mitochondria-related proteins that disturbed normal functioning of mitochondria (36), (e) increased production of lactic acid from glucose pumped out of cell (37), (f) knock-down of *ADH* (*Alcohol dehydrogenase*) and pyruvate decarboxylase that diminished virus replication rates (38), and, (g) interplay between glycolysis and fermentation that is suggested to serve metabolome channeling for virus replication highlighted as paradigm change (39). Bojkova et al. (40) observed that blocking of glycolysis resulted in prevention of SARS-CoV-2 replication in infected cells. Lactate present in the circulating system had been identified besides interleukin-6 as independent prognostic factors for disease progression in a relatively small clinical data collection. In addition, increased lactate was found to impair antiviral immunity (41). Subsequent to the appearance of symptoms, disease severity was classified by monitoring disease progression during 24h to 48h and beyond this duration of disease progression, lactate was not found to be useful to predict fatality (survivor vs non-survivor) (42). In primary lung epithelial cells validated by biopsies of COVID-19 patients, SARS-CoV-2 induced oxidative stress due to mitochondrial dysfunction (32). In these studies, endoplasmic stress symptoms were related to modulation of lipid metabolism. Transcriptional responses in primary lung epithelial cells and data from biopsies were shown to be predominantly of metabolic nature (59% to 65% of all differentially expressed genes). They indicated upregulation of glycolysis and dysregulation of citric acid cycle, which was mediated by the transcription factors *NF-kB* and *RELA*. In patients, elevated blood glucose was found to be a risk factor independently from diabetes. SARS-CoV-2 infection was associated to marked increase in intracellular glucose and transcriptional modulation in glucose metabolism along with elevated lactate levels (32). Several authors report that in virus research transcriptome data and a focus on gene sets revealed to be critically relevant for cell and organism performance (43–47).

Host Microtubule Assembly Is Critical for Virus Entry, Replication and Spread

Microtubules (MT) interfere with viral infection process right from the very start. Thus, the aspects related to regulation of biosynthesis

and organization of the MT assembly determines critically the frame for reprogramming and the acquiring of basal defense of the host cells. Virus attach and enter at specific sites of human host cells in relation to the spatial organization of surface receptors and entry factors, which is partially controlled by MT and their polarization (48). MT-driven transport within the cells by dynein and kinesin family of motors has been shown to have crucial role in virus replication and spread (49). Interaction of alpha-corona virus spike (S) proteins with tubulin has been shown to support S protein transport and incorporation into virus particles (50). Virus can promote MT polymerization, stabilization or disruption and actin-MT crosstalk depending on the cellular status with regard to the stage in viral infection. Receptor engagement by viruses' influences MT dynamics facilitating viral infection during the early stages of infection. MTs or MT-like proteinaceous filaments are found to be highly relevant for infections by diverse viral classes and across kingdoms and species (48). α-Tubulin has been shown to be involved in regulating cell death and program decisions linked to cell cycle activation (51, 52). Enzymes catalyzing post-translational MT modifications are realized to be suitable targets for drug development for combating viral infection (52).

Viruses Can Influence Host Cell Cycle in Favor of Their Own Replication

Viruses can affect host cell cycle regulation in favor of viral replication, which might seriously affect host cell physiology with impacts on pathogenesis (53–55). Viruses were shown to influence different stages of the host cell cycle resulting in either progression or arrest of cell cycle with the involvement of homologous proteins (54, 56). Cellular levels of phosphorylated retinoblastoma tumor suppressor protein (pRb) were found to be critical for the progression of cell division to S phase (57). Hyperphosphorylation of Rb allows activation of E2F family of transcription factors, resulting in the transcription of genes associated with S phase of the cell cycle (58). In Rb-deficient mouse embryos, mutation of E2F1 was found to markedly suppress apoptosis and also entry of cells into S-phase resulting in prolonged cell survival (59). Impressively, TOR-suppression in plants either by silencing or by inhibitor treatment (AZD8055) was shown to reduce virus replication, leading to conferring virus resistance in the host plant or even to total elimination of viral infection [reviewed in (60)]. Corona viruses have been shown to notably arrest cells in G0/G1 stage of cell cycle. Enrichment of coronavirus-infected cells had also been found in G2/M stage (54). SARS-CoV is known to produce 3b and 7a non-structural proteins, which together decrease level of cyclin D3 and dephosphorylation of pRb in order to arrest cell cycle at the S phase (61). SARS-CoV-induced synthesis of protein 3a was reported to arrest cell cycle at the G1 stage *via* the operation of the earlier mentioned pathways (62). Recently, Laise et al. (55) identified 12h, 24h, and 48h signatures from calu-3 lung adenocarcinoma cells infected with SARS-CoV, which included genes related to cell cycle progression, viz., E2F and mTOR.

Host Microbiome Can Influence Viral Infection Progress

Host microbiomes are individual specific. They can affect viral infection either negatively and positively (63, 64). Host microbiome can increase virion stability, provide DNA replication machinery, stimulate the lytic phase of virus and counteract host immune responses. Further, microbiota can even enhance viral genetic recombination through adhering host cells and facilitating thereby infection of two or more viruses in a single cell (65). In contrast, probiotic bacteria, such as *Bifidobacterium* and *Lactobacillus*, increased cellular biosynthesis of cytokines and interleukins upon viral infection (66, 67). Additionally, *Lactobacillus vaginalis* inhibited infection through human immunodeficiency virus (HIV) by producing lactic acid and maintaining acidic environment in vagina (68). Vaginal lactic acid can profoundly increase biosynthesis of anti-inflammatory cytokines, which was shown to help preventing infection by Herpes virus (69). These observations demonstrate that it can be important to better understand the interaction of microbiota with virus-induced early reprogramming in target cells and to apply this knowledge when prophylactic and early therapies are required.

MATERIAL AND METHODS

Gene Expression Analyses of RNA-Seq Data From *Arabidopsis thaliana* and Virus-Infected Human Cells

Genes for expression analyses correspond to standard profile of genes selected for studying virus-induced early reprogramming (named 'ReprogVirus') [(21), in press]. For plant material, 'ReprogVirus' was complemented by alternative oxidase (*AOX*) and genes of plant secondary metabolism. In summary, the selected genes related to ROS/RNS equilibration (*AOX*, *ADH2*/*ADH5*), anti-oxidant activities (*SOD*, *Cat*, *GPX*, *GSR*, plant-*PAL*, *CHS*, *C3H*, *CAD*), NO production (plant-*NR*, *NOS*), glycolysis (*Hexokinase*, *PFK-M*, *GAPDH*, *enolase*, *pyruvate kinase*), *G6PDH*, *MDH1/2*, fermentation (*LDH*, *ADH1*), structural cell organization (*alpha-tubulin*, *actin*), energy status-signaling (*SNRK*), cell cycle regulation (*TOR/mTOR*, *E2F1-3*, *E2F5*) and regulation of apoptosis/cell death (*BAG*, *Meta-CASP*, *Caspase In*, *Caspase Ex*, *Bcl-xL*). In the case of virus-infection trials, a set of additional genes from the immune system (*IRF9* and *IRF3*) and the two transcription factors NF-KB1 and NF-KB-RELA were included. For SARS-CoV-2related profiles, we added receptor ACE2 and the priming protease *TMPRSS2*. Additionally, we searched transcriptomes of virus-infected cells for the melatonin synthesis-related gene *ASMT* (N-acetylserotonin O-methyltransferase). *ASMT* is involved in melatonin synthesis in human cells (70).

Gene expression was evaluated in specific RNA-seq experiments, which were collected from Sequence Read Archive (SRA) database of GenBank, NCBI. Expression analyses were performed from *Arabidopsis* and virus infected human cells. Experimental and projects details were given in **Supplementary Table S11**. Specific regions (3' end) of each cDNA were aligned against RNA-seq experiments using mega BLASTn tool (71) to obtain the mapped reads according to Saraiva et al. (72). Specific parameters of mega BLASTn as word size were adjusted to allow specific read detection of each gene. The mapped reads were also verified using the Magic-Blast (73), a more recent tool with accurate features for RNA-seq

data. The number of mapped reads (on each gene/experiment) was normalized using the RPKM (Reads Per Kilo base of transcript per Million of mapped reads) method (74). The following equation was applied: RPKM = (number of mapped reads X 10^9)/(number of sequences in each database X number of nucleotides of each gene). According to the mined datasets, transcript levels of *Arabidopsis* seedlings consisted of the values of three biological replicates. All viral infection experiments had three biological replicates, except RSV infection to A459 cells, which had only two biological replicates. The number of reflected technical replicates varied in the RSV datasets between one and three.

All Selected Genes Are Listed Below:

For *Arabidopsis*: total *AOX* (Alternative oxidase) [*AOX1a* (AT3G22370.1); *AOX1b* (AT3G22360.1); *AOX1c* (AT3G27620.1); *AOX1d* (AT1G32350.1); *AOX2* (AT5G64210.1)], *LDH* (Lactate dehydrogenase) (AT4G17260.1), *ADH1* (Alcohol dehydrogenase) (AT1G77120.1), Total enolase [*cyt-ENO1* (AT2G29560.1); *cyt-ENO2* (Cytosolic-enolase) (AT2G36530.1); *plast-ENO1* (Plastidial-enolase) (AT1G74030.1)], *cyt-Fe-SOD1* (Cytosolic-iron-Superoxide dismutase 1) (AT4G25100.1), *mt_Mn-SOD1* (mitochondrial manganese superoxide dismutase 1) (AT3G10920.1), *CAT3* (catalase), (AT1G20620.1), Total *GPX* (Gluthatione peroxidase) [*GPX1* (AT2G25080.1); *GPX2* (AT2G31570.1); *GPX3* (AT2G43350.1); *GPX4* (AT2G48150.1); *GPX5* (AT3G63080.1); *GPX6* (AT4G11600.1); *GPX7* (AT4G31870.1); *GPX8* (AT1G63460.1)], *Cyt-GSR1* (Gluthatione reductase) (AT3G24170.1), total *PAL* (Phenylalanine ammonia lyase) [*PAL1* (AT2G37040.1); *PAL2* (AT3G53260.1); *PAL3* (AT5G04230.1); *PAL4* (AT3G10340.1)], total *CHS* (Chalcone synthase) [*CHS-A* (AT1G02050.1); *CHS-B* (AT4G34850.1); *CHS-C* (AT4G00040.1); *CHS-D* (AT5G13930.1)], *C3H* (p-coumarate 3-hdroxylase) (AT2G40890.1), *CAD* (cinnamyl alcohol dehydrogenase) [*CAD4* (At3g19450); *CAD5* (At4g34230); *CAD7* (At4g37980); *CAD8* (At4g37990)], total *NR* (nitrate reductase) [*NIA1* (AT1G77760.1); *NIA2* (AT1G37130.1)], *ADH2* (AT5G43940.2), total *ACT* (actin) [*ACT1* (AT2G37620.1); *ACT2* (AT3G18780.1); *ACT3* (AT3G53750.1); *ACT4* (AT5G59370.1); *ACT7* (AT5G09810.1); *ACT8* (AT1G49240.1); *ACT9* (AT2G42090.1); *ACT11* (AT3G12110.1); *ACT12* (AT3G46520.1); *ACT* (AT2G42170.1)], total alpha-tubulin [*TUA1* (AT1G64740.1); *TUA2* (AT1G50010.1); *TUA3* (AT5G19770.1); *TUA4* (AT1G04820.1); *TUA5* (AT5G19780.1); *TUA6* (AT4G14960.2)], *SNRK* (sucrose non-fermenting related kinase) [*KIN10* (AT3G01090.1); *KIN11* (AT3G29160.1)], *TOR* (target of rapamycin) (AT1G50030.1), *E2F* [E2F1 (AT5G22220.2); *E2F3* (AT2G36010.1); *E2F5* (AT1G47870.1)], total *BAG* (Bcl-2 associated gene) [*BAG1* (AT5G52060.1); *BAG2* (AT5G62100.1); *BAG3* (AT5G07220.1); *BAG4* (AT3G51780.1); *BAG5* (AT1G12060.1); *BAG6* (AT2G46240.1); *BAG7* (AT562390.1)], Meta-caspase (*MC-1* (AT1G02170.1); *MC-2* (AT4G25110.1) *MC-3* (AT5G64240.1); *MC-4* (AT1G79340.1); *MC-5* (AT1G79330.1); *MC-6* (AT1G79320.1); *MC-7* (AT1G79310.1); *MC-8* (AT1G16420.1); MC-9 (AT5G04200.1)], *ASMT* (AT4G35160).

For *Homo sapiens*: total *LDH* [*LDH-A* (NM_005566.4); *LDH-B* (NM_002300.8); *LDH-C* (NM_002301.4); *LDH-AL6A* (NM_144972.5); *LDH-AL6B* (NM_033195.3)], *SOD1* (NM_000454.5), *SOD2* (M36693.1), *Catalase* (NM_001752.4), total *GPX* [*GPX-1* (NM_000581.4); *GPX-2* (NM_002083.4); *GPX-3* (NM_002084.5); *GPX-4* (NM_002085.5); *GPX-5* (NM_001509.3); *GPX-6* (NM_182701.1); *GPX-7* (NM_015696.5); *GPX-8* (NM_001008397.4)], *GSR* (NM_000637.5), *NOS1* (nitric oxide synthase) (NM_000620.5), *NOS2* (NM_000625.4), *NOS3* (NM_000603.5), *ADH5* (NM_000671.4), *Hexokinase* [*HK1* (NM_000188.3); *HK2* (NM_000189.5); *HK3* (NM_002115.3)], *PFK-M* (NM_001166686.2), *GAPDH* (NM_002046.7), *Enolase* [*Eno1* (NM_001428.5); *Eno2* (NM_001975.3); *Eno3* (NM_001976.5)], *Pyruvate kinase* [*PKLR* (XM_006711386.4); *PKM* (NM_002654.6)], *G6PDH* (NM_000402.4), *MDH1* (NM_005917.4), *MDH2* (NM_005918.4), *SNRK* (NM_017719.5), *mTOR* (NM_004958.4), *E2F1* (NM_005225.3), *Actin* [*ACT-A1* (NM_001100.4); *ACT-B* (NM_001101.5); *ACT-G1* (NM_001199954.2)], *IRF9* (NM_006084.5), *IRF3* (NM_001571.6), *NF-KB1* (NM_003998.4), *NF-KB-RELA* (NM_021975.4), *Caspase in* [*CASP8* (NM_001228.4); *CASP9* (NM_001229.5); *CASP10* (NM_032977.4)], *Caspase ex* [*CASP3* (NM_004346.4); *CASP6* (NM_001226.4); *CASP7* (NM_001227.5)], *BCL-xL* (Z23115.1), *ACE2* (NM_001371415.1), *TMPRSS2* (NM_001135099.1), *ASMT* (NM_001171038.2).

Induction of Somatic Embryogenesis in *Daucus carota* L. for the Identification of Markers for Early Reprogramming

Carrot seeds (cv. Kuroda) were surface-sterilized with 75% ethanol for 1 min and 4% sodium hypochlorite solution for 20 min. Later, seeds were washed thrice with sterile water and dried on filter paper under laminar air flow. Dried seeds were collected into sterile screw-cap tubes and stored in dark at room temperature. Surface sterilized seeds were inoculated on B5 solid medium supplemented with 0.5 mg l^{-1} 2,4-D according to Mohanapriya et al. (75), also supplemented with 0% and 2% sucrose. Plates were incubated in culture room at 22-25˚C with 16h/8h photoperiod. Triplicates of 40 seeds per plate were maintained for each treatment. At 0, 6, 12, 24, 48 and 72 hours after inoculation (HAI) numbers of seeds with induced callus were recorded and samples were collected simultaneously for ADH assay. The embryogenic nature of calli was routinely evaluated by microscope till 45 days after inoculation. With 0% sucrose, non-embryogenic calli were induced, while in presence of 2% sucrose, embryogenic calli were obtained. In this system, the efficiency of SE induction can be optimized by increasing sucrose to 3%, that induces a significantly higher number of calli leading to SE when compared to 2% sucrose (unpublished data).

For studying the influence of the *AOX*, inhibitor salicyl hydroxamic acid (SHAM) and its interaction with sucrose on the morphogenetic effect of auxin-treatment, seeds were inoculated on 0.5 mg l^{-1} 2,4-D supplemented solid B_5 medium at 0% and 3% sucrose along with 10 mM SHAM (dissolved in 50% DMSO and sterilized by using 0.22 μm filters). Seeds were harvested at 0, 6 and 12HAI, and 10 days after inoculation, and then shifted into SHAM-free media. Emergence of calli was recorded at every 24h intervals until 240 HAI (24, 48, 72, 96, 120, 144, 168, 192, 216, and 240 HAI). Experiments of callus differentiation consisted in two sets of 40 seeds each per

treatment, and ADH measures were performed with duplicated pools of 40 seeds, according to the description of the experiment.

Alcohol Dehydrogenase (ADH) Assay Performed During SE Induction From Carrot Seeds to (a) Confirm Generality of the Observed Increase in ADH Expression at Transcriptome Level in the Model Plant Arabidopsis in a Second Plant System Using a Biochemical Approach and (b) to Study the Link Between Early ADH Enzyme Expression, External Sucrose and Initiation of Cell Proliferation

Forty seeds were collected at 0, 12, 24, 30, 48, and 72 HAI grown on 0.5 mg l^{-1} 2,4-D supplemented solid B_5 medium. They were bulked and ground into powder in a sterile mortar and pestle by using liquid nitrogen. Fine powder was extracted using 1 mL of 1X phosphate saline buffer (pH 7.2) and centrifuged for 30 min at 10,000 rpm at 4°C. The obtained supernatant was used as crude protein extract for enzyme assay. The assay was performed according to the protocol given in Kagi and Vallee (76). Briefly, the reaction mixture consisted of 1.3 ml 50 mM sodium phosphate buffer (pH 8.8), 0.1 ml 95% ethanol, 1.5 ml 5 mM *ß*-nicotinamide adenine dinucleotide (*ß*-NAD^+) and 0.1 ml of crude protein extract. This mixture was immediately inversed several times for homogenization and absorbance at 340 nm was recorded from 0 to 6 min. Alcohol dehydrogenase from *Saccharomyces cerevisiae* was used as positive control. Active enzyme units (IU - international units) were calculated according to the following formula:

$$\frac{(\Delta A340/minTest - \Delta A340/minBlank)(3.0)(df)}{(6.22)(0.1)}$$

3.0 = Total volume (ml) of assay

df = Dilution factor

6.22 = millimolar extinction coefficient of reduced ß-nicotinamide adenine dinucleotide (ß-NAD^+) at 340 nm

0.1 = Volume (ml) of crude extract used

In **Figure 1A.2**, the calculated IU of ADH was multiplied by a factor (x5) for better visualization of the relationship between ADH (IU) and number of seeds with showing callus.

Bacterial Endophyte and Mycorrhiza Treatments of *D. carota* L. Seeds to Explore Whether Microbiota Might Potentially Interact With Oxidative Metabolism/Redox Status Regulation During Early Cell Reprogramming and Whether This Might Depend on the Individuality of Organisms

Seeds of two carrot varieties, Kuroda and Early Nantes, were used for the experiments. At first, they were pretreated to remove native endophytes by an established protocol (75). Briefly, stock solutions of 1 mg ml^{-1} tetracycline (antibiotic) and miconazole nitrate (antifungal) were prepared and 5 ml of each solution was transferred onto a Petri plate. 200 mg of carrot seeds were weighed and added into the plate containing antibiotic and antifungal solutions and kept under constant shaking (100 rpm) for 6 hrs. Thereafter, seeds were washed with sterile deionized water for three times and kept under laminar air flow until they were completely dried. To confirm the effectiveness of the treatment, few seeds were collected randomly and cut into small pieces. These pieces were placed on nutrient agar/potato dextrose agar (Himedia, India) and incubated at 35°C/28°C for overnight (bacteria) to 2 days (fungi). Absence of bacterial/fungal growth indicated that seeds were free from native endophytes.

For bacterial endophyte treatment, Single colonies of native bacterial endophytes (EN1, EN2, EN3) were inoculated in 100 ml of autoclaved nutrient broth (HiMedia, India) and incubated at 35°C overnight. Overnight grown bacterial cultures were centrifuged for 5 min at 4000 rpm to collect the pellet. The pellet is diluted with sterile deionized water until the optical density (O.D) reached to 0.2 (2 x 10^8 cells ml^{-1}). The endophyte-free seeds were immersed in 20 ml of each bacterial culture and kept under shaking at 100 rpm for 2 h. Then, seeds were dried under laminar air flow to remove excess moisture.

For arbuscular mycorrhizal fungi (AMF) treatments, 90 days old root organ culture (ROC; stock culture) of *Rhizophagus irregularis* and *Rhizophagus proliferus* was obtained from the Centre for Mycorrhizal Culture Collection (CMCC), TERI, India. The stock cultures of AMF1 and AMF2 were harvested at 25°C in 100 ml sodium citrate buffer using a shaker (Kuhner Shaker, Basel, Switzerland) (78). After deionization, buffer containing harvested spores (without roots) was sieved through a 325 British Standard (300 µm) Sieve (BSS) (Fritsch, Idar-Oberstein, Germany). Spores retained on the sieve mesh were washed with sterile distilled water (twice). Finally, the spores were collected in 20 ml of sterile distilled water (all steps were performed under aseptic conditions) (79).

For experimental set-up, autoclaved germination papers (Glasil Scientific Industries, New Delhi, India) were placed onto Petri dishes and moisturized with 5 and 10mM SHAM. Forty bacterial endophyte-treated seeds were placed onto dishes using sterile forceps. In the case of the AMF1 and AMF2 treatments, 40 endophyte free seeds were placed onto the dishes and 5 spores *of R. irregularis* were inoculated per seed. All treatments were setup with three replicates. Triplicates of controls were maintained by inoculating seeds in sterile distilled water. After inoculation, seeds were kept in darkness for 40 h and then transferred to light conditions. The plates were incubated in a culture room at 22-25°C with a 16h/8h photoperiod. Germinated seeds were counted and data were recorded at 24, 30, 48, 72, 96 and 120 HAI. Seed germination was identified by radicle emergence.

Statistical Analyses

Normality and homogeneity of variances from the samples were tested with Shapiro-Wilk test and Bartlett or Levene tests, respectively. If data were parametric, Student's t-test (two populations) or one-way ANOVA were used. ANOVA was followed by Tuckey´s *post hoc* test when a significant effect of

the tested factor was detected. When pre-requisites for parametric analyses were not kept, variable transformations were made [ln(x) and sqrt(x)] to perform parametric analyses, or non-parametric tests were used. These last tests were performed only for ADH levels in auxin-induced seeds (Wilcoxon test, **Figure 1B**) and transcript levels obtained after viral infections of MRC5 cells (Krukal-Wallis test, **Figure 1B.4**). After ANOVA, *post hoc* analyses (Tukey's) were performed. Statistical packages used were InfoStat v. 2018 and R v. 4.0.2.

We highlight that we interpret our data as 'real' observations under the employed conditions involving only small samples, which certainly provide insights that cannot get relevance or not relevance by using significance calculation. Nevertheless, we applied significance calculations at usual p-values for biological research as an aid to appropriately focus our insights. This is the reason for describing our observations in figure legends by reporting the attributes of the data as either significant or non-significant only as additional information in parenthesis. Readers are encouraged to making themselves familiar with the current paradigm change related to the usage of statistical significance (80–84).

RESULTS & DISCUSSION

Following the perspective of Arnholdt-Schmitt et al. [(21), in press], our principle goal of this study on coronaviruses with special reference to SARS-CoV-2 was the validation of the hypothesis that transcript level profiles of justified target genes established from *in vitro* somatic embryogenesis (SE) induction in plants compared to virus-induced profiles can identify differential virus signatures that link to harmful reprogramming.

Thus, we first established a reference transcript profile from a plant model experimental system and subsequently compared this reference profile step-by-step along the outlined five characteristics with transcriptome profiles of corona virus-infected cells. As a consequence, we were able to extract targets promising for developing a strategy to avoid virus spread already in primary infected cells.

In **Figure 1**, we present functional transcriptome profiles related to reprogramming under SE-inducing conditions in the model species *Arabidopsis thaliana* (**Figure 1A.1**) and from corona virus-induced human cell lines (**Figure 1B.1**–**4**). Results of detailed observations and complementing wetlab research results are highlighted in the subfigures and in Figure legend. In the main text, we focus on the principle line of this research.

In summary, we found (A) that the reference plant transcript level profile marked early *de novo* cell programming by five salient characteristics as follows:

(1) Modified complex oxidative stress signaling pattern and a special role for increased superoxide dismutase (SOD) as stress indicator
(2) Decreased transcript levels of NO-producing NR
(3) Signals that indicate arrested cell cycles at reduced alpha-tubulin transcript levels
(4) Transient increase in aerobic fermentation connected to enhanced glycolysis
(5) Activation of the cell death-regulating system without cell death down-regulation

By comparing this reference profile step-by-step along these outlined five characteristics with transcriptome profiles of corona virus-infected cells, we (B) noted the following observations:

(for 1) increased *SOD2* transcript levels marked also early corona viral infection in cultured human cells (MERS-CoV, SARS-CoV and SARS-CoV-2). However, considering the pattern of anti-oxidant enzymes, virus-infected cells responded with differential complex patterning depending on infection pressure, hours post infection, virus type and host cell type;

(for 2) in MERS-CoV infected calu-3 cells at MOI 4.0, up-regulation of *NOS3* transcript level was observed already at 6hpi, which strongly increased at 24hpi. However, in SARS-CoV-2 infected NHBE cells at MOI 2.0, no transcription of *NOS* genes was observed;

(for 3) as in the reference profile, SARS-CoV-2 infected A459 cells at low MOI (0.2) signaled arrested cell cycle at significantly reduced alpha-tubulin transcript levels. However, SARS-CoV-2 infected NHBE cells at 10 times higher MOI (2.0) signaled cell cycle progression combined with significant increase in tubulin transcript levels. Also, MERS-CoV-infected calu-3 cells at high MOI (4.0) signaled cell cycle progression early at 6hpi, but in the presence of already significantly down-regulated tubulin transcripts. At 24hpi, arrested cell cycles were indicated, which was then combined with more drastic down-regulation of tubulin transcript levels. In agreement with the latter result, in MERS-CoV- and SARS-CoV-infected MRC5 cells at 24hpi and 48hpi, no sign of cell cycle progression or proliferation was found, which was independent on lower (0.1) or higher (3.0) MOI. Cell cycle arrest-signaling was combined with down-regulated tubulin transcript levels as also seen in the reference profile;

(for 4) SARS-CoV-2 infected NHBE cells (MOI 2.0) signaled significant increase in aerobic fermentation through increased *LDH* transcript levels, which was linked to enhanced transcript levels for enzymes associated with glycolysis. In MERS-CoV-infected calu-3 cells (MOI 4.0) at 6hpi, *LDH* levels were found to be basal. This was linked to reduced transcript levels for glycolysis. At 24hpi, transcript levels of *LDH* and glycolysis related genes were drastically reduced

(for 5) SARS-CoV-2 infected A549 cells at low MOI (0.2) indicated cell death arrest at 24hpi. NHBE cells that were infected at 2.0 MOI with SARS-CoV-2, signaled at 24hpi activation of the cell death-regulating system. RSV-infected A549 cells at 15 MOI indicated at 24 hpi cell death arrest. MERS-CoV infected calu-3 cells (MOI: 4.0) indicated strong induction of the cell death regulatory system at 6hpi and suppression of the system at 24hpi. Also, in MERS-CoV infected MRC5 cells, suppression for the cell death-regulating system was observed at 24hpi and at 48hpi at

MOI 0.1 and 3.0. SARS-CoV infected MRC5 cells under the same experimental conditions, showed much lower replication rates than MERS-CoV infection (transcriptional ORF reads, not shown). It might be due to this context that initial activation of the cell death regulatory system is indicated at 24hpi also in SARS-CoV-2 infected cells, but that this initial activation was still followed at 48hpi by a trend for increasing caspase (initiator and executor) transcript levels.

As a consequence from our observations described under A and B, we (C) could extract as main result the following targets promising for developing a strategy to avoid early virus spread:

1. Balanced pattern of oxidative stress pattern
2. Decreased NO production
3. Avoidance of cell cycle progression by disconnecting increased aerobic fermentation from energy canalization to alpha-tubulin-based cell restructuration early during viral infection
4. Avoidance of prolonged cell death promotion
5. Considering the unique holobiont nature of individuals in firstly virus-infected cells

Taken together, this complex approach resulted in arriving at two principle conclusions:

- Comparing *in vitro* coronavirus-induced transcriptome profiles with plant cell transcript profiles during *de novo* programming as a reference enabled to identify main characteristics for early SARS-CoV-2-induced transcript changes. Collectively, they indicate one *m*ajor *c*omplex *t*rait for *e*arly *de novo* programming, named here as 'CoV-MAC-TED': unbalanced ROS/RNS levels connected to increased aerobic fermentation that links to alpha-tubulin-based cell restructuration and cell cycle progression.
- In plant systems, it could be shown that the extent of aerobic fermentation induced during *de novo* programming that linked to the initiation of embryonic or non-embryonic cell proliferation was regulated by interacting sucrose- and AOX-levels. Early up-regulation of alcoholic and lactic aerobic fermentation was connected to higher glycolysis and oxidative stress levels. This was associated with increased *AOX* transcript accumulation. Furthermore, our results suggested that a mutant's capacity for more efficient reprogramming compared to wild type (WT) was linked to the capacity of limiting aerobic fermentation, which associated positively to *AOX* transcription levels.

These results are in good agreement with several studies, which showed the occurrence of ROS/RNS disturbances during early viral infection, a potential role of hijacked aerobic fermentation for virus replication, involvement of cytoskeleton during viral infection and virus-induced cell cycle modulation (see in background). The findings further pinpoint to the beneficial role of AOX for plant resilience that is related to both ROS/RNS equilibration and redox homeostasis, able to avoid acidification and excess of toxic ethanol, and regulating at the same time adaptive energy supply for growth performance [(21), in press, (77), preprint]. Interestingly, Ito et al. (85) discovered in *Arum* that temperature-dependent switching between critical *AOX* polymorphisms in the binding site for AOX-pyruvate can determine energy-related metabolic regulation, which in turn results in plant performance. This striking finding underlines again the multifunctional role of AOX that includes besides ROS/RNS equilibration adjusting energy metabolism at the threshold between mitochondrial respiration and aerobic fermentation. *AOX* polymorphisms have been identified and widely explored as genetic trait (86–96), besides being epigenetic (97) and developmental manifestation (98, 99). Also, polymorphisms in neighboring regions of conserved functional sites had been shown to discriminate AOX isoenzymes (100). Furthermore, it was found that *AOX* polymorphisms could be used to distinguish individual plants from the same species (101). Symbiotic AMF revealed substantial *AOX* gene polymorphism within and between spores (102), an observation which awaits to be functionally explored in relation to adaptive plant holobiont performance (103–107).

Our results demonstrated that endophytes can interfere with the redox biology of the host system related to the initiation of cell proliferation (**Figure 1A.4**). Plant-mycorrhizal fungi interaction is suggested to involve *AOX* from both symbiotic partners (102–104, 106). Consequently, the holobiont nature of primary virus-infected cells should be considered as influencer on the impact of early virus infections on cell redox status. We suppose that endophyte interaction is important for program initiation/realization rather than for early program induction (75, 109). This view is in agreement with Visser-Tenyenhuis et al. (110), who observed enhanced SE by bacterial co-cultivation, although bacteria *per se* could not induce SE. Bharadwaj et al. [(77), preprint] extended here reported results from endophyte effects during germination and could show that sucrose critically affected early endophyte impacts on the initiation of cell division growth dependent on the quality of microbiota. It was concluded that endophytes and symbiotic fungi can buffer negative effects of excess in sucrose during early reprogramming. In this way, microbiota and AOX are supposed to interact in support of equilibrated rapid adaptation to sugar-transmitted inner and outer environment signaling [see concept in (77), preprint].

The sophisticated role of *AOX* in plants had developed along evolution in the context of complex holobiont systems. Currently, AOX is developed as a tool for understanding the functionality of its beneficial mechanisms also in mammals that naturally do not enclose AOX [see references in (21), in press]. It showed good integration into normal physiology when constitutively overexpressed in animals. However, mammals did not evolve AOX genes as an integral part in their complex metabolic and multi-organism networks. Thus, whether AOX could be useful in therapy as proposed for mitochondrial respiratory deficiency diseases (111–116)) will, in our view, crucially depend on its adaptive (positive and negative) regulation, which includes also interaction with endophytes. In

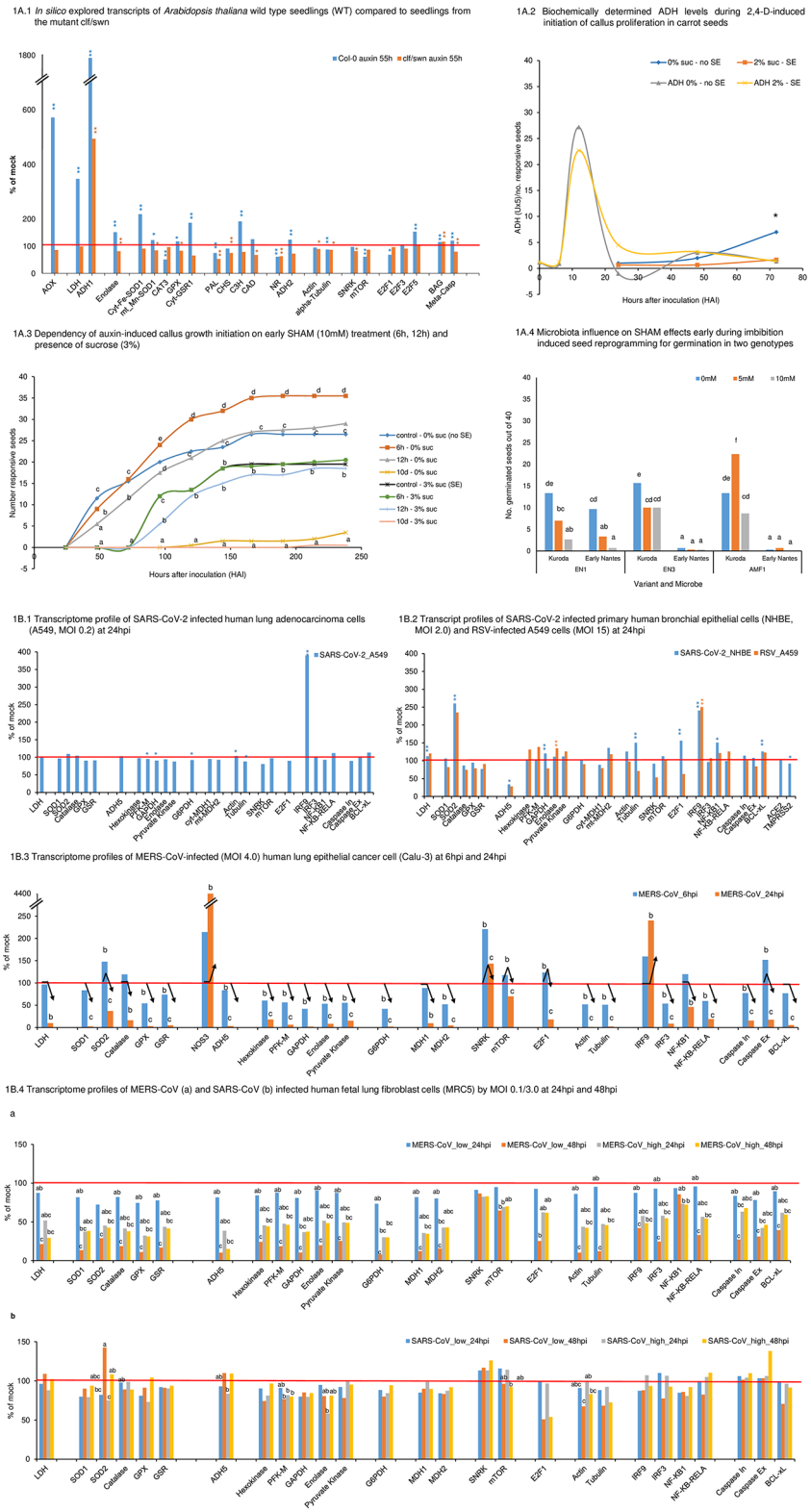

FIGURE 1 | Continued

FIGURE 1 | *In silico* explored transcripts of *Arabidopsis thaliana* wild type (WT) seedlings compared to seedlings from the mutant clf/swn. Results (1): At 55h of auxin treatment, the mutant did not show signs of oxidative stress: none of the selected transcripts involved in oxidative stress regulation were increased against the control and *AOX* remained close to control level. In contrast, the according transcript profile of WT seedlings indicated at that time high oxidative stress. *AOX* shows strongly enhanced transcript accumulation against the control (5.71 folds, significant) along increased complex transcript levels of several anti-oxidants (*SOD*, *GPX*, *GSR1*, *CH3*, *CAD*). (2) WT displays at 55h of auxin treatment strongly decreased *NOS1*- and also significantly reduced *NR* - transcript levels, which together indicate down-regulation of NO production. Significantly decreased *NOS1* transcript accumulation was also observed for the mutant though less pronounced. *NR* transcript level was indicated, but non-significant. No significant differences to the controls were observed for *ADH2/GSNOR* in both variants. (3) In both variants, we observed after 55h of auxin-treatment similarly reduced transcript levels for alpha-tubulin (significant). For actin, the mutant shows the same significant reduction in transcript levels as for tubulin, while in WT a reduction for actin is only indicated (non-significant). In WT, increased transcript levels for *E2F5* (cell cycle suppressor gene) associated to reduced levels of cell cycle activator *E2F1* (both significant) and equal-to-control levels for *E2F3* (activator) at almost unchanged SNRK but lowered TOR transcript levels (significant). All together, these results indicate cell cycle suppression for WT at this early stage of reprogramming. In contrast, the mutant showed only slightly decreased *TOR* and *E2F1/3* transcript levels (all non-significant) and no increase in *E2F5* transcripts, but significantly reduced *SNRK* transcripts. Collectively, these results signal that induction of cell cycle progression towards embryogenic callus growth were for the mutant already more advanced than non-embryogenic callus growth induction for WT. However, in both cases, cell cycle arrest was indicated for that time point. (4) In WT, fermentation-related *ADH1* and *LDH* gene transcripts were strongly enhanced (17.8 folds and 3.5 folds, both significant), which associated to increased glycolysis presented by *Enolase* transcripts (1.5 fold significant). In the mutant, the only profile components that increased against the respective mock control were *ADH1* and *BAG* (both significant). However, at 55h of auxin-treatment this increase in *ADH1* in the mutant (4.9 folds of mock) was clearly and significantly less than the increase observed for WT (17.8 folds). In **Figure 1** and **Supplementary Tables S1**-**S5** (both in **Supplementary files**), it can be seen that the mutant showed in the control higher transcript levels than WT control for *ADH1* (3.9 folds of WT, significant), *LDH* (2.4 folds of WT, non-significant) and *Enolase* (1.3 folds of WT, non-significant). However, after auxin treatment, the increased absolute *ADH1* transcript levels had been similar between both variants (1.1 fold of WT), whereas *LDH* levels remained basal and linked to decreased enolase. Higher levels of transcripts related to aerobic fermentation in the mutant controls were connected to higher *AOX* transcript levels (1.37 folds, significant). However, during auxin treatment *AOX1* transcript levels were strongly increased in WT (significant), indicating a stress situation, while no further increased *AOX1* transcript accumulation was observed in the mutant. This demonstrates that increased *AOX* transcript levels in the WT during auxin-induced reprogramming corresponded to acute metabolic requirements that were not given in the mutant. Overall, these results point to a higher basal capacity of the mutant to limit aerobic fermentation as relevant factor for the mutant's generally higher efficiency for auxin-inducible reprogramming and an associated role for AOX. This capacity was associated to already higher transcript levels of *AOX* in the mutant control. (5) A more advanced stage of reprogramming for the mutant is also obvious by looking to apoptosis- or, in general, cell death-related genes, such as *BAG* (Bcl-2-related genes) and *Meta-caspase*. *BAG* transcript levels were similarly enhanced in both variants (significant). However, while meta-caspase gene transcripts in WT were increased to about the same level as for *BAG* (significant), in the mutant, *Meta-caspase* transcript levels were down-regulated (significant). This observation indicates that **(a)** activation of the complex cell death-related regulatory system formed part of the reprogramming process and that **(b)** cell death-promoting enzymes were down-regulated during the later phase of reprogramming. **Figure 1A.2** Biochemically determined ADH levels during 2,4-D-induced initiation of callus cell proliferation in carrot seeds. Results: Sucrose postponed initiation of callus growth from second day onwards (significant at 72HAI). The initial arrest of callus growth associated to a lower ADH peak at 12h. Although the discrepancy in ADH peaks here shown was non-significant, we had observed in further trials that higher sucrose supply (3%) further reduced this ADH peak level at 12h [(52), preprint]. In conclusion, these results together with the findings in **Figure 1A.1** confirm that reprogramming is, in general, linked to temporarily up-regulated ADH. It indicates a general role for early regulated aerobic fermentation in reprogramming. In the three experimental systems described in the two figures, which include also different plant species and genotypes, cell proliferation was suppressed at the earliest stage during de novo reprogramming and this was independently on later cell destinies. These results also show that sucrose can be a critical factor for fermentation-related reprogramming during its early phase. **Figure 1A.3** Dependency of auxin-induced callus growth initiation on early SHAM (10mM) treatment (6h, 12h) and presence of sucrose (3%). Results: SHAM significantly affected callus growth initiation at 0% sucrose. At 48HAI and 72HAI, it could be observed that SHAM suppressed emergence of callus growth with time of treatment duration (6HAI, 12HAI to its permanent presence). In contrast, from 96HAI onwards, a short initial pulse of SHAM (6h) was sufficient to increase rate of callus emergence (significant). On the other hand, if SHAM supply was prolonged to 12hrs, callus emergence rate was similar to the control. However, in the presence of external sucrose (3%), short SHAM pulses of 6 or 12h did not affect callus initiation. Callus emergence was postponed to about the same degree as observed at 3% sucrose without SHAM and the growth curves were similar. This indicates that oxidative stress regulation and AOX involvement interact with sucrose. When SHAM was present during all 10 days of the trial, callus growth was suppressed in control and sucrose-containing media, and thus, also embryogenic development was suppressed. Overall, these results point to a superimposed role of fine-tuned oxidative metabolism/redox status regulation for hormone-dependent metabolic reprogramming during early induction and also during later growth initiation and highlight its interaction with sucrose. These results also validate central AOX functionality for efficient cell reprogramming under stress, which is highly relevant for breeding on plant resilience (75, 87). **Figure 1A.4** Microbiota influence on SHAM effects early during imbibition-induced seed reprogramming for germination in two genotypes. Results: SHAM had differential effects on root emergence monitored at 48 hours after imbibition dependent on microbiota treatment. In cultivar Kuroda, 5mM SHAM together with AMF improved germination, whereas treatment of 5mM SHAM together with EN1 and EN3 reduced root emergence. While SHAM effects had been dose-dependent for EN1 and AMF, under EN3 + AMF treatment, the higher concentration of SHAM did not lead to less germination. The cultivar Early Nantes is germinating later and only under EN1 treatment, SHAM reduced germination in this genotype. We identified main effects for all three factors (plant genotype, microorganism and SHAM concentration), and interactions for all factor combinations. These results point to the general importance of the holobiont nature of cells and individual organisms when considering oxidative metabolism/redox status regulation. They also support that genotype-dependent, differential AOX levels during early germination impact predictability of plant resilient performance (75, 152). **Figure 1B.1** Transcriptome profile of SARS-CoV-2 infected human lung adenocarcinoma cells (A549, MOI 0.2) 24 hours post infection (hpi). Results: SARS-CoV-2 infection stimulated the immunological system, presented here by interferon regulator factor IRF9 (significant), and transcript factor NF-KB-RELA (112%, non-significant), although multiplicity infection rates were low (MOI 0.2) and *ACE2* and *TMPRSS2* could not be identified in A549 cells. Down-regulation of caspase initiator gene transcript levels, stable levels of caspase executor genes and up-regulated levels of Bcl-xL (all non-significant) are conform with arrested apoptosis activity in the host cell. Down-regulated *SNRK* (non-significant), unchanged transcript levels of *mTOR* and reduced E2F1 cell cycle activator (non-significant) point together also to arrested cell cycle activity. This coincided with down-regulated GAPDH (significant), Pyruvate Kinase (non-significant) and G6PDH (significant) as well as *mt-MDH2* (non-significant). In this situation, LDH transcript level was found equal to control. However, transcript levels for SOD2 as a biomarker for oxidative stress regulation was slightly increased (non-significantly) and for GPX as well as GSR down-regulated, whereas *SOD1* and *Catalase* showed control level. ADH5/GSNOR kept control level, but NOS1 was slightly down-regulated (non-significant). This together with significantly down-regulated tubulin indicates the start of adaptive complex signaling and induced structural host cell reorganization. *IRF9* demonstrated early response of the immune system, which might qualify as functional marker candidate. *IRF3* remained at mock control level. ACE2 (*Angiotensin-converting enzyme 2*), *TMPRSS2* (transmembrane protease serine 2) gene expression was not detected in the analysis and genes were not denoted in the **Supplementary Table S6**. **Figure 1B.2** Transcript profiles of SARS-CoV-2 infected primary human bronchial epithelial cells (NHBE, MOI 2.0) and respiratory syncytial virus (RSV) - infected A549 cells (MOI 15) at 24hpi Results: In NHBE cells, *ACE2*

and *TMPRSS2* were identified. While the transcript level of *ACE2* remained unchanged, a significant decrease for ACE2-priming molecule TMPRSS2 was observed. Nevertheless, SARS-CoV-2 infection stimulated again the immunological system, presented here by *IRF9*, and *NF-KB1* (both significant). However, in this context different from **Figure 1B.1**, an equally strong increase in *SOD2* transcript level compared to *IRF9* was observed along with slightly up-regulated *SOD1*. This was accompanied by down-regulated levels of catalase, *GPX* and to a higher extent *GSR* (all non-significant) (**Supplementary Table S7**). Combining these last results, they signal changed oxidative stress level and complex fine-tuning activities. *NOS1* transcript level seems to be unchanged regarding the control. However, *ADH5/GSNOR* was down-regulated to 34% (significant), signaling a change in NO homeostasis. Increased *mTOR* transcript level and at the same time down-regulated *SNRK* level (both non-significant) coincide with significant *E2F1* transcript level increase, which goes along with a similarly strong transcript level increase of *Tubulin* (significant) and also of *Actin* (non-significant). Overall, this points to rapidly induced cell cycle activity. This picture is supported by an increase in *LDH* transcript level (significant) linked to an increase in glycolysis enzyme transcripts from *GAPDH* (significant) onwards and also *mt-MDH2* (non-significant), whereas *ct-MDH1* transcripts were reduced (non-significant). *G6PDH* transcript level was unchanged. The transcript level of anti-apoptotic *Bcl-xL* is significantly up-regulated, but also caspase initiator and caspase executor transcription were up-regulated though to an obviously lesser extent (both non-significant). In comparison, RSV-infected A549 cells responded under the applied experimental conditions strikingly similar in relation to the most pronounced host cell responses due to increased transcript levels of *mt-SOD2* and *IRF9* (number of replicates for transcripts was in part less for this experimental system, which can be responsible for missing significances). In both variants, *IRF3* remained basal. However, the overall response was differential and varying transcript level changes were also indicated for *NF-KB-RELA* and *NF-KB1*. Anti-oxidative enzyme transcript levels were more reduced apart from higher transcript accumulation for *GSR*. Also, *NOS1* transcripts were in this case down-regulated to 72.5% (non-significant). *ADH5/GSNOR* was reduced to a similar level (28%) as seen for SARS-CoV-2 infected NHBE cells. Although *LDH* transcripts were up-regulated to a similar extent as observed for SARS-CoV-2-infected NHBE cells, and again linked to up-regulation of the glycolysis pathway, the overall response was different. *GAPDH* transcript level was down-regulated and in this case, *Hexokinase* and *PFK-M* were up-regulated and enolase (significant) and pyruvate kinase showed higher transcript levels and *G6PDH* was down-regulated. A striking difference comes by the observation that *SNRK* was more strongly down-regulated, but *mTOR* was not up-regulated and *E2F1* did also not show up-regulation. This together linked to contrasting down-regulation of transcript levels for tubulin and obviously unchanged actin transcription. Collectively, it indicates that in this system at the given time point (24hpi), cell reprogramming was taking place, but cell cycle progress was not stimulated. No sign of cell death up- or down-regulation can be recognized. The transcript level of anti-apoptotic *Bcl-xL* is again significantly up-regulated, caspase initiator level remained basal and caspase executor transcription was down-regulated (non-significant). **Figure 1B.3** Dependency of auxin-induced callus growth initiation on early SHAM (10mM) treatment (6h, 12h) and presence of sucrose (3%). Results: SHAM significantly affected callus growth initiation at 0% sucrose. At 48HAI and 72HAI, it could be observed that SHAM suppressed emergence of callus growth with time of treatment duration (6HAI, 12HAI to its permanent presence). In contrast, from 96HAI onwards, a short initial pulse of SHAM (6h) was sufficient to increase rate of callus emergence (significant). On the other hand, if SHAM supply was prolonged to 12hrs, callus emergence rate was similar to the control. However, in the presence of external sucrose (3%), short SHAM pulses of 6 or 12h did not affect callus initiation. Callus emergence was postponed to about the same degree as observed at 3% sucrose without SHAM and the growth curves were similar. This indicates that oxidative stress regulation and AOX involvement interact with sucrose. When SHAM was present during all 10 days of the trial, callus growth was suppressed in control and sucrose-containing media, and thus, also embryogenic development was suppressed. Overall, these results point to a superimposed role of fine-tuned oxidative metabolism/redox status regulation for hormone-dependent metabolic reprogramming during early induction and also during later growth initiation and highlight its interaction with sucrose. These results also validate central AOX functionality for efficient cell reprogramming under stress, which is highly relevant for breeding on plant resilience (75, 87). **Figure 1B.4** Transcriptome profiles of MERS-CoV **(a)** and SARS-CoV **(b)** infected human fetal lung fibroblast cells (MRC5) by MOI 0.1/3.0 at 24hpi and 48hpi Results: For MRC5 cells, ACE2 was not identified. MERS-CoV-infection observed at 24hpi and 48hpi (**Figure 1B.4a**) showed for all profile components down-regulation at different degrees of significance (see letters in figure). This included also *SOD2* and *IRF9* (both with significant reduction at 48hpi and low MOI). *NOS1* seemed to be slightly less affected (39.5%, significant) than mt-SOD2 (29%). However, *ADH5/GSNOR* levels are significantly reduced to 10%. At 24hpi, low MOI (0.1) showed within each component always highest transcript levels by comparing MOI and infection times. In general, at higher MOI the effect of time seemed to be reduced. *SNRK* responded exceptionally among all components. Transcript levels stayed comparatively more stable across all variants and had not been significantly reduced to control level at any time. *mTOR* showed a small reduction to the control at low MOI at 24hpi (non-significant), but transcripts were significantly reduced by time and also at higher MOI at 24hpi. *E2F1* showed a parallel pattern to *mTOR*, but was at 48hpi and low MOI more drastically reduced (25% to control) (significant) than *mTOR* (65% to control) (significant). Again, *GAPDH* was the most affected enzyme from the glycolysis path at both MOI (10.5% and 37.5%) and *G6PDH* was decreased to slightly lower extent (8% and 30%) (significant at 48hpi for both, low MOI). *Tubulin* and *Actin* revealed similarly drastic and significant reduction in transcript levels at low MOI 48hpi (to 12% and 10%). *LDH* transcript levels decreased with time at both MOI (significant for 48hpi, low MOI) (**Supplementary Table S9**). In SARS-CoV-infected MRC5 cells (**Figure 1B.4b**), we found from transcriptional ORFs much lower virus replication for SARS-CoV than for MERS-CoV (data not shown). This might have contributed to only moderate broad down-regulation in comparison to MERS-CoV infection. Under these conditions, we see in SARS-CoV infected cells up-regulated SOD2 at 48hpi at both MOI and differential down-regulation of most of the anti-oxidant components (non-significant). *NOS1* was downregulated with time and MOI (significant), but 48hpi at high MOI transcript levels were increased again to basal. Also *ADH5/GSNOR* transcript levels were down-regulated at higher MOI, but, in general, demonstrated slightly increased levels at 48hpi (non-significant). *IRF9* was slightly up-regulated (non-significant) only at 24hpi and higher MOI. *SNRK* was consistently up-regulated (113 - 126%, non-significant). To the contrary, initial *mTOR* values above the control were down-regulated with time at high MOI. Further, strong down-regulation was observed in mean values for *E2F1* at both MOI levels (non-significant). Tubulin and actin transcript levels also tended to decrease with time at both MOI levels. Though all these observations were separately not significant, together they suggest early energy depletion and suppression of cell cycle progression or cell proliferation at 48hpi. In agreement with this observation, *LDH* transcripts seemed to be unchanged to the control (no significant differences) (**Supplementary Table S10**). Nevertheless, a tendency of enhanced transcription of *LDH* was observed at 48hpi at both MOI, which went along with increased levels for enzymes related to glycolysis. Together, these observations indicate increased energy-dependent metabolic reprogramming with time. The anti-apoptosis gene *Bcl-xL* tends to be reduced with time. Both caspases remained at control level at low MOI, but both indicate a tendency for increasing transcription levels with time, which is more obvious, though non-significant, for the executing caspase (138%). Thus, it seems that apoptosis/cell death was stimulated by time: *Significant ($p < 0.05$) and ** highly significant ($p < 0.01$) differences regarding control. Letters indicates significant differences betweet treatments (A.3, A.4) or HPI/MOI (B.3, B.4). Arrows indicate the behaviour of transcript levels along time, considering statistical (B3). Leter a is not labeled and correspond to the mock treatment (100%) (B.4), except in B.4B for SOD2 and mTOR, which labels for mock are abc and ab, respectively, as indicated.

this context, it is also pertinent to mention that recently an AOX-degrading protein has been discovered that might be added as a tool in potential AOX-based therapies (85).

In search for similarities between the beneficial role of AOX in plants in relation to adaptive oxidative stress level equilibration relevant for virus tolerance and that of natural agents in human cells, melatonin seems to be a strong candidate. Melatonin is a natural hormone in humans, which has also been recognized as a phytohormone (117). It is produced in most organs and cells (70, 117–120), including also human salivary gland cells (121).

Melatonin is known to possess anti-oxidant properties and shows high fluctuation in its cellular concentration. In plants, melatonin seems not to enhance *AOX* transcription (117) as it has been shown for auxin (75). In turn melatonin interacts with other enzymes involved in ROS/RNS balancing and was suggested to be in plants mainly involved in biotic stress defense rather than in growth regulation (117). Further, melatonin was reported to enhance the induction of adventitious roots through interaction with auxin-mediated signaling, which suggests a role for melatonin also in early reprogramming (122). All evidence put together, it could be suggested that melatonin might substitute early anti-oxidant function of AOX during reprogramming (75, 99, 123). By looking at the transcription of melatonin synthesis-related genes *ASMT* (*N*-acetylserotonin O-methyltransferase) and *NAT* (serotonin *N*-acetyltransferase) in WT *A.thaliana* our model system showed that auxin-induced *AOX* transcript level changes, we found up-regulated *ASMT*/*NAT* transcript levels 55 hours after 2,4-D treatment though at a low level (not shown). This preliminary observation might lead to future interest and encourages further studies. Currently, the molecular-physiological and clinical relevance of melatonin is found to be a hot topic. It was highlighted to show important functionality in physiology, pathophysiology and chronobiology. Since long melatonin was also recognized as a beneficial agent for managing viral infections (124). However, despite its widespread use as a drug for many other purposes, its functional role and application still needs stronger confirmation through extensive biochemical and clinical research (125–127). Apart from acting as an anti-oxidant, melatonin demonstrates anti-inflammatory activity and immune-enhancing features besides interacting with ACE2 (118, 124, 127, 128). Recently, the proposed rationale for employing melatonin as a potential anti-viral agent in general and anti-SARS-CoV-2 agent in particular has been indicated by Zhang et al. (127). Extensive efforts are being made to identify drugs for treating SARS-CoV-2 infections *via* a network-based drug repurposing (127). By adopting this methodology, melatonin has been identified as a promising drug, which can be administered either alone or in combination with immune-suppressant agents. In our studies, we focused additionally on melatonin-synthesis related genes to work-out its drug potentials. Results of the present study showed enhanced *ASMT* transcript accumulation at low level in SARS-CoV-2-, RSV-, MERS-CoV- and SARS-CoV-infected human cells of various origins (**Figures 2A, B**). Related to MERS-CoV-infected MRC5 cells, it can be seen that *ASMT* transcript levels were increasing dependent on MOI level and infection time (**Figure 2B**). However, we did not identify *ASMT* transcripts in MERS-CoV-infected calu3 cells (MOI 4.0). Nevertheless, these observations might encourage further research on the significance of melatonin actions during early viral infection in the primarily affected nose and mouth cells.

Another interesting approach in agreement with the focus of our study is the potential anti-viral use of ergothioneine (ET) (129). ET is a natural amino acid that enters *via* diet into the human body and can be found in all animal cells. It has multifunctional characteristics of interest for anti-viral functionality that includes flexible regulation of redox biology. ET makes an attractive option for its use as an alternative to AOX functionality in plants essentially due to its highly plastic distribution based on an appropriate adaptive transporter system (OCTN1) and its ubiquitous availability. However, in order to be functional during the early phase in mouth and nose cells ET needs to have direct effect on avoiding virus-induced reprogramming that connects to initiating viral replication, which awaits future research (129).

In plants, impressive success has been achieved in confronting viral infections by applying TOR inhibitors or TOR silencing [reviewed in (60)]. Avoiding cell cycle progression in primary virus-threatened nose and mouth cells could be critical during the early phase of virus entry. Drugs that target mTOR and tubulin are available (55, 128) and they might be repurposed and adapted to early treatment of primary corona virus-threatened cells in mouth and nose. However, prolonged suppression of cell renewal in nose and mouth could be fatal due to collateral bacterial or other infections or contaminations. Consequently, pulsed treatment strategies could be considered to avoid virus-induced cell progression that benefits virus replication, but at the same time allowing intermittent relaxation from cell cycle inhibition for cell renewal. This type of treatment might enable 'growing-out of danger' of host mouth/nose cells. This is a general strategy followed in plants *in vivo* and in plant biotechnology systems in order to get rid of virus spread. Comparably, pulsed treatment strategy might contribute to stimulation of cell proliferation in a way that virus replication cannot follow in the same velocity as healthy new cells are emerging.

In plant biotechnology, it is well established that phytohormones and their relative intracellular concentrations are essentially involved, either directly or indirectly, in program decisions about growth and differentiation. However, the impact of phytohormones depends on available bioenergy as the critical frame for program realization. Phytohormones are known to be critical for *de novo* membrane-based cell restructuring ('innovation') during quiescent cell reprogramming, whereas sucrose is required for structural organization ('performance') (130). In human cells, the entry receptor of SARS-CoV-2, i.e. ACE2, is also known to function as a negative regulator of the hormonal renin-angiotensin system (RAS). RAS plays an important role in regulating cellular growth, proliferation, differentiation, and apoptosis besides extracellular matrix remodeling and inflammation (131). The receptor has been shown to protect lung from injury (132). However, for SARS-CoV infection and the Spike protein it had been shown that they reduce *ACE2* expression. Spike injection into mice worsened acute lung failure *in vivo*, which could be attenuated by blocking the renin-angiotensin path (133). Actual studies are being focused to enable efficient anti-SARS-CoV-2 treatment *via* competing soluble ACE2 drugs (hrsACE2) (132). We are in favor of this approach and suggest extending studies on hrsACE2 by considering the early indicated metabolic effects, which are presented as complex trait 'CoV-MAC-TED'.

Rodríguez et al. (134) showed that differential action of phytohormones on the induction of new program (adventitious organogenesis) was epigenetically regulated. RAS action has also been linked to changes in the epigenome (135). During auxin-induced *de novo* cell programming, genome-wide

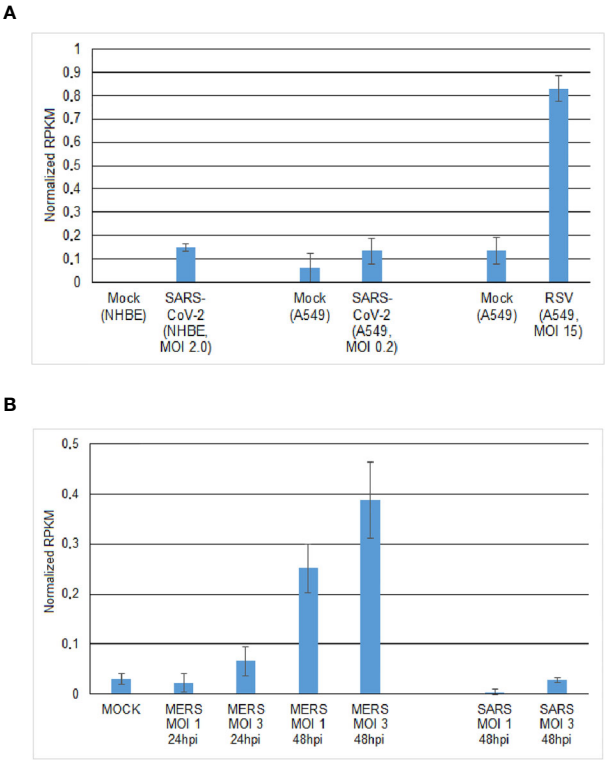

FIGURE 2 | Expression of ASMT (acetylserotonin O-methyltransferase) gene involved in 1041 melatonin biosynthesis in virus-infected human cells. **(A)** ASMT transcript levels in SARS-CoV-2- and RSV-infected cells at 24hpi. **(B)** ASMT transcript levels in MERS-CoV- and SARS-CoV-infected human lung fibrolast cells (MRC5) depends on MOI level (1.0;30) and infection time (24hpi; 48hpi).

hyper-methylation is known to take place [firstly reported for SE by (136)]. Gross hyper-methylation combined with genome-wide reduction in the amount of repetitive DNA linked to the occurrence of small DNA fragments had systematically been observed before cell cycle progression started (137). Therefore, we would like to suggest exploring the effect of soluble ACE2 drugs also on the level of epigenetic events that occur early during reprogramming before the cell cycle activation. Genome-wide repetitive DNA reduction linked to an increase in small DNA-fragments was also reported to happen before actual cell cycle start as a determinant for cell proliferation (137–139) and is supposed to be another powerful candidate linked to the induction of cell proliferation.

The possibilities of using controlled pH to support these strategies by early testing and treatment, should, in our view, be explored with priority. Cell reprogramming and its relation also to cell death (140), changes in anti-oxidant activity also related to AOX (141–143), aerobic fermentation (144) and viral infections (144–148) are all influenced or indicated by extracellular pH. Extracellular pH might also by its interaction with intracellular pH influence complex tasting, which includes not only sour tasting (149, 150). The relation between smell and pH is, to our knowledge, not sufficiently explored yet. However, SARS-CoV-2 entry proteins were found to be expressed in olfactory epithelial cells (151).

CONCLUSION

This is the first research to start validating hypothesis and wider perspective published by a non-institutional, voluntary competence focus under the title 'From plant survival under severe stress to anti-viral human defense – a perspective that calls for common efforts'

We identified a corona virus-induced complex trait named 'CoV-MAC-TED' (*co*rona*v*irus *ma*jor *c*omplex *t*rait for *e*arly *de novo* programming). It is well established during first few hours of viral infection and consists of unbalanced ROS/RNS levels connected to increased aerobic fermentation that links to alpha-tubulin-based cell restructuration and cell cycle progression.

Primary infected nose and mouth cells become rapidly 'super-spreaders'. This can have serious consequences for neighboring cells, the infected organism and for the environment.

Virus structures as non-living particles would be harmless, if the entry cells could ignore their presence. It is the proper host cell-reaction that is required and 'abused' for virus replication. Consequently, as long as we cannot avoid virus structures from establishing contact 'with us', we need to support the entry cells to become ignorant. If possible, this should happen proactively or, at least very rapidly and much before any symptoms of viral infection appears.

Considering our results, the first fingerprint of viral infection is complex and needs combined strategies that rigorously target the components of 'CoV-MAC-TED' in primary infected nose and mouth cells.

Thus, it is the need of the hour to develop efficient strategies to early equilibrate or rebalance ROS/RNS levels and to control cell progression in nose and mouth cells. We have discussed some promising approaches that are in agreement with our observations.

As a wider conclusion from our observation of the importance of balanced redox biology during early virus infection, we want to highlight our view that de-stressing in disease and social handling and communication should not be ignored as meaningless 'luxury', but rather considered as being essential part of an overall strategy to promote healthiness.

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 coordinated and performed transcriptome analyses supported by KT and SA and developed together with BA-S the scientific approach. GM performed carrot SE lab analyses and helped together with RB in figure design and formal manuscript organization, RB together with SS and AAd were responsible for lab studies on endophytes and AMF. SR together with RB and GM analyzed the experimental data and helped BA-S in overall *FunCROP* group coordination. RB wrote together with BA-S part of the manuscript and prepared overall manuscript for submission. RS supervised lab work of GM and RB. CN by support of MO performed all statistical analyses and was strongly engaged to improve performance and data interpretation. KG together with AK helped designing manuscript parts related to NO metabolism. All authors contributed to the article and approved the submitted version.

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The authors want to dedicate their work to FREEDOM and ETHICS.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.673692/full#supplementary-material

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ORIGINAL RESEARCH
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Critically Ill *vs*. Non-Critically Ill Patients With COVID-19 Pneumonia: Clinical Features, Laboratory Findings, and Prediction

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Objectives: The objective of this study was to investigate the clinical features and laboratory findings of patients with and without critical COVID-19 pneumonia and identify predictors for the critical form of the disease.

Methods: Demographic, clinical, and laboratory data of 63 COVID-19 pneumonia patients were retrospectively reviewed. Laboratory parameters were also collected within 3–5 days, 7–9 days, and 11–14 days of hospitalization. Outcomes were followed up until March 12, 2020.

Results: Twenty-two patients developed critically ill pneumonia; one of them died. Upon admission, older patients with critical illness were more likely to report cough and dyspnoea with higher respiration rates and had a greater possibility of abnormal laboratory parameters than patients without critical illness. When compared with the non-critically ill patients, patients with serious illness had a lower discharge rate and longer hospital stays, with a trend towards higher mortality. The interleukin-6 level in patients upon hospital admission was important in predicting disease severity and was associated with the length of hospitalization.

Conclusions: Many differences in clinical features and laboratory findings were observed between patients exhibiting non-critically ill and critically ill COVID-19 pneumonia. Non-critically ill COVID-19 pneumonia also needs aggressive treatments. Interleukin-6 was a superior predictor of disease severity.

Keywords: COVID-19, infection, pneumonia, severity, critically ill, predictor

HIGHLIGHTS

1. The mortality rate in critically ill patients is low.
2. Different severity of diseases has different clinical and laboratory results.
3. Non-critically ill COVID-19 pneumonia also needs aggressive treatments.
4. Interleukin-6 is a good predictor of critically ill COVID-19 pneumonia.
5. Interleukin-6 is associated with length of hospitalization

INTRODUCTION

Novel coronavirus (COVID-19) pneumonia is a newly recognized disease that has spread rapidly throughout China, originating from Wuhan (Hubei province) and expanding to other provinces within the country and around the world (Yang et al., 2020). The current novel coronavirus has surpassed Severe Acute Respiratory Syndrome (SARS) in terms of the number of recorded cases and deaths from the disease (Peeri et al., 2020). The clinical spectrum of COVID-19 pneumonia ranges from mild to critically ill (Yang et al., 2020). Yang et al. have reported on clinical courses and outcomes of critically ill patients with COVID-19 pneumonia in Wuhan, China (Yang et al., 2020). There has been no comparison of data between patients with and without severe COVID-19 pneumonia. In addition, critically ill patients had a high mortality rate of 61·5% and often had to be transferred to an intensive care unit (ICU). Therefore, it is important to recognize predictors of disease severity in the early phase of COVID-19 infection. This could help select patients who could benefit from close surveillance or aggressive interventions. Early case recognition and classification of disease severity improves clinical outcomes (Liao et al., 2020).

Therefore, our study aimed to investigate the clinical features of COVID-19 pneumonia in patients who were critically ill *vs.* those who were non-critically ill and identify possible predictors of disease severity.

MATERIALS AND METHODS

Study Design, Subject Selection

We conducted a retrospective cohort study in the First Affiliated Hospital of Wenzhou Medical University in mainland China. All patients with confirmed COVID-19 pneumonia between January 29, 2020 and March 12, 2020 were eligible for inclusion in this study. A confirmed case of COVID-19 was defined as exhibiting a positive result on high-throughput sequencing or real-time reverse-transcriptase–polymerase chain reaction (RT-PCR) assay of nasal and pharyngeal swab specimens (Guan et al., 2020). Exclusion criteria were the unavailability of chest computed tomography (CT) scans.

Definition of Severity

According to the China Guidelines for the Diagnosis and Treatment Plan of COVID-19 Infection (2020; Xu Y. H. et al., 2020), COVID-19 infections are classified into four types on admission: critically severe type with any of the following: respiratory failure needing mechanical ventilation, shock, or combination with different organ failure requiring admission into an intensive care unit (ICU); severe type with any of the following: respiratory distress with respiratory rate>30 times/minutes, oxygen saturation at rest <93%, or PaO2/FiO2 <300 mmHg; common with fever, respiratory symptoms, and imaging presentations of pneumonia; and, mild with slight clinical symptoms but no imaging presentations of pneumonia (2020).

For comparative analysis, we also defined the degree of severity of critically ill *vs.* non critically ill cases of COVID-19 according to a previous study (Kumar et al., 2009). Critically ill patients were defined as those admitted to the ICU and either required mechanical ventilation or had a fraction of inspired oxygen (FiO2) value of at least 60% or more during hospitalization (Kumar et al., 2009; Yang et al., 2020). The date of disease onset was defined as the day when the symptoms were first noticed (Wang et al., 2020).

Data Collection, Follow up and Ethics

The epidemiological, clinical, laboratory, radiologic, and treatment and outcomes data during the course of hospitalization were obtained with data collection forms from electronic medical records. The date of disease onset was defined as the day when the symptoms were noticed (Wang et al., 2020). Fever was defined as any value over normal body temperature (>37.0°C) during the time from symptoms to admission. Also, body temperature was measured at admission as a sign of the disease. Chronic concomitant diseases, alcohol consumption and smoking were also recorded at the time of admission (Hong et al., 2017; Hong et al., 2020). Longitudinal data of laboratory parameters at different time points, i.e. within 24 h, 3–5 days, 7–9 days, and 11–14 days after admission, were collected and analyzed. Outcomes were followed up until March 12, 2020.

This study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. It was performed according to the principles expressed in the Declaration of Helsinki, and informed consent was obtained from all the subjects.

Statistical Analysis

The Shapiro-Wilk test was used to determine if the continuous data follow a normal distribution (Hong et al., 2020). Continuous values were expressed by mean ± SD or median and Inter Quartile Range (IQR) and compared using one-way analysis of variance (ANOVA) or the Kruskal-Wallis non-parametric test. Categorical values were described by count and proportions and compared by the $\chi 2$ test or Fisher's exact test.

The area under the receiver operating characteristic (ROC) curve (AUC) was used to evaluate the performance of the predictors. A larger AUC value is indicative of greater diagnostic accuracy of a variable (Hong et al., 2011). An AUC

above 0·8 indicates good diagnostic accuracy for a variable (Hong et al., 2017). The best cut-off point was set as the point where the number of false positives is as low as possible (specificity>95%) and is determined by selecting a threshold value at the point where the longest increase in the sensitivity of the slope declines (Hong et al., 2017). The sensitivity, specificity, negative predictive value, positive predictive value, positive likelihood ratio, negative likelihood ratio, and diagnostic accuracy were all calculated for the various corresponding cut-off values.

Pearson correlation and linear regression analysis were used to investigate the relationship between predictor and length of hospitalization. The variables with skewed distribution were log-transformed for correlation analysis when necessary. Differences were considered to be statistically significant if the two-tailed P value was less than 0.05.

RESULTS

Clinical Characteristics

A total of 63 hospitalized patients confirmed to have COVID-19 pneumonia were enrolled in our study. Twenty-eight patients were imported cases who had traveled from Wuhan City (**Supplementary Figure 1**). None of the medical staff were infected. As shown in **Table 1**, the mean age of the pneumonia patients was 55·9 ± 15·3 (range: 17-92) years. Forty-one (65·1%) patients were men. The mean time from onset of symptoms to admission into our hospital was 6·9 ± 3·7 days. A few of the patients had a history of smoking (17·5%) and alcohol intake (16·0%). Twenty-seven (42·9%) patients had chronic concomitant diseases. The most common symptoms at the onset of the illness were fever (98·4%), cough (61·94%) and sputum (34·9%). However, 16 (25·4%) patients had normal body

TABLE 1 | Baseline characteristics of 63 patients infected with COVID-19 pneumonia on admission.

Characteristic	Total number (N=63)	Non- Critically ill (N=41)	Critically ill (N=22)	P-value
Median age, years (IQR)	56 ± 15	52·9 ± 14·9	61·5 ± 14·7	0·034
Male sex, N (%)	41 (65·1)	26 (63·4)	15 (68·2)	0·705
Time from symptoms to admission, days	6·9 ± 3·7	6·9 ± 4·0	6·8 ± 2·8	0·939
Epidemic data				0.847
Travelled from Wuhan, N (%)	25 (38·1)	16 (39·0)	9 (40·9)	
Contacted to case, N (%)	14 (22·2)	10 (24·4)	4 (18·2)	
Occult history, N (%)	24 (39·7)	15 (36·6)	9 (40·9)	
Median BMI	25·2 (22·3–26·9) (N=40)	24·5 (22.1–26.4) (N=28)	25·9 (24·5–29·0) (N=12)	0·165
Smoking, N (%)	11 (17·5)	6 (14·6)	5 (22·7)	0·420
Alcohol, N (%)	10 (16·0)	6 (14·6)	4 (18·2)	0·713
Chronic concomitant diseases, N (%)	27 (42·9)	15 (36·6)	12 (54·6)	0·170
Hypertension, N (%)	21 (33·3)	12 (29·3)	9 (40·9)	0·407
Diabetes mellitus, N (%)	9 (14·3)	4 (9·8)	5 (22·7)	0·256
Malignancy, N (%)	2 (3·2)	0 (0)	2 (9·1)	0·118
Cardiovascular, N (%)	2 (3·2)	2 (4·9)	0 (0)	0·538
Neurologic, N (%)	1 (1·6)	1 (2·4)	0 (0)	1·000
Pulmonary, N (%)	1 (1·6)	0 (0)	1 (4·5)	0·349
Hepatitis virus carrier, N (%)	1 (1·6)	1 (2·4)	0 (0)	1·000
Symptoms				
Fever, N (%)	62 (98·4)	40 (97·6)	22 (100)	1·000
Cough, N (%)	39 (61·9)	21 (51·2)	18 (81·8)	0·028
Sputum, N (%)	22 (34·9)	12 (29·3)	10 (45·5)	0·269
Dyspnoea, N (%)	17 (27·0)	4 (9·8)	13 (59·1)	<0·001
Chills, N (%)	15 (23·8)	12 (29·3)	3 (13·6)	0·222
Fatigue, N (%)	8 (12·7)	4 (9·8)	4 (18·2)	0·434
Sore throat, N (%)	7 (11·1)	6 (14·6)	1 (4·6)	0·405
Headache, N (%)	3 (4·8)	3 (7·3)	0 (0)	0·546
Myalgia, N (%)	4 (6·4)	2 (4·9)	2 (9·1)	0·606
Diarrhea, N (%)	3 (4·8)	2 (4·9)	1 (4·6)	1·000
Signs				
Distribution of temperature				0.128
<37·0, N (%)	16 (25·4)	14 (34·1)	2 (9·1)	
37·0-37·4, N (%)	17 (27·0)	9 (22·0)	8 (36·4)	
37·5-38·0, N (%)	15 (23·8)	10 (24·4)	5 (22·7)	
38·1-39, N (%)	14 (22·2)	7 (17·1)	7 (31·8)	
>39, N (%)	1 (1·6)	1 (2·4)	0 (0)	
Mean arterial pressure (IQR), mmHg	99·0 ± 11·5	98·6 ± 11·4	99·6 ± 11·9	0·741
Heart rate, bpm	87·1 ± 16·0	88·2 ± 16·7	84·9 ± 14·7	0·438
Respiratory rate	20 (20–23)	20 (20–20)	23·5 (20–28)	0·004
Location of CT findings				0·538
Unilateral pneumonia	2 (3·2)	2 (4·9)	0 (0)	
Bilateral pneumonia	61 (96·8)	39 (95·1)	22 (100)	

Data are shown either as the number of observations, percentage, or median and interquartile range.

temperatures at admission. This means that the fever maybe not persist from onset to admission. Sixty-one (96·8%) of the patients had bilateral involvement of pneumonia in chest CT images (**Table 1**). The typical findings from chest CT images upon admission were local high-density patches, masses (**Supplementary Figure 2A**), and ground-glass opacity (**Supplementary Figure 2B**).

As shown in **Figure 1**, upon admission, there were 13, 42, and 8 patients with common type, severe type, and critically severe type pneumonia, respectively, in our study. One COVID-19 pneumonia patient from the common type group and 13 COVID-19 pneumonia patients from the severe type group progressed to the critically ill type during hospitalization. Therefore, of the 63 patients, 22 (34·9%) required high-flow nasal cannula at a later stage or higher-level oxygen support measures to correct hypoxemia during hospitalization and were classified as critically ill patients, while the remainder (41 patients) were recorded as non-critically ill patients.

As shown in **Table 1**, in comparison with the non-critically ill patients, patients with critical illness were generally older and were more likely to report cough and dyspnoea with higher respiratory rate frequency.

Laboratory Findings

Upon admission, data regarding levels of D-dimer (full data available for 58 patients), B-type natriuretic peptide (full data available for 62 patients), and interleukin-6 (IL-6) (full data available for 46 patients) were obtained. As shown in **Table 2**, in comparison to the non-critically ill patients, patients with critical illness had higher white blood cell and neutrophil counts, as well as higher levels of aspartate transaminase, blood urea nitrogen, creatine, D-dimer, creatine kinase, B-type natriuretic peptide, C-reactive protein, procalcitonin, and interleukin-6. Besides, patients with severe pneumonia had more severe hypoalbuminemia than patients exhibiting less critical forms of the disease.

As shown in **Figure 2**, longitudinal data revealed that statistical difference was still significant between COVID-19 pneumonia patient groups (critically ill *vs* non-critically ill) in terms of counts or levels of white blood cells, neutrophils, aspartate transaminase, blood urea nitrogen, and D-dimer

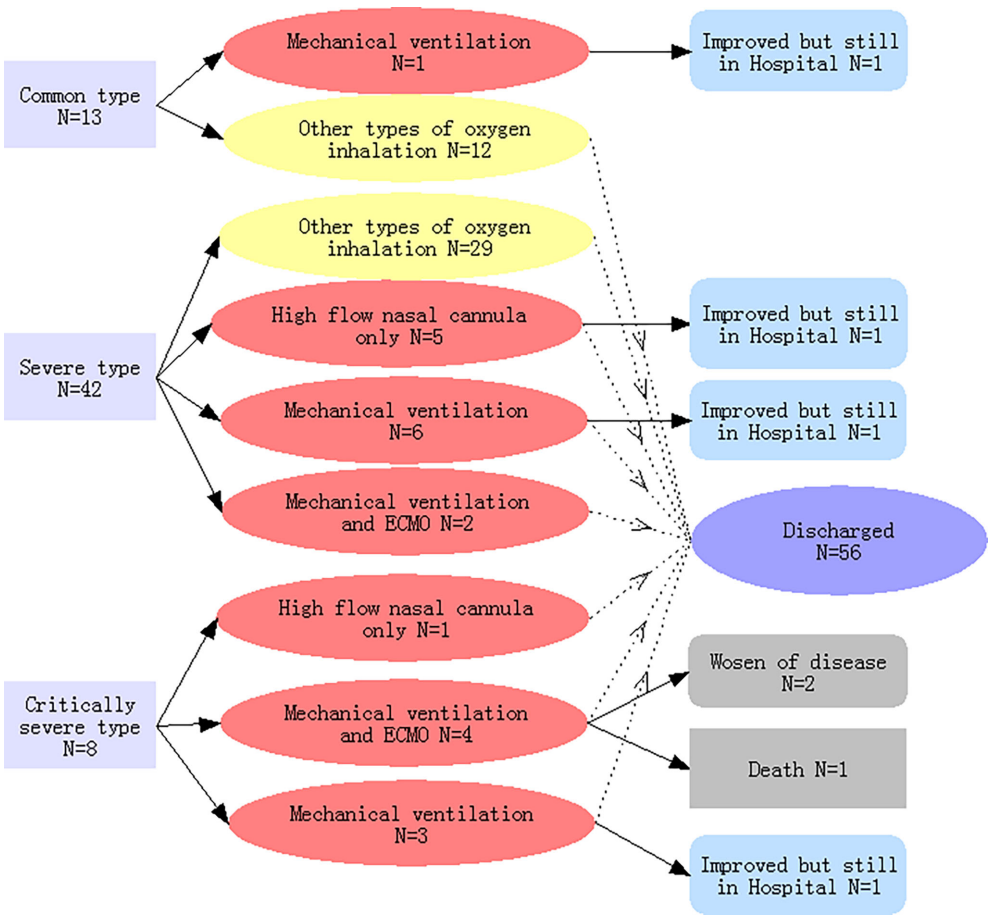

FIGURE 1 | Classifications of the severity of COVID-19-related pneumonia on admission, types of oxygen inhalation, and clinical outcomes. Light yellow indicates COVID-19 pneumonia patients with non-critical illness; light red indicated patients with critical illness.

TABLE 2 | Comparison of laboratory findings, treatment measures and clinical outcomes between critically and non-critically ill COVID-19 pneumonia patient groups.

Characteristic	Normal range	Non-Critically ill (N=41)	Critically ill(N=22)	P-value
Laboratory findings				
Leukocyte (10^9/L)	3.5–9.5	5·1 (4·3–7.2)	9·5 (7·8–12·4)	0·001
Lymphocyte (10^9/L)	1.1–3.2	0·9 (0·72–1·17)	0·67 (0·44–1·07)	0·059
Neutrophil (10^9/L)	1.80–6.3	3·69 (2·81–5·45)	8·06 (5·13–9·84)	0·002
Platelet (10^9/L)	125–350	219 (168–296)	196 (155–231)	0·056
Total bilirubin, mmol/L	0–20	11 (8–15)	12·5 (8–17)	0·406
Alanine aminotransferase, U/L	9–50	24 (20–42)	38·5 (21–69)	0·217
Aspartate transaminase, U/L	15–40	28 (23–38)	50 (35–83)	0·001
Albumin, (mg/dL)	40.0–55.0	34·4 ± 4·6	30·8 ± 3·7	0·002
Blood urea nitrogen, mmol/L	2.8–7.2	4·7 (3·4–5·9	5·5 (4·9–6·9)	0·014
Creatinine, μmol/L	44–97	60 (55–67)	68·5 (58–83)	0·030
Glucose, mmol/L	3.9–6.1	7·4 (5·7–10·7)	9·3 (8–9·6)	0·121
Prothrombin time	11.5–14.6	15·9 (15·3–16·8)	16·2 (15·7–16·8)	0·829
Fibrinogen (N=57), g/L	2.00–4.00	5·35 (4·65–6·35) (N=38)	6·05 (5·16–6·84) (N=19)	0·101
D-dimer (N=58), mg/L	0.00–0.50	0·68 (0·48–0·98) (N=36)	1·14 (0·68–1·47) (N=22)	0·009
Creatine kinase, U/L	0.00–4.87	69 (52–90)	147·5 (64–283)	0·014
B-type natriuretic peptide (N=62), pg/ml	0.00–125.0	18·5 (10–53·5) (N=40)	64·5 (19–152) (N=22)	0·002
C-reactive protein, mg/L	0.0–6.0	20·4 (14·9–47·6)	54 (24·1–90)	0·021
Procalcitonin, ng/mL	0-0.5	0·06 (0·04–0·08)	0·12 (0·06–0·20)	0·001
Interleukin-6 (N=46), pg/ml	<3	8·4 (4·0–32·2) (N=30)	76·1 (32·2–103·1) (N=16)	<0·001
Treatment*				
High flow nasal cannula, N (%)		0	18 (81·8)	<0·001
Mechanical ventilation, N (%)		0	16 (72·7)	<0·001
Non-invasive, N (%)		0	14 (63·6)	<0·001
invasive, N (%)		0	10 (45·5)	<0·001
Extracorporeal membrane oxygenation, N (%)		0	6 (27·3)	<0·001
Antibiotics, N (%)		31 (75·6)	21 (95·5)	0·079
Antiviral, N (%)				
Kaletra, N (%)		33 (80·5)	19 (86·4)	0·733
Arbidol, N (%)		39 (95·1)	21 (95·5)	1·000
methylprednisolone, N (%)		8 (19·5)	19 (86·4)	0·001
thymosin alpha 1, N (%)		10 (24·4)	16 (72·7)	<0·001
Intravenous immunoglobulin, N (%)		8 (19·5)	13 (59·1)	<0·001
blood plasma transfusion, N (%)		1 (2·4)	11 (50·0)	<0·001
Intravenous albumin, N (%)		22 (53·7)	21 (95·5)	0·001
Outcomes				
Hospitalization, N (%)		0 (0)	5 (22·7)	0·004
Discharge, N (%)		41 (100)	15 (68·2)	<0·001
Hospital days, N (%)		17·2 ± 6·7	24·1 ± 5·5	<0·001
Death, N (%)		0 (0)	1 (4.5)	0·118

**Patients may receive more than one treatment item.*

within 3–5 days, 7–9 days, and 11–14 days of hospitalization. There was a significant statistical difference recorded for albumin and B-type natriuretic peptide levels between both groups within 3–5 or 7–9 days of hospitalization, but not within 11–14 days of hospitalization. A significant statistical difference was also observed for the interleukin-6 levels between the two groups within 7–9 days and 11–14 days of hospitalization. Compared to patients not critically ill with pneumonia, critically ill patients had lower lymphocyte counts within 3–5 days, 7–9 days, and 11–14 days of hospitalization. And the lymphocyte numbers gradually increased with the duration of hospitalization in pneumonia patients irrespective of disease severity.

Treatment and Outcomes

A high flow nasal cannula was initially used in the treatment of 18 patients, of which 6 belonged to the critically ill group and strictly required a high flow nasal cannula, while the rest (12 patients) were switched to mechanical ventilation or extracorporeal membrane oxygenation (ECMO) (**Table 2** and **Figure 1**). Ultimately, mechanical ventilation was performed in 16 patients, 6 of whom received ECMO as rescue therapy (**Figure 1**).

A total of 52 (82·5%) patients received empirical antibiotic therapy (moxifloxacin, 33 [52·4%]; tazocin, 18 [18·6%]; ceftazidime, 6 [9·5%]; and Piperacillin, 6 [9·5%]) and antiviral therapy (Kaletra, 52 [82·5%] and Arbidol, 60 [95·2%]). Twenty-seven (42·9%) patients were given systematic methylprednisolone, while 26 (41·3%) patients received thymosin alpha 1. Additionally, intravenous immunoglobulin, blood plasma transfusion, and intravenous albumin were administered in 21 (33·3%), 12 (19·1%), and 43 (68·3%) patients, respectively. When compared to the non-critically ill group of patients, critically ill patients were given a higher dose of methylprednisolone, thymosin alpha 1, intravenous immunoglobulin, blood plasma transfusion, and intravenous albumin therapy (**Table 2**).

As of March 12, 2020, six critically ill patients were still hospitalized. Among these, four had been transferred to the

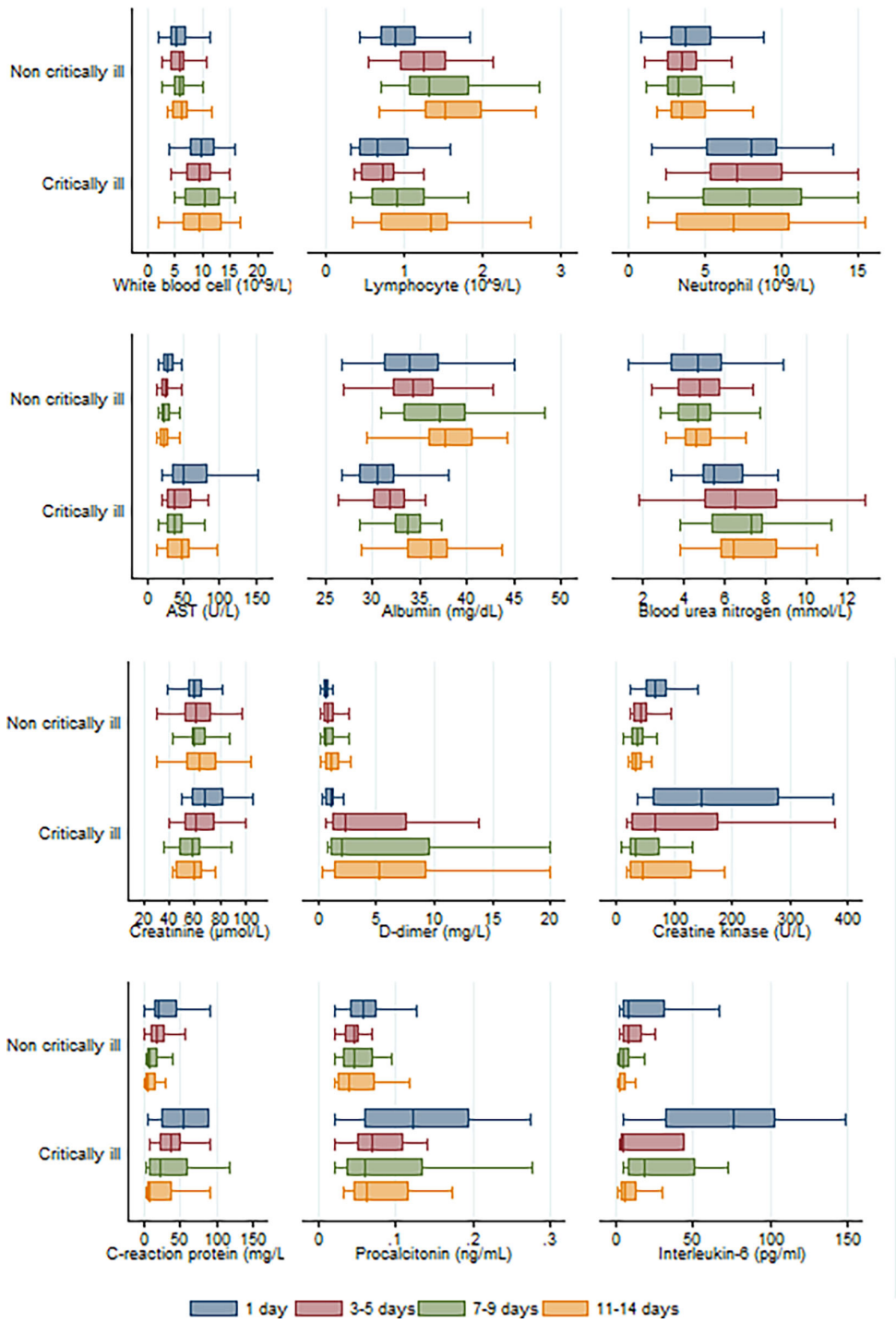

FIGURE 2 | Longitudinal analysis of twelve laboratory parameters altered during the hospitalization in critically and non-critically ill patients.

general wards because of improved condition, while the remaining two patients, who are 79 and 92 years old, respectively, and are both supported by ECMO, are still in the ICU. A total of 56 patients (88.9%) have been discharged, and one patient (1·6%) died. The patient who died was 79 years old and underwent ECMO therapy before death. When compared with the non-critically ill patient group, critically ill patients had a lower discharge rate (68·2% *vs.* 100%, $p<0·001$), longer hospital stays (24·1 *vs.* 17·2 days, $p<0·001$), and a trend towards higher mortality (4·5% *vs.* 0, $p=0·349$) (**Table 2**).

Interleukin-6 in Predicting the Severity of COVID-19 Pneumonia

Upon admission, the parameters that reached a statistically significant difference between patients with and without critical illness include age, presence of cough and dyspnoea, respiratory rate, white blood cell counts, neutrophil counts, and levels of aspartate transaminase, albumin, blood urea nitrogen, creatine, D-dimer, creatine kinase, B-type natriuretic peptide, C-reactive protein, procalcitonin, and interleukin-6. These were evaluated as potential predictors of severe COVID-19 pneumonia. Based on the ROC curve analysis, among single predictors, interleukin-6 has the highest AUC (0·85), indicative of excellent diagnostic accuracy (**Figure 3**). Further analysis revealed that there was a positive correlation between IL-6 levels and duration of hospital stay (R=0·58, p=0·0001) (**Figure 4**).

It was also inferred from the ROC curve analysis that the optimum cut-off value for interleukin-6 was 77·5 pg/mol. The sensitivity, specificity, positive likelihood ratio, negative likelihood ratio, positive predictive value, negative predictive value, and diagnostic accuracy were 50, 96·7, 15, 0·52, 88·9, 78·4, and 80·4%, respectively.

Using a cut-off value of 77·5 and the incidence of severe pneumonia (34·8% in this study) as the pre-test probability, the resulting Fagan plot (**Figure 5**) shows that interleukin-6 levels can be clinically informative, as it increases the probability of the patient being classified into the hypoxemia group by up to 89% when positive, and lowers the probability by up to 22% when negative.

DISCUSSION

The earliest patient was an imported case who had a history of travel from Wuhan City, and the latest patient had an occult epidemic history (**Supplementary Figure 1**). A total of 28 (38·1%) patients were imported cases that had immediate travel history from Wuhan city. Fourteen (22·2%) patients had a history of direct exposure to confirmed patients. The rapid human-to-human transmission among close contacts is an important feature of COVID-19 infection (Wang et al., 2020). Early restriction of patient contact may reduce transmission of

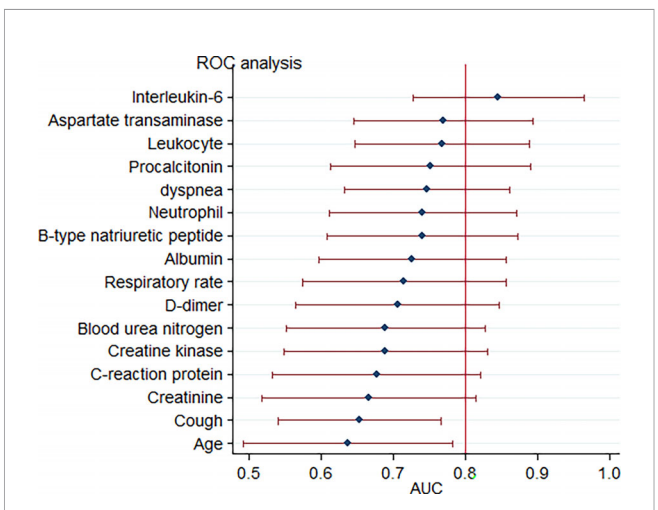

FIGURE 3 | Forest plot for accuracy of various markers in predicting critical COVID-19-related pneumonia. Each marker is plotted as an area under the curve of the receiver operating characteristic curve (AUC) with a 95% confidence interval.

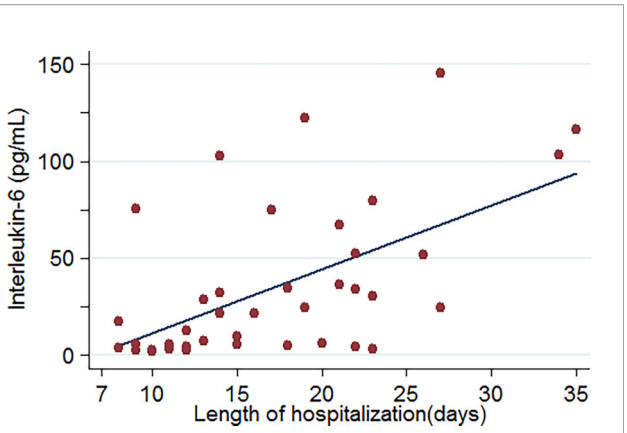

FIGURE 4 | Relationship between IL-6 levels and length of hospitalization of COVID-19-related pneumonia patients (data were available for 46 patients).

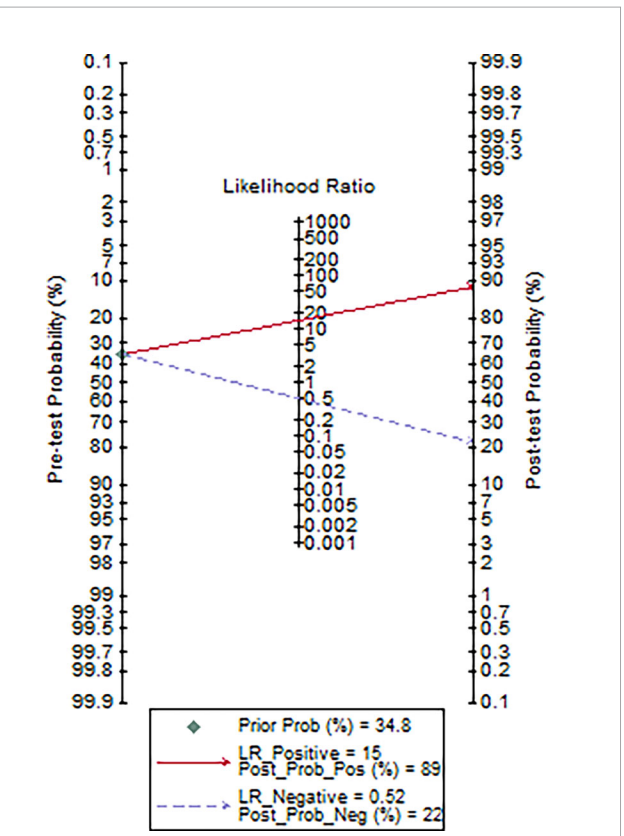

FIGURE 5 | Fagan plot of IL-6 for the hospital stay prediction of critically ill COVID-19 patients (data were available for 46 patients).

the disease. If individuals come into contact with confirmed patients with a history of travel to epidemic areas, such as Wuhan, they would be quarantined at designated hospitals for at least two weeks. Twenty-four (39·7%) patients had an occult epidemic history. This highlights the importance of not neglecting the possibility of transmission from asymptomatic patients, since most (80%) patients with COVID-19 infection would be mild and asymptomatic (Tian et al., 2020).

On average, critically ill patients were shown to be older than non-critically ill patients (61·5 *vs.* 52·9 years old, $p<0·034$) (**Table 1**). As of March 12, 2020, one patient died, while two were still alive but critically ill (**Figure 1**). The patient who died was 79 years old and underwent ECMO therapy before death. Zhou et al. (2020) showed that older age is one of the risk factors for mortality in adult inpatients with COVID-19 in Wuhan. In our cohort, fever was the most common symptom at the onset of the illness in patients with COVID-19 pneumonia (**Table 1**) occurring in 62 patients (98·4%), followed by cough (61·94%) and sputum (34·9%), which is in accordance with previous studies (Guan et al., 2020; Wang et al., 2020). The remaining patient was asymptomatic and had no fever from the moment of admission until the time he was discharged. He was hospitalized because he had a history of direct exposure to confirmed patients and evidence of pneumonia based on chest CT images. In addition, 16 (25·4%) patients had normal body temperatures at admission. These findings suggest that a normal fever scan cannot accurately diagnose COVID-19 pneumonia. An RT-PCR test and chest CT images may be useful in identifying COVID-19 pneumonia early and accurately in individuals that have an exposure history, although it is not known whether these measures are cost-effective or not. It also implies that measuring only the body temperature may not successfully identify all patients among individuals in public places such as airports, hospitals, and schools; 52·4% of patients had a temperature of <37·5°C, and only 1·6% of patients had a high temperature, i.e. >39·0°C (**Table 2**). These findings are consistent with a previous report by Guan et al. (2020). When compared with the non-critically ill patients, the patient group exhibiting critical illness had a higher incidence of cough and dyspnoea and a higher respiratory rate. These symptoms could be partly explained by histological findings from COVID-2019 pneumonia patients, in which lung tissue displayed pulmonary edema with hyaline membrane formation and desquamation of pneumocytes, indicating acute respiratory distress syndrome (Xu Z. et al., 2020).

Patients belonging to the critically ill group had higher white blood cell counts, neutrophil counts, and D-dimer levels than patients in the non-critically ill group. This indicated that severely ill patients get a stronger inflammatory response, as well as activation of coagulation induced by viral infection. Lymphocytopenia may be associated with cellular immune deficiency. Yang et al. suggested that the severity of lymphocytopenia may reflect the severity of COVID-2019 infection (Yang et al., 2020). Our study found that there was a trend for lower total lymphocytes in patients belonging to the critically ill group than those in the non-critically ill group, although it did not reach statistical significance upon admission ($p=0·059$). Longitudinal data indicated that when compared to non-critically ill COVID-19 pneumonia patients, critically ill patients had lower lymphocyte counts within 3–5 days, 7–9 days, and 11–14 days of hospitalization (**Figure 2**). Critically ill patients also had hypoalbuminemia and increased levels of aspartate transaminase, blood urea nitrogen, creatine, creatine kinase, B-type natriuretic peptide, which are more indicative of severe hepatic, kidney and myocardial injury (Wang et al., 2020). It has been shown that, similar to SARS-CoV, the COVID-19 virus exploits the angiotensin converting enzyme 2 (ACE2) receptor to gain entry into the cells (Baig et al., 2020). The most remarkable finding was the surface expression of ACE2 protein on lung alveolar epithelial cells and enterocytes of the small intestine (Hamming et al., 2004). Furthermore, ACE2 was present in arterial and venous endothelial cells and arterial smooth muscle cells in all tissues studied, including liver, kidney, cardiovascular, and brain tissues (Hamming et al., 2004).

Rapid production of IL-6 contributes to host defense during infection and tissue injury, but excessive IL-6 production causes severe inflammatory diseases (Kang et al., 2019). Our study showed that patients who were in critical condition had higher IL-6 levels than patients who were not (76·1 vs. 8·4 pg/mL, $p<0·001$) (**Table 2**). This means that severe COVID-19 infection could result in a cytokine storm. Our study showed that there was a good positive correlation between IL-6 levels and the length of hospitalization. Higher IL-6 levels were associated with longer hospital stays (R=0·58, $p=0·0001$) (**Figure 4**). Based on the ROC curve analysis, the AUC and the optimum cut-off value of interleukin-6 as a predictor of critical COVID-19 pneumonia cases were 0·85 and 77·5 pg/mol, respectively. The sensitivity, specificity, and diagnostic accuracy were 50, 96·7, and 80·4%, respectively. Using the incidence of critically ill COVID-19 pneumonia as the pre-test probability, the resulting Fagan plot shows that interleukin-6 can be clinically informative, as it increases the probability of a patient being classified into the hypoxemia group by up to 89% when positive and lowers the probability to 22% when negative (**Figure 5**). Longitudinal data also indicated a significant IL-6 decrease after hospitalization in both patients with and without critical stage COVID-19 pneumonia (**Figure 2**). There is evidence that IL-6 blockade strategy is beneficial for several inflammatory diseases, such as rheumatoid arthritis, juvenile idiopathic arthritis (Kang et al., 2019). It would be interesting to conduct a randomized clinical trial to test whether interfering with the IL-6 signalling axis in COVID-19 pneumonia patients is effective in treating the disease.

Yang et al. suggested that for non-critically ill patients, close follow-up is likely to be sufficient to manage the disease (Yang et al., 2020). Our study showed that patients with non-critical illness upon admission may advance to a critical stage later on. As shown in **Figure 1**, one COVID-19 pneumonia patient in the common type group and 13 patients in the severe type group advanced to the critically ill stage during hospitalization. This means that both critically ill and non-critically ill COVID-19 pneumonia patients need close monitoring and aggressive treatments.

Until now, no specific treatment has been recommended for coronavirus infections, except for meticulous supportive care (Wang et al., 2020). Mechanical ventilation is the main supportive treatment for critically ill patients (Yang et al., 2020). Three (50%) of the six patients who used ECMO recovered and got discharged. ECMO is an excellent candidate for refractory hypoxemia caused by severe acute respiratory distress syndrome, if it can be delivered in a medical center that has experience of using this form of therapy (Mi et al., 2018). Even without solid evidence for the effectiveness of antibacterial or antiviral therapies in the treatment of COVID-19 infections, 82·5% (52/63) of patients received antibacterial agents and nearly all the patients received antiviral therapy. For this study, we used Kaletra and Arbidol. A study on the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) showed that the timing of the start of treatment with antiviral agents is important in most viral infections, and starting antiviral treatment early might lead to better outcomes (Momattin et al., 2013).

When compared with the non-critically ill patients in our study, the critically ill ones received higher levels of methylprednisolone (86·4% *vs.* 19·5%, p=0·001). The dose of methylprednisolone varied depending on disease severity (Wang et al., 2020). Systemic corticosteroids were shown to delay viral clearance in critically ill patients with MERS-CoV (Memish et al., 2020). However, a study on severe acute respiratory syndrome (SARS) suggested that the use of interferon alfacon-1 plus corticosteroids was associated with reduced disease-associated impaired oxygen saturation and more rapid resolution of radiographic lung abnormalities (Loutfy et al., 2003). Wu et al. reported that treatment with methylprednisolone may be beneficial for patients with COVID-19 pneumonia who develop acute respiratory distress syndrome (Wu et al., 2020).

When compared with the non-critically ill patients, critically ill ones had a lower discharge rate (68·2% *vs.* 100%, p<0·001) and longer hospital days (24·1 *vs.* 17·2 days, p<0·001), as well as a trend towards higher mortality (4·5% *vs.* 0, p=0·349). The mortality of critically ill patients in our study was significantly lower than that reported by Yang et al. in Wuhan (4·5% *vs.* 61·5%) (Yang et al., 2020). Early isolation, early diagnosis, and early management might have collectively contributed to the reduction in mortality (Guan et al., 2020). In addition, thymosin alpha 1, intravenous administration of immunoglobulin, blood plasma transfusion and intravenous albumin therapy may also improve the clinical outcome.

The limitation of this study is that it is a retrospective study from a single center, and the sample size was small. Patients had a variety of different treatments (for example: antivirals, antibiotics, intravenous immunoglobulin, blood plasma transfusion, and intravenous albumin). This made it very difficult to interpret any other outcome data objectively. Therefore, it would be interesting to conduct a randomized clinical trial to assess the effect of different therapeutic strategies for patients with or without COVID-19 pneumonia in future studies. Additionally, only patients with pneumonia were enrolled, so our results may be not applicable to patients without pneumonia.

CONCLUSIONS

In conclusion, both critically ill and non-critically ill COVID-19 pneumonia patients need close monitoring and aggressive treatments. There was a statistically significant difference between critically ill and non-critically ill COVID-19 pneumonia patients in terms of age, some symptoms, laboratory findings, treatments, and outcomes. Interleukin-6 levels upon admission is a good predictor of the disease, and it is associated with the length of hospitalization.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. The committee decided to waive the need for written informed consent from the participants studied in this analysis as the data were analyzed retrospectively and anonymously.

AUTHOR CONTRIBUTIONS

WH conceived the study and carried out the majority of the work. WH, QC, SQ, YW, and JP participated in data collection. WH conducted data analysis and drafted the manuscript. ZB, VZ, MZ, and JP helped to finalize 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/fcimb.2021.550456/full#supplementary-material

Supplementary Figure 1 | Bar graph depicting travel history of patients with COVID-19-related pneumonia.

Supplementary Figure 2 | Representative computed tomography images from a patient with COVID-19-related pneumonia. **(A)** High-density local patches and masses are seen, and the edges are blurred and thickened in the left lung; **(B)** Ground glass opacities in two lungs.

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METHODS
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Planning as Inference in Epidemiological Dynamics Models

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In this work we demonstrate how to automate parts of the infectious disease-control policy-making process via performing inference in existing epidemiological models. The kind of inference tasks undertaken include computing the posterior distribution over controllable, via direct policy-making choices, simulation model parameters that give rise to acceptable disease progression outcomes. Among other things, we illustrate the use of a probabilistic programming language that automates inference in existing simulators. Neither the full capabilities of this tool for automating inference nor its utility for planning is widely disseminated at the current time. Timely gains in understanding about how such simulation-based models and inference automation tools applied in support of policy-making could lead to less economically damaging policy prescriptions, particularly during the current COVID-19 pandemic.

Keywords: public health preparedness, epidemiological dynamics, Bayesian inference, probabilistic programming, COVID-19

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1 INTRODUCTION

Our goal in this paper is to demonstrate how the *"planning as inference"* methodology at the intersection of Bayesian statistics and optimal control can directly aid policy-makers in assessing policy options and achieving policy goals, when implemented using epidemiological simulators and suitable automated software tools for probabilistic inference. Such software tools can be used to quickly identify the range of values towards which controllable variables should be driven by means of policy interventions, social pressure, or public messaging, so as to limit the spread and impact of an infectious disease such as COVID-19.

In this work, we introduce and apply a simple form of planning as inference in epidemiological models to automatically identify policy decisions that achieve a desired, high-level outcome. As but one example, if our policy aim is to contain infectious population totals below some threshold at all times in the foreseeable future, we can condition on this putative future holding and examine the allowable values of controllable behavioural variables at the agent or population level, which in the framework of planning as inference is formalized in terms of a *posterior distribution*. As we already know, to control the spread of COVID-19 and its impact on society, policies must be enacted that reduce disease transmission probability or lower the frequency and size of social interactions. This is because we might like to, for instance, not have the number of infected persons requiring hospitalization exceed the number of available hospital beds.

Throughout this work, we take a Bayesian approach, or at the very least, a *probabilistic* consideration of the task. Especially in the early stages of a new outbreak, the infectious

dynamics are not known precisely. Furthermore, the spread of the disease cannot be treated deterministically, as a result of either fundamental variability in the social dynamics that drive infections, or of uncertainty over the current infection levels, or of uncertainty over appropriate models for analyzing the dynamics. Therefore, developing methods capable of handling such uncertainty correctly will allow for courses of action to be evaluated and compared more effectively, and could lead to "better" policies: a policy that surpasses the desired objective with 55% probability, but fails with 45% probability, may be considered "worse" than a policy that simply meets the objective with 90% probability. Bayesian analysis also offers a form of probabilistic inference in which the contribution of individual variables to the overall uncertainty can be identified and quantified. Beyond simply obtaining "the most effective policy measure," this may be of interest to analysts trying to further understand *why* certain measures are more effective than others.

We first show how the problem of policy planning can be formulated as a Bayesian inference task in epidemiological models. This framing is general and extensible. We then demonstrate how particular existing software tools can be employed to perform this inference task in preexisting stochastic epidemiological models, without modifying the model itself or placing restrictions on the models that can be analyzed. This approach is particularly appealing, as it decouples the specification of epidemiological models by domain experts from the computational task of performing inference. This shift allows for more expressive and interpretable models to be expediently analyzed, and the sophistication of inference algorithms to be adjusted flexibly.

As a result, the techniques and tools we review in this paper are applicable to simulators ranging from simple compartmental models to highly expressive agent-based population dynamics models. In the former, the controls available to policy-makers are blunt–e.g., "reduce social interactions by some fractional amount"–but how best to achieve this is left as an exercise for policy-makers. In the latter, variables like "probability of individuals adhering to self-isolation" and "how long should schools be closed if at all" can be considered and evaluated in combination and comparison to others as potential fine-grained controls that could achieve the same policy objective more efficiently.

When governments impose any such controls, both citizens and financial markets want to know how draconian these measures must be and for how long they have to be in effect. Policy analysis based on models that reflect variability in resources such as healthcare facilities in different jurisdictions could hopefully make the answers to these questions more precise and the controls imposed more optimal in the utility maximizing sense. The same holds for the difference between models that can or cannot reflect variations in population mixing between rural and urban geographic areas. A person living in a farming county in central Illinois might reasonably wonder if it is as necessary to shelter in place there as it is in Chicago.

Current approaches to model-based policy-making are likely to be blunt. Simple models, e.g., compartmental models with few compartments, are rapid to fit to new diseases and easy to compute, but are incapable of evaluating policy options that are more fine-grained than the binning used, such as regionalized measures. The net effect of being able to only consider blunt controls arguably has contributed to a collective dragging of feet, even in the face of the current COVID-19 pandemic. This delayed reaction, combined with brute application of control, has led to devastating socioeconomic impact, with many sectors such as education, investment markets and small-medium enterprises being directly impacted.

We can and should be able to do better. We believe, and hope to demonstrate, that models and software automation focused on planning as inference for policy analysis and recommendation is one path forward that can help us better react to this and future pandemics, and improve our public health preparedness.

We upfront note that the specific models that we use in this paper are far from perfect. First, the pre-existing models we use to demonstrate our points in this paper are only crudely calibrated to present-day population dynamics and specific COVID-19 characteristics. We have made some efforts on the latter point, in particular sourcing a COVID-19 adapted compartmental model (Ogilvy Kermack and McKendrick, 1927; Blackwood and Childs, 2018b; Hill et al., 2020) and parameters from (Bhalchandra and SnehalShekatkar, 2020; Ferguson et al., 2020; Magdon-Ismail, 2020; Massonnaud et al., 2020; Peng et al., 2020; Riou and Althaus, 2020; Rovetta et al., 2020; Russo et al., 2020; Traini et al., 2020; Wen et al., 2020), but we stress this limitation. In addition, in simple cases, the type of problems for which we discuss solutions in this paper may be solved with more straightforward implementations involving parameter sweeps and "manual" checking for desired policy effects, albeit at potentially higher human cost. In this sense, our goal is not to claim fundamental novelty, uniqueness or superiority of any particular inference technique, but rather to raise awareness for the practical feasibility of the Bayesian formulation of planning as inference, which offers a higher level of flexibility and automation than appears to be understood widely in the policy-making arena.

Note also that current automated inference techniques for stochastic simulation-based models (Toni et al., 2009; Kypraios et al., 2017; McKinley et al., 2018; Chatzilena et al., 2019; Minter and Retkute, 2019) are computationally demanding and are by their very nature approximate. The academic topic at the core of this paper and the subject of a significant fraction of our academic work (Paige et al., 2014; Wood et al., 2014; Tolpin et al., 2015; van de Meent et al., 2015; Paige and Wood, 2016; Rainforth et al., 2016; Rainforth et al., 2018; Naderiparizi et al., 2019; Zhou et al., 2019; Warrington et al., 2020) deals with this challenge. Furthermore, the basic structure of simulators currently available may lack important policy-influenceable interaction parameters that one might like to examine. If viewed solely in light of the provided examples, our contribution could reasonably be seen both as highlighting the utility of inference for planning in this application setting, and as automating the manual selection of promising policy parameters. The tools we showcase are capable of significantly more; however, for expediency and clarity, we have focused on control as inference, an application that has seen relatively little specific coverage in the literature, and

the simplest possible inference methods which do not require familiarity with the technical literature on approximate Bayesian inference. We leave other straightforward applications of automated inference tools in this application area, like parameter inference from observed outbreak data (Toni et al., 2009; Kypraios et al., 2017; McKinley et al., 2018; Chatzilena et al., 2019; Minter and Retkute, 2019), to others.

That being said, our hope is to inform field epidemiologists and policy-makers about an existing technology that could, right now, be used to support public policy planning towards more precise, potentially tailored interventions that ensure safety while also potentially leading to fewer economic ramifications. Fully probabilistic methods are apparently only relatively recently being embraced by the epidemiology community (Lessler et al., 2016; Funk and King, 2020), while the communities for approximate Bayesian inference and simulation-based inference have remained mostly focused on the tasks of parameter estimation and forecasting (Toni et al., 2009; Kypraios et al., 2017; McKinley et al., 2018), rather than control as inference. Beyond this demonstration, we hope to encourage timely and significant developments on the modeling side, and, if requested, to actually aid in the fight against COVID-19 by helping arm policy-makers with a new kind of tool and training them how to use it rapidly. Finally, we hope to engage the machine learning community in the fight against COVID-19 by helping translate between the specific epidemiological control problem and the more general control problem formulations on which we work regularly.

2 ASSUMPTIONS AND FINDINGS

We start with the assumption that the effectiveness of policy-making can be significantly improved by consulting the outputs of model-based tools which provide quantitative metrics for the ability of particular policy actions to achieve specific formalized goals. In particular, we imagine the following scenario. There exists some current population, and the health status of its constituents is only partially known. There exists a disease whose transmission properties may be only partially known, but whose properties cannot themselves be readily controlled. There exists a population dynamic that can be controlled in some limited ways at the aggregate level. There exists a "policy goal" or target which we will refer to as the allowable, allowed, or goal set of system trajectories. An example of this could be "the total number of infectious persons should not ever exceed some percentage of the population" or "the first date at which the total number of infectious persons exceeds some threshold is at least some number of days away." We finally assume an implied set of allowable policy prescriptions, defined in the sense that population dynamics behaving according to such policies will be exactly the ones to attain the goal with high probability. In general, this set of allowable policies is intractable to compute exactly, motivating the use of automated tools implementing well established *approximate Bayesian inference* methods. We explicitly do not claim "completeness" of the stochastic dynamic models in any realistic sense, disregarding complications such as potential agent behaviour in strategic response to regionalized policies, and do not attempt to quantify all costs and benefits of the considered policies, for example economic or cultural impacts. Rather, formulating the problem described above in terms of Bayesian inference results in a *posterior distribution* over policies which have been conditioned on satisfying the formalized policy desiderata within the formalized dynamical model. Effectively, this is to be understood as "scrutinizing," "weighting," "prioritizing," or "focusing" potential policy actions for further consideration, rather than as an "optimal" prescription. When selecting a policy based on the posterior distribution, policy-makers are expected to account for additional, more complex socio-economic phenomena, costs and benefits using their own judgment.

The *only* things that may safely be taken away from this paper are the following:

- Existing compartmental models and agent-based simulators can be used as an aid for policy assessment via a Bayesian planning as inference formulation.
- Existing automated inference tools can be used to perform the required inferential computation.
- Opportunities exist for various fields to come together to improve both understanding of and availability of these techniques and tools.
- Further research and development into modeling and inference is recommended to be immediately undertaken to explore the possibility of more efficient, less economically devastating control of the COVID-19 pandemic.

What should *not* be taken away from this paper are any other conclusions, including in particular the following:

- Any conclusion or statements that there might exist less aggressive measures that could still be effective in controlling COVID-19.
- Any substantial novelty, uniqueness or performance claims about the particular numerical methods and software implementations for Bayesian inference which were used for the purpose of demonstrating the findings above. In particular, on the one hand, similar results could have been obtained using other software implementation strategies in principle, and on the other hand, more advanced inference methods could have been applied using the same software tools at the expense of rendering the conceptual exposition less accessible to audiences outside of the Bayesian inference community.

As scientists attempting to contribute "across the aisle," we use more qualifying statements than usual throughout this work in an attempt to reduce the risk of misunderstandings and sensationalism.

3 APPROACH

In this section we formalize the policy-making task in terms of computing conditional probabilities in a probabilistic model of

the disease spread. While the technical description can get involved at times, we emphasize that in practice the probabilistic model is already defined by an existing epidemiological simulator and the probabilistic programming tools we describe in this paper provide the ability to compute the required conditional probabilities automatically, including automatically introducing required approximations, so the users only need to focus on identifying which variables to condition on, and feeding real-world data to the system. Readers familiar with framing planning as inference may wish to skip directly to **Section 4**.

Being able to perform probabilistic inference is crucial for taking full advantage of available simulators in order to design good policies. This is because in the real world, many of the variables crucially impacting the dynamics of simulations are not directly observable, such as the number of infectious but asymptomatic carriers or the actual number of contacts people make in different regions each day. These variables are called *latent*, as opposed to *observable* variables such as the number of deaths or the number of passengers boarding a plane each day, which can often be directly measured with high accuracy. It is often absolutely crucial to perform inference over some latent variables to achieve reliable forecasts. For example, the future course of an epidemic like COVID-19 is driven by the number of people currently infected, rather than the number of people currently hospitalized, while in many countries in the world currently only the latter is known.

While performing inference over latent variables is very broadly applicable, the scenario described above being but one example, in this paper we primarily address the problem of choosing good policies to reduce the impact of an epidemic which can also be formulated as an inference problem. This choice of problem was driven by the hypothesis that the search for effective controls may not in fact be particularly well-served by automation at the current time. In the epidemiological context, the questions we are trying to answer are ones like "when and for how long do we need to close schools to ensure that we have enough ventilators for everyone who needs them?" While obviously this is overly simplistic and many different policy decisions need to be enacted in tandem to achieve good outcomes, we use this example to illustrate tools and techniques that can be applied to problems of realistic complexity.

Our approach is not novel, it has been studied extensively under the name "control via planning as inference" and is now well understood (Todorov, 2008; Toussaint, 2009; Kappen et al., 2012; Levine, 2018). What is more, the actual computations that result from following the recipes for planning as inference can be, in some cases readily, manually replicated. Again, our aim here is to inform or remind a critically important set of policy-makers and modellers that these methodologies are extremely relevant to the current crisis. Moreover, at least partial automation of model-informed policy-guidance is achievable using existing tools, and, may even lead to sufficient computational savings to make their use in current policy-making practical. Again, our broader hope here is to encourage rapid collaborations leading to more targeted and less-economically-devastating policy recommendations.

3.1 An Abstract Epidemiological Dynamics Model

In this work we will look at both compartmental and agent-based models. An overview of these specific types of models appears later. For the purposes of understanding our approach to planning as inference, it is helpful to describe the planning as inference problem in a formalism that can express both types of models. The approach of conducting control via planning as inference follows a general recipe:

1) Define the latent and control parameters of the model and place a prior distribution over them.
2) Either or both define a likelihood for the observed disease dynamics data and design constraints that define acceptable disease progression outcomes.
3) Do inference to generate a posterior distribution on control values that conditions both on the observed data and the defined constraints.
4) Make a policy recommendation by picking from the posterior distribution consisting of effective control values according to some utility maximizing objective.

We focus on steps 1–3 of this recipe, and in particular do not explore simultaneous conditioning. We ignore the observed disease dynamics data and focus entirely on inference with future constraints. We explain the rationale behind these choices near the end of the paper.

Very generally, an epidemiological model consists of a set of global parameters and time dependent variables. Global parameters are (θ, η), where θ denotes parameters that can be controlled by policy directives (e.g. close schools for some period of time or decrease the general level of social interactions by some amount), and η denotes parameters which cannot be affected by such measures (e.g. the incubation period or fatality rate of the disease).

The time dependent variables are (X_t, Y_t, Z_t) and jointly they constitute the full state of the simulator. X_t are the latent variables we are doing inference over (e.g. the total number of infected people or the spatio-temporal locations of outbreaks), Y_t are the observed variables whose values we obtain by measurements in the real world (e.g. the total number of deaths or diagnosed cases), and Z_t are all the other latent variables whose values we are not interested in knowing (e.g. the number of contacts between people or hygiene actions of individuals). For simplicity, we assume that all variables are either observed at all times or never, but this can be relaxed.

The time t can be either discrete or continuous. In the discrete case, we assume the following factorization

$$p(\theta, \eta, X_{0:T}, Y_{0:T}, Z_{0:T}) = p(\theta)p(\eta)\, p(X_0, Y_0, Z_0 \mid \theta, \eta) \cdot \prod_{t=1}^{T} p(X_t, Y_t, Z_t \mid X_{0:t-1}, Y_{0:t-1}, Z_{0:t-1}, \theta, \eta). \quad (1)$$

Note that we do not assume access to any particular factorization between observed and latent variables. We assume that a priori the controllable parameters θ are independent of non-controllable parameters η to avoid

situations where milder control measures are associated with better outcomes because they tend to be deployed when the circumstances are less severe, which would lead to erroneous conclusions when conditioning on good outcomes in **Section 3.3**.

3.2 Inference

The classical inference task (Toni et al., 2009; Kypraios et al., 2017; McKinley et al., 2018; Chatzilena et al., 2019; Minter and Retkute, 2019) is to compute the following conditional probability

$$p(\eta, X_{0:T} \mid Y_{0:T}, \theta) = \int p(\eta, X_{0:T}, Z_{0:T} \mid Y_{0:T}, \theta)\, dZ_{0:T}. \quad (2)$$

In the example given earlier X_t would be the number of infected people at time t and Y_t would be the number of hospitalized people at time t. If the non-controllable parameters η are known they can be plugged into the simulator, otherwise we can also perform inference over them, like in the equation above. This procedure automatically takes into account prior information, in the form of a model, and available data, in the form of observations. It produces estimates with appropriate amount of uncertainty depending on how much confidence can be obtained from the information available.

The difficulty lies in computing this conditional probability, since the simulator does not provide a mechanism to sample from it directly and for all but the simplest models the integral cannot be computed analytically. The main purpose of probabilistic programming tools is to provide a mechanism to perform the necessary computation automatically, freeing the user from having to come up with and implement a suitable algorithm. In this case, approximate Bayesian computation (ABC) would be a suitable tool. We describe it below, emphasizing again that its implementations are already provided by existing tools (Toni et al., 2009; Kypraios et al., 2017; McKinley et al., 2018; Chatzilena et al., 2019; Minter and Retkute, 2019).

The main problem in this model is that we do not have access to the likelihood $p(Y_t \mid X_t, \theta, \eta)$ so we cannot apply the standard importance sampling methods. To use ABC, we extend the model with auxiliary variables $Y^{obs}_{0:T}$, which represent the actual observations recorded, and use a suitably chosen synthetic likelihood $p(Y^{obs}_t \mid Y_t)$, often Gaussian. Effectively, that means we're solving the following inference problem,

$$p\left(X_{0:T} \mid Y^{obs}_{0:T}, \theta\right) = \iiint p\left(\eta, X_{0:T}, Y_{0:T}, Z_{0:T} \mid Y^{obs}_{0:T}, \theta\right) dY_{0:T}\, dZ_{0:T}\, d\eta, \quad (3)$$

which we can solve by importance sampling from the prior. Algorithmically, this means independently sampling a large number N of trajectories from the simulator

$$\left(\eta^{(i)}, X^{(i)}_{0:T}, Y^{(i)}_{0:T}, Z^{(i)}_{0:T}\right) \overset{\text{i.i.d.}}{\sim} p(\eta, X_{0:T}, Y_{0:T}, Z_{0:T} \mid \theta) \quad \text{for } i \in \{1, \ldots, N\}, \quad (4)$$

computing their importance weights

$$w_i = \frac{p\left(Y^{obs}_{0:T} \mid Y^{(i)}_{0:T}\right)}{\sum_{j=1}^{N} p\left(Y^{obs}_{0:T} \mid Y^{(j)}_{0:T}\right)}, \quad (5)$$

and approximating the posterior distribution

$$p\left(X_{0:T} \mid Y^{obs}_{0:T}, \theta\right) \approx \hat{p}\left(X_{0:T} \mid Y^{obs}_{0:T}, \theta\right) = \sum_{i=1}^{N} w_i \delta_{X^{(i)}_{0:T}}(X_{0:T}), \quad (6)$$

where δ is the Dirac delta putting all the probability mass on the point in its subscript. In more intuitive terms, we are approximating the posterior distribution with a collection of weighted samples where weights indicate their relative probabilities.

3.3 Control as Inference: Finding Actions That Achieve Desired Outcomes

In traditional inference tasks we condition on data observed in the real world. In order to do control as inference, we instead condition on what we *want* to observe in the real world, which tells us which actions are likely to lead to such observations. This is accomplished by introducing auxiliary variables that indicate how desirable a future state is or is not. In order to keep things simple, here we restrict ourselves to the binary case where $Y_t \in \{0, 1\}$, where 1 means that the situation at time t is acceptable and 0 means it is not. This indicates which outcomes are acceptable, allowing us to compute a distribution over those policies, while leaving the choice of which specific policy is likely to produce an acceptable outcome to policy-maker(s). For example, Y_t can be 1 when the number of patients needing hospitalization at a given time t is smaller than the number of hospital beds available and 0 otherwise.

To find a policy θ that is likely to lead to acceptable outcomes, we need to compute the posterior distribution

$$p(\theta \mid \forall_{t>0} : Y_t = 1). \quad (7)$$

Once again, probabilistic programming tools provide the functionality to compute this posterior automatically. In this case, rejection sampling would be a simple and appropriate inference algorithm. The rejection sampling algorithm repeatedly samples values of θ from the prior $p(\theta)$, runs the full simulator using θ, and keeps the sampled θ only if all Y_t are 1. The collection of accepted samples approximates the desired posterior. We use rejection sampling in our agent-based modeling experiments, but emphasize that other, more complex and potentially more computationally efficient, approaches to computing this posterior exist.

This tells us which policies are most likely to lead to a desired outcome but not how likely a given policy is to lead to that outcome. To do that, we can evaluate the conditional probability $p(\forall_{t>0} : Y_t = 1 \mid \theta)$, which is known as the model evidence, for a particular θ. A more sophisticated approach would be to condition on the policy leading to a desired outcome with a given probability p_0, that is

$$p\left(\theta \mid p(\forall_{t>0} : Y_t = 1 \mid \theta) > p_0\right). \quad (8)$$

For example, we could set $p_0 = 0.95$ to find a policy that ensures availability of hospital beds for everyone who needs one with at least 95% probability. The conditional probability in **Eq. 8** is more difficult to compute than the one in **Eq. 7**. It can be approximated by methods such as nested Monte Carlo (NMC) (Rainforth et al., 2018), which are natively available in advanced probabilistic programming systems such as Anglican (Tolpin et al., 2016) and WebPPL (Goodman and Stuhlmüller, 2014a) but in specific

cases can also be implemented on top of other systems, such as PyProb (Le et al., 2017), with relatively little effort, although using NMC usually has enormous computational cost.

To perform rejection sampling with nested Monte Carlo, we first draw a sample $\theta_i \sim p(\theta)$, then draw N samples of $Y_{0:T}^{(j)} \overset{\text{i.i.d.}}{\sim} p(Y_{0:T} \mid \theta_i)$ and reject θ_i if fewer than $p_0 N$ of sampled sequences of Ys are all 1s, otherwise we accept it. This procedure is continued until we have a required number K of accepted θs. For sufficiently high values of N and K, this algorithm approximates the posterior distribution **Eq. 8** arbitrarily well.

However we compute the posterior distribution, it contains multiple values of θ that represent different policies that, if implemented, can achieve the desired result. In this setup it is up to the policy-maker(s) to choose a policy $\theta^\star$ that has support under the posterior, i.e., yields the desired outcomes, taking into account some notion of utility.

Crucially, despite their relative simplicity, the rejection sampling algorithms we have discussed evaluate randomly sampled values of θ. This is a fundamental difference from the commonly used grid search over a deterministic array of parameter values. In practical terms, this is important because "well distributed" random samples are sufficient for experts to gauge the quantities of interest, and avoid grid searches that would be prohibitively expensive for θ with more than a few dimensions.

3.4 Stochastic Model Predictive Control: Reacting to What's Happened

During an outbreak governments continuously monitor and assess the situation, adjusting their policies based on newly available data. A convenient theoretical and general framework to formalize this is that of model predictive control (Camacho and Alba, 2013). In this case, Y_t consists of variables Y_t^{data} that can be measured as the epidemic unfolds (such as the number of deaths) and the auxiliary variables Y_t^{aux} that indicate whether desired goals were achieved, just like in **Section 3.3**. Say that at time $t = 0$ the policy-maker(s) choose a policy to enact θ_0^* based on the posterior distribution

$$p\left(\theta \mid Y_0^{data}, \forall_{t>0}\colon Y_t^{aux} = 1, \eta\right) = \iint p\left(\theta \mid X_0, Z_0, Y_0^{data}, \forall_{t>0}\colon Y_t^{aux} = 1, \eta\right) \hat{p}\left(X_0, Z_0 \mid Y_0^{data}, \eta\right) dX_0\, dZ_0. \tag{9}$$

Then at time $t = 1$ they will have gained additional information Y_1^{data}, leading to a new belief over the past and current states that we denote as $\hat{p}(X_{0:1}, Z_{0:1} \mid Y_{0:1}^{data}, \theta_0^*, \eta)$, for which we give a formula in the general case in **Eq. 11**. The policy-maker(s) then choose the policy θ_1^* from the posterior distribution $p(\theta \mid Y_{0:1}^{data}, \forall_{t>1}\colon Y_t^{aux} = 1, \theta_0^*, \eta)$.

Generally, at time t we compute the posterior distribution conditioned on the current state and on achieving desirable outcomes in the future

$$p\left(\theta \mid Y_{0:t}^{data}, \forall_{t'>t}\colon Y_{t'}^{aux} = 1, \theta_{0:t-1}^*, \eta\right) = \iint p\left(\theta \mid X_{0:t}, Z_{0:t}, Y_{0:t}^{data}, \forall_{t'>t}\colon Y_{t'}^{aux} = 1, \theta_{0:t-1}^*, \eta\right) \hat{p}\left(X_{0:t}, Z_{0:t} \mid Y_{0:t}^{data}, \theta_{0:t-1}^*, \eta\right) dX_{0:t}\, dZ_{0:t}. \tag{10}$$

Policy-maker(s) then can use this distribution to choose the policy θ_t^* that is actually enacted. The current belief state is computed by inference

$$\hat{p}\left(X_{0:t}, Z_{0:t} \mid Y_{0:t}^{data}, \theta_{0:t-1}^*, \eta\right) \propto p\left(X_t, Z_t, Y_t^{data} \mid X_{0:t-1}, Z_{0:t-1}, Y_{0:t-1}^{data}, \theta_{0:t-1}^*, \eta\right) \hat{p}\left(X_{0:t-1}, Z_{0:t-1} \mid Y_{0:t-1}^{data}, \theta_{0:t-2}^*, \eta\right). \tag{11}$$

Eq. 11 can be computed using methods described in **Section 3.2**, while **Eq. 10** can be computed using methods described in **Section 3.3**. As such, the policy enacted evolves over time, as a result of re-solving for the optimal control based on new information.

We note that what we introduce here as MPC may appear to be slightly different from what is commonly referred to as *model predictive control* (García et al., 1989). Firstly, instead of solving a finite-dimensional optimization problem over controls at each step, we perform a Bayesian update step and sample from the resulting posterior distribution over controls. Secondly, in more traditional applications of MPC a *receding horizon* (Jacob et al., 2011) is considered, where a finite and fixed-length window is considered. The controls required to satisfy the constraint over that horizon are then solved for and applied–without consideration of timesteps beyond this fixed window. At the next time step, the controls are then re-solved for. In this work, we rather consider a constant policy for the remaining $T - t$ time steps, as opposed to allowing a variable policy (discussed below) over a fixed window. We note that time-varying controls are fully permissible under the framework we present, as we demonstrate in **Section 3.5**. Furthermore, we could easily consider a fixed horizon, just by changing the definition of $Y_{t'}^{aux}$ to be equal to 1 for $t' > t + p$, where p is the length of the window being considered. Both of these extensions are provisioned for under the framework we provide, and could be used to implement what might be considered as more conventional stochastic MPC, but with all the auxiliary benefits of Bayesian inference.

Before we proceed, it is critical to note that the posterior $p(\theta \mid \forall_{t>0}\colon Y_t^{aux} = 1)$ describes the probability, given the dynamic model and the currently available information, for the parameters θ to achieve the policy goals. As such, our approach provides a method for "screening" policies such that candidate policies that achieve the required outcomes are found. What it does not take into account is the relative "cost" of each policy, or, how best to achieve the required parameter values. For instance, in the SEI^{3}R examples we present later, we learn the reduction in transmission required to avoid exceeding an infection threshold. The reduction in transmission can be achieved (for instance) by encouraging hand-washing and social distancing. While the method will identify whether or not a particular level of transmission will achieve the desired outcome, it does not indicate the optimal trade-off between the cost of a particular policy, the amount that the policy exceeds the required outcome, and the probability of the outcome. A human must therefore select, from the set of compliant policies identified, the policy that maximizes utility while minimizing cost. Therefore, this method is an important component of a wider policy making toolkit, as opposed to an oracle that can dictate the optimal policy decisions.

3.5 Time-Varying Control: Long Term Planning

It is also possible to explicitly model changing policy decisions over time, which enables more detailed planning, such as when to enact certain preventive measures such as closing schools. Notationally, this means instead of a single θ there is a separate θ_t for each time t. We can then find a good sequence of policy decisions by performing inference just like in **Section 3.3** by conditioning on achieving the desired outcome

$$p(\theta_{0:T} \mid \forall_{t>0} : Y_t = 1). \tag{12}$$

The inference problem is now more challenging, since the number of possible control sequences grows exponentially with the time horizon. Still, the posterior can be efficiently approximated with methods such as Sequential Monte Carlo.

It is straightforward to combine this extension with model predictive control from **Section 3.4**. The only required modification is that in **Eq. 10** we need to compute the posterior over all future policies,

$$\begin{aligned} &p\Big(\theta_{t:T} \mid Y_{0:t}^{data}, \forall_{t'>t} : Y_{t'}^{aux} = 1, \theta_{0:t-1}^{*}, \eta\Big) \\ &= \iint p\Big(\theta_{t:T} \mid X_{0:t}, Z_{0:t}, Y_{0:t}^{data}, \forall_{t'>t} : Y_{t'}^{aux} = 1, \theta_{0:t-1}^{*}, \eta\Big) \\ &\quad \hat{p}\Big(X_{0:t}, Z_{0:t} \mid Y_{0:t}^{data}, \theta_{0:t-1}^{*}, \eta\Big)\, dX_{0:t}\, dZ_{0:t}. \end{aligned} \tag{13}$$

At each time t the policy-maker(s) only choose the current policy θ_t^*, without committing to any future choices. This combination allows for continuously re-evaluating the situation based on available data, while explicitly planning for enacting certain policies in the future. While here we explicitly consider only open-loop control policies, this re-planning allows new information to be taken into account, and facilitates reactionary policy decisions to evolving situations.

In models with per-timestep control variables θ_t, it is very important that in the model (but not in the real world) the enacted policies must not depend on anything else. If the model includes feedback loops for changing policies based on the evolution of the outbreak, it introduces positive correlations between lax policies and low infection rates (or other measures of severity of the epidemic), which in turn means that conditioning on low infection rates is more likely to produce lax policies. This is a known phenomenon of reversing causality when correlated observational data is used to learn how to perform interventions (Pearl, 2000).

We note another potential pitfall that may be exacerbated by time-varying control: if the model used does not accurately reflect reality, any form of model-based control is likely to lead to poor results. To some extent, this can be accounted for with appropriate distributions reflecting uncertainty in parameter values. However, given the difficulty of modeling human behavior, and especially of modeling people's reactions to novel policies, there is likely to be some mismatch between modeled and real behavior. To give an example of a possible flaw in an agent-based model: if a proposed fine-grained policy closed one park while keeping open a second, nearby, park, the model may not account for the likely increase in visitors to the second park. The larger and more fine-grained (in terms of time or location) the space of considered policies is, the more such deficiencies may exist. We therefore recommend that practitioners restrict the space of considered policies to those which are likely to be reasonably well modeled.

3.6 Automation

We have intentionally not really explained how one might actually computationally characterize any of the conditional distributions defined in the preceding section. For the compartmental models that follow, we provide code that directly implements the necessary computations. Alternatively we could have used the automated inference facilities provided by any number of packages or probabilistic programming systems. Performing inference as described in existing, complex simulators is much more complex and not nearly as easy to implement from scratch. However, it can now be automated using the tools of probabilistic programming.

3.6.1 Probabilistic Programming

Probabilistic programming (van de Meent et al., 2018) is a growing subfield of machine learning that aims to build an analogous set of tools for automating inference as automatic differentiation did for continuous optimization. Like the gradient operator of languages that support automatic differentiation, probabilistic programming languages introduce observe operators that denote conditioning in the probabilistic or Bayesian sense. In those few languages that natively support nested Monte Carlo (Goodman and Stuhlmüller, 2014b; Tolpin et al., 2016), language constructs for defining conditional probability objects are introduced as well. Probabilistic programming languages (PPLs) have semantics (Staton et al., 2016) that can be understood in terms of Bayesian inference (Bishop, 2006; Gelman et al., 2013; Ghahramani, 2015). The major challenge in designing useful PPL systems is the development of general-purpose inference algorithms that work for a variety of user-specified programs. The work in the paper uses only the very simplest, and often least efficient, general purpose inference algorithms, importance sampling and rejection sampling. Others are covered in detail in (van de Meent et al., 2018).

Of all the various probabilistic programming systems, only one is readily compatible with inference in existing stochastic simulators: PyProb (Le et al., 2019). Quoting from its website[1] "PyProb is a PyTorch-based library for probabilistic programming and inference compilation. The main focus of PyProb is on coupling existing simulation codebases with probabilistic inference with minimal intervention." A textbook, technical description of how PyProb works appears in (van de Meent et al., 2018, Chapt. 6).

Recent examples of its use include inference in the standard model of particle physics conditioning on observed detector outputs (Baydin et al., 2018; Baydin et al., 2019), inference about internal composite material cure processes through a composite material cure simulator conditioned on observed

[1]https://github.com/pyprob/pyprob

surface temperatures (Munk et al., 2020), and inference about malaria spread in a malaria simulator (Gram-Hansen et al., 2019).

3.6.2 Alternative Approaches

It is important to note upfront that our ultimate objective is to understand the dependence of the policy outcome on the controllable parameters (for instance, as defined by **Eqs 7**, **8**). There are a litany of methods to quantify this dependency. Each method imposes different constraints on the model family that can be analyzed, and the nature of the solution obtained. For instance, the fully Bayesian approach we take aims to quantify the entire distribution, whereas traditional *optimal control* methods may only seek a pointwise maximizer of this probability. Therefore, before we introduce the models we analyze and examples we use to explore the proposed formulation, we give a brief survey of alternative approaches one could use to solve this problem, and give the benefits and drawbacks of our proposed formulation compared to these approaches.

The traditional toolkits used to analyze problems of this nature are *optimal control* (Vinter and Vinter, 2010; Lewis et al., 2012; Sharomi and Malik, 2017; Bertsekas, 2019) and *robust control* (Zhou and Doyle, 1998; Hansen and Sargent, 2001; Green and Limebeer, 2012). Most generally, optimal control solves for the control inputs (here denoted θ) such that some outcome is achieved (here denoted $\forall_{t>0}$: $Y_t^{aux} = 1$) while minimizing the associated cost of the controls. An example of this may be successfully maintaining stable flight while minimizing fuel expenditure. However, for the controls to be optimal, this assumes the model is correct. Alternatively, robust control does not assume that the model is correct, and instead solves for controls that are maximally robust to the uncertainty in the model, while still achieving the desired outcome.

Many traditional control approaches can exploit a mathematical model of the system to solve for controls that consider multiple timesteps, referred to as *model predictive control* (MPC) (Morari and Lee, 1999; Rawlings, 2000; Camacho and Alba, 2013). Alternatively, control methods can be *model-free*, where there is no notion of the temporal dynamics of the system being controlled, such as the canonical PID control. Model-based methods often solve for more effective control measures with lower overall computational costs, by exploiting the information encoded in the model. However, model predictive control methods are only applicable when an (accurate) model is available.

One could re-frame the objective as trying to maximize the *reward* of some applied controls. Here, reward may be a 0–1 indicator corresponding to whether or not the hospital capacity was exceeded at any point in time. The reward may also be more sophisticated, such as by reflecting the total number of people requiring hospital treatment, weighted by how critical each patient was. Reward may also internalize some notion of the *cost* of a particular control. Shutting workplaces may prevent the spread of infection, but has economic and social implications that (at least partially) counteract the benefit of reducing the infection rate. This can be implemented as earning progressively more negative reward for stronger policies.

This type of analysis is formalized through *reinforcement learning* (RL) (Sutton, 1992; Kaelbling et al., 1996; Sutton and Barto, 2018; Bertsekas, 2019; Warrington et al., 2021). In RL, a *policy*, here θ, is solved for that maximizes the expected reward. RL is an incredibly powerful toolkit that can learn highly expressive policies that vary as a function of time, state, different objectives etc. RL methods can also be described as model-based (Moerland et al., 2021) and model-free (Bertsekas, 2019) analogously to traditional control methods. While we do not delve into RL in detail here, it is important to highlight as an alternative approach, and we refer the reader to Levine (2018) for a more detailed introduction of RL, to Bertsekas (2019) for a discussion of the relationship between RL and control methods, and to Levine (2018) for a discussion of the relationship between RL and planning-as-inference.

However, a critical drawback of both control and RL is the strong dependence on the loss functions or specific models considered, both in terms of convergence and the optimal solution recovered (Adam and DeJong, 2003). This dependence may therefore mean that the solution recovered is not representative of the "true" optimal solution. Furthermore, RL approaches can be incredibly expensive to train, especially in high-dimensional time series models, with a large array of controls and sparse rewards. More traditional control-based methods can be limited in the models that they can analyze, both in terms of the transition distribution and the range of controls that can be considered. Finally, both RL and control-based approaches offer limited insight into *why* certain controls were proposed. This can limit the ability of researchers and modellers to expediently investigate the emergent properties of complex, simulation-based models. Furthermore, this lack of transparency may be disadvantageous for policy-makers, who may be required to justify why certain policy decisions were made, and the confidence with which that policy decision was believed to be optimal.

All of these methods, at least in terms of their core intentions, are very similar. Different methods impose different restrictions on the models that can be analyzed, and the nature of the solution obtained. In this work, we take the approach of framing control and planning as fully Bayesian inference with a binary objective. We choose this formulation as it places very few restrictions on the model class that can be analyzed, allows uncertainty to be succinctly handled throughout the inference, a broad range of objectives to be defined under the same framework, and correctly calibrated posterior distributions over all random variables in the model to be recovered. Furthermore, this formulation allows us to access the ever-growing array of powerful inference algorithms to perform the inference. While naive approaches such as grid-search or random sampling may be performant in low-dimensional applications, they do not scale to high dimensions through the *curse of dimensionality* (Köppen, 2000; Owen, 2013). Therefore, we focus on methods that can scale to high-dimensional applications (Owen, 2013). Finally, Bayesian inference methods are, arguably, the most complete formulation. Once the full posterior or joint distribution have been recovered, many different methodologies, constraints, objectives etc. can be formulated and evaluated *post facto*. This

flexibility allows for fine-grained analysis to be conducted on the inferred distributions to provide analysts with a powerful and general tool to further analyze and understand the model. Less myopically, this analysis and the understanding garnered may be more beneficial to our broader understanding of outbreaks and the feasibility and efficacy of particular responses.

Finally, we note that while throughout the experiments presented in **Section 5.2** we use the PyProb framework, PyProb is not the *only* framework available. Any inference methodology could be used to compute the probability in **Eq. 7**. However, PyProb is a natural choice as it allows for greater flexibility in interfacing between black-box simulator code, and powerful and efficiently implemented inference algorithms, without modifying either simulator or inference code. This reduces the implementation overhead to the analyst, and reduces the scope for implementation bugs and oversights to be introduced.

4 MODELS

Epidemiological dynamics models can be used to describe the spread of a disease such as COVID-19 in society. Different types span vastly different levels of fidelity. There are classical compartmental models (SIR, SEIR, etc.) (Blackwood and Childs, 2018a) that describe the bulk progression of diseases of different fundamental types. These models break the population down to a series of compartments (e.g. susceptible (S), infectious (I), exposed (E), and recovered (R)), and treat them as continuous quantities that vary smoothly and deterministically over time following dynamics defined by a particular system of ordinary differential equations. These models are amenable to theoretical analyses and are computationally efficient to forward simulate owing to their low dimensionality. As policy-making tools, they are rather blunt unless the number of compartments is made large enough to reflect different demographic information (age, socio-economic info, etc.), spatial strata, or combinations thereof.

At the other end of the spectrum are agent-based models (Hunter et al., 2017; Badham et al., 2018; Hunter et al., 2018; Tracy et al., 2018) (like the University of Pittsburgh Public Health Dynamics Laboratory's FRED (Grefenstette et al., 2013) or the Institute for Disease Modeling's EMOD (Anna et al., 2018)) that model populations and epidemiological dynamics via discrete agent interactions in "realistic" space and time. Imagine a simulation environment like the game Sim-City^{TM}, where the towns, populations, infrastructure (roads, airports, trains, etc.), and interactions (go to work, school, church, etc.) are modeled at a relatively high level of fidelity. These models exist only in the form of stochastic simulators, i.e., software programs that can be initialized with disease and population characteristics, then run forward to show in a more fine-grained way the spread of the disease over time and space.

Both types of models are useful for policy-making. Compartmental models are usually more blunt unless the number of compartments is very high and it is indexed by spatial location, demographics and age categories. Increasing the number of compartments adds more unknown parameters which must be estimated or marginalized. Agent-based models are complex by nature, but they may be more statistically efficient to estimate, as they are parameterized more efficiently, often directly in terms of actual individual and group behaviour choices. In many cases, predictions made by such models are more high fidelity, certainly more than compartmental models with few compartments, and this has implications for their use as predictive tools for policy analysis. For instance, policies based on simulating a single county in North Dakota with excellent hospital coverage and a highly dispersed, self-sufficient population could lead to different intervention recommendations compared to a compartmental model of the whole of the United States with only a few compartments.

4.1 A Compartmental Model of COVID-19

We begin by introducing a low-dimensional compartmental model to explore our methods in a well-known model family, before transitioning to a more complex agent-based simulator. The model we use is an example of a classical SEIR model (Ogilvy Kermack and McKendrick, 1927; C Blackwood and Childs, 2018; Hill et al., 2020). In such models, the population is subdivided into a set of compartments, representing the susceptible (uninfected), exposed (infected but not yet infectious), infectious (able to infect/expose others) and recovered (unable to be infected). Within each compartment, all individuals are treated identically, and the full state of the simulator is simply the size of the population of each compartment. Our survey of the literature found a lack of consensus about the compartmental model and parameters which most faithfully simulate the COVID-19 scenario. Models used range from standard SEIR (Liu et al., 2020a; Massonnaud et al., 2020; Rovetta et al., 2020; Traini et al., 2020), SIR (Jia et al., 2020; Pujari and Shekatkar, 2020; Teles, 2020; Traini et al., 2020; Weber et al., 2020), SIRD (Anastassopoulou et al., 2020; Liu et al., 2020b; Caccavo, 2020), QSEIR (Liu et al., 2020c), and SEAIHRD (Arenas et al., 2020). The choice depends on many factors, such as how early or late in the stages of an epidemic one is, what type of measures are being simulated, and the availability of real word data. We opted for the model described in this section, which seems to acceptably represent the manifestation of the disease in populations. Existing work has investigated parameter estimation in stochastic SEIR models (Lekone and Finkenstädt, 2006; Roberts et al., 2015). Although we will discuss how we set the model parameters, we emphasize that our contribution is instead in demonstrating how a calibrated model could be used for planning.

4.1.1 Model Description

We use an SEI^{3}R model (Hill et al., 2020), a variation on the standard SEIR model which allows additional modeling freedom. It uses six compartments: susceptible (S), exposed (E), infectious with mild (I_1), severe (I_2) or critical infection (I_3), and recovered (R). We do not include baseline birth and death rates in the model, although there is a death rate for people in the critically infected compartment. The state of the simulator at time $t \in [0, T]$ is $X_t = \{S_t, E_t, I_{1,t}, I_{2,t}, I_{3,t}, R_t\}$ with S_t, E_t, $I_{1,t}$, $I_{2,t}$, $I_{3,t}$, and R_t indicating the population sizes (or

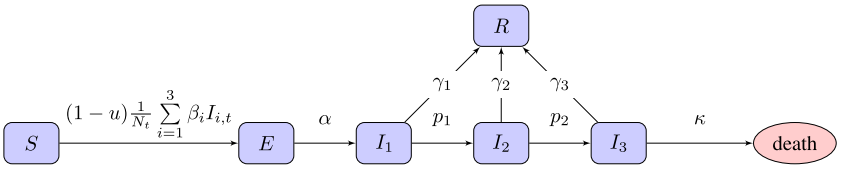

FIGURE 1 | Flow chart of the SEI^{3}R model we employ. A member of the susceptible population *S* moves to exposed *E* after being exposed to an infectious person, where "exposure" is defined as the previous susceptible person contracting the illness. After some incubation period, a random duration parameterized by α, they develop a mild infection (I_1). They may then either recover, moving to *R*, or progress to a severe infection (I_2). From I_2, they again may recover, or else progress further to a critical infection (I_3). From I_3, the critically infected person will either recover or die.

proportions) at time *t*. The unknown model parameters are $\eta = \{\alpha, \beta_1, \beta_2, \beta_3, p_1, p_2, \gamma_1, \gamma_2, \gamma_3, \kappa\}$, each with their own associated prior. To the model we add a free control parameter, denoted $u \in [0, 1]$, that acts to reduce the transmission of the disease. Since u is the only free parameter, $\theta = u$. An explanation of u is given later in the text. There are no internal latent random variables (Z_t) in this model. In this paper we do not demonstrate inference about θ given Y^{obs} within this model, and so do not consider Y^{obs} here. We do, however, consider Y^{aux} to perform policy selection, and discuss the form of Y^{aux} later. Defining the total live population (i.e., the summed population of all compartments) at time t to be N_t, the dynamics are given by the following equations, and shown in **Figure 1**.

$$\frac{\mathrm{d}}{\mathrm{d}t}S_t = -(1-u)\frac{1}{N_t}\sum_{i=1}^{3}\beta_i I_{i,t} S_t \tag{14}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}E = (1-u)\frac{1}{N_t}\sum_{i=1}^{3}\beta_i I_{i,t} S_t - \alpha E_t \tag{15}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}I_{1,t} = \alpha E_t - p_1 I_{1,t} - \gamma_1 I_{1,t} \tag{16}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}I_{2,t} = p_1 I_{1,t} - p_2 I_{2,t} - \gamma_2 I_{2,t} \tag{17}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}I_{3,t} = p_2 I_{2,t} - \kappa I_{3,t} - \gamma_3 I_{3,t} \tag{18}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}R_t = \gamma_1 I_{1,t} + \gamma_2 I_{2,t} + \gamma_3 I_{3,t}. \tag{19}$$

For the purposes of simulations with this model, we initialize the state with 0.01% of the population having been exposed to the infection, and the remaining 99.99% of the population being susceptible. The population classified as infectious and recovered are zero, i.e., $X_0 = \{0.9999, 0.0001, 0, 0, 0, 0\}$ and $N_t = 1$.

4.1.2 Example Trajectories

Before explaining how we set the SEI^{3}R model parameters, or pose inference problems in the model, we first verify that we are able to simulate feasible state evolutions. As we will describe later, we use parameters that are as reflective of current COVID-19 epidemiological data as possible at the time of writing. **Figures 2A,B** show deterministic simulations from the model with differing control values u. Shown in green is the susceptible population, in blue is the exposed population, in red is the infectious population, and in purple is the recovered population. The total live population is shown as a black dotted line. All populations are normalized by the initial total population, N_0. The dashed black line represents a threshold under which we wish to keep the number of infected people at all times. The following paragraph provides the rationale for this goal.

4.1.3 Policy Goal

As described in **Section 3.4**, parameters should be selected to ensure that a desired goal is achieved. In all scenarios using the SEI^{3}R model, we aim to maintain the maximal infectious population proportion requiring healthcare below the available number of hospital beds per capita, denoted *C*. This objective can be formulated as an auxiliary observation, $Y^{aux}_{0:T}$, introduced in **Section 3**, as:

$$Y^{aux}_{0:T} = \mathbb{I}\left[\left(\max_{t\in\{0,\ldots,T\}}\left(I_{1,t} + I_{2,t} + I_{3,t}\right)\right) < C\right], \tag{20}$$

where $I_{1,0:T}$, $I_{2,0:T}$ and $I_{3,0:T}$ are sampled from the model, conditioned on a θ value. This threshold value we use was selected to be 0.0145, as there are 0.0029 hospital beds per capita in the United States (World Bank, 2020), and roughly 20% of COVID-19 cases require hospitalization. This constraint was chosen to represent the notion that the healthcare system must have sufficient capacity to care for all those infected who require care, as opposed to just critical patients. However, this constraint is only intended as a demonstrative example of the nature of constraints and inference questions one can query using models such as these, and under the formalism used here, implementing and comparing inferences under different constraints is very straightforward. More complex constraints may account for the number of critical patients differently to those with mild and severe infections, model existing occupancy or seasonal variations in capacity, or, target other metrics such as the number of deceased or the duration of the epidemic.

The constraint is not met in **Figure 2A**, but is in **Figure 2B**, where a greater control input u has been used to slow the spread of the infection. This is an example of the widely discussed "flattening of the curve." As part of this, the infection lasts longer but the death toll is considerably lower.

4.1.4 Control Input

As noted before, we assume that only a single "controllable" parameter affects our model, u. This is the reduction in the "baseline reproductive ratio," R_0, due to policy interventions. Increasing u has the same effect as reducing the infectiousness parameters β_1, β_2 and β_3 by the same proportion. u can be interpreted as the effectiveness of policy choices to prevent new infections. Various policies could serve to increase u,

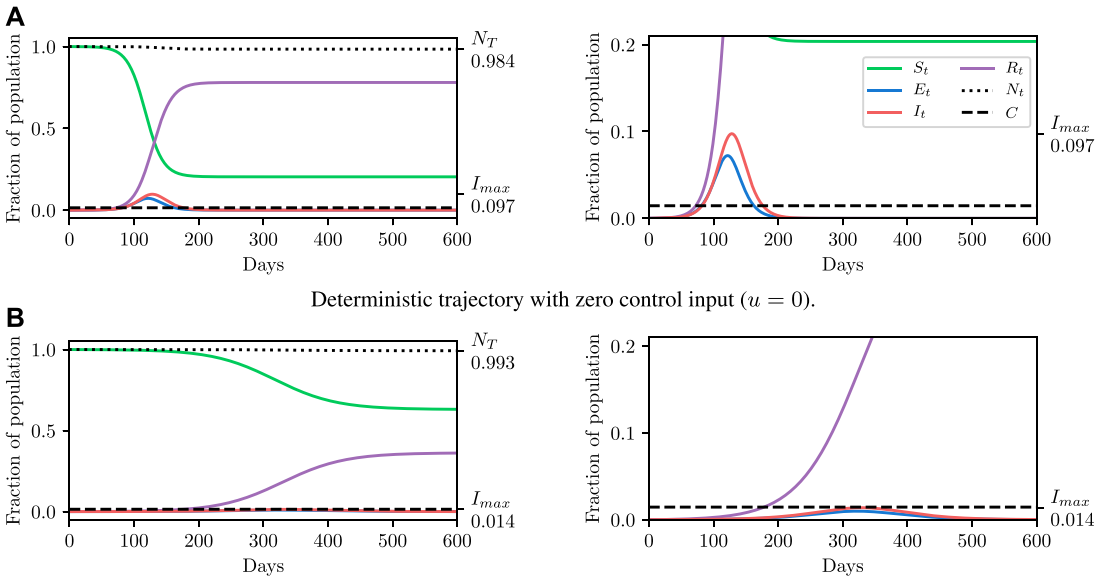

FIGURE 2 | Populations per compartment during deterministic SEI^{3}R simulations, both without intervention **(A)** and with intervention **(B)**. Plots in the left column show the full state trajectory, and in the right column are cropped to more clearly show the exposed and infected populations. Without intervention, the infected population requiring hospitalization (20% of cases) exceeds the threshold for infected population (0.0145, black dashed line), overwhelming hospital capacities. With intervention ($u = 0.37$) the infected population always remains below this limit. Note that we re-use the colour scheme from this figure through the rest of the paper.

since it is a function of both, for example, reductions in the "number of contacts while infectious" (which could be achieved by social distancing and isolation policy prescriptions), and the "probability of transmission per contact" (which could be achieved by, e.g., eye, hand, or mouth protective gear policy prescriptions). It is likely that both of these kinds of reductions are necessary to maximally reduce u at the lowest cost.

For completeness, the baseline reproductive ratio, R_0, is an estimate of the number of people a single infectious person will in turn infect and can be calculated from other model parameters (Hill et al., 2020). R_0 is often reported by studies as a measure of the infectiousness of a disease, however, since R_0 can be calculated from other parameters we do not explicitly parameterize the model using R_0, but we will use R_0 as a convenient notational shorthand. We compactly denote the action of u as controlling the baseline reproductive rate to be a "controlled reproductive rate," denoted $\hat{R}_0$, and calculated as $\hat{R}_0 = (1-u)R_0$. This is purely for notational compactness and conceptual ease, and is entirely consistent with the model definition above.

4.1.5 Using Point Estimates of Model Parameters

We now explain how we set the model parameters to deterministic estimates of values which roughly match COVID-19. The following section will consider how to include uncertainty in the parameter values. Specifically, the parameters are the incubation period α^{-1}; rates of disease progression p_1 and p_2; rates of recovery from each level of infection, γ_1, γ_2, and γ_3; infectiousness for each level of infection, β_1, β_2, and β_3; and a death rate for critical infections, κ. $u \in [0, 1]$ is a control parameter, representing the strength of action taken to prevent new infections (Boldog et al., 2020). To estimate distributions over the uncontrollable model parameters, we consider their relationships with various measurable quantities

$$\text{incubation period} = \alpha^{-1} \tag{21}$$

$$\text{mild duration} = \frac{1}{\gamma_1 + p_1} \tag{22}$$

$$\text{severe duration} = \frac{1}{\gamma_2 + p_2} \tag{23}$$

$$\text{critical duration} = \frac{1}{\gamma_3 + \kappa} \tag{24}$$

$$\text{mild fraction} = \frac{\gamma_1}{\gamma_1 + p_1} \tag{25}$$

$$\text{severe fraction} = \frac{\gamma_2}{\gamma_2 + p_2} \cdot (1 - \text{mild fraction}) \tag{26}$$

$$\text{critical fraction} = 1 - \text{severe fraction} - \text{mild fraction} \tag{27}$$

$$\text{fatality ratio} = \frac{\kappa}{\gamma_3 + \kappa} \cdot \text{critical fraction}. \tag{28}$$

Given the values of the left-hand sides of each of **Eqs 21–28**, (as estimated by various studies) we can calculate model parameters α, p_1, p_2, γ_1, γ_2, γ_3 and κ by inverting this system of Equations. These parameters, along with estimates for β_1, β_2, and β_3, and a control input u, fully specify the model. Hill et al. (2020) use such a procedure to deterministically fit parameter values. Given the parameter values, the simulation is entirely deterministic. Therefore, setting parameters in this way enables us to make deterministic simulations of "typical" trajectories, as shown in **Figure 2**. Specifying parameters in this way and

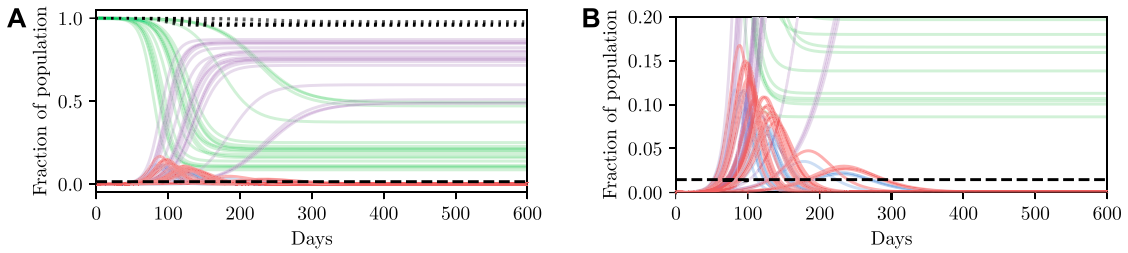

FIGURE 3 | Stochastic simulations from the SEI^{3}R model. **(A)** shows the full trajectory while **(B)** is cropped to the pertinent region. Compared to the deterministic simulations in **Figure 2A**, stochastic simulations have the capacity to be much more infectious. Therefore, fully stochastic simulations are required to accurately quantify the true risk in light of the uncertainty in the model. As a result of this, Bayesian methods, or at least methods that correctly handle uncertainty, are required for robust analysis. We reuse the colour scheme defined in **Figure 2A** for trajectories.

running simulations in this system provides a low overhead and easily interpretable environment, and hence is an invaluable tool to the modeller.

4.1.6 Dealing With Uncertainty About Model Parameter Values

Deterministic simulations are easy to interpret on a high level, but they require strong assumptions as they fix the values of unknown parameters to point estimates. We therefore describe how we can perform inference and conditioning in a stochastic model requiring less strict assumptions, and show that we are able to provide meaningful confidence bounds on our inferences that can be used to inform policy decisions more intelligently than without this stochasticity. As described in **Section 3**, stochasticity can be introduced to a model through a distribution over the latent global parameters η. Examples of stochastic simulations are shown in **Figure 3A**. Clearly there is more capacity in this model for representing the underlying volatility and unpredictability of the precise nature of real-world phenomena, especially compared to the deterministic model.

However, this capacity comes with the reality that increased effort must be invested to ensure that the unknown latent states are correctly accounted for. For more specific details on dealing with this stochasticity please refer back to **Section 3**, but, in short, one must simulate for multiple stochastic values of the unknown parameters, for each value of the controllable parameters, and agglomerate the many individual simulations appropriately for the inference objective. When asking questions such as "will this parameter value violate the constraint?" there are feasibly some trajectories that are slightly above and some slightly below the trajectory generated by the deterministic simulation due to the inherent stochasticity (aleatoric uncertainty) in the real world. This uncertainty is integrated over in the stochastic model, and hence we can ask questions such as "what is the probability that this parameter will violate the constraint?" Using confidence values is this way provides some measure of how certain one can be about the conclusion drawn from the inference–if the confidence value is very high then there is a measure of "tolerance" in the result, compared to a result with a much lower confidence.

We define a joint distribution over model parameters as follows. We consider the 95% confidence intervals of β_1, β_2, and β_3 and the values in the left-hand sides of **Eqs 21–24**, and assume that their true values are uniformly distributed across these confidence intervals. Then at each time t in a simulation, we sample these values and then invert the system of **Eqs 21–28** to obtain a sample of the model parameters. More sophisticated distributions could easily be introduced once this information becomes available. We now detail the nominal values used for typical trajectories (and the confidence intervals used for sampling). The nominal values are mostly the same as those used by (Hill et al., 2020). We use: an incubation period of 5.1 days (4.5–5.8) (Lauer et al., 2020); a mild infection duration of 6 days (5.5–6.5) (Wölfel et al., 2020); a severe infection duration of 4.5 days (3.5–5.5) (Sanche et al., 2020); a critical infection duration of 6.7 days (4.2–10.4); fractions of mild, severe, and critical cases of 81, 14, and 5% (Wu and McGoogan, 2020); and a fatality ratio of 2% (Wu and McGoogan, 2020). We also use $\beta_1 = 0.33$/day (0.23–0.43), and $\beta_2 = 0$/day (0–0.05), and $\beta_3 = 0$/day (0–0.025). Where possible, the confidence intervals are obtained from the studies which estimated the quantities. Where these are not given, we use a small range centred on the nominal value to account for possible imprecision.

4.2 Agent-Based Simulation

While compartmental models, such as the SEIR model described in **Section 4.1**, provide a mathematically well understood global approximation to disease dynamics, due to their coarse-grained statistical nature they cannot capture many important aspects and local details of the physical and social dynamics underlying the spread of a disease. These aspects include geographic information, spatio-temporal human interaction patterns in social hubs such as schools or workplaces, and the impact of individual beliefs on transmission events. To address these limitations, agent-based simulators (ABS) have been introduced. Such simulators have practically no restrictions in terms of expressiveness, i.e., they can make use of all features of modern Turing-complete programming languages, at the significant computational cost of simulating all details involved.

4.2.1 FRED: Fine-Grained Simulation of Disease Spreading

FRED[2] (Grefenstette et al., 2013) is an instance of the class of epidemiological agent-based simulators that are currently available for use in policy-making. FRED is an agent-based modeling language and execution platform for simulating changes in a population over time. FRED represents individual persons, along with social contacts and interactions with the environment. This enables the model to include individual responses and behaviors that vary according to the individual's characteristics, including demographics (age, sex, race, etc.), as well as the individual's interactions with members of various social interaction groups, such as their neighborhood, school or workplace. The FRED user can define and track any dynamic condition for the individuals within the population, including diseases (such as COVID-19), attitudes (such as vaccine acceptance), and behaviors (such as social distancing).

FRED captures demographic and geographic heterogeneities of the population by modeling every individual in a region, including realistic households, workplaces and social networks. Using census-based models available for every state and county in the US and selected international locations, FRED simulates interactions within the population in discrete time steps of 1 h. Transmission kernels model the spatial interaction between infectious places and susceptible agents. These characteristics enable FRED to provide much more fine-grained policy advice at either the regional or national level, based on socio-economic and political information which cannot be incorporated into compartmental models.

We chose to use FRED in this work because it has been used to evaluate potential responses to previous infectious disease epidemics, including vaccination policies (Lee et al., 2011), school closure (Potter et al., 2012), and the effects of population structure (Kumar et al., 2015) and personal health behaviors (Kumar et al., 2013; Liu et al., 2015).

After 10 years of develoment as an academic project, FRED has been licensed by the University of Pittsburgh to Epistemix [3], to develop commercial applications of the FRED modeling technology. In turn, Epistemix has developed a detailed COVID-19 model in FRED, which is used in the experiments described here.

The FRED COVID-19 model includes three interconnected components: *1*) The natural history of COVID-19; *2*) The social dynamics/behavior of individuals; and *3*) The Vaccination Program. The COVID-19 model was designed using the latest scientific data, survey information from local health authorities, and in consultation with expert epidemiologists. This model has been used to project COVID-19 cases in universities, K-12 school districts, large cities, and offices.

The FRED COVID-19 natural history model represents the period of time and trajectory of an individual from infection or onset to recovery or death. In the current version of the model, when an individual, or an agent, is exposed to SARS-CoV-2, the virus that causes COVID-19, the individual enters a 2-day latent period before they become infectious. In the infectious state, individuals can either be asymptomatic, symptomatic, or hospitalized. The probability of entering any of these infectious states is based on the individual's age, infection history, and vaccination history. Individuals have a duration of illness (i.e., number of days they can transmit the virus) which is dependent on infectious state, a severity of disease (i.e., magnitude of transmissibility), and a disease outcome (recovery or death).

When an agent is exposed to SARS-CoV-2 and becomes symptomatic, the individual chooses whether or not to isolate themselves from normal activities. Approximately 20% of individuals continue regular daily activities while symptomatic. Agents who are exposed and develop an asymptomatic infection do not isolate themselves and go about their regular activities. This introduces both symptomatic and asymptomatic forms of transmission into the model.

Prevalence of mask wearing and adherence to social distancing are unique to each location and change over time. The level of compliance to these behaviors is set based on the number of active infections that were generated from reported cases in the previous 2 weeks. Social distancing is assumed to reduce the number of contacts between agents in each place the agent attends.

Following the general recipe for framing planning as inference in **Section 3.1**, the following section defines what a prior on controls θ is in terms of FRED internals, how FRED parameters relate to η, and how to condition FRED on desirable future outcomes $Y^{aux}_{0:T}$. **Section 4.2.3** describes results of performing automated inference in this stochastic simulation-based model using the probabilistic programming system PyProb. The main point of this section is to illustrate how a stochastic simulator can be seen as a probabilistic model and, when integrated with an appropriate probabilistic programming system, can be repurposed to perform automatic inference, in this instance for planning as inference. The FRED-specific recipe we provide below should be read with the understanding that it is easy to apply the same recipe to most if not all existing epidemiological simulators, as their fundamental computational structure is regular.

4.2.2 Turning FRED Into a Probabilistic Program

The FRED simulator has a parameter file which stipulates the values of θ and η. In other words, both the controllable and non-controllable parameters live in a parameter file. FRED, when run given a particular parameter file, produces a sample from the distribution $p(X_{0:T}, Z_{0:T}|\theta, \eta)$. Changing the random seed and re-running FRED will result in a new sample from this distribution.

The difference between $X_{0:T}$ and $Z_{0:T}$ in FRED is largely in the eye of the beholder. One way of thinking about it is that $X_{0:T}$ are all the values that are computed in a run and saved in an output file and $Z_{0:T}$ is everything else.

In order to turn FRED into a probabilistic programming model useful for planning via inference several small but

[2]https://fred.publichealth.pitt.edu/

[3]http://www.epistemix.com/

consequential changes must be made to it. These changes can be directly examined by browsing one of the public source code repositories accompanying this paper.[4] First, the random number generator and all random variable samples must be identified so that they can be intercepted and controlled by PyProb. Second, any variables that are determined to be controllable (i.e., part of θ) need to be identified and named. Third, in the main stochastic simulation loop, the state variables required to compute Y_t^{aux} and Y_t^{obs} must be extracted. Fourth, these variables must be given either synthetic ABC likelihoods or given constraints in the form of likelihoods. Finally, a mechanism for identifying, recording, and or returning $X_{0:T}$ to the probabilistic programming system must be put in place. FRED, like many stochastic simulators, includes the ability to write-out results of a run of the simulator to the filesystem. This, provided that the correspondence between a sample $\theta^{(i)}$ and the output file or files that correspond to it is established and tracked, is how $X_{0:T}^{(i)}$ is implicitly defined.

In the interest of time and because we were familiar with the internals of PyProb and knew that we would not be using inference algorithms that were incompatible with this choice, the demonstration code does not show a full integration in which all random variables are controlled by the probabilistic programming system. Instead, it only controls the sampling of θ and the observation of Y_t^{aux}. Notably, this means that inference algorithms like lightweight Metropolis Hastings (Wingate et al., 2011), which are also included in PyProb, cannot be used with the released integration code.

4.2.3 Details of FRED+PyProb Integration

Our integration of PyProb into FRED required only minor modifications to FRED's code base, performed in collaboration with the FRED developers at Epistemix. More details about the integration of FRED and PyProb include:

1) The simulator is connected to PyProb through a cross-platform execution protocol (PPX[5]). This allows PyProb to control the stochasticity in the simulator, and requires FRED to wait, at the beginning of its execution, for a handshake with PyProb through a messaging layer.
2) PyProb overwrites the policy parameter values θ with random draws from the user-defined prior. While PyProb internally keeps track of all random samples it generates, we also decided to write out the updated FRED parameters to a parameter file in order to make associating $\theta^{(i)}$ and $X_{0:T}^{(i)}$ easy and reproducible.
3) For each daily iteration step in FRED's simulation, we call PyProb's observe function with a likelihood corresponding to the constraint we would like to hold in that day.

With these connections established, we are able to select an inference engine implemented by PyProb to compute the posterior. We use a particularly simple algorithm, namely rejection sampling, in order to focus our exposition on the conceptual framework of planning as inference. PyProb implements multiple other, more complex, algorithms, which may be able to better approximate the posterior with a given computational budget. However for the inference task we consider, in which we attempt to infer only a small fraction of the random variables in the simulator, we find that rejection sampling is sufficiently performant.

We also remind the reader that, like in **Section 3.5**, more complex controls can be considered, in principle allowing for complex time-dependent policies to be inferred. We do not examine this here, but note that this extension is straightforward to implement in the probabilistic programming framework, and that PyProb is particularly well adapted to coping with the additional complexity. Compared to sampling parameter values for FRED at the beginning of the simulation, such time-varying policies could be implemented through changing the FRED model source code directly. This approach will be explored in future research.

5 EXPERIMENTS

We now demonstrate how inference in epidemiological dynamics models can be used to inform policy-making decisions. We organize this section according to a reasonable succession of steps of increasing complexity that one might take when modeling a disease outbreak. We again stress that we are not making COVID-19 specific analyses here, but instead highlight how framing the task as in **Section 3** allows existing machine learning machinery to be leveraged to enhance analysis and evaluation of outcomes; and avoid some potential pitfalls.

We begin by showing how a simple, deterministic compartmental SEIR-based model can be used to inform policy-making decisions, and show how analysis derived from such a deterministic model can fail to achieve stated policy goals in practice. Next, we demonstrate how using a stochastic model can achieve more reliable outcomes by accounting for the uncertainty present in real world systems. While these stochastic models address the limitations of the deterministic model, low-fidelity SEIR models are, in general, not of high enough fidelity to provide localized, region-specific policy recommendations. To address this we conclude by performing inference in an existing agent-based simulator of infectious disease spread and demonstrate automatic determination of necessary controls.

5.1 SEI^{3}R Model

The most straightforward approach to modeling infectious diseases is to use low-dimensional, compartmental models such as the widely used susceptible-infectious-recovered (SIR) models, or the SEI^{3}R variant introduced in **Section 4.1**. These models are fast to simulate and easy to interpret, and hence form a powerful, low-overhead analysis tool.

5.1.1 Deterministic Model

The system of equations defining the SEI^{3}R model form a deterministic system when global parameter values, such as the mortality rates or incubation periods, are provided.

[4]https://github.com/plai-group/FRED
[5]https://github.com/pyprob/ppx

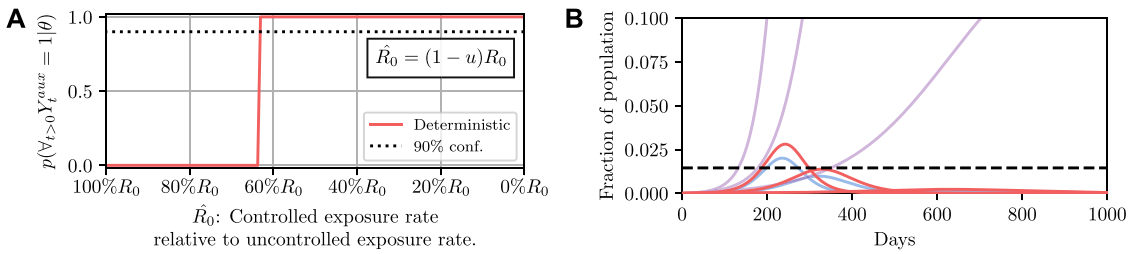

FIGURE 4 | Here we demonstrate planning using the deterministic SEI^{3}R model. **(A)** shows, in red, the probability that the constraint is met using the deterministic simulator. The probability jumps from zero to one at a value of approximately $u = 0.37$. **(B)** then shows trajectories using three salient parameter values, specifically, $u = [0.3, 0.37, 0.45]$. Each parameter value corresponds to more control than the previous, and is evident as the peak infection fraction drops from approximately 0.03 with $u = 0.3$, to 0.014 with $u = 0.37$ and almost zero with $u = 0.45$. These values were selected as values just below, on, and above the threshold seen in **(A)**. We reuse the colour scheme defined in **Figure 2A**.

However, the precise values of these parameter values are unknown, and instead only confidence intervals for these parameters are known, i.e., the incubation period is between 4.5 and 5.8 (Lauer et al., 2020). This variation may be due to underlying aleatoric uncertainty prevalent in biological systems, or epistemic uncertainty due to the low-fidelity nature of SIR-like models. We do not discuss them here, but work exists automatically fitting point-wise estimates of model parameter values directly from observed data (Wearing et al., 2005; Mamo and Koya, 2015).

Regardless of whether one obtains a point estimate of the parameter values by averaging confidence intervals, or by performing parameter optimization, the first step is to use these values to perform fully deterministic simulations, yielding simulations such as those shown in **Figure 2A**. Simulations such as these are invaluable for understanding the bulk dynamics of systems, investigating the influence of variations in global parameter values or investigating how controls affect the system. However, the ultimate utility in these models is to *use* them to inform policy decisions to reduce the impact of outbreaks. As eluded to above, this is the primary thrust of this work, combining epidemiological simulators with automated machine learning methodologies to model policy outcomes, by considering this problem as *conditioning* simulations on outcomes.

To demonstrate such an objective, we consider maintaining the infected population below a critical threshold C at all times. In a deterministic system there are no stochastic quantities and hence whether the threshold is exceeded is a deterministic function of the controlled parameters, i.e., the value of $p(\forall_{t>0}: Y_t^{aux} = 1|\theta)$ (related to **Eq. 7** via Bayes rule) is binary in a deterministic system and hence takes a value of either 0 or 1. Therefore, we can simply simulate the deterministic system for a finite number of θ values, and select those parameter values that do not violate the constraint. We vary the free parameter $u \in [0, 1]$, where u is a scalar value that reduces the baseline reproduction rate as $\hat{R}_0 = (1-u)R_0$. We define u in this way such that u represents an *intervention*, or change from normal conditions. The parameter u is the only parameter we have control over, and hence $\theta = u$.

Results for this are shown in **Figure 4**. It can then be read off that under the deterministic model $\hat{R}_0$ must be reduced by at least 37.5% of R_0 to satisfy the constraint. **Figure 4** shows trajectories simulated using insufficient intervention with $u = 0.3$ ($\hat{R}_0 = 70\%R_0$), acceptable intervention of $u = 0.375$ ($\hat{R}_0 = 62.5\%R_0$), and excessive intervention of $u = 0.45$ ($\hat{R}_0 = 55\%R_0$), and show that these parameters behave as expected, violating the constraint, remaining just under the threshold and remaining well beneath the threshold respectively.

5.1.2 Stochastic Simulation

While the above example demonstrates how parameters can be selected by conditioning on desired outcomes, we implicitly made a critical modeling assumption. While varying the free parameter u, we fixed the *other* model parameter values (α^{-1}, γ_1, etc.) to single values. We therefore found a policy intervention in an unrealistic scenario, namely one in which we (implicitly) claim to have certainty in all model parameters except u.

To demonstrate the pitfalls of analyzing deterministic systems and applying the results to an inherently stochastic system such as an epidemic, we use the permissible value of u solved for in the deterministic system, $u = 0.375$, and randomly sample values of the remaining simulation parameters. This "stochastic" simulator is a more realistic scenario than the deterministic variant, as each randomly sampled η represents a unique, plausible epidemic being rolled out from the current world state.

The results are shown in **Figure 5A**. Each line represents a possible epidemic. We can see that using the previously found value of u results in a large number of epidemics where the infectious population exceeds the constraint, represented by the red trajectories overshooting the dotted line. Simply put, the control parameter we found previously fails in an unacceptable number of simulations.

This detail highlights the shortcomings of the deterministic model: in the deterministic model a parameter value was either accepted or rejected with certainty. There was no notion of the variability in outcomes, and hence we have no mechanism to concretely evaluate the risk of a particular configuration.

Instead, we can use a stochastic model which at least does account for some aleatoric uncertainty about the world. We

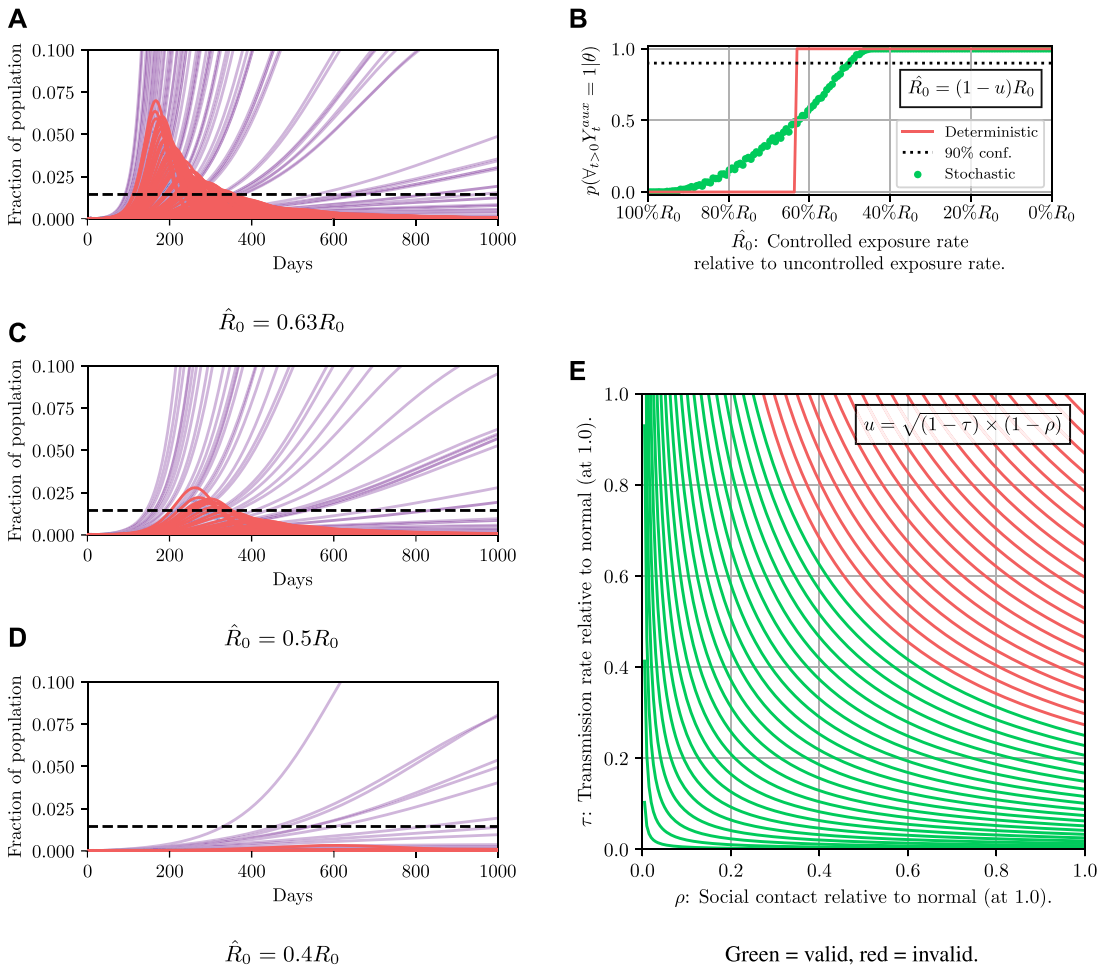

FIGURE 5 | Comparison of stochastic and deterministic SEI^{3}R models for policy selection. We reuse the colour scheme defined in **Figure 2A** for trajectories. **(A)** shows a stochastic simulation using $\hat{R}_0 = 0.63R_0$, identified as an acceptable parameter value under the deterministic model. However, once used in a stochastic system, the parameter performs poorly, yielding many simulations that violate the constraint. This highlights why analyses in deterministic systems can yield poor results. **(B)** repeats the analysis in **Figure 4A** adding planning in the stochastic simulation, where the *y*-coordinate can now be interpreted as the confidence level. **(C)** shows a simulation using the lowest valid value of *u*, representing the "weakest" valid policy. This value, approximately $\hat{R}_0 = 0.5R_0$, renders most of the trajectories under the threshold, with only a small fraction above, implying that it will satisfy the criteria with high probability. **(D)** shows that using $\hat{R}_0 = 0.4R_0$ effectively reduces the level of infection to near zero. **(E)** illustrates an example of how policy-level variables create model-level parameter values. Shown are the level-sets of the free parameter $u \in [0, 1]$, which acts to reduce the baseline reproduction rate R_0 as $\hat{R}_0 = (1 - u)R_0$. We suggest that the reduction in the (unknown) reproduction rate is given by the root of the product of two factors controllable through policy. Green level sets indicate that the value of *u* was effective and achieved a 90% confidence that the trajectory does not violate the constraint, whereas red curves do not satisfy this.

repeat the analysis picking the required value of *u*, but this time using the stochastic model detailed in **Section 4.1**. In practice, this means the (previously deterministic) model parameters detailed in **Eqs 21–28** are randomly sampled for each simulation according to the procedure outlined following the equations.

To estimate the value of $p(\forall_{t>0}: Y_t^{aux} = 1|\theta)$, for a given *u* value, we sample *M* stochastic trajectories from the system. We then simply count the number of trajectories for which the condition $\forall_{t>0}: Y_t^{aux} = 1$ holds, and divide this count by *M*. Intuitively, this operation is simple: for a given θ, simulate a number of possible trajectories, and, as the number of simulations *M* tends to infinity, the fraction that satisfy the constraint is the desired probability value. We note that this operation corresponds to an "inner" Monte Carlo expectation, sampling under the distribution of simulator trajectories conditioned on θ, evaluating the expected number of trajectories that do not violate the threshold. This value is then passed through a non-linear indicator function extracting those parameters that yield a confidence above a certain threshold. We are then free to use any method we please for exploring θ space, or, evaluating additional Monte Carlo expectations under the resulting θ distribution. As such, this system is a nested Monte Carlo sampler (Rainforth et al., 2018).

The results are shown in **Figure 5B**. The likelihood of the result under the stochastic model is not a binary value like in the deterministic case, and instead occupies a continuum of values

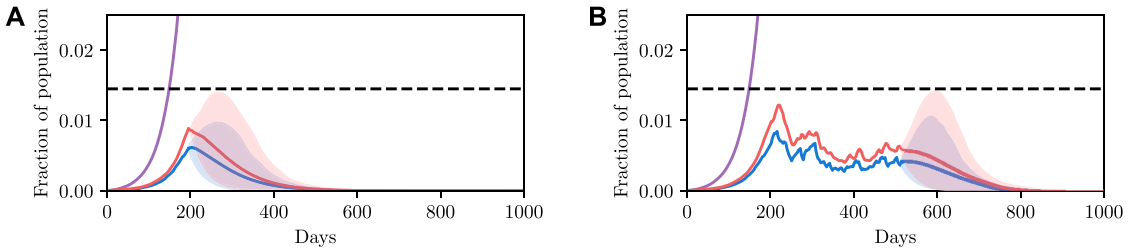

FIGURE 6 | Here we briefly demonstrate the capacity of the SEI^{3}R model in a model predictive control setting. **(A)** shows the state when we begin controlling the system at $t = 200$ with some level of infection already present. We solve for the minimum required control such that the constraint is satisfied. We plot the 90% confidence interval over trajectories conditioned on this control value. We then step through the system, randomly sampling continuations, and adapting the controls used such that the constraint is always met **(B)**. We uncover that stronger controls must be applied early on to reduce the infected population, but that the amount of control required can then reduce over time as herd immunity becomes a stronger effect. We reuse the color scheme defined in **Figure 2A** for plotting trajectories.

representing the confidence of the results. We see that the intersection between the red and green curves occurs at approximately 0.5, explaining the observation that approximately half of the simulations in **Figure 5A** exceed the threshold. We can now ask questions such as: "what is the parameter value that results in the constraint not being violated, with 90% confidence?" We can read off rapidly that we must instead reduce the value of $\hat{R}_0$ to 50% of its original value to satisfy this confidence-based constraint. Repeating the stochastic simulations using these computed values confirms that very few simulations violate the constraint (**Figure 5C**). The ability to tune the outcome based on a required level of confidence is paramount for safety-critical applications, as it informs how sensitive the system is to the particular parameter choice and is more resilient to model misspecification.

5.1.3 Model Predictive Control

We have shown how one can select the required parameter values to achieve a desired objective. To conclude this example, we apply the methodology to iterative planning. The principal idea underlying this is that policies are not static and can be varied over time conditioned on the current observed state. Under the formalism used here, re-evaluating the optimal control to be applied, conditioned on the new information, is as simple as re-applying the planning algorithm at each time step. Note that here we consider constant control. However, more complex, time-varying control policies can easily be considered under this framework. For instance, instead of recovering a fixed control parameter, the parameter values of a polynomial function defining time-varying control input could be recovered, or, a scalar value determining the instantaneous control input at each time-step. This is a benefit of the fully Bayesian and probabilistic programming-based approach we have taken: the model class that can be analyzed is not fixed and can be determined (and easily changed and iterated on) by the modeller, and the inference back-end cleanly and efficiently handles the inference.

We show a demonstration of this in **Figure 6**. In this example, we begin at time $t = 200$ with non-zero infection rates. We solve for a policy that satisfies the constraint with 90% certainty, and show this confidence interval over trajectories as a shaded region. We then simulate the true evolution of the system for a single step sampling from the conditional distribution over state under the selected control parameter. We then repeat this process at regular intervals, iteratively adapting the control to the new world state. We see that the confidence criterion is always satisfied and that the infection is able to be maintained at a reasonable level. We do not discuss this example in more detail, and only include it as an example of the utility of framing the problem as we have, insomuch as iterative re-planning based on new information is a trivial extension under the formulation used.

5.1.4 Policy-Based Controls

We have illustrated how simulations can be used to answer questions about the suitability of parameter values we can influence, while marginalizing over those parameters we do not have control over. However, u is not something that is *directly* within our control. Instead, the value of u is set through changing policy level factors. As an exploratory example we suggest that the value of u is the square root of the product of two policy-influenceable factors: the fractional reduction in social contact, ρ, below its normal level (indicated as a value of 1.0), and the transmission rate relative to the normal level, τ, where we again denote normal levels as 1.0. This relationship is shown in **Figure 5E**.

We indicate u level sets that violate the constraint in red, and valid sets in green. We suggest taking the least invasive, valid policy, being represented by the highest green curve. Once the analysis above has been performed to obtain a value of u, that satisfies the required infection threshold, it defines the set of achievable policies. Any combination of τ and η along this curve renders the policy valid. Here, additional factors may come into consideration that make particular settings of τ and η more or less advantageous. For instance, wearing more PPE may be cheaper to implement and less economically and socially disruptive than social distancing, and so higher values of τ may be selected relative to η. This reduces to a simple one-dimensional optimization of the cost surface along the level-set.

While we have simply hypothesized this as a potential relationship, it demonstrates how policy level factors influence

TABLE 1 | Prior over FRED control parameter $\theta = (\theta_1, \ldots, \theta_5)$ for influenza simulations.

Prior	Control
$\theta_1 \sim$ Uniform(0, 14)	shelter-in-place duration
$\theta_2 \sim$ Uniform(0, 1)	shelter-in-place compliance rate
$\theta_3 \sim$ Uniform(0, 1)	isolation rate
$\theta_4 \sim$ Uniform(0.01, 0.21)	school closure attack rate threshold
$\theta_5 \sim$ Uniform(0, 1)	hand washing compliance rate

simulations and outcomes. While the SEIR model family is an invaluable tool for analyzing and understanding the bulk dynamics of outbreaks, it is too coarse-grained for actual, meaningful, localized policy decisions to be made, especially when those policy decisions are directly influencing populations. Further, these notions of "policy" are somewhat abstract here because of the high-level nature of the SEI^{3}R model used. We now go on to resolve these issues by using the more sophisticated agent-based simulator FRED, where simulations are able to represent localized variations, and where real policy measures are more easily defined.

5.2 FRED Simulator

In this section, we turn to agent-based simulators. We showcase how control as inference might possibly be used to inform *regional* policy decisions through two scenarios presented in **Sections 5.2.1**, **5.2.2**. In the first scenario, we consider an influenza outbreak and in the second a COVID-19 outbreak is simulated. In both scenarios the policy makers wish to "flatten the curve" by limiting some statistic of the infected population under a certain threshold.

5.2.1 Influenza Simulation

In this section, we consider a scenario where an *influenza* (not COVID-19) outbreak has occurred in Allegheny County (similarly to (Grefenstette et al., 2013)), and policy makers wish to limit the number of infections to less than 10% of the county's population. To achieve this hypothetical goal, policy-makers might consider the following controls among others (corresponding to the θ parameter defined in **Table 1**):

- A "social distancing" policy which mandates all citizens stay home for a fixed period of time. Here, policy makers must influence:
  1) θ_1, a *shelter-in-place duration*, or the length of time a social distancing policy must be in place.
  2) θ_2, a *shelter-in-place compliance rate*, or the percentage of the population to which this policy applies.
- θ_3, a *symptomatic isolation rate*, the fraction of symptomatic individuals that self-isolate during an epidemic.
- θ_4, a *school closure attack rate threshold*, a threshold on the total percentage of people infected that automatically triggers a 3-week school closure when exceeded.
- θ_5, a *hand washing compliance*, the percentage of the population that washes their hands regularly.

For simplicity of results interpretation we have put uniform priors, appropriately continuous or discrete, on intervals of interest for these controllable parameters.

Also relative to **Eq. 7** we choose Y_t to be a binary variable indicating if the proportion of the county's infected population is below 10% on day t. By inferring $p(\theta \mid \forall_{t>0} : Y_t = 1)$, we characterize which control values will lead to this desired outcome.

Using the model described in **Section 4.2** with parameters as defined in this section, we used PyProb to perform automated inference over the policy parameters described in **Table 1**. To reiterate, we infer the posterior distribution over control parameters θ which satisfy the goal of limiting the instantaneous number of infections to less than 10% of the county's population at all times up to a maximum of 150 days. In conducting our experiments we generated one million samples, 40% of which satisfied the policy goal.

5.2.1.1 Simulator Configuration

We simulate Allegheny County, Pennsylvania, using 2010 U.S. Synthetic Population data. We use an older, publicly available version of FRED[6] and its default parameters, which simulates a model of influenza "in which infectivity starts 1 day before symptoms and lasts for 7 days. Both symptoms and infectivity ramp up and ramp down."[7] All simulations begin with 10 random agents seeded with the virus at day zero. Person-to-person contact rates for various environments (household, office, etc.) were calibrated to specifically model Allegheny County. Parameter files for the four policies under consideration are available online[8].

5.2.1.2 Results

Figure 7 shows the SEIR statistics for both the uncontrolled (left) and controlled (right) simulations. We see that the confidence interval in red stays beneath our infection rate constraint indicated by the dashed black line, while in the uncontrolled scenario the confidence band well exceeds the constraint. We also observed the overall number of exposed and infectious populations is much lower using samples of control variables from the inferred posterior, which indicates that control variable values drawn from this posterior are indeed effective in limiting the spread of the virus.

While **Figure 7** indicates that the inferred controls can achieve the desired aims, it doesn't indicate *which* policy to choose. To answer this, we plot the two-dimensional marginal distributions over controlled policy parameters in **Figure 8**. Policy-makers could use a figure like this, coupled with a utility function, to generate policy recommendations. Interpreting **Figure 8**, first we see the importance of influencing hand washing compliance and quick school closures. This plot indicates that hand washing compliance must be driven above 50% and the school closure attack rate threshold beneath 7% in order to achieve our stated

[6]https://github.com/PublicHealthDynamicsLab/FRED

[7]https://github.com/PublicHealthDynamicsLab/FRED/blob/FRED-v2.12.0/input_files/defaults

[8]https://github.com/plai-group/FRED/tree/FRED-v2.12.0/params

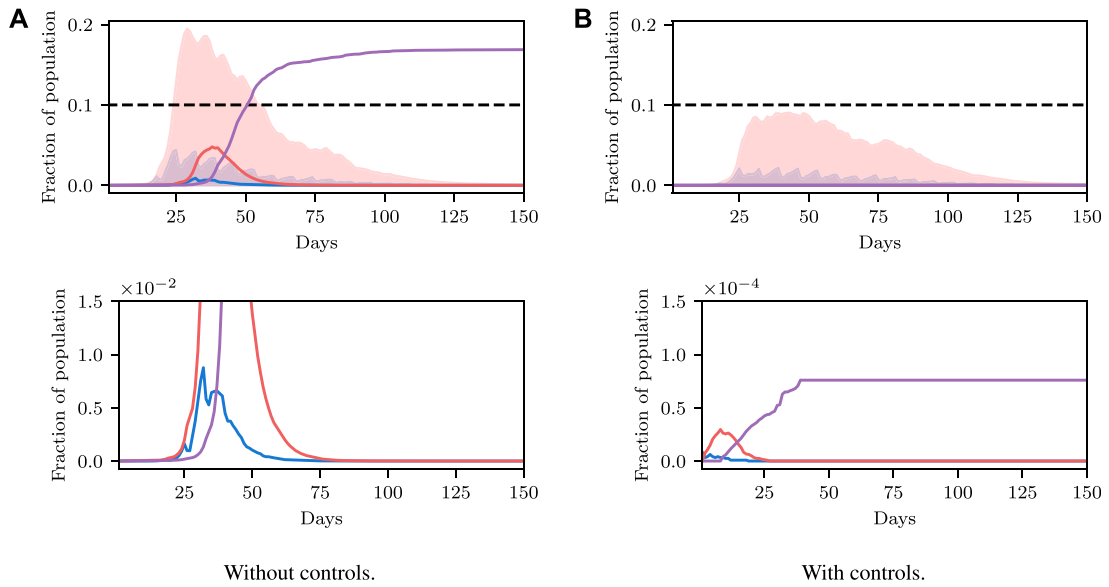

FIGURE 7 | Aggregated SEIR statistics extracted from 100,000 FRED simulations of an influenza (not COVID-19) outbreak in Allegheny County. The objective in this scenario is to keep the number of infectious people (I in SEIR terminology, shown here in red) below 10%, indicated by the black dotted line. We plot the median and confidence intervals between the 3rd and 97th percentiles, shown by the shaded areas. To avoid clutter and focus on relevant dynamics, the susceptible statistic (S in SEIR terminology, shown previously in green) and confidence intervals for the Recovered (R in SEIR terminology, shown here in purple) statistic are omitted. Blue shows the fraction of the population that has been exposed (E in SEIR terminology). The bottom row shows a zoomed-in version of the top row, where all confidence intervals have been removed, for ease of visual inspection. **(A)** shows the evolution of the outbreak when no controls are applied. **(B)** shows the evolution of the outbreak when controls, solved for using our method, are applied. When no controls are applied we see the number of infectious people often exceeds the allowable threshold, whereas, when optimal controls are applied, the confidence interval for infectious people (red) stays below our constraint.

goal. We also note the correlation between hand washing compliance and isolation rate. If we can achieve only 70% hand washing compliance then the isolation rate must be driven high, however, if hand washing compliance is very good then a lower isolation rate is tolerable. The importance of hygiene and long-term school closures has also been noted in the epidemiology literature (Lee et al., 2010; Saunders-Hastings et al., 2017; Germann et al., 2019).

A final interpretation of the results of **Figure 8** can be provided in terms of the outcome of a hypothetical influenza policy-making scenario. A recommendation one might extract from this visualization of the posterior inference results is a conjunction of the following controls:

1) Ensure that all schools close as soon as 2% of the population contracts the virus.
2) Attempt to drive the hand washing compliance to 80%.
3) Attempt to drive the symptomatic isolation rate to 50%.
4) No amount of sheltering in place is required.

Such a recommendation corresponds to what one could imagine would be, in comparison to more draconian options, a relatively mild, economically speaking, policy response that still attains the objective. Of course, in the presence of a real influenza outbreak, vaccination would be among the most critical controls to consider. We have intentionally excluded it here in order to be in some small way more reflective of the COVID-19 pandemic which is emerging at the time of conducting this experiment.

The results of applying this policy are shown in **Figure 9**, where we show a single simulation of the spread of influenza in Allegheny county over time in both the uncontrolled (left) and controlled (right) cases. Each red dot shows the household of an infectious person. This policy recommendation can be seen to work as it reduces the maximum number of infected households from 215,799 on day 29 in the uncontrolled case to 65,997 on day 83 in the uncontrolled case. The maximum in the controlled case actually occurs during a second "outbreak" after schools re-open (noticeable around day 90 in **Figure 9C**). While the controlled policy results in two "spikes," our inference procedure accounts for this and correctly controls the maximum number of infectious persons to remain below 10% of the total at all times.

5.2.2 COVID-19 Simulation

In this section, we consider COVID-19 outbreaks of/in the Seattle metropolitan area. The controls we consider in these experiments are the following (corresponding to the θ parameter defined in **Table 2**):

- θ_1, a *social distancing*, or the fraction of population practising social distancing.
- θ_2, a *workplace closure*, or the fraction of businesses deemed essential and exempt from workplace closure mandates.
- θ_3, a *school closure attack rate threshold*, a threshold on fraction of population hospitalized per day. Schools get closed if the daily hospitalization rate exceeds this threshold.

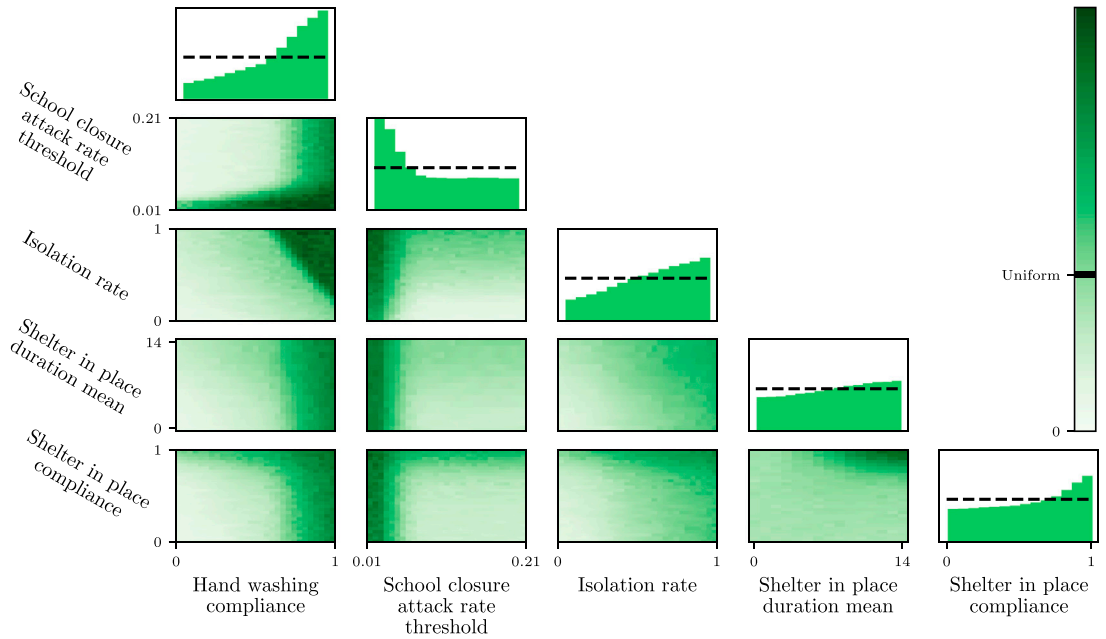

FIGURE 8 | Empirically determined marginals of the full posterior distribution over controllable policy parameters that lead to desired outcomes (*see* **Eq. 7**) for a simulated influenza epidemic in Allegheny county. Along the diagonal: one-dimensional marginals of each control parameter with a uniform prior indicated by the dashed line. We can clearly see the efficacy of high rates of hand washing and a quick school closure policy, as indicated by the non-uniformity of the marginal distributions. The remaining array of plots show two-dimensional marginal distributions of any two parameters where the dark green color indicates higher probability density. For reference, the color corresponding to a uniform prior on the two-dimensional plots is indicated on the color bar on the right. This illustrates policy-level outcomes such as there being a strong interaction when jointly enforcing high *isolation rate* and *hand washing compliance*. In contrast, the effectiveness of *school closure attack rate threshold* is largely independent of the *isolation rate* (and indeed most other parameters). Such richly structured information is paramount for making effective and *justifiable* policy decisions, and is only provided through fully Bayesian analysis, as opposed to simpler reinforcement learning or optimal control methodologies which may only provide a point estimate of the optimal control to be applied, with no quantification of uncertainty or the joint interaction of parameters.

Similar to **Section 5.2.1**, we have put uniform priors on these controllable parameters, as described in **Table 2**.

We put thresholds on the number of daily hospitalizations and daily deaths in different experiments. Hence, Y_t in **Eq. 7** is a binary variable indicating if any of the thresholds are exceeded.

5.2.2.1 Simulator Configuration

Our simulations presented here are on a synthetic population of the Seattle metropolitan area incorporated in the FRED simulator (a population size of 3,416,570). The synthetic population closely matches the 2010 census data for the United States with high spatial resolution, differing from the American Community Survey by less than 1% for large counties. A detailed description of the methodology and further comparisons are provided in (Wheaton et al., 2009). The parameters of the disease are calibrated to COVID-19, as explained in **Section 4.2**. We have conducted simulations in three different time periods and the initial conditions of each simulation (number of active infections, total hospitalizations, total deaths and vaccinated people) is set according to the numbers reported by U.S. health officials for the chosen dates (*see* **Table 3**).

5.2.2.2 Results

Figure 10 shows the number of daily cases, hospitalizations, and deaths (note that this is not the same as SEIR) for all time periods considered in this experiment. Similar to SEIR plots, it shows results for both the uncontrolled (left) and controlled (right) simulations.

Figure 11 shows samples from two-dimensional marginals of the distribution over controlled policy parameters. According to these results, social distancing is the most important control among the three and policy makers should make sure more than 70% of the population practice it. Although enforcing a more strict school or workplace closure helps as well and allows a slightly wider range for social distancing compliance, they are far less effective than social distancing. Adding more relative control parameters (for example, public mask wearing, recommended vaccine booster shots, restricting large events, imposing travel restrictions, etc.) are planned extensions of future research.

We have conducted COVID-19 simulations for three different scenarios:

- The 6 months following March 1, 2020 when the outbreak is in its early stages with a few cases.
- The 6 months following December 1, 2020 when there is a large number of cases, hospitalizations and deaths.
- The 12 months following June 1, 2021 when the number of hospitalizations is the largest among our simulations and there are more than 3000 active infections and near

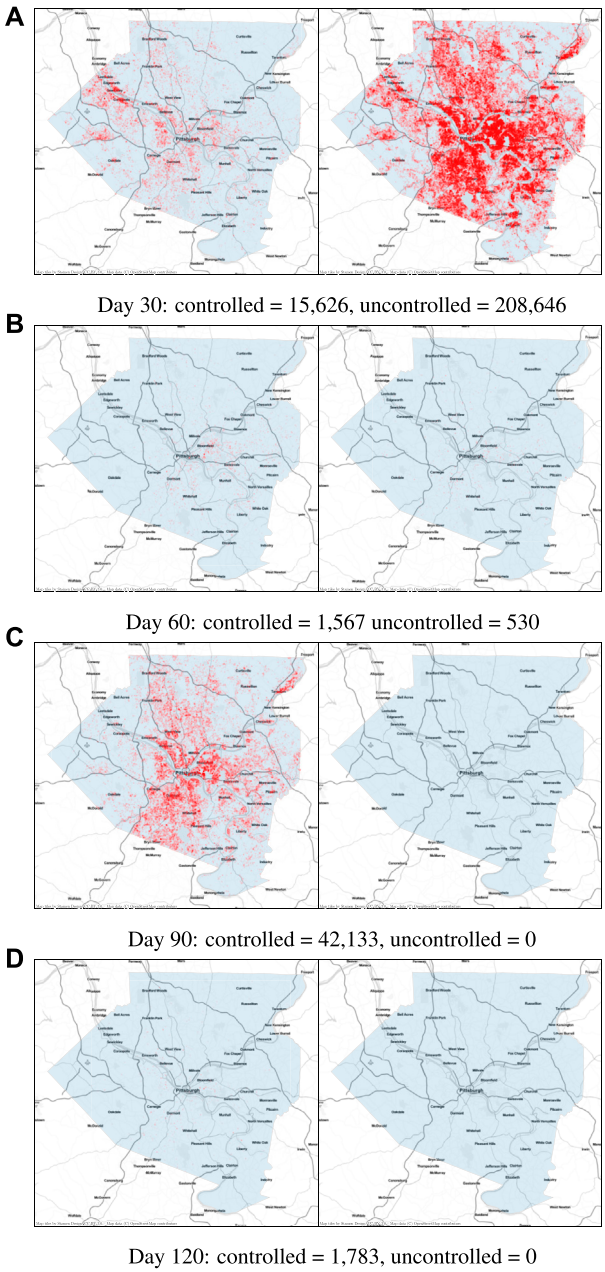

FIGURE 9 | Progression of a simulated influenza epidemic in Allegheny county under controlled **(left)** and uncontrolled **(right)** scenarios. Each red dot represents the household of an infectious person. The count of the number of infected households in each scenario appears in the captions below each row. The peak number of cases in the uncontrolled scenario is 215, 799 on day 29, while the peak number of cases in the controlled scenario is 65, 997 on day 83. We see that the controls we solve for successfully "flatten the curve," indicated by a much lower density of red dots. There is also a second spike predicted, visible in **(C)**, where as controls are removed there is an increase in cases throughout the susceptible portion of the population. However, this second spike is still below the required threshold.

3000 deaths. However, in this case 47% of the population are vaccinated at the beginning of the simulation.

TABLE 2 | Prior over FRED control parameter $\theta = \{\theta_1, \theta_2, \theta_3\}$ for COVID-19 simulations.

Prior	Control
$\theta_1 \sim$ Uniform(0, 1)	social distancing
$\theta_2 \sim$ Uniform(0, 1)	workplace closure
$\theta_3 \sim$ Uniform(1, 1.5×10^{-4})	school closure attack rate threshold

The goal in the first two scenarios is to avoid the number of daily hospitalizations and daily deaths exceeding 409 and 100 people, respectively, while these numbers are 102 and 20 for the third scenario. For the third scenario our results in **Figure 11** show the goals are achieved even with a slightly looser set of controls over the population despite the large number of active infections and hospitalizations and tighter goals. This is indeed the effect of higher vaccination rates in the initial conditions for the third scenario.

Finally, as a part of this project we have created a website[9] where users can perform planning as inference in COVID-19 simulations for different locations in the U.S. and Canada. It allows users to choose initial conditions of the simulations, policy goals and the set of control parameters (or interventions) to explore. The website automatically runs simulations on the population of the specified location and produces an interactive version of **Figures 10**, **11**. It also provides the location of infections on the selected location's map, similar to **Figure 9**. We are working on expanding the features and set of controllable parameters on the website as well.

6 SOFTWARE

In this paper, we have introduced and reviewed control as inference in epidemiological dynamics models and illustrated how this technique can be used to substantially increase the level of automation and precision of some aspects of policy-making using models at two extremes of expressivity and fidelity–compartmental and agent-based. We are releasing accompanying source code for our SEIR-type COVID-19 model along with bespoke inference code that researchers in the machine learning, control, policy-making, and approximate Bayesian inference communities can use immediately in their own research. We are also releasing code that demonstrates how a complex, existing, agent-based epidemiological dynamics model can be quickly interfaced to an existing probabilistic programming system so as to automate, even in such a complex model, the inference tasks resulting from the problem formulation of control as inference. Again, we stress that we do not report methodological innovation per se, as there are other ways of recombining existing inference packages and existing models to approach the control as inference

[9]https://covid19ideas.cs.ubc.ca/

TABLE 3 | Initial conditions and policy goals used in the COVID-19 simulations in the Seattle metropolitan area.

Simulation dates	Active infections	Total hospitalizations	Total deaths	% Vaccinated	Policy goals	
					Hospitalizations	Deaths
1 Mar. 2020–1 Sep. 2020	400	0	1	0	409	100
1 Dec. 2020–1 Jun. 2021	8017	2096	1459	0	409	100
1 Jun. 2021–1 Jun. 2022	3024	8313	2867	47	102	20

problem. In this sense, our principal goal is to demonstrate the feasibility of, and raise interdisciplinary awareness for, such approaches, rather than to champion any specific implementation.

The first part of the software release accompanying this paper is a pure Python implementation of the SEI^{3}R model adapted to COVID-19 from **Section 4.1**. It is contained inside an online software version control repository and consists of the model, "custom" inference code that implements control as inference via approximate nested Monte Carlo (NMC), and code that shows how to perform stochastic model predictive control using the model, i.e., the code used to produce **Figure 6**.

The second part of the software release accompanying this paper encompasses an influenza model implemented using an older, open source version of the agent-based simulation platform FRED (Grefenstette et al., 2013), as well as the modifications necessary for the latter to be interfaced with the probabilistic programming system PyProb for simulator-based approximate Bayesian inference. This code is contained in three separate repositories: a fork of the FRED repository with PyProb integration[10], a fork of the PyProb code with slight additions required for FRED integration[11], and a collection of scripts for orchestrating and plotting artifacts from simulation and inference[12]. This last repository also contains a Singularity container (Kurtzer et al., 2017) image bundling all of the necessary software dependencies, including the repositories above. This should significantly reduce the setup time for interested parties, as Singularity images are supported at a large number of existing high performance computing facilities. While the current FRED source code is proprietary, we are happy to discuss the process of PyProb+FRED integration with any interested parties.

We believe that model-inference combinations such as FRED+PyProb could provide formidable policy analysis tools with potentially significant societal benefits, particularly because they would allow high-fidelity assessment of region-specific targeted policies. We expect that the set of effective policy interventions might differ across the regional characteristics of counties, states or countries, as well as their transient epidemiological situations. Testing this hypothesis depends upon expanding the number of counties, provinces, and countries with simulation profiles in FRED, or in any other agent-based epidemiological model that can be interfaced with a universal and language-agnostic probabilistic programming system such as PyProb.

[10]https://github.com/plai-group/FRED

[11]https://github.com/plai-group/pyprob

[12]https://github.com/plai-group/covid

7 DISCUSSION

Our experience in conducting this research has led us to identify a number of opportunities for improvement in the fields of simulation-based inference and control.

7.1 Software Tools

Building a simple SEIR-type model with very few compartments is a project of the scope of graduate homework. Building and maintaining a simulator like FRED or the US National Large Scale Agent Model (Parker and Epstein, 2011) is a massive undertaking, which would be prohibitive to replicate or significantly extend in a crisis situation. As far as we could find with limited search effort when conducting this work in March 2020, there was neither a central repository of up-to-date open-source agent-based epidemiological models, nor an organizing body we could interface to immediately.

7.2 Methodology

There appear to be practically very consequential conceptual gaps between the fields of control, epidemiology, statistics, policy-making, and probabilistic programming. To quote Lessler et al. (Lessler et al., 2016), "the historic separation between the infectious disease dynamics and 'traditional' epidemiologic methods is beginning to erode." We, like them, see our work as a new opportunity for "cross pollination between fields." As discussed earlier, the most closely related work that we found in the literature is all focused on automatic model parameter estimation from observational data (Toni et al., 2009; Kypraios et al., 2017; McKinley et al., 2018; Chatzilena et al., 2019; Minter and Retkute, 2019). These methods and the models to which they have been applied could be repurposed for planning, as we have demonstrated, simply by changing the random variables they condition on to include safety or utility metrics. Our emphasis therefore lies on the feasibility of planning as inference using existing software tools for approximate Bayesian inference.

Looking closer at the implementation choices, we found at least two existing papers that explore using probabilistic programming coupled to epidemiological simulators; (Funk and King, 2020) which used Libbi (Murray, 2015) and (Gram-Hansen et al., 2019) which used PyProb. The latter is an example of work that "hijacks" a malaria simulator in the same way we "hijacked" the FRED

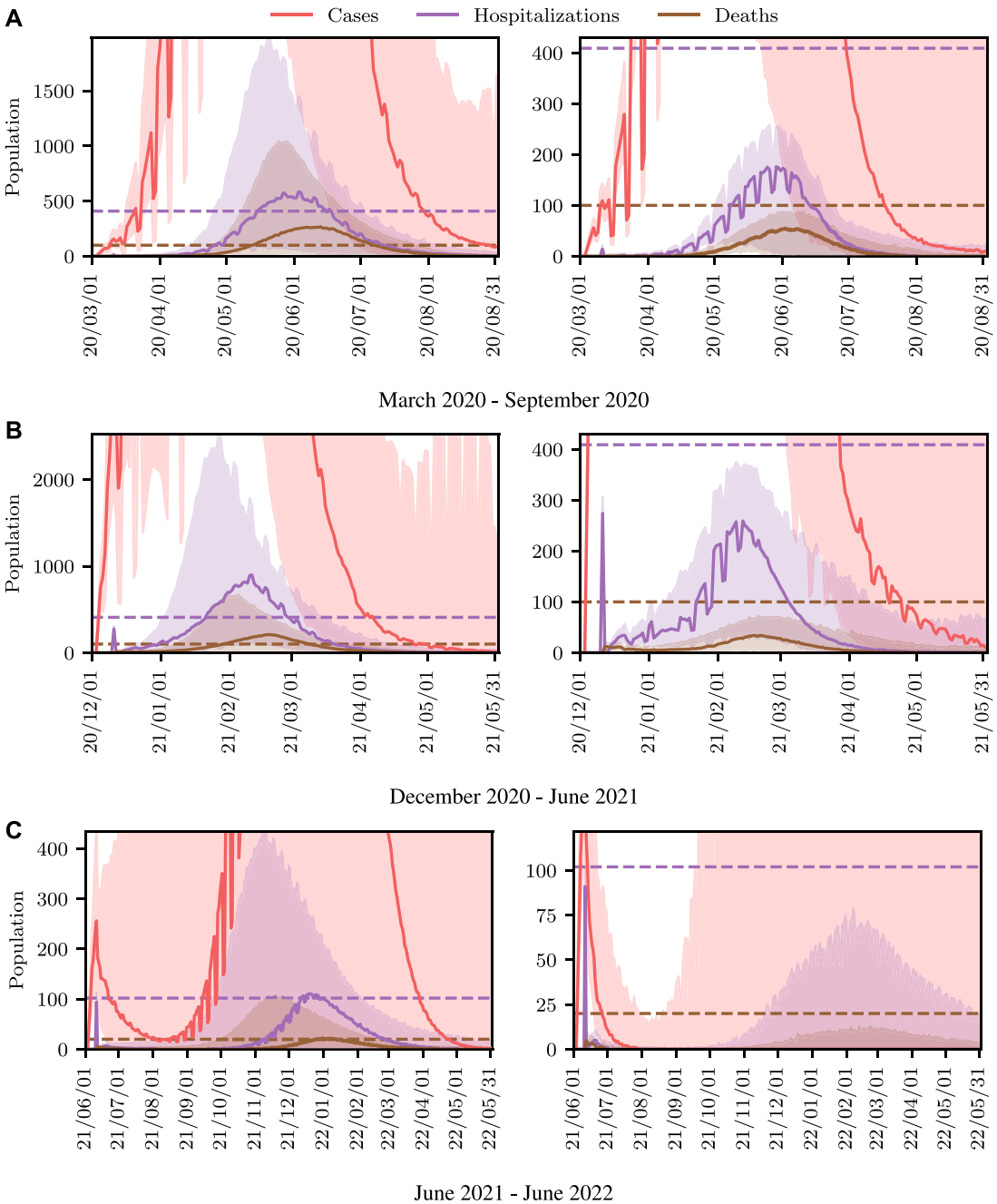

FIGURE 10 | Aggregated statistics from 1,000 FRED simulations of a COVID-19 outbreak in the Seattle metropolitan area. We plot the median and confidence intervals between the 3rd and 97th percentiles of the *daily* cases, hospitalizations, and deaths. Each row shows a different period of time with different starting conditions and policy goals. The goal in these simulations is to keep the number of daily hospitalizations and deaths below the thresholds specified by dashed lines in matching colors. The left column shows the evolution of the outbreak when no control is applied. The right column shows the evolution of the outbreak when controls provided by our method are applied. As expected, without control the number of hospitalizations and deaths quickly exceeds the thresholds whereas in the controlled simulations it is ensured that all the desired conditions are met throughout the simulation period.

simulator in this paper. Neither explicitly addresses control. Chatzilena et al., (2019) use the probabilistic programming system STAN Carpenter et al. (2017) to address parameter estimation in SEIR-type models, but it too does not explore control, nor could it be repurposed to control an agent-based model with non-differentiable joint densities or, more generally, any external simulator not directly defined in the STAN language.

Like (Toni et al., 2009; Kypraios et al., 2017; McKinley et al., 2018; Chatzilena et al., 2019; Minter and Retkute, 2019) we too could have demonstrated automated parameter estimation in both of the

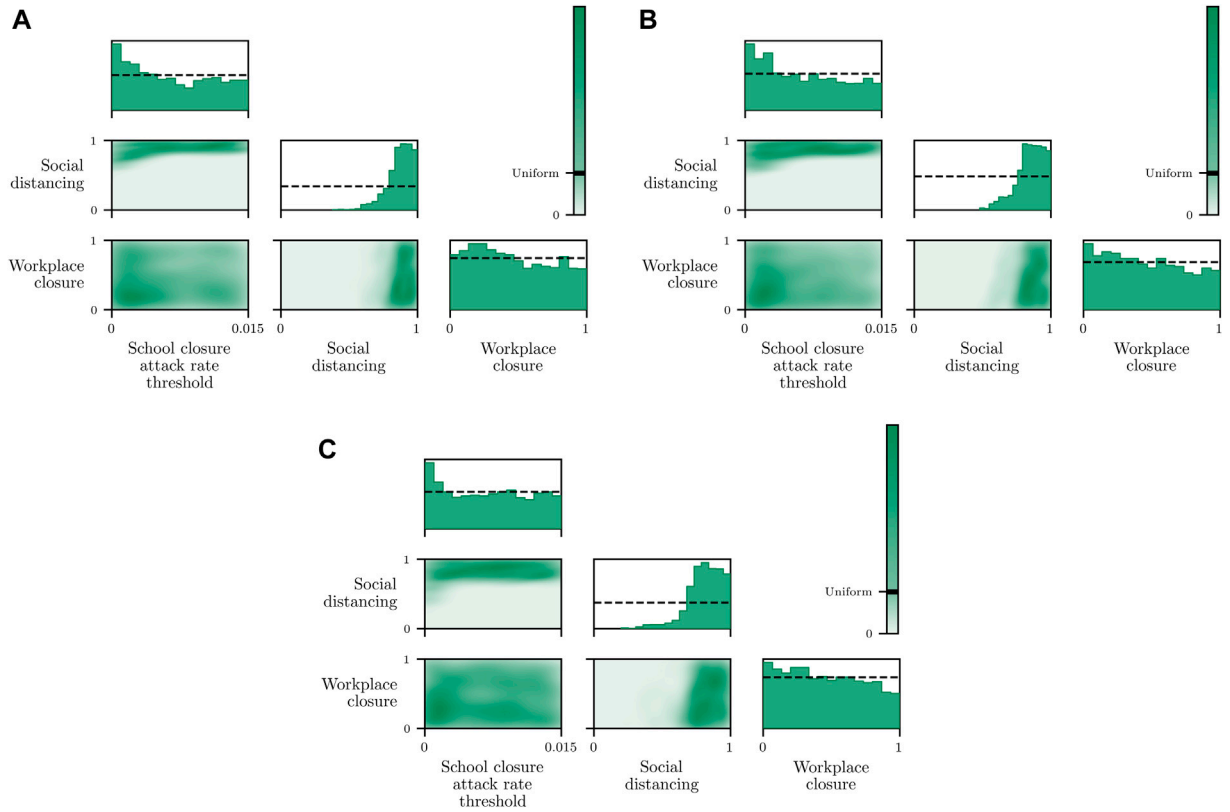

FIGURE 11 | Empirically determined marginals of the full posterior distribution over controllable policy parameters that give rise to appropriately controlled outcomes (*see* **Eq. 7**) for simulations of COVID-19 of/in the Seattle metropolitan area. Similar to **Figure 8**, the diagonal of each figure shows one-dimensional marginals for each policy parameter, with the uniform prior indicated by the dashed line. The remaining array of plots shows kernel density estimation (KDE) plots fitted to samples from the two-dimensional marginals of the joint posterior distribution. For reference, the color corresponding to a uniform prior on the two-dimensional plots is indicated on the color bar on the right. Each of the Subfigures **(A-C)** shows the results of simulations for a different time period as indicated. The initial parameters and policy goals for each plot are reported in **Table 3**. We see that among the three policy parameters, social distancing is the most effective in all time periods. Furthermore, putting a more restrictive policy on closing schools or workplaces leads to a wider range of acceptable social distancing policies. Finally, note that in the June 2021 - June 2022 period, when the vaccination rates are high, a looser set of policy enforcements is acceptable even though the desired outcomes for this experiment are much tighter (*see* **Table 3**).

models that we consider, for instance to automatically adjust uncertainty in disease-specific parameters by conditioning on observable quantities such as death counts that are measured online during a breakout. However, as the epidemiological community already relies upon long-standing methods established for estimating confidence intervals for model parameters during evolving pandemics, we exclusively restricted ourselves to demonstrating how to achieve control via inference, assuming that the disease parameter priors have been obtained from established parameter estimation techniques. Combining the two kinds of observations in a single inference task is technically straightforward but does require care in interpretation.

8 FINAL THOUGHTS

At the time of writing all authors, apart from JG and DC at Epistemix Inc., Pittsburgh, were principally affiliated with the "Programming Languages for Artificial Intelligence" (PLAI) research group at UBC, Vancouver, which is primarily involved in developing next-generation probabilistic AI tools and techniques. We felt, however, that the acute circumstances demanded we lend whatever we could to the global fight against COVID-19. Beyond the specific contributions outlined above, our secondary aim in writing this paper was to encourage other researchers to contribute their expertise to the fight against COVID-19 as well. We believe the world will be in a better place more quickly if they do.

DATA AVAILABILITY STATEMENT

The FRED simulator and model configurations used for the COVID-19 experiments are proprietary products of Epistemix Inc. All the other source codes, parameters and datasets presented in this study can be found at the open source repository https://github.com/plai-group/covid.

AUTHOR CONTRIBUTIONS

FW - conception, formulation, SEIR, FRED, writing; AW - conception, formulation, SEIR, writing; SN - conception, formulation, FRED, writing; CW - conception, formulation, FRED, writing; VM - conception, formulation, FRED, writing; WH - conception, formulation, SEIR, writing; AS - conception, formulation, writing; BB - conception, formulation, writing; DC - FRED; JG - FRED, writing; SAN - conception, writing.

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