Oxidative Medicine and Cellular Longevity

Oxidative Stress in the Pathogenesis of COVID-19

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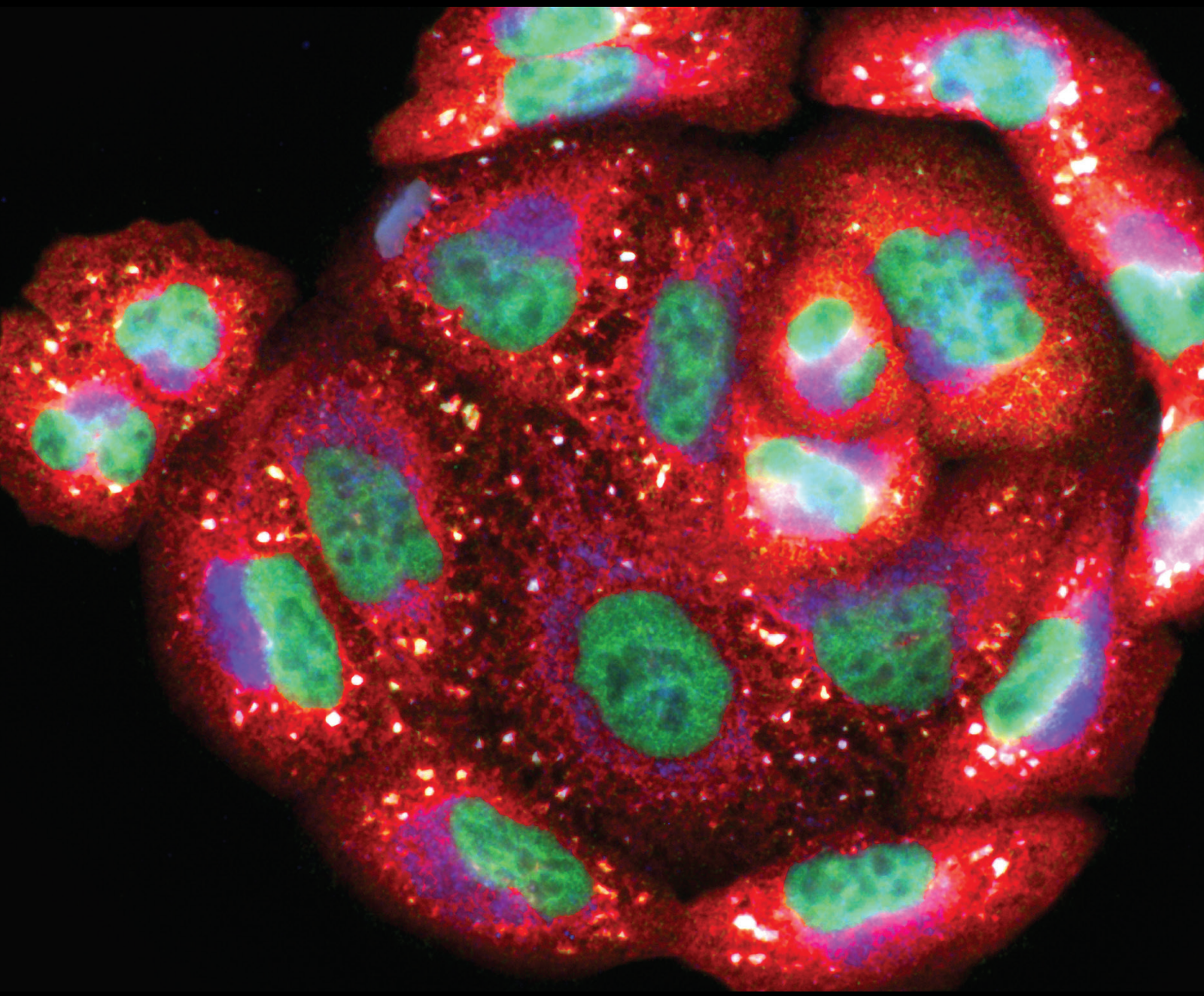

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Review Article

Oxidative Stress-Related Mechanisms in SARS-CoV-2 Infections

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The COVID-19 pandemic caused relatively high mortality in patients, especially in those with concomitant diseases (i.e., diabetes, hypertension, and chronic obstructive pulmonary disease (COPD)). In most of aforementioned comorbidities, the oxidative stress appears to be an important player in their pathogenesis. The direct cause of death in critically ill patients with COVID-19 is still far from being elucidated. Although some preliminary data suggests that the lung vasculature injury and the loss of the functioning part of pulmonary alveolar population are crucial, the precise mechanism is still unclear. On the other hand, at least two classes of medications used with some clinical benefits in COVID-19 treatment seem to have a major influence on ROS (reactive oxygen species) and RNS (reactive nitrogen species) production. However, oxidative stress is one of the important mechanisms in the antiviral immune response and innate immunity. Therefore, it would be of interest to summarize the data regarding the oxidative stress in severe COVID-19. In this review, we discuss the role of oxidative and antioxidant mechanisms in severe COVID-19 based on available studies. We also present the role of ROS and RNS in other viral infections in humans and in animal models. Although reactive oxygen and nitrogen species play an important role in the innate antiviral immune response, in some situations, they might have a deleterious effect, e.g., in some coronaviral infections. The understanding of the redox mechanisms in severe COVID-19 disease may have an impact on its treatment.

1. Introduction

Patients with pneumonia of unknown etiology had been diagnosed in mid-December 2019 in Wuhan (Hubei province, China). Later, the SARS-CoV-2 (severe acute respiratory syndrome) coronavirus started to spread all over the world, without any exemptions. As for today, more than 182 million of patients have been infected, and more than 3.9 million died due to COVID-19 [1], providing an estimate of the mortality rate at 3.3%. When compared to the seasonal flu, COVID-19 related mortality is at least 60 times higher. Seasonal flu outbreak annually causes the infection of 3 to 5 million people, both asymptomatic and symptomatic, with mortality rate not exceeding 0.05% [2]. Clinical course of COVID-19 may, in most cases, consist of three periods [3]. After a short incubation period lasting from 2 to 5 days, patients become symptomatic, with the loss of sense of taste and olfactory dysfunction, dry cough, fever exceeding 38°C, and dyspnoea. Other symptoms, including headache, fatigue, diarrhoea, and conjunctivitis, are less frequent. Additionally, most patients develop a bilateral interstitial pneumonia [4]. After 7-10 days, dyspnoea decreases in majority of patients, inflammatory changes in the lungs resolving to some extent, and the patients are free from the virus in most cases. In severe COVID-19, the pneumonia causes a rapid drop in arterial pO_2 levels with the transcutaneous saturation measurement, usually below 60% when breathing ambient air. The progressive respiratory failure due to the loss of lung active surface of gas exchange and vascular abnormalities leads to the need of noninvasive ventilation support. In most severe cases, patients suffer from disseminated intravascular coagulation (DIC) or a septic shock and have to be sedated and undergo ventilation support [5].

Some experimental data available so far has suggested that the severe COVID-19 course might be related to the viral load during the SARS-CoV-2 exposure [6]. A recent study performed in 1145 patients suggested a significant independent association between viral load and mortality (with the hazard ratio of 1.07 [95% CI 1.03–1.11], $p = 0•0014$) implying that the 7% increase in mortality risk was

present for each log transformed copy of viral RNA per mL of nasopharyngeal swab sample [7]. Another important factor, probably protecting from the development of severe COVID-19, is a normal to high level of serum vitamin D [8, 9]. Smoking cigarettes, however, may increase the risk of severe course of the disease, even in the absence of smoking-related disease [10–12]. The well-known and widely accepted hypothesis is that the male sex, hypertension, COPD, diabetes, or cancer may deeply influence the severity of the disease [13–16].

Today, it is not clear whether bronchial asthma may have any effect on the infection rate or the severity of COVID-19. Moreover, the question of how and why the viral pneumonia leads to DIC and septic shock with cytokine and bradykinin storms remains to be elucidated. ROS and RNS play an important role in the innate immune response, which is also directed against viruses [17]. In this review, we focus on the possible role of ROS and RNS in severe COVID-19 pathogenesis.

2. Antiviral Immune Response Mechanisms

The immune system has the potential to effectively control viral infections, and thus, it can limit their effect on the host organism. The processes of virus entry into the host cell, its replication, stimulation, and regulation of the antiviral immune response trigger a complex series of interactions between the virus and the host [18] (Figure 1(a)). There are two defense mechanisms: specific, acquired immunity and nonspecific, innate immunity. Nonspecific immunity is the first line of defense against infection and does not depend on prior contact with the pathogen. Mast cells, NK (natural killers), NKT (natural killer T cells), NHC (natural helper cells), natural lymphoid cells, granulocytes, macrophages, and monocytes are responsible for innate immunity. The pathophysiology of the extremely high pathogenicity of coronaviruses is not fully understood [19, 20]. It is worth noting that the immune system must develop a specific cytotoxic T cell (CTL) response. CTLs have the ability to recognize the viral-derived peptide on the surface of the infected cell, specifically in the MHC (the major histocompatibility complex) class I binding groove. Then, lymphocytes recognize the infected cell and destroy it by secreting cytolytic granules or activating programmed death in the cell through receptors such as FAS. In parallel with the development of the cellular response, a humoral immune response develops—associated with the activation of B cells and the subsequent release of specific antibodies. The helper T cells are at the center of the activation of adaptive immunity.

The lung epithelium is the largest surface that comes into contact with the environment. In the airways, viruses are detected by airway epithelial cells, mast cells, and cells of the mononuclear phagocyte system. The sensor cells are equipped with pattern recognition receptors such as Toll-like receptors (TLR). PAMPs (pathogen associated molecular patterns), derived from viruses, trigger a specific combination of PRRs (pattern recognition receptors) and adapter molecules, leading to the immune response adapted to the pathogen [21]. Coronavirus replication leads to, e.g., disruption of lysosomes, damage of mitochondria or/and imbalanced ion concentrations [22, 23]. As a consequence, pyroptosis occurs, which initiates the secretion of proinflammatory molecules of the interleukin-1 family [24, 25] (Figure 1(b)). Coronavirus SARS-CoV-2-induced cell death releases histones and a high-mobility group box 1, which are normally hidden from recognition by PRRs. Then, additional proinflammatory cytokines and chemokines are produced, e.g., IL-6, IP-10, MIP1$\alpha\beta$ (macrophage inflammatory proteins-1$\alpha\beta$), and MCP1 (monocyte chemoattractant protein-1). Only in theory, detection of CoVs by pattern recognition receptors triggers an innate immune response that would be effective to limit viral replication. Interferons (IFN)-α, β, and type III are released to help control/eliminate viral infection. Their function is to remove the virus from infected cells by activating ISGs (IFN-stimulated genes) which exert direct antiviral effects, i.e., recruit antiviral immune effector cells. It has been observed that during zoonosis, the antiviral immune response can be detrimental to the body if the timing and target tissue of the immune response are inadequate [26].

The mechanism of innate immunity leads to inflammation, release of IFN-$\alpha\beta$, and activation of NK cells, which allows the suppression of local infection. Unfortunately, coronaviruses have developed strategies to protect themselves or their by-products from being recognized by the host [27]. In addition, viruses inhibit interferon induction and block IFN signaling. For example, SARS-CoV-1 (the coronavirus emerged in 2003, causing severe acute respiratory syndrome coronavirus) can effectively suppress interferon expression by nonstructural and structural proteins [28]. Coronaviruses circumvent the early phase of the innate immune response. Generalizing, the virus is recognized due to the stimulation of Toll-like receptors located on the epithelium and on dendritic cells, which are designed to inform B and T lymphocytes about the invasion of the pathogen. In the case of coronaviruses, these are Toll-like receptors 7 and TLR8 receptors that recognize viral RNA. Viral proteins are recognized by TLR2 and TLR4 receptors. During SARS-CoV-2 infection, the level of these receptors decreases, and their expression is lower in the elderly. SARS-CoV-2 infection is dangerous when a patient lacks specific antibodies and specific CTLs, because it can progress to severe pneumonia and ARDS [29].

3. The Role of ROS and RNS in Antiviral Response

The generation of ROS is one of the major mechanisms leading to infected cell death through apoptosis or necrosis, specifically during the very early stages of the immune response [30]. Both ROS and RNS also play an important role in signal transduction. Viral proteins or nucleic acids triggering the pattern recognition receptors lead to activating the interferon response through TIR-domain-containing adapter-inducing interferon (TRIF) and interferon regulatory factors (IRFs) as well to increasing in the inducible nitric oxide synthase (iNOS) expression and activity through the myeloid differentiation primary response-88 (MyD-88) adapter protein [31]. These

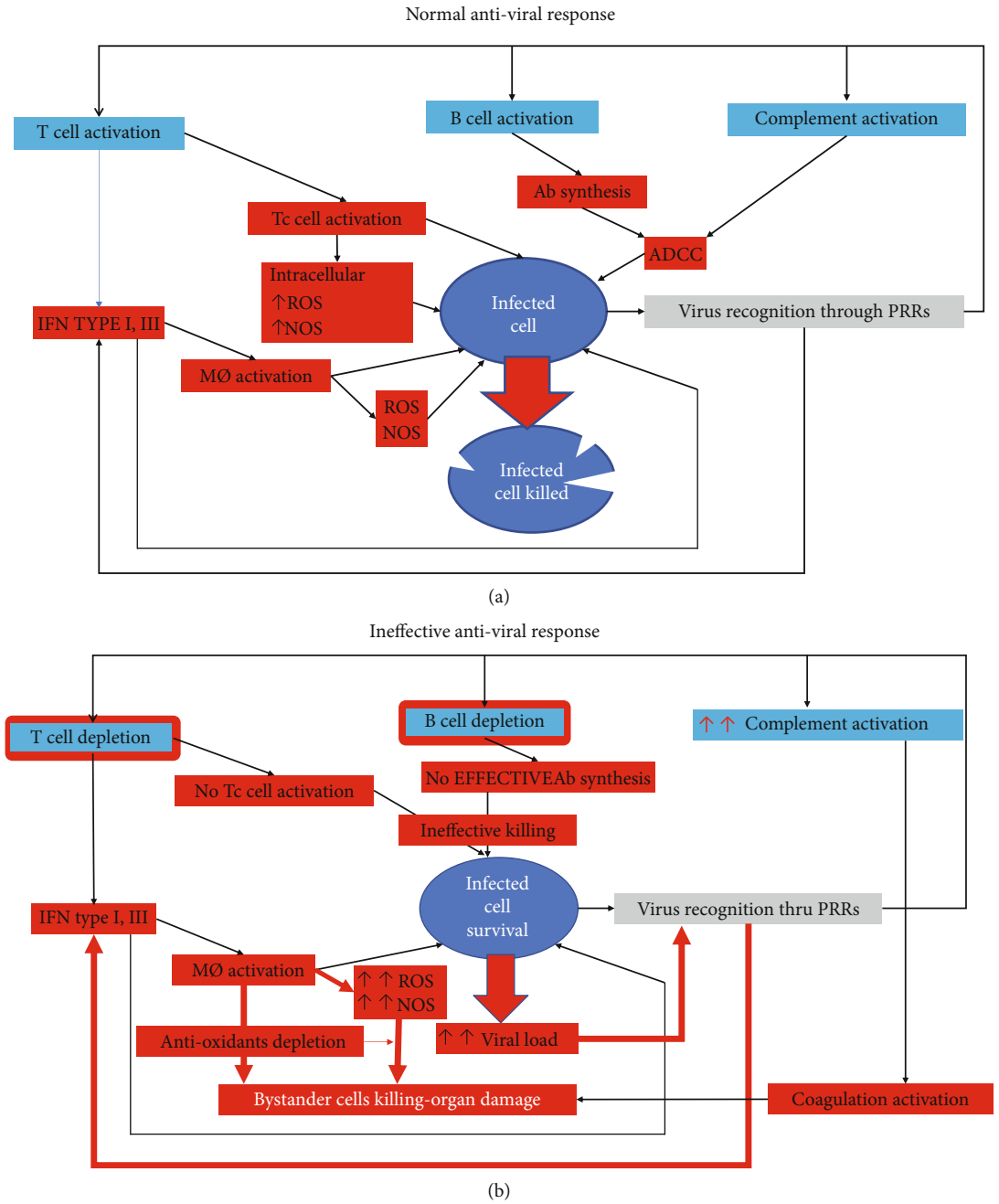

Figure 1: Normal (a) and ineffective (b) antiviral response. Under normal conditions, the presence of a virus activates various pathways leading to its killing by killing the infected cell: activation of complement, B and T lymphocytes, secretion of interferons, antibodies production, and macrophages activation, which result in an increase in ROS and NOS concentrations that help kill the infected cell. An ineffective antiviral response may occur when the responses to the presence of the virus are unnaturally enhanced, resulting in damage to surrounding tissues and as a consequence, organ damage. ADCC: Antibody-dependent cellular cytotoxicity.

processes lead to an increase in the RNS production. The RNS might inhibit viral proliferation in infected cells [32, 33]. Similarly, both PRRs and interferon type I pathways lead to an increase in ROS production from the xanthine oxidase, nitric oxide synthase, or the mitochondrial respiratory reactions. These processes have been crucial in the innate immune response to various viruses including human respiratory viruses (influenza viruses, HRSV (human respiratory syncytial virus), and rhinoviruses).

ROS are signaling molecules regulating a wide variety of physiological functions. ROS are a part of the mechanisms leading to the elimination of virus-infected cells and patient recovery. In some rare cases, specifically in the case of influenza infection, a severe course of the disease develops, leading to a severe adult respiratory distress syndrome (ARDS) with significant mortality [34]. Why ARDS is more frequent in some coronavirus infections (SARS, MERS (Middle East respiratory syndrome coronavirus), and SARS-CoV-2)

remains unknown. Therefore, ROS and RNS might be at least one of the important diseases modifying pathways in severe COVID-19.

The effects caused by the reactive forms of oxygen and nitrogen might depend on the source of their origin. For instance, many RNA viruses activate endosomal NADPH (nicotinamide adenine dinucleotide phosphate hydrogen) oxidase *via* Toll-like receptor 7 mechanism, activated in turn by binding to single-stranded RNA [35, 36]. This is likely because these viruses, when attached to the cell, are built into the endosomes and their RNA can interact with TLR7; SARS-CoV-2 might activate Nox2 (NADPH oxidase 2) through TLR7 and that might have a negative impact on the defense mechanism against viruses. This is due to the fact that Nox2 activation is used by viruses in order to restrain immune reactions and develop the infection [36, 37].

Overproduction of toxic ROS and excessive inflammation are harmful for tissues and may cause their damage [38–41]. As a result of an uncontrolled inflammatory response, oxidative stress (an imbalance between oxidants and antioxidants) arises, which in turn stimulates inflammatory cells to further produce cytokines and a "vicious circle" occurs (Figure 2).

The characteristic features of the severe form of COVID-19 include, but are not limited to, severe lymphopenia, lung tissue damage, and a "cytokine storm" leading to acute respiratory distress and multiorgan failure. Despite a central role of mitochondria in ROS generation, many questions remain unanswered about their role during the "cytokine storm" and pathogenesis of infections with coronaviruses. Lymphopenia causes, among others, a defect in the regulation of antiviral immunity. The cytokine storm begins with the intense activation of cytokine-secreting cells with innate and acquired immune mechanisms [42] (Figure 3). It should be pointed out that in the case of a "cytokine storm", neutrophil apoptosis does not occur. Patients have a huge amount of neutrophils that have undergone NETosis (NET-neutrophil extracellular traps). During NETosis, neutrophil extracellular trap is formed, and along with the "spilling out" of neutrophil DNA outside the cell, toxic enzymes are released, such as elastase, which damages lung tissue [43]. Moreover, microclots in the pulmonary circulation are formed. In the blood of COVID-19 patients, immune changes characteristic of viral infections were observed, i.e., increased levels of ASC-producing cells, activated CD4+ T cells and CD8+ T cells and IgM and IgG antibodies [44, 45]. Importantly, "cytokine storm" may occur, responsible for lung tissue damage during viral respiratory infections [46, 47]. Such sustained ROS production leads to the vicious circle that results in inflammatory damage but also hinders treatment of damage [48].

4. ROS and RNS Generation in SARS, MERS, and COVID-19

The high mortality rates of SARS-CoV-1, SARS-CoV-2, and MERS motivate scientists to study these infections in a variety of ways to find any effective therapeutic options. While numerous studies confirm a strong association between oxidative and nitrosative stress and severity of various viral infections (HCV (hepatitis C virus) [49], HBV (Hepatitis B virus) [50], and HRSV [51]), there is still limited clinical data showing such dependence in case of the SARS-CoV, SARS-CoV-2, and MERS infection—their severity or progression [52]. Previous research demonstrated that in SARS-CoV-infected human lung samples, explicit production of oxidized phospholipids followed by ROS generations was observed in the injured air spaces, pneumocytes, and alveolar macrophages [53]. Moreover, in macrophages, the oxidized phospholipids have been shown to modulate lung injury severity by TLR4-TRIF-TRAF6 expression and trigger cytokine production [22]. Lin et al. published a study showing that the ROS-activated NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signal transduction pathway is induced by SARS-CoV-1 protease-3CLpro and therefore might be involved in the SARS-CoV infection development [54].

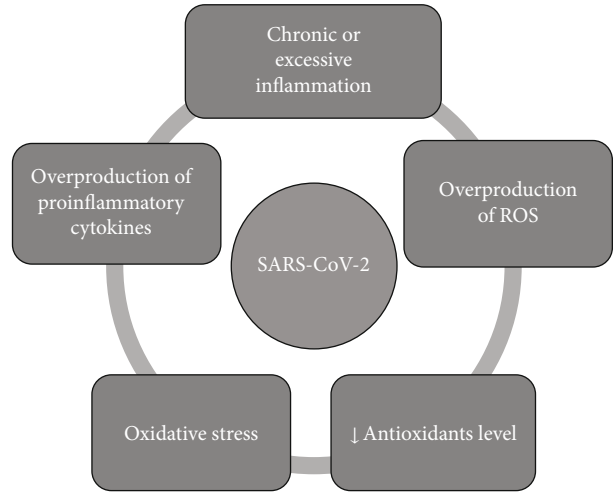

Figure 2: Molecular vicious circle of SARS-CoV-2 infection. Chronic or excessive inflammation damages tissues due to huge amounts of various toxic substances mainly ROS overproduced by cells of the immune system (neutrophils and macrophages). Activated phagocytes can also release prooxidant cytokines, e.g., TNF-α (tumor necrosis factor-alpha) and IL-1, which promote iron uptake by the reticuloendothelial system. The consequence of an uncontrolled inflammatory reaction is oxidative stress, which in turn, stimulates the inflammatory cells to further produce cytokines. Release of interleukins, e.g., 1β, 2, 6, 7, 12, 17, and TNF-α has been observed in COVID-19 [13], resulting in a vicious circle.

Angiotensin converting enzyme-2 (ACE2), known as the cell entry receptor of the SARS-CoV-2, is a multifunctional transmembrane protein. ACE2 plays a double-edged role in SARS-CoV-2 infection, and apart from being the cellular receptor for SARS-CoV-2 spike proteins, it is the critical molecule in combating inflammatory and oxidative damage of tissues by COVID-19. This enzyme decreases angiotensin II which is stimulant of NADPH oxidase. In addition, the product of ACE2 enzymatic activity, angiotensin 1-7, has a strong antioxidant effect [55, 56].

The virus binding to ACE2 receptor initiates its entry to the cell, and after attachment and virion-membrane fusion, ACE2 expression is downregulated [57, 58]. The viral

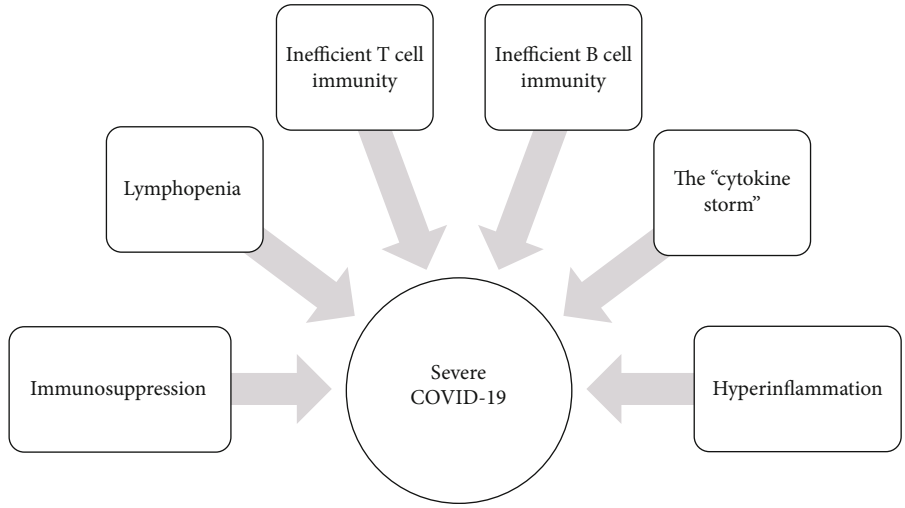

Figure 3: Immune changes characteristic of serve COVID-19. Deregulation of cytokines and influx of inflammatory cells can lead to lung infiltration and critical symptoms; a "cytokine storm" may lead to a dramatic disruption of the homeodynamics of the whole organism and, consequently, even death of the patient [24].

protein Spike interaction with ACE2 leads to an excessive production of angiotensin II (Ang II) and activation of NADPH oxidase which subsequently results in enhancing oxidative stress mechanisms (in contrast to what happens during other viral infections) but also releasing inflammatory molecules [59]. In the course of SARS-CoV-2 infection, angiontensin II availability is increased through the high affinity and resulting binding the virus to ACE2 [60]. ACE2 in SARS-CoV-infected cells has been shown to be also involved in postinfection regulation, including immune response, viral genome replication, and cytokine secretion [61]. A previous study demonstrated that overexpression of ACE2 prevents Ang II-induced Nox2 expression and ROS generation in endothelium [62]. In healthy individuals, ACE2 supports lung homeostasis *via* the production of angiotensin 1–7 and controls inflammation and blood pressure. However, ACE2 downregulation may prevent SARS-CoV-2 host cell interaction in chronic respiratory conditions [63]. ACE2 is expressed in a variety of cells. It has been shown that many factors can influence the changes in ACE2 expression and the progression of COVID-19, including gender and age [64].

The severity of coronavirus infections is generally age related [65], which might be attributed to a disruption in the redox balance, i.e., accumulated oxidative damage and a deteriorated antioxidative defense system followed by increased reactive oxygen species [66]. As a consequence, induction of proinflammatory cytokine expression occurs (such as TNF-α, interleukin (IL) 6, IL-8, and IL-1β), *via* redox-sensitive transcription factors, e.g., NF-κB [67, 68]. Previous genomic analyses of SARS-CoV-1 on aged macaques demonstrated that old subjects presented stronger host response to virus and more severe infection pathology than young ones; this was associated with a reduced expression of type I interferon and an increase in the differential expression of inflammatory genes related to NF-κB [66].

Recent study demonstrated that patients suffering from severe COVID-19 disease, requiring intensive care unit treatment, presented higher levels of Nox2 activation, and thus, Nox2 seems to be a pivotal agent in COVID-19 aggravation [37]. However, Li et al. published data suggesting that the SARS-CoV nonstructural protein nsp10 might impair the redox system in the mitochondria, another ROS source, by a loss in the cellular inner mitochondrial membrane potential. This effect probably enhanced the cytopathic effect of SARS-CoV-1 [69]. Interestingly, it has been shown recently that coronaviruses, thanks to the protein nsp10 in combination with nsp16, can methylate the 5 $'$ends of their mRNAs, thus resembling the host mRNA and protecting them from the innate immune response [70].

Moreover, inflammatory cytokines-TNF-α and IL-6, which may initiate mitochondrial ROS production and are associated with ATP production, were found in COVID-19 serum (Figure 4) [71, 72]. In fact, Saleh et al. proposed recently a hypothesis that, apart from the intracellular mitochondria failure that plays a key role in COVID-19 disease, the extracellular mitochondria are important mediators [73–76]. They provoke the immune response, regulate cell-to-cell communication, and danger sensing [77]. According to the authors, this complex interplay between platelet mitochondrial dysfunction, oxidative stress, and mitophagy would provide useful therapeutic strategies [73]. The excess of ROS can oxidize biomolecules (lipids, proteins, and DNA) or it can structurally modify proteins and genes to trigger signaling cascades that can lead to an inflammatory response. SARS-CoV-2 infection intensifies the already existing oxidative stress in patients of older age with comorbidities, e.g., diabetes, hypertension, and cardiovascular diseases and that is one of the possible explanations for the severity of COVID-19 in these categories of patients [52, 78].

The above mentioned Nox2 is a multisubunit protein, and its activation requires translocation of the cytosolic subunits—p47phox, p67phox, and Rac to the NOX/p22phox membrane complex [79]. Superoxide produced by Nox2 is implicated in influenza-mediated lung pathology [80]. Tang et al. published studies suggesting that endosomes are the

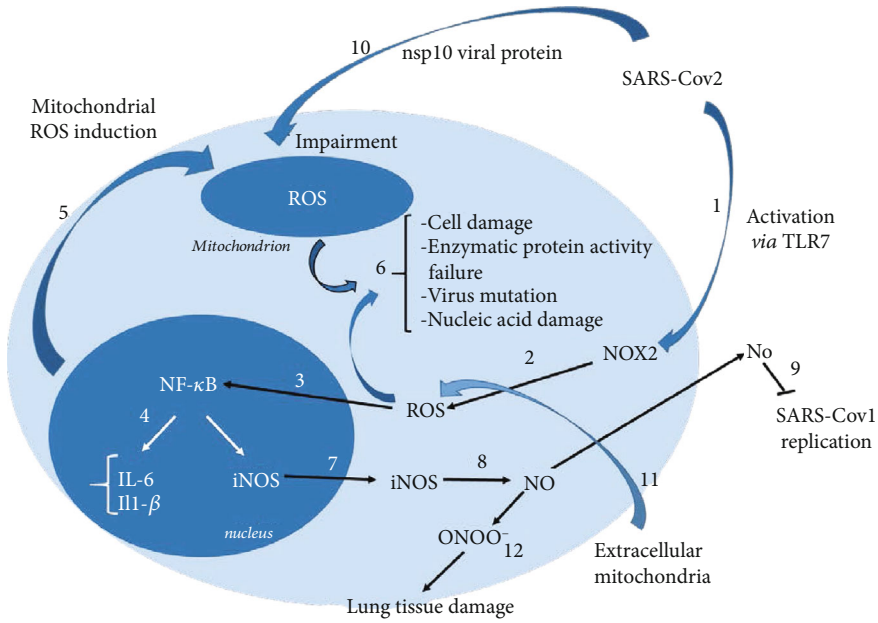

Figure 4: Action of SARS-CoV-2 in proposed mechanisms in the context of reactive oxygen and nitrogen species. SARS-Cov-2 may affect the induction of reactive oxygen species by inducing both of their sources—NADPH oxidase and mitochondria. The increase in Nox2 activity in COVID-19 patients may be related to the activation of this enzyme by TLR7 (1), as is the case with other RNA viruses. Activated NADPH oxidase is responsible for the production of ROS (2), which are related to the activation of NF-κB (3). The activity of this transcription factor results in the expression of proinflammatory cytokines like IL-6 and-1β (4), which in turn can induce the production of mitochondrial ROS (5). On the other hand, ROS, if produced in excess, regardless of the source, may cause cell damage, enzymatic protein activity failure, virus mutation, and nucleic acid damage (6). NF-κB, activated by ROS, has been proved to induce the expression of iNOS (7). The enzyme, responsible for the production of nitric oxide (8), has been shown to inhibit SARS-Cov virus replication (the coronavirus causing severe acute respiratory syndrome coronavirus, emerged in 2003), (9). Based on the analogy and similarity between SARS-Cov and SARS-Cov-2, it may be assumed that the nonstructural protein nsp10 causes mitochondrial impairment (10). Additionally, extracellular mitochondria, which are also ROS source, are able to provoke the immune response, regulate cell-to-cell communication and danger sensing (11). Peroxynitrite is formed by the reaction of nitrite ($NO^{\bullet}$) and hydrogen peroxide (12), and it has been proved to damage lung tissue and thus playing an important role in lung destruction in viral infections.

main site of ROS production under the influenza virus infection [46]. In addition, the authors indicated that ROS generation might be triggered by influenza virus in endosome *via* four different ways, one of which is TLR7 activation through the single-stranded RNA and protein kinase C activation. This results in phosphorylation of p47phox and by the assembly of the Nox2 oxidase complex at the endosomal membrane. The importance of Nox2 in influenza A infection was confirmed by literature, showing that in the absence of Nox2, influenza A virus results in lower viral burden and consequently results in significantly less lung injury, suggesting that ROS generated by Nox2 promotes rather than inhibits viral infection [80–83] (Figure 5.).

As mentioned earlier, apart from Nox2, also Nox1, Nox 4, and Duox2 might play a role in the ROS formation of viral infections [84–86]. Nox1 was shown to critically inhibit the early burst of proinflammatory cytokine expression in the lung and subsequently—oxidative stress followed by influenza A virus infection [85]. Nox1 oxidase has been proved to suppress early proinflammatory cytokine expression burst. Taking into consideration that ROS contribute to dysfunction and injury of the lung during influenza virus infection, this role of Nox1 seems surprising [85]. On the contrary, the study of Hofstetter et al. demonstrated that Nox1 presents activity promoting mortality during the peak of influenza infection, through restrain of the early phase of the adaptive immune response [87].

One of the key mediators of cytokines/chemokines induction is NF-κB. The pathway of this transcription factor is directly activated by ROS and by certain proinflammatory cytokines, such as TNF-α and IL-1β. A wide spectrum of cytokines and chemokines may be expressed as a consequence of NF-κB action, including IL-1β, IL-6, and IL-8, produced by most viruses (e.g., influenza virus, HBV, HIV (human immunodeficiency virus), EBV, SARS-CoV-2, and MERS); moreover, many respiratory viruses induce NF-κB signaling both *in vitro* and *in vivo* in a ROS-dependent fashion [88–92]. During viral infections, NF-κB binds to distinct sites of the iNOS promoter, causing iNOS enhanced expression. NO overproduction is predominantly caused by iNOS, which might be expressed, e.g., by inflammatory phagocytic cells [93–95]. Reactive nitrogen species play an important role in viral infections, in fact, some viruses, e.g., HCV, HRSV, or HIV, might upregulate the expression of iNOS [96–98]. On the other hand, IL-10, produced by many viruses (e.g., EBCV, HBV, HIV, SARS-CoV-2, and MERS), indirectly inhibits iNOS by inducing arginase, which reduces the availability of L-arginine, the substrate of iNOS [99, 100]. A previous study shows that HRSV directly upregulated iNOS in human type 2 alveolar epithelial cells, suggesting

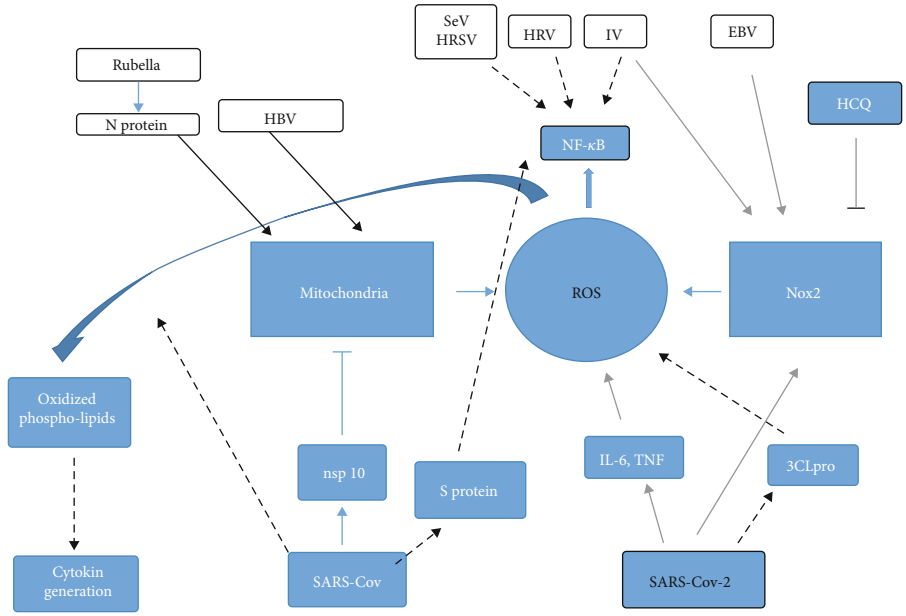

Figure 5: Chosen features of ROS/RNS metabolism during the COVID-19 in comparison to other viral infections. Nox 2, as the reactive oxygen species source, has been reported to be increased in COVID-19 patients [46], and, hydroxychloroquine (HCQ), the antimalarial drug, was demonstrated to inhibit Nox2 activity [48]. Nox increased activity is also a common feature of EBV and influenza virus infection, and Nox2 inhibition in IV infection lowers viral burden. On the basis of feedback, viruses (HRSV, HRV, and SeV), cause ROS induction via Nox2 [45, 74–76, 86]. In serum of COVD-19 patients, inflammatory cytokines (TNF-α and IL-6) were increased, possibly taking part in the initiation of mitochondrial ROS production [52, 53] (thick grey lines). Coronavirus SARS-CoV-2 protease–3CLpro has been shown to act through mitochondrial ROS, inducing NF-κB signal transduction pathway [20]. This pathway is closely related to many other viral infections, e.g., SeV, HRV, IV, and SARS-Cov (via S protein) [74–76, 89]. The latter virus, by induction of ROS, and subsequent generation of oxidized phospholipids, may not only modulate the severity of acute lung injury but also directly induce inflammatory cytokine production in macrophages [19] (black, dotted line). Mitochondria are proved to be important ROS source in rubella and HBV infection [79, 80], as they induce ROS production, either directly, or via viral N protein (in case of rubella, black thick lines).

that the expression increase might be associated with interferon regulatory factor 1, instead of cytokines [101]. The regulatory factor mentioned has been involved in iNOS expression activation together with NF-κB and double-stranded RNA-activated protein kinase, as shown previously in influenza virus infection [102].

5. Molecular Mechanisms of ROS and RNS Generation in Response to Viruses

Reactive oxygen and nitrogen radicals are generated, among others, in viral infections. In conditions of infection, ROS and RNS production might be activated either by viral components or by cytokines, in response to the pathogen. Apart from the influenza virus, several viruses are associated with ROS generation. Epstein-Barr virus (EBV) may induce generation of ROS by NADPH oxidase in B lymphocytes through upregulating Nox2 [103]. In turn, Nox4 is activated by core protein of HCV, but this oxidase initiates mitochondrial ROS production, showing that this virus induces other proteins to trigger ROS generation [104, 105]. Mitochondrial ROS generation pathway is activated by viruses in various ways—the rubella virus produces N protein which induces the production of ROS by increasing the activity of mitochondrial respiratory chain [106]. Yuan et al. demonstrated that hepatitis B virus increases mitochondrial ROS generation resulting in the elevated IL-6 expression [107].

Apart from mitochondrial source, ROS are produced by enzymatic activity of NADPH oxidases in a highly regulated manner and play roles in both physiology and disease [108]. Out of seven NADPH oxidase homologs, four are implicated in ROS generation under viral infections: Nox1, Nox2, Nox4, and Duox2, but the primary source of inflammatory cell ROS is the Nox2 oxidase enzyme [36, 109, 110]. Although Nox2 is a phagocytic enzyme playing a role in killing bacteria and fungi, it is also known for contributing to virus-induced ROS production during viral infections, e.g., with IV (Influenza virus) [111], HRSV [91], HRV (human rhinovirus) [112], SARS-CoV-2 [37], and SeV (sendai virus) [91, 92].

In severe COVID-19, the major cytokines generated as part of immune response are IL-1β, IL-2, IL-6, and TNF. Also, IFN-Υ seems to play an important role in antiviral response, although the data may suggest some defective interferon synthesis and release in severe patients infected with SARS-CoV-2. IL-1β is a well-known ROS and RNS generation activator [113]. Similarly, IL-2 stimulates RNS to generate nitrogen radicals [114]. Interleukin-6 activates human neutrophils and monocytes increasing the generation of free oxygen radicals [115]. Similarly, IFN-Υ and TNF stimulate the generation of RNS in human [114]. On

the other hand, free oxygen radicals may increase IL-6 production and free nitrogen radicals are responsible, at least in part for its synthesis [116, 117]. Moreover, high levels of IL-6 are associated with the higher mortality rate in ICU-(intensive care unit-) treated COVID-19 patients [118, 119].

The effects of the antiviral potential of nitric oxide (NO) against SARS coronavirus have been described in Vero E6 cells and revealed, that NO donor, S-nitroso-N-acetylpenicillamine inhibited the replication cycle of SARS-CoV in a dose-dependent manner [67]. In patients with SARS, NO was associated with oxygenation amelioration. Moreover, endogenous but also exogenous NO inhibited SARS-CoV viral replication [120–122]. NO reacts with superoxide radicals yielding peroxynitrite, and both peroxynitrite and NO are toxic to mitochondria.

Apart from iNOS induction in response to viruses and viral components, interferon gamma has been reported as a major cytokine to induce iNOS and NO overproduction in the pathogenesis of virus infection [123, 124]. This cytokine is associated to Th1 cell response, as it is acknowledged that antiviral adaptive response is Th1 type [125]. Nevertheless, some viruses (such as influenza virus and HSV) might inhibit Th1 response through downregulation of interferons production. This type of immune response manipulation may prominently influence the consequence of the infection [126, 127]. Moreover, produced in excess during viral infection, reactive nitrogen species, are likely to influence mutagenesis in the virus [128].

6. The Possible Therapeutic Approach Related to Oxidative Stress Tampering in COVID-19

Several strategies for treating the SARS-CoV-2 infection are currently under consideration. Scientists and doctors have developed therapies based on the use of interferons, antibodies, inhibitors of viral/host proteases, and host-directed therapies. To date, no clinically effective antiviral therapy against SARS-CoV-2 has been confirmed; therefore, patients receive mainly supportive treatment which is often supplemented with various drug combinations. Many authors have documented elevated chemokines and interleukins levels in COVID-19 patients, so future efforts should focus inter alia on drugs that can be rapidly deployed and have immunomodulatory properties [129–132]. The use of interleukin 1 receptor antagonist in nine patients with moderate to severe COVID-19 pneumonia was effective in improving clinical and biological indices [133]. IL-1 receptor blocker reduced the need for invasive mechanical ventilation in the intensive care unit as well as mortality in patients with severe COVID-19 [134]. Shakoory et al. [135] in their randomized controlled trial confirmed that the inhibition of IL-1 receptor significantly decreased mortality in sepsis patients with features of macrophage activation syndrome. Patients who received IL-6 receptor antagonists had a marked reduction in pyrexia within days after treatment and a reduction in oxygen demand [136]. In the TESEO (the tocilizumab in patients with severe COVID-19 pneumonia) study, the use of a recombinant humanized antihuman IL-6 receptor monoclonal antibody (i.v. or s.c.) was associated with a reduced risk of mechanical ventilation and death [137]. Another IL-6 receptor blocker was effective only in critically ill COVID-19 patients requiring mechanical ventilation or high-flow oxygenation or requiring intensive care treatment [138]. Recent studies have highlighted the role of optimal nutritional status in boosting the immune system, focusing on the most important ingredients that reduce inflammation and oxidative stress parameters [139]. Interestingly, hydroxychloroquine (HCQ), the antimalarial drug, used to treat COVID-19, has been recently demonstrated to inhibit Nox2 activity through the ability to alkalize endosomes and therefore impedes antiphospholipd antibody activity (aPL) [35, 140]. The aPL, as a proinflammatory factor, has been proved to act *via* the pathway in which NADPH oxidase takes part [141]. There are many mechanisms for neutralizing free radicals, e. g., glutathione which is capable of affecting viral replication; the glutathione peroxidase/reductase enzyme system that allows reduced glutathione to bind to free radicals to produce oxidized glutathione, which is then regenerated to GSH; peroxyredoxin system that neutralizes lipid peroxidation; superoxide dismutase neutralizing superoxide anion; catalase eliminating hydrogen peroxide; carotenoids and polyphenols with scavenging effects; vitamins E and C; and finally, zinc and selenium, which have antioxidant properties as cofactors of antioxidant enzymes [142]. Providing substances that strengthen the antioxidant system will reduce the level of oxidative stress parameters during infection. Moreover, the use of molecular techniques to target antioxidants to organs of interest is an approach that might enhance the effectiveness of the antioxidant and circumvent toxicity [143].

Resveratrol is a wide studied antioxidative agent, which plays a role in mitochondria-derived ROS [144] but also down regulates the expression and activity of the NADPH oxidase [145]. In the case of MERS-CoV, resveratrol appeared to inhibit MERS-CoV infection. Moreover, the authors of a recently published study point out that as MERS-CoV infection leads to inflammatory cytokines production, resveratrol, via hindering NF-κB pathway, may reduce the inflammation [146–148]. They also found that the expression of the nucleocapsid (N), which is essential for MERS-CoV replication, was decreased after resveratrol treatment [61]. MERS-CoV next to SARS-CoV-1 and SARS-CoV-2 has been demonstrated to depend on TMPRSS2 (transmembrane serine protease 2) which plays an important role during the virus entry to the cell. Presumably, TMPRSS2 might regulate mitochondrial function [149–151].

Recently, many others antioxidants have been tested for the highly conserved SARS-CoV-2 main protease using molecular docking. Of all the compounds that were investigated, the lowest predicted IC50 value was observed for taxifolin. Moreover, taxifolin along with other compounds such as eriodictyol did not show any toxicity against the toxicity parameters used in the experiment [152]. This flavonoid was found to be a powerful antiradical and antioxidant activities in different *in vitro* bioassays when compared with standard antioxidant compounds [153]. This compound inhibits NF-

κB pathway and downregulates STAT3 of the JAK/STAT pathway [154]. Thus, taxifolin could be a potential inhibitor against Mpro but further *in vivo* studies are needed [155]. Another analyzes also point to the natural compounds, taxifolin and rhamnetin, as potential inhibitors of Mpro [156]. Rutin (a polyphenolic flavonoid) may able a potential inhibitor as it is able to form several hydrogen bonds and σ-π stacking interactions with various amino acids of Mpro in anchoring and blocking the substrate into the active pocket of the catalytic center [157]. *In vivo* and in silico studies have demonstrated that silymarin and its derivative silybin (a flavonoid from the group of flavonolignans) are able to inhibit SARS-CoV-2 main protease [158]. Another authors found luteolin to be effective in blocking the S2 protein of SARS-CoV [159]. It is already known that the SARS-CoV and SARS-CoV-2 S proteins share about 76% amino acid similarity [142]. Several other herbal compounds like quercetin, naringenin, kaempferol, allicin, demethoxycurcumin, catechin, apigenin-7-glucoside, oleuropein, curcumin, zingerol or gingerol have been also investigated [57].

The approach of using antioxidants both to reduce viral replication and to reduce viral-induced oxidative damage may prove to be particularly useful for those viruses, which have thus far eluded attempts at antiviral therapies.

7. Conclusion

In conclusion, the literature demonstrates an important role of reactive oxygen and nitrogen species during SARS-CoV-2 infections, associated with a weakened antioxidant defense. Nevertheless, it must be noted that some of the understanding, background, and supporting data presented in the current review come from the experience with other human coronaviruses or viruses, such as RSV/HBV/HCV, and may not necessarily be known to be appropriate with respect to SARS-CoV-2.

The oxidative stress mechanism coupled with innate immunity activates transcription factors, such as NF-κB, which results in an exacerbated proinflammatory host response. The importance of ROS and RNS is also connected with the fact that this virus is especially dangerous for the elderly, and their deteriorated antioxidative/nitrosative defense system affected by increased reactive oxygen and nitrogen species. Moreover, only treatments diminishing the ROS and RNS production such as dexamethasone and tocilizumab deliver substantial benefits to severe COVID-19 patients. Therefore, there is a strong need to deeply investigate this issue, as it would be of interest to use the antioxidants as potential therapeutic tools.

Abbreviations

ACE2: Angiotensin-converting enzyme 2
ADCC: Antibody dependent cellular cytotoxicity
aPL: Antiphospholipd antibodies
ARDS: Acute respiratory distress syndrome
CTL: Cytotoxic T leukocytes
DIC: Disseminated intravascular coagulation
EBV: Epstein-Barr virus
HBV: Hepatitis B virus
HCV: Hepatitis C virus
HCQ: Hydroxychloroquine
HIV: Human immunodeficiency virus
HRSV: Human respiratory syncytial virus
HRV: Human rhinovirus
ICU: Intensive care unit
IFN: Interferon
IL: Interleukin
iNOS: Inducible nitric oxide synthase
IRFs: Interferon regulatory factors
ISGs: IFN-stimulated genes
IV: Influenza virus
MCP-1: Monocyte chemoattractant protein-1
MERS: Middle East respiratory syndrome coronavirus
MHC: Major histocompatibility complex
MIP1$\alpha\beta$: Macrophage inflammatory proteins-1$\alpha\beta$
MyD-88: Myeloid differentiation primary response-88
NADPH: Nicotinamide adenine dinucleotide phosphate hydrogen
NET: Neutrophil extracellular traps
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NHC: Natural helper cells
NK: Natural killers
NKT: Natural killer T cells
NOX2: NADPH oxidase 2
PAMPs: Pathogen associated molecular patterns
PRR: Pattern recognition receptors
SARS: Severe acute respiratory syndrome
SeV: Sendai virus
TLR: Toll-like receptors
TMPRSS2: Transmembrane serine protease 2
TNF-α: Tumor necrosis factor alpha
TRAF6: TNF receptor associated factor 6
TRIF: TIR-domain-containing adapter-inducing interferon-β.

Data Availability

The data supporting this review article are from previously reported studies and datasets, which have been cited. The processed data are available from the corresponding authors of upon request.

Conflicts of Interest

The authors declare no conflict of interest.

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Research Article

D-dimer, CRP, PCT, and IL-6 Levels at Admission to ICU Can Predict In-Hospital Mortality in Patients with COVID-19 Pneumonia

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Introduction. Health care workers have had a challenging task since the COVID-19 outbreak. Prompt and effective predictors of clinical outcomes are crucial to recognize potentially critically ill patients and improve the management of COVID-19 patients. The aim of this study was to identify potential predictors of clinical outcomes in critically ill COVID-19 patients. *Methods*. The study was designed as a retrospective cohort study, which included 318 patients treated from June 2020 to January 2021 in the Intensive Care Unit (ICU) of the Clinical Hospital Center "Bezanijska Kosa" in Belgrade, Serbia. The verified diagnosis of COVID-19 disease, patients over 18 years of age, and the hospitalization in ICU were the criteria for inclusion in the study. The optimal cutoff value of D-dimer, CRP, IL-6, and PCT for predicting hospital mortality was determined using the ROC curve, while the Kaplan-Meier method and log-rank test were used to assess survival. *Results*. The study included 318 patients: 219 (68.9%) were male and 99 (31.1%) female. The median age of patients was 69 (60-77) years. During the treatment, 195 (61.3%) patients died, thereof 130 male (66.7%) and 65 female (33.3%). 123 (38.7%) patients were discharged from hospital treatment. The cutoff value of IL-6 for in-hospital death prediction was 74.98 pg/mL (Sn 69.7%, Sp 62.7%); cutoff value of CRP was 81 mg/L (Sn 60.7%, Sp 60%); cutoff value of procalcitonin was 0.56 ng/mL (Sn 81.1%, Sp 76%); and cutoff value of D-dimer was 760 ng/mL FEU (Sn 63.4%, Sp 57.1%). IL-6 ≥ 74.98 pg/mL, CRP ≥ 81 mg/L, PCT ≥ 0.56 ng/mL, and D-dimer ≥ 760 ng/mL were statistically significant predictors of in-hospital mortality. *Conclusion*. IL-6 ≥ 74.98 pg/mL, CRP values ≥ 81 mg/L, procalcitonin ≥ 0.56 ng/mL, and D-dimer ≥ 760 ng/mL could effectively predict in-hospital mortality in COVID-19 patients.

1. Introduction

In December 2019, SARS-CoV-2 was identified for the first time as a cause of COVID-19 disease by Chinese scientists [1]. However, after more than a year since the pandemic's beginning, we still do not have a complete picture of the disease itself.

Initially, COVID-19 was considered a respiratory disease, with pneumonia being the most common and deadliest complication. However, SARS-CoV-2 has been shown to

trigger an excessive and uncontrolled immune-hemostasis response that causes many complications, such as thrombosis, tissue damage, ARDS, DIC, and MODS; therefore, not only it is necessary to understand COVID-19 as a respiratory, but also as a potential multisystem disease [2, 3].

Studies from the beginning of pandemic estimated overall hospital mortality from COVID-19 are approximately 15% to 20%, but up to 40% among patients requiring ICU admission; however, mortality rates vary across age cohorts, from 5% among patients younger than 40 years to greater than 60% for patients aged 80 to 89 years [4]. In contrast, a recent study suggested lower mortality due to the presence of appropriate treatment and vaccination [5].

It is well known that thrombosis is an important complication that significantly increases the risk of a deadly outcome. A great number of thrombosis was verified in SARS-COV2-positive patients [6–8]. Even, despite standard thromboprophylaxis doses of LMWH, 31% of individuals with proven COVID-19 pneumonia developed thrombosis in ICU [9]. Significant changes in coagulation parameters were verified in patients with COVID-19. Concerning the patients, higher values of D-dimer were observed in persons requiring treatment in ICU [10]. Consequently, higher D-dimer values were associated with severe clinical presentation of COVID-19 disease, as mentioned above [11–14].

Considering all of this, D-dimer and other inflammatory parameters such as IL-6, CRP, and PCT might be used to predict mortality. Predictors of mortality among laboratory parameters are important as they can reflect possible mechanisms of disease progression and give important information on potentially useful therapeutic modalities [15]. Adequate and precise predictors are crucial, especially in the pandemics era.

The aim of this study was to identify potential biochemical predictors of in-hospital mortality among COVID-19 patients and to determine their predictive cutoff values.

2. Methods

2.1. Study Design and Participants. The study was designed as a retrospective cohort study, which included 318 patients treated from June 2020 to January 2021 in the Clinical Hospital Center "Bezanijska Kosa" ICU in Belgrade. The criteria for inclusion in the study were the verified diagnosis of COVID-19 disease, patients over 18 years of age, and hospitalization in the ICU. The criteria for excluding patients from the study were incomplete data, the patient's stay in the ICU for some other reasons not due to complications of COVID-19 (e.g., postoperative treatment of patients), and transfer of patients to other medical institutions for COVID-19 treatment.

2.2. Definitions, Diagnosis, and Outcomes. COVID-19 diagnosis was made based on the clinical symptoms and signs of the disease, with/without a positive radiological finding (X-ray, CT), and a positive result of the nasopharyngeal swab SARS-CoV-2, detected by the RT-PCR method. The main clinical criteria for Respiratory ICU admission was radiographic or CT scan severity score progression, peripheral oxygen saturation (Sp02) below 93% despite maximal conventional supportive oxygen therapy (up to 15 L/min through a nasal cannula, conventional oxygen, or nonrebreather mask), laboratory test results, mainly an increase of inflammatory parameters after repeated controls, and arterial blood gas test. Critically ill patients on invasive, noninvasive ventilation and high flow oxygen therapy with moderate and severe ARDS were selected for the study according to the Berlin definition of ARDS [16]. The primary outcome of interest was in-hospital mortality and was stratified as deceased or discharged from the hospital. Survivors refer to participants who were discharged from the hospital, and no survivors refer to deceased participants. All patients were followed until their outcomes.

2.3. Treatment. During the hospitalization, patients were treated according to the adjusted National protocol of the Republic of Serbia to treat COVID-19 infection [17]. Antiviral agents (favipiravir, remdesivir) were used 5-7 days from symptom onset in patients on supportive oxygen therapy and with radiographically verified severe bilateral pneumonia. Corticosteroids (prednisone 0.5 mg/kg in two doses, methylprednisolone 1-2 mg/kg, and dexamethasone 6 mg/day) were used in patients with moderate to severe clinical image with signs of clinical deterioration or in patients with incipient or developed ARDS. Anticoagulant therapy was used in the standard prophylactic dose of LMWH for patients with multiple risk factors and conventional oxygen therapy. According to the anti-Xa levels, therapeutical doses were used for patients in the ICU requiring mechanical ventilation or high-flow oxygen therapy, those on long-term anticoagulant therapy, or those with suspectable or confirmed thrombosis. Antibiotics were used empirically or according to the antibiogram. The main criteria for tocilizumab administration were an increase in IL-6 values above 40 pg/mL and CRP values above 50 mg/L or a threefold increase during the last 48 h in patients with clinical worsening with more than 25 resp/min, saturation below 93%, and partial presure of oxygen below 8.66 kPa without supportive oxygen therapy. Convalescent plasma was used in patients with rapid worsening, positive PCR test for SARS-CoV-2 virus, in the first two weeks from symptom onset. The indication was established according to the specific scoring system with different variables, including the patient's clinical status, a form of the disease, time from symptom onset, respiratory status, radiographic findings, comorbidities, and applied therapy. Inotropic agents, noradrenaline, dobutamine, vasopressin, and adrenaline were used in a standard dosage.

2.4. Data Collection. The necessary data were obtained from the health information system of the Clinical Hospital Center "Bezanijska Kosa" (Heliant, v7.3, r48602). The data includes demographic data (age, gender), laboratory values (IL-6, CRP, PCT, ferritin, D-dimer, lymphocytes, thrombocytes, PT, aPTT, and fibrinogen), and the outcome of the treatment. Past medical history (hypertension, diabetes mellitus, COPD, coronary heart disease, obesity, heart failure, cardiomyopathy, and chronic kidney disease) was obtained from participants' medical documentations and was filed in

the health information system. Clinical and laboratory parameters were followed upon admission to the hospital and ICU, with specific parameters followed during hospitalization.

2.5. Statistical Analysis. Descriptive statistics methods were used to process and present the results. Continuous variables were presented as the median and IQR and as the frequency (%) for categorical variables. The Mann–Whitney *U* test and Pearson's chi-square test were used to compare the data. The optimal cutoff value of D-dimer, CRP, IL-6, and PCT for predicting hospital mortality was determined using the ROC curve, while the Kaplan-Meier method and log-rank test were used to assess survival. A value of $p < 0.05$ was considered statistically significant.

3. Results

The study included 318 patients, 219 (68.9%) male and 99 (31.1%) female. The median age of patients was 69 (60-77) years. During the treatment, 195 (61.3%) patients died: thereof 130 were male (66.7%), and 65 were female (33.3%). 123 (38.7%) patients were discharged from the treatment. Age, gender, comorbidities, laboratory parameters, and CT score of patients are shown in Table 1.

C-indices for IL-6, CRP, PCT, and D-dimer are presented in Table 2. PCT has the highest *C*-index (0.77) to predict in-hospital mortality in COVID-19 patients.

Cutoff values of the analyzed parameters were obtained using the ROC curve. The cutoff value of IL-6 for in-hospital death prediction was 74.98 pg/mL (Sn 69.7%, Sp 62.7%); cutoff value of CRP was 81 mg/L (Sn 60.7%, Sp 60%); cutoff value of PCT was 0.56 ng/mL (Sn 81.1%, Sp 76%); and cutoff value of D-dimer was 760 ng/mL FEU (Sn 63.4%, Sp 57.1%). ROC curves are presented in Figure 1.

Using the Kaplan-Meier survival curve and log-rank test, it was shown that IL-6 higher or equal to 74.98 pg/mL was a statistically significant predictor of in-hospital mortality ($p = 0.04$). In addition, CRP values higher or equal than CRP 81 mg/L, PCT higher or equal than 0.56 ng/mL, and D-dimer higher or equal than 760 ng/mL FEU represent significant predictors of in-hospital mortality (CRP, $p = 0.02$; PCT, $p < 0.001$; and D-dimer, $p = 0.04$) (Figure 2).

4. Discussion

First, our National protocol is mainly following the WHO treatment guidelines [18]. However, we would like to address a few differences between our National protocol and the WHO treatment guidelines. According to our National protocol, the main difference is the usage of favipiravir and remdesivir. The use of systemic corticosteroids, monoclonal antibodies, and IL-6 receptor blockers was in accordance with the WHO treatment guidelines. This slight discordance between protocols should not affect the discussion of our results with results in the literature.

The study indicated significant disorders of laboratory parameters in patients with COVID-19 treated in ICU. Elevated levels of IL-6, CRP, PCT, D-dimer, and lower serum albumin levels were detected in subjects with fatal disease outcomes during treatment. Significantly higher in-hospital mortality was observed in individuals whose IL-6 values were equal to or higher than 74.98 pg/mL, followed by CRP values higher than 81 mg/L, PCT values equal to or higher than 0.56 ng/mL, and D-dimer values equal to or higher than 760 ng/mL FEU. However, 5 out of 318 participants with IL-6, CRP, PCT, and D-dimer values above cutoff value survived. These findings suggest a good prediction of in-hospital mortality in patients with COVID-19 who require admission to the ICU, especially when jointly using all four cutoff values.

The cytokine storm is one of the most critical factors contributing to COVID-19 mortality. Elevated values of various cytokines, such as IL-1, IL-2, IL-6, IL-7, IL-8, IL-12, IFN, MCP-1, and TNF-α, were observed. In SARS-CoV-2-positive patients, cytokine storm is characterized by high serum concentrations of IL-6 and TNF-α predominantly [19, 20]. Our study observed higher mortality in patients with IL-6 concentrations higher than 74.98 pg/mL. In addition, other studies also favoured a more severe form of the disease and higher mortality of patients with higher values of IL-6 [21–23]. Patients whose maximum IL-6 values exceeded 80 pg/mL had a significantly higher probability of need of invasive mechanical ventilation. Hyperinflammatory response in the setting of COVID-19 could also be responsible for the potential multiorgan failure and various life-threatening complications, including ARDS, myocardial damage, and kidney and liver failure. Also, a significant predictor was elevated values of CRP [24].

In addition to IL-6, CRP is a significant marker of COVID-19 inflammation. Higher levels of serum CRP are associated with higher mortality in people with severe COVID-19 disease [25], more specifically, CRP values above 77.35 mg/L [26]. On the other hand, the CRP threshold, which was found as a predictor of in-hospital mortality by Du et al., was lower, and it was 10 mg/L [27]. Wang confirmed a positive correlation between CRP values and CT findings in the lungs in the initial stages of the disease. Their findings could give grounds for the connection between high CRP values and a more severe form of the disease [28]. Furthermore, CRP had a significantly better effect in predicting death than age, neutrophil count, and platelet count [29].

Therefore, it is essential to recognize the hyperinflammatory syndrome in COVID-19 patients, primarily over the previous quoted inflammatory parameters, and apply the anti-inflammatory therapy right on time. Corticosteroids, as anti-inflammatory drugs, have shown significant positive effects in patients with a hyperinflammatory response to SARS-CoV2 by reducing mortality, decreasing hospital stay, and increasing ventilator-free days [30, 31]. The anti-inflammatory effects of corticosteroids are proven by inducing the synthesis of anti-inflammatory proteins and, on the other hand, by inhibiting the synthesis of proinflammatory proteins [32]. It is crucial to start corticosteroid treatment at the right time and in the right patient since early administration and administration to patients with asymptomatic and milder forms of the disease may have adverse effects [33].

Table 1: Age, comorbidities, laboratory parameters, and CT score of patients participated in the study. Results are expressed in *n* (%) and median (IQR).

	Total (*n* = 318)	No survivor (*n* = 195)	Survivor (*n* = 123)	*p* value
Age (years)	69 (60-77)	72 (64-79)	63 (51-73)	**<0.001**
Males, *n* (%)	219 (68.9)	130 (59.4)	89 (40.6)	0.286
Females, *n* (%)	99 (31.1)	65 (65.7)	34 (34.3)	
Comorbidities				
Hypertension, *n* (%)	223 (70.1)	140 (71.8)	83 (67.5)	
Diabetes mellitus, *n* (%)	100 (31.4)	54 (27.7)	46 (37.4)	
Coronary disease, *n* (%)	62 (19.5)	37 (19)	25 (20.3)	
Obesity, *n* (%)	40 (12.6)	21 (10.8)	19 (15.4)	
Cardiomyopathy, *n* (%)	27 (8.5)	18 (9.2)	9 (7.3)	
COPD, *n* (%)	19 (6)	13 (6.7)	6 (4.9)	
Asthma, *n* (%)	14 (4.4)	9 (4.6)	5 (4.1)	
Laboratory parameters				
IL-6 (pg/L)	110.8 (44.1-399.6)	160.7 (71.4-812.3)	66.8 (29.7-239)	**<0.001**
CRP (mg/L)	88 (53.8-191.5)	103.4 (61.1-210.1)	75.5 (41.7-177.2)	**<0.001**
Lymphocyte (%)	0.7 (0.5-1.1)	0.7 (0.5-1)	0.8 (0.5-1.2)	0.063
Serum ferritin (μg/L)	822 (415.5-1478)	766.5 (374-1409.2)	760.5 (306.7-1416)	0.673
PCT (ng/mL)	1.1 (0.2-9)	3.27 (0.8-17.9)	0.2 (0.1-0.7)	**<0.001**
D-dimer (ng/mL)	829 (497-2759.5)	1121.5 (594-3212.2)	666 (353.5-1317)	**<0.001**
Platelet count ($\times 10^9$/L)	225 (161.5-303)	204.5 (146-281)	234 (179.7-339.5)	0.022
INR	1.1 (1-1.3)	1.1 (1-1.3)	1.1 (1-1.2)	0.341
aPTT (s)	25.6 (22.5-30.3)	26 (22.7-30.3)	24.6 (22.4-28.4)	0.073
Fibrinogen (g/L)	4.1 (3.5-4.9)	4.1 (3.5-5)	4.2 (3.4-5.1)	0.921
Albumin (g/L)	32 (29-35)	31 (27.5-33)	35 (32-38)	**<0.001**
CT score	17 (5-22)	17 (1.5-22)	17 (8-22)	0.96

Table 2: C-statistic of IL-6, CRP, PCT, and D-dimer to predict mortality in patients with COVID-19.

Predictor value	*C*-index	95% confidence interval
IL-6	0.64	0.57–0.71
CRP	0.62	0.56–0.69
PCT	0.77	0.71–0.83
D-dimer	0.64	0.57–0.7

Elevated PCT has been detected in individuals treated for COVID-19 disease. PCT equal to or higher than 0.56 ng/mL is associated with higher mortality. Elevated PCT levels in individuals are primarily caused by bacterial coinfections, showing a good role in detecting bacterial coinfections and consequently initiating an antibiotic therapy [34]. A meta-analysis that analyzed four studies proved that an increase in PCT is associated with a five times higher risk of a more severe COVID-19 presentation (OR, 4.76; 95% CI, 2.74–8.29) [35]. Furthermore, another meta-analysis, which included over 10 thousand patients, indicated the importance of elevated PCT values as a predictor of fatal disease outcomes. The same study showed that lymphopenia, thrombocytopenia, elevated D-dimer, elevated CRP, then elevated CK, AST, ALT, LDH, and creatinine are independent predictors of deadly disease outcomes [36].

Our study proved that the concentration of D-dimer above 760 ng/mL FEU, measured on admission to the ICU, was associated with a higher risk of death during hospitalization. High values of D-dimer in COVID-19 patients are associated with local pulmonary thrombosis, which occurs as an immune-hemostatic response to prevent and limit further spread of the virus. The elevated D-dimer values exist due to a breakdown of these microthrombi [37, 38]. Elevation of D-dimer during the disease carries a higher risk of progression to severe form and mortality [3]. In particular, elevated values of D-dimer and fibrin degradation products and prolonged prothrombin time were measured at admission in the deceased subjects compared to the cured ones [39]. The study by Klok et al. [9] demonstrated that D-dimer values above 1 μg/mL, measured on admission to COVID-19 treatment facilities, were associated with an eighteen times higher risk of death. Further, any increase in D-dimer values by 1 μg/mL on admission is associated with an increase in the risk of death by 6%, as well as an increase in the probability (8%) of treatment with mechanical ventilation [40]. Creel-Bulos et al. showed a variation in D-dimer values in the first twenty-five days of hospitalization [41]. There is an almost linear trend of D-dimer increase in the first ten days of treatment, after which D-dimer levels are flattened. Moreover, a steeper D-dimer growth curve was observed in individuals with detected deep vein thrombosis

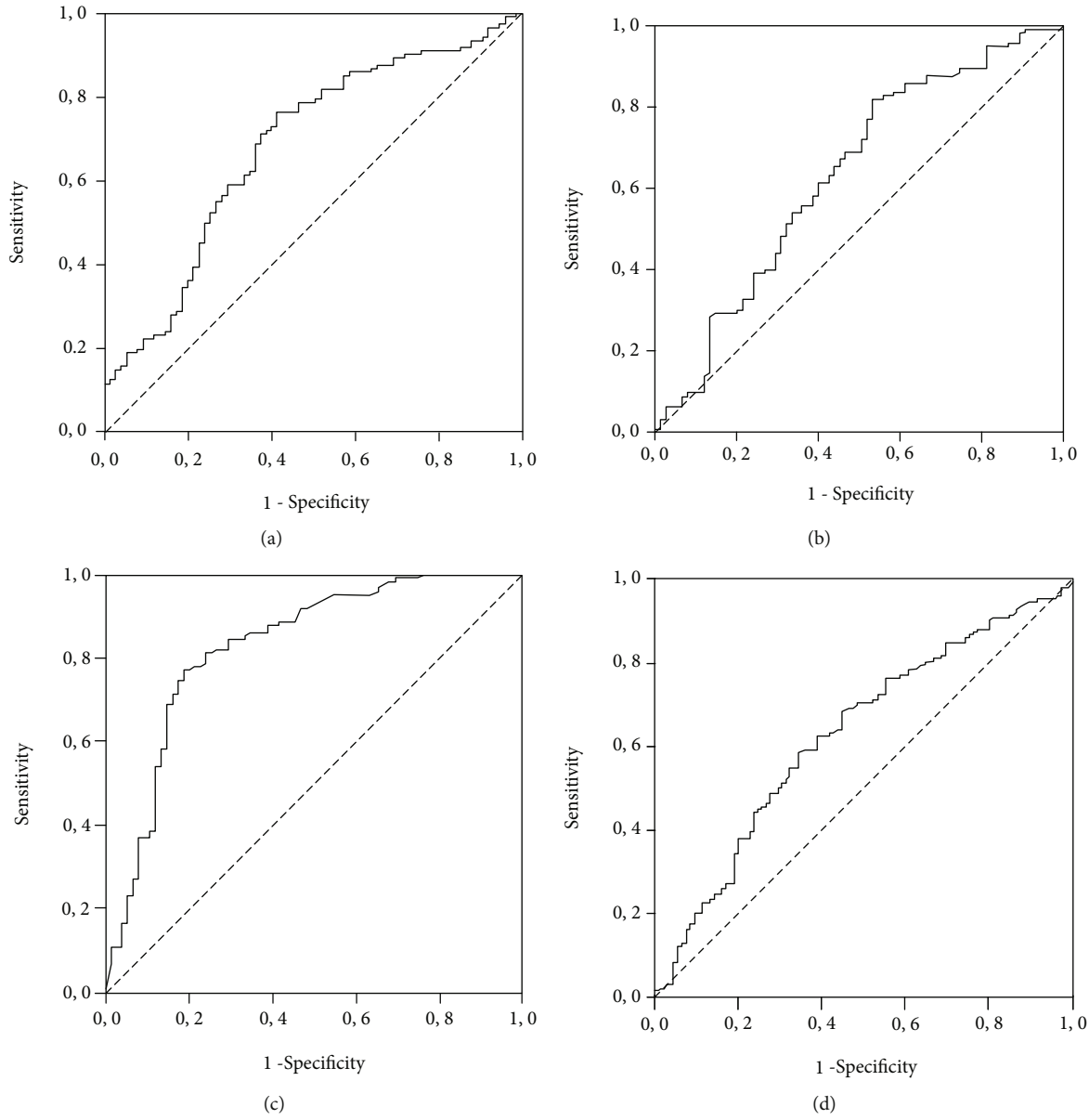

Figure 1: Receiver operator characteristic curve for (a) IL-6, (b) CRP, (c) PCT, and (d) D-dimer to predict deaths. The optimum cutoff point, identified as the point closest to the upper left corner, was for IL-6 (74.98 pg/mL), CRP (81 mg/L), PCT (0.56 ng/mL), and D-dimer (760 ng/mL FEU).

during treatment. In contrast, differences in D-dimer growth curves were not seen in the deceased and those discharged from treatment. Zhang et al. [42] concluded that a D-dimer higher than 2 μg/mL on admission could be considered a predictor of mortality during hospitalization. On the other hand, the study by Soni et al. did not prove that the values of the same parameter above 2 μg/mL measured at admission were mortality predictors [43]. Still, it demonstrated that D-dimer higher than 2 μg/mL during hospitalization is a mortality predictor if the highest measured values are viewed.

The literature has scarce data regarding IL-6, CRP, PCT, and D-dimer values variance in COVID-19 pneumonia and non-COVID pneumonia. Currently, the best-compared variance of mentioned parameters is between patients with COVID-19 and patients with influenza. Therefore, significantly higher CRP values on hospital admission were detected in influenza-positive subjects after comparing those groups of patients [44]. In their study, Kuang et al. have evinced the higher incidence of influenza patients detected on admission with CRP value above 10 mg/dl and PCT value above 0.5 ng/mL, compared to COVID-19 patients [45]. On the other hand, a significant increase in IL-6 level was observed in COVID-19 patients compared to patients with influenza [46]. Values of D-dimer were high on admission in both groups, COVID-19 and influenza [47]. During the

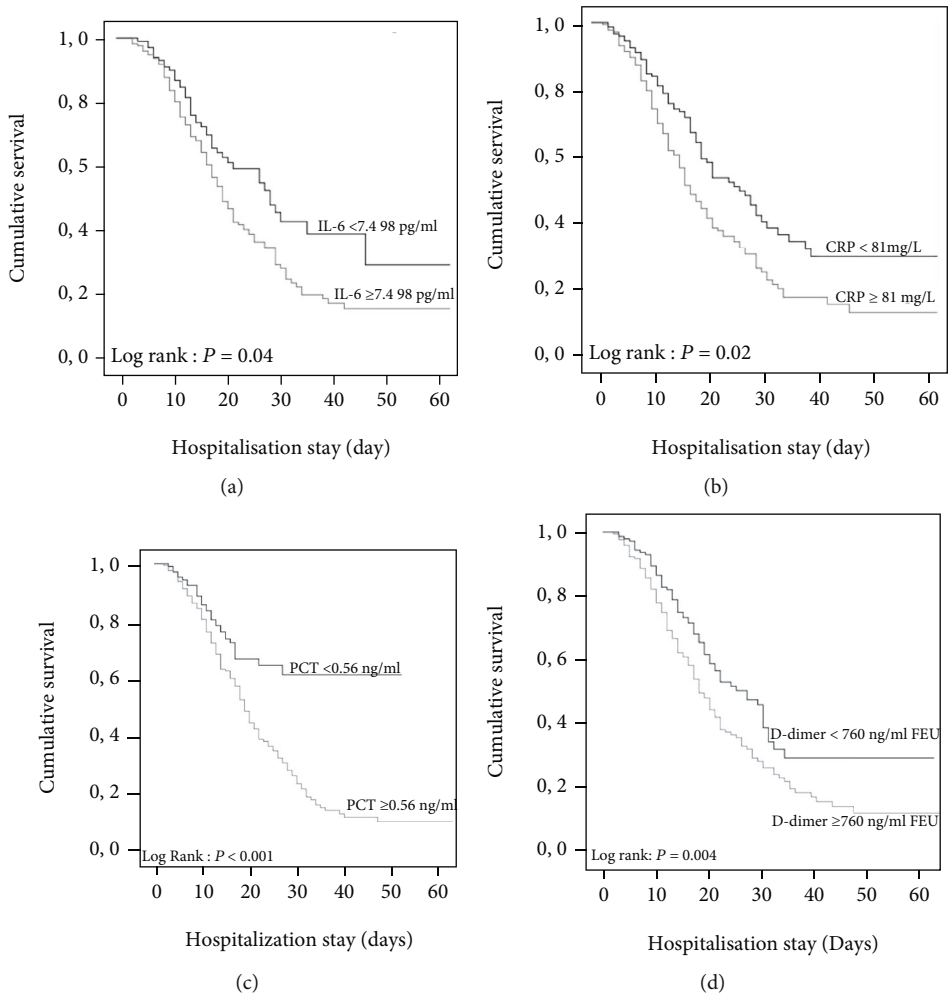

Figure 2: Kaplan-Meier survival curves for (a) IL-6, (b) CRP, (c) PCT, and (d) D-dimer levels on admission to the ICU.

14-day monitoring of D-dimer values, a significant increase was detected in COVID-19 patients in comparison to influenza patients [48]. Considering everything, COVID-19 and influenza present a potentially life-threatening disease, and therefore, both should be treated with caution. D-dimer dynamics measurement may be used to distinguish COVID-19 infection and influenza infection.

The impact of gender on survival is still debated. Males have a higher chance for severe pneumonia, and therefore, they have a greater chance to be admitted to the ICU [49]. Potential gender-specific mechanisms modulating the course of the disease Gebhard et al. explain with a hormone-regulated expression of genes encoding for the SARS-CoV2 entry receptors ACE 2 receptor and TMPRSS2 as well as sex hormone-driven innate and adaptive immune responses and immunoaging [50]. They stressed out also elucidating the impact of gender-specific lifestyle, health behaviour, psychological stress, and socioeconomic conditions on COVID-19 [50]. Our study included 318 patients, 68.9% male and 31.1% female, admitted to ICU. These findings are in obedience to the previous study. On the contrary, we did not find a statistically significant difference between genders regarding in-hospital mortality; this statement is supported by the result in Zhou et al.'s study [11]. This can imply that biochemical parameters on admission to the ICU as predictors of in-hospital mortality should be used in all patients with the same prognostic value.

This study has several limitations that should be addressed. First, it is a single-center, retrospective study. The sample size is relatively small. Therefore, the study has limited power to detect the difference between groups. Selection bias is also presented due to the exclusion of patients without D-dimer level on admission to the ICU. Another limitation is the lack of inclusion of some data in the study, such as partial pressure of O_2, CO_2, BMI, etc. The reason for this is their absence in the health information system. Furthermore, we did not perform dynamic D-dimer, CRP, IL-6, and PCT measurements because of the study's retrospective design. Incorporating these data might disclose more information and give more power to our study. Unmeasured confounders such as therapy delay, previous corticosteroids use, and BMI could give us residual

confounding. Finally, performing a multiple-parameter prediction model including D-dimer, CRP, IL-6, and PCT could better predict in-hospital mortality.

5. Conclusion

This study supported a growing body of literature regarding hyperinflammatory syndrome and diffuse microvascular thrombosis as predictors of poor clinical outcomes in COVID-19 patients. Proper and on-time differentiation patients with lower survival chances may be crucial for starting anti-inflammatory therapy such as corticosteroids, which may reduce in-hospital mortality. In particular, IL-6 $\geq$ 74.98 pg/mL, CRP $\geq$ 81 mg/L, PCT $\geq$ 0.56 ng/mL, and D-dimer $\geq$ 760 ng/mL on admission to the ICU could effectively predict in-hospital mortality in COVID-19 patients. Using these laboratory parameters single or in combination may help identify patients with lower survival chances and, on time, improve further treatment. Further prospective multicenter studies are necessary to confirm our findings.

Abbreviations

SARS-CoV-2:	Severe acute respiratory syndrome coronavirus 2
COVID-19:	Coronavirus disease 2019
ARDS:	Acute respiratory distress syndrome
DIC:	Disseminated intravascular coagulation
MODS:	Multiorgan dysfunction syndrome
LMWH:	Low molecular weight heparin
ICU:	Intensive Care Unit
IL-6:	Interleukin 6
CRP:	C-reactive protein
PCT:	Procalcitonin
CT:	Computed tomography
RT-PCR:	Reverse transcription-polymerase chain reaction
BMI:	Body mass index
COPD:	Chronic obstructive pulmonary disease
PT:	Prothrombin time
aPTT:	Activated partial thromboplastin time
IQR:	Interquartile range
Sn:	Sensitivity
Sp:	Specificity
IL-1, IL-2, IL-7, IL-8, and IL-12:	Interleukins 1, 2, 7, 8, and 12
IFN:	Interferon
MCP-1:	Membrane cofactor protein 1
TNF-α:	Tumor necrosis factor α
CK:	Creatine kinase
AST:	Aspartate Aminotransferase
ALT:	Alanine Aminotransferase
LDH:	Lactate dehydrogenase
ACE 2:	Angiotensin converting enzyme 2
TMPRSS2:	Transmembrane Serine Protease 2

Data Availability

The data used to support the findings of this study are available from the corresponding author (AH) upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

Combating Oxidative Stress and Inflammation in COVID-19 by Molecular Hydrogen Therapy: Mechanisms and Perspectives

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COVID-19 is a widespread global pandemic with nearly 185 million confirmed cases and about four million deaths. It is caused by an infection with the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), which primarily affects the alveolar type II pneumocytes. The infection induces pathological responses including increased inflammation, oxidative stress, and apoptosis. This situation results in impaired gas exchange, hypoxia, and other sequelae that lead to multisystem organ failure and death. As summarized in this article, many interventions and therapeutics have been proposed and investigated to combat the viral infection-induced inflammation and oxidative stress that contributes to the etiology and pathogenesis of COVID-19. However, these methods have not significantly improved treatment outcomes. This may partly be attributable to their inability at restoring redox and inflammatory homeostasis, for which molecular hydrogen (H_2), an emerging novel medical gas, may complement. Herein, we systematically review the antioxidative, anti-inflammatory, and antiapoptotic mechanisms of H_2. Its small molecular size and nonpolarity allow H_2 to rapidly diffuse through cell membranes and penetrate cellular organelles. H_2 has been demonstrated to suppress NF-κB inflammatory signaling and induce the Nrf2/Keap1 antioxidant pathway, as well as to improve mitochondrial function and enhance cellular bioenergetics. Many preclinical and clinical studies have demonstrated the beneficial effects of H_2 in varying diseases, including COVID-19. However, the exact mechanisms, primary modes of action, and its true clinical effects remain to be delineated and verified. Accordingly, additional mechanistic and clinical research into this novel medical gas to combat COVID-19 complications is warranted.

1. Introduction: Clinical Challenges and Dilemma of COVID-19 Treatments

COVID-19 (initially named 2019 novel coronavirus, or 2019-nCoV disease, after the first reported outbreak in 2019) has become the most widely spread global pandemic in the past century [1]. It has affected 189 countries and regions with nearly 185 million confirmed cases and about four million reported deaths worldwide as of current statistics [2]. The novel coronavirus responsible for this disease was named by the World Health Organization the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) for its genetic similarity to the coronavirus that caused the SARS outbreak in 2003 (SARS-CoV) [1]. While not

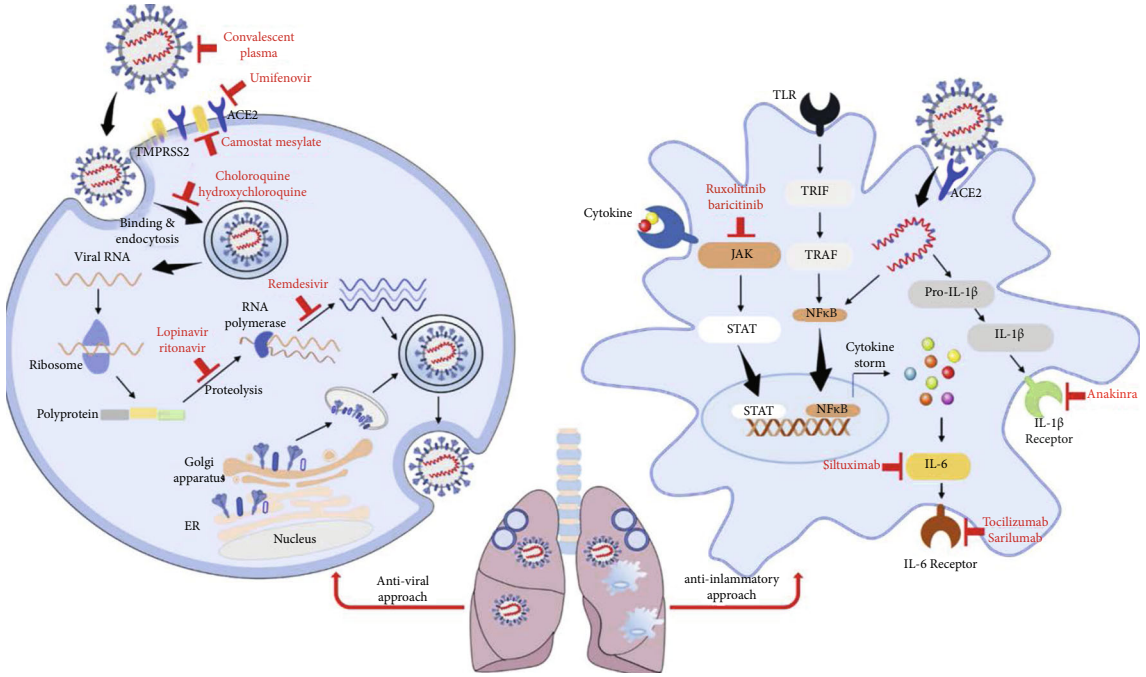

Figure 1: Illustration of various pharmacological therapies proposed and investigated to treat COVID-19 patients classified as two categories: antiviral approach and anti-inflammatory approach. The antiviral approach includes the use of agents that block viral binding, entry, fusion, RNA duplication, viral assembly, or exocytosis. The anti-inflammatory approach includes the application of agents that inhibit various inflammatory pathways, reduce cytokine production, and block cytokine receptors. Reproduced with permission from the publisher [4].

ominously fatal as contracting SARS-CoV, COVID-19's mild symptoms and asymptomatic transmission, coupled with long incubation time and long fomite survival time of the virus have complicated epidemic control globally.

Most COVID-19 cases manifest as a respiratory illness with vague symptomatology, starting with a fever, dry cough, and fatigue, followed by shortness of breath with worsening disease. About 80% of infected people may recover from the illness without hospitalization; yet, the remainder (20%) progress to pneumonia and severe acute respiratory distress syndrome (ARDS) [3]. An estimated 5% of patients require treatment in an intensive care unit (ICU), requiring ventilation for oxygenation and intubation to support life [3]. Of these critically ill patients in ICU, approximately half eventually die of infection-associated complications, typically following multiple organ injury and failure [3]. COVID-19 complications have been correlated to underlying medical conditions, particularly older adults with hypertension, diabetes, and/or other cardiovascular diseases.

In contrast, cytokine storms, caused by an overactive host immune system to any infection, are most responsible for mortality in young and middle-aged patients without medical histories. Current treatment modalities, including antiviral, anti-inflammatory (Figure 1), antimalarial, immunoregulatory therapeutics, ventilation, and extracorporeal membrane oxygenation (ECMO), attempt to mitigate the sequelae caused by infection (Table 1), but they cannot fully address the upstream factors that lead to "cytokine storms," which contribute to multiple organ failure and sudden deaths.

The coronavirus appears to exploit angiotensin-converting enzyme II (ACE2) as a receptor for cell binding and entry. ACE2 is expressed abundantly on epithelial cells in certain mucosal tissues [6]. Of note, the oral and nasal mucosa, eyes, and upper respiratory tract are the primary anatomical inoculation points for viruses that are mainly transmitted *via* aerosol droplets, propagated from human carriers in close proximity. The infection progresses to lower airways, particularly to alveolar epithelial cells that are susceptible to viral entry. When this occurs, alveolar macrophages and infiltrated immune cells are activated, which then increases oxygen consumption exacerbating alveolar hypoxia [7]. Activated alveolar macrophages also release proinflammatory cytokines within alveoli and pulmonary microvessels, which then enter the systemic circulation. Because injured lungs cannot effectively deliver oxygen or eliminate carbon dioxide from the bloodstream, systemic hypoxia (namely, hypoxemia) and hypercapnia develop. Both alveolar hypoxia and hypoxemia further induce inflammatory cascades, leading to the production of excess reactive oxygen species (ROS) and activation of hypoxia-inducible factors (HIF-1α), nuclear factor-kappa-light-chain-enhancer of activated B cells (NF-κB), and proinflammatory cytokines [7]. Thus, oxygen inhalation and anti-inflammatory therapy are considered essential for severe COVID-19, in addition to other potentially useful therapies.

However, in severe COVID-19 pneumonia, inflammation of the respiratory tract and exudation of viscous mucus in bronchioles and alveoli make oxygenation of blood inefficient. Despite high-speed oxygen ventilation, oxygen cannot

Table 1: Selected treatments investigated in clinical trials for COVID-19.*.

Antiviral	Anti-inflammatory	Anticoagulation and antivasculopathy
Arbidol	Acalabrutinib	Argatroban
Azithromycin (antibiotic)	Anakinra	Alteplase
Camostat mesilate (reduce viral infection)	Aviptadil	Alteplase
Chloroquine or hydroxychloroquine	Baricitinib (janus kinase (JAK) inhibitor)	Acetylsalicylic acid
Clevudine	Chlorpromazine	Atorvastatin (HMG-CoA inhibitor)
Darunavir/Cobicistat (Prezcobix; Rezolsta)	Colchicine	Bevacizumab (antivascular endothelial growth factors (VEGF))
Favipiravir (Avigan®)	Deferoxamine	Clopidogrel
Interferon	Dexamethasone	Crizanlizumab (vasculopathy)
Ivermectin plus nitazoxanide	Dornase alfa (Pulmozyme®)	Dapagliflozin (sodium-glucose transporter-2 inhibitor)
Lactoferrin	Duvelisib	Enoxaparin
Lopinavir-ritonavir (Kaletra®)	Eculizumab	Fondaparinux
Nafamostat (blocks TMPRSS2 activation and SARS-CoV-2 cell entry)	Famotidine	Heparin
Oseltamivir	Hydrocortisone	Losartan (angiotensin II receptor blocker (ARB))
Remdesivir	Imatinib	Nitric oxide (inhalation) Nicotine
	Infliximab	Ramipril (angiotensin-converting enzyme inhibitor (ACEi))
	Isotretinoin	Rivaroxaban (direct oral anticoagulant (DOAC))
	Leflunomide	Sulodexide
	Methylprednisolone Morphine	Telmisartan (ARB)
Umifenovir	Ozanimod	
	Plitidepsin	
	Prednisolone	
	Ruxolitinib (JAK inhibitor)	Valsartan (ARB)
	Sarilumab	
	Sirolimus	
	Tocilizumab (IL-6 inhibitor)	
	Tofacitinib (JAK inhibitor)	
Antioxidant treatment	*Traditional Chinese medicine*	*Oxygen therapy*
Vitamin A	Single herbs	Oxygen inhalation
Vitamin C	Chinese patent formulas	Mechanical ventilation
Vitamin D		Prone position ventilation
Vitamin E		Hyperbaric oxygen therapy
Glutathione	Chinese herbal compounds	
N-Acetyl-L-cysteine (NAC)		Oxyhydrogen inhalation *via* a nebulizer
Melatonin		
Zinc		
Vaccine and antibodies	*Extracorporeal membrane oxygenation support*	
mRNA, recombinant protein, vector	Oxygenation, removal of CO_2, filtrating proinflammatory cytokines via a filter	
Pamrevlumab		
Anti-SARS-CoV-2 convalescent plasma		

*Selected from 9149 total COVID-19 studies, including 6115 from COVID-19 NIH registered clinical trials [5] and the rest registered outside of the USA found from WHO International Clinical Trials Registry Platform (ICTRP) database [6–8].

easily penetrate mucus plugs; in fact, high airflow may instead further condense the plugs. It is also speculated that the positive pressure of ventilation may break the already-fragile alveolar sacs [9]. Moreover, ventilation of highly concentrated oxygen in patients with low SpO_2 levels may produce harmful superoxide free radicals like what happens in ischemia reperfusion.

2. Treatments Proposed and Investigated for COVID-19

Current guidelines for COVID-19 critical care involve general supportive measures such as hemodynamic support with a vasopressor (usually norepinephrine), corticosteroids to treat refractory shock, continuous renal replacement therapy (CRRT) or intermittent renal replacement (IRR) for acute renal failure, and mechanical ventilation to treat severe ARDS. However, the clinical benefit for patients with severe disease that requires aggressive oxygen management, such as invasive or noninvasive mechanical ventilation, high-flow oxygen, or ECMO, is uncertain. Given the high cost, procurement hurdles, and pending research, health agencies have restricted distribution to hospital systems for patients 12 years of age or older requiring supplemental oxygen without aggressive oxygen management [10].

No single pharmacotherapy has shown sufficient clinical efficacy for routine use; at the clinician's discretion, however, select patients with severe disease may receive a trial of remdesivir and/or immunomodulatory therapy (such as corticosteroids) [10].

2.1. Antiviral Therapies. Some preliminary studies suggest that antiretroviral remdesivir (Veklury™) may modestly shorten recovery time. However, despite its *in vitro* activity against SARS-CoV-2, its effect on mortality rate for patients with severe COVID-19 is uncertain [11–13]. Remdesivir, an adenosine analog, purportedly targets viral RNA to cause premature termination of reverse transcription [13] (Figure 1).

Other antivirals, such as lopinavir/ritonavir (Kaletra®), oseltamivir, or ribavirin, showed no clinical benefit in mortality [14–16]. Some studies combining lopinavir/ritonavir and ribavirin, however, have suggested a reduction in mortality and ARDS risk [14, 15]. Anti-infectives chloroquine and hydroxychloroquine have been studied exhaustively with clinical evidence suggesting no mortality benefit yet potential harm due to cardiac conduction abnormalities [17]. These results were negative despite their potent *in vitro* inhibitory effect on SARS-CoV-2 by raising host endosomal pH and preventing viral entry [13], though a study exploring their prophylactic role in healthcare workers is currently ongoing (NCT04334148). With similar publicity, the role of azithromycin remains contentious with the COALITION II trial, suggesting no clinical benefit when combined with hydroxychloroquine [18].

2.2. Immunomodulatory Therapies. Given the lack of effective antiviral treatments, some groups have investigated convalescent plasma (CP) as an interim treatment. Historically, CP has been used for various other infections (such as diphtheria, hepatitis A and B, rabies, or polio) for which at some time periods, like COVID-19, lacked any suitable pharmacological treatment [19]. In theory, immunocompetent COVID-19 survivors could produce immunoglobins as part of acquired immunity, which can then be purified and transfused. Its efficacy is heralded by reports that reinfection with COVID-19 is rare, indicating that these antibodies may be highly effective in preventing or treating severe COVID-19 [20]. While some preliminary studies have demonstrated reduced mortality, reduced oxygen requirements, and reduced viral load, with mostly minor adverse events, large-scale and high-quality clinical research is lacking [20]. Furthermore, some hypothesize that, as with infections similar to SARS and Middle East respiratory syndrome (MERS) [21, 22], conferred immunity will only last for a limited number of months and may not be effective in the long term. With the lack of viral-targeted treatments, the clinical focus has since shifted more towards preventing complications in advanced disease, with promise in treating with corticosteroids.

Corticosteroids were previously avoided due to the potential decrease in immune responses and viral clearance and increase in osteopenia and osteoporosis observed in patients with SARS and MERS [23]. Preliminary studies, however, have suggested that corticosteroids may mitigate the sequelae that lead to multisystem organ failure and lung injury observed in severe COVID-19. In particular, clinicians have closely observed the preliminary results of an open-label trial, RECOVERY ($n = 4321$), which suggested a clinically significant decrease in mortality for patients requiring oxygen and ventilation when treated with a 10-day course of dexamethasone 6 mg (NNT = 8 for ventilated patients, 34 for nonventilated oxygen therapy). No mortality benefit was observed for patients with early disease, or mild to moderate disease not requiring oxygen therapy, suggesting that dexamethasone works against the inflammatory response in later stages of disease rather than reducing the viral load [24]. Other corticosteroids were also briefly studied and are used clinically with benefit [25], but were stopped early pending the RECOVERY trial publication: these included hydrocortisone in the REMAP-CAP and CAPE COVID trials [26] and methylprednisolone [27]. Given the promiscuous anti-inflammatory nature and risks of corticosteroids, including dysglycemia, immunosuppression, latent infection reactivation particularly with *Strongyloides* [28], and agitation, research interest blossomed in pharmacotherapies that target specific anti-inflammatory pathways.

Clinicians have reported cytokine storms manifesting in patients with severe COVID-19, which has promoted additional research into molecules that target proinflammatory pathways to treat ARDS and multiorgan sequelae [29]. Some of these molecules include interleukins, such as anakinra (anti-IL-1), aviptadil (anti-IL-6 and antitumor necrosis factor (TNF)), monoclonal antibodies (anti-IL-6; tocilizumab, sarilumab, and siltuximab), and JAK inhibitors (anti-IL-6; ruxolitinib baricitinib); general anti-inflammatories such as colchicine; and steroid-sparing immunosuppressives such

as sirolimus and tacrolimus. Some of these studies have suggested potential clinical benefit in COVID-19. For instance, anakinra 5 mg/kg twice daily may improve survival for patients with moderate to severe ARDS compared to a historical cohort [30]. Similarly, studies with tocilizumab for patients experiencing cytokine storms have suggested potential benefit with one or two doses of 400 to 800 mg [31, 32]. However, treatment with tocilizumab in some cases worsened COVID-19 infections, likely because of immunosuppression [33]. Similarly, studies with other molecules have suggested no clinical effect or potential harm due to immunosuppression (such as with sarilumab) [34], or insufficient power of statistical analysis to measure a mortality benefit (such as with colchicine) [35].

2.3. Therapies with Ancillary Benefits from Other Mechanisms of Action. Molecules targeting other host pathways are also being investigated, and many studies, as shown in Table 1, are still ongoing. Murine studies have suggested, for instance, that lung sequelae such as leukocyte infiltration and acute lung failure from the related SARS-CoV from the 2003 pandemic could be reduced with angiotensin II receptor blocker (ARB) losartan 15 mg/kg, secondarily to inhibiting ACE2 binding of viral Spike-Fc [36]. Similarly, famotidine, a histamine 2-receptor blocker used for treating acid reflux disease, may inhibit viral replication by a mechanism still being investigated. Famotidine therapy was correlated with reduced inpatient mortality or intubation [37], with some cases of reduced outpatient symptom severity reported [38]. Furthermore, recent developments in coagulopathy secondarily to cytokine storms that expose the basement membrane and activate coagulation cascades have honed research in targeting VEGF (with bevacizumab), tissue plasminogen activators (alteplase) [39], and anticoagulants (argatroban, enoxaparin, fondaparinux, heparin, and rivaroxaban) [40].

Interestingly, some molecules have been investigated based on retrospective observations of patients with polypharmacy. Many of which seem to correlate with drugs that reduce inflammation and oxidative stress. For instance, some studies have suggested that sodium-glucose cotransporter-2 (SGLT2) inhibitors, a class of multifunctional antihyperglycemics, may prevent respiratory failure associated with endothelial disruption, inflammation, and oxidative stress by purportedly reducing serum lactate production and cytokines. Studies with dapagliflozin in patients with or without diabetes are currently underway (NCT04350593) [41]. Additionally, past studies with antilipidemic "statin" drugs (e.g., atorvastatin) have suggested improved symptom management in patients with concurrent viral infections with the annual avian influenza and the 2009 H1N1. These effects may be ascribed to their anti-inflammatory, antioxidant, and ACE2-downregulatory effects, which have prompted further clinical studies with atorvastatin [42]. In fact, this projection may be supported by observations from the use of statins in, for instance, the prevention of cytokine and oxidative stress-mediated iodinated contrast-induced nephrotoxicity [43].

Some think tanks have considered incidental findings of morphine and its inhibitory effects on cytokine production, particularly in dyspneic patients. Studies have found decreased levels of IL-12, TNF, and interferons, albeit inconsistently, when morphine is used in patients with chronic obstructive pulmonary disease (COPD) [44]. Other effects observed from morphine use may be translatable to similar features in the collection of syndromes related to COVID-19. One such study explored the prevention of mitochondrial-related reperfusion injury secondarily to post-myocardial infarction percutaneous intervention. By preventing the influx of reactive oxygen species and eventual cell death, morphine could have some effect in preventing damage after restoration of oxygen status to cells [45]. Studies with the use of morphine in dyspnea have been recruiting at the time of this article (NCT04522037).

Despite the current developments outlined, and over 9100 registered clinical trials to date [5–7], the vast research vision has tunneled to individual mechanisms that include viral entry, replication inhibition, or cytokine attenuation [46].

3. Importance and Possible Mechanisms of Molecular Hydrogen in COVID-19 Treatment

Alveolar hypoxia, alveolar macrophages, and reactive oxygen species (ROS) cause an inflammatory response which may lead to ARDS. Excess proinflammatory cytokine secretion may further damage multiple organs. To address all of these contributing factors to cytokine storm in COVID-19, inhalation of molecular hydrogen may offer an effective solution to tackle both hypoxia and oxidative stress, thereby reducing downstream cytokine secretion. Many reports described possible mechanisms of molecular hydrogen actions against different diseases [47–60]. The majority of these reports revealed three main effects of molecular hydrogen in pathophysiology: antioxidative stress, anti-inflammatory, and antiapoptotic effects. However, these three categories also include many subgroups of different effects of molecular hydrogen observed in various studies, for example, the regulation of oxidative stress, regulation of endoplasmic reticulum stress, regulation of mitochondria, inhibition of overactivation of the immune system, prevention of apoptosis, regulation of autophagy, reduction of pyroptosis-related inflammation, protection of cells from pyroptosis, positive regulation of ferroptosis, and potential regulation of the circadian clock. In 2020, Yang et al. listed the possible mechanisms of molecular hydrogen in 10 main disease systems [48]. In 2011, Ohta summarized the diseases and the organs targeted by molecular hydrogen treatment. After the appearance of the COVID-19 disease, many global efforts were applied to fight this pandemic [61]. In China, the famous epidemiologist Dr. Zhong Nanshan has applied H_2/O_2 inhalation for treating more than 2000 COVID-19 patients with very positive and effective outcomes [62, 63]. Additionally, a global scientific discussion has been launched on the ResearchGate platform about the possibility of the use of molecular hydrogen in COVID-19 treatment [64]. Several articles have been published about the potential benefits of molecular hydrogen therapy for COVID-19 [48, 65, 66],

including its ability to combat effects of fatigue [67]. Although its beneficial effects have been reported in the literature and demonstrated in some clinical trials, a systemic review of the properties and underlying mechanisms of molecular hydrogen is necessary to broaden the utility of its positive effects in treating COVID-19. Currently, there is no report that fully elucidates the mechanisms behind the positive influence of molecular hydrogen in COVID-19 treatment.

4. Physical, Chemical, and Biological Properties and Safety of Molecular Hydrogen

4.1. Physical Properties of Molecular Hydrogen. Hydrogen is the most abundant element in the universe especially in stars. It combines with another hydrogen atom to form molecular hydrogen, with the chemical symbol of H_2. H_2 is the smallest and lightest molecule with a density of 0.08988 g/L at standard temperature and pressure (STP). However, molecular hydrogen is rare in Earth's atmosphere at a level of about 0.53 ppm [68]. Hydrogen is physically characterized as a nontoxic, colorless, odorless, tasteless, and nonmetallic gas at standard temperature and pressure. H_2 has a lower solubility in water compared to oxygen and carbon dioxide with 0.8, 1.3, and 34.0 mmol/L at 20°C, respectively [69]. The hydrogen-saturated water contains 0.78 mM (1.6 mg/L) of hydrogen at 25°C. It was estimated that 2–5% of H_2 is lost every 3 min when hydrogen-rich water is kept in an open container [70]. To preserve the levels of hydrogen in hydrogen-rich water during storage, the product must be filled in a metal package such as aluminum as plastics are permeable to H_2 [51].

4.2. Chemical Properties of Molecular Hydrogen. The earliest known chemical property of hydrogen is that it burns with oxygen to form water. Under ordinary conditions, hydrogen gas is a loose aggregation of hydrogen molecules, each molecule consisting of a pair of hydrogen atoms, to form the diatomic molecule, H_2 [71]. Additionally, molecular hydrogen can react with many elements and compounds, but at room temperature, the reaction rates are usually so low as to be negligible due to its very high dissociation energy [72].

In food processing, H_2 is classified as a food additive with E949, and in the European Union (EU), it is permitted in part C group I of regulation 1129/2011 additives permitted at *quantum satis* [73]. At normal temperature and pressure, H_2 is considered a noncorrosive and not very reactive substance (inert gas). It is used to store foodstuffs in packages under modified atmosphere beside CO_2 and N_2, and so protects them from undesirable chemical reactions such as food spoilage and oxidation during subsequent transport and storage [74, 75]. The addition of molecular hydrogen, i.e., hydrogenation, is used to produce margarine and vegetable shortening by converting unsaturated liquid animal and vegetable oils and fats to a saturated solid form. These processes require a catalyst, and high temperatures and pressures to overcome the activation energy of the stable nonpolar covalent bond that holds the hydrogen atoms together. Moreover, hydrogen is used to reduce aldehydes, fatty acids, and esters to the corresponding alcohols.

4.3. Biological Properties of Molecular Hydrogen. Intestinal bacteria in humans naturally produce hydrogen at about 50 to 1,000 mg/day [76, 77] via degradation of oligosaccharides [78]. However, the amount of H_2 produced by colonic fermentation is partially consumed by bacterial flora in the colon [70]. The ingestion of hydrogen-rich water was reported to increase both hydrogen peaks and the area under the curve (AUC) of breath hydrogen in a dose-dependent manner [79] within 10 min of ingestion [70]. It was estimated that approximately 41% of ingested H_2 via hydrogen-rich water was kept in the body [70]. The loss of H_2 from the skin surface is negligible (less than 0.1%). Hydrogen may be transferred to the milk when the mother drinks hydrogen-rich water [80]. H_2 has no adverse effects on the saturation level of arterial oxygen (SpO_2) and hemodynamic parameters [81]. The inhalation of H_2/O_2 mixed gas did not interfere with any vital signs of the body including respiratory rate, heart rate, blood pressure, and pulse oximetry [82].

4.4. Safety Property of Molecular Hydrogen. The American Conference of Governmental Industrial Hygienists classifies hydrogen as a simple asphyxiant and describes its major hazard due to its flammable and explosive properties [83]. Hydrogen is highly flammable at a range of 4-75% (*v*/*v*) in air, and it explodes in the air at the range of 18.3-59% (*v*/*v*) [84, 85]. However, the dilution of hydrogen with nitrogen lowers the risk of explosion [86]. Additionally, the autoignition temperature (the temperature at which spontaneous combustion will occur) of hydrogen is quite high, i.e., 500°C.

5. Redox-Related Mechanisms in the Pathophysiology of COVID-19

The cellular redox status can affect the structural composition of various sensitive components found inside or on the surface of the cell. These redox-sensitive components include many proteins/enzymes composed of sulfur-containing amino acids/peptides (SH and S-S) making them sensitive to the redox state of the environment. Methionine, cysteine (Cys), cystine, homocysteine, glutathione, and hydrogen sulfide are the common sulfur-containing compounds impacting protein regulation and cell signaling. Furthermore, the cofactors such as Fe, Zn, Mg, and Cu found in their oxidized or reduced form, make the cellular enzymes susceptible to the redox change in the environment. In the same manner, we can discuss the effect of redox value on various redox-sensitive molecules located on the surface of the cell such as enzymes, proteins, phospholipids, and saturated and unsaturated fatty acids, which could become targets for the redox change in the environment/cytoplasm. The modification in the structure of these components can directly affect different functional and structural cellular systems such as cellular transport and bioenergetics.

The cell possesses a redox homeostasis system that regulates many key functions such as protein synthesis, enzyme activity, metabolic pathways, and transport across the membrane. This redox homeostasis can be regulated by different factors such as oxidoreductases (catalase (CAT), superoxide

dismutase (SODs), and glutathione peroxidase (GPXs)), metallic ions (Fe, Cu, Mg, etc.), metabolites (adenosine triphosphate/adenosine monophosphate (ATP/AMP), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and tricarboxylic acid cycle (TCA) intermediates), gaseous-signaling molecules (ROS, H_2, H_2S, CO, $NO^{\bullet}$, etc.), and internal antioxidants (ascorbate, vitamin E, β-carotene, urate, and thiols). Amino acids and their macromolecules, i.e., peptides and proteins, can affect and be affected by the redox state of the cytoplasm and environment. The amino acids, peptides, and proteins containing thiols (SH) form the targets for oxidants such as ROS [49]. The production of ROS and/or the change in the thiols/disulfide ratio lead to the perturbation of the intracellular redox homeostasis. This critical situation leads the cell to sense redox signaling, and thus regulate the cellular redox state [49]. When the levels of the generated ROS are high, the cell can use the redox-sensitive signaling pathways and transcription factors to upregulate genes encoding reductants such as thiols, enzymes, thioredoxin (Trxs), and glutaredoxins (Glrxs) that will reset redox homeostasis [49]. However, when the situation is more severe with very high levels of ROS, for example, during acute injury or inflammation, damage occurs to various macromolecules and cellular structures and functions, which can lead to irreversible injury and cell death. The presence of molecular hydrogen in the last case can mitigate the cytotoxic effects of ROS by reducing only the most aggressive ones, i.e., $^{\bullet}OH$ and $ONOO^{-}$, without affecting the physiologically beneficial ROS-dependent signaling molecules, i.e., $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}NO$, and thus, maintaining redox homeostasis of the cell [52].

The modification of the structural composition of proteins due to the change of thiol (SH) to the disulfide (S-S) form impairs molecular chaperoning, translation, metabolism, cytoskeletal structure, cell growth, and signal transduction. Additionally, the formation of disulfide bonds affects the conformation of redox-sensitive proteins [58]. It was reported that in an oxidizing medium, the sulfur group in cysteine can form intramolecular disulfide bonds creating a reversible cross-link that can be broken in the presence of a reducing agent [87]. Oxidative stress conditions are characterized by a high generation of ROS and are related to many diseases involving disulfide bond formation [87]. Thiol-disulfide reactions follow an exchangeable and rate-dependent bond rupture mechanism [87].

5.1. Importance of Thiols for Cellular Redox Status. Thiols have been shown to play a key role in many functional processes in cellular physiology. Glutathione (GSH), for example, was identified as a crucial intracellular antioxidant thiol that plays an essential role in protection against environmental oxidant-mediated injury in addition to its role in the redox signaling process [88]. The increase in the intracellular content of GSH leads to a decrease in the release of cytokines and chemokines from lung cells by decreasing NF-κB activation. This property was related to the antioxidant activity of GSH [88]. Normally, glutathione disulfide (GSSG) represents less than 1% of the total cellular GSH pool. The perturbation to the GSH/GSSG ratio due to the excessive generation of ROS can alter signaling pathways that play key roles in many physiological responses such as cell proliferation, autophagy, apoptosis, and gene expression.

It was reported that the activation of redox-sensitive transcription factors such as nuclear factor erythroid 2-related factor 2 (Nrf2), NF-κB, and activator protein 1 (AP-1) differentially regulate the genes for proinflammatory cytokines as well as the protective antioxidant genes [88]. Moreover, GSH is considered a crucial factor for the enzymatic activity of GPx, which is a major contributor to the cellular enzymatic antioxidant defense [89]. Sustained oxidative challenge leads to depletion of lung GSH along with other antioxidants forming the main reasons for many lung diseases, e.g., ARDS, chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis (CF), idiopathic pulmonary fibrosis (IPF), and neonatal lung disease [88]. Moreover, GSH levels were found to be depleted in several viral infections such as infection with HIV, influenza A virus, hepatitis C virus, and herpes simplex virus-1 [90]. On the other hand, the decrease in the levels of GSH in the lung lining fluid have been shown in various pulmonary diseases such as IPF, ARDS, CF, lung allograft patients, and patients with human immunodeficiency virus (HIV) [88]. This observation was explained by the formation of disulfide bonds due to the huge generation of ROS. Accordingly, several approaches have been studied to increase the cellular GSH levels to improve the cell's ability to cope with the increased ROS production. The administration of GSH itself has been shown to have limited therapeutic value due to its short plasma half-life, i.e., <30 min, and its inability to pass the cell membrane. Therefore, other strategies have been evaluated to increase intracellular GSH pools.

One of the most studied pro-GSH molecules is N-acetyl-L-cysteine (NAC). Roederer et al. demonstrated in 1992 that NAC inhibited HIV replication *in vitro* [91]. NAC, ascorbic acid, and vitamin E were reported to decrease both viral replication and inflammation in cells of mice infected with influenza (IV) and/or human respiratory syncytial (HRSV) respiratory viruses [92]. Although the treatment of NAC *in vitro* and *in vivo* experiments showed an increase in GSH levels that reduced the viral load by inhibiting viral replication in several viruses, e.g., influenza A (H3N2 and H5N1), the protective effect of NAC alone appeared weak or null in some models with a variation in its efficacy depending on the infecting viral strain [93]. Based on a trial study of 198 patients with COVID-19, a noticeable increase of glutathione reductase levels occurred in around 40% of COVID-19 patients [93] suggesting an increase in GSH metabolism. However, although NAC may be effective in this case, its antioxidant and therapeutic benefits may be strain specific. Therefore, clinical evidence is required before NAC supplementation can be recommended. Moreover, there is currently no COVID-specific evidence for the use of NAC [93].

5.2. Potential Use of Molecular Hydrogen to Improve Cellular Redox Status. A favorable GSH balance was reported to ameliorate bronchial asthma by suppressing chemokine production and eosinophil migration itself [88]. The latter authors

Table 2: Summary of some possible mechanisms related to the positive effects of molecular hydrogen in different diseases and COVID-19 treatment.

Possible Mechanism	Type of Study	Principle	Reference
Molecular properties-related mechanisms	In vivo	Unlike most antioxidants, can penetrate biomembranes and diffuse into the cytosol, mitochondria and nucleus and reach cell organelles Has a rapid gaseous diffusion rate making it highly effective for reducing cytotoxic radicals Regulates the redox homeostasis after a ROS-related dissipation stage Mild enough not to disrupt metabolic oxidoreduction reactions or interrupt ROS-induced disruption of cell signaling Selectively reduce the strongest cytotoxic oxidants, $^{\bullet}OH$ and $ONOO^{-}$; whereas, the biological useful oxidants such as superoxide, hydrogen peroxide, nitric oxide are not altered	[50]
	in silico	Protects nuclear DNA and mitochondria Protects cells and tissues against strong oxidative stress Decreases production of ROS Reduces the reversible cross-linked intramolecular disulfide bonds formed after an oxidative stress e.g. ROS Decreases the energy barrier of disulfide rupture Balances the S-S/SH in favor of thiols	[86]
Redox-related mechanisms	In vivo	Protects Inositol 1, 4, 5-trisphosphate receptors (IP3Rs) function Protects the ATP-induced Ca^{2+} signal by reducing the H_2O_2-induced disulfide bonds in IP3Rs and restores protein function Activates glutathione/thioredoxin systems involved in the modulation of disulfide bond formation during oxidative stress leading to reduced H_2O_2-induced disulfide bond formation Repairs the processes of cell injury produced through high ROS generation Mitigates the oxidative damage selectively reduces $^{\bullet}OH$ attenuating ischemia/reperfusion-Induced organ damage	[58]
	Animal	Increases superoxide dismutase (SOD) activity against ROS-mediated cellular damage Increases activities of antioxidant enzymes Can significantly decrease levels of oxidative products	[98]
	Human	Induces superoxide dismutases (SODs) activity to quench ROS production	[99]
	Human	Decreases ROS levels via upregulating superoxide dismutase (SOD) and glutathione (GSH) as well as downregulating NADPH oxidase (NOX 2) expression	[100]
	Animal	Decreases oxidative damage	[98]
Inflammatory reactions and apoptosis-related mechanisms	Animal	Inhibits the over-expression of inflammatory factors (IL-6, IL-8 and TNF-α) Downregulates the expression of proapoptotic Fas proteins Up-regulates the expression of the anti-apoptotic protein Bcl2 Ameliorates LPS-induced bronchopulmonary dysplasia Reduces LPS-induced oxidative stress production	[98]
Lung and alveoli-related mechanisms	Animal	Ameliorates LPS-induced suppression of genes encoding fibroblast growth factor receptor 4 (FGFR4), VEGFR2, and HO-1, as well as LPS-induced overexpression of inflammatory marker proteins (TNFα and IL-6) Suppresses the induced expressions of inflammatory marker proteins (TNFα and IL-6)	[79]
	Animal	Reduces ROS production in alveolar epithelial cells Attenuates septic shock-induced organ injury Decreases neutrophil infiltrate in the alveoli	[98]

Table 2: Continued.

Possible Mechanism	Type of Study	Principle	Reference
		Reduces alveolar damage	
		Reduces levels of high-mobility group box 1 in serum and lung tissue improving the survival rate of mice with sepsis	
		Reduces the levels of IL-6, IL-8 and TNF-α	
		Down-regulates the levels of Fas protein and up-regulates the levels of Bcl2 protein, which may inhibit ALI by inducing apoptosis, and may protect lung function	
		Effectively prevents enterogenous sepsis	
		Significantly decreases the level of MDA and MPO	
		Protects against the alveolar destruction attenuating oxidative DNA damage and SIPS in the lungs	
	Animal	Decreases the markers of oxidative DNA damage such as phosphorylated histone H2AX and 8-hydroxydeoxyguanosine, and senescence markers such as cyclin-dependent kinase inhibitor 2A, cyclin-dependent kinase inhibitor 1, and b-galactosidase	[101]
		Restores static lung compliance	
		Reduces airspace enlargement and parenchymal destruction	
		Attenuates cigarette smoke-induced oxidative DNA damage and premature senescence in the lungs	
	Animal	Enhances phagocytic activity of alveolar macrophages	[102]
		Attenuates lung injury	
		Attenuates alveolar epithelial barrier damage	
	Animal	Improves alveolar gas exchange	[60]
		Reduces cell damage caused by alveolar epithelial cell apoptosis and excessive autophagy	
	Human	H_2/O_2 mixture relieves dyspnea and alleviates patient discomfort during the perioperative period	[81]
Small intestine injury-related mechanisms	Animal	Protects the intestinal mucosa from mechanical injury	[98]
		Reduces the pathological changes of the small intestine	
		Inhibits bacterial translocation	
		Protects the function of other organs in the body	

revealed that small changes in the cellular redox status may alter signaling pathways, and the GSH/GSSG ratio can serve as a good indicator of the cellular redox state. While the increase in the GSH/GSSG leads to proliferation, the decrease in the GSH/GSSG causes apoptosis. GSH/GSSG and Cys/CySS were found to be decreased in some oxidative-related diseases such as smoking, diabetes, obesity, and pneumonia [94]. Those most susceptible to developing COVID-19 and serious illness are those with underlying pathologies such as obesity, which is associated with impaired redox and inflammatory homeostasis [95]. Another beneficial role of hydrogen in oxidative stress-related diseases may be attributed to balancing the S-S/SH in favor of thiols. Previous reports indicate that the presence of reducing agents decreased the number of disulfide bonds, resulting in a loss of cross-link-induced stability produced by the chemical microenvironment [58]. In 2012, Keten et al. reported that the stability of the disulfide bond may mildly be influenced by the redox value of the chemical microenvironment where the concentration of reducing agents can trigger various fractures in the protein by decreasing the energy barrier of disulfide rupture [87]. They performed a simulation of disulfide rupture in the presence of a hydrogen molecule, illustrating the reduction mechanism of the disulfide bond. This phenomenon was explained by the elongation of the disulfide bond leading to a weakening of the bond followed by a reduction of the sulfur atoms and fracture of the protein at the S-S bond. The authors assumed that the reaction of the hydrogen molecule with a disulfide bond occurs violently once they are near each other [87] (Table 2). However, there is no evidence that this hypothetical mechanism is responsible for the observed biological effects of molecular hydrogen at improving the GSH/GSSG ratio. However, H_2 can increase GSH levels [96] by activating the Nrf2 pathway [97]. A nonsignificant increase in GSH, GSH/GSSG, and GSH peroxidase combined with a decrease in GSSG levels in rat livers fed with hydrogen-rich water compared to control was reported [98].

Interestingly, both endogenous and exogenous oxidants have been shown to need hours to significantly affect GSH levels in the majority of cells [88]. This is a double-edged

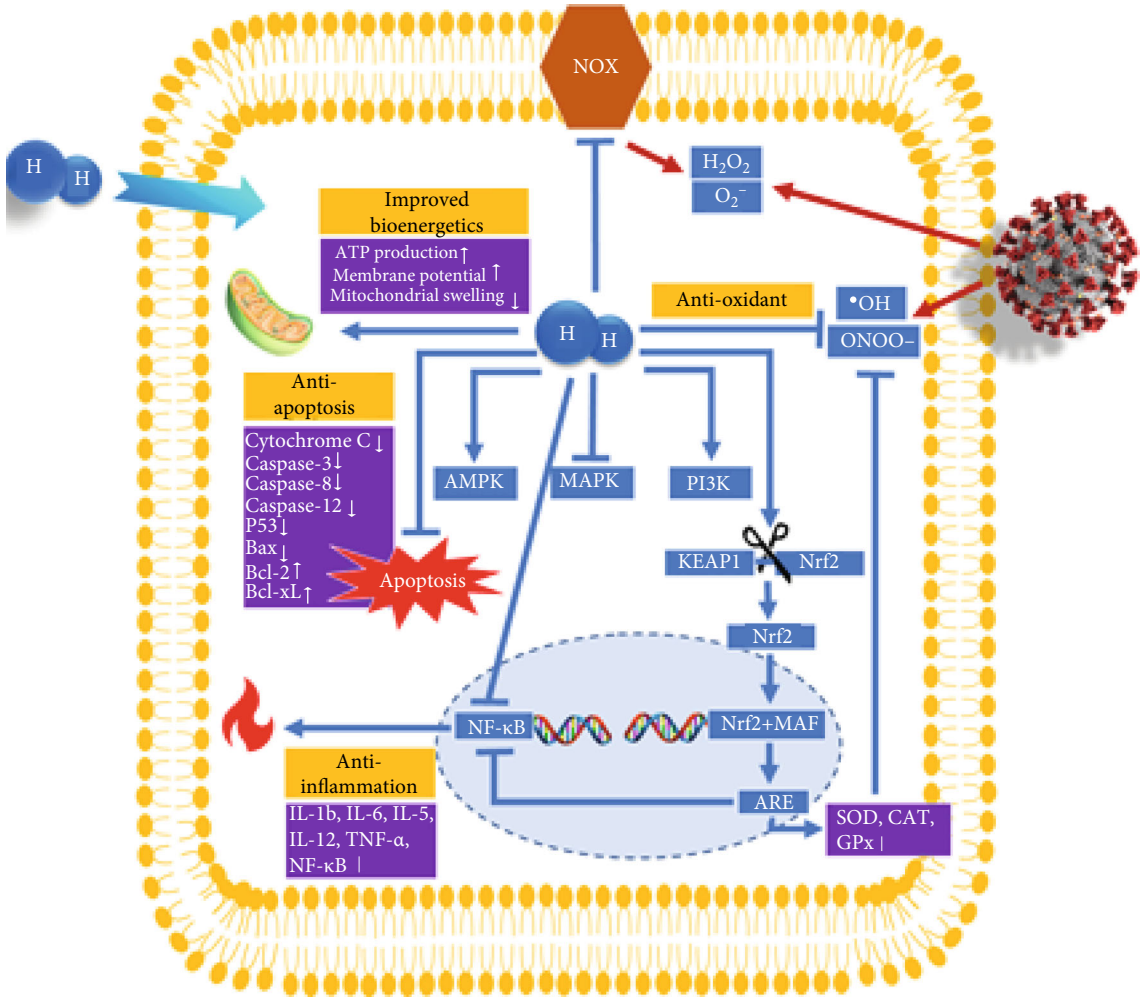

Figure 2: Possible mechanisms of alleviation properties of molecular hydrogen on COVID-19 patients. •OH: hydroxyl radical; O_2^-: superoxide anion; $ONOO^-$: peroxynitrite anion; H_2O_2: hydrogen peroxide; H_2: molecular hydrogen; NOX: nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; AMPK: 5′ adenosine monophosphate- (AMP-) activated protein kinase; MAPK: mitogen-activated protein kinase; PI3K: phosphatidylinositol 3-kinase; Keap1: Kelch-like ECH-associated protein 1; Nrf2: nuclear factor erythroid 2-related factor 2; MAF: small MAF protein; ARE: Nrf2-antioxidant response element; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; P53: tumor protein; Bax: BCL2-associated X protein; Bcl-2: B-cell lymphoma 2 protein; Bcl-XL: B-cell lymphoma–extra-large protein; IL-12: interleukin 12; IL-1β: interleukin 1-beta; IL-6: interleukin 6; IL-8: interleukin 8; TNF-α: tumor necrosis factor α; NF-κB: nuclear factor-kappa-light-chain-enhancer of activated B cells.

sword because, on the one hand, the redox status stays in the range of homeostasis despite a significant amount of oxidative stress. On the other hand, by the time the GSH/GSSG ratio has changed enough to be detected, it may be too late and/or too difficult to reestablish homeostasis by pharmacological interventions. Once the GSH levels are depleted, the antioxidant redox cycling is also negatively impacted, potentially rendering pharmacological interventions or antioxidant supplementation less effective. However, premature ingestion of reducing substances either orally or intravenously may exacerbate the redox condition. In contrast, molecular hydrogen is capable of reaching any organelle in the cell within minutes and does not perturb the GSH/GSSH ratio from optimal homeostasis. Instead, H_2 modulates signal transduction and maintains optimal redox homeostasis within the cell (Table 2). In this way, H_2 has the ability to act as a reducing agent at low concentration with the ability to antagonize the ROS-induced deleterious effects on cell signaling [50]. H_2 has been characterized by its ability to decrease ROS levels via upregulating superoxide dismutase (SOD) and glutathione (GSH) as well as downregulating NADPH oxidase (NOX 2) expression in a rat model [101] (Table 2 and Figure 2).

An additional but crucial role of hydrogen was found in repair processes of cell injury produced through high ROS generation. H_2 can induce heat shock proteins (HSPs) and suppress ROS production [58]. For example, the activation of glutathione/thioredoxin systems, which reduces H_2O_2-induced disulfide bond formation, is another possible mechanism underlying the H_2-induced elimination of ROS damage of inositol 1,4,5-trisphosphate receptors (IP3Rs) [58]. H_2O_2 is a highly reactive molecule capable of oxidizing

sulfhydryl groups of cysteine and methionine in proteins and forming sulfenic acid or disulfide [49, 58]. This modification in the structure induces dysfunction of proteins leading to the impairment of many physiological processes. By this phenomenon, H_2O_2 was able to decrease the Ca^{2+} signal by triggering IP3R disulfide bond formation. However, the IP3R function was partially protected by treatment with H_2 [58]. In other words, the H_2-containing medium protected the ATP-induced Ca^{2+} signal by reducing the H_2O_2-induced disulfide bonds in IP3Rs.

SARS-CoV-2 infection was reported to evoke free radical-associated damage in the body by targeting different molecules. Therefore, all therapeutic means that can alleviate free radicals may be considered for COVID-19 patients to conquer the inflammation-induced burst of free radicals [104]. The rapid gaseous diffusion of H_2 makes it highly effective for penetrating the subcellular compartments of the body. Importantly, H_2 was identified as clinically more effective than two ROS scavengers for the treatment of cerebral infarction, i.e., edaravone and FK506, in alleviating oxidative injury [105]. In addition to the greater benefit compared to other ROS scavengers, H_2 is considered mild enough not to affect the ROS that play essential roles in signal transduction such as H_2O_2, $NO^{\bullet}$, and $O_2^{-\bullet}$ [50]. H_2 can react with only the strongest oxidants, i.e., $^{\bullet}OH$ and $ONOO^-$, which are considered the most reactive ROS (Figure 2). Additionally, H_2 does not reduce the oxidized form of some biomolecules/cofactors involved in metabolic oxidoreduction reactions, e.g., NAD^+, FAD, or the oxidized form of cytochrome C [50] (Table 2).

5.3. Alveolus-Related Mechanism of Molecular Hydrogen-Based COVID-19 Treatment. Pulmonary surfactants play various crucial roles in the function of alveoli. The surfactants prevent lung collapse, increase the gas exchange, and contribute to the elastic properties of the lungs. These functions of surfactants can be accomplished due to their ability to reduce the surface tension inside the alveoli. These surfactants are composed of lipids, phospholipids, and proteins synthesized and secreted by alveolar type II cells that line the alveolar surfaces of the lungs [106]. The fluid lining alveolar surfaces contains different antioxidants such as GSH, vitamin C, and ceruloplasmin, which can quench free radicals [106]. The content of GSH in the respiratory tract lining fluids (RTLFs) was reported to be subnormal in various diseases such as acute immunodeficiency syndrome (AIDS), idiopathic pulmonary fibrosis, cystic fibrosis, acute respiratory disease syndrome, and in lung allograft patients [107]. The SOD and CAT were reported to be found in both surfactant and lung epithelial lining fluid, and take part in the regulation of postnatal lung vascular development and the protection of microvasculature from ROS-induced injury [108].

The oxidative modification of surfactants due to the effect of ROS on phospholipids, lipids, proteins, and biophysical activity can lead to dysfunction and several lung diseases such as acute lung injury and acute respiratory distress syndrome [109]. ROS production can lead to an increased lipid peroxidation and destruction of the cell membrane of the alveolar epithelial cells, and an increased membrane permeability [99].

Two factors were reported to promote the oxidation of surfactant lipids. First, the excessive production of ROS makes the antioxidant defenses incapable of providing protection. Secondly, the major antioxidants in the alveoli may be excluded from the microenvironment [106]. The ROS or reactive nitrogen species (RNS), especially $ONOO^-$, produced during lung injury can cause surfactant inactivation leading to increased leakage of proteins into the alveoli [110]. This latter situation prolongs the need for supplemental oxygen and assisted ventilation. It was reported that, once the SARS-CoV-2 enters the respiratory tract, it reaches the alveoli where its primary target is the type II pneumocyte, thus impairing surfactant production [111]. It was reported that both SARS-CoV-2 and SARS-CoV-1 viruses perturb alveoli to produce the major pathology in the lung, resulting in increased fluid entry, cell death, and inflammation, along with a reduction in gas exchange and levels of surfactant [112] (Figure 3).

Different antioxidants were proposed to prevent lipid peroxidation of lung surfactants such as melatonin-ebselen and vitamin E [106]. Importantly, it was reported that the continuous exposure (24 hours) to 10% hydrogen decreased the production of ROS in A549 human lung epithelial cells [80]. It was also revealed that inhalation of 2% hydrogen attenuated septic shock-induced organ injury and decreased neutrophil infiltrate in the alveoli, and reduced alveolar damage [99]. On the other hand, inhalation of H_2/O_2 mixed gas has been shown to reduce the inspiratory effort in patients with acute severe tracheal stenosis [82]. Moreover, hydrogen-rich water was reported to protect against the alveolar destruction attenuating the oxidative DNA damage and swimming-induced pulmonary edema (SIPS) in the lungs of COPD model mice [102]. Furthermore, hydrogen-rich water was found to attenuate lung injury by inhibiting lipid peroxidation [103]. Hydrogen-rich saline was also reported to reduce ROS production in alveolar epithelial cells, attenuate the alveolar epithelial barrier damage, improve alveolar gas exchange, and reduce cell damage caused by alveolar epithelial cell apoptosis and excessive autophagy [60] (Table 2).

6. Conclusion and Perspectives

An explanation for the advantageous effects of molecular hydrogen in COVID-19 treatment is related to the different properties of molecular hydrogen: (1) the small molecular size and nonpolarity of H_2 allow it to rapidly permeate the tissues and cells, (2) it can selectively reduce only the cytotoxic ROS, (3) it can suppress the excessive production of otherwise good ROS, (4) it can suppress proinflammatory cytokines, (5) it can induce cytoprotective heat shock proteins, (6) it can improve mitochondrial bioenergetics, and (7) it has no known toxic effects even at very high levels [114]. These properties may explain the improvement in the conditions of COVID-19 patients treated by inhalation of H_2/O_2 mixed gas (67% H_2/33% O_2), who felt reduction in chest pain and cough, and easier deeper breathing and

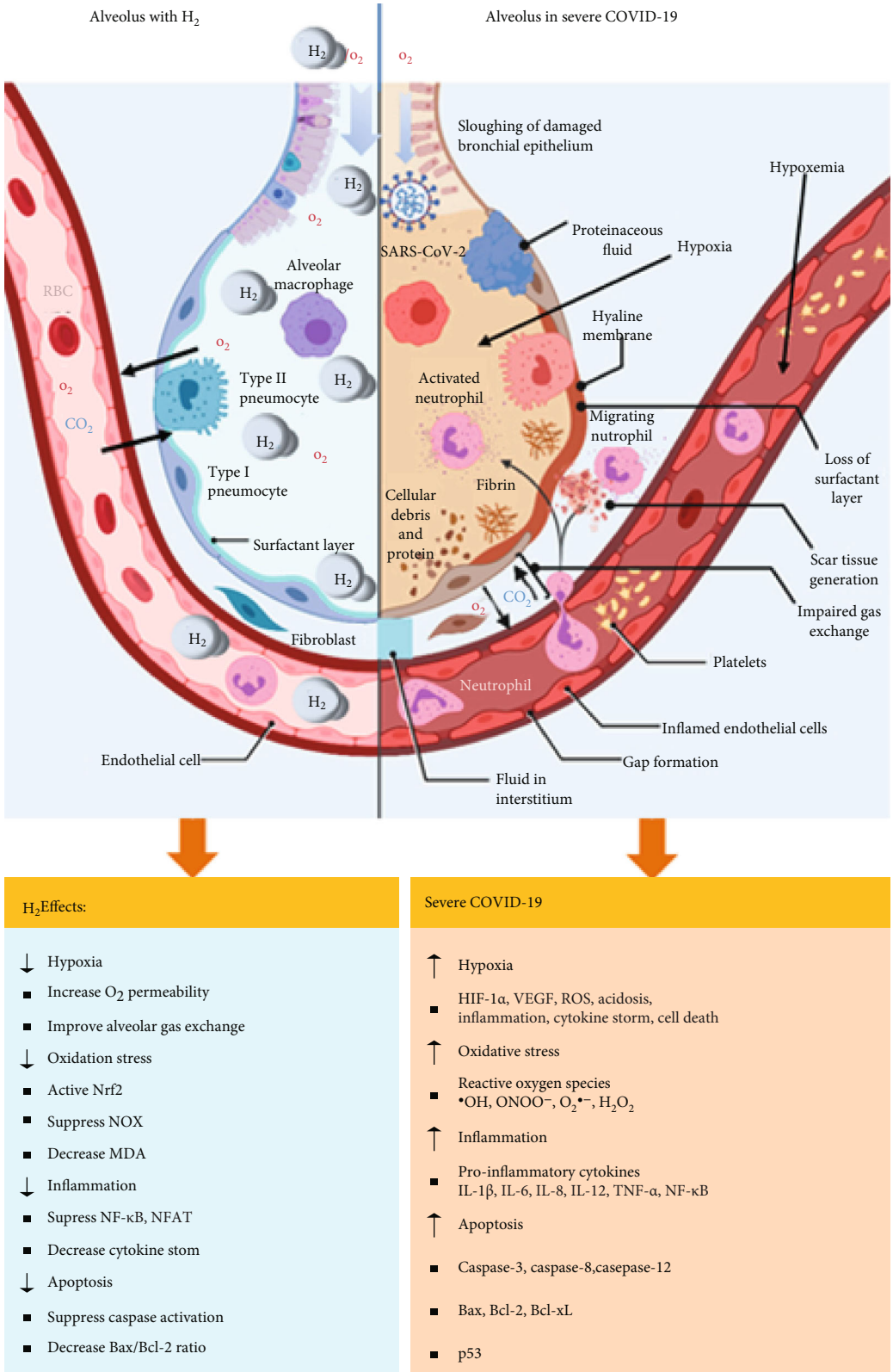

Figure 3: Alveolar changes due to SARS-CoV-2 infection in severe COVID-19 cytokine syndrome-induced acute respiratory distress syndrome (ARDS). Reproduced and modified from [113].

comfort sensation [62, 63]. The positive results of the pilot study led Dr. Zhong Nanshan, the epidemiologist who discovered the SARS virus (SARS-CoV-1) in 2003, to recommend the H_2/O_2 inhalation therapy for COVID-19 patients [115] and prompted more clinical trials using H_2/O_2 mixed gas [116–118].

Currently, there are twenty registered clinical trials on the use of H_2 for COVID-19. Of these, four are registered at the Centre for Evidence-Based Medicine (Oxford) using H_2/O_2 mixed gas inhalation [116], five clinical trials are registered at ClinicalTrials.gov of the US National Library of Medicine for inhalation [118], eight clinical trials are registered at ICTRP (WHO) with six for inhalation and two trials for hydrogen-rich water [8], and three clinical trials, related to the use of either inhalation or ingestion of hydrogen-rich water, are registered at the Chinese Clinical Trial Registry center [117]. Up to date, the reported benefits of H_2 therapy in COVID-19 patients are limited to the symptomatic description. To expand the utility of H_2 therapy in COVID-19, more thorough understanding of the underlying mechanism of H_2 in patients is required. Therefore, accurate analysis of a broad spectrum of biomarkers is highly recommended to delineate the correlation between clinical and biochemical presentations and the proposed biological effect of H_2.

According to the report of WHO, data from China and around the world suggest that the majority of people with COVID-19 have a mild illness, about 15% of them have a severe illness requiring oxygen therapy, and 5% are critically ill requiring mechanical ventilation. Owing to the widespread transmissibility and emergence of more infectious variants of SARS-CoV-2, many hospitals have been overwhelmed by the crush of new COVID-19 patients and have exhausted ICU beds and ventilators in some regions. Therefore, an alternative yet effective treatment, e.g., H_2/O_2 gas inhalation, would ease the pressure on hospitals and prevent severe illness of COVID-19 patients.

The medical model of H_2/O_2 mixed gas machine is small, portable, and safe [119]. It costs about one-tenth of the price of a ventilator. The H_2/O_2 inhalation treatment may be performed in regular wards or by outpatients at home isolation using a portable H_2/O_2 generating and inhalation device. This kind of treatment may reduce hospitalization time for a high number of patients. This strategy could decrease the pressure of massive patient numbers on hospitals. It is important to mention that, although molecular hydrogen is not considered a drug, its intake in different ways such as drinking hydrogen-rich water or inhaling H_2/O_2 gas may be beneficial in preventive medical health in addition to its therapeutic use. Due to the high safety profile and favorable preliminary results in preclinical and clinical studies, application and additional research of molecular hydrogen therapy for COVID-19 are encouraged.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Research Article

Association of Low Molecular Weight Plasma Aminothiols with the Severity of Coronavirus Disease 2019

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Objective. Aminothiols (glutathione (GSH), cysteinylglycine (CG)) may play an important role in the pathogenesis of coronavirus disease 2019 (COVID-19), but the possible association of these indicators with the severity of COVID-19 has not yet been investigated. *Methods*. The total content (t) and reduced forms (r) of aminothiols were determined in patients with COVID-19 ($n = 59$) on admission. Lung injury was characterized by computed tomography (CT) findings in accordance with the CT0-4 classification. *Results*. Low tGSH level was associated with the risk of severe COVID-19 ($tGSH \leq 1.5\,\mu M$, mild vs. moderate/severe: risk ratio (RR) = 3.09, $p = 0.007$) and degree of lung damage ($tGSH \leq 1.8\,\mu M$, CT < 2 vs. CT ≥ 2: RR = 2.14, $p = 0.0094$). The rGSH level showed a negative association with D-dimer levels ($\rho = -0.599$, $p = 0.014$). Low rCG level was also associated with the risk of lung damage ($rCG \leq 1.3\,\mu M$, CT < 2 vs. CT ≥ 2: RR = 2.28, $p = 0.001$). Levels of rCG ($\rho = -0.339$, $p = 0.012$) and especially tCG ($\rho = -0.551$, $p = 0.004$) were negatively associated with platelet count. In addition, a significant relationship was found between the advanced oxidation protein product level and tGSH in patients with moderate or severe but not in patients with mild COVID-19. *Conclusion*. Thus, tGSH and rCG can be seen as potential markers for the risk of severe COVID-19. GSH appears to be an important factor to oxidative damage prevention as infection progresses. This suggests the potential clinical efficacy of correcting glutathione metabolism as an adjunct therapy for COVID-19.

1. Introduction

The treatment of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has become a focus of medical research since 2020. Endothelial dysfunction plays a key role in the pathogenesis of this condition, and the disease develops as a result of the disruption of the surface protein angiotensin II-converting enzyme (ACE II). This triggers numerous pathways that dysregulate the homeostasis of vascular tone and permeability and leads to impaired lung function and in some cases to multiple organ failure [1].

Markers of COVID-19 severity and prognosis are being actively studied. Several studies have suggested that a precise disulfide-thiol balance is crucial for viral entry and fusion to the host cell and that oxidative stress generated from free radicals can affect this balance [2]. Low molecular weight aminothiols (LMWTs: cysteine (Cys), cysteinylglycine

(CG), glutathione (GSH), and homocysteine (Hcy)) play an important role in biochemical processes involved in the key mechanisms of the body's response to COVID-19; therefore, they can also be considered potential biomarkers of the severity of COVID-19. GSH is the main intracellular antioxidant, and glutathionylation of proteins is one of the important mechanisms of posttranslational regulation of their function [3]. Low GSH levels are associated with a predisposition to respiratory tract infections and cardiometabolic disorders [4, 5]. GSH and Cys were found as independent factors of atherothrombotic events [6]. In plasma, GSH is hydrolyzed to CG. For the synthesis of GSH, Cys is required, which can be formed as a result of hydrolytic cleavage of proteins, acquired from the extracellular environment or synthesized from Hcy by the so-called transsulfuration pathway. Hcy is formed from methionine via the intermediates S-adenosylmethionine and S-adenosylhomocysteine. Due to the fact that all transmethylation reactions use S-adenosylmethionine as a methyl group donor, Hcy has an impact on many vital processes, including the regulation of gene expression for cytokines, inflammatory proteins, and proliferation of viral particles.

Elevated Hcy levels (hyperhomocysteinemia (HHcy)) may be an important factor affecting the negative course of COVID-19 since it affects key pathophysiological mechanisms: oxidative stress (OS), endothelial dysfunction, thrombosis, and activation of type 1 receptors to angiotensin II (AT1R) [7–9]. In contrast, OS, which is characteristic of systemic inflammatory diseases, negatively affects the basic pathways of Hcy utilization (methionine synthase and betaine homocysteine methyltransferase activity, which can lead to an increase in Hcy plasma levels [10–12]. In blood plasma, LMWTs exist mainly in the oxidized form, and only a small proportion is in the reduced (*r*) form [13]. The sum of these forms is the total (*t*) content of LMWTs.

Despite the high interest in COVID-19, its effects on the LMWT system have not yet been reported. Several studies have suggested that high Hcy and especially a GSH deficit are risk factors for the severity of COVID-19 or its complications [2, 14–20]. One study demonstrated that a high tHcy can predict severe pneumonia on chest CT in COVID-19 patients [21]. The same study found that a tHcy level exceeding 15.4 μM increases the probability of COVID-19 progression to extremely severe forms of severe acute respiratory syndrome by 3.2–3.5-fold.

In this study, we investigated the plasma levels of the *r*- and *t*-forms of Hcy, Cys, CG, and GSH in COVID-19 patients, in order to identify their associations with traditionally used laboratory parameters and advanced oxidation protein products (AOPP, a marker of oxidative stress (OS)) and to identify the possible impact of these LMWTs on COVID-19 severity and level of lung injury.

2. Methods

2.1. Patients. This study included 59 COVID-19 patients who were admitted to the pulmonary department of the Burdenko Main Military Clinical Hospital from August 24 to November 13, 2020. The study was approved by the local institutional ethics committee. Informed written consent was obtained from each patient. A graphical scheme of study design is presented in Figure 1.

The patients were diagnosed according to the World Health Organization interim guidance for COVID-19. The main inclusion criterion was a confirmed primary SARS-CoV-2 infection. Exclusion criteria included the following: exacerbations of cardiovascular disease, HIV infection, hepatitis B and C, terminal cancer, and decompensated renal failure. All patients included in the study were discharged with recovery from infection and improvement in their general condition.

Chest CT scans were performed on the 48 h of patients' admission using the Optima CT660 tomograph (GE Healthcare, USA), from the level of the thoracic entrance to the level of the diaphragm, and completed at the end of inspiration. The scanning parameters were as follows: tube voltage 120 kV, tube current 114~350 mA, and layer thickness 5 mm. At the end of scanning, a thin layer image with a layer thickness of 2.5 mm is automatically reconstructed and recorded as DICOM image data. The reconstruction algorithm used is with a field of view of 360 mm × 360 mm and a matrix of 512 × 512. Image browsing and multiplane reconstruction were performed using GE AW VolumeShare software v.4.6; images of the lungs (window width 1500, window level 500) and the mediastinum (window width 350, window level 35-40) were also observed using the same software. Image analysis was performed based on the standard protocol as described elsewere [22]. The degree of lung damage then was assessed using the following scoring system based on percentage of lobar involvement: <5% (CT0), 5-25% (CT1), 26-49% (CT2), 50-75% (CT3), and >75% (CT4) [23]. Based on the data of an objective study, respiratory function, and blood oxygen saturation, patients were categorized as having mild, moderate, or severe COVID-19 using previously described criteria above [24].

2.2. Laboratory Procedures. Venous blood samples were collected upon admission in tubes containing sodium citrate (0.105 M) and centrifuged at 3000*g* for 15 minutes. Then, plasma (1450 μl) was mixed with 3 M acetic acid (50 μl) and samples were frozen at -80°C and stored until LMWTs determination.

All patients were confirmed by viral detections using the SARS-CoV-2 nucleic acid detection kit "AmpliPrime® SARS-CoV-2 DUO" (Next Bio, Russia) and PCR analyzer RotorGene Q (Qiagen, Germany). Hematology analyzer MD-7600 (Meredith Diagnostics, United Kingdom), automatic biochemistry analyzer Ellipse (Analyzer Medical System, Italy), Biosen C line (EKF Diagnostics, Germany), express immunochemiluminescent analyzer PATHFAST (Mitsubishi Chemical Medience Corporation, Japan), and erythrocyte sedimentation rate analyzer ESR 3000 (SFRI, France) were used for routine blood analysis.

LMWTs were determined by liquid chromatography as described early with some modifications [25]. An UPLC ACQUITY system (Waters, Milford, MA) with a PDAλ UV-detector (λ = 330 nm) and FTN Sample manager was used.

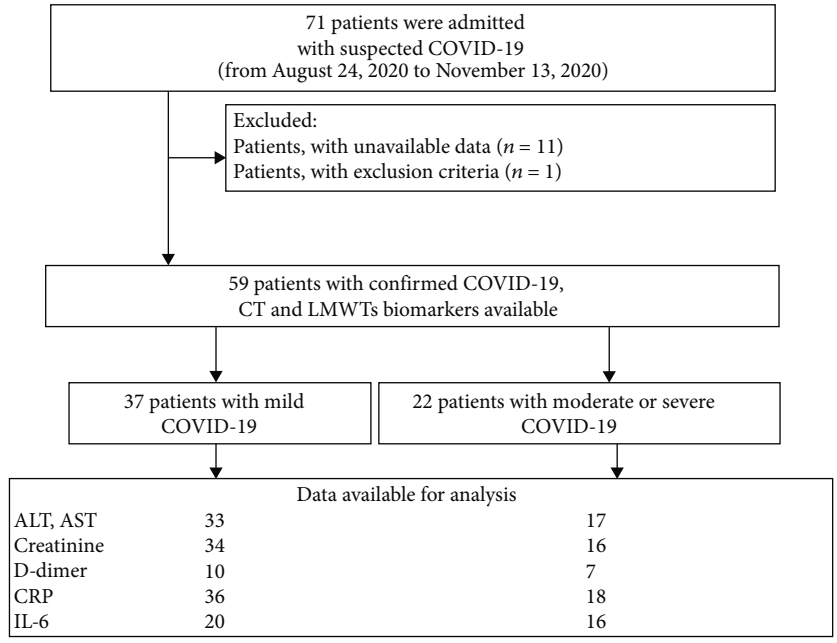

Figure 1: Patient flow diagram.

For total LMWT determination, we mixed 5 μl of 1 mM penicillamine (internal standard), 5 μl of 0.2 M dithiothreitol, and 10 μl of 0.4 M Na-phosphate buffer pH 8.0, containing 50 mM ethylenediaminetetraacetic acid disodium salt, with 50 μl of blood plasma. These mixtures were incubated for 30 minutes at 37°C, and 200 μl of 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB) in acetonitrile was added. Probes were mixed intensively and centrifuged for 5 minutes at 15,000g. We then added 10 μl of 1 M HCl and 200 μl $CHCl_3$ to each supernatant. Probes were mixed intensively and centrifuged for 1 min at 4,000g. The upper phase was diluted 3 times and injected (10 μl) in chromatograph. A Zorbax Eclipse Plus C18 Rapid Resolution HD 100 × 2 mm and 1.8 μm column (Agilent, Santa Clara, CA) was used for quantitation of total LMWTs. The flow rate was 0.15 ml/min, and t = 35°C. Mobile phases were 0.15 M NH_4 acetate with 0.075% (v/v) formic acid and acetonitrile. Chromatography was performed using a linear acetonitrile gradient (3%–13%) for 4.5 minutes. The column was regenerated with 50% acetonitrile for 0.5 minute and equilibrated with 3% acetonitrile for 6.5 minutes.

For reduced LMWT determination, 200 μl of plasma was mixed with 400 μl 2.5 mM DTNB in acetonitrile and 12 μl of 1.5 M NaOH. Afret 5 sec 20 μl of 0.1 M iodoacetamide was added. After 10 minutes, incubation samples were centrifuged for 5 minutes at 15,000g. Then, the supernatant (200 μl) was mixed with 50 μl of internal standard (10 μM penicillamine+200 μM DTNB) and 1200 μl water, and the mixture was passed through a diethylaminoethyl cellulose column (40 mg). The column was flushed with 2 ml water, and analytes were eluted with 400 μl 0.2 M HCl with 0.2 M NaCl. A Zorbax Eclipse Plus C18 Rapid Resolution HD 150 × 3 mm and 1.8 μm column (Agilent, Santa Clara, CA) was used for quantitation of reduced LMWTs. The flow rate was 0.4 ml/min, and t = 40°C. Mobile phases were 0.05 M NH4 acetate with 0.15% (v/v) formic acid and acetonitrile. Chromatography was performed using a linear acetonitrile gradient (4%–11.5%) for 3 minutes. The column was regenerated with 50% acetonitrile for 0.5 minute and equilibrated with 4% acetonitrile for 4.2 minutes.

Advanced oxidation protein product (AOPP) level in plasma was determined by the Witko-Sarsat method [26] with little modifications. After centrifugation (1 min, 1000g), the plasma sample (80 μl) was mixed with PBS+0.05% Nonidet P40 (320 μl), KI (1.16 M, 20 μl), and acetic acid (40 μl). Optical density (OD) was immediately measured at 340 nm against a blank sample (PBS with KI and acetic acid) in a 1 cm path cuvette. Chloramine B (0–62.5 μM) in PBS was used as calibration standards. Its absorbance was linear within this range ($OD = 0.0156 \cdot C^{chloramine} + 0.0059$, $R^2 = 0.999$).

2.3. Statistical Analysis. Data collection and primary processing (identification and integration of the chromatographic peaks) were performed in MassLynx v4.1 (Waters, USA). Statistical data analysis was performed using SPSS Statistics v. 22 (IBM, USA). Data on age, clinical findings, biochemical tests, and LMWT levels were expressed as medians (1st; 3rd quartile). Differences in the levels of these parameters between the patient groups were determined using the Mann–Whitney U tests. Spearman's correlation coefficient (ρ) was used to measure the degree of association between two variables. Binomial indicators (bivariable analysis) were compared by calculating the relative risk (RR) and odds ratio (OR). A p value < 0.05 was considered to indicate a significant difference.

3. Results

The general characteristics of patients are presented in Table 1. Most patients (46 of 59) were men. We found no statistically significant difference in sex distribution in the mild and moderate/severe groups. The median patient age was 61 (range, 20–88) years. No smokers or regular users of alcohol or drugs were identified. Most of the admitted patients had mild COVID-19 (68%) and no more than 50% lung damage (75%). Only three (5%) patients had severe COVID-19, and two of them had a degree of lung damage that corresponded to CT4. Therefore, the groups with moderate and severe COVID-19 and CT3 and CT4 were subsequently merged. On admission, two patients underwent resuscitation/intensive therapy. A significant proportion of patients were previously diagnosed with arterial hypertension (24 out of 59, 41%) and atherosclerosis (17 out of 59, 29%). Sixteen (27%) patients were diagnosed with HHcy (tHcy > 10 μM), mostly mild (<15 μM). Only 6 patients had a tHcy level > 15 μM.

Spearman's rank correlation revealed a number of associations between clinical laboratory parameters and LMWTs in all the patients. The tCys level had a negative impact on hemoglobin (HGB) level ($\rho = -0.330$, $p = 0.0093$, $n = 59$). The tCG level was positively associated with hematocrit (HCT: $\rho = 0.395$, $p = 0.0128$, $n = 39$) and had a rather significant negative effect on platelet count (PLT: $\rho = -0.551$, $p = 0.00041$, $n = 37$). Level of rCG was also negatively associated with PLT ($\rho = -0.339$, $p = 0.0121$, $n = 54$). A negative relationship was found between tGSH levels and mean erythrocyte volume (MCV: $\rho = -0.425$, $p = 0.0011$, $n = 56$) and mean erythrocyte hemoglobin (MCH: $\rho = -0.449$, $p = 0.00051$, $n = 56$). Interestingly, a fairly close negative association was observed between D-dimer and rGSH levels ($\rho = -0.599$, $p = 0.0142$, $n = 16$), although D-dimer level determination was performed in a limited number of patients ($n = 16$).

Since there were only 3 patients with severe infection in the cohort, the patients were stratified into two groups based on disease severity (mild and moderate+severe). No significant differences in age and sex were found in these groups. As shown in Table 2, patients with moderate or severe disease were characterized by increased incidence of severe lung injury (CT3-4), hemoconcentration (increased HCT), increase in the leukocyte index, and decrease in tGSH levels.

When comparing patients with different degrees of lung damage, significant differences were observed in a number of indicators (Table 3). The erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) level significantly increased in the CT0-CT4 series. Among LMWTs, it can be noted that tGSH and rCG levels were lower in CT2-4 patients than in CT0-1 patients.

The impact of LMWTs (tGSH, rCG) as risk factors for the severity of COVID-19 and lung injury is presented in Tables 4 and 5. As shown in Table 4, tGSH levels $\leq 1.5\,\mu M$ corresponded to 3-fold higher risk of moderate/severe COVID-19. Approximately 80% of patients with moderate/-severe COVID-19 had tGSH levels ≤ 1.5 μM. Low levels of rCG (≤1.3 μM) and tGSH (≤1.8 μM) were also associated

Table 1: General characteristics of patients*.

N	59
Age, y	61 [51; 67.5]
Sex, man (%)	46 (78%)
Condition upon admission	
Mild	40 (68%)
Moderate	16 (27%)
Severe	3 (5%)
Severity of the infection:	
Mild	37 (63%)
Moderate	19 (32%)
Severe	3 (5%)
Arterial hypertension (%)	24 (41%)
Diabetes mellitus (%)	7 (12%)
Atherosclerosis (%)	17 (29%)
Lung CT level:	
0	2
1	24
2	17
3	13
4	2
HHcy: tHcy > 10 μM (%)	16 (27%)
HGB (g/l)	144 [126; 154]
HCT (%)	42 [36.9; 44.5]
RBC (10^{12}/l)	4.97 [4.16; 5.20]
MCV (fl)	86 [83; 89]
MCH (pg)	30.0 [28.6; 30.7]
MCHC (g/dl)	346.5 [335.0; 358.5]
PLT (10^9/l)	260 [204; 312]
WBC (10^9/l)	5.8 [3.9; 8.8]
LI	3.2 [1.9; 4.9]
ESR (mm/h)	46 [21; 76]
Glucose (mM)	5.55 [4.68; 7.03]
Creatinine (μM)	93 [84; 112]
D-dimer (mg/l)	0.83 [0.48; 1.53]
CRB (mg/l)	23.5 [3.7; 55.7]
IL-6 (ng/l)	7.4 [2.9; 22.9]
LMWTs	
tCys (μM)	227 [205; 249]
tCG (μM)	13.6 [11.0; 16.3]
tGSH (μM)	1.24 [0.92; 1.94]
tHcy (μM)	7.9 [6.2; 10.4]
rCys (μM)	14.8 [9.5; 16.9]
rCG (μM)	1.43 [1.18; 1.90]
rGSH (nM)	163 [90; 223]
rHcy (nM)	93 [74; 127]
AOPP (μM chloramine B equivalents)	41.8 [31.7; 60.7]

*Data are presented as median [Q1; Q3].

Table 2: Comparative characteristics of patients with different severity of the course of coronavirus infection*.

	Mild	Moderate+severe	p
N	37	22	
Age, y	63 [53; 68]	57.5 [50.5; 64.0]	0.373
Sex, man (%)	27	19	0.308
HHcy (tHcy > 10 μM)	8	8	0.219
Arterial hypertension (%)	14 (38%)	10 (45%)	0.562
Diabetes mellitus (%)	3 (8%)	4 (18%)	0.246
Atherosclerosis (%)	11 (30%)	6 (27%)	0.84
Lung CT:			
0,1	19	7	0.144
2	12	6	0.67
3,4	6	9	0.045
HCT (%)	41 [33; 44]	43 [42; 45]	0.006
HGB (g/l)	138 [122; 152]	149 [141; 154]	0.038
LI	2.6 [1.6; 3.3]	4.1 [2.6; 5.7]	0.011
tGSH (μM)	1.72 [1.10; 2.29]	0.99 [0.68; 1.40]	0.008

*Data are presented as median [Q1; Q3].

Table 3: Comparative characteristics of patients with different degrees of lung damage.

	CT0, 1	CT2	CT3,4
N	26	18	15
Age, y	64.5 [51.3; 71.8]	60.5 [53.0; 66.5]	57.0 [49.5; 63.5]
Sex, man (%)	18 (69%)	13 (72%)	15 (100%)‡
HHcy: tHcy > 10 μM (%)	4 (15%)	6 (33%)	6 (40%)
Arterial hypertension (%)	13 (50%)	6 (33%)	5 (33%)
Diabetes mellitus (%)	3 (12%)	2 (11%)	2 (13%)
Atherosclerosis (%)	9 (35%)	3 (17%)	5 (33%)
HGB (g/l)	140 [128; 161]	130 [120; 161]	147 [142; 149]
HCT (%)	42 [37; 45]	41 [36; 46]	42 [41; 43]
RBC (10^{12}/l)	5.0 [4.3; 5.2]	5.0 [3.8; 5.3]	4.9 [4.5; 5.1]
PLT (10^9/l)	261 [216; 326]	237 [169; 286]	271 [214; 317]
WBC (10^9/l)	6.14 [4.68; 8.88]	5.2 [3.6; 5.9]	7.7 [4.1; 10.9]
ESR (mm/h)	34 [19; 52]	46 [27; 72]	82 [76; 86] $^{\ddagger,£,*,**}$
D-dimer (mg/l)	0.93 [0.51; 1.53]	0.71 [0.46; 0.82]	1.23 [0.83; 1.53]
CRB (mg/l)	7.4 [1.8; 24.0]	38.9 [6.6; 56.0]‡	61.1 [32.0; 117]‡,*,**
IL-6 (ng/l)	4.85 [3.00; 18.5]	6.40 [3.48; 26.0]	13.97 [5.34; 50.6]
tGSH (μM)	1.81 [1.04; 2.34]	1.15 [0.85; 1.76]	1.22 [0.76; 1.42]*
rCG (μM)	1.59 [1.31; 1.98]	1.30 [1.12; 1.78]	1.29 [1.08; 1.81]*
AOPP (μM chloramine B equivalents)	39.7 [29.5; 53.2]	42.0 [30.0; 61.1]	47.1 [34.3; 71.8]

*CT0, 1 vs. CT2-4, **CT0-2 vs. CT3,4, $^{\ddagger}p < 0.05$ compared with the "CT0,1" group. $^{£}p < 0.05$ compared with the "CT2" group.

with at least the double risk of moderate-to-severe lung damage (Table 5). Most of the CT2-4 patients (83%) had low tGSH and rCG levels. The RR of high lung damage (CT3-4) in patients with low tGSH and rCG levels was 3-fold higher than that in patients with tGSH > 1.8 μM or rCG > 1.3 μM.

We did not find any significant differences in AOPP levels when comparing patients with different severity or degrees of lung injury. We also did not find any significant association of this indicator with r- and t-forms of LMWTs and their r/t ratio in the entire cohort of patients. However, it was found that in patients with severe lung injury (CT3,4), there is a strong negative association between AOPP and r/t of the GSH ratio (i.e., its redox status), which is absent in patients with moderate lung injury CT0-2 (Figure 2(a)). In addition, a negative association of AOPP with tGSH was

Table 4: Effect of rCG and tGSH levels on the severity of lung injury.

rCG (μM)	N_{total}	N^{CT2-4}	w (%)	RR	p	OR	95% CI
≤1.3	23	18	78	2.28	0.001	6.9	2.05-23.2
>1.3	35	12	34				
tGSH (μM)							
≤1.8	40	27	68	2.14	0.0094	4.5	1.39-14.5
>1.8	19	6	32				
	N_{total}	N^{CT3-4}					
tGSH ≤ 1.8 and rCG ≤ 1.3 μM	18	8	44	3.04	0.0132	4.67	1.3-16.6
tGSH>1.8 or rCG> 1.3 μM	41	6	15				

Table 5: Influence of tGSH level on the severity of the course of coronavirus infection.

tGSH (μM)	N_{total}	N^*	w (%)	RR	p	OR	95% CI
≤1.5	35	18	51	3.09	0.0068	5.29	1.50-18.7
>1.5	24	4	17				

*Moderate+severe COVID-19.

observed in the group of patients with moderate or severe infection, which was not observed in patients with mild COVID-19 (Figure 2(b)).

4. Discussion

In a previous large study of 273 patients with COVID-19, negative progression in the lungs on CT was associated with the level of tHcy and the role of HHcy as a factor for progression to severe COVID-19 [21]. In contrast, in our work, there was no significant effect of tHcy and rHcy on the severity of infection, and there was no significant association of these parameters with the results of a clinical blood test. However, it is worth noting that the frequency of HHcy in patients with moderate/severe COVID-19 at admission was twice as high as that in patients with mild COVID-19 (42 vs. 20%, $p = 0.075$), but the sample size was not large enough for this difference to be significant. In addition, there were no patients in our sample whose disease progressed and who did not recover.

OS plays an important role in the pathogenesis of atherosclerosis and inflammatory lung diseases. Although the details of GSH involvement in these processes are not yet fully understood, the importance of the protective function of this aminothiol is emphasized both by its direct antioxidant activity and the key role of GSH-dependent enzymes that carry out (de)glutathionylation of proteins and the GSH hydrolysis. The role of glutathione S-transferase (GST) P1, glutathione transferase omega 1 (GSTO1-1), γ-glutamyl transpeptidase (GGT), and glutaredoxin in the activation of endothelial cells, smooth muscle cells, and macrophages is being actively studied [27–30]. The deficiency of the GSH redox cycle (GSH-peroxidase and GST) enzymes observed in the atherosclerotic plaques area contributes to the creation of a prooxidant environment within the vascular wall [31]. The protective role of GST P1 was also demonstrated in a model of endotoxinemia, where it was shown that activation of this enzyme causes inhibition of MAPKs and NFκB, which leads to suppression of the expression of proinflammatory factors TNFα, IL-1β, MCP-1, and overproduction of NO [32].

In addition, it was found that the effect of oxidized low-density lipoproteins (LDL) in macrophages leads to an increase in the level of glutathionylation of their proteins and promotes cell death [33]. It was revealed that GSTO1-1 plays an important role in the activation of macrophages by deglutathionylation of proteins such as caspase-1, STAT3, and hypoxia-inducible factor 1α [28], and GSH protects macrophages from oxidized LDL-induced cell injury [31]. On the other hand, the ApoB100 protein, which is part of LDL, is itself a target of glutathionylation, but the pathological significance of this modification has not yet been adequately studied. Glutathionylation of proteins is a mechanism actively involved in the formation of atherosclerotic plaque and ED in general, including oxidation of LDL, modulates cell response to OS in key events of plaque initiation (monocyte recruitment and differentiation), and progression (macrophage activation and death) [31, 33]. This is confirmed by a clinical study in which the positive correlation between atherosclerosis progression and the level of protein glutathionylation was found [34]. Among the numerous targets, one can distinguish Ca^{+2} ATPase, whose glutathionylation is a cGMP-independent mechanism of vasodilation, impaired in atherosclerosis [35]; the regulatory protein Ras is activated upon glutathionylation under the action of various atherogenic stimuli (angiotensin II, peroxynitrile, and oxidized LDL) and triggers the activation of Akt and ERK [33]. Interestingly, the Ras mutation, which prevents its glutathionylation, blocks the development of OS mediated by angiotensin II [36]. Glutathionylation of glutaredoxin-1 is also likely to be involved in the regulation of the Akt-dependent signaling pathway and is important for maintaining the physiological level of vascular permeability [33, 37]. In addition, the disturbance of laminar flow, observed in the areas most susceptible to atherosclerotic changes, negatively affects the activity of glutaredoxin-1 [30].

Our results showed that low tGSH levels could be considered a marker for the risk of developing severe COVID-19, severity of lung damage, and course of COVID-19. Although there is very little data on the role of GSH during COVID-19, there are some reasons that this metabolite may

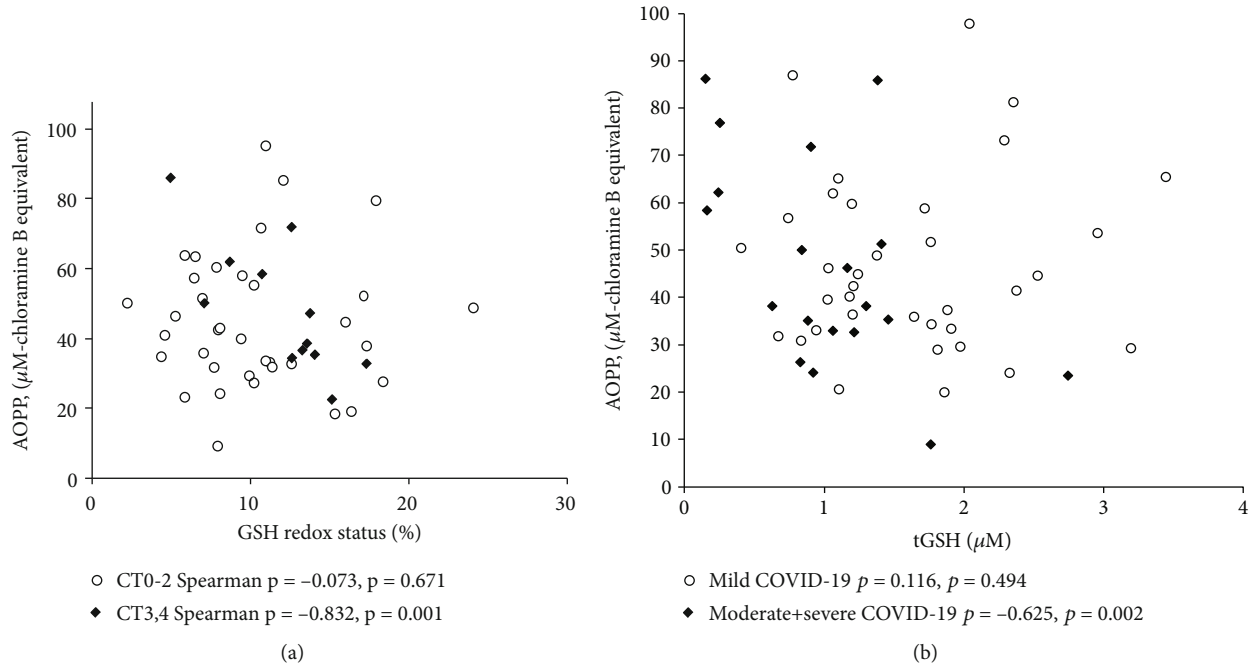

Figure 2: Association of AOPP with GSH redox status (a) and tGSH level (b).

play an important role in viral replication and resistance to infection [20]. It is known that loss of GSH affects the Na^+ H^+ membrane antiport with the decrease in intracellular pH, which facilitates both virus endocytosis and its replication [5]. It has been concluded from previously published literature that various risk factors associated with high mortality rates of COVID-19 are usually associated with low baseline GSH levels or impaired GSH metabolism; thus, GSH depletion may play a central role in COVID-19 mortality and pathophysiology [19]. The role of GSH in the protection of DNA from peroxynitrile-mediated damage, which is characteristic of acute inflammatory reactions, has been shown early [16].

AOPP was proposed in 1996 by Witko-Sarsat et al. as a surrogate marker of OS [26]. AOPP are formed mainly due to the oxidation of tyrosine residues and SH-groups of plasma proteins by hypochlorous acid (HClO), which, in turn, is formed by the reaction of H_2O_2 with Cl^-, catalyzed by myeloperoxidase. The appearance of a close negative association of this indicator with the level of plasma tGSH and GSH redox status in patients with high level of lung injury indicates that GSH is becoming a really important determinant that prevents oxidative damage to proteins. An indirect confirmation of the results obtained is the close correlation between the level of rGSH in blood and the level of SH-groups of proteins in critically ill adult patients hospitalized for severe COVID-19 [38].

With COVID-19, there is a shift in the balance between angiotensin II, which has a prooxidant effect, and angiotensin 1-7, the ACEII-mediated product of angiotensin II cleavage, which inhibits reactive oxygen species (ROS) generation. Overall, the formation of OS is favored. This can enhance the invasion of SARS-CoV-2 due to the oxidation of cysteine residues in ACE-II and the virus's spike glycoproteins [39]. Angiotensin II-mediated activation of NADPH oxidase can have a profound effect on GSH homeostasis, since the restoration of GSH by GSH reductase requires NADPH. It is interesting to note that in addition to these, in principle, nonspecific mechanisms, two more mechanisms related to GSH metabolism, specific for COVID-19, have been suggested [17]. First, it was hypothesized that the major protease SARS-CoV-2 has the ability to break down GSH peroxidase, the main enzyme that GSH uses to detoxify cells from ROS. Second, it has been suggested that this major protease can cleave glutamate-cysteine ligase, which is necessary for the synthesis of GSH. In addition, SARS-CoV-2-mediated activation of tumor growth factor (TGF-β) suppresses the expression of glutamate-cysteine ligase [40]. Finally, it is known that many viruses are capable of activating mechanisms aimed at reducing the synthesis and redox status of GSH by increasing the expression of NADPH oxidases, NF-κB, and inhibition of NRF2 expression [5].

Low GSH levels inhibit T lymphocyte proliferation and subsequently disrupt the immune response [41, 42]. GSH depletion is necessary for apoptosis to be triggered in lymphocytes, regardless of the presence of ROS [43]. GSH may also contribute to the increased risk of severe COVID-19 with age, since in the elderly, there is a decrease in the GSH level in erythrocytes, lymphocytes, and plasma [44–46].

Although the results of studies on the role of vitamin D deficiency in the severity of COVID-19 are ambiguous [47, 48], it has been shown that SARS-CoV-2 infection can inhibit the activity of the vitamin D receptor [49]. GSH deficiency can alter genes that work together to synthesize vitamin D, vitamin D-binding proteins, and receptors, but supplementation with L-cystine, a precursor for GSH, increases the levels of vitamin D and its binding proteins [20, 50].

The importance of the protective role of GSH in the development of SARS is confirmed by a number of works that show the effectiveness of the use of the GSH precursor N-acetylcysteine for the prevention of this complication in patients at high risk [51, 52]. Experimental work has also shown that GSH/N-acetylcysteine has antiviral activity toward a wide range of viruses [19, 20].

Numerous studies show that the fibrin degradation fragment, D-dimer, is a useful clinical indicator of thromboembolism, predictor of mortality, and marker for the progression of COVID-19 [20, 53, 54]. In this regard, it is interesting to identify a rather close negative association of rGSH with the D-dimer level, which may indicate a significant effect of GSH in the regulation of blood coagulation activity in COVID-19.

To date, data on the possibility of using GSH as a diagnostic marker or therapeutic target in COVID-19 are scarce. The first study that proposed a negative role of GSH deficiency in COVID-19 included a sample of only four patients, two of whom had severe and moderate-to-severe disease [18]. It was reported that these patients had a decreased plasma GSH/ROS ratios, but the method used to determine these values was not described. In patients in the intensive care unit (ICU), the level of rGSH in whole blood was reduced, and the GPX activity, on the contrary, was increased compared with reference levels [38]. So far, there are several reports of the successful use of N-acetylcysteine or GSH in patients with COVID-19 [55, 56].

In addition, of interest is the negative association of tGSH level with MCV and MCH in COVID-19 patients. In the literature, we did not find data on whether such an association exists in healthy individuals; therefore, we cannot argue that this relationship is a characteristic feature of COVID-19. However, it was previously shown that MCVs were significantly higher in COVID-19 nonsurvivors than in survivors, which indicates the importance of GSH as a protective factor [57].

The present study also found that patients with lung damage > 25% (CT2-4) had low plasma rCG levels. CG is a product of plasma GSH cleavage by the surface protein GGT. Decreases in rCG levels may be due to a shift in the redox status of LMWTs, a decrease in plasma rGSH (or tGSH) levels, a decrease in GGT activity, or an increase in dipeptidase levels. According to our data, nothing is currently known about changes in the activity of GGT and dipeptidase in COVID-19. Some clinical studies have revealed a positive association of GGT with the arterial hypertension, risk for cardiovascular diseases, and mortality [29]. Apparently, an increase in GGT activity is the body's response to OS [58], which is confirmed by the close relationship between the GGT level and CRP [59]. The level of Cys in the plasma and thus the synthesis of GSH largely depend on the activity of GGT and dipeptidase, since neither GSH itself nor CG is transported into cells. However, CG also has prooxidant properties, since, being a strong reducing agent, it is able to reduce Fe+3 to Fe+2. Oxidation of Fe+2, in turn, causes the appearance of ROS (superoxide anion, H_2O_2). It is with this mechanism that the participation of CG and GGT in atherosclerosis is associated [29]. It is also difficult to explain the negative association of rCG and tCG levels with PLT, especially in the latter case. It can be assumed that the activation of hemostasis, leading to a decrease in the PLT level, is accompanied by the release of GSH from platelets, which is rapidly metabolized to CG. Thus, the question of the association of CG with platelet functions requires additional research.

5. Conclusions

In the present work, it was shown that the levels of tGSH and rCG can be considered potential risk markers for the severity of COVID-19 and lung damage upon admission. GSH appears to be an important factor to oxidative damage prevention as infection progresses. Further, the association of GSH and CG with hematological parameters and D-dimer levels indicates the potential clinical efficacy of correcting GSH metabolism as an adjunct therapy for COVID-19. Since a decrease in GSH levels is characteristic of aging and comorbidities (diabetes mellitus, obesity, and hypertension), which can have a major impact on the development of COVID-19 severity [5], targeted studies of aminothiols among such patient groups are of interest.

Abbreviations

ACE II: Angiotensin II-converting enzyme
AOPP: Advanced oxidation protein products
AT1R: Type 1 receptors to angiotensin II
COVID-19: Coronavirus disease 2019
CT: Computed tomography
Cys: Cysteine
CG: Cysteinylglycine
CRP: C-reactive protein
DTNB: 5,5′-Dithiobis(2-nitrobenzoic) acid
ICU: Intensive care unit
GGT: γ-Glutamyl transpeptidase
GGT: γ-Glutamyl transpeptidase
GSH: Glutathione
GST: Glutathione S-transferase
GSTO1-1: Glutathione transferase omega 1
HCT: Hematocrit
HGB: Hemoglobin
Hcy: Homocysteine
HHcy: Hyperhomocysteinemia
LDL: Low-density lipoproteins
LMWTs: Low molecular weight aminothiols
MCH: Mean erythrocyte hemoglobin
MCV: Mean erythrocyte volume
OS: Oxidative stress
PLT: Platelet count
ROS: Reactive oxygen species
SARS-CoV-2: Acute respiratory syndrome coronavirus-2
TGF-β: Tumor growth factor beta.

Data Availability

Data are available on request.

Conflicts of Interest

The authors have declared no conflict of interest.

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Review Article

The Antiviral Roles of Hydrogen Sulfide by Blocking the Interaction between SARS-CoV-2 and Its Potential Cell Surface Receptors

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The ongoing coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is posing a great threat to the global economy and public health security. Together with the acknowledged angiotensin-converting enzyme 2, glucose-regulated protein 78, transferrin receptor, AXL, kidney injury molecule-1, and neuropilin 1 are also identified as potential receptors to mediate SARS-CoV-2 infection. Therefore, how to inhibit or delay the binding of SARS-CoV-2 with the abovementioned receptors is a key step for the prevention and treatment of COVID-19. As the third gasotransmitter, hydrogen sulfide (H_2S) plays an important role in many physiological and pathophysiological processes. Recently, survivors were reported to have significantly higher H_2S levels in COVID-19 patients, and mortality was significantly greater among patients with decreased H_2S levels. Considering that the beneficial role of H_2S against COVID-19 and COVID-19-induced comorbidities and multiorgan damage has been well-examined and reported in some excellent reviews, this review will discuss the recent findings on the potential receptors of SARS-CoV-2 and how H_2S modulates the above receptors, in turn blocking SARS-CoV-2 entry into host cells.

1. Introduction

The ongoing coronavirus disease 2019 (COVID-19) pandemic has now spread worldwide to more than 200 countries/regions and has caused over 180 million infections, and over 4 million deaths globally (as of 10 July 2021), which continues to rise rapidly. It is posing a great threat to the global economy and public health security. The current pandemic is caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a lipid-enveloped positive-sense RNA virus belonging to the β-coronavirus genus. Similar to other β-coronaviruses, spike (S) protein mediates attachment and membrane fusion of virus particles with target cells in SARS-CoV-2 infection [1]. The S protein is a typical type I fusion protein and is composed of two functional subunits: S1, which contains the receptor binding domain (RBD) to mediate receptor binding, and S2, which contains the transmembrane domain to mediate virus-cell fusion [2]. Together with the acknowledged angiotensin-converting enzyme 2 (ACE2), [3] glucose-regulated protein 78 (GRP78), [4] transferrin receptor (TFR), [5] AXL, [6] kidney injury molecule-1 (KIM-1) [7], and neuropilin 1 (NRP1) [8] are identified as additional potential receptors to mediate SARS-CoV-2 infection. The first step of SARS-CoV-2 infection in humans is the binding of RBD in the S1 subunit to the host's cell surface receptors, which plays a decisive role in the invasion and spread of viruses, and, in turn, affects the clinical symptoms of patients. Therefore, how to inhibit or delay the binding of RBD with the abovementioned receptors is a key step for the prevention and treatment of COVID-19.

For a long time, hydrogen sulfide (H_2S) was known as a poisonous gas to life and the environment. However, since the pioneering work by Abe and Kimura in which it was reported as a neuromodulator, H_2S has been recognized as the third gasotransmitter akin to nitric oxide and carbon monoxide [9]. In biologic systems, H_2S is endogenously synthesized by three enzymes, namely, cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST) and elicits its effect through four distinct pathways: (1) scavenging reactive oxygen species (ROS); (2) posttranslational modification, termed S-sulfhydration or persulfidation, on protein cysteine residues; (3) binding of metalloprotein centers; and (4) interaction with inter- or intramolecular disulfide bonds. [10–12] It is becoming increasingly clear that H_2S plays an important role in many physiological processes, and disturbances of the endogenous H_2S production are associated with the onset of several diseases, such as hypertension, diabetes, cancer, and viral infection. [13–15] Recently, Renieris et al. found that survivors had significantly higher H_2S levels in COVID-19 patients and mortality was significantly greater among patients with a decrease of H_2S levels. [16] Combined application of N-acetylcysteine, a potential H_2S-releasing donor, improved the symptoms in COVID-19 patients [17]. Furthermore, a beneficial role of H_2S against COVID-19 and COVID-19-induced comorbidities and multi-organ damage had been well examined and reported in some excellent reviews [18, 19]. Here, this review will discuss the recent findings on the potential receptors of SARS-CoV-2 and how H_2S modulates the abovementioned receptors, in turn blocking SARS-CoV-2 entry into host cells.

2. Organ Damage of the SARS-CoV-2 and the Protective Effect of H_2S

Similar to other coronaviruses, direct organ damage will be induced by the SARS-CoV-2 replication once it has invaded the host cell. [20–22] Then, it also can induce organ damage indirectly by the systemic inflammatory response (also called as cytokine storm) [23], endothelial dysfunction, [24] hypoxia, [25] and sympathetic overactivation. [26] Although high H_2S concentration is cytotoxic by inhibition of mitochondrial respiration, the physiological concentration of H_2S has been reported to protect multiple organs from injury by its broad spectra of bioactivities, including antiviral, alleviation of inflammation, restoration of endothelial function, inhibition of the hypoxia or ischemia injury, and normalization of sympathetic activities. [19, 27–29] Firstly, accumulated evidence has demonstrated that H_2S significantly decreased viral replication and improved lung functions in mice, while blockage of CSE activity or knockout of CSE expression increased viral replication and enhanced lung damage. [30] H_2S also upregulated ACE2 expression to reduce organ damages that were exacerbated by Ang II accumulation after ACE2 internalizaion [31]. Secondly, after being released, viral RNA, as a pathogen-associated molecular pattern, was recognized by a variety of pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) in an immune cell. Then, large amounts of proinflammatory cytokines and chemokines were secreted in an unrestrained way causing a cytokine storm and serious organ damage [32]. H_2S was found to reduce the expression of TLRs to prevent TLR-mediated inflammatory response. [33] H_2S was also found to inhibit the secretion of virus-induced chemokines and cytokines by inhibiting the activation and nuclear translocation of NF-κB and then reducing the transcription of proinflammatory genes [34]. Thirdly, Varga et al. verified that endothelial cells were directly infected by SARS-CoV-2 and caused diffuse endothelial inflammation, which induced endothelial dysfunction, thereby worsening organ damage. [35] H_2S was reported in various studies to ameliorate endothelial dysfunction in cardiovascular disorders such as hypertension, atherosclerosis, and metabolic syndrome, [13, 27] which would be beneficial for COVID-19 treatment. Fourthly, in addition to virus-related lung damage, macro- and microvascular thrombosis induced by inflammation and endothelial dysfunction could cause tissue hypoxia and aggravate organ damage [36]. It was worthy of mentioning that H_2S has been identified as an excitatory mediator of hypoxic sensing in the carotid bodies to elicit its protective roles under hypoxic conditions. [37] H_2S was also found to attenuate ferric chloride-induced arterial thrombosis and enhance the blood flow. [38] In addition, it promoted angiogenesis and increased capillary density to limit damages in the ischemic tissues. [39] Finally, it was indicated that patients with preexisting cardiovascular diseases, including hypertension, diabetes mellitus, and ischemic heart disease, which were characterized by increased sympathetic activity, seem to have a higher risk of morbidity and mortality in COVID-19. Conversely, COVID-19 also increased sympathetic overactivation inducing organ damage. [40] The vicious circle between COVID-19 and sympathetic overactivation might exacerbate the organ damage and comorbidities. However, H_2S could break this vicious circle by inhibiting sympathetic activation in the significant central sympathetic sites [41, 42].

3. The Potential Receptors of SARS-CoV-2 and H_2S

The virus-induced organ damage as described above is initiated by the binding of S protein with the host's cell surface receptors. Here, the important entry receptors including ACE2, GRP78, TFR, AXL, KIM-1, and NRP1 have been outlined, and the possible mechanism of H_2S in blocking SARS-CoV-2 entry has been discussed (Table 1).

4. ACE2 and H_2S

ACE2, a type I integral membrane glycoprotein composed of a single extracellular N-terminal domain containing the active catalytic site domain, a C-terminal membrane anchor, and a HEXXH zinc-binding domain, is widely expressed in a variety of tissues and cell types, including those in the lungs, heart, kidneys, gut, and brain [86]. Known as a typical zinc metallopeptidase, ACE2 counterregulates the renin-angiotensin-aldosterone system (RAAS) by converting Ang II to Ang 1-7 or Ang I to Ang 1-9, thus maintaining blood

Table 1: Potential host receptors and possible mechanism of H_2S in blocking SARS-CoV-2 entry.

Potential receptors	Possible regulatory mechanism of H_2S	Key references
ACE2	Interaction with intramolecular disulfide bonds	[43–49]
GRP78	Downregulation of its expression by inhibiting the related signal pathways	[50–53]
TFR	Downregulation of its expression at transcriptional levels	[54–56]
	Downregulation of its expression at posttranscriptional levels	[57–60]
AXL	Downregulation of its expression by S-sulfhydrating its transcription factor	[61, 62]
	Downregulation of its expression by inhibiting its transcription factor translocation	[63–65]
	Downregulation of its expression by histone acetylation of its promoter	[66–68]
KIM-1	Downregulation of its expression by reducing the phosphorylation of its transcription factor	[69–72]
	Downregulation of its expression by reducing ROS or ERK1/2 pathway	[73–75]
NRP1	Downregulation of its expression by S-sulfhydrating its transcription factor	[62, 76]
	Downregulation of its expression by inhibiting its transcription factor translocation	[64, 65, 77]
	Inhibition of cytokines	[78–81]
	Downregulation of its expression by inhibiting the related signal pathways	[82–85]

pressure homeostasis and fluid and salt balance. It also functions as an amino acid transporter or a functional receptor for MERS-CoV and SARS-CoV. [87] Like in SARS-CoV infections, ACE2 serves as a major entry receptor for SARS-CoV-2 in humans by binding to its S protein. [88] ACE2 has a 10- to 20-fold higher affinity for SARS-CoV-2 S than for SARS-CoV, which results in a higher SARS-CoV-2 infection efficiency. [89] Recent evidence in the literature indicated that intra- and intermolecular disulfides in both ACE2 and SARS-CoV-2 S protein had an important role for the binding process, which was regulated by the thiol-disulfide balance of the extracellular environment. [43, 44] Using molecular dynamics simulations revealed that the reduction of all disulfides into the sulfydryl groups completely impaired the binding of the SARS-CoV-2 S protein to ACE2. When the disulfides of only ACE2 were reduced to sulfydryl groups, the binding became weaker, while the reduction of disulfides of the SARS-CoV-2 S protein had a comparatively less effect [45]. Recently, several reducing agents including N-acetylcysteine amide and L-ascorbic acid were reported to inhibit viral entry by the disruption of disulfides [46]. As a weak reducing agent, H_2S activated vascular endothelial growth factor receptor 2 (VEGFR2) to promote angiogenesis by breaking the Cys1045-Cys1024 disulfide bond within the receptor [47]. H_2S also targeted the Cys320/Cys529 motif and broke the disulfide bonds in Kv4.2 to inhibit I_{to} potassium channels in cardiomyocytes and regularize fatal arrhythmia in myocardial infarction [48]. Moreover, H_2S was used to break mucin disulfide bonds, making the mucus less viscous and easier to be expelled by the respiratory ciliary apparatus, facilitating the elimination of potentially harmful viruses or extraneous particle [49]. Thus, H_2S is hypothesized to exhibit antiviral activity by interfering with the combination of ACE2 and SARS-CoV-2.

5. CS-GRP78 and H_2S

GRP78, also known as immunoglobulin heavy-chain-binding protein (BiP) or heat shock protein A5 (HSPA5), is a well-characterized endoplasmic reticulum (ER) chaperone protein whose function is to translocate nascent polypeptides across the ER membrane and facilitates the correct folding and assembly of proteins in normal cells. When misfolded proteins accumulate in the ER following ER stress, GRP78 is upregulated and plays a pivotal role in the unfolded protein response (UPR) by binding to misfolded proteins initiating the refolding or degradation mechanisms [90]. Conversely, under the ER stress, overexpressed GRP78 can escape the ER retention and translocate to the cell surface, termed cell surface CS-GRP78, where it functions as a multifunctional receptor to regulate cellular signaling, proliferation, migration, invasion, apoptosis, inflammation, and immunity [91]. CS-GRP78 is also reported to play a critical role in viral and fungal infections. Viruses including Coxsackie virus, Zika virus, dengue virus, and Borna disease virus recognize CS-GRP78 for entry or invasion into the host cells. [92] CS-GRP78 was reported to facilitate MERS-CoV entry into permissive cells by augmenting virus attachment in the presence of DPP4 [93]. Recently, a molecular dynamics study combined with molecular docking revealed the existence of H-bonds or hydrophobic contacts between GRP78 and C480-C488 of SARS-CoV-2 S protein [4, 94], which might be related to viral infection. A better binding was also found between GRP78 and the new UK variant of SARS-CoV-2. [95] In addition, COVID-19 patients had higher gene expression and serum concentrations of GRP78 [96]. Considering that virus invasion was associated with elevated levels of CS-GRP78 expression, inhibiting overexpressed GRP78 would be a promising strategy to reduce virus infection. H_2S has been reported to downregulate the expressions of ER stress-related proteins, including GRP78, in multiple diseases by different pathways. Our study found that the ER stress markers, including GRP78, CHOP, and active caspase-12 levels, were significantly elevated in the calcified rat aorta and H_2S alleviated vascular calcification by inhibiting ERS through the Akt signaling pathway activation [50]. In uranium-treated kidney cells, H_2S downregulated the expressions of GRP78 and CHOP and attenuated ER stress via 20S proteasome involved in Akt/GSK-3β/Fyn-Nrf2

signaling axis. [51] In hyperhomocysteinemia-induced cardiomyocyte injury, H_2S supplementation decreased the expressions of ER stress-associated proteins, including GRP78, while the inhibition of endogenous H_2S production further increased the expressions of those proteins [52]. H_2S was also reported to inhibit cigarette smoke-induced overexpression of ER stress-associated proteins in bronchial epithelial cells [53]. Therefore, H_2S may block the SARS-CoV-2 from entering the host cells by inhibiting the ES stress and reducing the expression of CS-GRP78.

6. TFR and H_2S

TFR is a membrane receptor playing a critical role in the maintenance of body iron homeostasis. The TFRs have two subtypes: TFR1 and TFR2. TFR1 is ubiquitously expressed at different levels on normal cells and serves as a gatekeeper regulating the cellular uptake of iron from transferrin, while TFR2 is specially expressed in hepatocytes and serves as an iron sensor [97]. TFR1 has attracted more attention than TFR2 by having diverse functions. TFR1, also known as cluster of differentiation 71 (CD71), is a homodimeric type II transmembrane glycoprotein involved in the cellular iron uptake through a constitutive clathrin-dependent endocytosis mechanism. It is expressed at low levels in most normal cells and at greater levels in rapidly proliferating cells and energy-requiring cells owing to the increased iron requirements. So, it may play additional roles in cell growth and proliferation [98]. Given that it is a ubiquitously and abundantly expressed cell surface membrane protein, TFR1 is a vulnerable target for pathogens to initiate host cell infection. It has been documented that multiple viruses, including New World hemorrhagic arenaviruses, hepatitis C virus, and human adenoviruses, recognize and bind with the apical domain of TFR1 to enter cells without interfering with iron delivery. [99] In this way, the viruses infect rapidly proliferating and iron-acquiring cells, which can facilitate their replication. Furthermore, endocytosed TRF1 recycles back to the cell surface in a constitutive manner but is not downregulated by viruses' infection, which may cause the superinfection. [100] Recently, it was reported that TFR1 directly interacted with the S protein of SARS-CoV-2 to mediate virus entry, while it was blocked by interfering TFR-spike interaction. Furthermore, anti-TFR antibody showed the promising antiviral effects in mouse model. [5] Considering that it is another receptor for SARS-CoV-2 entry, downregulating the expression of TFR1 or preventing the translocation of TFR1 to plasma membrane may be effective strategies to prevent virus invasion. Various molecular mechanisms are involved in the regulation of its expression at both the transcriptional and posttranscriptional levels. At the transcriptional level, TFR1 gene transcription has been shown to be stimulated by the transcription factor c-Myc. [54] However, the impact of H_2S on the c-Myc remains controversial. Zhang et al. reported that exogenous H_2S activated the ERK1/2/c-Myc pathways and restored postconditioning-mediated cardioprotection in the aged cardiomyocytes [101]. Contrastingly, Song et al. reported that H_2S donor inhibited the cell proliferation by downregulating the expression of proliferation-related proteins including c-Myc. [55] Moreover, diallyl disulfide, a potential H_2S donor, decreased telomerase activity in U937 cells by reduced binding of c-Myc to their respective binding sites on the promoter. [56] At the posttranscriptional level, TFR1 expression is finely regulated in an intracellular iron-dependent manner by the iron-responsive element/iron-regulated protein (IRE/IRP) system. [57] In case of cellular iron deficiency, the two IRPs (IRP1 and IRP2) bind to the multiple IRE motifs by the -SH residues in the $3'$ untranslated region of TFR1 mRNA and inhibit their degradation by a steric hindrance mechanism, thus increasing TFR1 protein expression. Conversely, in the presence of excess iron, IRP1 becomes an aconitase with the binding of a 4Fe-4S cluster, while IRP2 is degraded after ubiquitination, leading to the disappearance of IRE binding activity and degradation of TFR1 mRNA. Reactive oxygen species (ROS), including superoxide anion and hydrogen peroxide, was found to promote the loss of the 4Fe-4S cluster and enhance the IRE binding activity of IRP1, resulting in TFR1 translation [58]. Since it has long been assumed to be an antioxidant, H_2S may inhibit the IRP binding activity and downregulated TFR1 protein by scavenging ROS. H_2S was also reported to regulate the bioactivities of multiple proteins via S-sulfhydration of cysteine residues, [59] so H_2S might S-sulfhydrate cysteine residues of IRP1 to prevent its IRE binding activity, thus downregulating TFR1 protein. In addition, the Na^+/H^+ exchanger enhanced TFR1 translocation to the membrane of microvascular endothelial cells at the blood-brain barrier, [60] which might be inhibited by H_2S [102].

7. AXL and H_2S

AXL, also known as UFO, ARK, Tyro7, or JTK11, belongs to the tumor-associated macrophage (TYRO3, AXL, and MERTK) family receptor tyrosine kinases (RTKs). After binding with its ligand, growth arrest-specific protein 6 (GAS6), it leads to the activation of several downstream signaling pathways, including the Ras/Raf/MEK/ERK cascade and PI3K/Akt signaling pathways, and transduces signals from the extracellular matrix into the cytoplasm. [103] AXL has been originally detected as an unidentified transforming gene in chronic myeloid leukemia. Since then, AXL is found to be overexpressed in many types of cancer and is associated with therapy resistance, adverse prognosis, and worse outcome [104]. Under normal physiologic conditions, AXL is ubiquitously expressed in a wide variety of organs and cells originating from hematopoietic, epithelial, and mesenchymal sources and regulates many important physiological processes, including taming inflammation, clearing apoptotic cells, maintaining vascular integrity, and regulating cell survival, proliferation, and differentiation. Moreover, AXL has been found to be a candidate entry receptor for West Nile, Ebola, and Zika viral infections and its specific inhibitors reduced viral infectivity [105]. Most recently, Wang et al. found that AXL specifically interacted with the N-terminal domain of the spike glycoprotein in SARS-CoV-2, which colocalized mainly to the cell membrane, and it was a novel entry receptor for SARS-CoV-2

which played an important role in promoting viral infection to the human respiratory system. [6] In line with it, gilteritinib, an AXL inhibitor for acute myeloid leukemia treatment, was recently demonstrated to possess antiviral efficacy against SARS-CoV-2 infection in Vero E6 cells [106]. After virus infection, the TLR-mediated immune network is stimulated by viral particles, and then, the consequent type I interferon (IFN) antiviral response upregulates AXL expression, [107] which further promotes virus infectivity. Huang et al. reported that H_2S downregulated TLR4, inhibited its downstream NLRP3 inflammasome activation, and alleviated high glucose-induced cardiac injury [108]. H_2S was also able to ameliorate LPS-induced inflammation through TLR4/NF-κB signaling pathway inhibition [109]. In addition, polysulfide donors were reported to protect the mice from lethal endotoxin shock by inhibiting TLR signaling. [110] Several transcription factors, including specificity protein 1 (Sp1) [61] and hypoxia-inducible factor 1α (HIF-1α), [63] have been shown to directly upregulate AXL expression at transcriptional levels. H_2S-mediated S-sulfhydration of the Sp1 has been shown to decrease its binding activity to the gene promoter region, thus preventing myocardial hypertrophy [62]. H_2S also suppressed HIF-1α translation or activation under hypoxia [64, 65]. Conversely, reduced H_2S levels increased the levels of HIF-1α via increased ROI levels in infected CSE KO macrophages [111]. Histone acetylation can also affect AXL transcript levels. Reduced histone acetylation of the AXL promoter led to the upregulation of AXL expression that correlated with therapy resistance and adverse prognosis in some types of cancers [66]. AOAA, the inhibitor of endogenous H_2S production, has been reported to reduce histone acetylation, and H_2S donor increased H3 and H4 acetylation in LPS-treated cell [67]. H_2S also suppressed the endothelial dysfunction and prevented the occurrence of hypertension by inhibiting HDAC6 expression that removes acetyl groups from lysine residues of histone to reverse histone acetylation [68]. In addition, AXL mRNA expression is inhibited by miR-34a which has identified target sequences in the AXL 3′ untranslated region. [112] miR-34a expression was found to upregulate diallyl disulfide-treated MDA-MB-231 cells [113].

8. KIM-1 and H_2S

KIM-1, also known as TIM-1, is a single-pass type I cell membrane glycoprotein with an extracellular six-cysteine immunoglobulin-like (Ig V) domain topping a domain characteristic of mucin-like O-glycosylated proteins. It is virtually undetectable in normal kidney tissues, but its expression is dramatically upregulated in the apical membrane of the proximal tubule to reduce the innate immune response and regulate the regeneration and repair of the damaged epithelial cells after acute ischemic or toxic kidney injury. However, prolonged KIM-1 expression may be maladaptive and may lead to interstitial inflammation and fibrosis in chronic kidney disease. Therefore, it is recognized as a robust and reliable biomarker for early diagnosis, prognosis, and monitoring of therapeutic effects in various kidney diseases [114]. Moreover, KIM-1 is also identified as a hepatitis A virus cell receptor 1 (HAVCR-1) that is expressed by on the surface of different epithelial cells and facilitates cellular entry of several viruses, including Ebola virus, dengue virus, West Nile virus, and hepatitis A virus, via the IgV domain [115]. A recent report suggested that KIM-1 was not only a biomarker for COVID-19-associated acute kidney injury (AKI) [116] but also a potential receptor for SARS-CoV-2. [7] SARS-CoV-2 was reported to directly infect the renal tubules by ACE2 and induced AKI, which is one of the most prevalent complications among hospitalized COVID-19 patients [117]. After upregulated expression induced by AKI, KIM-1 could directly bind to SARS-CoV-2 S protein which was inhibited both by anti-KIM-1 antibodies and TW-37, an inhibitor of KIM-1 [118]. Another study suggested that SARS-CoV-2 RBD bind with KIM-1 and ACE2 via two distinct pockets, implicating that KIM-1 and ACE2 may synergistically mediate the invasion of SARS-CoV-2 in kidney cells [119]. The above "vicious cycle" exacerbates SARS-CoV-2 infection and KIM-1 may offer a new therapeutic target that can minimize injuries due to SARS-CoV-2. It was reported that H_2S treatment downregulated KIM-1 expression in hyperglycemic condition by inhibiting Ca^{2+}-induced mitochondrial permeability transition pore opening [120]. Dopamine decreased KIM-1 levels and preserved renal integrity during deep hypothermia and rewarming likely by maintaining the expression of renal H_2S-producing enzymes and serum H_2S. [121] A previous report had shown that nuclear signal transducer and activator of transcription 3 (STAT3) could bind to the KIM-1 promoter and increased its mRNA and protein levels. [69] In our study, PPG, the inhibitor of endogenous H_2S production, increased phosphorylation of STAT3 and aggravated vascular remodeling, while NaHS decreased phosphorylation of STAT3 and improved vascular remodeling [70]. The AMPK pathway might mediate the inhibition of STAT3 phosphorylation by H_2S during inflammation [71]. Recently, polysulfides were also reported to attenuate diabetic renal lesions via the inactivation of STAT3 phosphorylation/acetylation through S-sulfhydrating SIRT1. [72] In addition, the increased KIM-1 expression was also mediated by the ROS or ERK1/2 pathway, [73, 74] whereas H_2S not only attenuated ROS production but also abolished ERK1/2 activation, which possibly decreased KIM-1 expression. [75]

9. NRP1 and H_2S

Neuropilins (NRPs) are highly conserved single-pass transmembrane glycoproteins that are expressed by a wide variety of cell types, including neurons, blood vessels, immune cells, and multiple tumor cells in mammals. To date, two homologous NRP isoforms have been identified, namely, NRP1 and NRP2, which share 44% sequence homology and have a similar domain structure. The NRPs are composed of a large extracellular domain, a transmembrane domain, and a short cytoplasmic domain that lacks enzymatic activity. Despite being devoid of an intracellular kinase domain, NRPs act predominantly as a multifunctional coreceptor to bind with various ligands including class 3 semaphorins (SEMA3s), vascular endothelial growth factor, fibroblast

growth factor, and transforming growth factor-β1 (TGF-β1) by their well-structured extracellular part. As such, NRPs mediate a wide range of signaling pathways and play critical roles in the physiological and pathological processes, including nervous and vascular development, immune response, and tumor progression. [122] Moreover, NRPs have been shown to mediate cellular entry and infectivity of viruses such as Epstein-Barr virus (EBV), human T cell lymphotropic virus-1 (HTLV-1), and murine cytomegalovirus (MCMV) [123–125]. Recent literature has established NRP1 as a coreceptor that facilitated SARS-CoV-2 cell entry and infectivity, and NRP1 mRNA expression was elevated in SARS-CoV-2-infected cells, but not in uninfected cells from severe COVID-19 patients [126]. Studies based on X-ray crystallography and biochemical approaches also showed that the SARS-CoV-2 S proteins directly bind with extracellular domain of NRP1 by electrostatic attraction and infected human cells. [8] So NRP1 could be an ideal therapeutic target against SARS-CoV-2 infections. Although it lacks a direct study, H_2S may indirectly regulate NRP1 expression by affecting its transcription factors or some cytokines. It had been demonstrated that NRP1 was the downstream target of transcription factor Sp1 or HIF-1α [76, 77]. However, as mentioned above, H_2S inhibited the downstream protein expression by regulating these two transcription factors [62, 64, 65]. Cytokines, such as TNF-α and TGF-β, were reported to induce NRP1 mRNA and protein expressions, [78, 79] while, as indicated by the plethora of evidence, H_2S downregulated TNF-α and TGF-β expressions in a variety of pathological conditions [80, 81]. In addition, NRP1 was upregulated by Wnt/β-catenin signaling and sonic hedgehog (SHH)/GLI1 signaling in mammary development and tumorigenesis [82, 83]. However, diallyl trisulfide, a H_2S donor, was found to inhibit breast cancer stem cells via suppression of the Wnt/β-catenin pathway, and sulforaphane, another H_2S donor, significantly inhibited the SHH/GLI1 pathway and its downstream target gene expression to regulate self-renewal of pancreatic cancer stem cells [84, 85].

10. Conclusion

This review summarizes the potential receptors for entry of SARS-CoV-2, including GRP78, TFR, AXL, KIM-1, and NRP1, in addition to ACE2. Meanwhile, the potential mechanism by which H_2S regulates the abovementioned receptors to block the binding of SARS-CoV-2 has been discussed. Although inorganic sulfide salts (NaHS and Na_2S) have been the most widely employed in biological and preclinical studies, none of them are unlikely to be a suitable clinical option for a number of reasons, including poor water solubility, fast and uncontrollable release, and unpleasant odor. Given that it is not trivial to synthesize a clinically suitable H_2S donor in a short time, three types of potential H_2S donors or drugs should be considered to block viral entry: (1) natural H_2S donors (e.g., garlic and onions) [127, 128] or dietary micronutrients (e.g., L-cysteine and taurine) [129, 130], (2) H_2S-donating derivatives of clinically used drugs that link various H_2S donating groups to clinically used drugs (e.g., ATB-346 [131] and GIC-1001 [132] which has completed phase 2 clinical trial), and (3) several clinically used drugs that have been verified to increase H_2S levels (e.g., α-lipoic acid, [133] sodium thiosulfate, [134] zofenoprilat, [135] and N-acetylcysteine [136]). Sodium thiosulfate has been proposed as an inhalation therapy for COVID-19, [137] and it was confirmed that the combined application of N-acetylcysteine improved the symptoms in COVID-19 patient. [17] However, the abovementioned is just a stopgap; developing new clinically suitable H_2S donor drugs is necessary, and the clinical application of H_2S-targeted therapeutics to fight against diseases that are not limited to COVID-19 should advance.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Comprehensive Analysis of the Systemic Transcriptomic Alternations and Inflammatory Response during the Occurrence and Progress of COVID-19

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The pandemic of the coronavirus disease 2019 (COVID-19) has posed huge threats to healthcare systems and the global economy. However, the host response towards COVID-19 on the molecular and cellular levels still lacks full understanding and effective therapies are in urgent need. Here, we integrate three datasets, GSE152641, GSE161777, and GSE157103. Compared to healthy people, 314 differentially expressed genes were identified, which were mostly involved in neutrophil degranulation and cell division. The protein-protein network was established and two significant subsets were filtered by MCODE: ssGSEA and CIBERSORT, which comprehensively revealed the alternation of immune cell abundance. Weighted gene coexpression network analysis (WGCNA) as well as GO and KEGG analyses unveiled the role of neutrophils and T cells during the progress of the disease. Based on the hospital-free days after 45 days of follow-up and statistical methods such as nonnegative matrix factorization (NMF), submap, and linear correlation analysis, 31 genes were regarded as the signature of the peripheral blood of COVID-19. Various immune cells were identified to be related to the prognosis of the patients. Drugs were predicted for the genes in the signature by DGIdb. Overall, our study comprehensively revealed the relationship between the inflammatory response and the disease course, which provided strategies for the treatment of COVID-19.

1. Introduction

The global pandemic of the coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), exhibits high levels of mortality and morbidity and has posed huge threats to healthcare systems and the global economy [1, 2]. The current COVID-19 pandemic is unprecedented; globally, there have been over 108 million confirmed cases of COVID-19 that have led to over 2.37 million deaths, released by the World Health Organization (WHO) on February 14, 2021 (https://www.worldometers.info/coronavirus/). It is urgent to understand the molecular mechanisms of COVID-19 and identify the patients' susceptibilities so as to find therapeutic interventions.

SARS-CoV-2 belongs to the family of single-stranded RNA viruses known as coronavirus. Its cellular entry requires angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2) for membrane fusion or through the endosomal pathway to infect the host [3–5]. With an oxidative stress and excessive inflammatory response, COVID-19 is being regarded as a systemic disease. With diverse clinical manifestations, COVID-19 patients may present as asymptomatic, with mild respiratory tract

infection, acute respiratory distress syndrome, respiratory failure, or even death [6–8]. The imbalance of host immune response and the activation of inflammatory cytokines are called "cytokine storm," which is related to the severity of the disease and poor prognosis [9].

So far, several immunological characteristics of COVID-19 patients have been demonstrated. Serum c-reactive protein (CRP) and interleukin-6 (IL-6) will increase, while CD4+ and CD8+ T lymphocytes decrease [10–12]. Elevated levels of other inflammatory cytokines and chemokines such as interleukin-2 (IL-2) and interleukin-8 (IL-8), accompanied by increased neutrophils and eosinophils, may also lead to abnormal immune function in COVID-19 patients, further causing more immune cells to be activated and recruited into the lungs, causing "cytokine release syndrome" (CRS) [13–15]. The ratio of macrophages and CD14+ monocytes in PBMC increased, especially in patients with severe COVID-19 in the disease progression stage [16]. At the same time, the number of B cells in the peripheral blood of patients with severe COVID-19 increased significantly but the number of T cells and DC decreased [17]. With a lower baseline levels and functionally exhausted in CD8+ T cells and NK cells, the imbalance of patients in the intensive care unit (ICU) is more prominent [18]. Inflammation is further aggravated by the activation of humoral immunity and the complement system, and the weakening of some classical immune negative signals exacerbates inflammation [9, 19, 20].

Furthermore, several analyses of the transcriptome with high throughput have been conducted to identify the molecular signature of COVID-19 patients [21–25]. However, different studies may have distinct results due to the cohort size and sample heterogeneity. In our study, we aimed to integrate different high-throughput studies to unveil the transcriptomic alterations and differences of immune cell infiltration in the peripheral blood of COVID-19 patients. We uncovered the differentially expressed genes between the healthy people and patients, as well as the DEGs between non-ICU and ICU patients, which underwent comprehensive functional annotation and PPI network construction. We applied ssGSEA and CIBERSORT to evaluate the immune cell infiltration, and the DGIdb database was utilized to predict the drug-gene interaction. By profiling the characteristics of COVID-19 patients with different courses, we hoped to provide new insights into molecular pathogenesis and potential therapeutic targets of COVID-19.

2. Materials and Methods

The workflow of the study was shown in Figure 1.

2.1. Data Processing. Two gene expression series, GSE152641 and GSE161777 [26, 27], which contained blood samples from healthy controls and patients, were downloaded from the Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) database on January 3rd and February 23 publicly. GSE152641 contained RNA sequencing data from 62 COVID-19 patients and 24 healthy controls in the form of count. 27 samples of GSE161777 were selected in the form of count, including 13 patients (the first blood sample collection after diagnosis) and 14 healthy controls. Furthermore, GSE157103 [28], another RNA-seq profile in the form of TPM (trans per million), containing peripheral blood leukocyte samples as well as various clinical information from 50 ICU and 50 non-ICU COVID-19 patients was also downloaded from the GEO database publicly for further exploration on January 26.

2.2. Identification of Differentially Expressed Genes (DEGs). The limma [29], limma_voom [30], and edgeR [31] package of R were employed to perform the identification of DEGs; the first one was for data in the TPM format and the latter two were for data in the count format. We consider genes with $|\log_2 \text{fold change (FC)}| > 1$ and an adjusted p value < 0.05 is differentially expressed between two groups. These genes were counted and included in the Venn diagram by the Venndiagram [32] package of R to distinguish the repeated ones.

2.3. Pathway and Functional Enrichment Analyses. The clusterProfiler [33] package of R was applied to perform the pathway and functional enrichment analyses, based on the Gene Ontology (GO) database [34] (http://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database [35] (https://www.genome.jp/kegg/). GO is a platform constructed from the cellular component (CC), molecular function (MF), and biological process (BP). KEGG is a database widely used to carry out the biological pathway enrichment. Reactome [36] enrichment and UniProt [37] database annotation are directly available on Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, http://string.embl.de/) online database for further functional and pathway enrichment analyses.

2.4. Protein-Protein Interaction (PPI) Network Generation and MCODE Analysis. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) was also exploited to generate a protein-protein interaction (PPI) network, for the online biological database is based on known and potential protein-protein interaction [38]. Only those genes with interaction scores higher than 0.7 would be picked up and put into Cytoscape software [39] for further visualization analysis. The plug-in Molecular Complex Detection (MCODE) [40] was designed to seek subnets of PPI networks from the STRING online database, and we set all the parameters to default to identify significant subnets.

2.5. Evaluation of Immune Cell Abundance. Single-sample gene set enrichment analysis (ssGSEA) was applied to quantify the abundance of infiltration of different types of immune cells through the GVSA [41] package of R. For every single sample, we conducted standardization in order of the gene expression amount and calculated the enrichment scores (ES) by empirical cumulative distribution function, which can finally be transformed into the abundance of infiltration of 28 types of immune cell, and the immune cells gene sets were obtained from a recent study [42]. CIBERSORT [43] (https://cibersort.stanford.edu/), an analytical tool (R script version was utilized) which can estimate the

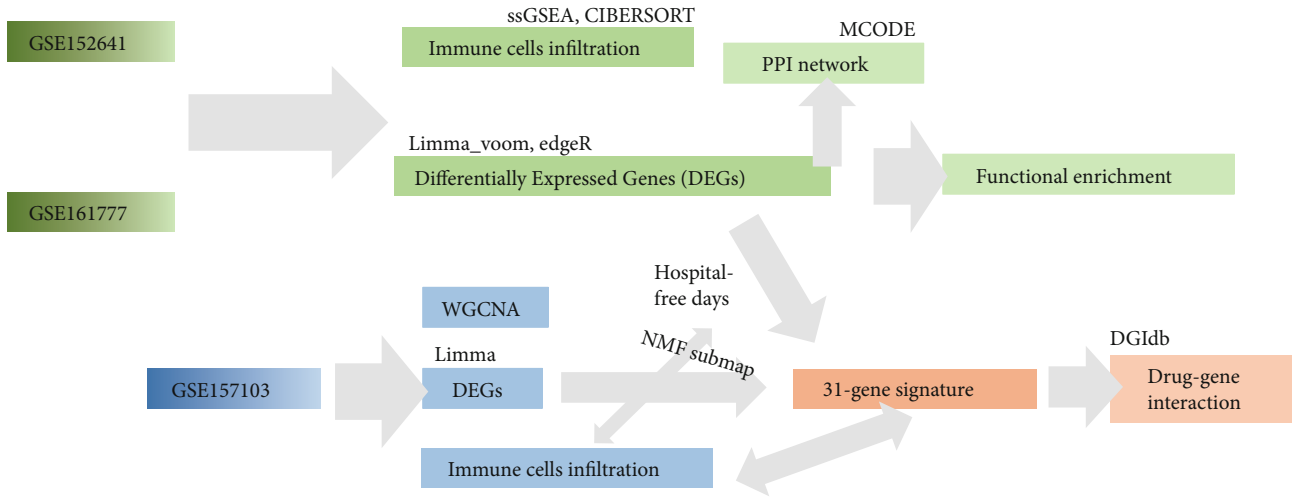


Figure 1: Workflow in the present study.

abundances of certain cell types in a mixed cell population, was employed to reveal the proportion of 22 types of immune cells.

2.6. Weighted Gene Coexpression Network Analysis (WGCNA). Weighted gene coexpression network analysis (WGCNA) was aimed at seeking for coexpressed gene modules and exploring the connection between gene networks and the traits being studied. First, according to the expression of genes in different samples, the correlation between any two genes, calculated by Pearson correlation analysis [44], was collected to form a similarity matrix. At the same time, the topological overlap matrix (TOM) method was employed to take both direct and indirect relationships into account. Then, the hierarchical cluster tree would generate whose division of gene modules was based on the TOM value between genes [45]. The module with the highest correlation with sample characteristics was selected for further analysis.

2.7. Clustering and Subclass Mapping. Nonnegative matrix factorization (NMF) clustering was conducted by the NMF package in R [46]. Briefly, the best number of clusters was chosen according to the cophenetic value. Then, NMF was conducted with the best rank and the method set to "brunet." Submap [47] in the GenePattern online tool (https://cloud.genepattern.org/gp) was applied to evaluate the similarities between the clusters identified by NMF and the clinical traits. Patients were classified into 4 groups (divided by the median and the upper and lower quantiles) according to the hospital-free days, which were named B1, B2, B3, and B4. The p value in the result was corrected by the Bonferroni method.

2.8. Drug-Gene Interaction Prediction. The open-source database named the Drug Gene Interaction Database (DGIdb, https://dgidb.genome.wustl.edu) [48] was utilized to show the known or potential interaction between drugs and genes by entering a list of genes. DGIdb covers over 100000 drug-gene interactions and 42 potentially druggable gene categories involving more than 40000 kinds of genes and 10000 types of drugs, based on PharmGKB, DrugBank, Chembl, TTD, Drug Target Commons, and others. Here, we only included the drug which had been approved and had a certain interaction (activator or inhibitor) with the gene. Then, the interaction network downloaded was visualized by Cytoscape.

2.9. Statistical Analysis. The Wilcoxon test was applied to judge whether a statistically significant difference exists among groups. Pearson correlation analysis was employed to conduct the correlation analysis in WGCNA and Spearman correlation coefficient to evaluate the correlation between genes, immune cells, and hospital-free days. All of these statistical analysis were performed in R 4.0.3 version.

3. Result

3.1. Transcriptomic Alternations and Functional Enrichment in COVID-19 Patients. Firstly, GSE152641, containing blood samples from 62 COVID-19 patients and 24 healthy controls, were obtained from the GEO database in the form of count. Then, the result of featureCounts of GSE161777 provided by the authors was merged, in which blood samples of 14 healthy people and 13 patients (first blood collection in the trial) were selected for subsequent analysis. We applied limma_voom and edgeR for each of the two datasets to increase the reliability of differentially expressed analysis. Totally, 253 genes were found to be upregulated after intersection of 4 DEG results, and 61 genes were downregulated (Figure 2(a)). GO analysis revealed that the 253 upregulated genes were enriched in the different process associated with neutrophil activation and miosis in the BP module, which was validated in the CC and MF module (Figure 2(b)). Similar results were gained in KEGG enrichment (Figure S1a). However, not significant pathways were enriched in GO and KEGG for the 61 downregulated genes (Figure S1b-c). In all, peripheral blood of COVID-19 patients might be

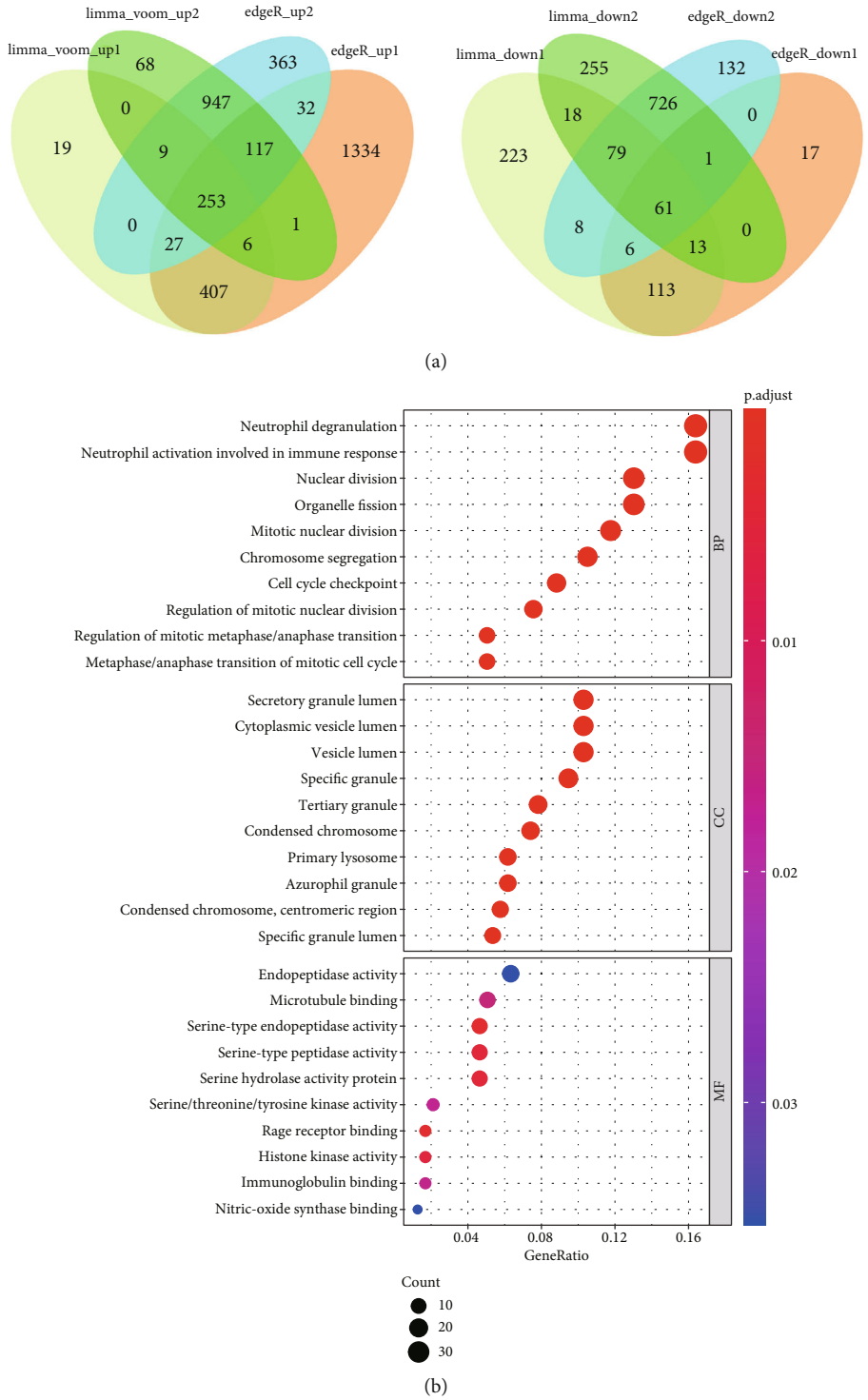


Figure 2: Transcriptomic alternations and functional enrichment in COVID-19 patients. (a) Differentially expressed genes (DEGs) upregulated ($n = 278$) and downregulated ($n = 59$) in COVID-19 patients compared with healthy cohort. (b) Gene Ontology analysis for the 278 upregulated genes.

characterized by neutrophil activation and cells were in a state of hyperproliferation.

3.2. Protein-Protein Interaction (PPI) Network for the DEGs. To explore the important genetic interaction of the occurrence of COVID-19, we utilized the STRING database to construct the PPI network of the 314 DEGs and only the genes with interaction scores larger than 0.7 were extracted, which was then put into Cytoscape. The PPI network was visualized containing 153 nodes and 1253 edges (Figure S2). The size and

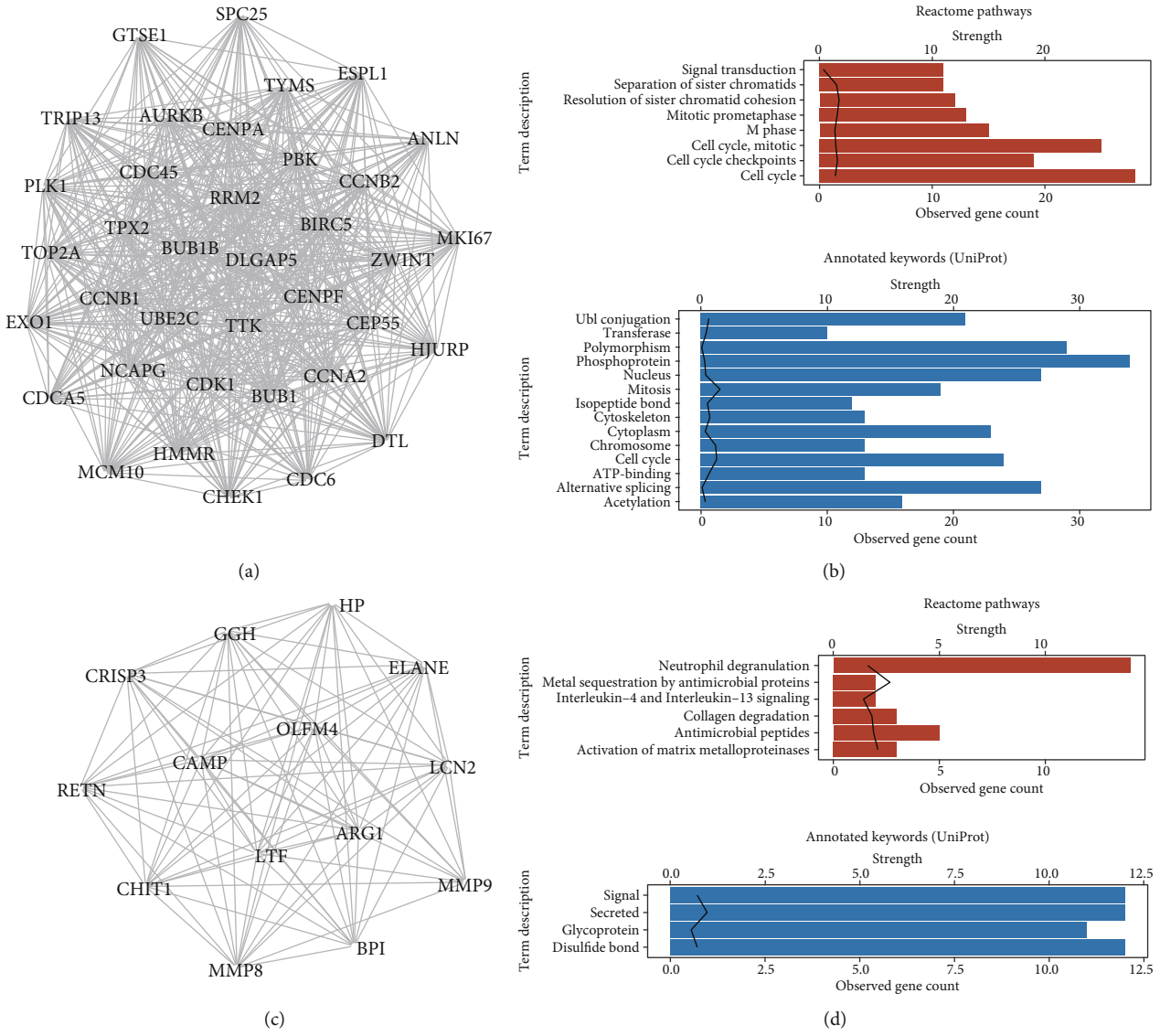


Figure 3: Protein-protein interaction (PPI) network for the DEGs. (a) Cluster 1 (score = 34.3, node = 37, and edge = 618) detected by molecular complex detection (MCODE) of Cytoscape. (b) Reactome pathway enrichment and UniProt database annotation for the genes in cluster1; line represented strength value. (c) Cluster2 (score = 13.5, node = 14, edge = 88) identified by MCODE. (c) Reactome pathway enrichment and UniProt database annotation for the genes in cluster2.

color of the nodes as well as edges had been mapped according to the statistic results by NetworkAnalyzer. Furthermore, MCODE was used to identify the key subnets, with all the parameters set to default. We then presented the first two significant clusters, which were again put into STRING for the functional enrichment. Genes in cluster1 (score: 34.3, 37 nodes, and 618 edges) mostly involved in the cell cycle according to Reactome Pathway enrichment; protein-annotated keyword by UniProt showed the similar results (Figures 3(a)–3(b)). Genes in cluster2 (score: 13.5, 14 nodes, and 88 edges) involved in Neutrophil degranulation, which are mostly secretory protein and signaling protein (Figures 3(c)–3(d)). Totally, there is more evidence to support that neutrophil degranulation and the strong cell proliferation status were the significant characteristics of the infection of SARS-CoV-2 (early stage).

3.3. Difference of Immune Cell Abundance between COVID-19 Patients and Healthy People. In order to clarify the alteration of infiltration of different types of immune cells in the peripheral blood, we applied ssGSEA and CIBERSORT to evaluate the immune cell abundance. For both of the two datasets, ssGSEA identified the increase of activated CD4 T cell, gamma delta T cell, type 2 T helper cell, activated dendritic cell, macrophage, and neutrophil (Wilcox test, p value < 0.05) in COVID-19 patients compared to healthy people. ssGSEA also identified the decrease of activated B cell, activated CD8 T cell, immature B cell, and natural killer cell (Figures 4(a) and 4(c)). As for CIBERSORT, for both of the two datasets, plasma cells, macrophages M0, and neutrophils were identified to be upregulated in COVID-19 patients significantly (Wilcox test, p value < 0.05) but naïve B cells; T cells CD8 were detected to be downregulated

(Figures 4(b) and 4(d)). Overall, we supposed that the occurrence of COVID-19 was accompanied by activation of neutrophil and macrophage, especially neutrophil. However, there was a dramatic alteration of lymphocytes, CD 8 cells, and naïve B cells that were considered to be downregulated in the COVID-19 patients.

3.4. Relationship between Transcriptomic Alternations and Severity of Patients. The transcriptomic and immune cell infiltration alternations during the occurrence of the disease have been revealed in the above study, but we wondered the immunological factors in disease progression. GSE157103, containing RNA-Seq data of peripheral blood leukocyte samples and various clinical data from 50 ICU and 50 non-ICU COVID-19 patients, was downloaded from the GEO database in the form of TPM. Limma package for DEG analysis identified 376 DEGs, including 67 upregulated genes and 309 downregulated genes, which were enriched in neutrophil degranulation and T cell activation in BP of GO, respectively (Figures 5(a) and 5(b)). To verify the DEGs, next, WGCNA was conducted on the top 5000 genes with the max median absolute deviation. 7 modules were clustered under the power value set to 30 (Figure 5(c), 1, Figure S3a). The grey module presented the highest correlation with the clinical traits (correlation efficient = 0.65) (Figure 5(c), 2). Thus, genes in the grey module were extracted for GO and KEGG analysis, which still showed that neutrophil degranulation and neutrophil activation involved in immune response played a crucial role (Figure 5(d), Figure S3).

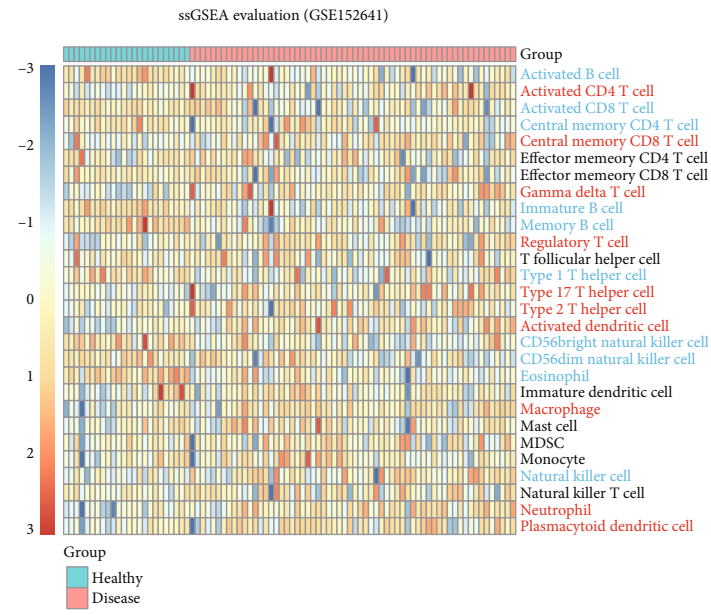
3.5. Drug-Gene Interaction Analysis for Genes Related with Hospital-Free Days. In order to establish a gene signature representing the occurrence and development of COVID-19, we designed a pipeline for constructing the signature (Figure 6(a)). Firstly, we intersected the 314 and 376 DEGs gained from the above analysis. The 42 genes represented the molecule made sense both in the occurrence and progress of the disease. Then, the correlation coefficients between the 42 genes and the hospital-free days during 45 days of follow-up were calculated. And 31 genes with the coefficient larger than 0.4 or smaller than −0.4 were selected, which were considered as the factors that had influence on the clinical outcome. We regarded the 31 genes as a "signature" always active in COVID-19 (Table 1).

Next, the DGIdb database was used to predict the drug-gene interaction. All of the 31 genes were input and only drugs that had been approved and had a clear pharmacological effect (inhibitor or activator) were included. Drugs targeting 5 of the 31 genes, including CA4, S100A12, MMP8, MMP9, and FCER1A were identified (Figure 6(b)). We had gotten that CA4, S100A12, MMP8, and MMP9 were related to a longer hospital day (inhibitor needed) while FCER1A was related to a longer hospital-free days (activator needed). For CA4, 16 kinds of inhibitors were found. Trichlormethiazide and bendroflumethiazide were the top two with the highest query score and interaction score. For S100A12, olopatadine and amlexanox tended to be the inhibitors. Doxycycline and doxycycline calcium were found to target MMP8, while glucosamine, minocycline, and captopril targeted MMP9, and benzylpenicilloyl polylysine can act as an agonist for FCER1A.

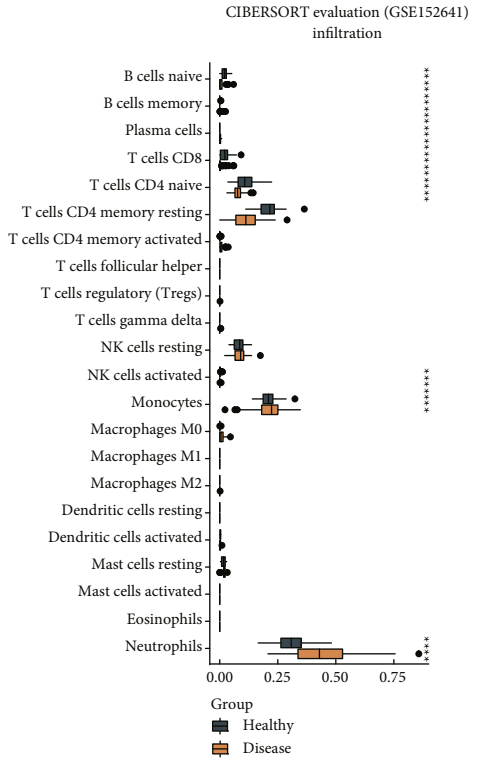
3.6. Difference of Immune Cell Abundance between ICU Patients and Non-ICU Patients and Immune Subtypes. To explore the immunological changes during the progress of the disease, we again utilized the ssGSEA and CIBERSORT for the evaluation of immune cell infiltration on GSE157103. Interestingly, we observed a significant decline of different types of immune cells in the ICU patients based on ssGSEA. CIBERSORT also implicated the decrease of different types of immune cells, including T cell CD8 and T cell CD4 memory-activated and NK cells resting, but showed an increase of neutrophils (Wilcox test, p value < 0.05). Therefore, the COVID-19 patients in the ICU might show less activation of the immune system (Figures 7(a)–7(b)).

Next, to preliminarily demonstrate the impact of immune cells on clinical prognosis, we utilized the ssGSEA result to conduct the NMF clustering. 4 clusters were identified as shown in the heat map (Figure 7(c), 1). We noticed that only plasmacytoid dendritic cell, neutrophil, activated dendritic cell, MDSC, monocyte, activated CD8 T cell, activated B cell, and immature B cell were included in the clustering. Cluster3 was the subtype abundant of the first 3 cells and poor of the latter 5 cells, and cluster4 was opposite (Figure 7(c), 2). Then, patients were divided into 4 groups according to the hospital-free days during the 45 days of follow-up: 0 days (always in hospital), 0–26 days, 26–38 days, and 38–45days. Submap was applied to evaluate the similarity of gene expression characteristics between cluster1–4 and B1–4. Interestingly, the Bonferroni-corrected p value hinted that cluster3 (subtype of abundant plasmacytoid dendritic cell, neutrophil, and activated dendritic cell but poor of others) could be mapped to the patients with short hospital-free days (Bonferroni corrected $p = 0.02$), while cluster4 could be mapped to the patients with a relatively good prognosis (Figure 7(d)).

3.7. Immune Cell Abundance Was Closely Related with Hospital-Free Days and Gene Signature. The correlation coefficient between different types of immune cell infiltration and the hospital-free days during 45-day follow-up was calculated; ssGSEA identified 10 types of immune cells which could ameliorate the patient's hospitalization (Figure 8(a)). Unfortunately, most of them degraded in the ICU patients compared to non-ICU patients. Furthermore, CIBERSORT identified a negative impact of neutrophils on the hospital days (Figure 8(b)). Integrated with the previous analysis, it was credible that lymphocytes, especially CD8 T cells, were a protective factor of COVID-19 and the neutrophil could be a risk factor. Additionally, to understand the mechanism of the effect of the immune cells, we listed the correlation between the immune cells and the 31-gene signature as well as the correlation between the cells and the 5 genes with targeted drugs (Figure S4a-b). Whether in ssGSEA or CIBERSORT, it was implicated that CA4, S100A12, MMP8, and MMP9 were related with the regression of lymphocytes, especially CD8 T cells, while related with the activation of neutrophil. Conversely,



(a)



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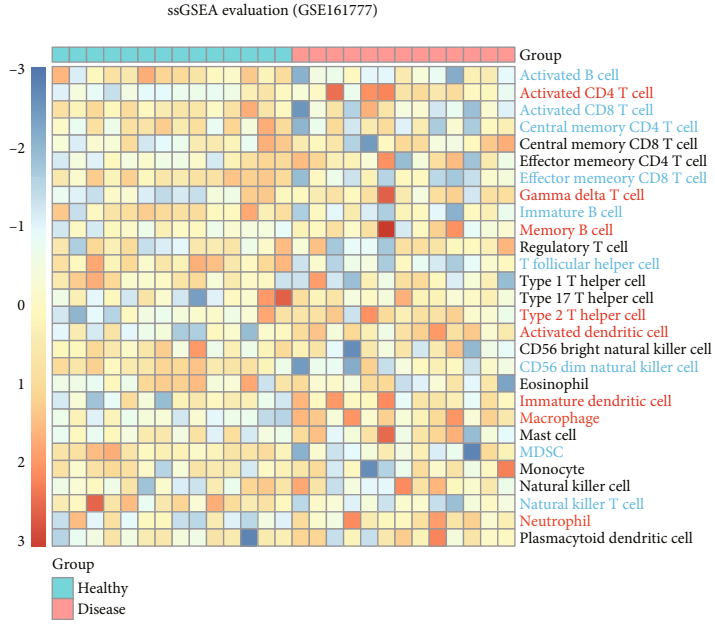
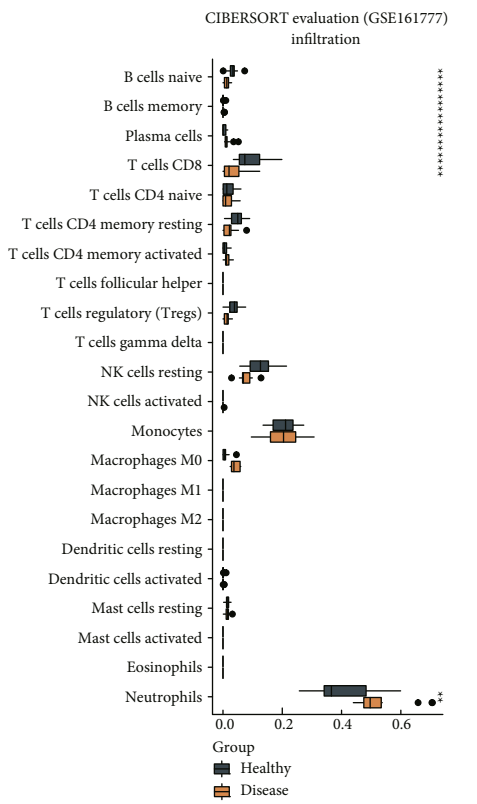


(c)



(d)

Figure 4: Difference of immune cell abundance between COVID-19 patients and healthy people (a) ssGSEA for evaluation of 28 immune cell infiltration in GSE152641. (b) CIBERSORT for evaluation of 22 immune cell infiltration in GSE152641. (c) SsGSEA for evaluation of 28 immune cell infiltration in GSE161777. (d) CIBERSORT for evaluation of 22 immune cell infiltration in GSE161777.

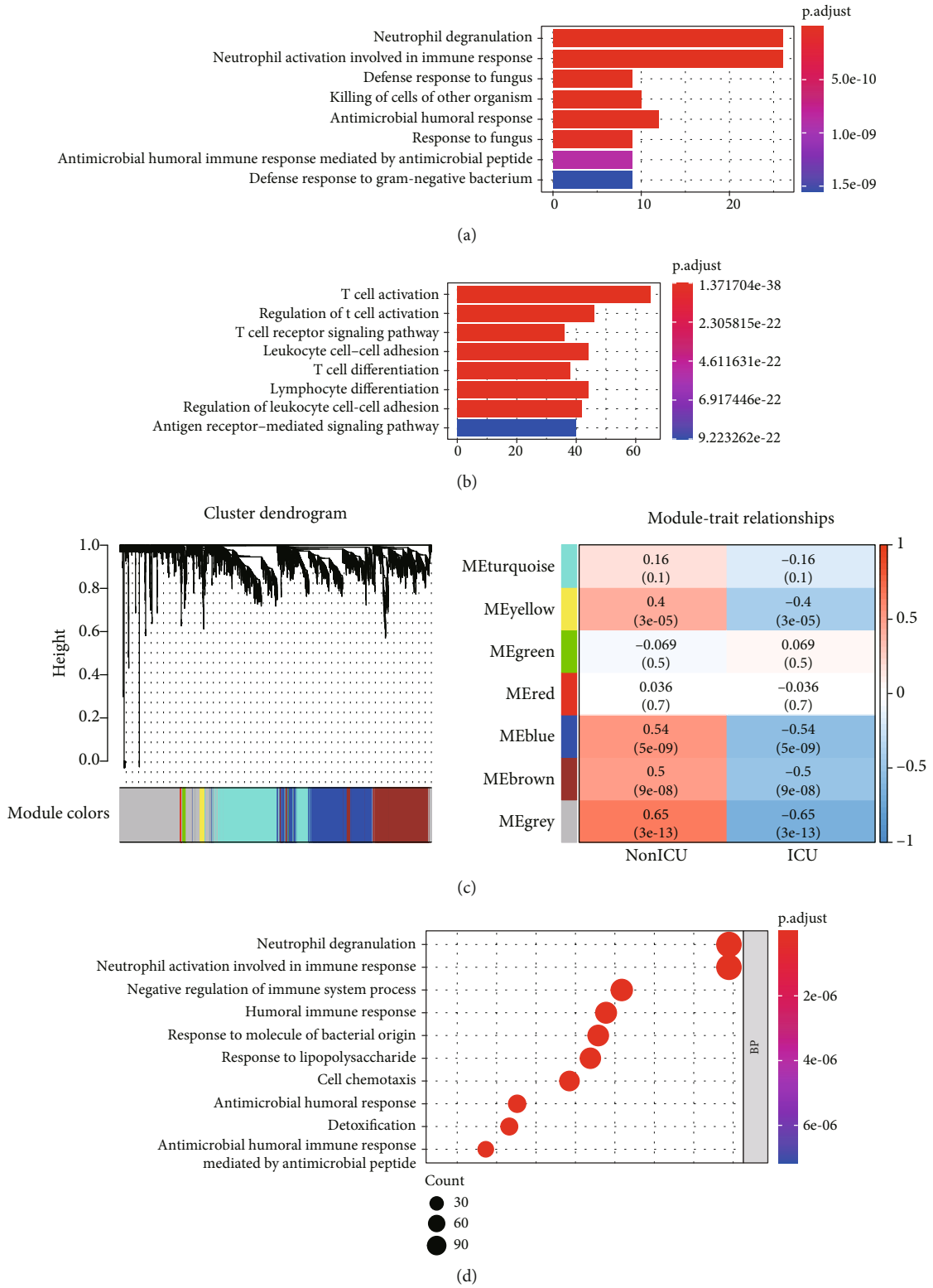


Figure 5: Relationship between transcriptomic alternations with severity of patients. (a) Biological process (BP) of GO analysis for genes upregulated ($n = 67$) in ICU patients compared to non-ICU patients in GSE157103. (b) BP of GO analysis for genes downregulated ($n = 309$) in the ICU patients. (c) Modules clustering and their relationship with clinical traits in WGCNA. (d) BP of GO analyses for genes in the grey module.

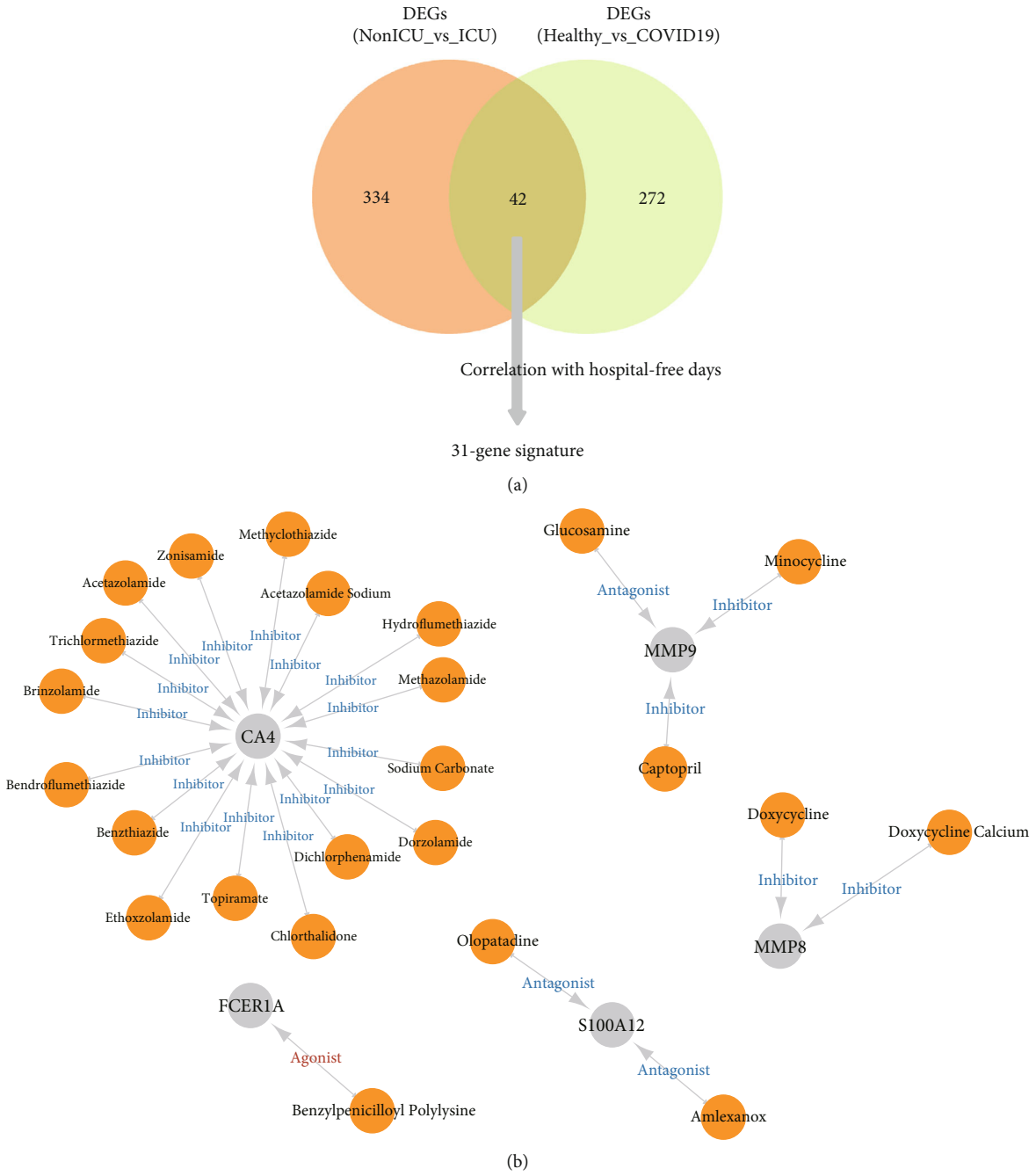


Figure 6: Drug-gene interaction analysis for genes related with hospital-free days. (a) Pipeline for the selection of the gene signature. (b) Drug-gene interaction predicted by DGIdb.

FCER1A was related to the activation of various kinds of immune cells but was negatively correlated with neutrophil infiltration (Figure 8(c)). In short, studies at the level of transcriptome and immune cells can be integrated, which also provided an explanation for the predicted drugs.

4. Discussion

Although the pandemic of COVID-19 has threatened the health of the world, the host immune response to SARS-CoV-2 infection still lacks full demonstration. Up to now, evidence showed that an imbalanced immune response to inflammation is a major trigger of COVID-19 and the dysfunction of local and systemic immune responses had been implicated in the disease outcome and prognosis. Thus, identifying transcriptomic and immunological alternations may not only be significant for a better comprehensive understanding of the mechanisms of the disease but also help to effective therapy excavation and individualized management.

In the present study, we paid close attention to both the occurrence (comparison1: healthy vs. patients) and the

Table 1: 31 Genes in the signature of COVID-19.

Gene	Correlation coefficient	p value
PID1	0.693520919	1.27*E*−15
P2RY10	0.679852476	7.38*E*−15
CD40LG	0.668959371	2.81*E*−14
FCER1A	0.654672468	1.49*E*−13
CD5	0.645849101	4.00*E*−13
TCF7	0.636922512	1.05*E*−12
FAM102A	0.624610777	3.80*E*−12
TRABD2A	0.623338096	4.32*E*−12
NELL2	0.618426883	7.08*E*−12
CPA3	0.593173164	7.88*E*−11
TPPP3	0.584820798	1.67*E*−10
HDC	0.57412751	4.24*E*−10
MAL	0.536815499	8.54*E*−09
PRSS33	0.521039587	2.73*E*−08
ALOX15	0.516734511	3.72*E*−08
VSIG4	−0.429548434	8.21*E*−06
MMP8	−0.472200179	7.05*E*−07
ANXA3	−0.517766599	3.46*E*−08
CHIT1	−0.529930553	1.43*E*−08
ADAMTS2	−0.53487937	9.89*E*−09
PCOLCE2	−0.544461309	4.76*E*−09
TPST1	−0.551635666	2.71*E*−09
WFDC1	−0.555936581	1.92*E*−09
IL18R1	−0.561055133	1.27*E*−09
CA4	−0.581762072	2.19*E*−10
MMP9	−0.583963653	1.80*E*−10
CD177	−0.594606481	6.91*E*−11
ARG1	−0.60433151	2.79*E*−11
OLAH	−0.619818119	6.16*E*−12
S100A12	−0.646161289	3.87*E*−13
MCEMP1	−0.683772837	4.50*E*−15

progress (comparison2: non-ICUers vs. ICUers) of COVID-19. 253 upregulated genes and 61 downregulated genes were identified to be differentially expressed during the occurrence of the disease. GO, KEGG, Reactome, and UniProt were used to annotate the function of DEGs, and the PPI network was constructed, with 2 crucial subnets identified. WGCNA was used to find the significant gene modules. ssGSEA and CIBERSORT revealed that neutrophil activation and CD8+ T cell downregulations were two reliable changes in both of the comparisons. Novelly, several drugs were predicted and the pharmacological effects were understood.

Combined with the enrichment result and the evaluation of immune cells, it was clear that peripheral blood of the COVID-19 patients was in a state of hyperproliferation and immune cells show overall activation. Such changes had been considered to be strongly related to oxidative stress, which strengthened the immune system but could also cause excessive inflammatory and respiratory failure [49, 50]. Inferred from two comparisons, the beneficial immune defense gradually transformed into an excessive inflammatory response, while the immune system would be in a state of exhaustion. On the other hand, neutrophils, which was believed to play a key role during the disease course in the present study, could also lead to damage through oxygen species (ROS) [51].

Here, we will review the genes that had potential drugs, which owned a close relationship with oxidative stress and inflammation. CAs, which catalyze the interconversion of water and carbon dioxide into dissociated ions of carbonic acid, are a kind of zinc metalloenzymes broadly engaged in various biological processes [52–54]. There are 14 isozymes of CAs altered genetically in the pathological status in human [55], and CA4 is the most widely distributed one [56]. CA4 plays an important role in the bicarbonate reabsorption of the kidney [57]. During acidosis, its competence is enhanced to generate more H+ to relay the acidosis [58]. We assume that CA4 on blood cells can act likely in the acidotic status resulting from hypoxia created by COVID-19. And CA4 may affect the function of neutrophils by modulating altering pH [59].

Next, MMPs are an enzyme family majorly correlated with the remodeling of extracellular matrix (ECM) components [60]. MMP9 (or gelatinase B) is one of the main types of MMPs and can be found in diverse cells like monocytes, macrophages, and neutrophils. MMP9 are highly expressed in pathological processes including inflammation [61], as is also discovered in this study. MMP9 is an inflammatory cytokine, acting as a regulator to promote the secretion of other cytokines by leukocytes. Besides, MMP9 itself is also regulated by the degranulation from neutrophils, which is induced by other various types of chemotactic factors. Moreover, MMP9 can truncate IL-8, the major human neutrophil chemoattractant, into a tenfold more potent form, creating a positive feedback loop for neutrophil activation and chemotaxis [62]. MMP8 is majorly synthesized and archived in neutrophils [63]. Circulating MMP8 has been found to closely related with lung fibrosis in COVID-19 patients [64] and serves as member of a 5-protein classifier to predict the prognosis of idiopathic pulmonary fibrosis (IPF) [65].

Besides, S100A12 is a member of the S100 protein family of calcium-binding ability and is predominantly secreted by neutrophils [66, 67]. As an emerging biomarker for inflammatory diseases, the level of S100A12 in serum can reflect the systemic inflammatory status in acute otitis media, cystic fibrosis, respiratory distress syndrome, and dermatomyositis-associated interstitial lung disease [67, 68]. Besides, S100A12 is also found to herald worse cardiac output and mortality in pulmonary hypertension [69], which is also common in COVID-19 [70]. Moreover, SA100A12, together with S100A8 and S100A9, which are also both released by neutrophils, can activate airway epithelial cells to produce MUC5AC, a major mucin protein in the respiratory tract [71], partly interoperating the excessive mucus discovered in the necropsy of COVID-19 patients [72]. And compared with S100A8 and S100A9, SA100A12 is more considered as a marker for respiratory diseases with neutrophilic inflammation [73].

Furthermore, FCER1A encodes a subunit of FcεR that can bind with IgE [74] and can be found on the surface of

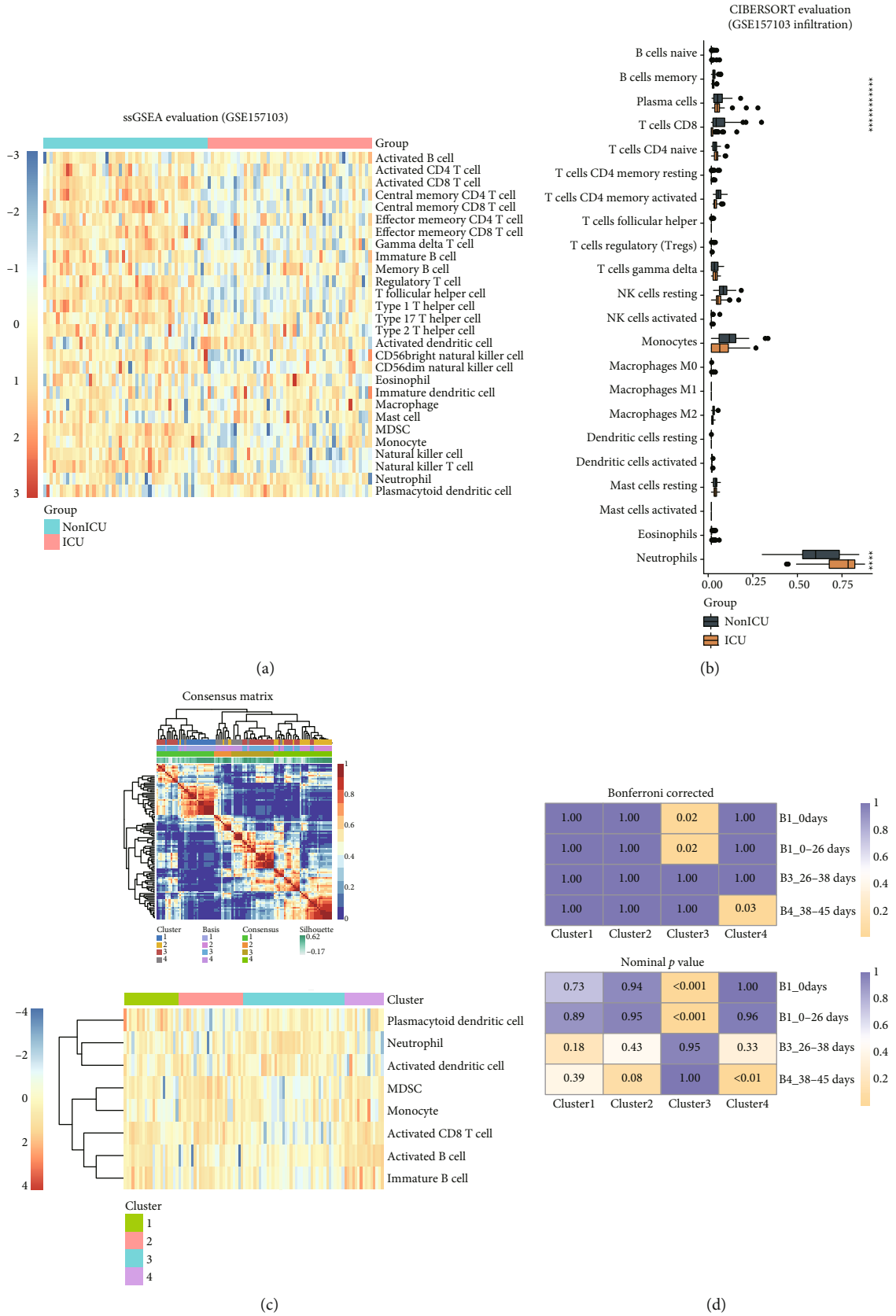


Figure 7: Difference of immune cell abundance between ICU patients and non-ICU patients and immune subtypes. (a) ssGSEA for evaluation of 28 immune cell infiltration in GSE157103. (b) CIBERSORT for evaluation of 22 immune cell infiltration in GSE157103. (c) 4 clusters were identified in the NMF clustering using the ssGSEA result. (d) Bonferroni-corrected and nominal p value of the submap result.

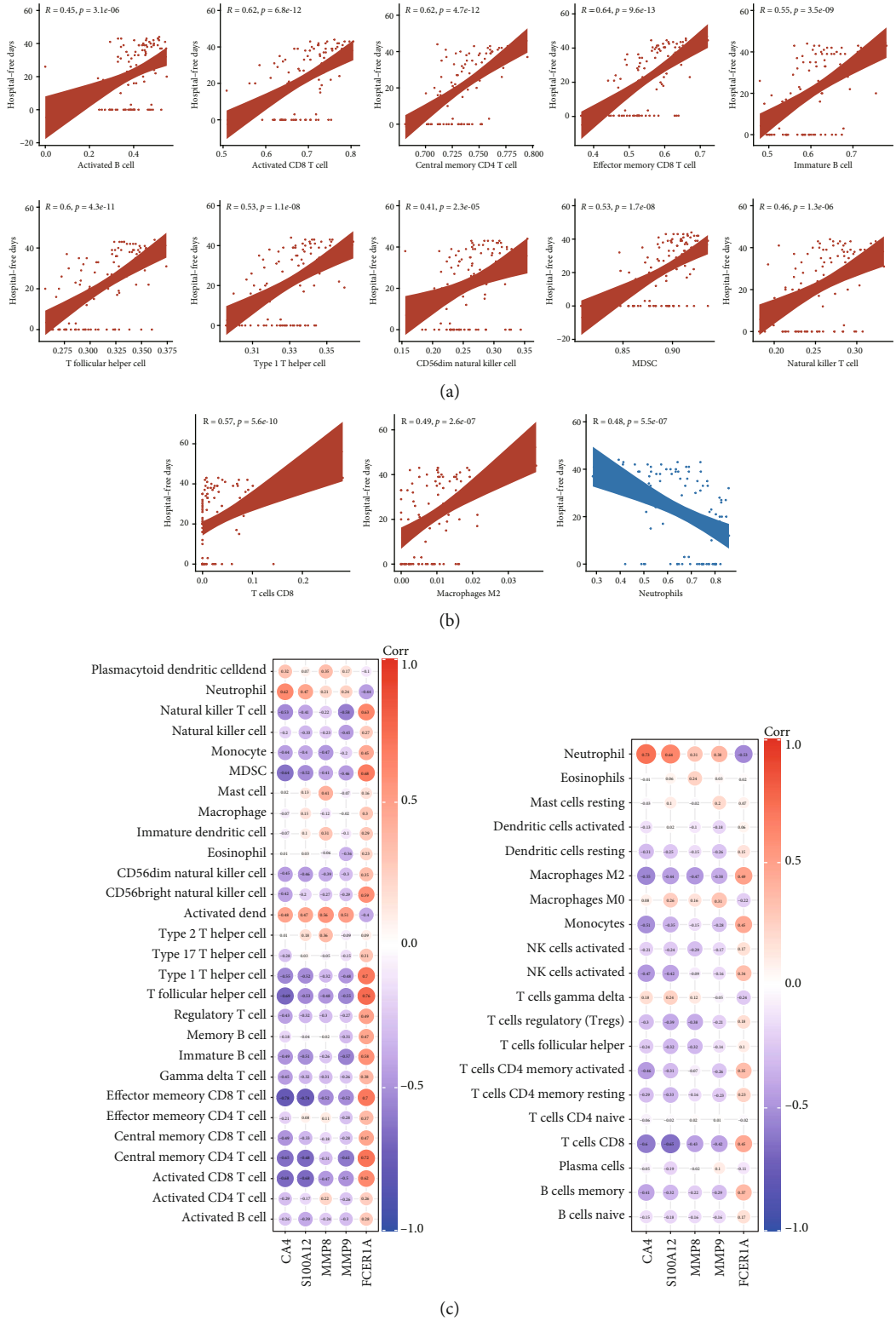


Figure 8: Immune cell abundance was closely related with hospital-free days and gene signature. (a) Immune cells were significantly related with hospital-free days (|Spearman correlation coefficient|>0.4) in ssGSEA. (b) Immune cells were significantly related with hospital-free days (|correlation coefficient|>0.4) in CIBERSORT. (c) Correlation between the expression level of CA4, S100A12, MMP8, MMP9, and FCER1A and immune cell infiltration evaluated by ssGSEA. (d) Correlation between the expression levels of CA4, S100A12, MMP8, MMP9, and FCER1A and immune cell infiltration evaluated by CIBERSORT.

various kind of cells like basophils, mast cells, monocytes, dendritic cells, and neutrophils [75]. Studies looking into FCER1A in the respiratory system mainly focus on mast cell and basophils, two major cells involved in allergy [76]. But the decreased level of FCER1A in COVID-19 patients seemed to not explain the potential role of the activation of mast cell or that basophil plays in hyperinflammation with patients [77, 78], which deserves to be further studied.

With regard to the immune cell infiltration of the blood, the elevation of the abundance of neutrophils is universally observed in various studies [79]. This study also suggested the important role of neutrophils in the pathological process of COVID-19. Infected lung cells are found to express neutrophil-attracting chemokines, and attracted neutrophils can attract even more neutrophils that might finally result in the excessive activation and degranulation of neutrophils, contributing to neutrophil-related lung damage [80, 81]. Several possible mechanisms concerning neutrophils are proposed. Neutrophil extracellular traps (NETs), which refer to web-like chromatin structures derived from dead neutrophils [82], might be one of the most prevalent ones [83]. MMP8, MMP9, and S100A12, three genes that we found significantly upregulated in COVID-19 patient, are also common components in NETs [84–86], which further demonstrates the vital roles of NETs in COVID-19 development.

On the other hand, the abundance of CD8+ T cells was reported to decrease in COVID-19 patients and exhibit functional exhaustion molecules, such as NKG2A, PD-1, and TIM-3 [87]. And neutrophil-to-lymphocyte can also increase as a result of systemic inflammation serving as a prognostic marker [88]. Single-cell RNA sequencing of bronchoalveolar cells depicted a more complicated landscape of CD8+ T cells in COVID-19 patients, further pointing out the heterogeneity of cell numbers and clonal expansion of different CD8+ T cell clusters [89].

Herein, we can understand the mechanisms of the drugs which were predicted in the present study based on the above discussion. Trichlormethiazide and bendroflumethiazide are both inhibitors for CAs [90, 91]. Application of CA inhibitors in COVID-19 individuals can block the discharge of H+ in the kidney and worsen the acidotic status in patients [92]. Besides, application of the CA inhibitor can rescue the decrease of IL-8, the most important chemotactic for neutrophils in hypercarbia, which might deteriorate the overactivation of neutrophils [59]. Olopatadine is an antiallergic drug antagonizing the histamine H(1) receptor [93]. Amlexanox is a small-molecule targeted therapy used to treat atopic diseases [94]. Both olopatadine and amlexanox were found to have the ability to suppress the migration of monocytes induced by proinflammatory S100A12 [95]. Doxycycline has antimicrobial effect as well as potent anti-inflammatory activity [96]. And doxycycline can downregulate MMP8 both in mRNA and protein levels [97]. Minocycline is another kind of common antibiotic used in bedside and it was found to reduce the level of MMP9 [98, 99]. Captopril is one of angiotensin-converting enzyme inhibitors (ACEIs) usually used to relieve hypertension [100] and can also downregulate the expression of MMP9 and reactive oxygen species (ROS) [101].

5. Conclusion

Based on 3 dependent RNA-seq of COVID-19 patients, we learned that the neutrophil degranulation was significant in the occurrence of the disease, during which the peripheral blood was in a hyperproliferative state. Neutrophil activation and the inactivation of CD8+ T cells played a key role during the progress of the disease and 4 immune subtypes were identified. A 31-gene composed signature was established which was crucial during the course of the disease. Several drugs were predicted for the therapies of COVID-19 based on the prognostic value of the genes in the signature. In short, we believe that our study shed light on the understanding and treatment of COVID-19.

Data Availability

Three datasets were obtained publicly from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/); further inquiries for the codes or other data can be directed to the corresponding author.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors' Contributions

S.M. was responsible for the data analysis and the writing of results. L.D. completed the discussion section. Y.W. completed the methods section. B.S. completed the introduction section. Z.Y. was responsible for the literature search. W.G. was responsible for the design and part of the data analysis of the study.

Supplementary Materials

The Supplementary Material for this article can be found online. Supplementary Figure 1: functional enrichment for the DEGs (a) KEGG analysis for the 253 up-regulated genes. (b) GO analysis for the 61 downregulated genes. (c) KEGG analysis for the 61 downregulated genes. Supplementary Figure 2: the PPI network for the total 314 genes. Supplementary Figure 3: WGCNA for the top 5000 genes with the max median absolute deviation. (a) Selection for the best power value. The R^2 threshold was set to 0.8. (b) KEGG analysis for the genes in grey module. Supplementary Figure 4: correlation between genes in the signature and immune cells. (a) Correlation between the 31 genes in the signature and 28 immune cells evaluated by ssGSEA. (b) Correlation between the 31 genes in the signature and 22 immune cells evaluated by CIBERSORT. *(Supplementary Materials)*

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Review Article

Endothelial Dysfunction, Inflammation, and Oxidative Stress in COVID-19—Mechanisms and Therapeutic Targets

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The outbreak of the COVID-19 pandemic represents an ongoing healthcare emergency responsible for more than 3.4 million deaths worldwide. COVID-19 is the disease caused by SARS-CoV-2, a virus that targets not only the lungs but also the cardiovascular system. COVID-19 can manifest with a wide range of clinical manifestations, from mild symptoms to severe forms of the disease, characterized by respiratory failure due to severe alveolar damage. Several studies investigated the underlying mechanisms of the severe lung damage associated with SARS-CoV-2 infection and revealed that the respiratory failure associated with COVID-19 is the consequence not only of acute respiratory distress syndrome but also of macro- and microvascular involvement. New observations show that COVID-19 is an endothelial disease, and the consequent endotheliopathy is responsible for inflammation, cytokine storm, oxidative stress, and coagulopathy. In this review, we show the central role of endothelial dysfunction, inflammation, and oxidative stress in the COVID-19 pathogenesis and present the therapeutic targets deriving from this endotheliopathy.

1. Introduction

The SARS-CoV-2 virus, responsible for COVID-19 disease, can evolve with a wide range of clinical manifestations, from mild forms manifesting as fever, dyspnea, cough, and loss of smell and taste to severe forms, especially in the elderly with comorbidities, characterized by respiratory failure due to severe alveolar damage [1]. In the extremely severe forms of the disease, rapidly progressive multiple organ failure occurs, which manifests through complications such as shock, acute cardiac injury, acute respiratory distress syndrome (ARDS), disseminated intravascular coagulopathy (DIC), and acute kidney injury, which may ultimately prove fatal [2]. Recent studies have demonstrated that respiratory failure occurring in COVID-19 is due not only to acute respiratory distress syndrome but also to macro- and

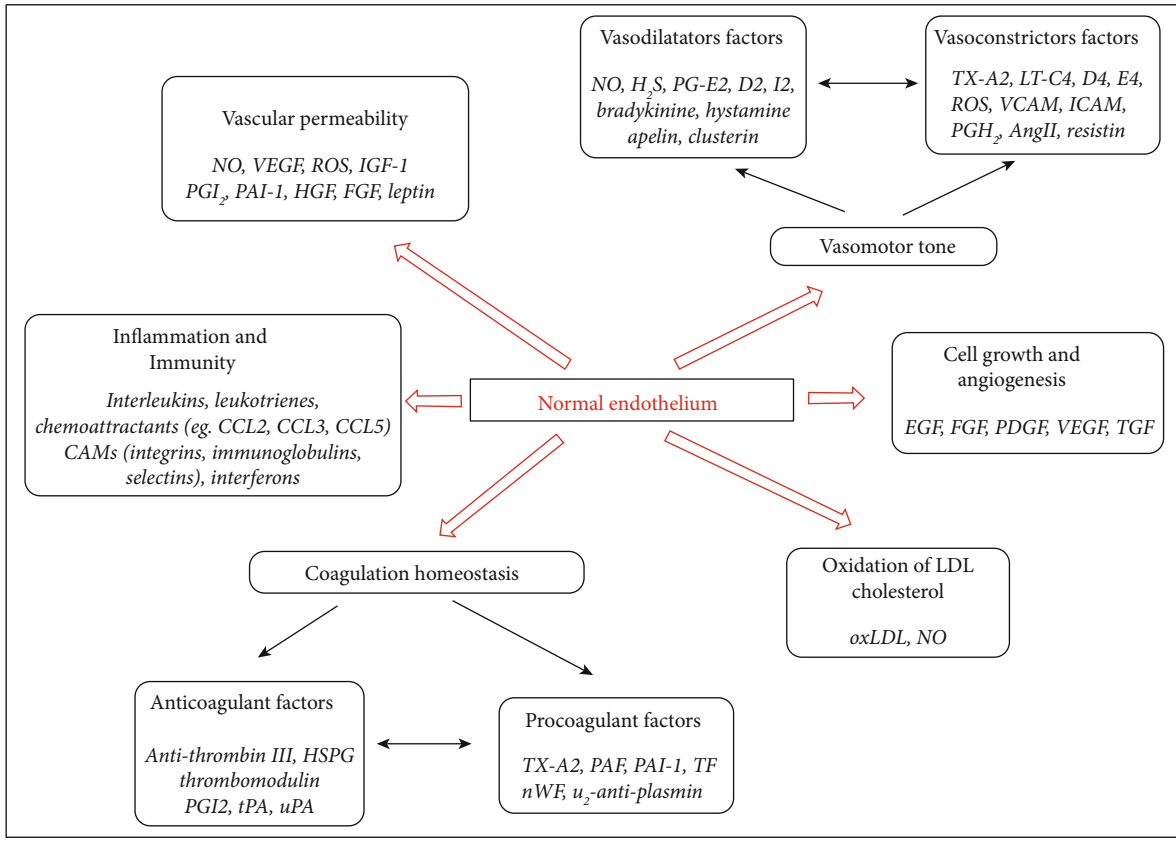

Figure 1: Functions of vascular endothelium. Endothelium cells produced some vascular mediators/factors that accomplished the six major functions of normal endothelium (modulation of vascular permeability and vasomotor tone modulation, coagulation homeostasis, inflammation and immunity regulation, cell growth regulation, and oxidation of LDL cholesterol) by which the vascular homeostasis is maintained (adapted after [9]).

microvascular involvement [3–5], a particular role being played by vascular endothelial damage [6, 7]. New observations show that COVID-19 is an endothelial disease [8] and that endotheliopathy is responsible for inflammation, cytokine storm, oxidative stress, and coagulopathy. An argument of this theory is the fact that patients who have endothelial dysfunction due to various comorbidities (obesity, hypertension, and diabetes) develop more severe forms of COVID-19, explained by an additional alteration of the already dysfunctional vascular endothelium [7].

In this review, we show the central role of endothelial dysfunction, inflammation, and oxidative stress in the development of complications of SARS-CoV-2 infection and their pathophysiological consequences, and examine the main therapeutic targets deriving from this endotheliopathy.

The endothelium, one of the largest organs of the human body, is capable of producing a wide variety of molecules, with effects that are often contradictory, with a role in maintaining homeostasis, such as vasodilator and vasoconstrictor, procoagulant and anticoagulant, inflammatory and anti-inflammatory, fibrinolytic and antifibrinolytic, and oxidant and antioxidant substances [9].

The normal endothelium regulates vascular homeostasis through six major functions: (1) modulation of vascular permeability, (2) modulation of vasomotor tone, (3) modulation of coagulation homeostasis, (4) regulation of inflammation and immunity, (5) regulation of cell growth, and (6) oxidation of LDL cholesterol (Figure 1). These functions are achieved through numerous mediators, of which the most studied is nitric oxide (NO) [9].

Nitric oxide is the most important vasodilator substance produced by endothelial cells. NO also has an antithrombotic action, inhibiting the fibrotic properties of angiotensin II and endothelin I by downregulating the receptors for these molecules. NO is synthesized in endothelial cells from L-arginin under the action of the endothelial NO synthase (eNOS) [10]. This reaction requires the presence of molecular oxygen and certain cofactors, including calmodulin, tetrahydrobiopterin (THB4), NADPH (adenine dinucleotide phosphate), flavin adenine dinucleotide, and flavin mononucleotide. From this reaction, L-citrulline as a by-product results [11].

Endothelial dysfunction is defined as a reduction in the bioavailability of vasodilator substances, especially NO, and an increase in vasoconstrictor substances.

The reduction of NO bioavailability can be due to a decrease in eNOS production (lack of cofactors necessary for eNOS synthesis) on the one hand, and to an increase in excessive NO degradation or inactivation by reactive oxygen species (ROS), on the other hand [12]. The increase in the

production of ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^\bullet$), hypochlorous acid (HOCl), and lipid superoxide radical, represents the main cause of the decrease in NO bioavailability in cardiovascular diseases [13]. Under physiological conditions, ROS production is controlled by an effective system of antioxidants, molecules that are capable of neutralizing ROS, thus preventing oxidative stress. In tissues, natural enzymatic antioxidants, such as superoxide dismutase (SOD), glutathione peroxidase, and catalase, play an important role in the conversion of ROS to oxygen and water. In pathological conditions, ROS can be present in excess relatively to the existing antioxidant capacity. This alteration of the balance in favor of oxidation termed "oxidative stress" may have negative effects on cell and tissue function [9].

Endothelial cells (EC) possess a number of mechanisms that reduce local oxidative stress. When subjected to shear stress, the endothelium produces SOD, which eliminates ROS [14]. The endothelial cell can also express glutathione peroxidase, which can mitigate oxidative stress [15]. Similarly, haem-oxygenase provides another mechanism by which the endothelial cell can resist to local oxidative stress [16, 17].

In contrast, proinflammatory cytokines can stimulate endothelial cells to mobilize NADPH-oxidase that generates superoxide anions, amplifying local oxidative stress [18, 19].

2. COVID-19-Associated Endotheliopathy and Oxidative Stress

Endothelial dysfunction or endotheliopathy is an important pathological characteristic in COVID-19 [20]. Electron microscopy of blood vessels in autopsy samples from patients with COVID-19 revealed the presence of endothelial cell degradation and apoptosis [21, 22]. Endothelial dysfunction biomarkers, such as thrombomodulin, von Willebrand factor (vWF), angiopoietin 2, and PAI-1, are frequently increased in patients with COVID-19 compared to healthy persons and seem to have prognostic significance, being associated with more severe forms of the disease and high mortality [23, 24]. Endothelial dysfunction is an important factor in the pathophysiology of thrombotic complications associated with COVID-19, including myocardial infarction and stroke [23, 24].

At present, it is uncertain whether endotheliopathy associated with COVID-19 is the result of direct endothelial cell viral infection, as reported in some autopsy studies [21, 25] or is a consequence of the inflammatory response induced by the virus.

Many pathophysiological mechanisms have been described which explain the implication of endothelial dysfunction in the occurrence of microvascular involvement in COVID-19 infection. Microvascular cerebral involvement in COVID-19 as a result of age-related endothelial dysfunction is an important challenge for research [20]. Overactivation of poly-(ADP-ribose) polymerase 1, as can be observed in viral infections, can lead to NAD+ depletion and subsequent endothelial dysfunction [26, 27]. In addition, the dysfunction of the nuclear factor erythroid 2-related factor 2 (NRF2) antioxidant defense pathway in endothelial cells might also play a role in the COVID-19 associated endotheliopathy [28]. The pharmacological activators of NRF2 were proposed as potential treatment options for COVID-19 [29]. NRF2 has strong anti-inflammatory and antiapoptotic effects in endothelial cells. It should be noted that NRF2 dysfunction exacerbates the deleterious effect of hypertension and diabetes on the endothelium, conditions known for the increase in the COVID-19-related risk of death [29].

Oxidative stress is generated by high Ang II concentrations and low Ang 1-7 concentrations (Figure 2). These ROS can oxidize cysteine residues in the peptidase domain of receptors ACE2 and RBD of proteins SARS-CoV and SARS-CoV-2, maintaining them in oxidized forms (disulfide), unlike reduced forms (thiol) [30]. It is possible that oxidation of these thiols to disulfides, through an oxidative stress mechanism, may increase the affinity of proteins SARS-CoV and SARS-CoV-2 S for ACE2 receptors and, consequently, increase the severity of COVID-19 infection [31].

The relationship between Ang II and NADPH-oxidase was investigated using murine smooth vascular muscle cells. When the cells were exposed to Ang II, the researchers observed an increased activity of NADPH-oxidase, as well as an increased production of superoxide anions. The exact mechanisms for the stimulation of NADPH-oxidase are complex, genetically mediated, at transcriptional and post-transcriptional level, and involve numerous signaling molecules and scaffolding proteins/platforms [32]. Inactive NADPH-oxidase contains two subunits: glycoprotein (gp) 91phox and p22phox. In the presence of Ang II, NADPH-oxidase is activated through the involvement of additional subunits p67phox, p47phox, p40phox, and Rac1. Activated NADPH-oxidase can generate superoxide anions. Studies in mice have shown that increased NADPH-oxidase activity can be found even in the absence of ACE2 [33, 34]. Since binding of SARS-CoV-2 to ACE2 receptor inhibits the catalytic activity of the enzyme, i.e., the conversion of Ang II to Ang 1-7, the activity of NADPH-oxidase increases in patients with SARS-CoV-2, subsequently leading to an increase in oxidative stress [35].

In a recently published study [36], the long-term effects of SARS-CoV-2 virus on oxidative stress and vascular endothelium were discussed. Thus, it was proposed that SARS-CoV-2, by inducing mitochondrial dysfunction and oxidative stress, can initiate a feedback loop promoting a chronic state of inflammation and endothelial dysfunction even after the viral particles have been eliminated from the body. In this proposed mechanism, SARS-CoV-2 first induces activation of NADPH-oxidase, which produces superoxide (O_2^-), a ROS that is involved in reactions which deteriorate the electron transport chain (ETC) [32, 37].

Increased oxidative stress and inflammation resulting from this mitochondrial dysfunction subsequently initiate a feedback loop that perpetuates NADPH-oxidase activation, mitochondrial dysfunction, inflammatory cytokine production and loss of identity of EC [36]. Considering these hypothetical long-term consequences of SARS-CoV-2 infection on blood vessels, the treatment of chronic oxidative stress

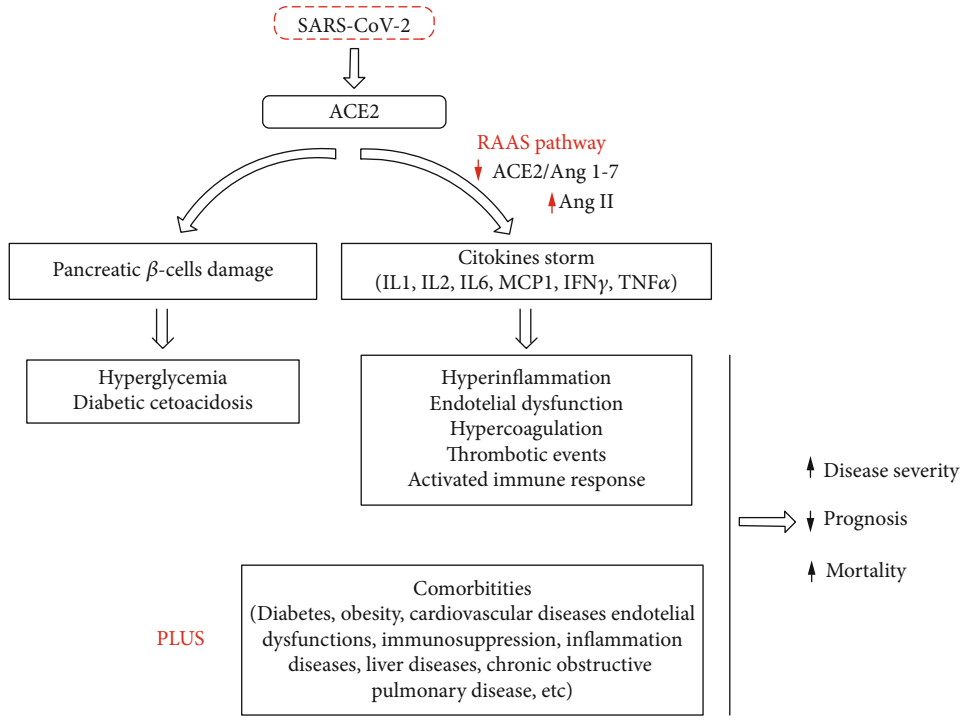

Figure 2: SARS-CoV-2 enters the human body by binding to ACE2. Activation of RAAS produced a cytokine storm, resulting in the secretion of proinflammatory cytokines/chemokines such as interleukins (ILs), interferon-gamma (IFN-γ), monocyte chemoattractant protein-1 (MCP1), and tumor necrosis factor-alpha (TNF-α). This storm produces a pleiades of phenomena which is associated with preexistent comorbidities that lead to an increase in disease severity (adapted after [31]).

and inflammation in EC can be essential in preventing future complications among millions of persons currently diagnosed with COVID-19 [38].

3. COVID-19 Endotheliitis

Numerous postmortem histopathological examinations in patients who died of COVID-19 not only revealed the presence of endotheliitis in the key organs affected by SARS-CoV-2, but also demonstrated the presence of viral structures within the endothelial cells by electron microscopy [21, 25, 39, 40]. By analyzing samples from the transplanted kidney in a COVID-19 patient who developed multiorgan failure, Varga et al. [25] demonstrated the capacity of the virus to invade endothelial cells. In the same patient, histological findings showed the inflammatory infiltrate of the endothelium and the morphological changes that occur during apoptosis in the heart, small bowel, and lungs. Furthermore, they proved the presence of endotheliitis in the lung, heart, kidney, liver, and small intestine of two other COVID-19 patients by postmortem analysis [25]. The wide distribution of ACE2 receptor in endothelial cells explains the multiorgan affinity of the virus, confirmed once more in a study by Puelles et al. The presence of viral particles in the pharynx, lungs, heart, blood, liver, kidneys, and brain was established despite the level of viral load [39].

The electron microscopy studies performed by Ackermann et al. [21] proved the presence of SARS-CoV-2 within the endothelial cells and in the extracellular space; furthermore, ultrastructural injury of the endothelium was also present. The authors of the aforementioned study compared the histological changes that occur in the lungs of SARS-CoV-2 patients with those occurring in acute respiratory distress syndrome caused by influenza A (H1N1) and ten uninfected control lungs. The results revealed that the lungs of COVID-19 patients presented disseminated alveolar injury associated with necrosis, lymphocytic inflammation, and microthrombosis. In addition, the expression of angiotensin-converting enzyme 2 (ACE2) investigated by immunohistochemical analysis was present in lymphocytes only in the COVID-19 and influenza groups [21].

The postmortem electron microscopy analysis of the kidney tissue of 26 patients with COVID-19 from China revealed the presence of coronavirus-like particles in the renal tissue. Furthermore, the SARS-CoV-2 receptor ACE2 was upregulated in these patients. This study conducted by Su et al. confirms once more the virus tropism for kidney tissue [40].

Menter et al. identified in patients who died with COVID-19 the presence of capillaritis and microthrombi in the lungs, and showed diffuse vascular damage in other organs highly suggestive of vascular dysfunction [41].

Cutaneous biopsies from the skin lesions associated with SARS-CoV-2 were also performed. The optical microscopy findings of a biopsy from a chilblain-like lesion in a 23-year-old patient diagnosed with coronavirus disease revealed the presence of inflammatory infiltrate, consisting especially of lymphocytes, which were "tightly cuffing the vessels" [42]. Kanitakis et al. accomplished histological,

immunofluorescence, and immunohistochemical studies in seventeen cases of acral chilblain-like skin lesions in patients with suspected, but not confirmed, coronavirus disease, and endotheliitis was present in 65% of cases [43]. The association of COVID-19 with chilblain-like skin lesions is still conflicting. Initially, acral lesions were thought to be related to SARS-CoV-2 infection, but more recent case studies could not sustain an association between them [43, 44].

All data collected from the autopsies indicate that changes in the endothelium are not limited to the lungs and suggest that COVID-19 is a whole-body disease.

Numerous symptoms of SARS-CoV-2-positive patients could be assigned to multiorgan endotheliitis and subsequent endothelial dysfunction.

As mentioned above, tropism for the kidneys, lungs, and cardiovascular system of the novel coronavirus was demonstrated. This explains the respiratory and cardiocirculatory events associated with the disease. Several hypotheses were proposed in order to explain other organ specific symptoms. The early neurological manifestations (hyposmia, anosmia, dysgeusia, or hypogeusia) which have been frequently described in these patients together with life threatening events such as stroke and intracerebral or subarachnoid hemorrhage could represent a consequence of endotheliitis [45]. In a short communication, Benger et al. made a detailed analysis of 5 patients with COVID-19 and intracerebral hemorrhage. They suggest that endothelial damage and endotheliitis along with a prothrombotic state and proinflammatory cytokine production are responsible for intracerebral hemorrhage, which occurred in younger individuals. Hemorrhage affected the anterior cerebral circulation [46].

In addition to the detrimental effect on blood vessels, the heart also represents a target for SARS-CoV-2. The main cardiovascular manifestations of COVID-19 are cardiac arrhythmias, caused by the inflammation of the myocardium and metabolic dysregulation [47]. It has been suggested that both direct and indirect viral injury is responsible for COVID-19-associated myocarditis [48].

The emerging evidence recognizes the endothelium as a key factor in the pathophysiological chain in COVID-19 [49]. Therefore, arterial and venous thrombosis, pulmonary embolism [49], central nervous system acute hemorrhagic events, and multiorgan failure associated with SARS-CoV-2 infection [50] might be the aftermath of subsequent endotheliitis and endothelial dysfunction associated with a procoagulant state. Endothelial cell damage together with endotheliitis also explains the predisposition for severe manifestations of the disease in patients with preexisting endothelial dysfunction caused by chronic pathologies such as hypertension [47].

While the major role of endothelial cells in the pathophysiology of COVID-19 is a compelling subject for ongoing research projects, the hypothesis according to which the endothelium could represent a therapeutic target in critically ill patients is intensely analyzed [49].

4. COVID-19-Renin-Angiotensin System

The role of the renin-angiotensin-aldosterone system (RAAS) in COVID-19 infection has been taken into consideration from the beginning of the pandemic, since one of the first known facts was that ACE2 (angiotensin-converting enzyme 2) is the receptor that allows SARS-CoV-2 to enter human cells.

RAAS is a natural protective mechanism for maintaining circulatory volume. Renal hypoperfusion stimulates renin release from the juxtaglomerular apparatus. Renin cleaves angiotensinogen to angiotensinogen I, and ACE hydrolyzes Ang I to Ang II. Ang II binds to angiotensin II type 1 receptor (AT1R) and promotes aldosterone production, leading to sodium retention, water reabsorption, and vasoconstriction. On the other arm of the cascade, ACE2 is maintaining the equilibrium by converting Ang II to angiotensin 1-7. Angiotensin 1-7 binds to the Mas receptor and mediates anti-inflammatory, antioxidative, and vasodilatory effects. In the case of insufficient ACE2, Ang II binding AT1R prevails and exerts vasoconstrictive and proinflammatory effects [51].

Angiotensin-converting enzyme 2 (ACE2) is expressed in the human vascular endothelium, respiratory epithelium, and other types of cells, and represents a primary mechanism for the entry and infection of SARS-CoV-2 virus. In a physiological state, ACE2 through the activity of carboxypeptidase generates angiotensin fragments (Ang 1-9 and Ang 1-7) and plays an essential role in the renin-angiotensin system (RAS), which is an important regulator of cardiovascular homeostasis. SARS-CoV-2 through its surface glycoprotein interacts with ACE2 and invades the host cells.

For SARS-CoV-2 infection, in addition to ACE2, one or more proteases including transmembrane protease serine 2 (TMPRSS2), basigin (also known as CD147), and potentially cathepsin B or cathepsin L are required [52].

ACE2 is expressed as a transmembrane protein whose active site is exposed at the extracellular surface and resides in the lung alveolar epithelial cells, heart, kidneys, vessels, and gastrointestinal system [53]. ACE2 can be cleaved and circulates in small amounts in the blood stream, but its role is uncertain [54–57].

While ACE2 is clearly responsible for facilitating cell insertion, it may also be the cause of individual variation in disease severity. The polymorphism of ACE2 in the population could impact the affinity for the virus's spike protein and make the infection more likely or more severe [57]. Also, the ACE2 gene is X-linked, and this could explain the slight protective effect in the female sex observed in COVID-19. Besides these genetic variations, ACE2 gene expression is increased in diabetes, CVD, and hypertension [58]. Several researches indicate that RAAS-modulating drugs could also modulate ACE2 expression and activity in various ways. Animal model studies have shown that ACE inhibitors (ACEIs) and angiotensin II receptor blockers (ARBs) upregulate ACE2 cell expression, and ARBs and mineralocorticoid receptor antagonists (MRA) increase ACE2 activity, [59, 60]. However, simultaneously, ACEIs reduce Ang II synthesis, and consequently, in the absence of excess Ang II, AT1R is thought to interact with ACE2 [61]. This interaction could reduce the affinity of COVID S protein to ACE2 and then reduce COVID-19 viral entry [61].

SARS-CoV-2 spike protein binding to ACE2 in alveolar epithelial cells downregulates ACE2 expression. Without ACE2 to lead Ang II to angiotensin 1-7, Ang II binds to AT1R, leading to a hyperaldosteronism state, materialized as hypokalemia in severe cases of COVID-19 infection [62], vasoconstriction, fibrosis, and inflammatory cell proliferation [63]. Murine studies proved that loss of ACE2 expression enhances vascular permeability, increases lung edema and neutrophil accumulation, and hence worsening lung function [64].

One of the earliest researches of Chinese scientists empowers the theory that excessive Ang II leads to a bad outcome. Liu et al. observed in a small cohort of COVID-19 patients that the plasma concentrations of Ang II were significantly higher than in healthy individuals and also that Ang II levels in COVID-19 patients were correlated with viral load and lung injury [65].

Besides exacerbated inflammation and hypoxemia through vasoconstriction in small pulmonary vessels, Ang II induces plasminogen activator inhibitor-1 (PAI-1) expression in endothelial cells via the AT1 receptor. PAI-1 leads to unresolved fibrin deposits in the alveoli of patients with both SARS and COVID-19 infection [51]. Also, excessive Ang II can be metabolized to angiotensin IV [66], which enhances thrombosis development [67, 68]; since hypercoagulability has been noticed in many severe cases, it can be hypothesized that a reduction in ACE2 contributes to increasing thrombotic risk.

Since ACE2 has been recognized as the gate of SARS-CoV-2, worldwide medical boards raised the question if RAAS modulators—ACEIs and ARBs—increase the risk of developing severe forms of COVID-19 infection. The rationale behind this concern was based on some experimental animal models which have shown increasing numbers of ACE2 after intravenous infusion of ACEIs and ARBs [59].

In order to establish whether RAAS modulators are harmful or not, scientists firstly compared the outcomes of COVID-19 patients with arterial hypertension and different treatments. Shyh et al. found that those on ARBs are significantly less likely to develop COVID-19, while ACEIs did not show a similar effect, considering that they do not directly affect ACE2 activity [69]. On the other hand, patients taking calcium channel-blockers (CCBs) had a significantly increased risk of manifesting symptoms of COVID-19.

Several other retrospective multicenter studies [63, 70] looked for an association between in-hospital use of ACEIs/ARBs and all-cause mortality of COVID-19 among patients with hypertension. Their results show that COVID-19 hypertensive patients treated with ACEIs/ARBs had a better outcome than COVID-19 patients without ACEIs/ARBs or treated with a different class of other antihypertensive agents. On a molecular basis, they identified that patients on ACEIs/ARBs had lower levels of IL-6, decreased cytokine production, and decreased viral load during hospitalization, and peripheral T cells were significantly higher than in the non-ACEI/ARB group [70].

Researchers' restless work not only offered substantial information about the role of ACE2 in COVID-19 infection, but also brought up several potential therapeutic approaches: spike protein-based vaccine, inhibition of transmembrane protease serine 2 (TMPRSS2-human proteinase which facilitates viral spike protein binding to ACE2) activity, blocking ACE2 receptor, and delivering an excessive soluble form of ACE2 [71]. It was postulated that delivering excessive soluble ACE2 would capture most of the viral load, restricting their fixation on cell membrane ACE2, and therefore limit the infection and also keep the balance of the 2 RAAS arms, preventing severe inflammatory tissue lesions [72, 73]. Most of these theories are based on animal model or in vitro studies and, needless to say, require extensive research and trials before becoming available therapies.

5. Cytokine Storm Associated with SARS-CoV-2 Infection

About 5% of the patients infected with SARS-CoV-2 develop critical disease forms manifesting by respiratory failure, shock, or multiple organ failure [74]. The presence of these disease forms does not seem to be correlated with viral load. Although these patients have a high viral load, the same load is found in patients having mild forms of the disease and even in asymptomatic persons [75]. Thus, the hypothesis was advanced that abnormal immune response, manifesting as a "cytokine storm," is the main determining factor of disease severity [76].

Cytokine storm associated with COVID-19 is similar to other clinical entities, such as cytokine release syndrome observed following CAR-T cell therapy [77], primary or secondary hemophagocytic lymphohistiocytosis (HLH), sepsis caused by Herpesviridae and other pathogens [78], and macrophage activation syndrome that occurs in various autoimmune diseases [79].

This progressive systemic inflammation leads to the loss of vascular tone clinically manifesting by a decrease in blood pressure, vasodilatory shock, and progressive organ failure. In the context of cytokine storms associated with highly pathogenic viruses such as SARS-CoV-2, SARS-CoV, and MERS-CoV, the greatest impact is on the lungs, where acute respiratory distress syndrome (ARDS) occurs which is the main cause of death. The effects are not limited to the lungs; cardiac, renal, and central nervous system damage is also involved [80].

After receptor binding and complex internalization, the viral RNA is released into the cell cytosol, replicated, and finally removed by exocytosis.

Intracellular viral RNA is identified by the recognition mechanisms of the innate immune response through specific receptors: PRRs (pattern recognition receptors), TLRs (toll-like receptors), and NLRs (NOD-like receptors). The recognition of viral RNA by these receptors determines the activation of intracellular signaling pathways, such as NF-κB and IRF 3/7. NF-κB stimulates the transcription of proinflammatory cytokines such as TNF-alpha, IL-6, and IL-1 and activates the immune response mediated by T helper 1 and 17 lymphocytes. IRF 3/7 stimulates the production of type 1 IFN, which induces activation of the JAK1/TYK2-STAT1/2 pathway, the effect being the transcription

of interferon-stimulated genes (ISG), with a role in the secretion of cytokines and the activation of other immune system components to stop viral replication [81, 82].

Previous studies have shown that in some cases, coronaviruses can delay type I IFN response through various mechanisms, the result being a more severe form of the disease caused by ineffective viral replication control and paradoxical hyperinflammation caused by type I IFN. In the case of SARS-CoV-2, an altered response of type I IFN seems to occur. A study showed that serum IFN activity was significantly lower in patients with severe or critical forms of the disease compared to those with mild-moderate forms. Moreover, serum ISG and type I IFN values in patients who subsequently developed ARDS with the need for invasive ventilation indicated that a mitigated type I IFN response precedes clinical deterioration [83].

This abnormal response of interferon leads to a massive inflow of neutrophils and monocytes, which are a major source of proinflammatory cytokines, apoptosis of T lymphocytes, and epithelial and endothelial cells [81].

Lymphopenia occurs in about 80% of the patients infected with SARS-CoV-2 and is more marked in the severe forms of the disease. There are many causal hypotheses explaining this process. Firstly, the virus can directly infect T lymphocytes but cannot replicate inside these, thus leading to cell death through apoptosis, necrosis, or pyroptosis. Secondly, the first wave of cytokines released, described above, includes anti-inflammatory cytokines such as TNF-alpha and IL-10, which cause apoptosis, exhaustion, and inhibition of TL proliferation. Not the least, lymphopenia could be the result of redistribution in the lungs and lymphoid organs [81, 84].

In the most severe disease cases, a sudden and rapid clinical deterioration occurs, which is associated with increased levels of acute phase reactants, coagulopathy, and cell lysis, and high proinflammatory cytokine levels, suggesting a second wave of cytokines, responsible for the so-called cytokine storm [81].

The triggering factor of the cytokine storm seems to be immunodeficiency caused by the decrease in the number and the dysfunction of T lymphocytes. Although other innate immunity hyperactivation mechanisms are supposed to be responsible, the cytokine storm is much more likely to occur as a result of a delayed response of innate immunity, followed by persistent hypercytokinemia and an abnormal response of the acquired immune system through T lymphocytes. The result is the failure to eliminate apoptotic cells or macrophages migrated to the site of inflammation and continuous antigenic stimulation by failure of viral clearance. These cells will continue to secrete proinflammatory cytokines, of which the most important are IL-18 and IFN-γ, which restimulate macrophage activation. Thus, a vicious circle is created which culminates in cytokine secretion, hemophagocytosis, coagulopathy, and ARDS [82, 85].

5.1. Cytokines and the Correlation with the Severity of the Disease. The first evidence of this correlation comes from the study conducted by Huang et al. in a sample of 41 patients who had the plasma levels of several cytokines and chemokines measured. The authors observed that the initial plasma levels of IL-1B, IL-1RA, IL-7, IL-8, IL-9, IL-10, FGF, GCSF, GMCSF, IFN-γ, IP-10, MCP1, MIP1A, MIP1B, PDGF, TNF-α, and VEGF were higher in all COVID-19 patients compared to healthy persons, the plasma concentrations of IL-5, IL-12p70, IL-15, eotaxin, and RANTES were similar in patients infected with SARS-CoV-2 and healthy persons, and the levels of IL-2, IL-7, IL-10, GCSF, IP-10, MCP1, MIP1A, and TNF-α were significantly higher in patients with severe forms of the disease requiring intensive therapy compared to those with mild or moderate forms [86]. Since then, many studies have been conducted in the attempt to elucidate the pathogenic mechanisms of the exacerbated immune response associated with SARS-CoV-2 infection and in the attempt to identify laboratory markers that correlate with the severity and prognosis of the disease in order to achieve a stratification of patients for adequate management based on early therapeutic intervention.

A recently published meta-analysis of 50 studies showed statistically significantly higher values of IL-2, IL-2R, IL-4, IL-6, IL-8, IL-10, TNF-α, and INF-γ in patients with severe forms of the disease compared to the others. In contrast, there were no significant differences between IL-17 and IL-1β values. As it can be seen, in some cases, there is an excessive production of proinflammatory as well as anti-inflammatory cytokines (IL-2R, IL-10), which highlights the dual pathogenic mechanism responsible for the occurrence of the cytokine storm [87]. Another meta-analysis and extensive systematic analysis shows that in patients with severe forms of the disease, lymphocytopenia (decreased CD3, CD4, and CD8 T lymphocytes), leukocytosis, high values of ESR, procalcitonin, LDH, and ALT occur more frequently. The levels of inflammatory cytokines, especially IL-6, 8, 10, and 2R and TNF-alpha, were significantly increased [88].

Regarding the profile of leukocytes, both meta-analyses evidenced a significant decrease in CD4 and CD8 T lymphocytes in the group of patients with severe disease forms [87, 88].

The most studied interleukin is perhaps IL-6, given that tocilizumab, a monoclonal antibody directed against the IL-6 receptor, can be used as therapy for COVID-19 patients who present signs of hyperinflammation. Mojtabavi et al. show in their analysis of 11 studies that IL-6 values are significantly higher in patients with severe forms of COVID-19 compared to those with mild or moderate forms [89]. Furthermore, Laguna-Goya et al. elaborated a model for predicting the risk of mortality in hospitalized COVID-19 patients based on IL-6 values. This includes 5 parameters: $FiO_2/SatO_2$ ratio, neutrophil/lymphocyte ratio, IL-6 value, LDH value, and age. This model might help to stratify patients into more uniform groups from a clinical and biological point of view before their inclusion in randomized clinical trials evaluating the efficacy of tocilizumab or other drugs. Until completion of clinical trials, this model could be used to select patients that would benefit the most from immunomodulatory therapy [90].

The prognostic value of IL-6 was also demonstrated in another study, where it was incorporated along with

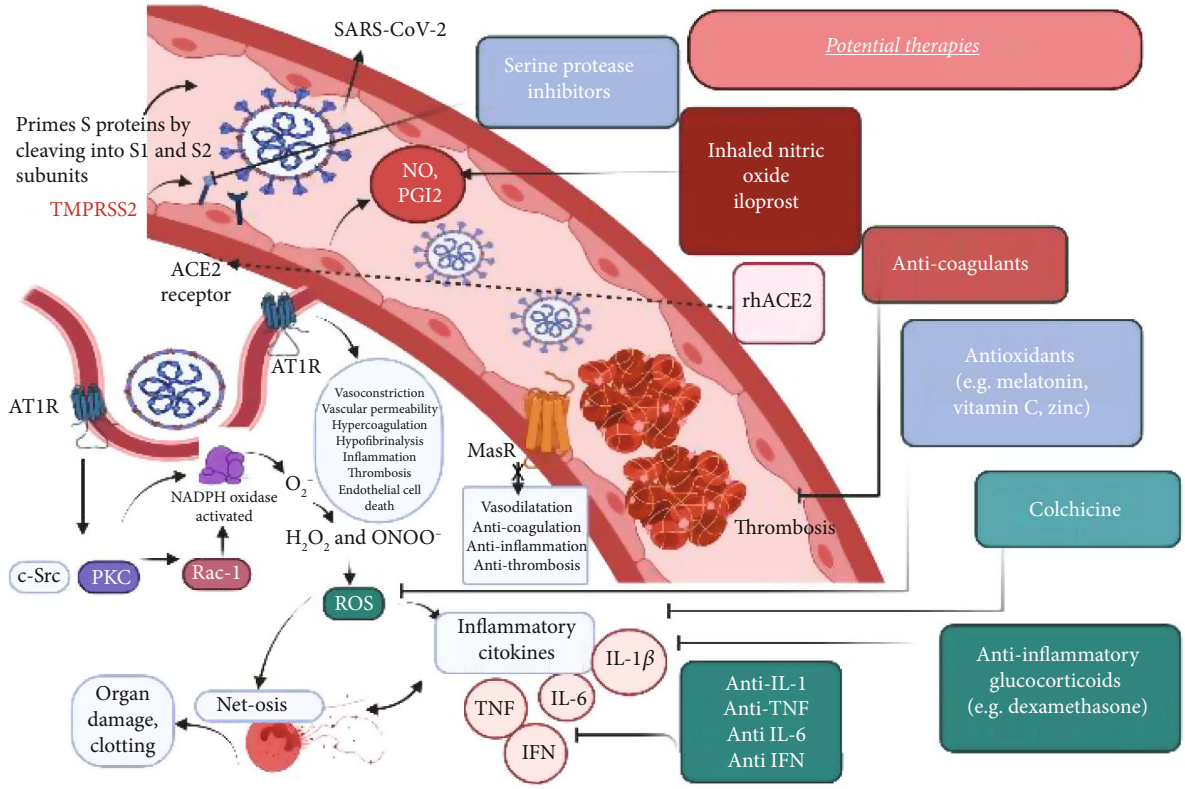

Figure 3: Mechanisms of endothelial dysfunction, inflammation, oxidative stress, and therapeutic targets in SARS-CoV-2 infection. SARS-CoV-2 infection begins when its peak proteins are proteolytically prepared by TMPRSS2, allowing them to bind to ACE2 and initiate viral endocytosis in the EC. This increases the amount of binding of Ang II to AT1R, which in turn activates NADPH-oxidase and subsequently induces an increased production of ROS. These excess ROS mediate signaling pathways that increase the production of inflammatory cytokines (such as IL-1β, IL-6, and TNF), decrease the bioavailability of NO and PGI2, and induce endothelial cell apoptosis, leading to endothelial damage and dysfunction. Furthermore, the release of proinflammatory and prothrombotic factors can lead to vascular inflammation, platelet aggregation, and thrombosis. These interactions increase the risk of thrombosis and lung damage in people infected with SARS-CoV-2. ROS also induce an overflow of NETs. There may be several positive feedback loops between cytokines (TNF-α, IL-1β) and ROS production as well as between cytokines (TNF-α, IL-1β) and NET formation. ROS, NETs, and proteolytic enzymes released by activated neutrophils also contribute to organ damage and clotting in vessels. Therapeutic targets address SARS-CoV-2-induced feedback loops in EC. Although there have been many therapies proposed to stop the spread of the coronavirus pandemic, those described here address feedback loops involving endothelial dysfunction, oxidative stress, and inflammation. TMPRSS2: transmembrane protease, serine 2; ACE2: angiotensin-converting enzyme 2; AT1R: angiotensin type 1 receptor; ROS: reactive oxygen species; c-Src: protooncogene tyrosine-protein kinase Src; PKC: protein kinase C; IL: interleukin; TNF: tissue necrosis factor; NO: nitric oxide; PGI2: prostaglandin I2 (also known as prostacyclin).

CD8+ TL into a prognostic model. The authors of the study showed that IL − 6 values > 20 pg/mL and CD8 + TL values < 165 cells/μL are correlated with mortality, being a better indicator of in-hospital mortality than the CURB-65 score [91].

Other cytokines were studied in the attempt to identify the prognostic factors of disease severity and prove their usefulness. An example is represented by IL-2R, included in several prognostic models such as the IL-2R/lymphocyte ratio, as demonstrated in the study conducted by Hou et al. [92], or the model developed by another group which incorporates IL-2R, the values of neutrophils, lymphocytes, and thrombocytes [93]. Another study proposes to monitor IP-10 and MCP-3 values early during the course of the disease in order to identify patients at risk for hyperinflammation and implicitly for more severe forms of the disease [94].

6. Therapeutic Targets for the Treatment of COVID-19

Numerous therapeutic targets (Figure 3) have been proposed taking into consideration the various mechanisms of action of SARS-CoV-2 on the endothelium. Regarding the key role of oxidative stress, endotheliopathy, and inflammatory mediators in the COVID-19 pathogenesis [8], we will further present the therapies that counteract the SARS-CoV-2-induced disturbances.

6.1. Interleukin-6 Inhibitors. As shown above, IL-6 plays an extremely important role in the occurrence and maintenance of the cytokine storm associated with COVID-19 and is correlated with disease severity, and thus it is an important therapeutic target. In addition, the inhibitors of IL-6 or its receptor proved to be effective in the treatment of other

similar syndromes such as HLH associated with Still's disease [95] or in the cytokine storm secondary to CAR-T cell therapy [96]. Regarding their use in COVID-19 patients, only data from case-control studies or case reports are currently available. It should be taken into consideration that these studies were extremely heterogeneous, performed on small samples, with divergent results concerning the monitored indicators (e.g., the need for invasive ventilation and the length of hospital stay). With respect to mortality, the majority showed an increase in survival or at least a favorable trend. Currently, many clinical trials are in progress to evaluate the efficacy and safety of using IL-6 inhibitors in this context. Experimental studies have shown that IL-6 can have a dual effect, both facilitating and suppressing viral replication [23], so that the optimal time of administration is another question that these clinical trials should answer [82, 97–100].

Tocilizumab, sarilumab, and siltuximab are Food and Drug Administration- (FDA-) approved IL-6 inhibitors evaluated for the management of patients with COVID-19 who have systemic inflammation. Tocilizumab is a recombinant humanized anti-IL-6 receptor monoclonal antibody that is approved by the FDA for use in patients with rheumatologic disorders and cytokine release syndrome (CRS) induced by chimeric antigen receptor T cell (CAR-T cell) therapy. Tocilizumab in combination with dexamethasone are indicated in certain hospitalized patients who are exhibiting rapid respiratory decompensation due to COVID-19 [101]. Further findings from REMAP-CAP and the RECOVERY study justify the use of tocilizumab in certain hospitalized patients with rapid respiratory decompensation due to COVID-19 [102].

Sarilumab is a recombinant humanized anti-IL-6 receptor monoclonal antibody that is approved by the FDA for use in patients with rheumatoid arthritis. It is available as an SQ formulation and is not approved for the treatment of CRS [101]. Preliminary efficacy results from REMAP-CAP for sarilumab were similar to those for tocilizumab. Compared to placebo, sarilumab reduced both mortality and time to ICU discharge, and increased the number of organ support-free days; however, the number of participants who received sarilumab in this trial was relatively small, limiting the conclusions and implications of these findings [102].

Siltuximab is a recombinant human-mouse chimeric monoclonal antibody that binds IL-6 and is approved by the FDA for use in patients with multicentric Castleman's disease. Siltuximab prevents the binding of IL-6 to both soluble and membrane-bound IL-6 receptors, inhibiting IL-6 signaling. Siltuximab is dosed as an IV infusion [103]. There are limited data describing the efficacy of siltuximab in patients with COVID-19 [104].

6.2. Interleukin-1 Inhibitors. Anakinra is a recombinant IL-1 receptor antagonist, currently approved in the treatment of a number of autoimmune diseases induced by excessive IL-1 secretion, with the aim of reducing inflammation and complications such as ARDS [105].

Starting from the data obtained from the use of anakinra in other similar syndromes such as secondary HLH or macrophage activation syndrome [105] and taking into consideration the high values of this interleukin reported in persons infected with SARS-CoV-2, it was supposed that IL-1 could be an important target in the management of the cytokine storm associated with SARS-CoV-2 as well. A retrospective study showed a clinical improvement in 72% of COVID-19 and ARDS patients treated with this drug [106]. Several randomized clinical trials that test anakinra in COVID-19 patients are underway.

Aside from anakinra, canakinumab, a high-affinity human monoclonal antibody [101], and rilonacept, a soluble IL-1 trap, represent therapeutic options for IL-1 inhibition [107].

Canakinumab counteracts the activity of IL-1 by blocking the interaction between IL-1β and its receptor [108]. The beneficial effect of canakinumab for COVID-19 patients results from the improvement of clinical status and reduction of invasive mechanical ventilation needed in these patients together with a prompt amelioration and maintenance in oxygenation levels [109, 110]. Furthermore, canakinumab ameliorates the prognosis of COVID-19 patients and prevents the clinical degradation by blocking the cytokine storm [110].

6.3. Anti-TNF-α. TNF-α is another cytokine with important inflammatory effects, whose increased serum values were also demonstrated in COVID-19 patients. Opinions diverge on the usefulness of anti-TNF-α monoclonal antibodies in this context. Infliximab, adalimumab, etanercept, certolizumab, and golimumab are the 5 most commonly prescribed TNFs inhibitors. On the one hand, TNF-α inhibition decreases IL-6 and IL-1 concentrations and reduces capillary permeability [111], and studies on animals have shown that the inhibition of this cytokine confers protection against SARS-CoV-2 infection. On the other hand, studies in which TNF-α inhibitors were used in syndromes similar to the cytokine storm have reported divergent results, some of them even demonstrating an aggravation of the disease [112].

6.4. Type I IFN. Considering the key role of IFN in antiviral response and its immunomodulatory effect, type I IFN seems to be an important potential therapeutic target. Type I IFN was studied both in vivo and in vitro, as monotherapy or in combination with antiviral drugs, in the treatment of SARS-CoV and MERS-CoV infection. Although interferon treatment was demonstrated to be efficient in vitro and in some studies on animals, in human studies the results were divergent. These results can be explained by the limited number of patients included and the heterogeneity of the studies, by the different inhibition mechanisms of the IFN signaling pathway used by the two viruses, as well as by the difficulty in assessing whether the clinical benefit observed was due to IFN or to the drugs with which it was used as part of combined therapy [113].

Another explanation for these results could be the subtype of IFN used as a therapeutic target. Compared to IFN-α, IFN-β seems to be a much more potent inhibitor of coronaviruses [114]. The time of administration seems to be an important element. Early administration was

associated with favorable results, while late administration was associated with significant adverse reactions without an effect on viral replication [115]. In addition, in vitro studies report viral replication inhibition by administration of prophylactic IFN in the case of SARS-CoV-2, while the same strategy is ineffective in the case of SARS-CoV and MERS-CoV [116–118]. A prospective study conducted in China on a sample of 2944 persons working in the health care system showed that interferon administered as a nasal spray is effective in the prophylaxis of SARS-CoV-2 infection [119].

Starting from the information obtained from previous studies on SARS-CoV and MERS-CoV and from the data regarding the pathology of SARS-CoV-2 infection, a number of clinical trials are in progress to test the efficacy of type I IFN in patients infected with SARS-CoV-2.

6.5. Inhibitor of Synthetic Serine Protease. Transmembrane protease serine 2 (TMPRSS2) represents the cornerstone in the SARS-CoV-2 S protein interaction with the endothelial cell [120]. TMPRSS2 is a protease that proved its capacity of preventing the cell invasion by SARS-CoV-2 in vitro [52].

Camostat mesylate, an inhibitor of synthetic serine protease infection, could block SARS-CoV-2 spreading in human tissue [120]. Taking into consideration the desirable effects in COVID-19 patients, TMPRSS2 has been approved for clinical use [52].

6.6. Recombinant Human ACE2 Protein (rhACE2). Taking into consideration that SARS-CoV-2 infection induces the depletion of ACE2 receptors, which contributes to systemic and especially pulmonary inflammation, the hypothesis was advanced that administration of recombinant human ACE2 protein can represent a therapeutic target. The causal mechanisms of immune dysfunction and hyperinflammation are multiple, so that the use of rhACE2 as monotherapy is probably insufficient, as demonstrated in patients infected with SARS-CoV in 2017 [76]. There is currently a clinical trial that studies the therapeutic efficacy of this molecule in COVID-19 patients.

6.7. JAK Inhibitors. The activated type I IFN JAK1/TYK2-STAT1/2 intracellular signaling pathway plays an important role in cytokine production, so that its inhibition might have a therapeutic effect in the cytokine storm associated with SARS-CoV-2.

Baricitinib is an inhibitor of JAK kinase currently used in the treatment of rheumatoid arthritis, which by selective and reversible binding to JAK receptors disrupts the transduction of the intracellular signal mediated by cytokines and thus attenuates the inflammatory response [121]. In addition, this compound is supposed to inhibit AAK1 receptor, required for viral endocytosis, also inhibiting in this way the entrance of the virus into the host cell [122].

At present, there are several ongoing clinical trials that investigate the efficacy of different JAK inhibitors in COVID-19 patients. An important aspect should be taken into account: the fact that SARS-CoV-2 infection predisposes to coagulopathy and formation of thrombi, and treatment with JAK inhibitors has been associated with an increase in thromboembolic risk [123].

6.8. Nitric Oxide. Inhaled nitric oxide (NO) proved its antiviral effects against various coronavirus strains together with the pulmonary vasodilation activity. Of great interest is the ability of NO in the prevention of the development of severe forms of the disease, if administrated at the proper time, at the early stage of COVID-19 [101].

6.9. Iloprost. The prostacyclin (PGI2) analogue, iloprost, showed beneficial effects in COVID-19 patients. Iloprost might represent a valuable therapeutic option for respiratory performance improvement [124]. Synthesized in the vascular endothelium, PGI2 plays a role not only in the endothelial barrier homeostasis and platelet aggregation, but it also has anti-inflammatory and vasodilatory effects. [125, 126].

In COVID-19 patients, iloprost could prevent the associated thrombotic events through its protective effects on the endothelium and the antithrombotic activity [124].

6.10. The Glycosaminoglycans. Another valuable therapeutic approach is represented by the glycosaminoglycans (GAGs), taking into consideration the double role they play in COVID-19 pathogenesis, their interaction with the chemokines, and the SARS-CoV-2 coreceptor function. Thus, the chemokine interaction with GAGs together with SARS-CoV-2 GAG-mediated cell entry might represent important targets in COVID-19 therapy [127].

6.11. Chemokine Receptor 5 Antagonism. The chemokine receptor 5 (CCR5) is a transmembrane structure expressed by several cells, including the endothelial cells [128], and it might be implicated in the SARS-CoV-2 invasion of the endothelial cells. By preventing the SARS-CoV-2 from entering the cell, the CCR5 antagonism could represent a valuable tool in preventing the severe inflammatory response characteristic for COVID-19-associated acute respiratory distress syndrome (ARDS) [127]. CCR5 antagonists proved their efficiency for preventing HIV-1 entry into the cells [129]. Maraviroc, a CCR5 antagonist, blocks the SARS-CoV-2 fusion with other cells (via S protein) and prevents its multiplication [130]. Leronlimab is a monoclonal IgG4 antibody which also has CCR5 as a therapeutic target. Leronlimab successfully reduced the IL-6 levels in patients with severe COVID 19 manifestations [131]. Taking into consideration the role of CCR5 in the COVID-19 pathogenesis and their expression by the endothelial cells, the CCR5 antagonism might represent a therapeutic option in the treatment of SARS-CoV-2-induced endotheliopathy.

6.12. The CXCL-8 Pathway. CXCL-8/IL-8 is an inflammatory chemokine that promotes the angiogenesis on endothelial cells via VEGF [132, 133]. The implication of the CXCL-8 pathway in SARS-CoV-2 infection pathogenesis results from its increased circulating levels identified in COVID-19 patients [134]. CXCL-8 is a powerful neutrophil chemotactic factor [135] and its high serum levels in COVID-19 patients might explain the associated neutrophilia. The neutralizing IL-8 antibody therapy and CXCL-8 receptor

(CXCR-2) antagonists might represent a therapeutic option for hospitalized COVID-19 patients [127].

7. Conclusions

This review summarized the relationship between COVID-19, endothelial dysfunction, inflammation, and oxidative stress. The implication of endothelium in SARS-CoV-2 pathogenesis remains a subject of interest which is intensely researched in current studies. Even though several studies place the endothelial dysfunction and oxidative stress as the main factors responsible for microvascular COVID-19-associated complications, the direct invasion of endothelial cells by SARS-CoV-2 remains disputable. An explanation for the severe COVID-19 manifestations in patients suffering from cardiovascular and metabolic comorbidities might be the endothelial dysfunction associated with the aforementioned conditions; thus, those patients are at high risk for developing pulmonary and extrapulmonary complications. The central role of endothelium in the COVID-19 pathogenesis remains of great interest particularly for its role as a valuable therapeutic target for the prevention and/or treatment of vascular complications in SARS-CoV-2 patients. With a plethora of physiopathological mechanisms, the SARS-CoV-2-induced endotheliopathy appears to play a central role in COVID-19 pathogenesis.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

The authors Adriana Fodor, Brandusa Tiperciuc, and Cezar Login have equal contribution.

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