SEPSIS AND COVID-19: CROSS-TALK IN SIGNALLING PATHWAYS AND IN THERAPEUTIC PERSPECTIVES

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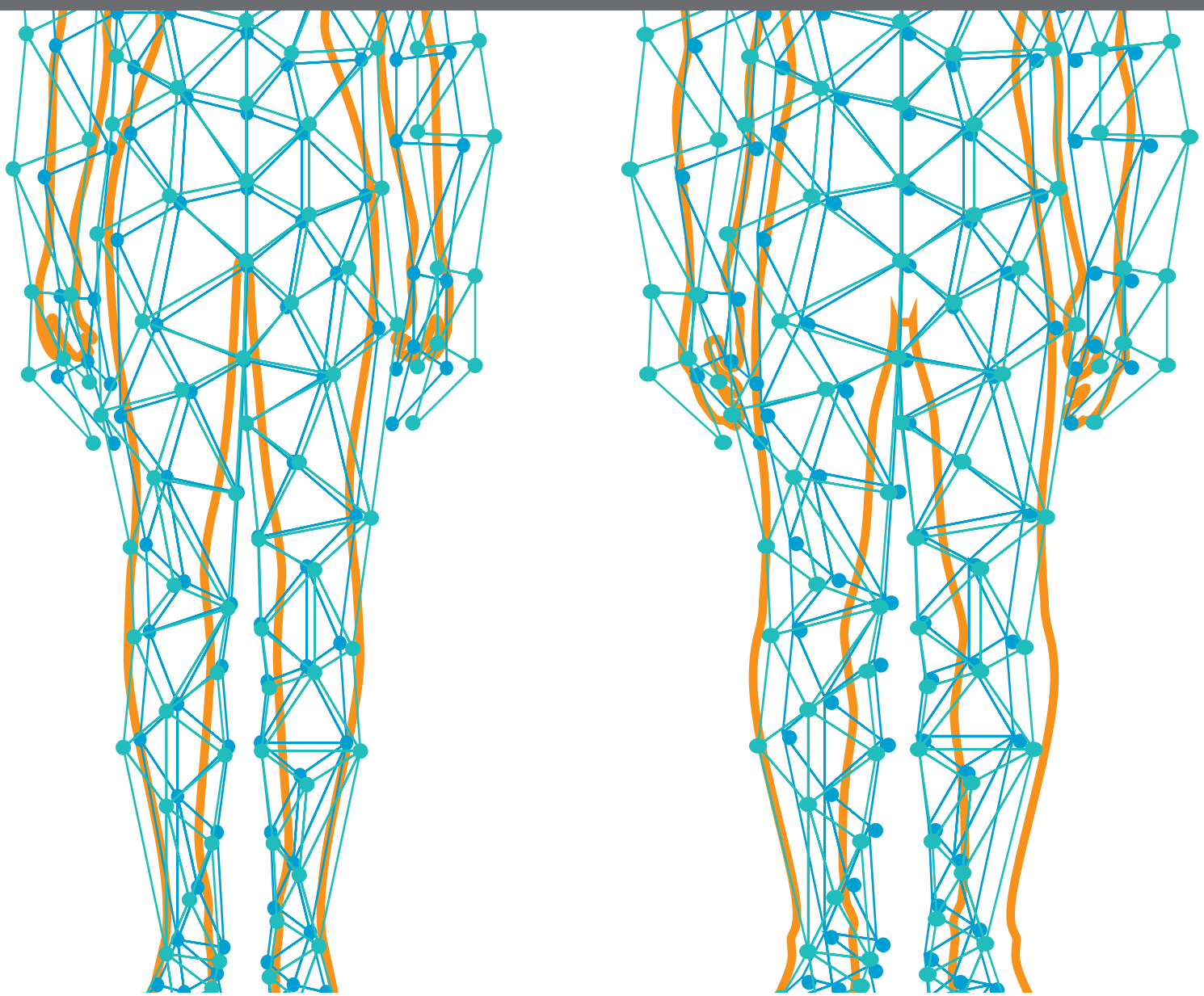

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SEPSIS AND COVID-19: CROSS-TALK IN SIGNALLING PATHWAYS AND IN THERAPEUTIC PERSPECTIVES

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EDITORIAL
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Editorial: Sepsis and COVID-19: Cross-Talk in Signaling Pathways and in Therapeutic Perspectives

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Editorial on the Research Topic

Sepsis and COVID-19: Cross-Talk in Signaling Pathways and in Therapeutic Perspectives

Sepsis, as a manifestation of several endemic and epidemic diseases, has had a profound impact on humankind's history. In the last decades, sepsis remained a major cause of morbidity and mortality worldwide. In December 2019, the city of Wuhan, in China, became the center of an outbreak of pneumonia of unknown cause, rapidly identified as triggered by a new coronavirus, the SARS-CoV-2 (from "severe acute respiratory syndrome coronavirus 2"). The disease was characterized as COVID-19 (an acronym for "coronavirus disease") by the World Health Organization (WHO February 11, 2020, and in just a month, on March 11, it was declared a global pandemic (WHO Director-General's opening remarks at the media briefing on COVID19 -March 2020). As of 8 April 2022, there have been circa 500 million confirmed cases of COVID-19, including over 6 million deaths reported to (1).

Patients infected with SARS-CoV-2 and progressing to critical COVID-19 illness are unequivocally presenting sepsis. However, there are important differences in the clinical trajectories and underlying mechanisms driving to critical disease between a COVID-19 patient and a regular septic patient. COVID-19 is a disease and sepsis is a syndrome. COVID-19 patients deteriorating to sepsis and septic shock show a typical clinical course, the medium time from symptoms' onset to dyspnea ranging from 5 to 8 days, the median time to acute respiratory distress syndrome (ARDS) from 8 to 12 days, and the median time to ICU admission ranging from 10 to 12 days. This is in frank contrast with the unpredictable or heterogeneous timeframe of events in a regular septic patient and has been a clue for a better understanding of the pathophysiological events as well as to achieve more success in adjunctive therapy strategies.

Although present important differences, the pathogenesis of sepsis and COVID-19 converges to a pivotal role in host systemic inflammatory response. Cytokines storm, procoagulant state, Toll-Like Receptor (TLR) signaling, Pathogen-associated Molecular Patterns (PAMPs) and Damage-Associated Molecular Patterns (DAMPs), neutrophil extracellular traps (NETs), inflammasome, changes in lipids profile are involved in both diseases. Thus, the sepsis literature quickly became COVID literature as well, and this overlap highlights our need to better phenotyping regular sepsis.

This Research Topic covered a wide scope of sepsis and COVID-19 interfaces, including clinical profiles and sequelae, translational research evaluating biomarkers and predictive outcomes, and mechanisms underlying the dysregulated inflammatory response.

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Abumayyaleh et al. developed a score aiming to help clinicians in identifying high-risk COVID-19 patients progressing to sepsis while Alencar et al. tested the performance of NEWS, qSOFA, and SIRS scores for assessing mortality, early bacterial infection, and admission to ICU in covid-19 patients admitted in the emergency department. Zhang et al. reported on clinical similarities and differences between acute lung injury (ALI) in COVID-19 and non-COVID-19 patients in the intensive care unit (ICU), and Prestes et al. showed that long term lung dysfunction is common in patients with severe COVID-19 and impacts negatively on activities of daily living

Pathogenetic changes are related to clinical outcomes or envisaged as oriented target therapy. Microangiopathy and thrombosis coupled with dysfunctional local inflammatory response are the basis for organ dysfunction in COVID-19. Here, Maldonado et al. observed increased concentrations of thrombomodulin, angiopoietin-2, human vascular endothelial growth factor, and human hepatocyte growth factor and a decrease in human tissue inhibitor of metalloproteinases-2 in COVID-19 patients, and demonstrated that early endothelial and angiogenic biomarkers could predict mortality in patients with COVID-19.

A dysregulated immune response with concomitant pro-inflammatory and immune suppressive responses is one of the fundamental changes observed in sepsis. Alon et al. bring evidence that CD45/TCR intracellular signaling is downregulated in peripheral blood mononuclear cells from COVID-19 patients and demonstrate that C24D, an immunomodulatory peptide, rescued CD45 signaling. On the other hand, the robust data on dysfunctional inflammatory response and the pivotal role of "NETosis", the process of neutrophil release of their extracellular traps, is discussed in a comprehensive review by Keane et al.

In an interesting approach, Bouwman et al. addressed signal transduction pathway activities in whole blood samples from patients with sepsis and observed increased activity in androgen (AR) receptor and TGFβ pathways. AR showed a good performance for diagnosing and predicting sepsis' outcomes.

There is increasing evidence that immune and metabolic response during an infectious process is under epigenetic control. Epigenetic changes play an important role in regulating DNA processes, such as transcription, repair, and replication. Falcão-Holanda et al. review the epigenetic changes reported in experimental and clinical studies and discuss their role in the pathogenesis of sepsis and as a potential target for adjunctive therapy.

The rapid development of vaccines against COVID-19, based on different platforms, from the traditional inactivated viruses to the new mRNA technology, was an unprecedented scientific achievement and a central approach to pandemic control. As of 5 April 2022, over 11 billion vaccine doses have been administered in the world (WHO, 2022). One concern with this massive immunization is the possible side effects, among others, related to thromboembolism, myocarditis/pericarditis, and neuropathies. Here, Di Mauro et al. focused on acute vertigo syndrome, which, as they pointed out, could represent an overlap between ear/labyrinth and nervous system disorders following COVID-19 immunization.

The pathogenesis-oriented target therapy also converges sepsis and COVID-19. In COVID-19, as was formerly the case with sepsis, a great enthusiasm was observed with multiple inflammation-based targets for intervention, the IL-6 inhibitors as an illustrating example. On the other hand, targeting the immunosuppressive state with IL-7 has undergone clinical trials as well. Again, deciphering the host-protective defense from the harmful response and identifying patients which would benefit from one or other approach are a pivotal research challenge.

We are in debt to the authors of the Research Topic for the excellence of their contributions and hope that the topic will contribute with our increasing knowledge about sepsis and COVID-19.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Epigenetic Regulation in Sepsis, Role in Pathophysiology and Therapeutic Perspective

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Sepsis is characterized by an initial hyperinflammatory response, with intense cell activation and cytokine storm. In parallel, a prolonged compensatory anti-inflammatory response, known as immunological tolerance, can lead to immunosuppression. Clinically, this condition is associated with multiple organ failure, resulting in the patient's death. The mechanisms underlying the pathophysiology of sepsis are not yet fully understood, but evidence is strong showing that epigenetic changes, including DNA methylation and post-translational modifications of histones, modulate the inflammatory response of sepsis. During the onset of infection, host cells undergo epigenetic changes that favor pathogen survival. Besides, epigenetic changes in essential genes also orchestrate the patient's inflammatory response. In this review, we gathered studies on sepsis and epigenetics to show the central role of epigenetic mechanisms in various aspects of the pathogenesis of sepsis and the potential of epigenetic interventions for its treatment.

Keywords: sepsis, epigenetics, chromatin remodeling, DNA methylation, histone modification

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INTRODUCTION

Sepsis is a syndrome that includes different abnormalities, described in 1992 as systemic inflammatory response syndrome. It was believed that its pathogenesis was mainly due to an unbalanced inflammatory response of the organism triggered by the presence of an infectious agent. This response is much more complex is characterized by the simultaneous exacerbation of inflammatory, metabolic, catabolic, and immunosuppressive pathways, with lingering effects and difficulty in restoring basal homeostasis (1, 2). The concept of sepsis and the understanding of its pathogenesis are continually evolving. Many of the changes considered a dysregulated host response to infection may be, at least in part, an effort to adapt to a hostile environment (3).

Despite all efforts to unravel the mechanisms that orchestrate sepsis, questions remain about its pathophysiology. Epigenetic mechanisms play a prominent role in regulating gene transcription, and gene transcription undergoes significant changes during sepsis. Therefore, epigenetic mechanisms are involved in the acute events of sepsis and in the long-standing post-septic effects on the host response.

DEFINITION OF EPIGENETIC MECHANISMS

Epigenetic changes are described in literature as chemical changes in chromatin, inherited during cell division, with a role in regulating gene expression and genome stability, without involving changes in the DNA sequence (4). The most studied epigenetic mechanisms are DNA methylation and post-translational modifications (PTMs) of histones but also include changes in chromatin remodeling and regulation by non-coding RNAs (ncRNA) (5). Information on the epigenetic changes plays an important role in regulating DNA processes, such as transcription, repair, and replication. As a result, abnormal expression patterns of gene changes in chromatin regulators may have discrepant results (6).

Gene activation or silencing is controlled by enzymes that add or remove chemical groups (acetyl, methyl, among others) in chromatin (**Figure 1**). These modifications interact with "reader" proteins that have unique structurally conserved domains present in various chromatin regulators and transcription factors, recruiting components of transcriptional machinery and chromatin remodeling complexes (6). These complexes can be subdivided into two main regions: heterochromatin, composed mainly of inactive genes, with late and highly condensed replication; and euchromatin, which contains most of the active genes and has the loosest chromatin (7).

DNA methylation and histone modifications are complementary dynamic processes that together determine the pattern of gene expression, essential in the development, differentiation, and cellular function (8); from the beginning of development and throughout an individual's life, they act regularly and physiologically. Epigenetic marks have plasticity in response to the cellular state and the environment. Epigenetic patterns are influenced by environmental factors during pregnancy, neonatal phase, puberty, and adulthood, and even by exposure to radiation and other chemical and physical agents. In addition, epigenetic errors are associated with the development of chronic diseases in humans (9, 10).

DNA Methylation

In mammals, DNA methylation occurs predominantly in cytosines that precede guanine, called CpG dinucleotides. DNA methyltransferases (DNMTs) are enzymes that catalyze the transfer of the methyl group ($-CH_3$) to carbon 5 of the cytosine, converting it to 5-methylcytosine (5mC) (11). DNMT1 is a maintenance methyltransferase that maintains the mitotic inheritance of the DNA bases through the preferential recognition of hemimethylated DNA during replication,

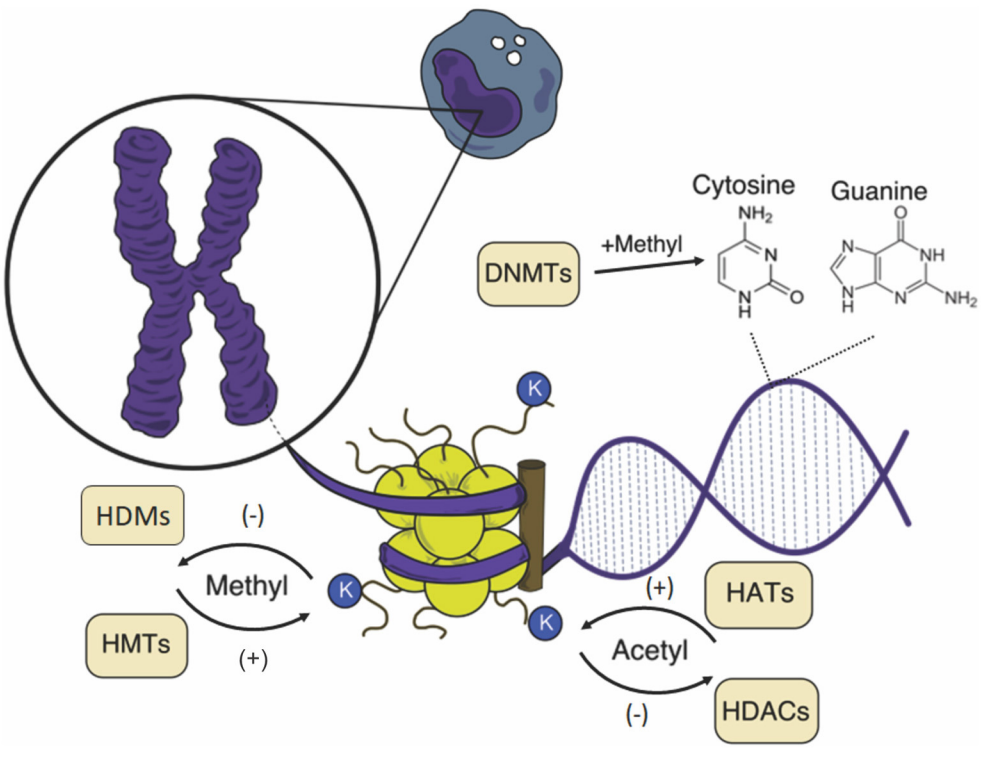

FIGURE 1 | Schematic representation of epigenetic changes in the mononuclear cell. The chromosome is composed of chromatin, a complex formed by DNA and nucleosomes, and the core is formed by an octamer of histones. Both DNA and histones can suffer the action of catalyzing enzymes of chemical groups that influence the chromatin structure, affecting gene expression. K, lysine; HATs, histone acetyltransferases; HDACs, histone deacetylases; HMTs, histone methyltransferases; HDMs, histone demethylases; DNMTs, DNA methyltransferases.

TABLE 1 | Effects of the most frequent histone changes in gene transcription.

Histone modification	Modifying enzymes	Function	Location
H3K4me1	SET1, SET7/9, MLL, SMYD2, PRDM9	Activation	Enhancers
H3K4me3	SET1, MLL1, MLL2, SMYD3, PRDM9	Activation	Promoters
H3K9ac	GCN5	Activation	Enhancers, promoters
H3K27ac	GCN5	Activation	Enhancers, promoters
H3K27me3	EZH1, EZH2	Repression	Promoters, gene-rich regions
H3K9me3	SUV39H1, SUV39H2	Repression	Satellite repeats, telomeres, pericentromeres

From references From references (18–20).

methylating the newly synthesized CpG dinucleotides, generating two new methylated DNA molecules (12). DNMT3a and DNMT3b can recognize any strand of unmethylated DNA and act mainly in establishing new methylation patterns, playing a fundamental role during embryogenesis (13).

More than half of all genes contain high concentrations of CpGs (CpG islands) in their promoters. Gene's promoters containing unmethylated CpGs give the gene a permissive state for transcription. In contrast, hypermethylation of these promoters may prevent binding of transcriptional factors and/or recruiting methyl-binding proteins and repressor complexes, resulting in gene silencing (14).

Histone Modification

Histones are proteins that compose nucleosomes H1, H2A, H2B, H3, and H4. They have amino acid residues, mainly in their N-terminal portions, subject to covalent modifications, such as acetylation, phosphorylation, methylation, and ubiquitination, which regulate the chromatin structure. Histone modifications can either modify their load or recruit proteins and complexes that affect the transcription of genes present in the region, DNA repair, and replication (6, 15).

Among these modifications, the acetylation of lysines in the N-terminal portions of histones is dynamic and catalyzed by histone acetyltransferase enzymes (HATs). Addition of acetyl groups neutralizes the positive charge of the lysine, weakening the electrostatic interaction between histones and negatively charged DNA, which favors transcriptional activation. Another family of enzymes that is also part of this process is histone deacetylases (HDACs), which have opposite effects to HATs and remove the acetyl group, restoring the positive charge of lysine (15).

Histone methylation occurs mainly in the side chain of lysine and arginine residues through the action of histone methyltransferases (HMTs). Lysines can receive more than one methyl group so that gene transcription can be suppressed or activated, depending on the number of methyl groups and the modified amino acid residue (16). In contrast to acetylation, histone methylation does not alter the general charge of the molecule. This modification was once considered static and stable. However, different families of histone demethylases (HDMs) enzymes act on the lysine residues (15, 17).

Table 1 shows the most frequent changes in histones, their function, the enzymes promoting the changes, and location (18–20).

Non-coding RNAs

In addition to the classic epigenetic mechanisms of DNA and histones, a new layer of complexity involving non-coding RNAs has emerged as an important post-transcriptional regulator of gene expression (21). Thus, ncRNAs are a group of RNAs that do not encode functional proteins, being broadly classified as short (<200 nucleotides) or long (more than 200 nucleotides), and these can be grouped by their genomic origin and biogenic processes (22).

MicroRNAs (miRNAs) belong to the most studied and highly conserved class of short ncRNAs, presenting 19–22 nucleotides (nt) in length, which destabilize messenger RNA (mRNA) by binding to 3′ untranslated regions (3′-UTR) or inhibiting protein translation (23, 24). In contrast, long ncRNAs (lncRNAs), generally not much conserved among species, have a multitude of roles, including gene expression regulation at epigenetic, transcriptional, and post-transcriptional levels (23, 25).

EPIGENETIC REGULATION OF THE IMMUNE SYSTEM

The immune system can recognize different agents and substances foreign to the body, triggering an immune response mediated by immediate reactions of innate immunity and late responses of adaptive immunity through signaling pathways that are strictly regulated at different levels. Epigenetic changes can also occur during an infectious process, so changes in the epigenome can affect the immune cell phenotype, interfering with the response to infection and contributing to inflammatory disorders (**Figure 2**) (26–28).

During sepsis, the host innate immune system cells release an excessive number of inflammatory mediators through recognizing the pathogen by pattern recognition receptors (PRRs) that identify the microorganism through pathogen-associated molecular patterns (PAMPs) and damages (DAMPs). These include Toll-like receptors (TLRs), cytosolic RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs), which induce complex intracellular signaling with complementary activities that activate transcriptional factors that regulate inflammatory response genes, generating dynamic changes in chromatin (26, 29–31).

Pathogens are capable of various epigenetic strategies to guarantee their survival and replication, in such a way that they decrease PRR detection and signaling pathways and modulate the

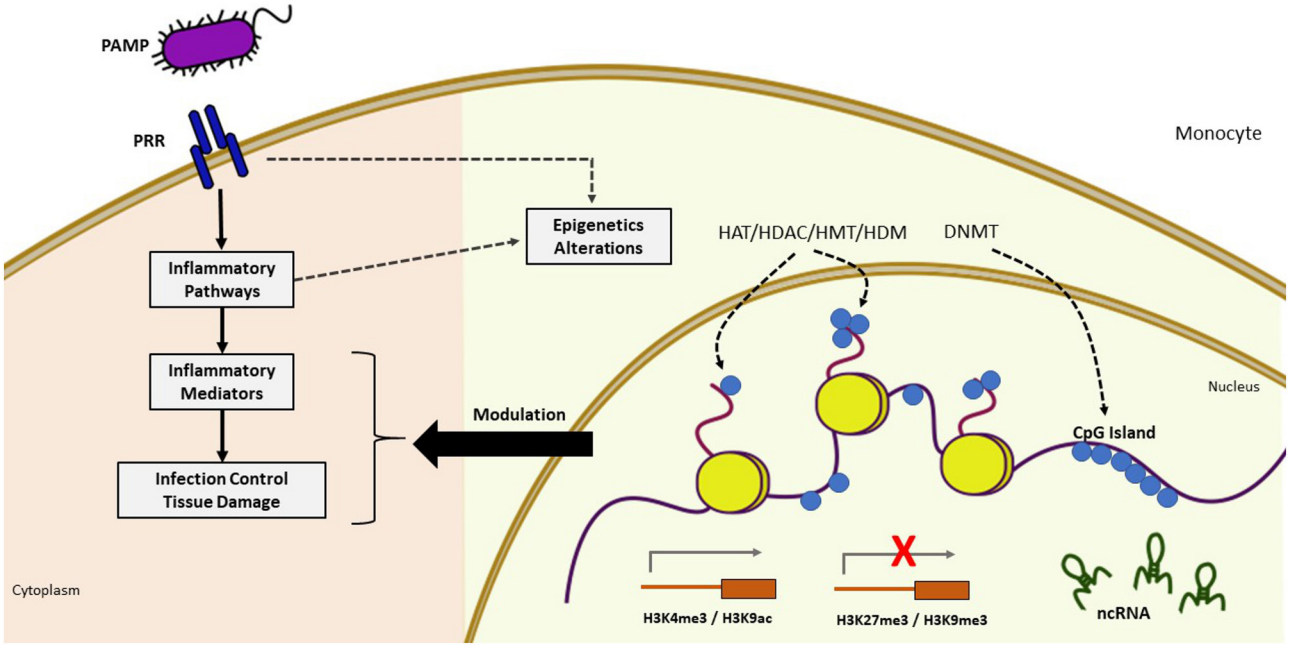

FIGURE 2 | Epigenetic modifications during infection. Pathogen recognition by PRR triggers cascades of intracellular signaling activating inflammatory mediators which induces dynamic changes in chromatin through epigenetic mechanisms, leading to increased or decreased gene activation. The pathogen can also directly alter chromatin through the influence of epigenetic modifiers. These epigenetic alterations (yellow background) may modulate the inflammatory response (pink background). Sepsis induces profound changes in gene expression involved in the inflammatory process and host defense. Epigenetic modifications play a central role in its regulation as evidenced by the presence of differentially methylated CpG islands, several modifications of histones with effects on gene activation (H3K4me, H3K9ac) and repression (H3K27me3, H3K9me3), and the presence of differentially expressed ncRNAs. PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; DNMT, DNA methyltransferase; HAT, histone acetyltransferase; HDAC, histone deacetylase; HDM, histone demethylase; HMT, histone methyltransferases; ncRNA, non-coding RNA.

expression of immunity-activating and -repressing substances. Thus, a chronic infection can induce epigenetic dysregulation, contributing to the pathogenesis of infectious diseases and even cancer. However, considering that epigenetic changes are potentially reversible, these could be reversed, allowing the host immunity to return to respond efficiently to stimuli (27).

The lipopolysaccharide (LPS) present in the cell wall of gram-negative bacteria binds to TLR-4 receptor, inducing the expression of several genes via the transcription factor NF-κB, such as tumor necrosis factor (TNF), interleukin 1 (IL-1), and IL-8. This activation generates a local and systemic inflammatory process, resulting in coagulation, vasodilation, endothelial escape, scrolling, and leakage of neutrophils and inflammatory mediators to the extravascular space, which can lead to organ dysfunction and hypotension (2, 3, 30–32). LPS stimulation in human monocytes results in the erasure of a repression marker, histone methylation into lysine 9 (H3K9me) in inducible inflammatory gene promoters, regulating these genes (33). In the human endotoxin model, transcriptome analysis revealed that 3,714 genes undergo transcriptional changes after 2 h of exposure, with changes in DNA methylation in several regions of the genome, correlating these results with the tolerance of the immune system and the increase in vulnerability to subsequent infections (34, 35).

Recent evidence has shown that the innate immune system can generate an immune memory mediated by epigenetic reprogramming of transcription pathways, known as trained immunity. This consists of the functional long-lasting reprogramming of innate immune cells in response to exogenous or endogenous stimuli, generating an altered response to a second challenge after returning to baseline (36). For example, individuals vaccinated with BCG (Bacille Calmette-Guérin) have monocyte epigenetic reprogramming throughout the genome, with increased H3K4 trimethylation activation mark (me3), increasing IL-1β production and protection against viral infections in an experimental model of yellow fever. These functional changes indicate trained immunity (37).

During sepsis, a phenomenon known as immunological tolerance occurs. The immune system of patients leaves the state of hyper-inflammation, called a cytokine storm, and goes to a dysfunctional state, where the innate cells do not respond adequately to posterior stimuli (3). In this process, there is a reorganization of the immune functions and metabolic processes of inflammatory cells, with suppression to subsequent challenges as part of this acute cellular reprogramming. Studies show epigenetic modifications are essential for establishing immunosuppression in late sepsis. These modifications include changes in histone marks, loss of activation marks in promoter

regions, and gene enhancers that are negatively transcribed into tolerant monocytes (2, 34, 38, 39).

In a model of tolerance induced by LPS, nuclear factor-κB (NF-kB)-activated genes are downregulated. In contrast, genes related to the p38 pathway are preserved, showing a different regulation from the TLR cascades during immunoparalysis (40). Austenaa and colleagues showed that the H3K4me3 epigenetic activation mark participates in regulating the TLR4 signaling pathway and described the profile of this modification in the mouse macrophage genome during the response to LPS (41).

T cells recognize antigens through the human leukocyte antigen (HLA) system [the major histocompatibility complex (MHC) in humans] that are expressed on the surface of host cells; research points to a decrease in the expression of HLA-DR in septic monocytes and DCs (2, 42, 43), combined with transcription reduction of the class II transactivator gene (CIITA), which is modulated by the action of HATs (44, 45).

In this context, the dysregulation of innate and adaptive immunity is associated with harmful consequences, which, together with organ failure, lead to increased short- and long-term mortality in septic patients (46).

Another fundamental mechanism for regulating the inflammatory response is cellular metabolism. During sepsis, innate immunity cells activate a series of intracellular cascades that result in cellular metabolism alterations. The metabolic shift from oxidative phosphorylation to glycolysis during acute inflammation provides the necessary energy for cell function and induces an accumulation of metabolites that function as cofactors of epigenetic enzymes (47).

Thus, a reduction in intracellular levels of acetyl-CoA can decrease histone acetylation. The accumulation of nicotinamide adenine dinucleotide (NAD)+ activates histone deacetylases of the sirtuin class, leading to lower acetylation levels. High concentrations of fumarate inhibit the histone demethylase enzyme KDM5, responsible for removing methyl groups. Therefore, several cellular metabolites can activate or inhibit different enzymes involved in epigenetic programming. They induce changes in chromatin and DNA, modulate gene transcription, and lead to different functional states during sepsis, such as excessive inflammation immunoparalysis (2, 33).

EPIGENETIC REGULATION IN SEPSIS

Different epigenetic changes have already been associated with immune activation and tolerance during sepsis, contributing to the process of prolonged inflammation, organ failure, persistent immunosuppression, development of severe secondary infections, and even death (36, 48, 49).

Much of the research that correlates epigenetics and sepsis has been with *in vitro* studies or animal models, with scarce data in septic patients (**Tables 2**, **3**).

Histone Modification and Sepsis

Foster and collaborators presented the first evidence linking tolerance to LPS with epigenetic mechanisms. They showed that in mouse macrophages, a different response occurs in genes induced by TLR4. These responses were divided into two classes: class T composed of pro-inflammatory genes, which were inhibited in tolerant macrophages; and the NT class genes, composed of antimicrobials that were not inhibited in these macrophages. In the promoters of inflammatory genes, the H3K4me3 activation marks and H4 acetylation were lost during a re-exposition to LPS, and the NT class genes remained with the presence of the activation marks after a second challenge (50). Also, monocytes exposed to LPS do not show active histone marks in the promoter region and in gene enhancers that participate in lipid metabolism and phagocytic pathways, resulting in transcriptional inactivity of these genes through new stimulus (31).

In human sepsis, selective and precise changes in chromatin occur in regulatory regions of genes that participate in the immune process. Chromatin immunoprecipitation combined with high-throughput sequencing showed that in the cells of septic patients, transcriptional activation marks (H3K4me3 and H3K9ac) increased in genes related to immune response; in contrast, genes involved in processing and presenting antigens gained the repression mark (H3K27me3) compared with healthy controls (64). Differences in epigenetic marks can be explained by their plasticity at different times of exposure to the pathogen. Zhao and colleagues found the presence of the activation mark H3K4me2 in bone marrow–derived macrophages (BMDMs) in mice after 30 min of stimulation with LPS, with a return to baseline levels after prolonged exposure to the stimulus (51).

Organ dysfunction occurs during sepsis due to the excessive initial response of cytokines that generate tissue damage. In a model of acute lung injury (ALI) induced by sepsis in mice, loss of histone acetylation was observed in promoters of the main angiogenic genes in the lung and extrapulmonary organs. This systemic response of negative transcription regulation has been included in the pathogenesis of microvascular leakage induced by sepsis and multiple organ dysfunction syndrome (MODS) (54), suggesting early intervention can preserve these epigenetic marks, maintaining endothelial integrity (68). Mice pretreated with HDAC inhibitors attenuated ALI during sepsis (57).

Epigenetic Regulators and Sepsis

The activity of the enzymes HATs and HDACs can be modulated by LPS, but their contribution to endotoxin tolerance is not yet clear. A study showed that the inhibition of acetyl-lysine binding domain, known as bromodomain and its subfamily bromo- and extra-terminal (BET), induces a negative regulation of inflammatory genes in activated macrophages, reducing inflammation in a model of bacterial sepsis in murine (69).

In the NF-kB activation signaling pathway, the CREB-binding protein (CBP)—a transcriptional coactivator of HAT function—contains bromodomains that bind to acetylated histones H3 and H4 in a way that allows the expression of pro-inflammatory cytokine genes (70). Exposure to LPS increases the stability of CBP by reducing interaction with the FBXL19 subunit of ubiquitin ligase 3 and activating the deubiquitylating enzyme

TABLE 2 | *In vitro* and *in vivo* experimental studies evaluating epigenetic modifications in LPS challenge and infection.

Study	Epigenetic modification	Experimental model	Results
In vitro			
(41)	Histone methylation	Macrophages of Wbp7 –/– mice exposed to LPS	Macrophages Wbp7 –/– show impaired responses to LPS, with loss of H3K4me3
(50)	Histone methylation histone acetylation	BMM stimulated with LPS	Epigenetic changes are associated with silencing of inflammatory genes and priming of antimicrobial effector
(51)	Histone methylation	Murine RAW264.7 cells and BMDMs upon LPS stimulation	LPS stimulation resulted in enhanced methylation at H3K4 and H3K9 in cells
(52)	Histone methylation	Raw264.7 macrophages LPS-treated	The JmjC-Jmjd3 domain protein is H3K27me macrophage-induced demethylase in the presence of bacterial products and inflammatory cytokines
(53)	Histone methylation	BMM stimulated with LPS	Jmjd3 interferes with the transcription of LPS-activated genes in an independent way to demethylate H3K27
In vivo			
(54)	Histone acetylation	ALI sepsis in murine	ALI sepsis reduces the levels of histone H3 lysine acetylation that permits the transcription of angiogenic genes in the lung, kidney, and liver
(55)	DNA methylation	ALI sepsis in rat	1,721 genes had aberrant methylation in the rat's lung tissue with acute LPS-induced injury
(56)	Histone acetylation DNA methylation	ALI sepsis in mice	Combined treatment of DNMTi and HDACi alleviates inflammation-induced pyroptosis and apoptosis during ALI
(57)	Histone acetylation	CLP-induced sepsis in mice	Pretreatment with HDACi 30 min before CLP resulted in decreased lung injury and increased survival
(58)	DNA methylation	CLP-induced sepsis in mice	Treatment with decitabine reduces DNMTs, minimizes NF-kB activation, and attenuates inflammatory cytokine levels, inhibiting sepsis progression
(59)	DNA methylation	Rat model of endotoxemia	Treatment with DMNTi (procainamide) reduced the levels of DMNT1 and 5-methylcytosine, improving inflammatory infiltrate and superoxide production in the lung
(60)	Histone acetylation	Mice injected with LPS	Prophylactic treatment with HDACi (SAHA) reduced levels of TNF-α, IL-1-β, IL-6, and IFN-γ induced by LPS
(61)	Histone acetylation	Mice injected with LPS	SAHA-treated mice had increased survival than untreated mice

LPS, lipopolysaccharide; BMM, bone marrow macrophage; BMDM, bone marrow–derived macrophage; ALI, acute lung injury–induced sepsis; DMNTi, DNA methyltransferase inhibitor; HDACi, histone deacetylase inhibitor; CLP, cecal ligation and puncture; SAHA, suberoylanilide hydroxamic acid; DMNT1, DNA methyltransferase 1; JMJD3, Jumonji domain-containing protein D3.

USP14, resulting in chromatin remodeling and cytokine gene expression (71).

The expression of the Jumonji domain-containing protein D3 (Jmjd3) enzyme, a H3K27me histone demethylase class, is induced in macrophages by the transcription factor NF-kB in response to LPS, and binds to genes targeting proteins of the Polycomb group, which belongs to the Chromobox family proteins and mediates gene silencing, regulating the levels of the repressor mark H3K27me3 and transcriptional activity, independent of H3K27 demethylation (52, 53).

Cellular bioenergetic changes during sepsis can also be coordinated by epigenetic mechanisms. During sepsis, sirtuin 1 (SIRT1) rapidly accumulates in the TNF-α and IL-1β gene promoters, deacetylating H4K16 and blocking NF-kB-dependent transcription (62). The presence of SIRT6 can also attenuate NF-kB signaling by deacetylating H3K9 in chromatin (72). Besides, in endotoxin tolerance, the interaction of DNA methylation with histone H3K9 methylation silences the expression of some pro-inflammatory genes (63, 73). LPS activates M1 macrophages, which present a high rate of glycolysis, leading to HDAC degradation, which interferes with the activity of inflammatory cytokines (74, 75). α-Ketoglutarate (αKG), a tricarboxylic acid cycle (TCA) intermediate, favors tolerance to endotoxin in inflammatory genes after M1 macrophages are activated, independently of Jmjd3 (76). Exposure to LPS also increases the metabolism of one-carbon, which produces S-adenosyl methionine, a potent methyl donor (77).

DNA Methylation and Sepsis

A pilot study involving septic and non-septic patients analyzed methylation throughout the genome in the samples of these individuals and found 668 differentially methylated regions (DMRs) between the septic vs. non-septic groups, among which 56 genes have already been associated with sepsis in literature (65). Blood transcriptome analysis of patients with community-acquired pneumonia identified several chromatin-modifying enzymes are differentially expressed in the initial sepsis, leading to chromatin reorganization and stimulating widespread transcriptional reprogramming (66).

TABLE 3 | Epigenetic modifications in human cells *in vitro* and in different clinical settings.

Study	Epigenetic modification	Model	Results
(33)	Histone methylation	Human monocytes exposed to LPS	Exposure to LPS changed the methylation pattern of H3K9 in a set of inflammatory gene
(34)	DNA methylation histone methylation	Human monocytes exposed to LPS	Exposure to endotoxin generated changes in DNA methylation, mainly demethylation, and a gain of acetyl in H3K27 and methyl H3K4 in cytokine promoters
(62)	Histone acetylation	Human monocyte cell model of endotoxin tolerance	SIRT1 coordinates the epigenetic and bioenergy shifts
(63)	DNA methylation histone methylation	Human monocyte cell line THP-1 incubated with LPS	In tolerant macrophages, the interaction of DNA methylation with H3K9 methylation silences TNF-α expression
(64)	Histone methylation histone acetylation	Monocytes from septic patients	Sepsis induces changes in chromatin, with selective and precise changes in promoter regions of immunological genes
(65)	DNA methylation	Adults patients with Sepsis	The DNA methylation profile showed 668 differentially methylated regions between patients with sepsis and patients with critical non-septic diseases
(66)	DNA methylation histone methylation	Adult patients with community-acquired pneumonia	Chromatin remodeling occurs in community-acquired pneumonia associated with extensive transcriptional deregulation of chromatin-modifying enzymes
(67)	DNA methylation	Neonates with bacterial sepsis	Analysis of the entire epigenome of whole blood samples reveals 81 differently methylated CpG sites in 64 genes, where functional analysis showed an enrichment of protocadherin genes in neonatal sepsis

LPS, lipopolysaccharide; SIRT1, sirtuin 1.

Retrospective research evaluated whether the DNA methylation pattern of CpG sites in the procalcitonin gene [polypeptide related to α calcitonin (CALCA)] could be used as an epigenetic biomarker for bacterial sepsis in premature newborns. These preterm patients showed variation in the DNA methylation status of the CALCA promoter in different types of bacterial sepsis, suggesting different regulation of this gene at the epigenetic level according to the type of infection (78). In a further approach searching for prognostic markers of neonatal sepsis, a small epigenome study analyzed the methylation status of CpGs in blood samples from 3 septic neonates and 3 non-septic and found 81 differently methylated CpG sites in 64 genes, whose functional analysis showed the enrichment of protocadherin genes in neonatal sepsis (67).

An experimental model of LPS-induced ALI found an increase in DNMT1 and 5-methylcytosine, accompanied by neutrophil infiltration and superoxide production in the lung tissue of endotoxemic rats (59). Another epigenomic analysis showed aberrant DNA methylation occurs in promoter regions of 1,721 genes, many of which participate in the hyperinflammatory response (55).

Non-coding RNAs and Sepsis

ncRNAs are also involved in the pathogenesis of sepsis. A study analyzing the co-expression network of protein-coding and lncRNAs in septic and healthy neutrophils showed that several lncRNAs are linked to genes differentially expressed during sepsis and appear to have a regulatory role in the translation of proteins, and participate in regulatory loops that are altered during sepsis (79). They were detected as sepsis regulators because of the interaction between lncRNAs and sepsis co-expression modules identified by whole blood RNA expression profile of septic patients (80). An analysis of the transcriptome in blood leukocytes of volunteers with sepsis and healthy individuals showed that both lncRNAs and, to a lesser extent, short ncRNAs undergo significant changes during sepsis in healthy individuals (81). So, different types of ncRNAs can serve as potential biomarkers for sepsis and as new therapeutic targets (82).

POTENTIAL EPIGENETIC THERAPIES FOR SEPSIS

Several studies evaluated the potential therapeutic effect of epigenetic drugs in modulating chromatin regulatory enzymes during sepsis. Animal research has shown that epigenetic mechanisms can mitigate the acute inflammatory response to endotoxins (39, 48). Many of these epigenetic therapies are undergoing clinical trials to treat different cancers (83–85). Some of these therapies have been approved by the Food and Drug Administration and are used in clinical practice.

Suberoylanilide hydroxamic acid (SAHA) is a histone deacetylase (HDACi) inhibitor known for its anti-tumor effects. Leoni and colleagues showed that SAHA could also reduce the production of pro-inflammatory cytokines. Mice treated with this inhibitor reduced the production of TNF-α, IL-1β, IL-6, and interferon gamma (IFN-γ) after a challenge with LPS (60). When mice were submitted to the polymicrobial sepsis model initiated by ligation and cecal puncture (CLP) (61, 86), survival improved and damage reduced.

Another HDACi that appears to be effective in improving the clinical outcomes of sepsis is Trichostatin A (TSA)

(86). TSA-pretreated mice submitted to CLP presented a protective effect during sepsis-induced lung injury, with reduced inflammatory infiltrate, decreased expression of the intercellular adhesion molecule-1 (ICAM-1) and E-selectin in lung tissue samples, and reduced plasma IL-6, with increased survival (57). Treatment with TSA, in combination with DNA methyltransferase inhibitor (DNMTi) 5-Aza 2-deoxycytidine (5-AZA-CdR), decreases apoptosis and inflammation in BMDMs of mice exposed to LPS (56). Also, TSA blocks the effect of endotoxin tolerance in reducing IL-6 production (87).

Valproic acid (VPA) and sodium butyrate (SB) also act as HDACi and have shown efficacy in experimental models of sepsis, with reduced expression of inflammatory genes and decreased organic damage (57, 88, 89); however, toxicity may prevent its use in clinical trials (90).

Studies show that different DNMTi can reverse some sepsis results in an endotoxemia model in rats. As an example, procainamide inhibited the increase in DNMT1 and decreased neutrophil infiltration in the lung of endotoxemic rats (59). Decitabine, a DNMTi, reduced NF-kB activation, decreased the levels of inflammatory cytokines, and inhibited sepsis progression in mice challenged with CLP (58).

The mechanisms by which DNMTi and HDACi act to reverse some of the consequences of sepsis are still not fully understood. These inhibitors are believed to prevent epigenetic changes and their modulating effects on gene expression (39). However, use of these inhibitors may become unfavorable because they reduce the expression of pro-inflammatory and anti-inflammatory cytokines and mediators, decreasing bacterial clearance (91).

Despite the effects of these enzymes' inhibitors in pre-clinical models of sepsis, one should be cautious to translate this approach as a potential clinical adjuvant therapy for sepsis. In most studies already mentioned here, the inhibitors were administered prophylactically, which does not mimic the setting of sepsis therapy. Few are those who demonstrate the benefits of late epigenetic drug use. One used the highly specific SIRT1 inhibitor, EX-527, in mice 24 h after onset of sepsis. All animals receiving this epigenetic agent survived sepsis with the reversion of endotoxin tolerance (92). Furthermore, other animal models should also be tested. For other potential uses of these drugs, the benefits must overcome the risks and toxicity.

CONCLUSION

During sepsis, dysregulated gene expression occurs, generating hyperinflammatory responses and, in parallel, persistent hypo-inflammatory reactions. Strong evidence points to epigenetic changes as one of the main factors influencing gene expression changes associated with this clinical condition. In this review, we summarize studies that highlight epigenetic mechanisms as essential events during sepsis pathology, changing as sepsis progresses. Thus, epigenetic regulation occurs mainly in transcriptional promoter regions or gene enhancers, leading to pathological and bioenergetic adaptations through specific enzymes that catalyze chemical groups. The use of epigenetic enzyme inhibitors is promising as a therapeutic target during sepsis, but further research is needed to understand their role in clinical settings.

AUTHOR CONTRIBUTIONS

RF-H wrote the first draft of the article. RF-H and RS reviewed and wrote the final draft of article. MJ and MB contributed to paper revision and to the final version. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.685333/full#supplementary-material

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BRIEF RESEARCH REPORT
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Downregulation of CD45 Signaling in COVID-19 Patients Is Reversed by C24D, a Novel CD45 Targeting Peptide

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CD45, the predominant transmembrane tyrosine phosphatase in leukocytes, is required for the efficient induction of T cell receptor signaling and activation. We recently reported that the CD45-intracellular signals in peripheral blood mononuclear cells (PBMCs) of triple negative breast cancer (TNBC) patients are inhibited. We also reported that C24D, an immune modulating therapeutic peptide, binds to CD45 on immune-suppressed cells and resets the functionality of the immune system via the CD45 signaling pathway. Various studies have demonstrated that also viruses can interfere with the functions of CD45 and that patients with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) are immune-suppressed. Given the similarity between the role of CD45 in viral immune suppression and our findings on TNBC, we hypothesized that the C24D peptide may have a similar "immune-resetting" effect on PBMCs from COVID-19 patients as it did on PBMCs from TNBC patients. We tested this hypothesis by comparing the CD45/TCR intracellular signaling in PBMCs from ten COVID-19 patients vs. PBMCs from ten healthy volunteers. Herein, we report our findings, demonstrating the immune reactivating effect of C24D via the phosphorylation of the tyrosine 505 and 394 in Lck, the tyrosine 493 in ZAP-70 and the tyrosine 172 in VAV-1 proteins in the CD45 signaling pathway. Despite the relatively small number of patients in this report, the results demonstrate that C24D rescued CD45 signaling. Given the central role played by CD45 in the immune system, we suggest CD45 as a potential therapeutic target.

Keywords: CD45, COVID-19, PBMC, Src family of tyrosine kinases, immunosuppression

INTRODUCTION

CD45 is a transmembrane protein tyrosine phosphatase receptor type C (PTPRC), expressed exclusively in leukocytes, with double opposing effects on T cell receptor (TCR) activity (1, 2). On the one hand, CD45 plays an inhibitory function involving the dephosphorylation of the tyrosine 394 (Y394) in the lymphocyte-specific protein tyrosine kinase (Lck), preventing its activation. On the other hand, CD45 plays the role of an activator when it dephosphorylates the tyrosine 505 (Y505), an inhibitory site at the C-terminal end of the non-receptor tyrosine-Src kinases.

Activated Lck phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) of the T cell receptor (TCR)/CD3 complex. The phosphorylated ITAMs recruit the Zeta-chain-associated protein kinase 70 (ZAP-70), via its Src homology 2 (SH2) domains. Finally, for TCR activation, CD3-bound ZAP-70 is activated by both Lck and (trans)-auto-phosphorylation at the ZAP70 tyrosine 493 (Y493) (3–5). The ZAP70 tyrosine kinase transmits a downstream signal leading to VAV-1 phosphorylation and activation (6, 7).

We recently reported an immune escape mechanism in TNBC patients showing that CD45's intracellular signals are inhibited (8). We also reported that C24D, a previously described immune modulating therapeutic peptide (9), binds to the CD45 receptor of the TNBC-suppressed immune cells and reverses immune-suppression, via the CD45 signaling pathway (8). C24D-binding to CD45 in the immune-suppressed cells resulted in immune reactivation and specific tumor killing.

Various studies have demonstrated that also viruses can interfere with the functions of CD45 (10). The underlying mechanism of the viral/CD45 immune-suppressive interaction was elucidated on cytomegaloviruses, adenoviruses and others (11, 12). The protein UL11 from the cytomegalovirus (CMV) and the protein E3/49K from adenovirus (AdV) are known to bind to CD45 (11, 12). The sec49K viral protein, derived from E3/49K, was found to affect CD45 in non-infected adjacent and distant cells (13). Additionally, functional studies showed that sec49K can suppress the activation, signaling, cytotoxicity and cytokine production of T and NK cells (13).

Coronavirus disease 2019 (COVID-19), first identified in December 2019 in Wuhan, China, is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). By March 2021, the number of COVID-19 confirmed cases globally was 113,467,303 and 2,520,550 deaths were reported (14, 15).

It has been shown that patients with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) are immune-suppressed (16–18). Given the similarity between the CD45 viral immune suppression reported by others and our oncology findings, we hypothesized that the C24D peptide may have a similar immune-resetting effect on PBMCs from COVID-19 patients as it did on PBMCs from TNBC patients. We demonstrate in this Brief Report the effect of adding C24D to PBMCs obtained from ten hospitalized COVID-19 patients on the phosphorylation of Lck, ZAP-70 and VAV-1 proteins in the CD45 signaling pathway.

METHODS

C24D Peptide Synthesis

The C24D peptide is a 25 amino acid homodimer peptide with a disulphyde bond at CG (CGHHLLRPRRRKRPHSIPTPILIFRSP), synthesized by Synpeptide Co., Ltd. (Shanghai, China). HPLC showed purity >97%.

PBMC Isolation

PBMC was isolated from blood samples of healthy female donors and hospitalized COVID-19 patients and was obtained from the Blood Bank Mada Tel HaShomer and Meir Medical Center, respectively. The protocol was approved by the Institutional Review Board at Meir Medical Center, Israel (0094-20-MMC). Samples were isolated by Ficoll–Hypaque density gradient ($d =$ 1,077 g/mL, Ficoll-Paque Plus, GE Healthcare, Upsalla, Sweden) by centrifugation at 650 × g for 30 min.

Patients and Data Collection

We conducted analyses of ten COVID-19 patients hospitalized in the Meir Medical Center, Kfar Saba, Israel. COVID-19 was diagnosed by RT-PCR, based on criteria issued by the National Health Commission of Israel. Only patients with a positive, laboratory-confirmed test for SARS-CoV-2 and who suffered from sufficiently serious COVID-19 symptoms to warrant hospitalization were included. Baseline and follow-up data for all patients was obtained from the electronic medical record system. All ten patients were still hospitalized at the time of blood extraction; five patients already had a negative confirmed RT-PCR test for SARS-CoV-2 at the time of blood extraction and five still had a positive result. The Ethics Committees of the Meir Medical Center and the Israel Ministry of Health approved the study and written informed consent was obtained from all subjects. All ten patients were categorized as non-severe cases.

CD45 Signal Transduction

C24D, at 10 μg/ml, was added immediately after PBMC isolation and incubated for 5, 15, 30, 60 min and 24 h at room temperature. PBMCs were centrifuged and re-suspended in 0.12 ml of lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM NaF, 2 mM Na_3VO_4, 1% NP40, 10 mM b-glycerophosphate, 30% glycerol, 1 mM EDTA, 0.5% sodium-deoxycholate, 0.5% protease inhibitor cocktail), followed by one freeze-thaw cycle of 20 min. Cells were harvested and centrifuged (14,000 rpm, 15 min, 4°C). The supernatants were collected, and aliquots were separated on 10% SDS PAGE, followed by Western blotting with anti-phospho-Lck Y505 (0.5 μg/ml, ab4901, Abcam, Cambridge, UK), anti-phospho-Lck Y394 (0.25 μg/ml, ab201567, Abcam), anti phospho-VAV-1 Y174 (0.23 μg/ml, ab76225, Abcam) and anti-phospho-ZAP70 Y493 (1 μg/ml, ab194800, Abcam). GAPDH (1 μg/ml, ab9485, Abcam) was added as a control for sample loading. After several washings, the secondary antibody, IRDye 800CW Goat anti-Rabbit or IRDye 680CW Goat anti mouse (1 μg/ml, LI-COR, Nebraska, USA) was added for 1 h.

Quantification Methods

The membrane was analyzed by Odyssey 2.1 (Infrared Imaging System) for specific band identification. Quantification of phosphorylation was done by Image J (NIH, USA). Percentage (%) of maximal phosphorylation of phosphorylated proteins were first normalized to the levels obtained with GAPDH, respectively, and the activation values were normalized for each time point vs. its control, without C24D (e.g., C24D + lymphocytes vs. lymphocytes control). The values obtained were then expressed as % of maximal activation that was observed in each experiment, at each time point. All the results were normalized with GAPDH as the reference protein.

Statistics

Data to compare results between the patients and healthy groups we used the independent (two-tailed) *t*-test. For multiple comparisons we used the One-Way ANOVA test. Significance was defined as $p < 0.05$.

RESULTS

No Correlation Between Clinical Characteristics of Patients and RT-PCR Results Was Found

Baseline clinical data and laboratory findings are shown in **Table 1**. The median age of the study population was 60.7 (age range, 42–79) years, 50% were female. Blood from five patients was obtained toward the end of hospitalization (RT-PCR negative) and from five patients shortly after being hospitalized (RT-PCR positive). All patients were hospitalized for a minimum of 1 week (range, 7–45 days). Main risk factors for severe Covid-19 disease in our patient population included obesity (four patients) and impaired glucose tolerance (three patients).

Addition of C24D to PBMCs From COVID-19 Patients Resets the Phosphorylation of Src Protein Kinases

Using western blot analysis, we determined the phosphorylation of proteins in the CD45 signaling pathway resulting from the addition of the C24D peptide to fresh PBMCs from ten COVID-19 patients. The results were compared to the same measurements using PBMCs from healthy volunteers (**Figure 1**). Addition of C24D for 5–60 min to fresh PBMCs from the COVID-19 patients resulted in activation of Lck (a member of the Src protein kinases).

Statistically significant differences were found between four groups: (1) patients, (2) healthy volunteers, (3) before and (4) after the addition of C24D to PBMCs, ($p < 6.8 \times 10^{-5}$, $p < 0.02$, $p < 0.017$, and $p < 0.05$) in Lck Y505, Lck Y394, ZAP-70 and VAV-1 phosphorylation, respectively, as determined by One-Way ANOVA. The statistically significant results correspond to the effect of the addition of C24D to PBMCs from COVID-19 patients.

As depicted in **Figure 1A**, a significant decrease in the phosphorylation of the inhibitory tyrosine 505 in Lck was observed (from 130.35 ± 12.08% to 103.12 ± 7.35%, $p < 0.01$) only in patients. In parallel, we observed a significant increase in the phosphorylation of the tyrosine 394 of Lck (from 130.19 ± 19.23% to 168.25 ± 25.69%, $p < 0.007$). Consequently, ZAP-70 was activated, as evidenced by a significant increase in the phosphorylation of the tyrosine 493 in ZAP-70 (from 77.97 ± 10.17% to 97.34 ± 10.14%, $p < 0.0001$). A similar pattern was observed for VAV-1 phosphorylation. Addition of C24D to fresh PBMCs from COVID-19 patients significantly increased VAV-1 phosphorylation (from 65.20 ± 3.99% to 78.73 ± 6.09%, $p < 0.005$, **Figure 1A**). In contrast to the C24D-induced immune reactivation observed in PBMCs from COVID-19 patients, no significant effect was seen on Lck (Y505, $p < 0.122$; Y394, $p < 0.301$), ZAP70 ($p < 0.08$) and VAV-1 ($p < 0.274$) phosphorylation when fresh PBMCs from healthy donors were incubated with C24D for 5 min to 24 h (**Figure 1A**). **Figure 1B** depicts the western blot results obtained from three representative Covid-19 patients. **Supplementary Figure 1** depicts the remaining 7 COVID-19 patients' western blot results.

To better understand the effect of the C2D peptide on the CD45 signaling pathway, we analyzed the percent change in phosphorylation for each of the 4 relevant proteins, for every PBMC sample from the ten COVID-19 patients, +C24D vs. –C24D (**Figure 2A**). The average change resulting from treatment with the C24D peptide on the phosphorylation of the inhibitory Lck tyrosine 505 and the immune-stimulating tyrosine 394 was −18.6 and +29.2%, respectively. ZAP-70 phosphorylation increased by an average of 24.8% and VAV-1 phosphorylation by 20.7%. Treatment of PBMCs from healthy volunteers with C24D did not cause a significant change in the phosphorylation of the four relevant proteins (**Figure 2B**). Thus, in this study, C24D specifically altered the phosphorylation pattern of key CD45-signaling molecules and did so only in PBMCs derived from COVID-19 patients.

Supplementary Figure 2 shows the pattern of phosphorylation of Lck, ZAP-70 and VAV-1 over time (from 5 to 30 min), obtained by western blot analysis, for each COVID-19 patient. Lck 505 de-phosphorylation and Lck 394 phosphorylation are completed 30 min after addition of C24D, to PBMCs of all patients. Five minutes and 15 min after treatment, ZAP-70 and VAV-1 are phosphorylated, respectively, in all tested PBMCs.

No Correlation Was Observed Between Clinical Characteristics and the Protein Phosphorylation Pattern

No correlation was observed between the clinical characteristics (age, weight, glucose and ferritin levels) of the ten hospitalized COVID-19 patients and the percentage of Lck505, Lck394, ZAP70, and VAV-1 phosphorylation induced by C24D, as determined by One-Way ANOVA test.

Interestingly, there was no difference in the C24D-induced CD45 signaling between the five hospitalized patients who already had negative RT-PCR results and the 5 hospitalized patients who still had positive RT-PCR results, implying that the patients whose RT-PCR results reverted to negative were still immunosuppressed.

We divided the patients into two significantly different age groups: ≥65 and <65 ($p < 5.73E\text{-}05$, **Figure 3A**). No correlation between the response to C24D in Lck, ZAP-70, and VAV-1 activation to the age of patients was found.

In spite of the small number of patients in each of the weight, glucose and ferritin values sub-groups, we nonetheless evaluated the effect of C24D on CD45 signaling. We found that the difference between the two weight sub-groups (obese vs. normal) was statistically significant ($p < 0.025$). However, no correlation between the phosphorylation of Lck505, Lck394, ZAP70, and VAV-1 and weight was observed (**Figure 3B**). Addition of C24D induced Lck, ZAP70 and VAV-1 activation equally in both sub-groups. A similar pattern of protein phosphorylation was

TABLE 1 | Clinical characteristics of the ten hospitalized Covid-19 patients.

Patient	Age	Gender	PCR	Weight	WBC x 10^3	RBC x 10^6	Hb	Glucose	Ferritin	Secondary infections	Days of hospitalization
1	78	M	Neg	76	7.14	4.04	12.2	107	760	None	45
2	79	F	Neg	54	11.99	4.29	12.6	N/A	N/A	None	30
3	55	M	neg	103	89.2	5.36	13.3	207	405	None	11
4	42	F	Pos	115	5.2	5.01	13.7	90	833	None	19
5	46	M	Pos	110	4.74	4.91	14.2	96	597	None	11
6	70	M	Neg	75	6.02	5.34	15.8	97	690	None	14
7	74	M	Pos	64	5.84	4.7	14.3	80	681	Klebsiella-colonization	16
8	68	F	Neg	n/a	9.8	5.27	12.2	119	147	Serratia bacteremia	12
9	42	F	Pos	63	7.71	4.11	12.3	94	N/A	None	8
10	53	F	Pos	n/a	4.57	4.46	12.6	96	398	None	8

WBC, White blood cell count; RBC, red blood cell count; Hb, Hemoglobin; PCR, RT PCR results for Covid-19 disease.

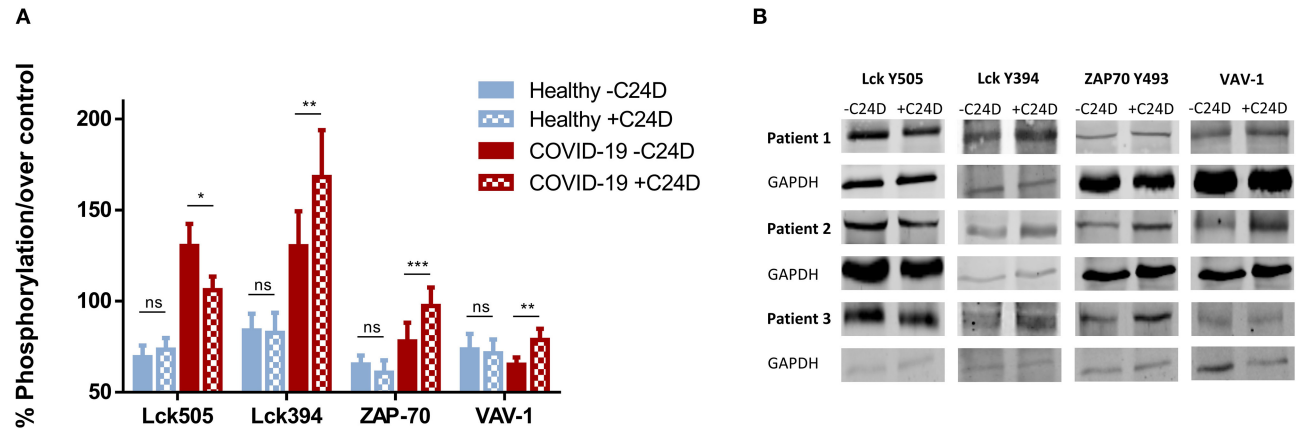

FIGURE 1 | C24D binding to CD45 triggers the CD45 signaling pathway: PBMCs from ten hospitalized patients, ± C24D treatment, were lysed, separated on 10% SDS and blotted with antibodies for the determination of phosphorylation of the tyrosine 505 and 394 in Lck, the tyrosine 493 in ZAP-70 and the tyrosine 174 in VAV-1. PBMCs from healthy volunteers served as controls. The percentage of phosphorylation was calculated individually vs. GAPDH as protein control. **(A)** Percentage change of protein phosphorylation in PBMCs from 10 patients, compared to 10 healthy donors, after addition of C24D for 5–60 min to patient PBMCs, or 24 h to PBMCs from healthy donor (values represent mean± SE, $*p < 0.01$, $**p < 0.005$, $***p < 0.0001$). **(B)** Western blot results obtained in three representative patients for each phosphorylated protein, ± C24D.

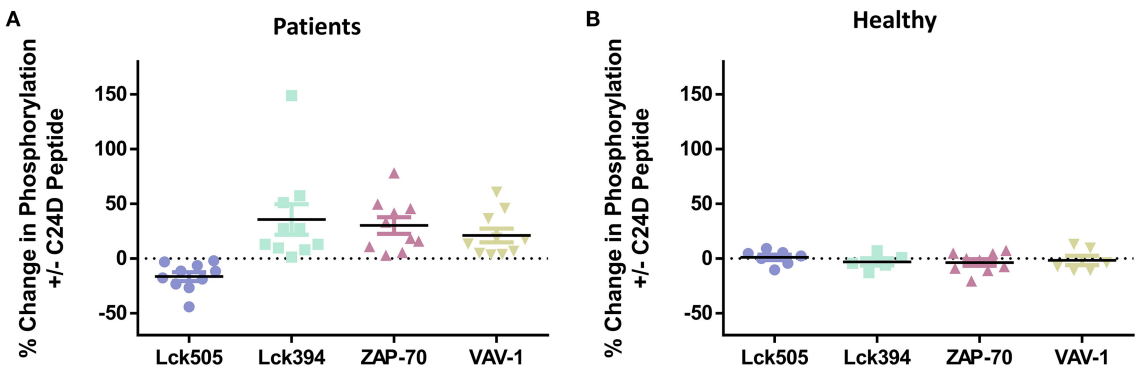

FIGURE 2 | C24D reversed CD45 immune suppression signaling only in patients: **(A)** Percentage change of Lck, ZAP-70 and VAV-1 phosphorylation in PBMCs from each of the 10 patients, after addition of C24D for 5–60 min, normalized to control without C24D. **(B)** Percentage change of Lck, ZAP-70 and VAV-1 phosphorylation in PBMCs from healthy donors, after addition of C24D for at least 24 h, normalized to control without C24D.

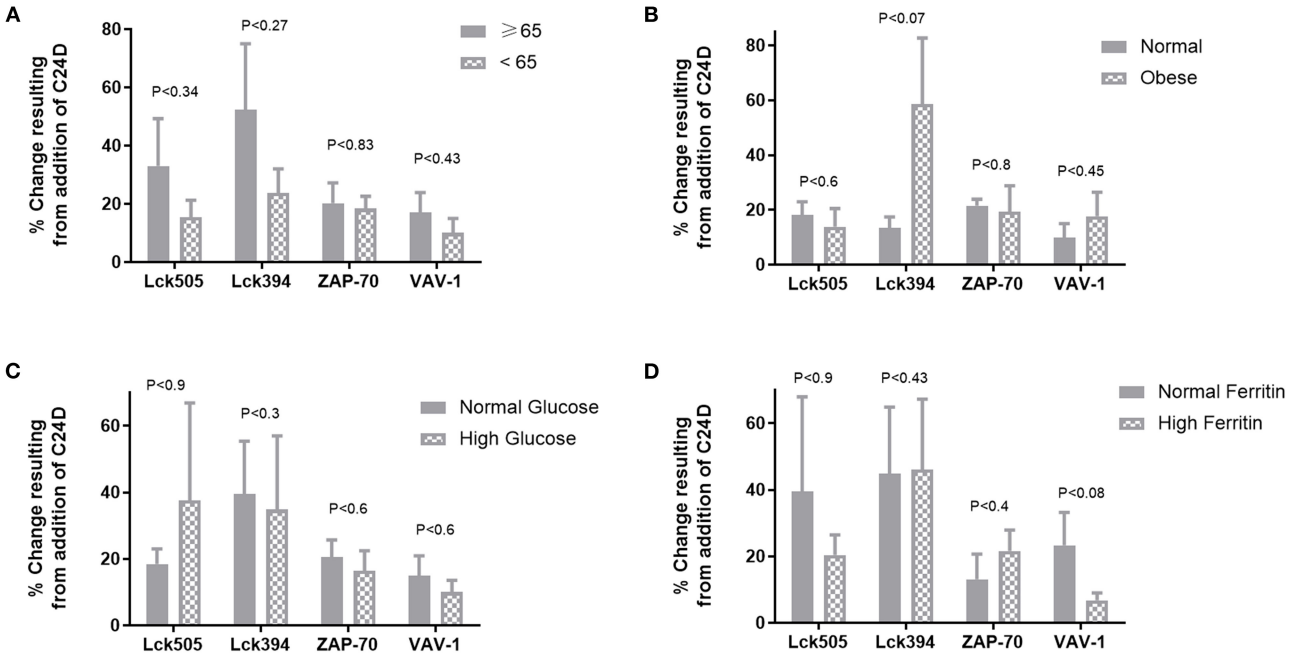

FIGURE 3 | Correlations between clinical characteristics and the protein phosphorylation pattern: no significant differences were found between age, weight, glucose levels or ferritin values and the change of phosphorylation in proteins, normalized to control without C24D. **(A)** Age, in two groups ≥65 vs. <65 years of age, $p < 5.7 \times 10^{-5.}$ **(B)** Weight, in two groups, normal weight: 66.4 ± 9.1 vs. obese: 109.3 ± 6.02, $p < 0.00004$ **(C)** Glucose, in two groups, normal glucose: 91.9 ± 0.02 vs. high glucose: 144.3 ± 54, $P < 0.027$ **(D)** Ferritin, in two groups, normal ferritin: 316 ± 145 vs. high ferritin: 712 ± 79, $p < 0.0028$.

observed in patients with normal and high glucose levels ($p < 0.0004$, **Figure 3C**) and in patients with normal and high ferritin values ($p < 0.003$, **Figure 3D**).

DISCUSSION

It has been reported that patients infected with the SARS-CoV-2 are immune-suppressed (16–20). In some studies, immunosuppression was described as a consequence of a drastic reduction in the number of both CD4+ and CD8+ T cells in moderate and severe COVID-19 patients (21). This is consistent with reports that viruses developed immune evasion strategies similar to those deployed by tumors (22, 23).

In this short report, we demonstrated that treatment of PBMCs from ten hospitalized COVID-19 patients with C24D resulted in the reactivation of CD45 key-signaling molecules: Lck, ZAP-70 and VAV-1. Binding of C24D to the CD45 receptor provoked a decrease in the phosphorylation of the inhibitory tyrosine 505 and an increase in the phosphorylation of the tyrosine 394 in Lck, inducing its activation. The tyrosine 493 in ZAP70 and tyrosine 174 in VAV-1 were phosphorylated, resulting in TCR activation (24, 25).

Given the pivotal role of CD45 in the immune system (26–28), it is not surprising that viruses interfere with the activity of CD45 to dampen the immune response.

Similar to our findings on TNBC tumors (8), viral interference with the functions of the receptor tyrosine phosphatase CD45 have been widely reported. It was demonstrated that CD45 functions are crucial for stimulating a protective immune response against Herpes simplex virus type 1 (HSV-1) (29). When CD45 is down-regulated, the immune system fails to control HSV-1 infection and to prevent HSV-1 associated encephalitis. In adenovirus, the secreted protein sec49K derived from the viral protein E3/49K was found to bind to CD45 receptor resulting in a significant decrease in the activation of Src tyrosine proteins kinases and ZAP-70, causing suppression of activation of T and NK cells (13).

In this study, we found that on binding to CD45's extracellular domain of PBMCs of COVID-19 patients, C24D reverses the deactivation of kinases involved in CD45/TCR signaling. Conversely, in PBMCs from healthy volunteers, C24D did not change the CD45 signaling pattern, suggesting that C24D acts only on immune-suppressed cells. The focus on T cell re-activation originated from reported studies which demonstrated that the severity of COVID-19 inversely correlates with T-cell immunity of the host. Although T cells cannot prevent infections, in COVID-19 patients, killer T cells mean the difference between a mild infection and a severe one (30, 31).

The five hospitalized COVID-19 patients who, at the time blood was drawn, already had negative RT-PCR results, presented the same response to C24D treatment as did the five RT-PCR positive Covid-19 patients. This suggests that some immunosuppression may endure for some time after the elimination of the SARS-CoV-2 virus.

Age-associated alterations in the immune system contribute to the increased incidence and severity of infectious diseases in elderly patients (32). There is a consensus that the elderly (≥65 age) is the population group most vulnerable to COVID-19. We found no statistically significant difference in the effect of C24D on CD45 signaling in COVID-19 patients under 65 and over 65 years of age, suggesting that C24D might be effective also in patients ≥65 years of age.

In each of the weight, ferritin and glucose values sub-groups, no statistically significant differences in the phosphorylation of Lck505, Lck394, ZAP70, and VAV-1 were observed. Ferritin values aroused our attention due to C24D being a peptide derived from the placental immunomodulatory ferritin (9).

Due to the small cohort of patients, it is possible that a correlation between the effect of treatment with the C24D peptide on CD45 downstream signaling and patients' clinical characteristics was masked.

Other report limitation was related to the hospital ethics committee that did not allow us to perform experiments with serum or plasma from COVID-19 patients due to standard biosecurity and institutional safety. The quantity of PBMCs recovered from each patient was minimal and lyzed for virus neutralization. The amount of cells only sufficed for the study of protein phosphorylation and not for functional assays.

COVID-19 is a multifaceted illness that affects different people in different ways (33). The long-term effectiveness of COVID-19 vaccines is yet to be determined and may be vulnerable to virus mutations (33). Thus, there is a need to find additional therapeutic strategies to tackle COVID-19.

In spite of the relatively small number of patients in this report, the results showed that C24D rescued CD45 signaling. T cell re-activation through the CD45 molecular pathway is potentially a new therapeutic strategy against immunosuppression induced by coronavirus. Given that some cases of immune overactivation (cytokine storm) have been reported (34, 35), a molecule such as C24D, which does not activate, but rather resets the immune system, could likely serve as an important therapeutic option in the treatment of the current, and possibly future generations of SARS-CoV-2.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Meir Medical Center and Israel Ministry of Health. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DA performed and managed the ethical approval for patient recruitment and sample collection. YP analyzed and contributed to data analysis and visualization. ER contributed to recruitment, sample collection and data analysis, and visualization. NG and JL performed the experiments described in this manuscript in patients and healthy volunteers' samples. RY and CC revised and critically edited the manuscript. AR wrote, reviewed and edited the manuscript, and analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.675963/full#supplementary-material

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Comparative Study of Acute Lung Injury in COVID-19 and Non-COVID-19 Patients

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Background: Amid the coronavirus disease 2019 (COVID-19) pandemic, we analyzed clinical characteristics of acute lung injury (ALI) in COVID-19 patients and reported their similarity and dissimilarity to those of non-COVID-19 patients in the intensive care unit (ICU).

Methods: We reported on 90 COVID-19 and 130 non-COVID-19 ALI patients in the ICUs of multiple centers. Demographic data, medical histories, laboratory findings, and radiological images were analyzed and compared between the two cohorts and within each cohort between survivors and non-survivors. For ALI survivors, clinical characteristics before and after treatment were also compared.

Findings: Aberrations in blood parameters, such as leukocytosis, neutrophilia, and thrombocytopenia, were observed in both cohorts. More characteristic abnormalities, including significantly higher red cell distribution width (RDW), C-reactive proteins, and lactic dehydrogenase (LDH) but lower troponin (TnT) and procalcitonin, were observed in the COVID-19 cohort than in the non-COVID-19 cohort, whereas D-dimer levels showed a similar elevation in both cohorts. The COVID-19 cohort also showed more diversified CT patterns where severe features such as consolidations and crazy paving patterns were more frequently observed. Multivariate analysis indicated that age, fever symptom, prothrombin time, procalcitonin, partial pressure of carbon dioxide, oxygenated hemoglobin, and crazy paving patterns in CT scans were independent risk factors associated with COVID-19.

Interpretation: Comparison of ALI characteristics between COVID-19 and non-COVID-19 patients in the ICU setting provided insight into the pathogenesis of ALI induced by different risk factors, suggesting distinct treatment plans.

Keywords: COVID-19, intensive care unit, acute lung injury, acute respiratory distress syndrome, treatment

BACKGROUND

Following the novel viral pneumonia that broke out in December 2019, the responsible pathogen was identified as severe acute respiratory syndrome (SARS) coronavirus 2 (SARS-CoV-2), and the illness was later named as coronavirus disease 2019 (COVID-19) (1). Strikingly, as of November 10, 2020, COVID-19 has swept across the world, infecting over 50 million people with a death rate exceeding 2.5% (2). With no valid treatment, the COVID-19 pandemic posed an unprecedented challenge to global public health.

Now, we learn that COVID-19 is far more than a typical pulmonary disease. Nevertheless, the highly infective SARS-CoV-2 is mainly transmitted *via* aerosol (3) with the infection beginning predominantly in the lungs, where acute lung injury (ALI) progressed as the illness worsened (4). ALI can develop into acute respiratory distress syndrome (ARDS) as hypoxemia worsens, leading to a high mortality rate among severe ALI patients (5). Studies in the early period of the COVID-19 breakout identified a 67–85% mortality in patients admitted to intensive care units (ICUs), which was attributed to ARDS (6–8). In contrast, general ARDS mortality in ICU patients was estimated as 35.3% (9). Moreover, ARDS mortality after ICU admission in SARS patients was 52.2% (10).

In this study, we focused on the comparison of ALI/ARDS characteristics between COVID-19 and non-COVID-19 patients in the ICU scenario, looking for insight into the heightened death incidence of COVID-19-induced ALI and propose an efficacious treatment plan.

METHODS

Study Design

In this retrospective study, we reported 90 COVID-19 ALI patients (admitted between January 2020 and April 2020) and 130 non-COVID-19 ALI patients (admitted between January 2017 and October 2019) from different ICUs of multiple centers. For all selected ICU patients, they were diagnosed with ARDS upon ICU admission. ARDS was defined when positive end expiratory pressure (PEEP) or continuous positive airway pressure (CPAP) was >5 cmH_2O and PaO_2/FiO_2 was <300 mmHg, following a classic Berlin Definition (11). Exclusion criteria were as follows: (1) pediatric patients <18 years old; (2) pregnant or lactating women patients; and (3) patients with malignant tumors, immunodeficiency, or terminal illness. A flowchart indicating the inclusion and exclusion criteria of patients is shown in **Figure 1**. As a result, 130 ALI patients admitted to the ICU in the Affiliated Hospital of Jiangsu University (TAHJU) were selected as the non-COVID-19 cohort. Patient consents were acquired, and the study was approved by the Medical Ethics Committee of TAHJU. In parallel, 90 ALI patients in the COVID-19 cohort were admitted to the First People's Hospital of Jiangxia District (TFPHJD) at Wuhan and Huangshi Central Hospital (HCH) at Huangshi city, both in the Hubei Province of China. ALI/ARDS management followed the published formal guidelines (12–14). Patient information remains anonymous and written consent was waived. The study was approved by the Ethics Commissions of TFPHJD and HCH. Patient data for comparison were gathered before and after stays in ICU. More details of the study design can be found in the **Supplementary Material**.

Procedure

Details of patient procedures can be found in the **Supplementary Material**. In particular, all COVID-19 patients were received at TFPHJD and HCH and diagnosed following the standard procedure, and all COVID-19 ICU patients were admitted following published criteria (15) and treated by following the published guidelines during early outbreak (16). For patients with clinical symptoms, such as fever, cough, and radiological abnormality, throat swabs were gathered for SARS-CoV-2 RNA detection by gene sequencing or by real-time RT-PCR as previously reported (7).

Statistical Analysis

The categorical variables were described as frequency rates and percentages, and continuous variables were applied to describe the median and interquartile range (IQR) values. All data were collected and compared between the COVID-19 cohort and the non-COVID-19 cohort. Comparison of continuous variables between the two cohorts was analyzed with Mann–Whitney test, and c^2 test was used to compare the categorical variables. These statistical methods followed a published method (17), and methodological details can be found in the **Supplementary Material**. For key variables with a $p < 0.05$ in the univariate analysis, multivariate logistic regression analysis was performed to explore the independent risk factors associated with either the COVID-19 or the non-COVID-19 cohort. A $p < 0.05$ was considered statistically significant.

RESULTS

A total of 220 ICU patients were hospitalized, namely, a COVID-19 cohort of 90 and a non-COVID-19 (non-viral) cohort of 130. Median age was 68.0 (IQR: 57.0–76.0); 33.2% were female and 31.8% had a history of smoking (**Table 1**). The median ICU stay was 13 days, and the eventual mortality rate reached 44.5%. Compared to the non-COVID-19 cohort, the COVID-19 cohort showed younger age, fewer male patients, shorter ICU stays, and higher death rate ($p < 0.05$), although smoking history had a similar effect on both cohorts; 74.5% of patients had comorbidity, with the COVID-19 cohort (65.5%) having a significantly lower proportion of patients with comorbidity than the non-COVID-19 cohort (80.8%). Hypertension, diabetes, bronchitis, and cardiovascular disease were the most common comorbidities. The frequency of each comorbidity showed no significant difference between the two cohorts. Despite different disease pathogeneses, patients in both cohorts experienced similar symptoms, including cough, fever, dyspnea, expectoration, fatigue, and vomiting (**Table 1**). Notably, the COVID-19 cohort had significantly more patients with fever but fewer showing expectoration. In our previous study, significantly fewer COVID-19 patients experienced

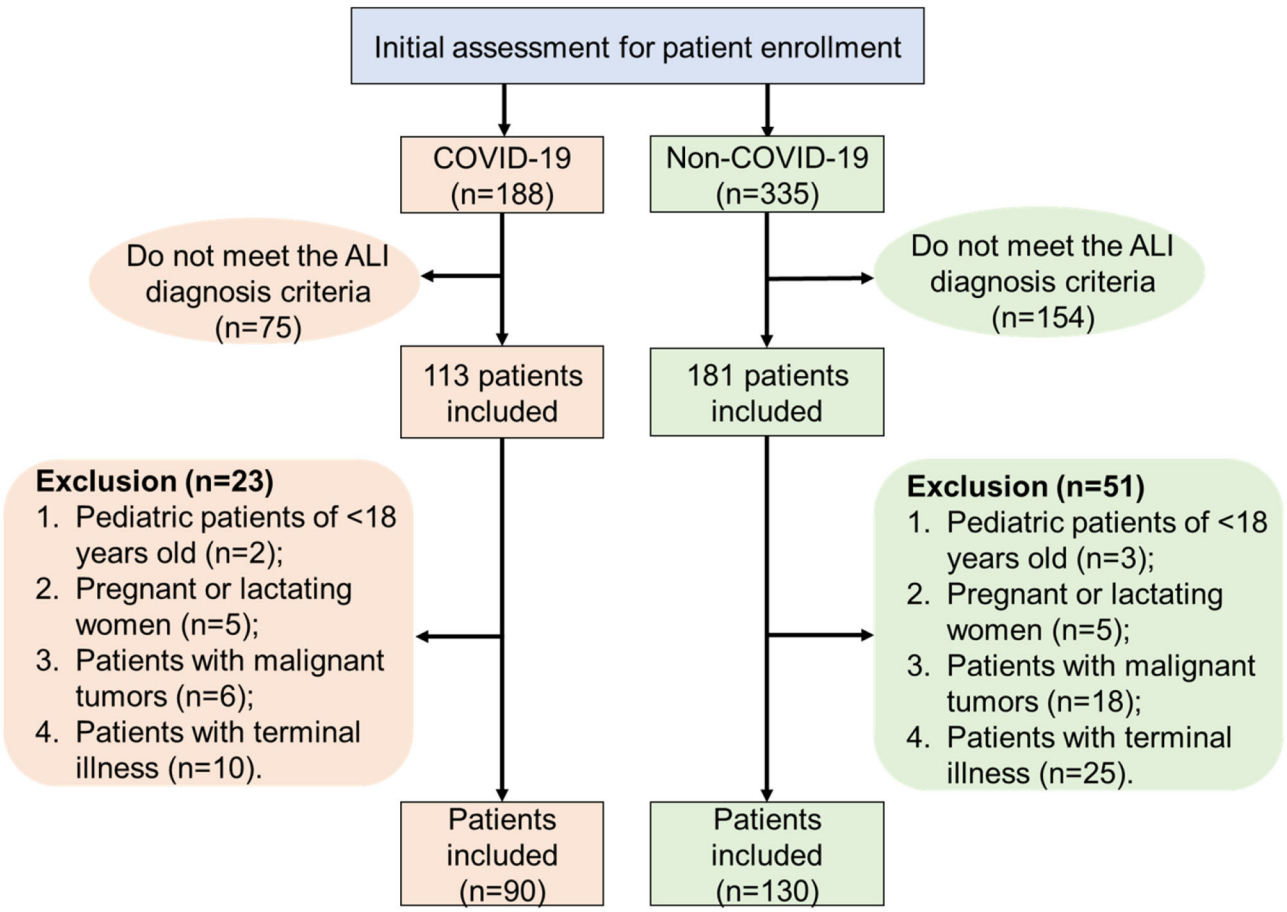

FIGURE 1 | A flowchart that illustrates patient inclusion and exclusion criteria in the patient selection procedure.

expectoration than influenza patients despite sharing flu-like symptoms (17).

Baseline blood characteristics for all patients upon ICU admission are shown in **Table 2**. Compared to the non-COVID-19 cohort, the COVID-19 cohort showed a higher proportion of patients with leukocytosis or thrombocytopenia, but a lower proportion with neutrophilia or monocytosis. Similarly, both cohorts exhibited an overwhelmingly low red blood cell count and low levels of hemoglobin or hematocrit, indicating serious anemia in ALI patients regardless of pathogenesis. Nevertheless, a notably higher proportion of COVID-19 patients with elevated values of red cell distribution width (RDW) compared with that of non-COVID-19 patients was found, establishing a distinctive feature of COVID-19 infection. This finding was consistent with another report (18). For coagulation factors, abnormally increased prothrombin time, activated partial thromboplastin time, thrombin time, D-dimer level, international normalized ratio, and decreased fibrinogen level were found in a substantial number of ALI patients in both cohorts. Among them, D-dimer elevation has been reported to correlate with the severity of COVID-19 (19, 20). In our study, most ALI patients showed heightened D-dimer levels, but these were indistinguishable between the COVID-19 or non-COVID-19 cohorts. In addition, ALI patients showed reduced protein and ionic concentrations, and augmented levels of many metabolic proteins and enzymatic biomarkers (**Table 2**), including C-reactive proteins (CRPs), bilirubin, ALT, AST, BUN, LDH, and CPK. Among them, compared to the non-COVID-19 cohort, COVID-19 patients demonstrated much higher levels of CRP and LDH, but a dramatically lower level of TnT and procalcitonin.

Next, arterial blood gas profiles were examined for all ICU ALI patients (**Table 3**). Compared to the non-COVID-19 cohort, the COVID-19 cohort exhibited similar levels of blood parameters such as acidity and base excess but significantly lower levels of actual bicarbonate, partial pressure of carbon dioxide or oxygen, oxygen saturation, and oxygenated hemoglobin. In parallel, CT examination was performed for all patients upon ICU admission and image patterns were compared between the two cohorts (**Table 4**). The COVID-19 cohort showed infections with substantially expanded lung involvement, with a significantly higher portion of ALI patients with bilateral lung involvement, multilobular lesions (with lobe number = 4, 5), and more lesions in each lobe. Specific CT patterns, such as consolidation and pleural effusion, were found significantly more frequently

TABLE 1 | Demographic data, medical history, and clinical symptoms of 220 ALI patients.

	Total ($n = 220$)	COVID-19 ($n = 90$)	Non-COVID-19 ($n = 130$)	p-value
Age	68.0 (57.0–76.0)	60.5 (46.8–71.3)	70.0 (63.8–78.0)	<0.0001
Gender, female *N* (%)	73 (33.2%)	39 (43.3%)	34 (26.2%)	0.008
Smoking history	70 (31.8%)	30 (33.3%)	40 (30.8%)	0.688
ICU stay, day	13.0 (9.0–23.8)	10.0 (6.0–20.3)	15.0 (12.0–27.0)	<0.0001
Mortality, *N* (%)	98 (44.5%)	52 (57.8%)	46 (35.4%)	0.001
Comorbidity				
Hypertension	86 (39.1%)	34 (37.8%)	52 (40.0%)	0.740
Diabetes	42 (19.1)	15 (16.7%)	27 (20.8%)	0.447
Bronchitis	31 (14.1%)	9 (10.0%)	22 (16.9%)	0.147
Cardiovascular diseases	24 (10.9%)	6 (6.7%)	18 (13.8%)	0.093
Hepatitis B	9 (4.1%)	3 (3.3%)	6 (4.6%)	0.741
Intracerebral hemorrhage	6 (2.7%)	4 (4.4%)	2 (1.5%)	0.229
Renal dysfunction	6 (2.7%)	2 (2.2%)	4 (3.1%)	1.000
Hypothyroidism	5 (2.3%)	1 (1.1%)	4 (3.1%)	0.651
Gallstone	4 (1.8%)	4 (4.4%)	0 (0)	0.027
Cholecystitis	3 (1.4%)	2 (2.2%)	1 (0.8%)	0.569
Renal calculi	3 (1.4%)	2 (2.2%)	1 (0.8%)	0.569
Gout	2 (0.9%)	2 (2.2%)	0 (0)	0.166
Symptoms				
Cough	144 (65.5%)	65 (72.2%)	79 (60.8%)	0.079
Fever	127 (57.7%)	67 (74.4%)	60 (46.2%)	<0.0001
Dyspnea	86 (39.1%)	33 (36.7%)	53 (40.8%)	0.540
Expectoration	77 (35.0%)	22 (24.4%)	55 (42.3%)	0.006
Fatigue	59 (26.8%)	31 (34.4%)	28 (21.5%)	0.034
Vomiting	33 (15.0%)	11 (12.2%)	22 (16.9%)	0.337
Diarrhea	20 (9.1%)	11 (12.2%)	9 (6.9%)	0.179
Chest pain	18 (8.2%)	3 (3.3%)	15 (11.5%)	0.043
Abdominal pain	15 (6.8%)	6 (6.7%)	9 (6.9%)	0.941

in the COVID-19 cohort. More characteristically, crazy paving patterns, linear opacity, rounded opacity, halo sign, nodules, tree-in-bud sign, air bronchogram, and interlobular septal thickening were more frequently observed in the COVID-19 cohort than the non-COVID-19 cohort, highlighting explicit CT features caused by SARS-CoV-2 infection.

Variables with a $p < 0.05$ in the previous univariate analysis were put into multivariate logistic regression analysis, and results are shown in **Table 5**. It can be concluded that age, fever symptom, prothrombin time, procalcitonin, $PaCO_2$, HbO_2, and crazy paving patterns in CT scans are independent risk factors for differentiating COVID-19 ALI patients from non-COVID-19 ALI patients. Compared to the non-COVID-19 cohort, the COVID-19 cohort exhibited more inclination to younger population, experiencing fever, lengthened prothrombin time, and augmented lung involvement and crazy paving patterns in CT features. In addition, the COVID-19 patients also showed higher disposition to demonstrate abnormally lower levels of procalcitonin, $PaCO_2$, and HbO_2.

Critically ill ALI patients typically developed hypoxemia, dyspnea, and even respiratory failure requiring invasive or non-invasive oxygen support (**Table 6**). For the COVID-19 cohort, patients were treated with an array of antiviral drugs, including 16.7% with oseltamivir, 44.4% with arbidol, 53.3% with ribavirin, and 61.1% with α-interferon. They were also given a variety of antibiotics, including 18.9% with sulbactam/cefoperazone sodium, 38.9% with piperacillin/tazobactam sodium, 43.3% with imipenem/cilastatin, and 50.0% with moxifloxacin. For the non-COVID-19 cohort, 50% of patients were given imipenem/cilastatin, 32.3% ceftazidime, 30.0% piperacillin/sulbactam sodium, and 26.2% tigecycline. As a result, mortality was 57.8% in the COVID-19 cohort and 35.4% in the non-COVID-19 cohort.

Within the COVID-19 cohort, baseline characteristics and radiological parameters were compared between survivors and non-survivors (**Supplementary Tables 1**–**5**). High age was found as a risk factor for mortality, while no substantial difference was found between survivors and non-survivors in their other demographic information, medical history, clinical symptoms, and CT patterns upon ICU admission. Between survivors and non-survivors, blood parameters were found to be similar; however, many arterial blood gas features were significantly different. Compared to survivors, non-survivors exhibited lower pH, PaO_2, SO_2, PaO_2/FiO_2, $aADO_2$, HbO_2, and tHb, but higher

TABLE 2 | Laboratory testing results of ALI patients in the COVID-19 and non-COVID-19 cohorts.

	Normal range	Total (n = 220)	COVID-19 (n = 90)	Non-COVID-19 (n = 130)	p-value
Blood count panel					
White blood cells, $\times10^9$/L	3.5–9.5	9.2 (5.9–14.7)	7.5 (4.8–14.3)	10.2 (6.9–15.3)	0.003
>9.5		107 (48.6%)	34 (37.8%)	73 (56.2%)	0.007
Neutrophils, $\times10^9$/L	1.8–6.3	8.4 (4.4–14.7)	5.9 (3.2–13.1)	9.6 (5.8–15.2)	0.0003
>6.3		136 (61.8%)	42 (46.7%)	94 (72.3%)	0.0001
Lymphocytes, $\times10^9$/L	1.1–3.2	0.6 (0.4–1.1)	0.6 (0.4–1.1)	0.6 (0.4–1.1)	0.871
>3.2		7 (3.2%)	5 (5.5%)	2 (1.5%)	0.125
Monocytes, $\times10^9$/L	0.1–0.6	0.5 (0.3–0.8)	0.3 (0.2–0.5)	0.6 (0.4–1.0)	<0.0001
>0.6		79 (35.9%)	15 (16.7%)	64 (49.2%)	<0.0001
Eosinophils, $\times10^9$/L	0.02–0.52	0.0 (0.0–0.04)	0.0 (0.0–0.03)	0.0 (0.0–0.05)	0.330
>0.52		8(3.6%)	2 (2.2%)	6 (4.6%)	0.476
Basophils, $\times10^9$/L	0–0.06	0.0 (0.0–0.02)	0.0 (0.0–0.01)	0.0 (0.0–0.02)	0.537
>0.06		18 (8.2%)	5 (5.6%)	13 (10.0%)	0.319
Red blood cells, $\times10^{12}$/L	4.3–5.8	3.1 (2.7–3.8)	3.4 (2.9–4.0)	3.0 (2.6–3.6)	0.001
<4.3		196 (89.1%)	78 (86.7%)	118 (90.8%)	0.337
Hemoglobin, g/L	130–175	104.0 (84.3–124.0)	105.5 (84.0–122.3)	103.0 (84.8–125.3)	0.761
<130		173 (78.6%)	73 (81.1%)	100 (76.9%)	0.456
Hematocrit, %	40–50	31.4 (26.4–37.9)	31.7 (26.4–36.8)	31.4 (26.5–38.6)	0.358
<40		177 (80.5%)	78 (86.7%)	99 (76.2%)	0.053
MCV, fL	82–100	91.8 (87.2–95.8)	90.0 (86.1–95.8)	92.6 (87.9–95.9)	0.128
<82		15 (6.8%)	7 (7.8%)	8 (6.2%)	0.639
MCH, pg	27–34	30.2 (29.1–31.5)	30.6 (29.6–32.2)	29.8 (28.9–31.3)	0.004
<27		21 (9.5%)	6 (6.7%)	15 (11.5%)	0.227
MCHC, g/L	316–354	326.0 (314.0–338.0)	331.0 (317.0–347.5)	322.0 (310.0–334.0)	0.001
<316		63 (28.6%)	19 (21.1%)	44 (33.8%)	0.040
RDW, %	11.5–17.8	17.0 (13.2–41.5)	42.4 (39.1–47.5)	13.7 (12.5–15.1)	<0.0001
>17.8		102 (46.4%)	89 (98.9%)	13 (10.0%)	<0.0001
Platelets, $\times10^9$/L	125–350	158.5 (88.5–242.3)	148.0 (76.0–275.0)	162.0 (99.8–233.3)	0.293
<125		84 (38.2%)	42 (46.7%)	42 (32.3%)	0.031
MPV, fL	7.4–12.5	11.0 (10.0–12.4)	10.8 (9.9–12.6)	11.1 (10.1–12.3)	0.497
>12.5		53 (24.1%)	22 (24.4%)	31 (23.8%)	0.919
PDW, %	9–17	16.4 (15.1–17.0)	16.4 (14.9–17.2)	16.4 (15.2–17.0)	0.948
>17		54 (24.5%)	23 (25.6%)	31 (23.8%)	0.772
Coagulation panel					
Prothrombin time, s	9–13	14.1 (12.4–15.7)	14.8 (13.5–17.4)	13.3 (11.6–15.3)	<0.0001
>13		145 (65.9%)	78 (86.7%)	67 (51.5%)	<0.0001
INR	0.8–1.2	1.2 (1.0–1.4)	1.2 (1.1–1.5)	1.2 (1.0–1.4)	0.497
>1.2		104 (47.3%)	42 (46.7%)	62 (47.7%)	0.881
aPPT, s	23.3–32.5	30.7 (26.6–37.0)	31.7 (28.6–37.1)	29.2 (24.5–37.7)	0.021
>32.5		92 (41.8%)	39 (43.3%)	53 (40.8%)	0.705
Thrombin time, s	14–21	18.1 (16.8–20.3)	17.4 (15.9–18.9)	18.9 (17.5–22.0)	<0.0001
>21		50 (22.7%)	10 (11.1%)	40 (30.8%)	0.001
Fibrinogen, g/L	2–4	4.3 (2.7–5.7)	4.8 (3.8–5.8)	3.8 (2.2–5.5)	0.012
<2		28 (12.7%)	5 (5.6%)	23 (17.7%)	0.008
D-dimer, mg/L	<0.55	3.4 (0.9–7.5)	3.6 (0.8–71)	2.8 (1.0–8.5)	0.738
>0.55		180 (81.8%)	71 (78.9%)	109 (83.8%)	0.349
Metabolic panel					
C-reactive protein, mg/L	0–10	26.7 (7.8–99.1)	45.8 (14.2–88.0)	18.9 (4.0–120.6)	0.026
>10		160 (72.7%)	78 (86.7%)	82 (63.1%)	0.0001
Total bilirubin, mmol/L	3–22	14.1 (7.9–23.1)	12.9 (7.7–22.9)	14.4 (8.0–23.3)	0.421

(Continued)

TABLE 2 | Continued

	Normal range	Total (n = 220)	COVID-19 (n = 90)	Non-COVID-19 (n = 130)	p-value
>22		58 (26.4%)	24 (26.7%)	34 (26.2%)	0.932
Direct bilirubin, mmol/L	0–5	4.7 (2.9–8.5)	5.3 (2.8–8.6)	4.5 (2.9–8.3)	0.838
>5		102 (46.4%)	46 (51.1%)	56 (43.1%)	0.240
Indirect bilirubin, mmol/L	0–19	11.1 (7.8–19.8)	9.7 (6.6–13.4)	13.2 (8.9–46.2)	<0.0001
>19		58 (26.4%)	5 (5.6%)	53 (40.8%)	<0.0001
ALT, U/L	9–50	41.1 (20.6–71.9)	40.7 (17.1–68.9)	42.5 (22.8–72.3)	0.672
>50		84 (38.2%)	32 (35.6%)	52 (40.0%)	0.505
AST, U/L	15–40	49.8 (29.0–79.4)	46.0 (25.8–81.7)	54.3 (30.2–79.3)	0.686
>40		130 (59.1%)	53 (58.9%)	77 (59.2%)	0.960
ALP, U/L	32–126	94.1 (65.0–146.5)	90.5 (65.0–124.3)	103.0 (63.8–164.3)	0.080
>126		72 (32.7%)	19 (21.1%)	53 (40.8%)	0.002
GGT, U/L	12–73	53.5 (31.0–88.8)	45.8 (29.3–81.3)	61.0 (31.8–91.5)	0.136
>73		78 (35.5%)	28 (31.1%)	50 (38.5%)	0.263
Total protein, g/L	65–85	55.1 (49.1–63.3)	57.5 (50.6–64.6)	54.5 (46.2–62.2)	0.010
<65		171 (77.7%)	68 (75.6%)	103 (79.2%)	0.520
Albumin, g/L	40–55	30.0 (26.3–34.7)	31.9 (28.9–35.9)	28.0 (24.4–32.6)	<0.0001
<40		202 (91.8%)	82 (91.1%)	120 (92.3%)	0.750
Globulin, g/L	20–40	24.5 (20.5–29.2)	22.7 (19.2–28.7)	25.5 (21.9–29.2)	0.028
<20		46 (20.9%)	26 (28.9%)	20 (15.4%)	0.015
BUN, mmol/L	2.86–8.2	8.4 (5.4–13.2)	7.7 (4.8–11.6)	8.9 (6.5–14.7)	0.014
>8.2		115 (52.3%)	40 (44.4%)	75 (57.7%)	0.053
Creatinine, mmol/L	31.7–133	71.3 (55.1–103.7)	69.5 (57.8–109.0)	72.2 (53.6–99.7)	0.676
>133		36 (16.4%)	17 (18.9%)	19 (14.6%)	0.400
Carbon dioxide, mmol/L	20–29	25.0 (19.5–29.3)	22.0 (18.4–27.1)	26.0 (21.5–30.3)	0.006
>29		57 (25.9%)	17 (18.9%)	40 (30.8%)	0.048
Glucose, mmol/L	3.89–6.11	8.4 (6.3–12.2)	8.5 (6.8–12.2)	8.1 (5.8–12.3)	0.316
>6.11		170 (77.3%)	76 (84.4%)	94 (72.3%)	0.035
Potassium, mmol/L	3.5–5.3	3.9 (3.5–4.4)	3.9 (3.5–4.4)	3.9 (3.5–4.4)	0.753
<3.5		50 (22.7%)	22 (24.4%)	28 (21.5%)	0.613
Sodium, mmol/L	137–147	138.0 (134.4–142.1)	139.0 (135.1–143.0)	136.7 (133.6–141.0)	0.029
<137		98 (44.5%)	31 (34.4%)	67 (51.5%)	0.012
Total calcium, mmol/L	2.08–2.6	2.0 (1.9–2.1)	1.9 (1.8–2.1)	2.1 (1.9–2.3)	<0.0001
<2.08		144 (65.5%)	71 (78.9%)	73 (56.2%)	0.001
Biomarkers					
LDH, U/L	80–285	282.5 (199.3–420.8)	404 (228.2–619.6)	246.5 (178.0–335.5)	<0.0001
>285		107 (48.6%)	59 (65.6%)	48 (36.9%)	<0.0001
TnT, ng/mL	0–0.4	0.13 (0.03–0.94)	0.04 (0.01–0.20)	0.36 (0.07–1.27)	<0.0001
>0.4		76 (34.5%)	13 (14.4%)	63 (48.5%)	<0.0001
Myoglobin, U/L	25–58	73.8 (25.8–217.0)	64.2 (21.1–225.1)	82.9 (27.4–207.3)	0.474
>58		125 (56.8%)	48 (53.3%)	77 (59.2%)	0.385
CPK, U/L	38–174	126.5 (65.0–328.0)	114.9 (53.0–272.8)	165.5 (71.0–328.0)	0.146
>174		89 (40.5%)	30 (33.3%)	59 (45.4%)	0.073
CK-MB, U/L	0–25	25.4 (15.9–43.2)	21.1 (13.1–32.1)	29.1 (18.2–60.7)	0.003
>25		111 (50.5%)	36 (40.0%)	75 (57.7%)	0.010
Homocysteine, mmol/L	0–15	15.0 (12.8–23.5)	15.1 (12.8–22.7)	15.0 (12.8–23.8)	0.997
>15		108 (49.1%)	46 (51.1%)	62 (47.7%)	0.618
Procalcitonin, ng/mL	<0.1	5.1 (0.6–20.9)	0.4 (0.1–1.6)	18.8 (5.5–25.8)	<0.0001
>0.1		201 (91.4%)	71 (78.9%)	130 (100.0%)	<0.0001

MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; MPV, mean platelet volume; PDW, platelet distribution width; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutamyl transferase; BUN, blood urea nitrogen; troponin T, TnT; CK-MB, creatine kinase isoenzyme; LDH, lactic dehydrogenase; CPK, creatine phosphokinase; INR, international normalized ratio; aPTT, activated partial thromboplastin time.

TABLE 3 | Arterial blood gas profiles for ALI patients in the COVID-19 and non-COVID-19 cohorts.

	Normal range	Total (n = 220)	COVID-19 (n = 90)	Non-COVID-19 (n = 130)	p-value
ICU panel					
pH	7.35–7.45	7.30 (7.25–7.33)	7.31 (7.26–7.34)	7.30 (7.25–7.32)	0.103
<7.35		192 (87.3%)	74 (82.2%)	118 (90.8%)	0.062
>7.45		11 (5.0%)	6 (6.7%)	5 (3.8%)	0.363
Base excess, mmol/L	−3–3	−5.2 (−8.1–1.8)	−5.8 (−9.4–1.5)	−4.5 (−7.3–1.9)	0.068
<-3		128 (58.2%)	58 (64.4%)	70 (53.8%)	0.117
>3		47 (21.4%)	19 (21.1%)	28 (21.5%)	0.939
[$aHCO_3^-$], mmol/L	22–27	20.9 (18.3–25.1)	19.7 (17.9–23.5)	22.6 (19.2–25.8)	0.004
<22		121 (55.0%%)	61 (67.8%)	60 (46.2%)	0.002
>27		40 (18.2%)	14 (15.6%)	26 (20.0%)	0.401
PaO_2, mmHg	80–100	62.6 (58.0–66.3)	60.7 (56.4–65.2)	62.7 (59.4–67.6)	0.006
<80		206 (93.6%)	90 (100.0%)	116 (89.2%)	0.001
$PaCO_2$, mmHg	35–45	49.3 (42.5–55.0)	47.4 (38.9–53.7)	50.5 (44.9–56.6)	0.004
<35		13 (5.9%)	10 (11.1%)	3 (2.3%)	0.009
>45		147 (66.8%)	50 (55.6%)	97 (74.6%)	0.003
SO_2, %	95–100	92.0 (88.2–94.0)	90.5 (87.0–93.3)	92.0 (90.0–94.0)	0.012
<95		186 (84.5%)	79 (87.8%)	107 (82.3%)	0.270
PaO_2/FiO_2	>300	217.3 (197.6–247.3)	218.6 (197.3–253.1)	211.9 (197.6–239.5)	0.367
≤300		220 (100.0%)	90 (100.0%)	130 (100.0%)	
$aADO_2$, mmHg	0–100	90.4 (78.4–107.4)	92.9 (79.7–108.5)	89.2 (76.8–105.7)	0.269
>100		78 (35.5%)	34 (37.8%)	44 (33.8%)	0.549
HbO_2,%	90–95	85.5 (80.9–89.3)	81.5 (76.5–85.5)	87.3 (84.8–90.4)	<0.0001
<90		176 (80.0%)	83 (92.2%)	93 (71.5%)	0.0002
MetHb, g/dL	0.2–0.8	0.4 (0.3–0.6)	0.4 (0.3–0.6)	0.4 (0.3–0.5)	0.094
<0.2		9 (4.1%)	3 (3.3%)	6 (4.6%)	0.741
tHb, g/dL	11.5–17.4	9.1 (8.2–9.9)	8.7 (7.5–10.0)	9.2 (8.3–9.8)	0.212
<11.5		208 (94.5%)	81 (90.0%)	127 (97.7%)	0.017

Actual bicarbonate, [$aHCO_3^-$]; $PaCO_2$, the partial pressure of carbon dioxide; PaO_2, the partial pressure of oxygen; SO_2, the oxygen saturation; PaO_2/FiO_2, the oxygenation index; $aADO_2$, alveolar-arterial oxygen pressure; tHb, the total hemoglobin; HbO_2, the oxygenated hemoglobin; MetHb, methemoglobin.

$PaCO_2$, portending more severely impaired gas exchange in their virus-infected lungs.

In parallel, within the non-COVID-19 cohort, baseline characteristics and radiological parameters were compared between survivors and non-survivors (**Supplementary Tables 6–10**). Instead of high age, male gender was found to be a risk factor for mortality, while no substantial difference was found between survivors and non-survivors in their other demographic information, co-existing disease, clinical symptoms, and most CT patterns. Paradoxically, many blood parameters were found to be worse in survivors than in non-survivors upon ICU admission, such as aberrantly higher values of white blood cells, neutrophils, D-dimers, LDH, CRP, and procalcitonin, and lower values of PaO_2/FiO_2, $aADO_2$, and HbO_2. This could be associated with various pathogeneses of non-COVID-19 ALI (**Supplementary Table 10**), including direct and indirect lung infection, mostly triggered by sepsis, and leading to various impacts on the patient after ICU admission.

After different treatment plans were adopted in the two cohorts, all arterial blood gas profiles in ALI survivors recovered well and their laboratory parameters and CT characters were significantly ameliorated upon transfer to non-ICU wards (**Supplementary Tables 11–16**). However, in the COVID-19 cohort, RDW, D-dimer, CRP, and procalcitonin were similarly abnormal compared to before treatment, showing a slow recovery in those values due to COVID-19 infection despite such ICU patients having been discharged from critical care. In contrast, D-dimer, CRP, and procalcitonin were significantly improved in survivors of the non-COVID-19 cohort after treatment.

DISCUSSION

As pulmonary injuries (e.g., pneumonia and aspiration) may cause direct damage to alveolar epithelium, extrapulmonary insults (e.g., systemic infection, trauma, or other non-pulmonary acute disease) could pose an indirect threat to the integrity of the capillary endothelium. Such impairment can lead to the production of pro-inflammatory cytokines, induction of cell death and leakage at intercellular junctions in the alveolar capillary membrane, and eventual migration of immune cells from microvessels into the alveolar airspace that initiates diffuse alveolar damage (DAD) (21). In the early stage of DAD, an exudative phase takes place where polymorphonuclear leukocytes (e.g., neutrophils and eosinophils), platelets, and plasma proteins

TABLE 4 | Radiological findings of ALI patients in the COVID-19 and non-COVID-19 cohorts.

	Total ($n = 220$)	COVID-19 ($n = 90$)	Non-COVID-19 ($n = 130$)	p-value
Lung involvement				
Unilateral	88 (40.0%)	21 (23.3%)	67 (51.5%)	<0.0001
Bilateral	132 (60.0%)	69 (76.7%)	63 (48.5%)	<0.0001
Number of lobes with lesions				
0	0	0	0	
1	42 (19.1%)	9 (10.0%)	33 (25.4%)	0.004
2	55 (25.0%)	11 (12.2%)	44 (20.0%)	0.0003
3	38 (17.3%)	13 (14.4%)	25 (19.2%)	0.356
4	68 (30.9%)	45 (50.0%)	23 (17.7%)	<0.0001
5	17 (7.7%)	12 (13.3%)	5 (3.8%)	0.018
Location of lesions				
Left upper lobe	79 (35.9%)	39 (43.3%)	40 (30.8%)	0.056
Left lower lobe	150 (68.2%)	69 (76.7%)	81 (62.3%)	0.025
Right upper lobe	75 (34.1%)	43 (47.8%)	32 (24.6%)	0.0004
Right middle lobe	151 (68.6%)	78 (86.7%)	73 (56.2%)	<0.0001
Right lower lobe	168 (76.4%)	81 (90.0%)	87 (66.9%)	<0.0001
Predominant distribution				
Central	41 (18.6%)	11 (12.2%)	30 (23.1%)	0.042
Peripheral	93 (42.3%)	38 (42.2%)	55 (42.3%)	0.990
Central + Peripheral	87 (39.5%)	42 (46.7%)	45 (34.6%)	0.072
Characteristic pattern				
Ground glass opacity (GGO)	93 (42.3%)	31 (34.4%)	62 (47.7%)	0.051
Consolidation	65 (29.5%)	34 (37.8%)	31 (23.8%)	0.026
GGO + Consolidation	50 (22.7%)	25 (27.8%)	25 (19.2%)	0.137
Crazy paving pattern	36 (16.4%)	32 (35.6%)	4 (3.1%)	<0.0001
Linear opacities	84 (38.2%)	58 (64.4%)	26 (20.0%)	<0.0001
Rounded opacities	49 (22.3%)	45 (50.0%)	4 (3.1%)	<0.0001
Halo sign	28 (12.7%)	25 (27.8%)	3 (2.3%)	<0.0001
Nodules	35 (15.9%)	29 (32.2%)	6 (4.6%)	<0.0001
Tree-in-bud sign	19 (8.6%)	16 (17.8%)	3 (2.3%)	<0.0001
Air bronchogram	44 (20.0%)	31 (34.4%)	13 (10.0%)	<0.0001
Interlobular septal thickening	66 (30.0%)	56 (62.2%)	10 (7.7%)	<0.0001
Bronchiolar wall thickening	42 (19.1%)	34 (37.8%)	8 (61.5%)	<0.0001
Cavitation	11 (5.0%)	7 (7.8%)	4 (3.1%)	0.129
Pleural effusion	53 (24.1%)	29 (32.2%)	24 (18.5%)	0.019
Pericardial effusion	16 (7.3%)	9 (10.0%)	7 (5.4%)	0.195

in the alveolar capillary are recruited across the damaged ACM to flood interstitium and airspace, interacting with resident macrophages and forming edema (22). Consisting of cell debris, surfactant, cytokines, and other proteins, edema further promotes the formation of hyaline membrane that deposits along the alveolar walls and becomes characteristic of DAD, radiologically featured as patchy ground glass densities (23, 24). During this phase, the initial inflammation from primary insults are exacerbated, and gaseous exchange is seriously impeded.

Then, a proliferative phase follows as a self-repair mechanism when type II pneumocytes start to proliferate and differentiate into type I pneumocytes, to pump the edema into interstitium for drainage, to reproduce surfactants to lower pulmonary tension, and to summon macrophages to clear cell fragments (25, 26). As a result, the permeability barrier of the ACM may recover with improved oxygenation. Conversely, inability to clear alveolar fluid will lead to hypoxemia and hypercapnic acidosis resulting in acute respiratory failure.

In this study, compared to the COVID-19 cohort, the non-COVID-19 patients exhibited higher age, higher male ratio, longer ICU stay, and lower death rate, suggesting a higher incidence of non-viral ALI associated with older age and male gender, consistent with a previous report (5). While common clinical symptoms may include fever, dry cough, dyspnea, fatigue, and diarrhea for both cohorts, a much higher proportion of COVID-19 patients may experience fever but not expectoration.

ALI in the COVID-19 cohort is induced by SARS-CoV-2 infection, a direct pulmonary injury to the patients. In

the non-COVID-19 (non-viral) cohort, due to the diversity of primary disease. ALI may be caused by trauma, surgery (non-thoracic or thoracic), and gastrointestinal bleeding (non-pulmonary sepsis), showing a mixture of extrapulmonary and pulmonary induction of acute injury. For hospitalized ALI patients, leading comorbidities include hypertension, diabetes, bronchitis, and cardiovascular diseases, indicating an elevated instability of ACM in those with compromised immune systems.

Besides commonly observed aberrations in blood parameters due to systemic infection, such as leukocytosis, neutrophilia, and thrombocytopenia, more characteristic abnormalities in COVID-19 ALI patients were noticed when compared to their non-COVID-19 counterparts, including significantly higher RDW, CRP, and LDH but lower TnT and procalcitonin, whereas D-dimer levels showed similar elevation between the two cohorts. Furthermore, debilitated oxygenation in arterial blood was noticed more commonly in the COVID-19 cohort than in the non-COVID-19 cohort. After individual treatment and discharge from ICU, those characteristic abnormalities were ameliorated in the non-COVID-19 cohort and to a much lesser degree in the COVID-19 cohort where the characteristic parameters remained markedly out of the normal range, demonstrating a more sluggish recovery from direct lung infection by SARS-CoV-2.

Thoracic CT scan has been recommended as a diagnostic standard of positive COVID-19 following initial nucleic acid testing of pathogen (15, 27). Both asymptomatic and symptomatic COVID-19 patients demonstrated abnormality in CT images, typically progressing from unilateral or bilateral and multifocal ground glass opacities (GGOs) to intensified consolidation, until formation of reticular pave pattern (28, 29). In our study, COVID-19 patients showed more diversified and complicated CT patterns, with severe features such as consolidations and crazy paving patterns in comparison with non-COVID-19 patients.

Possible correlation between specific genes and the incidence of ALI/ARDS was unclear, except that the angiotensin-converting enzyme 2 (ACE2), actively expressed in alveolar epithelial and endothelial cells, is responsible for adjusting alveolar permeability and repairing lung injury and was also identified as the viral entry receptor for SARS-CoV and SARS-CoV-2

TABLE 5 | Multivariate analysis of independent risk factors for differentiating COVID-19 ALI from non-COVID-19 ALI cases.

Variables	Odds ratio (OR)	95% confidence interval (CI)	*p*-value
Age	0.947	0.912–0.984	0.005
Gender	1.712	0.451–6.500	0.429
Fever	6.283	1.573–25.090	0.009
White blood cells	0.980	0.892–1.076	0.666
Prothrombin time	1.162	1.051–1.286	0.003
TnT	0.589	0.315–1.104	0.099
CK-MB	1.004	0.996–1.013	0.303
Procalcitonin	0.845	0.785–0.909	<0.001
$PaCO_2$	0.842	0.759–0.933	0.001
HbO_2	0.642	0.533–0.775	<0.001
Lung involvement	3.746	0.846–16.592	0.082
Crazy paving pattern	32.169	4.558–227.056	<0.001

TABLE 6 | Treatment of ALI patients in the COVID-19 and non-COVID-19 cohorts.

	Total ($n = 220$)	COVID-19 ($n = 90$)	Non-COVID-19 ($n = 130$)	*p*-value
Oxygen support				
Invasive	146 (66.4%)	56 (62.2%)	90 (69.2%)	0.279
Non-invasive	74 (33.6%)	34 (37.8%)	40 (30.8%)	
Antibiotics				
Sulbactam/cefoperazone sodium	17 (7.7%)	17 (18.9%)	0	
Moxifloxacin	45 (20.5%)	45 (50.0%)	0	
Piperacillin/tazobactam sodium	35 (15.9%)	35 (38.9%)	0	
Imipenem/cilastatin	104 (47.3%)	39 (43.3%)	65 (50.0%)	
Piperacillin/sulbactam sodium	39 (17.7%)	0	39 (30.0%)	
Ceftazidime	42 (19.1%)	0	42 (32.3%)	
Tigecycline	34 (15.5%)	0	34 (26.2%)	
Antiviral drugs				
Oseltamivir		15 (16.7%)	0	
Ribavirin		48 (53.3%)	0	
α-interferon		55 (61.1%)	0	
Arbidol		40 (44.4%)	0	
Sedatives				
Dexmedetomidine		0	79 (60.8%)	
Midazolam		0	35 (26.9%)	
Propofol		0	42 (32.3%)	

(30, 31). In our study, the COVID-19 cohort showed more severe ALI/ARDS with higher mortality and slower recovery among survivors, possibly because inhaled SARS-CoV-2 directly bound and downregulated ACE2, further weakening the lungs (32, 33). To further differentiate COVID-19 ALI characteristics from non-COVID-19 cases, multivariate analysis indicated that age, fever symptom, prothrombin time, levels of procalcitonin, $PaCO_2$ and HbO_2, and crazy paving patterns in CT manifestations are independent risk factors.

Antibiotics were commonly used in the ICU for ALI patients due to possible bacterial (co)infection. Here, in the COVID-19 cohort, highly effective and broad-spectrum antibiotics were recommended for treatment along with antiviral drugs. At the same time, mechanical ventilation, whether invasive or non-invasive, was applied to support respiration. However, antibiotic treatment did not improve the survival of severe COVID-19 patients, consistent with a recent report (34). Moreover, early administration of antibiotics in severe COVID-19 patients may cause antibiotic resistance in the late stage of treatment (35). Invasive and non-invasive oxygen support by mechanical ventilation did not significantly influence the survival of non-COVID-19 patients. Invasive but not non-invasive mechanical ventilation caused a higher fatality rate in the COVID-19 cohort, as described in other reports (36, 37). This suggested that intubation could further damage lung function and aggravate the condition of critically ill COVID-19 patients, whereas simpler, non-invasive respiratory support should be prioritized. Even with limited understanding of COVID-19 during the beginning of pandemic, antiviral treatments (α-interferon, ribavirin, arbidol, and oseltamivir) applied in our COVID-19 cohort enabled efficacious clearance of SARS-CoV-2 and improved patient prognosis. Validated treatment plans against COVID-19 extend to remdesivir, chloroquine, corticosteroids, convalescent plasma, and monoclonal antibodies (38).

This study has several limitations. First, the sample size is small, which leads to a high variability, causing bias. In this retrospective study, we collected patient data from three regional hospitals, where a limited number of ALI patients had been admitted. Second, given a large set of variables studied but the relatively small size of the sample, the validity of multivariate analysis may be weakened, although it did yield useful information. Third, most blood parameters were not continuously monitored or recorded in the ICU settings. This may restrict our understanding toward the disease development and so limit our conclusion.

In conclusion, our study highlights the distinction in ALI characteristics between severe COVID-19 and non-COVID-19 patients and demonstrated the efficacy of our current therapeutic regimen in the ICU scenario through improved survival of critically ill ALI patients. This work will enhance our understanding of this life-threatening illness and help develop refined treatment regimens leading to better outcomes.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: Data available on request due to privacy/ethical restrictions. Requests to access these datasets should be directed to Zhimin Tao, jsutao@ujs.edu.cn.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Affiliated Hospital of Jiangsu University, Jiangxia First People's Hospital, Huangshi Central Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JiaZ and ZT conceived the idea and designed the study. JiaZ, XH, DD, JinZ, LX, ZH, and ZT contributed to the data acquisition, processing, and table preparation. JiaZ, WX, and ZT contributed to the manuscript writing. JiaZ, XH, and ZT contributed to the statistical analysis. All authors reviewed and approved the manuscript submission.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Immune System Disequilibrium—Neutrophils, Their Extracellular Traps, and COVID-19-Induced Sepsis

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Equilibrium within the immune system can often determine the fate of its host. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the pathogen responsible for the coronavirus disease 2019 (COVID-19) pandemic. Immune dysregulation remains one of the main pathophysiological components of SARS-CoV-2-associated organ injury, with over-activation of the innate immune system, and induced apoptosis of adaptive immune cells. Here, we provide an overview of the innate immune system, both in general and relating to COVID-19. We specifically discuss "NETosis," the process of neutrophil release of their extracellular traps, which may be a more recently described form of cell death that is different from apoptosis, and how this may propagate organ dysfunction in COVID-19. We complete this review by discussing Stem Cell Therapies in COVID-19 and emerging COVID-19 phenotypes, which may allow for more targeted therapy in the future. Finally, we consider the array of potential therapeutic targets in COVID-19, and associated therapeutics.

Keywords: neutrophil, neutrophil extracellular trap, COVID-19, NETosis, immune system, innate, SARS-CoV-2

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INTRODUCTION

Equilibrium within the immune system can often determine the fate of its host. In sepsis, and many other inflammatory syndromes, the host's immune system performs a balancing act between the protection it offers through eradication of the offending pathogen, vs. the constant threat of an immune-mediated pathophysiological maelstrom.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the pathogen responsible for the coronavirus disease 2019 (COVID-19) pandemic (1).

SARS-CoV-2 breaches the alveolar epithelial membrane after binding to the human angiotensin-converting enzyme (ACE) 2 receptor. Subsequent viral RNAs serve as pathogen-associated molecular patterns (PAMPs), which are then sensed by Toll-like receptors (TLRs) (2). This results in epithelial cell activation, initiating a cascade of innate immune cell chemoattraction (**Figure 1**) (3). This immune cell infiltration causes acute respiratory distress syndrome (ARDS) locally in the lungs, and septic shock, coagulation dysfunction, and multiple organ dysfunction syndrome beyond the lungs (2). The mechanisms behind this distal organ injury are multiple, but immune dysregulation remains one of the main pathophysiological aetiologies. Neutrophil migration is affected with SARS-CoV-2 sepsis.

This review will focus on the innate immune system in COVID-19-induced sepsis and subsequently discusses stem cell therapies, emerging COVID-19 phenotypes and potential therapeutic targets.

THE INNATE IMMUNE SYSTEM

Once a pathogen enters the body, the innate immune system must recognise this as foreign and initiate an immune response, with a view to the pathogen's destruction or elimination. Cells of innate immunity originate largely from the common myeloid progenitor cells in the bone marrow before differentiating into cells such as macrophages, dendritic cells, and granulocytes, including neutrophils (4). These cells, amongst others, recognise PAMPs, which are then sensed by pathogen recognition receptors (PRRs) such as TLRs (2). This discriminates non-self from self and allows for phagocytosis, degradation and pro-inflammatory cytokine signalling to alert cells downstream to the invader.

One of the major weapons of the innate immune response is the macrophage, differentiated from the monocyte (5). Macrophages have a role in immune surveillance, phagocytosis of pathogens and clearance of cell debris or apoptotic cells (efferocytosis), as well as tissue remodelling after insult (6). They are activated through PAMPs or self-derived damage-associated molecular patterns (DAMPs) binding to PRRs like TLRs, NOD-like receptors (NLR), and RIG-I-like helicases. Macrophages then initiate signal transduction pathways, *via* mediators like myeloid differentiation primary response 88 (MyD88), that culminate in the production of pro-inflammatory cytokines and chemokines (7). Macrophages can be broadly separated into two opposing phenotypes, pro-inflammatory (M1) and anti-inflammatory (M2) (8). Originally, macrophages were thought to share their monocyte precursor with dendritic cells, displaying different cell surface markers like CD11b which aid their primary functions (6). However, more recent findings challenge this and suggest a lymphoid origin for dendritic cells (9). Dendritic cells (marked by CD11c) specialise in antigen presentation *via* major histocompatibility complex (MHC) molecules and serve as a link between the innate and adaptive immune system, recruiting lymphocytes (10).

Neutrophil maturation in the bone marrow, under the regulation of granulocyte colony stimulating factor (G-CSF), results in circulating short-lived mature neutrophils. PAMPs in infected tissue bind to PRRs, initiating a cascade of events, generating chemotactic, and haplotactic gradients (e.g., CXCL-2) that recruit activated neutrophils to the affected area (11). M2-like macrophages increase targeted neutrophil recruitment to injured tissue *via* CXCL-2 secretion. Corresponding CXCR-2 receptors on neutrophils bind CXCL-2, and appropriate transendothelial neutrophil migration occurs to the injured tissue (12). Once at the designated tissue, neutrophils have a variety of anti-microbial effector functions like phagocytosis, degranulation of toxic substances such as nitric oxide and reactive oxygen species, and the release of neutrophil extracellular traps (NETs) (11). Elimination of the invading organism can then successfully be achieved (13).

THE COMPLEMENT SYSTEM

Another component of the innate immune system is the complement system. It is an auxiliary defence mechanism of innate immunity. It was discovered in 1896 by Bordet and named for its ability to "complement" antibodies in their antimicrobial defence (14). It comprises of over 30 soluble serum proteins, mostly proteases, which are cleaved and activated in sequence to elicit an effect. Low-level complement system activity maintains homeostasis, with ability for rapid activation in response to trauma or infectious insults (15). Cellular invasion by SARS-CoV-2, and the subsequent "cytokine storm" results in an excessive and unsustainable complement system activation (16), with C3 activation resulting in the production of proinflammatory mediators and opsonisation of the pathogen, and the formation of the membrane attack complex (MAC) made up of C5–C9 (14).

Three pathways exist—the classical, lectin, and alternative pathways. They differ in their initial steps, with the classical pathway requiring C1q and an antibody-antigen interaction (17). The lectin pathway is immunoglobulin-independent, using PRRs like mannose-binding lectin to recognise foreign molecules (17). The alternative pathway is continuously activated by spontaneous hydrolysis of C3 and can be upregulated by bacterial endotoxins, yeasts and immunoglobulins (18). The pathways converge on C3 convertases, resulting in the production of proinflammatory mediators, opsonisation of the pathogen's surface with markers such as C3b and lastly, the formation of the membrane attack complex (MAC) made up of C5–C9 (14). The MAC inserts into the lipid bilayer, allowing the dysregulated transmembrane movement of water and ions and subsequent lysis of the target cell.

In COVID-19 infection, JAK-STAT signalling induces the expression of C3 and Factor B resulting in alternative pathway activation, and intracellular processing of complement proteins (19), while in the extracellular space SARS-CoV-2 activates the lectin pathway (20). Complement hyperactivation is key to the detrimental effects of COVID-19, shown in two recent studies where higher complement activation products correlated with increased disease severity (19, 21). Factor D, upregulated by COVID-19 and involved in the alternative pathway, is correlated with markers of endothelial cell injury (e.g., angiotensin 2) and coagulation (e.g., vWF), possibly contributing to the association between COVID-19 and coagulopathy (21). Potential therapeutic mechanisms to reduce or prevent complement-mediated damage in COVID-19 are discussed below.

SEPSIS AND COVID-19 CROSSTALK

There has been much advancement in the understanding of the host response to infectious disease in the last decade. It is now well accepted that the mechanisms of damage of pathogens are not limited to their direct virulence, but also the host's immune response to the pathogen. These secondary reactions can range from localised to systemic, and manifest in the form of sepsis — "a severe, potentially fatal, organic dysfunction caused by an

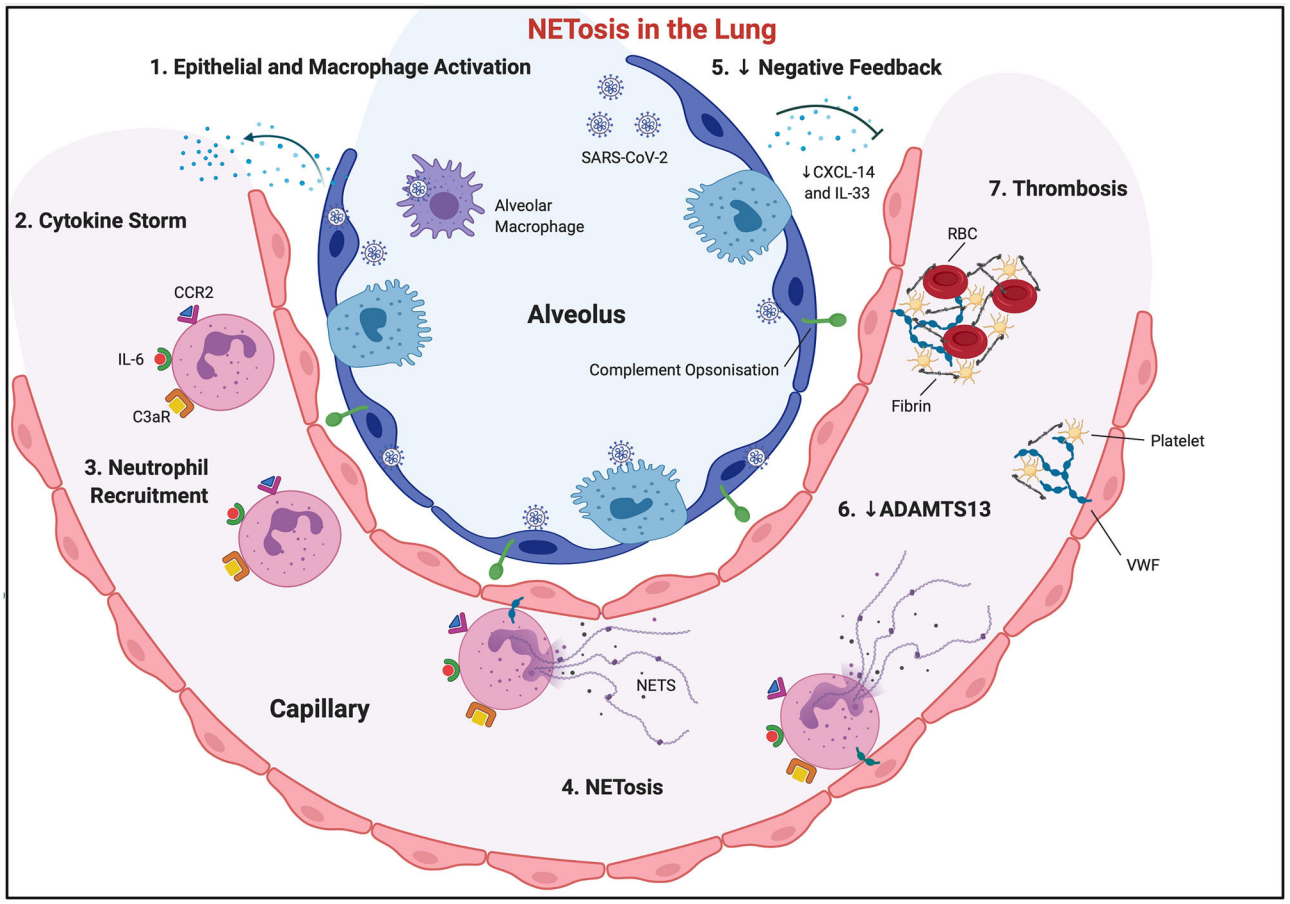

FIGURE 1 | NETosis in the lung. SARS-CoV-2 virus invades alveolar epithelial cells, activating them, along with local macrophage populations (1). This causes a surge in the production and systemic release of cytokines and chemokines (cytokine storm) (2), with subsequent neutrophil recruitment (3). Activated endothelial cells exocytose Von Willebrand Factor, which allows neutrophil and neutrophil extracellular trap adherence to the vascular wall. CCL2 and IL-6 encourage "NETosis" (4). Negative feedback loops may be inhibited, through reduced CXCL-14 and IL-33, allowing sustained and enhanced immune cell recruitment (5). Complement C3 is also released from NETs, further propagating "NETosis," and allowing opsonisation of surrounding tissue, which will ultimately necrose. NETs downregulate ADAMTS13, allowing VWF multimer development (6). Subsequent fibrinogen and platelet trapping occurs, which, along with red blood cells, encourages fibrin cross-linking, and ultimate vessel thrombosis.

inadequate or dysregulated host response to infection (sepsis-3)" (22). There were 48.9 million cases of sepsis worldwide in 2017, accounting for 20% of all deaths (23), marking this as an extremely important disease to better understand and manage.

The emergence of SARS-CoV-2 has dramatically changed the landscape of communicable disease. The most common causes of death in these patients are sepsis and respiratory failure. A relatively new phenomenon of viral sepsis is being widely seen (24) and is similar to the well-characterised bacterial sepsis in the literature. Scientific efforts are underway to understand the disease's effects on the body and immune system to repurpose and develop therapies to improve outcomes and save lives. The overlap between COVID-19 and sepsis for individual aspects of innate immunity is discussed below.

Cytokine Storm

Sepsis is a complex combination of various dysregulated immune response mechanisms. The cytokine storm occurs in the early phase (hours to days) of sepsis where PAMPs are recognised by PRRs on innate immune cells causing a "hyper-inflammatory" innate immune response (25). Influenza, a disease similar to SARS-CoV-2, was the first infectious disease where the cytokine storm was characterised in 2003 (26). Activated PRRs initiate signalling pathways, resulting in the production of proinflammatory cytokines like TNF-α, IL-1β, interferon regulatory factor 3 (IRF3), IRF7, or adaptor-protein 1 (AP-1), under the regulation of the transcription factor NF-κB (27). The activation of PRRs by SARS-COV-2 viral RNA (specifically TLR3, TLR7, TLR8, and TLR9) results in epithelial cell activation, and the production of numerous proinflammatory molecules

including TNF-α, IL-1α, IL-1β, IL-2, IL-6, IL-8 (CXCL-8), IFN-γ, and CCL-2 (**Figure 1**) (2). This cytokine milieu is involved in ARDS pathological propagation in COVID-19 populations (3, 28).

The result is the increased activation, proliferation, or migration of immune cells (**Figure 1**). In sepsis, PRR expression is dysregulated with higher levels of TLR4 mRNA and TLR2 receptors (29). Levels of IL-1β, amongst other pro-inflammatory cytokines, were found to be higher in patients who died of sepsis than in those who survived (30). Similarly, IL-6 overexpression has been associated with more severe sepsis and worse outcomes (31), potentially due to complement activation (32). In COVID-19, IL-6 becomes upregulated (TLR-8-induced in neutrophils, C5a-induced in monocytes/macrophages), enhancing neutrophil superoxide production, and delaying apoptosis (3). IL-6 production is thought to be a major initiator of the "cytokine storm" in COVID-19 (2) leading to repeated attempts to modulate IL-6 activity in sepsis, and more recently in COVID-19, with varying success (33, 34).

Cell death, caused by microbes as well as the host inflammatory response, releases endogenous DAMPs, further activating PRRs, auto-amplifying the cytokine storm (35) and initiating a cascade of innate immune cell chemoattraction (3). Chemokines also play a major role in immune cell chemoattraction in sepsis. They are small molecules specialised in the recruitment of leukocytes and their release from the bone marrow or spleen. C-X-C chemokine secretion from tissue-resident macrophages is also upregulated in COVID-19. Recruited neutrophils release CCL20, which *via* CCL6 attracts dendritic cells, memory T and B cells, and macrophages to the site of inflammation. A lack of chemokines, or their receptors, leads to an immunosuppressed state where the host is more susceptible to sepsis-induced death (36). Conversely, CXCL-14 potently inhibits epithelial cell chemotaxis, and is downregulated in COVID-19 allowing sustained and enhanced immune cell recruitment (3).

As detailed above, SARS-CoV-2 breaches the alveolar epithelial membrane after binding to the human ACE-2 receptor. The protein transmembrane protease serine 2 (TMPRSS2) is an essential facilitator of SARS-CoV-2 viral cell entry, in conjunction with ACE-2 (37). The SARS-CoV-2 spike protein subsequently interacts with ACE-2, downregulating it (38). Without ACE2, angiotensin-II concentrations and signalling potential increase, upregulating the activation of inflammatory pathways in epithelial and endothelial cells, particularly the p38/MAPK pathway (39). This intracellular inflammatory upregulation, combined with a downregulation of cytokine-release checkpoints (CXCL-14) contributes to the "Cytokine Release Syndrome" which is now well-described in COVID-19, and likely highly pathological. As described in a prior review (40), it is established that the severity of sepsis may be more linked to the host's response to the pathogen, rather than the virulence. Another study discusses the link between clinical manifestations and host gene transcription patterns in staphylococcal infection (41), and noted a significant link between pattern of cytokine gene expression and disease severity, regardless of the causative pathogen (42). This could be relevant in the study of COVID-19, where researchers hypothesise how a single pathogen can have such a varied effect on different individuals ranging from asymptomatic to devastating ARDS and multi-organ dysfunction syndrome (MODS).

The Inflammasome

The importance of the NOD-like receptor pyrin containing-domain 3 (NLRP3) inflammasome is becoming better understood in sepsis. This macromolecular protein complex converts pro-caspases to their mature form, inducing the release of pro-inflammatory cytokines like IL-1β (43). In sepsis, activation of TLRs primes the inflammasome through NF-κB, and it is activated by ROS release and mitochondrial damage by phagocytic cells, having a widespread effect on various systems (44). It is active in patients with COVID-19 and higher levels of IL-18 and Casp1p20 are correlated with COVID-19 severity and poor clinical outcome (45).

Mitochondria

Mitochondria also have a pivotal role in sepsis, well beyond their classical role in oxidative phosphorylation and ATP production. Research has shown sepsis-induced mitochondrial dysfunction may play a pathophysiological role in major organ dysfunction and death (46). For example, in sepsis, mitochondria increase free radical production, propagating the cytokine storm from Kupffer cells in the liver (47), and inducing caspase-mediated apoptosis in the heart causing cardiac dysfunction (48). There is also a dysregulated electron transport chain that may cause a rise in lactate (49). There is evidence of reduced mitochondrial gene expression in individuals who die of sepsis, signifying a loss of function in mitochondria (50). In COVID-19, there is widespread mitochondrial dysfunction caused by inflammation, cytokine storm, oxidative stress, microbiota dysregulation, iron overload, and ROS accumulation (51).

Immunosuppression

Equilibrium of the host's immune response to an offending pathogen is important. A balance must be struck between pro- and anti-inflammatory responses to effectively create an immune response to recognise and eliminate the microbial threat and prevent secondary infection, without excess damage to host cells and organs and to allow full resolution of inflammation. If a patient survives the initial cytokine storm, long-lasting immunosuppression may increase the complications of secondary infection, potentially leading to their death. It has been reported in the literature, agreeing with our clinical observations, that 15% of hospitalised COVID-19 patients, and 50% of those who subsequently die, acquire a secondary infection (52). The incidence of ventilatory-associated lower respiratory tract infections in SARS-CoV-2 patients is significantly higher than in patients with influenza (53). This is likely also the case for COVID-19 associated pulmonary aspergillosis (54). Through genome-wide transcription profiling, it has been possible to quantify downregulation of antigen presentation and suppression of T cell activation to a much greater degree in those who died from sepsis (55). We also know that much mechanistic

immunological research has demonstrated that an intact T cell mediated immune response is required for eliminating and suppressing viral infections (56).

Lymphopenia has consistently correlated with disease severity throughout COVID-19. It is rare in children, in whom COVID-19 mortality is very low, and much more common in the elderly, where higher mortality rates are seen (57). It is also seen that there is a consistent and marked reduction in T cell counts, which is not always the case with B cell counts (58), which may question the necessity of B cell involvement in mounting a successful response to COVID-19.

Several possible mechanisms exist for this lymphocyte depletion in COVID-19. The cytokine release syndrome, detailed above, especially cytokines IL-6 and TNF-α, may lead to massive lymphocyte death. Regulatory T cells seem to be spared however. These cytokines may also reduce the toxicity of T cells and NK cells (59). COVID-19 can also result in T cell exhaustion. This may be a result of neutrophils-induced apoptosis. There is upregulation of programmed death ligand 1 (PD-L1) and T cell immunoglobulin and mucin domain 3 (Tim-3), molecules that promotes the death of the target cell, which interacts with $CD4^+$ and $CD8^+$ lymphocytes to induce apoptosis (59, 60). SARS-CoV-2 may also infect T cells (61). Finally, SARS-CoV-2 may interfere with T cell expansion. MAP2K7 and SOS1, genes involved in T cell activation and function, may be downregulated in severe COVID-19 disease (62).

The subsequent alteration of the neutrophil-lymphocyte ratio is associated with increased nosocomial infection and mortality in severe sepsis (63).

Interestingly in sepsis, these pro- and anti-inflammatory phases appear to happen simultaneously as one response, and not as a distinct two-phase temporal relationship between pro- then anti-inflammatory immunity (55). Therefore, attempts to quantify patients into "hyperinflammatory" or "immunosuppressed" phenotypes may be an over-simplification of the host response, and a theranostic therapeutic approach may prove more difficult than initially proposed.

Monocytes

In sepsis, there is downregulation of the human leukocyte antigen (HLA)-DR molecule on the monocyte, necessary for antigen presentation (64). There is also a reduction in LPS-induced TNF-α secretion from monocytes in sepsis, and these patients may benefit more from an immune adjuvant therapy such as G-CSF (65). This "immunoparalysis" correlates with increased risk of septic complications and death (66). The monocyte's lifespan, like the neutrophil's, is significantly prolonged in sepsis (67). Interestingly, in sepsis, hepatocytes release large amounts of high mobility group box 1 (HMGB-1) (a potent DAMP) which is transported to the cytoplasm of macrophages where it induces pyroptosis (a lytic form of cell death) resulting in depletion of the macrophage population, shock, multiple organ failure, and death (68). The phenotypic switch from M1 to anti-inflammatory M2 macrophages in sepsis also likely contributes to an immune suppressed state (69). Dendritic cells are also decreased in patients with septic shock, and their depletion is associated with increased mortality and health care associated infection (70, 71).

Neutrophils

During septic shock, which may occur with COVID-19, neutrophils are systemically stimulated, which leads to impaired neutrophil migration to the infection focus. Bacterial components present in the blood activate TLRs expressed on neutrophils, leading to the upregulation of G protein-coupled receptor kinase 2 (GRK2), which induces internalisation of CXCR2 receptors on the neutrophil surface. Additionally, TLR activation induces the expression of TNF-α and iNOS (inducible nitric oxide synthase), the latter of which might also be activated by intracellular phosphatidylinositol-3-kinase (PI3K). Both TNF-α and NO (nitric oxide) can lead to upregulation of GRK2, exacerbating the downregulation of CXCR2 on the neutrophil surface. As a consequence, neutrophil trafficking is impaired in sepsis (72), reducing targeted microbial clearance. Furthermore, activation of TLRs also induces the expression of CCR2 on the surface of neutrophils. These activated neutrophils can migrate from inflamed tissues to other, non-infected, tissue and organ systems producing CCL2 (termed "reverse migration"), causing widespread host injury and organ dysfunction, potentially culminating in MODS (73, 74). It has been demonstrated that IL-33 can prevent the upregulation of GRK2 expression induced by TLR overactivation and consequently prevent the failure of neutrophil migration to the site of infection (73). This has not been described specifically in the novel disease process of COVID-19 but may outline the pathophysiologic mechanisms at play in this illness, and its propensity to induce distal organ injury.

Sepsis fundamentally alters the transcriptional profile of the innate immune system's key mediators—the macrophage and neutrophil. Upregulation of genes involved in inflammation and inhibition of apoptosis are seen in neutrophils in human subjects challenged with administration of endotoxins (75) as a model for bacterial sepsis. This response is similar to that seen in multi-trauma patients (76). In a non-septic patient, rapid apoptosis is seen within 24 h in 50% of neutrophils. A core difference in neutrophil activity consistently seen in sepsis is their ability to resist apoptosis with only 5–10% of neutrophils undergoing apoptosis in the first 24 h (77). This prolonged survival is mediated through alterations in gene expression with increases in key molecules like NF-κB (77), IL-1β (78), and PBEF/Nampt (79).

NEUTROPHIL EXTRACELLULAR TRAPS

Neutrophil extracellular traps (NETs) were first described by Brinkmann in 2004 (80). NETs (**Figure 1**) are structures released from neutrophils comprising a core of chromatin DNA and histones, surrounded by specific antimicrobial proteins (lactoferrin, cathepsin G, defensins, LL-37, and bacterial permeability increasing protein), proteases (neutrophil elastase, proteinase-3, and gelatinase), and reactive oxygen species-generating enzymes (myeloperoxidase) (81). NETs are extremely efficient in pathogen trapping, killing, and prevention of pathogen dissemination. "NETosis," the process of release of

these extracellular traps, may be a new form of cell death that is different from apoptosis (3). CCL2, as well as recruiting immune cells, also signals for extracellular trap release (from neutrophils, mast cells, monocytes/macrophages, and eosinophils) (3), as does IL-6, CXCL-8, TNF-α, and IL-1β (associated with mast cell extracellular trap release). Activated endothelial cells may also encourage NETosis, which will ultimately kill these cells. "NETotic" neutrophils do not release apoptotic signals, do not undergo membrane blebbing, or perform nuclear chromatin condensation (3).

Dysregulated NETosis may lead to the development and exacerbation of several autoimmune and chronic infectious or inflammatory diseases (82). NETS have also been associated with multiple types of neoplastic processes (83). NETs can be released in a process of *suicidal* NETosis, where the neutrophil ruptures, or *vital* NETosis, where NETs are exocytosed from neutrophils in vesicles (84). In suicidal NETosis, several gramme-negative bacteria activate NADPH oxidase 2, which induces NETosis *via* reactive oxygen species production, while a NADPH-independent pathway for suicidal NETosis also exists, involving TLR-4-platelet-neutrophil interaction (85). This TLR-4-platelet-neutrophil interaction may be especially important in the pathogenesis of NET-induced "immunothrombosis." Vital NETosis, however, also requires the presence of complement receptor-3 and TLR-2 (86). A recent paper highlights the key role that certain regulatory mitogen-activated protein kinases (MAPK), namely stress-activated protein kinase/c-Jun N-terminal Kinase (SAPK/JNK), play in regulating neutrophil survival. Specifically, a TLR-4/JNK activation axis exists, determining a neutrophil as NETotic or not (85).

Von Willebrand Factor (VWF) is exocytosed by activated endothelial cells onto their apical/luminal cell membrane, where the plasma glycoproteins then bind NETs *via* electrostatic bonds (84). VWF thrombogenic potential is tightly regulated in health by the metalloprotease ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motifs, member 13). NETs downregulate ADAMTS13 activity, promoting the formation, or inhibiting the degradation, of VWF multimers (84). NETs can be a significant source of enzymatic activity that may accelerate the formation of thrombi in blood vessels during infection (87, 88). As well as adhering NETs, VWF will also trap passing platelets and fibrinogen, allowing fibrin deposition and cross-linking, and ultimately vessel thrombosis (84). NETs also ultimately lead to alternative complement pathway activation, through neutrophil secretion of complement factor P, B, and C3, compounding the prothrombotic nature of NETs (**Figure 1**) (3, 89). This vicious cycle can potentially self-propagate unopposed in septic shock.

This process is supported by laboratory studies, where released NETs have been shown to disrupt alveolar epithelium and endothelium, and also degrade the thin alveolar basement membrane, culminating in epithelial necrosis, denudation of epithelial lining, vascular damage, pulmonary oedema, and haemorrhage in lethal influenza-infected mice (90). In humans, NETs have been shown to contribute to the development of ARDS in other severe viral respiratory infections, including H1N1 influenza (90). In COVID-19 pathogenesis, lung infection may accelerate local thromboembolic events, with neutrophils being a major contributor (91, 92). Mechanical ventilation may contribute however to an increased level of NETs markers in the alveoli of critically ill patients (93), compounding an already inflamed microenvironment. Another reason, perhaps, to be cautious regarding initiation of ventilation in COVID-19.

Extra-pulmonary injury from NETs has also been reported in COVID-19. Histone-induced tubular epithelial cell death results in acute kidney injury. Renal injury may be exacerbated with renal thrombosis due to NETs release. NETs may interact with hepatocytes *via* TLR2 and TLR4. Hepatocyte necrosis may occur secondary to damage from histones and C3a. Liver involvement also increases the propensity for thrombosis due to NETs (3).

STEM CELL THERAPIES

Stem cells (regardless of age of donor or source tissue) are undifferentiated cells with capacity to self-renew and/or generate more than one differentiated functional daughter cell type. Mesenchymal stem cells (MSCs) are a specific population of stem cells with much therapeutic potential for sepsis. They are relatively immune privileged, avoiding the need for immunosuppression during use. MSCs may re-programme the immune system to reduce host tissue damage while preserving a strengthened immune response to microorganisms. They have also been shown to enhance tissue and endothelial repair following sepsis and have an extensive and growing safety profile in clinical trials (13).

Multiple pre-clinical septic animal models demonstrate the potential for MSCs therapy to reprogram neutrophil function to reduce host injury while maintaining bactericidal function (94, 95). MSCs reduce the infiltration of neutrophils to target organs, including liver, lung, intestine, and kidney, reducing injury and improving the function of these organs in preclinical sepsis models (94–99). MSCs also enhance neutrophil-mediated phagocytosis, making them more effective in the clearance of bacteria (95). Neutrophil depletion, using anti-Ly6G antibody, completely abrogated the protective effect of MSCs in systemic sepsis (95), highlighting the pivotal MSC-neutrophil interaction to the resolution of sepsis.

The Cellular Immunotherapy for Septic Shock (CISS) Trial, an open label phase 1 dose escalation trial for early septic shock, has led to the phase 2 CISS Trial, assessing safety and efficacy. Other trials include French (CHOCMSC [NCT02883803]) and Russian (100) studies. One clinical trial using cell-based therapies has been completed in COVID-19, using exosomes (extracellular vesicles derived from MSCs) (101). It demonstrated safety of MSC-derived exosome use in COVID-19, and potential as a therapeutic for this disease. At least 17 other clinical trials are in progress assessing MSCs in COVID-19-induced ARDS, as recently reviewed by Gonzalez et al. (102).

PHENOTYPES

Phenotypic characterisation of illnesses may allow significant therapeutic advancement. In this regard, the identification of

sub-phenotypes or "endotypes" within the sepsis population has been undertaken in patients with ARDS by Calfee et al. (103). A related approach, termed "Theranostics," involves identifying biomarkers of therapeutic responsiveness. Man et al. (104) used this approach to identify potential subgroups of patients in the PROWESS-shock trial that may have benefited from activated Protein-C therapy (105). Similarly, Wong et al. (106) identified a paediatric septic shock subgroup that had a higher mortality from corticosteroid administration. Recently, Reddy et al. (107) published a review addressing subphenotypes in critical care, and how these can be translated into clinical practise.

IFN-γ and TNF-α drive a CXCL10/CCL2/macrophage phenotype seen in Crohns Disease and Rheumatoid Arthritis. Therefore, anti-TNF-α and janus kinase (JAK) inhibitors may be potentially successful therapeutic targets for COVID-19 (108). COVID-19 inflammatory phenotypes present in more severe illness progressing to mechanical ventilation have been described by Chua et al. (109). Several other authors have proposed clinical COVID-19 phenotypes (110–112), but the most extensive phenotypical characterisation to date is from Rodriguez et al. (113). Using unsupervised clustering analysis, Rodriguez characterised three novel clinical phenotypes. They associated the phenotypes with comorbidities and clinical outcome, using routinely available clinical and laboratory values, which may allow for easier and more economical future applicability of this model.

Septic patient populations can be divided into clinical or biomarker-driven subphenotypes, the latter focusing on more mechanistic and biologic categorisation. Translation of subphenotypes into clinical practise requires a better understanding of sepsis pathophysiology; how stable the subphenotypes are over time, how quickly, easily, and affordably we can diagnose them, and understanding the effect that multimorbidity has on these patient cohorts and their response to therapy. A theranostic approach may already have proven successful, by treating a specific subgroup of patients requiring oxygen in the first 24–48 h with anti-IL-6 therapy, leading to reduced mortality in a large trial by the REMAP-CAP group (33). However, this benefit in survival was not shared by the EMPACTA trial, investigating the same treatment with slightly different inclusion criteria (114).

POTENTIAL THERAPEUTIC TARGETS

Immune system disequilibrium is difficult to treat. To date, no specific anti-inflammatory treatment has been consistently successful in reducing morbidity or mortality in sepsis (115). Corticosteroids have shown much promise and act to inhibit NF-κB and AP-1 (116). Initially, low-dose corticosteroids were shown to reduce mortality in severe sepsis and septic shock by Annane et al. (117) but this was unable to be replicated in the larger CORTICUS randomised control trial, which showed no benefit (118). More specific blockade of proinflammatory molecules like TNF-α has also failed to show consistent success. A meta-analysis in 2013 showed a modest reduction in death in sepsis in patients given anti-TNF medications but concluded that larger trials with over 10,000 patients were needed to fully demonstrate this benefit (119). The benefit of immunomodulators in sepsis has been difficult to demonstrate for a variety of reasons, including difficulty with timing treatments, heterogeneity of the patient cohort, and variation of the underlying causes of sepsis (35). Many of these issues are not as prominent in COVID-19, with the disease course being more predictable, the typical patient cohort being slightly more homogenous, and the cause of the dysregulated inflammatory response being consistent. This may spell greater success for upcoming trials of immunomodulation in improving outcomes in COVID-19, many of which are discussed below.

With SARS-CoV-2 infection and immune system activation, many therapeutic targets exist. A theranostic approaches to finding a solution to the problems we have highlighted above may therefore succeed. Some old, yet rejuvenated, therapies, and some novel.

Approaches to altering the "Cytokine Release Syndrome" are 2-fold; block the action of a known cytokine propagator or increase the effects of an inflammatory down-regulator. Inhibition of the effects of IL-6, through blockade of its receptor (IL-6R) with Tocilizumab has received considerable attention, as IL-6 is thought to be a major initiator of the "cytokine storm" in COVID-19 (2). Initial trials in minority, non-ventilated populations failed (114), but more recent work by the REMAP-CAP (33) and RECOVERY (120) investigators in more critically ill patients has shown promise.

IL-17, produced by Th17 T-cells, is another proinflammatory cytokine (2). It is also produced by mast cells and NETs, and may play a role in thrombosis (3), as well as upregulating the production of other cytokines, most notably IL-6. Two monoclonal antibodies against IL-17, and one targeting the IL-17R have been successfully used in rheumatoid arthritis and psoriasis (2). The CXCL10-CXCR3 axis may also be a therapeutic target, especially blocking CXCL10 (eldelumab/MDX-1100) (121).

Corticosteroids have also been shown to reduce CXCL10 levels in COVID-19 (121), while separately, dexamethasone (122) and hydrocortisone (123) have been shown to reduce (rate ratio 0.83), and likely reduce (with a 93% probability) mortality, respectively. CXCL-14 potently inhibits epithelial cell chemotaxis, and is downregulated in COVID-19 allowing sustained and enhanced immune cell recruitment (3). This is, as yet an untargeted potential therapeutic.

Modulation of this overactive complement system has been attempted. Complement inhibition *via* AMY-101 (C3) or Eculizumab (C5) significantly reduced immune hyperactivation in severe COVID-19 (16). NLR was significantly altered by C3 inhibition, with reduced neutrophils and increased lymphocytes at day 7 compared to C5 inhibition. C3 inhibition resolved thrombocytopenia quicker than C5, and NETosis (*via* MPO-DNA levels) was reduced more profoundly, but not significantly, with C3 inhibition in both intubated and non-intubated patients. Ultimately, C3 inhibition may be better, preventing immune cell activation (*via* C3a–C3aR blockade), C3 opsonisation of epithelial or alveolar cells, and also the associated effects of C5 cleavage to C5a (C5a–C5aR inflammatory upregulation)

and C5b (C5b–C9 MAC and cell lysis). Reduced neutrophil and T-cell recruitment *via* reduced C3a and C5a was also seen. C5a induction of monocytes and macrophages upregulates IL-6 production (3). Therefore, the viability of targeting the complement system seems more profitable than targeting a single cytokine or its receptor, due to the multi-layered effects of activating this system (C3, C5, inflammatory cell activation, and the MAC). More clinical trials may shed light on this [NCT04346797]. In a lung epithelial cell line study, ruxolitinib, a JAK1/2 inhibitor normalised interferon gene and complement gene signals induced by SARS-CoV-2, and reduced C3a production (19), showing potential to move into clinical trials. Another JAK1/2 inhibitor, Baricitinib, in combination with the antiviral agent Remdesivir, has shown benefit in hospitalised patients with COVID-19 (124).

Several therapeutics targeting innate immune cell recruitment, effector-memory T cells, or their phagocytic products are under assessment. A study assessing Vitamin C and its effects on COVID-19 patients by reducing neutrophil influx, activation and NET-associated alveolar capillary damage was abandoned due to difficulty in recruitment [NCT04264533]. The CXCR2 antagonists AZD5069 (blocks neutrophil trafficking but preserves neutrophil-mediated host immunity) and Danirixin and SCH527123 (both reduce neutrophil influx/migration) may be of benefit here (2). Neutrophil Elastase antagonists are either in clinical trial or approved for clinical use as treatments of ARDS pre-COVID-19 (2). Melatonin, a chronobiotic hormone, rejuvenates exhausted glutathione redox system in neutrophils during infection (125). Melatonin [NCT04409522], along with colchicine [NCT04350320] may also induce blockade of the inflammasome, offering other potential therapeutic targets in COVID-19.

Augmentation of the adaptive immune system is of particular interest in COVID-19, given the marked lymphopenia seen, potentially *via* upregulation of PD-L1 that induces lymphocyte apoptosis (60, 126). Its blockade may be a potential target in COVID-19 to improve outcomes (127) [NCT04356508, NCT04413838, and NCT04268537].

PAD (peptidylarginine deiminase) 4 inhibitors block NETs formation and release in murine sepsis models (128). Dipyridamole can inhibit NETs by activation of adenosine A_{2A} receptors (129), blocking adenosine reuptake and being a non-selective PDE4 inhibitor. Disulfaram as a therapy for COVID-19 is in clinical trial as a gasdermin D inhibitor, also inhibiting NETs formation [NCT04485130]. Hydroxychloroquine and Azithromycin can inhibit IL-1β and NET formation, but have not been shown to improve patient outcome in COVID-19 (130). The peptide-based agent Lupuzor/P140, trialled successfully in Systemic Lupus Erythematosus, may be of benefit in COVID-19 by blocking NET release but hasn't been trialled (131). Other NET-inhibitors include GSK-484 and BMS-P5, which have not been used *in vivo* as of yet (2).

Finally, dornase alfa (Pulmozyme, recombinant human deoxyribonuclease I) may improve ARDS in patients with severe COVID-19 through reduced mucus accumulation, lung injury, and improved gas exchange (132). However, the fragmented DNA may risk spreading inflammation beyond the area of viral invasion. Nine clinical trials are currently in progress for this therapeutic in COVID-19 (132).

CONCLUSION

At the date of writing, global case incidence and related mortality of COVID-19 had surpassed 160 and 3.34 million, respectively. New, more transmissible strains of SARS-CoV-2 are now driving further waves of infection globally, and overwhelming health systems (133), with an inevitable surge in critically ill COVID-19 patients. With this, the vicious cycle of pulmonary epithelial cell infection and activation, cytokine and chemoattractant over-production, immune-cell recruitment, uncontrolled hyper-inflammation, and MODS continues. NETosis, while attempting to eradicate SARS-CoV-2, compounds this uncontrolled inflammation, with secondary "immunothrombosis" detrimental to the organ systems involved. Mechanical ventilation may compound this (93), and such support should be judiciously implemented. Emerging COVID-19 phenotypes may allow for more targeted therapy in the future. Currently, corticosteroids (122, 123), IL-6R antagonists (33, 120), and JAK inhibitors (124) are the only therapies showing promise for critically ill COVID-19 patients. Many hundreds of other clinical trials in COVID-19 maintain recruitment.

While vaccines against SARS-CoV-2 are being rolled out (134), further global pandemics are predicted (135). Future therapies against invasive pathogens revolve not only around their eradication but understanding better the deleterious effects they have on the human immune system, and how to regain and retain physiology over pathology. Perhaps trials using stem-cell-based therapies may shed some light.

AUTHOR CONTRIBUTIONS

CK conceived the presented idea and took lead in writing the manuscript. CK and MC wrote the manuscript in consultation with IM-L. All authors contributed to the article and approved the submitted version.

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Sepsis of Patients Infected by SARS-CoV-2: Real-World Experience From the International HOPE-COVID-19-Registry and Validation of HOPE Sepsis Score

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Background: Patients with sepsis with a concomitant coronavirus (COVID-19) infection are related to a high morbidity and mortality rate. We investigated a large cohort of patients with sepsis with a concomitant COVID-19, and we developed a risk score for the estimation of sepsis risk in COVID-19.

Methods: We conducted a sub-analysis from the international Health Outcome Predictive Evaluation Registry for COVID-19 (HOPE-COVID-19-Registry, NCT04334291). Out of 5,837 patients with COVID-19, 624 patients were diagnosed with sepsis according to the Sepsis-3 International Consensus.

Results: In multivariable analysis, the following risk factors were identified as independent predictors for developing sepsis: current smoking, tachypnoea (>22 breath per minute), hemoptysis, peripheral oxygen saturation (SpO_2) <92%, blood pressure (BP) (systolic BP < 90 mmHg and diastolic BP < 60 mmHg), Glasgow Coma Scale (GCS) <15, elevated procalcitonin (PCT), elevated troponin I (TnI), and elevated creatinine >1.5 mg/dl. By assigning odds ratio (OR) weighted points to these variables, the following three risk categories were defined to develop sepsis during admission: low-risk

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group (probability of sepsis 3.1–11.8%); intermediate-risk group (24.8–53.8%); and high-risk-group (58.3–100%). A score of 1 was assigned to current smoking, tachypnoea, decreased SpO_2, decreased BP, decreased GCS, elevated PCT, TnI, and creatinine, whereas a score of 2 was assigned to hemoptysis.

Conclusions: The HOPE Sepsis Score including nine parameters is useful in identifying high-risk COVID-19 patients to develop sepsis. Sepsis in COVID-19 is associated with a high mortality rate.

Keywords: sepsis, score, COVID-19, SARS-CoV-2, outcome

INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreak, which was first emerged in Wuhan, China, in December 2019, has spread rapidly and has had an immense impact on the whole world. Consequently, states have endeavored to slow down the progression of the disease.

The course of coronavirus infectious disease (COVID-19) caused by SARS-CoV-2 is mild in the majority of patients. In 5% of COVID-19 patients, multiorgan dysfunction with an overall mortality rate of 1–11% was observed (1–4). However, sepsis is the main cause of death from the infection, particularly if not diagnosed and treated promptly.

It was revealed that many patients with severe COVID-19 showed general signs of shock (5). These patients met the sepsis and septic shock criteria according to the Sepsis-3 International Consensus (6). However, there are no comparative data available about the incidence and mortality rate in patients suffering from sepsis in COVID-19. In addition, predictors of sepsis have not yet been investigated.

In the international Health Outcome Predictive Evaluation Registry for COVID-19 (HOPE-COVID-19-Registry) (7), we compared baseline characteristics and clinical, laboratory, and radiologic findings in COVID-19 patients suffering from sepsis with those without sepsis at admission. We developed the HOPE Sepsis Score to estimate the risk of developing sepsis during admission. Predictors of mortality were analyzed.

METHODS

Study Design and Patients

HOPE-COVID-19 (NCT04334291) is an international project. It is designed as a retrospective cohort registry without any financial compensation. The data of 5,837 consecutive hospitalized patients with COVID-19 were gathered. We analyzed all included patients from March 1, 2020, to June 2, 2020. An online database was built and completed by each participating center. Additional information on datasets of the HOPE-COVID-19-Registry is available at www.hopeprojectmd.com. The methodology of the HOPE-COVID-19-Registry has been described previously (7, 8). The study was approved by the Ethics Committee in all involved centers.

Sepsis Definition III

The third international Consensus Task Force defined sepsis as life-threatening organ dysfunction due to a dysregulated host response to the infection. Organ failure in patients with sepsis increases in-hospital mortality by greater than 10% (6).

Data Collection

Clinical laboratory investigation consisted of transaminases, glomerular filtration rate (GFR), creatinine, lactate dehydrogenase (LDH), electrolytes, coagulation profile, and complete blood count. Radiological imaging, such as chest radiography or CT, to detect bilateral or unilateral infiltrates was applied. Abnormal blood pressure (BP) was defined as systolic BP (SBP) less than 90 mmHg or diastolic BP (DBP) less than 60 mmHg. Glasgow Coma Scale (GCS) consisted of eye-opening, verbal, and motor responses. Elevated creatinine was defined as an elevation of more than 1.5 mg/dl, elevated troponin I (TnI) more than 0.05 μg/L, and procalcitonin (PCT) more than 0.5 ng/ml. We gathered as primary end point all-cause mortality. Oxygen therapy at admission including high nasal-cannula, non-invasive ventilation, and invasive mechanical ventilation, respiratory insufficiency, heart failure, upper respiratory tract involvement, clinically relevant bleeding, and embolic events as secondary end points were reported. Missing data are addressed in the tables.

Statistical Analysis

Data of continuous variables were performed as mean ± SD with a normal distribution, median (interquartile range) with a non-normal distribution, while categorical variables were presented as frequencies and percentages (%). The Kolmogorov-Smirnov test was used to test the normal distribution. The Mann-Whitney U-test and Student's *t*-test were used to compare normal or non-normal distributions of continuous variables, respectively. For distribution analysis of categorical variables, Fisher's exact test or chi-squared test was used. We applied a two-tailed Fisher's exact test in tests with a sample size of $n = 5$ or below. Results are performed with 95% CIs. We estimated the differences in both groups using Kaplan-Meier and applied Log-Rank statistics. Predictors of sepsis were identified by univariate analysis. Predictors with $p < 0.0001$ were analyzed by the logistic multivariate regression. These variables were used to build a Score system. The Score system was confirmed through comparison with random choice with 10% of all the participants. Harrell's C-index or the area under the receiver operating

characteristic curve (AUC-ROC) was used to evaluate the ability of risk scores to predict outcome (C-index measures the goodness of fit of a model, with 0.5 indicating no discrimination and 1.0 indicating perfect prediction). We estimated the mortality risk according to HOPE Sepsis Score using Kaplan-Meier and applied Log-Rank statistics. Sensitivity, specificity, and positive (PPV) and negative predictive values (NPV) of HOPE Sepsis Score to predict the sepsis in low-, intermediate-, and high-risk groups were calculated. Statistical analysis was showed with SPSS (IBM Statistics, Version 23.0. Armonk, NY: IBM Corp). $p < 0.05$ was recognized as statistically significant.

RESULTS

Comparison of Sepsis to Non-Sepsis Participants

At baseline, patients suffering from sepsis in COVID-19 were older than non-sepsis patients (≥65 years old; 66.3 vs. 52%; $p < 0.001$). Patients with sepsis showed more baseline comorbidities, such as arterial hypertension (65.2 vs. 46.9%; $p < 0.001$), dyslipidemia (41.9 vs. 32.8%; $p < 0.001$), diabetes mellitus (DM) (25.6 vs. 17.7%; $p < 0.001$), and current smoking (11.4 vs. 4.5%; $p < 0.001$), **Table 1**. Clinical presentations, such as dyspnoea (68.1 vs. 55%; $p < 0.001$), tachypnoea (46.3 vs. 23.5%; $p < 0.001$), hemoptysis (6.3 vs. 1.1%; $p < 0.001$), anosmia or hyposmia (10.4 vs. 5.9%; $p < 0.001$), and dysgeusia (11.7 vs. 6.3%; $p < 0.001$), were more observed in the sepsis group as compared to the non-sepsis group. Clinical parameters at admission were worse in patients with sepsis as compared to non-sepsis patients with a decrease in peripheral oxygen saturation (SpO_2) <92% and abnormal BP (systolic BP < 90 mmHg and/or diastolic BP < 60 mmHg; 61.1 vs. 31.1%; $p < 0.001$; and 16.8 vs. 5.8%; $p < 0.001$). Similarly, changes in laboratory parameters were also more pronounced in sepsis group (**Table 1**).

In-Hospital Course

Non-invasive ventilation and invasive mechanical ventilation were more often required in patients with sepsis as compared to those without sepsis, (34.2 vs. 11%; $p < 0.001$) and (32.5 vs. 4%; $p < 0.001$), respectively. Accordingly, the mortality rate was considerably higher in the sepsis group (61.2 vs. 15.2%; $p < 0.001$; **Table 1**).

Treatment Approaches

During hospital stay, patients with sepsis more often received glucocorticoids (44.4 vs. 25.1%; $p < 0.001$), interferon (28.2 vs. 11.5%; $p < 0.001$), tocilizumab (21.3 vs. 6.7%; $p < 0.001$), and antibiotics (89.4 vs. 74.2%; $p < 0.001$). Interestingly, hydroxychloroquine use and antiviral drugs, such as lopinavir and/or ritonavir use, were higher in the non-sepsis group (79.4 vs. 85%; $p < 0.001$ and 52.3 vs. 59.7%; $p = 0.35$). Angiotensin-converting enzyme inhibitor (ACEi) or angiotensin receptor blocker (ARB) treatment at admission was not different in both groups (18.7 vs. 19.9%; $p = 506$; **Table 1**).

Predictors of Sepsis, Development, and Validation of the HOPE Sepsis Score

Table 2 presents the result of univariable and multivariable analyses. The multivariable analysis identified the following nine independent predictors to developing sepsis: current smoking (odds ratio, OR 2.43, 95% CI: 1.77–3.33; $p < 0.001$), tachypnoea (OR 1.60, 95% CI: 1.31–1.96; $p < 0.001$), hemoptysis (OR 4.30, 95% CI: 2.66–6.96; $p < 0.001$), reduced SpO_2 < 92% (OR 2.11, 95% CI: 1.73–2.57; $p < 0.001$), reduced BP at admission (OR 1.87, 95% CI: 1.08–3.22; $p = 0.02$), reduced GCS (OR 1.89, 95% CI: 1.42–2.51; $p < 0.001$), elevated PCT (OR 2.44, 95% CI: 1.99–2.99; $p < 0.001$), TnI (OR 1.94, 95% CI: 1.48–2.54; $p < 0.001$), and creatinine (OR 2.24, 95% CI: 1.81–2.78; $p < 0.001$). We divided the OR value of each variable by the median value of the regression coefficients of all variables (rounded to nearest 0.5 points). A score of 1 was assigned to current smoking, tachypnoea, decreased SpO_2, decreased BP, decreased GCS, elevated PCT, TnI, and creatinine, whereas a score of 2 was assigned to hemoptysis. This score can be used to assess the risk for developing sepsis by assigning patients with COVID-19 to three risk groups: a low-risk group from 0 to 2 points, an intermediate-risk group from 3 to 5 points, and a high-risk group from 6 to 10 points (**Figure 1**). The probability of sepsis risk was 3.1–11.8% in the low-risk group, 24.8–53.8% in the intermediate-risk group, and 58.3–100% in the high-risk group.

The final model was applied to the validation cohort (random choice of 10% of all study participants). The C-index for the HOPE Sepsis Score was 0.763, while the C-index for the validation cohort was 0.77 (**Table 3**). In addition, the sensitivity of the HOPE Sepsis Score to predict sepsis was higher in the intermediate-risk group as compared to high-risk patients (81.1 vs. 34.3%). On the other hand, the specificity and PPV were lower in the intermediate-risk group than in patients with high risk for sepsis, respectively (specificity: 80.3 vs. 99.2% and PPV: 32.4 vs. 66.1%). In addition, estimating the risk of mortality in COVID-19 according to HOPE Sepsis Score was investigated (**Figure 2**). Clinical characteristics of the validated group, sensitivity, specificity, PPV, and NPV are presented in the **Supplementary Materials**.

DISCUSSION

HOPE-COVID-19-Registry shows real-world experience from data worldwide. The present study shows patient characteristics at baseline, in-hospital complications, and mortality, particularly in the participants with sepsis. The main findings of the study are that (1) patients suffering from sepsis in COVID-19 had higher rates of comorbidity, (2) the incidence of sepsis in COVID-19 is estimated at 11%, (3) predictors for developing sepsis are identified, and (4) HOPE Sepsis Score is developed to support physicians to early identifying of COVID-19 patients with sepsis on the basis of chronic conditions, clinical findings, hemodynamic, and laboratory parameters at admission.

TABLE 1 | Patients with Sepsis as compared to patients without Sepsis; Baseline characteristics, laboratory and radiographic findings, complications, and clinical outcomes.

Characteristic	Patients with Sepsis $N = 624$	Patients without Sepsis $N = 5213$	*P*-value*
Age – no. (%)			
<65	207/614 (33.7)	2458/5124 (47.9)	**<0.001**
≥65	407/614 (66.3)	2666/5124 (52)	**<0.001**
Male – no. (%)	417/624 (66.8)	3004/5213 (57.6)	**<0.001**
Duration of symptom onset to admission – days mean ± SD	5.9 ± 7.6	7.2 ± 6.5	**<0.001**
Duration of hospital stay – days mean ± SD	12.8 ± 11.7	9.8 ± 8.6	**<0.001**
Chronic conditions – no. (%)			
Arterial hypertension	407/624 (65.2)	2443/5213 (46.9)	**<0.001**
Dyslipidaemia	259/618 (41.9)	1643/5007 (32.8)	**<0.001**
Diabetes Mellitus	160/624 (25.6)	924/5213 (17.7)	**<0.001**
Obesity	126/497 (25.4)	890/4034 (22.1)	0.09
Current Smoking	71/624 (11.4)	235/5213 (4.5)	**<0.001**
Renal insufficiency ¥	75/624 (12)	306/5213 (5.9)	**<0.001**
Lung disease	126/624 (20.2)	933/5043 (18.5)	0.307
Cardiac disease	200/624 (32.1)	1129/5213 (21.7)	**<0.001**
Atrial Fibrillation	27/624 (4.3)	172/5043 (3.4)	0.24
Cerebrovascular disease	78/624 (12.5)	372/5213 (7.1)	**<0.001**
Connective Tissue disease	27/624 (4.3)	136/5213 (2.6)	**0.013**
Liver disease	30/624 (4.8)	182/5213 (3.5)	0.09
Cancer disease	131/624 (21)	639/5213 (12.3)	**<0.001**
Immunosuppression – no. (%) ≪	88/624 (14.1)	328/5213 (6.3)	**<0.001**
Prior tuberculosis – no. (%)	4/624 (0.6)	11/5043 (0.2)	0.074
Human Immunodeficiency virus – no. (%)	3/624 (0.5)	18/5043 (0.4)	0.498
Home Oxygen Therapy – no. (%)	31/624 (5)	140/5213 (2.7)	**0.002**
Premedication – no. (%)			
ASA Ω	148/624 (23.7)	720/5213 (13.8)	**<0.001**
Antiplatelet drug	36/579 (6.2)	166/4945 (3.4)	**0.001**
Oral Anticoagulation	98/624 (16.3)	494/5213 (9.5)	**<0.001**
Beta Blockers	147/601 (24.5)	761/4990 (15.3)	**<0.001**
Beta Agonist Inhalation Therapy	69/599 (11.5)	487/4983 (9.8)	0.178
Glucocorticoids Inhalation Therapy	58/604 (9.6)	438/4992 (8.8)	0.499
Vitamin D3	96/604 (15.9)	491/4966 (9.9)	**<0.001**
Benzodiazepine	115/606 (19)	729/4997 (14.5)	**0.004**
Antidepressant	104/603 (17.2)	625/4987 (12.5)	**0.001**
Symptomatic – no. (%)			
Asymptomatic	21/606 (3.5)	272/5012 (5.4)	**0.04**
Dyspnoea	425/624 (68.1)	2869/5213 (55)	**<0.001**
Tachypnoea > 22 breaths per minute	289/624 (46.3)	1226/5213 (23.5)	**<0.001**
Haemoptysis	39/624 (6.3)	57/5213 (1.1)	**<0.001**
Fatigue	315/589 (53.5)	2205/4896 (45)	**<0.001**
Anosmia / Hyposmia	65/624 (10.4)	310/5213 (5.9)	**<0.001**
Dysgeusia	73/624 (11.7)	329/5213 (6.3)	**<0.001**
Sorethroat	83/567 (14.6)	570/4779 (11.9)	0.062
Fever	511/614 (83.2)	3962/5003 (79.2)	**0.019**
Cough	398/606 (65.7)	3425/4992 (68.6)	0.143
Vomiting	49/586 (8.4)	358/4879 (7.3)	0.372
Diarrhea	105/581 (18.1)	965/4897 (19.7)	0.348
Erythromelalgia	157/579 (27.1)	1603/4880 (32.8)	**0.005**
Clinical parameters – no. (%)			

(Continued)

TABLE 1 | Continued

Characteristic	Patients with Sepsis *N* = 624	Patients without Sepsis *N* = 5213	*P*-value*
Peripheral Oxygen Saturation < 92 %	375/624 (60.1)	1619/5213 (31.1)	**<0.001**
Reduced Blood Pressure §	105/624 (16.8)	302/5213 (5.8)	**<0.001**
GCS ø < 15 – no. (%)	104/624 (16.7)	257/5213 (4.9)	**<0.001**
Laboratory parameters – no. (%) or median (IQR)			
Elevated Di-Dimer	410/624(65.7)	2773/5213(53.2)	**<0.001**
Elevated Procalcitonin	277/624 (44.4)	675/5213 (13)	**<0.001**
Elevated CRP ∂	581/624 (93.1)	4430/5213 (85)	**<0.001**
Elevated TnI ∞	126/624 (20.2)	279/5213 (5.4)	**<0.001**
Elevated Transaminases •	315/624 (50.5)	1836/5213 (35.2)	**<0.001**
Elevated Ferritin	272/390 (69.8)	1473/2552 (57.7)	**<0.001**
Elevated Triglyceride	100/345 (29)	416/2186 (19)	**<0.001**
Elevated LDH ◦	465/624 (82.2)	3247/5213 (62.1)	**<0.001**
Elevated Creatinine (>1.5 mg/dl)	211/624 (33.8)	596/5213 (11.4)	**<0.001**
Leukocytopenia (<4000 10E9/l)	79/619 (12.8)	739/4922 (15)	0.137
Lymphocytopenia (<1500 10E9/l)	469/605 (77.5)	3728/4832 (77.2)	0.839
Anemia hemoglobin (< 12 g/dl)	232/624 (37.2)	1229/5213 (23.6)	**<0.001**
Thrombocytopenia (<150000 10E9/l)	193/611 (31.6)	1199/4908 (24.4)	**<0.001**
Moderate Hyponatremia	38/400 (9)	188/3876 (4.9)	**<0.001**
Severe Hyponatremia	19/624 (3)	39/5213 (0.7)	**<0.001**
Complication			
Respiratory Insufficiency	503/616 (81.7)	2302/5033 (45.7)	**<0.001**
Heart Failure	115/611 (18.8)	241/5012 (4.8)	**<0.001**
Acute kidney Injury	293/611 (48)	609/5026 (12.1)	**<0.001**
Upper Respiratory-Tract Infection	119/575 (20.7)	596/4959 (12)	**<0.001**
Pneumonia	575/624(92.1)	4471/5213(85.8)	**<0.001**
SIRS π	333/601 (55.4)	747/4991 (15)	**<0.001**
Any relevant bleeding ç	44/594 (7.4)	100/4978 (2)	**<0.001**
Embolic event	34/600 (5.7)	85/4999 (1.7)	**<0.001**
Oxygen Therapy			
O2 at the admission	563/621 (90.7)	3388/4958 (68.3)	**<0.001**
High Flow Nasal Cannula	283/604 (46.9)	798/4942 (16)	**<0.001**
Non-Invasive Mechanical Ventilation	206/603 (34.2)	545/4984 (11)	**<0.001**
Invasive Mechanical Ventilation	193/594 (32.5)	198/4955 (4)	**<0.001**
Another Medication or Intervention Procedures during the Admission			
Prone Position	156/599 (26)	400/4937 (8.1)	**<0.001**
ECMO å	4/396 (1)	21/3549 (0.6)	0.320
Use of Glucocorticoids	267/601 (44.4)	1243/4955 (25.1)	**<0.001**
Use of Hydroxychloroquine	483/608 (79.4)	4259/5013 (85)	**<0.001**
Use of Antiviral Drugs ∑	319/610 (52.3)	2978/4991 (59.7)	0.35
Use of Interferon	166/589 (28.2)	566/4940 (11.5)	**<0.001**
Use of Tocilizumab	126/592 (21.3)	330/4958 (6.7)	**<0.001**
Use of Antibiotics	530/593 (89.4)	3507/4727 (74.2)	**<0.001**
ACEI/ARB's ≪	110/587 (18.7)	963/4840 (19.9)	0.506
Anticoagulation	269/366 (73.5)	2182/2929 (74.5)	**<0.001**
Discharge			
ACEI/ARB's	69/624 (11.1)	924/5042 (18.3)	**<0.001**
Antiplatelet Drug	38/361 (10.5)	367/4336 (8.5)	0.180
Anticoagulation Drug	76/602 (12.6)	1018/4934 (20.6)	**<0.001**
Death †	382/624 (61.2)	767/5043 (15.2)	**<0.001**

SD standard deviation. *¥ CrCL < 30. ≪ Immunosuppressive therapy for psoriasis arthritis, lung transplantation, kidney transplantation or systemic lupus erythematosus; oncological disease such as mamma-ca, prostate-ca, myelodysplastic syndrome or gammopathy; glucocorticoid therapy caused by COPD; dialysis; HIV or hepatitis. Ω Acetylsalicylic acid. § Systolic blood pressure < 90 mmHg or diastolic blood pressure < 60 mmHg. Ø Glasgow coma scale. ∂ C-reactive Protein. ∞ High sensitive Troponin I (cardiac injury; troponin > 99th percentile upper reference limit). • ALAT and ASAT. π Systemic inflammatory response syndrome. Ç Rectorrhagia, haematuria, epistaxis, and popliteal aneurysm bleeding with relevant decreased hemoglobin> 2 mg/l. å Extracorporeal membrane oxygenation. ∑ Lopinavir or /and Ritonavir. ≪ Premedication with ACEI/ARB's is not stopped. Significant p values are marked bold.*

TABLE 2 | Predictors of Sepsis, multivariate analysis.

Variable	*Univariate analysis*			*Multivariate analysis*		
	OR	**95% CI**	***P*-value**	**OR**	**95% CI**	***P*-value**
Patients characteristic						
Male	1.48	1.24–1.76	**<0.001**			
Age≪	1.81	1.52–2.16	**<0.001**			
Chronic conditions						
Hypertension	2.13	1.79–2.53	**<0.001**			
Diabetes Mellitus	1.60	1.32–1.94	**<0.001**			
Current Smoking	2.72	2.06–3.59	**<0.001**	2.43	1.77–3.33	**<0.001**
Renal insufficiency	2.19	1.68–2.86	**<0.001**			
Prior heart disease	1.71	1.43–2.05	**<0.001**			
Cerebrovascular disease	1.86	1.43–2.41	**<0.001**			
Prior cancer disease	1.90	1.54–2.35	**<0.001**			
Connective tissue disease	1.69	1.11–2.57	**0.013**			
Previous therapies						
Immunosuppression	2.46	1.90–3.15	**<0.001**			
Home oxygen therapy	1.81	1.22–2.69	**0.002**			
ASA	1.94	1.59–2.37	**<0.001**			
Oral anticoagulation	1.78	1.41–2.25	**<0.001**			
Symptomatic						
Dyspnoea	1.75	1.46–2.08	**<0.001**			
Tachypnoea	2.81	2.37–3.32	**<0.001**	1.60	1.31–1.96	**<0.001**
Anosmia / Hyposmia	1.84	1.39–2.44	**<0.001**			
Dysgeusia	1.97	1.50–2.57	**<0.001**			
Haemoptysis	6.03	3.98–9.14	**<0.001**	4.30	2.66–6.96	**<0.001**
Clinical parameters at admission						
SpO2 < 92%#	3.34	2.82–3.97	**<0.001**	2.11	1.73–2.57	**<0.001**
Reduced Blood Pressure §	3.29	2.59–4.18	**<0.001**	1.90	1.44–2.50	**<0.001**
Reduced GCS	3.86	3.02–4.93	**<0.001**	1.89	1.42–2.51	**<0.001**
Laboratory values						
Elevated CRP	2.38	1.74–3.29	**<0.001**			
Elevated Procalcitonin	3.84	3.20–4.61	**<0.001**	2.44	1.99–2.99	**<0.001**
Elevated Ferritin	1.88	1.59–2.24	**<0.001**			
Elevated LDH	1.77	1.47–2.14	**<0.001**			
Elevated Di-Dimer	1.69	1.42–2.01	**<0.001**			
Elevated TnI	4.47	3.56–5.63	**<0.001**	1.94	1.48–2.54	**<0.001**
Elevated Creatinine*	3.96	3.28–4.77	**<0.001**	2.24	1.81–2.78	**<0.001**
Elevated Transaminases	1.88	1.59–2.22	**<0.001**			
Anemia∑	1.91	1.61–2.28	**<0.001**			
Severe hyponatremia	4.17	2.39–7.26	**<0.001**			
X–Ray Abnormality						
Uni- or bilateral infiltrates	1.95	1.44–2.63	**<0.001**			

*HR, hazard ratio; EF, ejection fraction; CI, confidence interval; ≪, age ≥ 65; *, > 1.5 mg/dl; ∂, <1500 10E9/l; ∑, Hb <12 g/dl; # peripheral oxygen saturation;* **§** *Systolic blood pressure < 90 mmHg or diastolic blood pressure < 60 mmHg. Significant p values are marked bold.*

Comparison of COVID-19 Patients With Sepsis and Without Sepsis

Patients with sepsis were older and had more comorbidities as compared to patients with non-sepsis. The incidence of sepsis in COVID-19 is estimated at 11%. In addition, in the sepsis cohort, an increase of inflammatory markers, such as CRP, PCT, and ferritin, was more pronounced than in participants with non-sepsis. This phenomenon is known in patients with sepsis due to excessive inflammation (9). In patients with COVID-19, the immune response seems to be more pronounced and may be based on underlying pathomechanisms: macrophage-activation syndrome, viral sepsis-induced immune paralysis, and dysregulation of an intermediate functional state of the immune system in infected patients with SARS-CoV-2 (10–12). Other

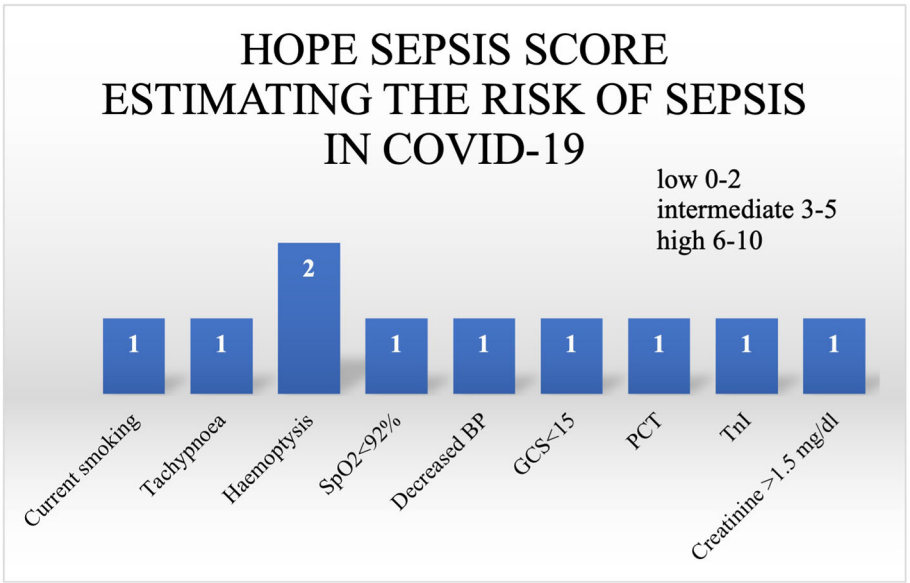

FIGURE 1 | HOPE Sepsis Score, C-index = 0.763 (*N* = 5,837); tachypnoea >22 breath per minute; SpO_2, peripheral oxygen saturation; BP, blood pressure; GCS < 15 (Glasgow coma scale); PCT, elevated procalcitonin; TnI, elevated troponin; creatinine, elevated creatinine > 1.5 mg/dl; HOPE, the international Health Outcome Predictive Evaluation.

laboratory abnormalities were more observed in participants with sepsis than those without sepsis, such as elevated d-dimer, transaminases, creatinine, LDH, anemia, thrombocytopenia, triglyceride, and hyponatremia. These abnormalities indicate that liver and kidney functions were impaired, such as coagulation disorder in patients with sepsis at admission. Clinical Data from 409 US hospitals from 2009 to 2014 in patients showed a slightly lower sepsis rate of 6% as compared to our data (13). Chen et al. reported that dead 119 patients with COVID-19 presented an increase of inflammatory parameters (14). The coagulation disorder may develop disseminated intravascular coagulopathy (DIC) in patients with sepsis. Therefore, it is proposed to establish prophylaxis against venous thromboembolism (VTE) (15). These changes, such as abnormal coagulation function, were observed in patients infected with SARS-CoV-2 (2, 14, 16). Additionally, COVID-19 patients have built antiphospholipid antibodies (17). However, the inflammation could increase procoagulant activity thereby contributing to thrombus formation (18). All these abnormalities may explain the higher rate of thromboembolism and multiorgan dysfunction in patients with sepsis.

HOPE SEPSIS SCORE

HOPE Sepsis Score is developed and validated to support physicians to identify COVID-19 patients with sepsis. The score integrates nine parameters ranging from medical history to clinical and laboratory findings. Collecting the clinical findings, such as current smoking, hemoptysis, tachypnoea, decreased BP, GCS, SpO_2, elevated PCT, TnI, and creatinine, at admission is relatively easy and promptly. Concerning this matter, a score of 2 is assigned to hemoptysis that represents an important predictor for developing sepsis. However, Hemoptysis is a less common symptom in patients with COVID-19 (1). As laboratory findings, the HOPE Sepsis Score represents TnI, PCT, and elevated creatinine as predictors for developing sepsis as compared to the sequential failure assessment (SOFA) score, which only included respiratory rate, GCS, BP, and elevated creatinine (6). To summarize, the HOPE Sepsis Score is also useful and feasible in identifying high-risk COVID-19 patients predicted to develop sepsis with a high mortality rate. The C-index for HOPE Sepsis Score was 0.763; the score can also be used to predict sepsis

TABLE 3 | The validation of HOPE Sepsis Score; the Risk of developing sepsis in COVID-19 in the validated group (*n* = 584) as compared to all patients (*n* = 5837).

Validated group ***N* = 584**	**Findings at admission**	**Points**
Chronic conditions	Current smoking	1
Symptoms	Tachypnoea	1
	Haemoptysis	2
Clinical findings at admission	SpO2 < 92%	1
	Decreased BP	1
	GCS<15	1
Laboratory values	PCT	1
	TnI	1
	Creatinine >1.5 mg/dl	1

C-index = 0.77 (N = 584); Tachypnoea >22 breath per minute; SpO2, peripheral oxygen saturation; BP, blood pressure; GCS, Glasgow coma scale; PCT, procalcitonin; TnI, troponin I.

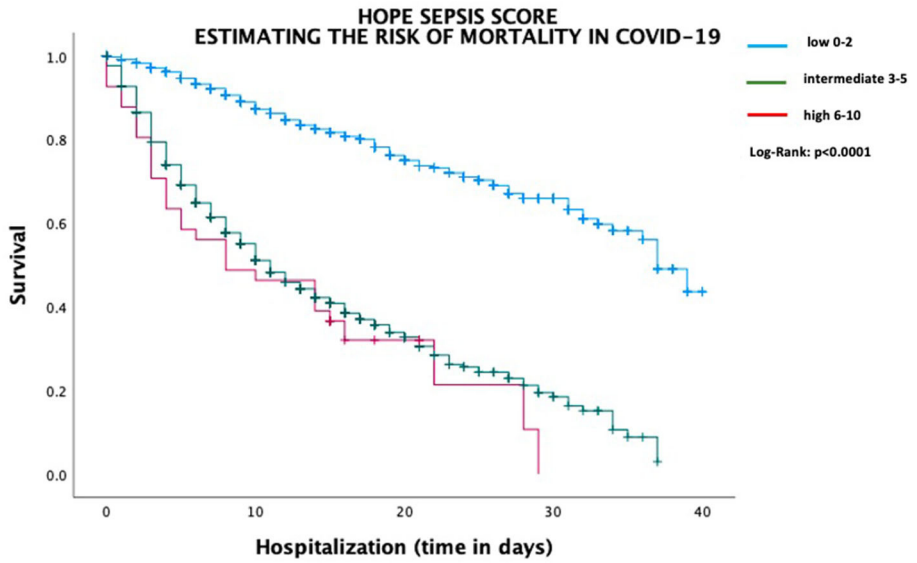

FIGURE 2 | Estimating the risk of mortality in COVID-19 according to HOPE Sepsis Score.

in COVID-19. The C-index of SOFA score in patients who required intensive care unit (ICU) was 0.74, while the C-index in other hospitalized patients was 0.79 (6). In addition, the C-index of qSOFA was 0.66 in ICU while it was 0.81 for non-ICU patients (19). The logistic organ dysfunction score (LODS) can be used to assessing the severity of sepsis in ICU. The C-index of LODS was 0.843 (20). In summary, the C-index of our score is comparable to the recently published scores. Additionally, the sensitivity of the HOPE Sepsis Score to predict sepsis was higher in intermediate as compared to high-risk patients (81.1 vs. 34.3%). On the other hand, the specificity and PPV of the HOPE Sepsis Score to predict the risk of sepsis were lower in patients with intermediate than those with high-risk for sepsis, respectively (specificity: 80.3 vs. 99.2% and PPV: 32.4 vs. 66.1%). However, the sensitivity and specificity of qSOFA ≥ 2 to predict in-hospital mortality were 69 and 55.5%, respectively (21). In 2,112 patients suffering from infections, the calculation of systemic inflammatory response syndrome (SIRS) and qSOFA showed a sensitivity of 52.8 and 19.5% and a specificity of 52.5 and 92.6% for 28-day mortality (22).

Therapeutic Approaches in Patients With Sepsis in COVID-19

The use of antibiotic treatment was significantly higher in patients with sepsis than those without sepsis, followed by hydroxychloroquine and then antiviral drugs. Prone position was more revealed in sepsis as compared to patients with non-sepsis. The co-infection among COVID-19 patients with diverse co-pathogens including bacteria was reported (23). In one observational study, the treatment with hydroxychloroquine was not associated with a lower mortality rate (24). RECOVERY trial did not show a reduction of 28-day-mortality in patients with COVID-19 after lopinavir-ritonavir treatment (25). However, these patients did not suffer from sepsis. In addition, the short duration of prone position associated with better oxygenation did not improve the mortality rate (26). In other clinical trials, prone positioning for 16 hours every day in patients with acute respiratory distress syndrome (ARDS) was reduced to 90-day mortality (27). However, further randomized clinical trials are needed to investigate the safety and efficacy of all treatment options in patients infected by SARS-CoV-2.

Outcomes of COVID-19 Patients With Sepsis

The mortality rate was significantly higher in patients with sepsis as compared to the non-sepsis group due to diverse complications (61.2 vs. 15.2%). In addition to respiratory insufficiency, other complications were more observed among patients with sepsis in comparison to non-sepsis participants; these included heart failure, acute kidney injury, pneumonia, bleeding, embolic event, and need for oxygen therapy including high flow nasal cannula, non-invasive, and invasive mechanical ventilation. In New York City, the mortality rate of COVID-19 patients, who received invasive mechanical ventilation, was less than the rate in our sepsis cohort (14.6%) but comparable with the non-sepsis group (28). Additionally, COVID-19 patients with cardiac injury presented a high mortality rate (51.2%) (29). In this regard, our data also showed that elevated TnI was associated with developing sepsis and consequently a high mortality rate. However, data in patients with sepsis with COVID-19 are limited.

At last, in comparison to SARS-CoV with 8,098 cases across 29 countries and Middle East respiratory syndrome (MERS) with 2,494 cases across 27 countries with the case-fatality rate (CFR) of 10% and 35%, the CFR of SARS-CoV-2 in Hubei was 2.9% and outside Hubei 0.4% with respect of challenges to identify all cases particularly with asymptomatic and mild courses (4, 30).

Therefore, patients with more comorbidities are susceptible to suffer from sepsis. Smokers who particularly suffering from hemoptysis and tachypnoea with decreased BP, SpO2, and GCS at admission who show abnormal laboratory as elevated PCT, TnI, and creatinine are more potential to develop sepsis when infected by SARS-CoV-2.

This study has some limitations. It has a retrospective character, not all laboratory tests were done in all patients. In addition, data about blood, urine, and stool culture are missing. External validation of our sepsis score is not performed.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University Medical Centre Mannheim, University of Heidelberg, Mannheim, Germany. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MA, IN-G, IE-B, and IA made substantial contributions to the study concept and design. All authors took obtaining ethical approval. Data were collected by MA, IN-G, IE-B, VE, VB-M, AU, IF-R, GF, RA-E, DT, JL, MP, RR, MO-A, MB, TA, FD'A, OF-A, AB, FM, DB, RS-G, CF, AF-O, and CM. MA, IN-G, and IA analysed all data. CW supported the descriptive statistics. IN-G approved the statistical analysis. MA, IN-G, IE-B, and IA prepared the manuscript. All authors contributed to the article and approved the submission version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.728102/full#supplementary-material

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Androgen Receptor Pathway Activity Assay for Sepsis Diagnosis and Prediction of Favorable Prognosis

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Introduction: Sepsis is a life-threatening complication of a bacterial infection. It is hard to predict which patients with a bacterial infection will develop sepsis, and accurate and timely diagnosis as well as assessment of prognosis is difficult. Aside from antibiotics-based treatment of the causative infection and supportive measures, treatment options have remained limited. Better understanding of the immuno-pathophysiology of sepsis is expected to lead to improved diagnostic and therapeutic solutions.

Functional activity of the innate (inflammatory) and adaptive immune response is controlled by a dedicated set of cellular signal transduction pathways, that are active in the various immune cell types. To develop an immune response-based diagnostic assay for sepsis and provide novel therapeutic targets, signal transduction pathway activities have been analyzed in whole blood samples from patients with sepsis.

Methods: A validated and previously published set of signal transduction pathway (STP) assays, enabling determination of immune cell function, was used to analyze public Affymetrix expression microarray data from clinical studies containing data from pediatric and adult patients with sepsis. STP assays enable quantitative measurement of STP activity on individual patient sample data, and were used to calculate activity of androgen receptor (AR), estrogen receptor (ER), JAK-STAT1/2, JAK-STAT3, Notch, Hedgehog, TGFβ, FOXO-PI3K, MAPK-AP1, and NFκB signal transduction pathways.

Results: Activity of AR and TGFβ pathways was increased in children and adults with sepsis. Using the mean plus two standard deviations of normal pathway activity (in healthy individuals) as threshold for abnormal STP activity, diagnostic assay parameters were determined. For diagnosis of pediatric sepsis, the AR pathway assay showed high sensitivity (77%) and specificity (97%), with a positive prediction value (PPV) of 99% and negative prediction value (NPV) of 50%. For prediction of favorable prognosis (survival), PPV was 95%, NPV was 21%. The TGFβ pathway activity assay performed slightly less for diagnosing sepsis, with a sensitivity of 64% and specificity of 98% (PPV 99%, NPV 39%).

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Conclusion: The AR and TGFβ pathways have an immunosuppressive role, suggesting a causal relation between increased pathway activity and sepsis immunopathology. STP assays have been converted to qPCR assays for further evaluation of clinical utility for sepsis diagnosis and prediction of prognosis, as well as for prediction of risk at developing sepsis in patients with a bacterial infection. STPs may present novel therapeutic targets in sepsis.

Keywords: sepsis, pathway activity assay, host response, infectious diseases, diagnosis, prediction, signal transduction pathways, androgen receptor pathway

INTRODUCTION

Sepsis is a life-threatening infection in which the immune response is dysregulated resulting in multi-organ dysfunction or failure (1). Sepsis is generally a complication of severe bacterial infection and characterized by a systemic inflammatory response leading to septic shock. Mortality rates range between 25 and 30% for sepsis and 40% to 70% for septic shock (2–4).

Aside from antibiotics and supportive measures to maintain blood circulation of internal organs, no treatments have proven to be effective, although it cannot be excluded that some treatments may benefit a subset of patients who so far cannot be identified (1). One reason for failure to develop effective treatments is the heterogeneity among sepsis patients, that is, variation in underlying medical conditions and use of drugs, and genetic variations influencing the immune response in an individual patient.

Detailed assessment of the functional immune response in a patient with sepsis may enable a personalized treatment approach and improve treatment efficacy. Diagnostic assessment of immune function is currently limited to routine blood measurements, such as numbers of immune cells and inflammation markers (e.g., C-reactive protein), but is not informative on the functional activity state of the various types of immune cells, responsible for the abnormal immune response in a sepsis patient.

The functional state of immune cells is determined by a small number of so-called cellular signal transduction pathways (STPs) (5–9). Recently, novel assays have been developed to quantitatively measure activity of STPs in cell and tissue samples, including blood samples (10–13). Measuring combined activity of these STPs in blood cells is expected to enable quantitative assessment of the innate and adaptive immune response in an individual patient (8, 14).

In this study, STP analysis was performed on publicly available gene expression data from multiple clinical sepsis studies. Measurement of activity of the androgen receptor (AR) pathway, and to a lesser extent the TGFβ pathway, in a whole blood sample is shown to have value for sepsis diagnosis and prediction of prognosis in a sepsis patient, and may lead to novel personalized treatment options. Measurement of STP activity to identify patients with a bacterial infection who are at high risk to develop sepsis is discussed.

METHODS

STP Assays to Determine Activity of AR, Estrogen Receptor (ER), FOXO-PI3K, JAK-STAT1/2, JAK-STAT3, Notch, Hedgehog (HH), TGFβ, and NFκB Pathways in Blood Cells

Development and validation of assays to quantify STP activity have been described before (10–13). In brief, target genes of transcription factors of the respective signal transduction pathways were identified, and a Bayesian network computational model was created for interpretation of measured mRNA levels of the pathway target genes to generate a quantitative pathway activity score (PAS). PAS are presented on a log2 odds scale as described (15, 16).

Affymetrix Expression Microarray Data Analysis

For analysis of clinical studies, STP assays were performed on public Affymetrix HG-U133 Plus2.0 microarray expression datasets from previously published clinical studies [deposited in the Gene Expression Omnibus (GEO) database (17)]. Quality control (QC) was performed on Affymetrix data of each individual sample prior to STP analysis, as described before (11), using available R packages (18, 19). Samples that failed QC were removed prior to data analysis.

A summary of clinical datasets used in this study is shown in **Supplementary Table 1**. All studies provided Affymetrix data from whole blood samples. Duplicate sample data between datasets of different studies were removed when such datasets were combined into one analysis or calculation, such as for determining normal pathway activity range thresholds and for sensitivity and specificity calculations. For these purposes, 8 duplicates from GSE26640, 28 duplicates from GSE8121, 10 duplicates from GSE9692, 48 duplicates from GSE13904, and 1 duplicate from GSE26378 were removed (specified in **Supplementary Table 1**).

Interpretation of Signal Transduction Pathway Activity Scores

An important and unique advantage of the STP assays is that they can in principle be performed on all cell types. A few

considerations to bear in mind when interpretating log2 odds PAS, as described before (11), are:

(1) On the same sample, log2 odds PAS cannot be compared between different signaling pathways, since each of the signaling pathways has its own range in log2 odds activity scores (11).
(2) The log2 odds range for pathway activity (minimum to maximum activity) may vary depending on cell type. Once the range has been defined using samples with known pathway activity, on each new sample the absolute value can be directly interpreted against that reference. If the range has not been defined, only differences in log2 odds activity score between samples can be interpreted.
(3) PAS are highly quantitative, and even small differences in log2 odds PAS can be reproducible and meaningful.
(4) A negative log2 odds ratio does not necessarily mean that the pathway is inactive.

Statistics

Boxplots were made using the Python data visualization library function seaborn. Statistical annotations were created using the Python package statannot (20, 21). Two sided Mann-Whitney-Wilcoxon testing was used to compare PAS across groups. For paired testing (dataset GSE95233) a one-sided *t*-test was used. *P*-values are indicated in the figures. In accordance with current consensus regarding the use of statistical parameters, a *p*-value of 0.01 (indicated as double asterisk in the figures) was considered significant (22). Receiver Operating Characteristics (ROC) curves and Area Under Curve (AUC) was calculated in R. Normal range thresholds for diagnosis and prognosis classification were based on the normal range for pathway activity in healthy individuals, as determined by mean ±2 standard deviations (SD) of pathway activity of healthy individuals. Based on these thresholds, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were determined, and Fisher exact tests were used to compare groups.

RESULTS

Measuring STP Activity in Whole Blood Samples of Pediatric and Adult Patients With Sepsis

PAS scores for the AR, ER, MAPK-AP1, FOXO-PI3K, JAK-STAT1/2, JAK-STAT3, Notch, Hedgehog, TGFβ, and NFκB pathways were measured using target gene expression data of whole blood samples from clinical studies on children (**Supplementary Figures 1–6**) and adults with sepsis and septic shock (**Supplementary Figures 7, 8**). An overview of included datasets is available in **Supplementary Table 1**.

AR and TGFβ Signal Transduction Pathway Activity Scores Are Higher in Patients With Sepsis/Septic Shock

Activity of the AR pathway, and with exception of clinical study GSE57065, also of the TGFβ pathway, were consistently and significantly increased in pediatric and adult patients with sepsis and septic shock (**Figures 1–4**). No consistent differences in AR and TGFβ pathway activity scores were observed between men and women (**Figures 1F**, **2F**, and **Supplementary Figure 6**), between patients categorized as "sepsis" vs. "septic shock" (**Figures 1D**, **2D**, and **Supplementary Figure 4**), nor between patients categorized as "SAPSII-low" and "SAPSII-high" (**Figures 3A**, **4A**, and **Supplementary Figure 7**). No difference was found between patients with gram-negative and gram-positive bacterial infections (clinical study datasets GSE4607 and GSE9692; data not shown).

Two adult sepsis clinical studies [GSE57065 (28); GSE95233 (29)] allowed investigation of STP activity at several time points after disease onset. Blood samples had been collected within 30 min, and at 24 and 48 h after septic shock onset (**Figures 3A**, **4A**, and **Supplementary Figure 7**) or at Day 1, Day 2, or Day 3 (**Figure 3B** and **Supplementary Figures 8, 11**). AR PAS were found to be significantly increased at first measurement and remained increased during subsequent days.

Normal Range AR Pathway Activity in Patients With Sepsis May Be Indicative for Survival

In pediatric sepsis patients who survived, AR (but not TGFβ) pathway activity showed a trend toward lower PAS, within the PAS range of healthy controls (**Figures 1A–C,F** and **Figures 2A–C**). Only one adult sepsis study allowed investigation of the relation between survival and STP activity [GSE95233 (29), **Figures 3B-F** and **Supplementary Figures 8, 11**]. AR pathway activity tended to be lower for sepsis survivors at day three after diagnosis, decreased between Day 1 and Day 3 of sepsis in survivor patients and (**Figures 3B-D** and **Supplementary Figure 11**). For the TGFβ pathway this was not found (**Figures 4B**, **3E,F**, and **Supplementary Figure 11**). Although not significant, results from these independent clinical studies (children and adults) suggest that AR pathway activity within the normal range may be favorable in patients with sepsis.

Activity of Other Signaling Pathways (Supplementary Figures 1–8)

ER pathway PAS were low and increased slightly in patients with sepsis, but the increase was not significant in dataset GSE95233 (pediatrics) and GSE9692 (adults). MAPK-AP1, FOXO-PI3K, JAK-STAT1/2, JAK-STAT3, NFκB and Notch signaling pathway PAS were either not significantly increased in sepsis patients, or not consistently increased across multiple independent clinical studies. Activity of the Hedgehog pathway tended to be lower in sepsis patients, only meeting statistical requirements in the adult sepsis studies (GSE57065 and GSE95233) (**Supplementary Figures 7, 8**).

Defining an Upper Threshold for Normal AR and TGFβ Signal Transduction Pathway Activity in Whole Blood Samples

To enable determination of STP assay performance parameters for diagnostic use in sepsis patients, normal STP PAS ranges in

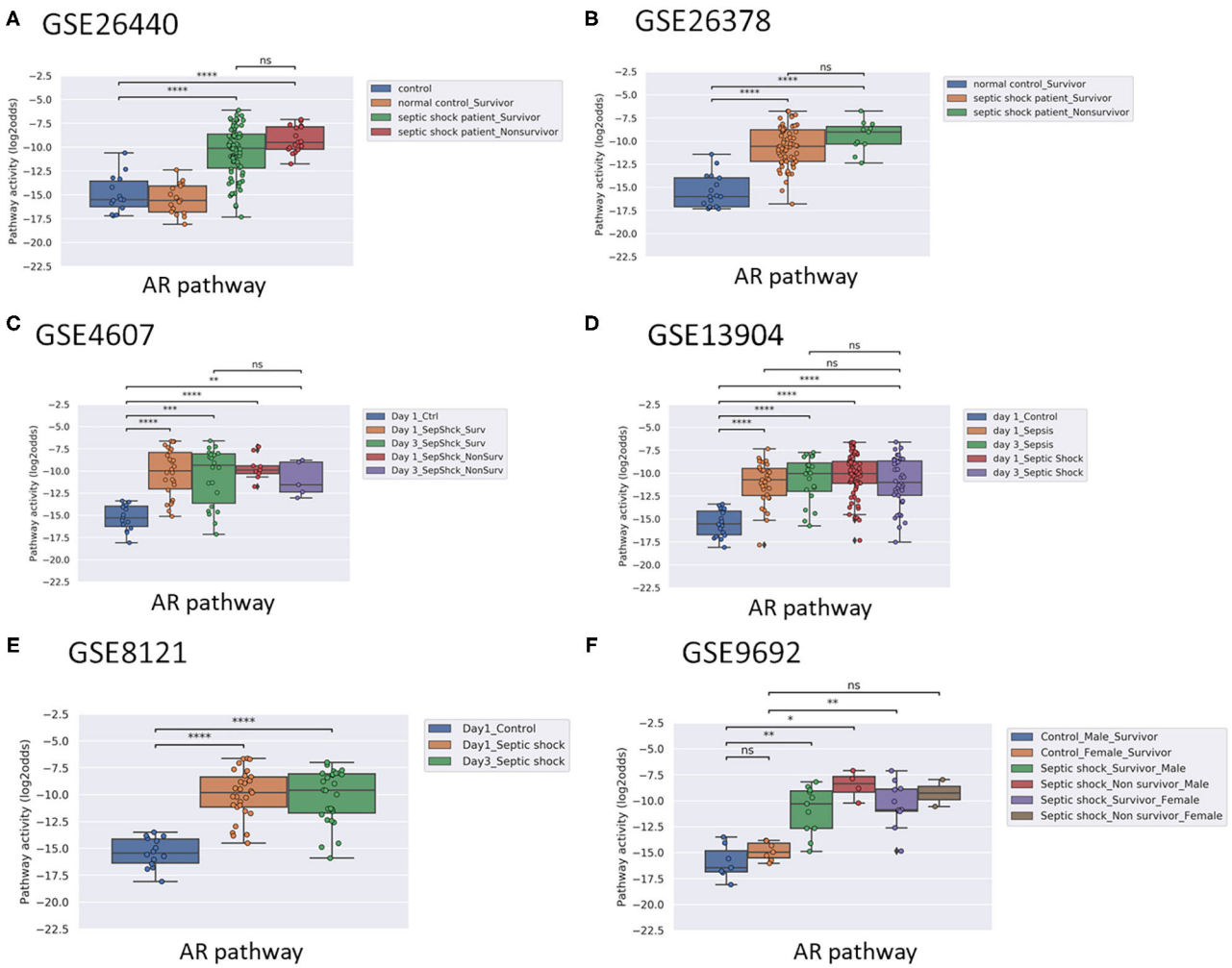

FIGURE 1 | AR pathway activity scores (PAS) of Affymetrix data of whole blood samples of children with sepsis (<10 years of age). Samples were obtained within 24 h of initial presentation to the pediatric intensive care unit (PICU) (all datasets). Indicated when applicable: survivors and non-survivors **(A–F)**; time (Day) on PICU **(C)**; and gender **(F)**. Control patients were recruited using the following exclusion criteria: a recent febrile illness (within 2 weeks), recent use of anti-inflammatory medications (within 2 weeks), or history of chronic or acute disease associated with inflammation. **(A)** Dataset GSE26440 (23). **(B)** GSE26378 (23). **(C)** Dataset GSE4607 (24). **(D)** Dataset GSE13904 (25). **(E)** Dataset GSE8121 (26). **(F)** Dataset GSE9692 (27). AR PAS on Y-axis on a log2 odds scale. Two sided Mann–Whitney–Wilcoxon statistical tests were performed; *p*-values are indicated in the figures as $^{*}p < 0.5$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ or ns (not significant).

whole blood samples were determined. Eight datasets contained samples of healthy individuals [GSE26440 (22), GSE26378 (23), GSE4607 (24), GSE13904 (25), GSE8121(26), and GSE9692 (27)] (**Supplementary Table 1**).

Mean AR PAS in healthy individuals was −15.37 (log2 odds scale, SD 1.52) for children ($n = 93$) and −12.44 (log2 odds scale, SD 1.44) for adults ($n = 37$) (**Supplementary Table 2**). Combining healthy pediatric and adult sample data ($n = 130$), mean AR PAS was −14.5 (log2 odds scale, SD 2.0). No significant differences between men and women were found. Using this information, normal range (healthy) STP PAS thresholds were determined for each STP, based on the mean STP PAS score in healthy controls ±2 SD for combined datasets (**Supplementary Table 2**) and for individual datasets (**Supplementary Table 4**). The upper AR PAS threshold for normal AR pathway activity was calculated as the mean +2SD, resulting in a threshold for pediatric patients of −12.33 (log2 odds scale), for adults −9.57 (log2 odds scale), and for pediatric and adults combined −10.54 (log2 odds scale). For the TGFβ pathway mean PAS in healthy individuals was −17.05 (log2 odds scale, SD 1.63) for children and −13.01 (log2 odds scale, SD 1.83) for adults (**Supplementary Table 2**). Combining healthy pediatric and adult sample data, mean TGFβ PAS was −15.9 (log2 odds scale, SD 2.49).

Applying the pediatric upper threshold for normal AR PAS on the pediatric study data showed that only 3 out of 45 (7%) children in the non-survivor groups had an AR pathway activity in the normal range, while 53 out of 210 (25%) survivor children had AR pathway activity in the normal range (Fisher exact test $p = 0.005$). For the TGFβ pathway

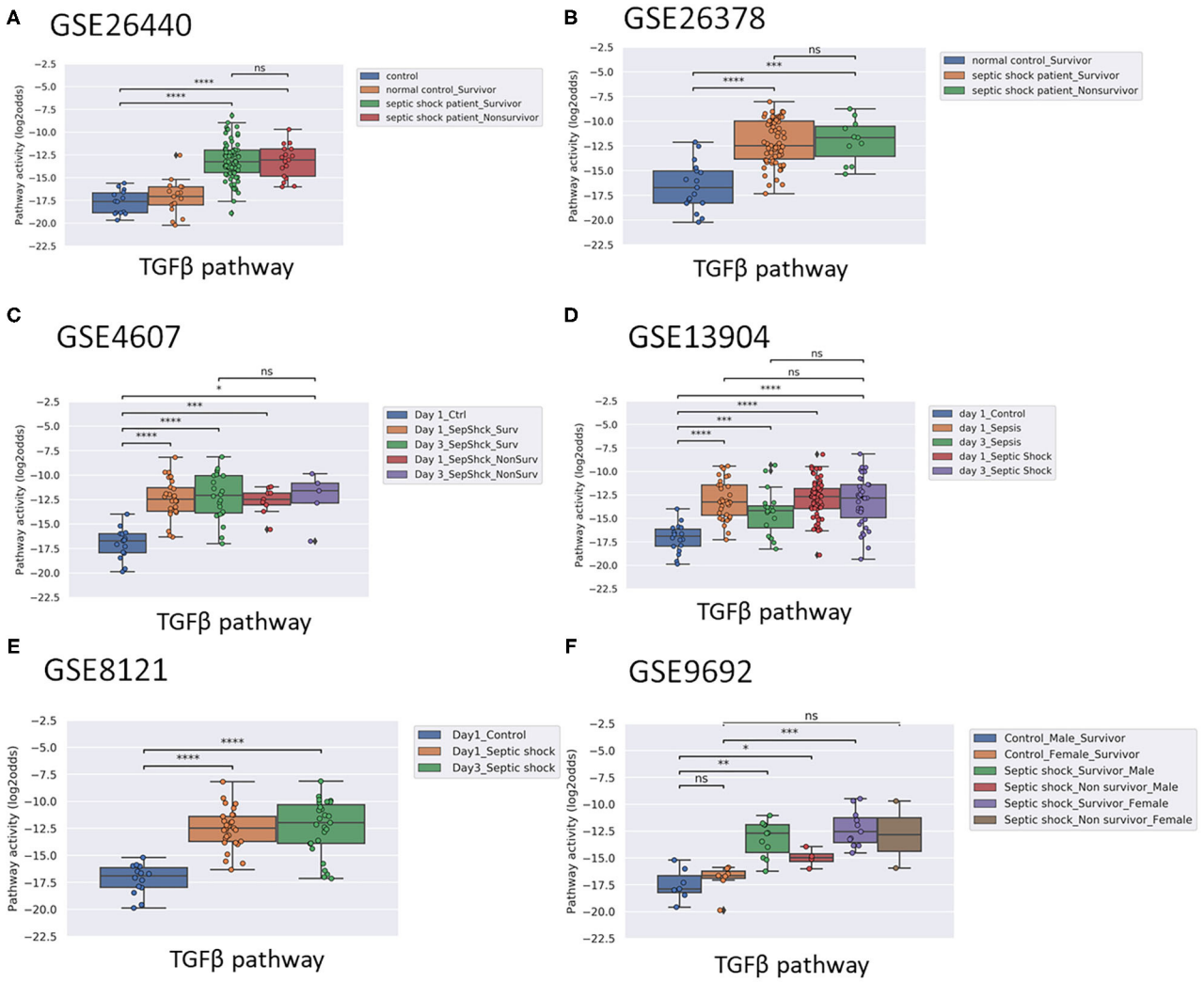

FIGURE 2 | TGFβ PAS, same datasets as in **Figure 1**. Two sided Mann–Whitney–Wilcoxon statistical tests were performed; *p*-values are indicated in the figures as $^{*}p < 0.5$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ or ns (not significant). (For explanation see **Figure 1** caption).

the percentages were respectively 31 and 32. Thus, AR PAS in the normal range was associated with sepsis survival, at least in children (**Figures 1A-C,F** and **Figures 2A-C, F**). We proceeded with calculating AR and TGFβ pathway assay performance parameters.

Sensitivity and Specificity of AR and TGFβ Pathway Activity Assays for Pediatric Sepsis Diagnosis and Prognosis Prediction

Using the pediatric upper threshold of the normal range of AR and TGFβ PAS, sensitivity, specificity, PPV, and NPV for diagnosis and prediction of survival in a pediatric sepsis patient were calculated (**Tables 1, 2**, and **Supplementary Tables 5, 6**), and a ROC curve was generated (**Figure 5**). For the same parameters of the other STPs, see **Supplementary Figures 9, 10**. For sepsis diagnosis, the AR pathway assay showed both a high sensitivity (77%) and specificity (97%), with a PPV of 99% and a NPV of 50%. AUC in the ROC curve was 0.94 for sepsis diagnosis. For prediction of favorable (survivor) prognosis, the PPV was 95%, indicating that the assay was highly specific (93%) in identifying survivor patients. The NPV was 21%, indicative of low sensitivity (25%) in predicting survivor patients. The TGFβ assay performed less, with a sensitivity for diagnosing sepsis of respectively 64% and specificity of 98%; all other STP assays performed less well (**Supplementary Figure 9**).

Comparison of Affymetrix Data Analysis Results, Described in the Original Publication Associated With the Dataset, With Results of STP Analysis

For each Affymetrix dataset, bioinformatics tools used for data analysis as reported in the associated publication were listed, together with reported functional gene annotations and/or identified "pathways" as defined by the used

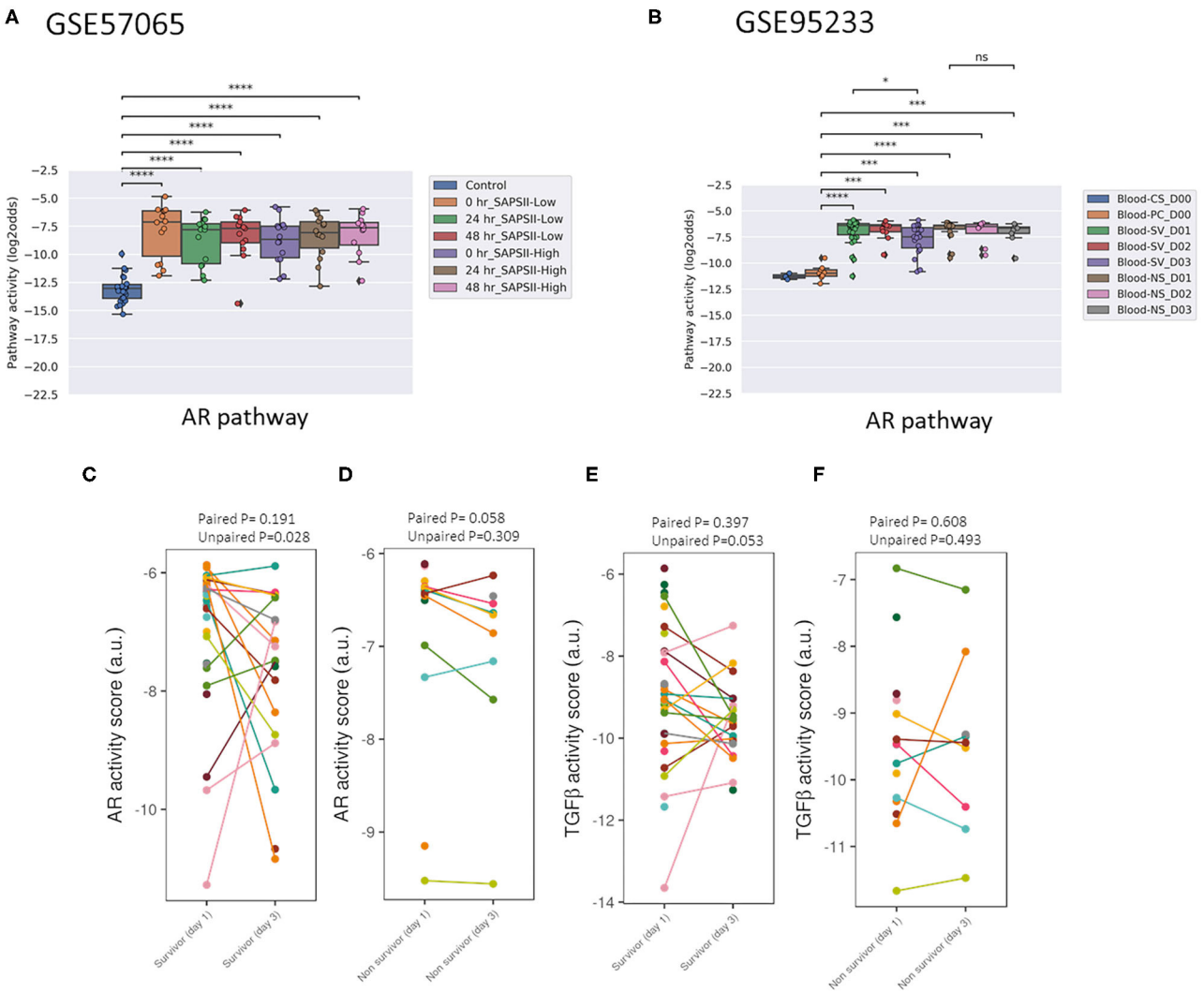

FIGURE 3 | AR PAS of datasets of whole blood samples from adults with sepsis and healthy individuals. **(A)** Dataset GSE57065 (28), containing ICU patients included at the onset of septic shock. Blood samples were collected within 30 min, and 24 and 48 h after diagnosis of sepsis. Sepsis patients were divided into SAPSII-low and SAPSII-high groups according to the median of SAPSII score (SAPSII scores of <45 and >45, respectively). **(B)** Dataset GSE95233 (29), containing ICU patients included at diagnosis of septic shock. Blood samples were collected at admission, and at day 2 (D2) or day 3 (D3). SV indicates survivor, NS indicates non-survivor sepsis patient, CS indicates control healthy individual, PC indicates patient control. **(C-E)** Dataset GSE95233 (29). AR **(C,D)** and TGFβ **(E,F)** PAS for sepsis survivor vs. non-survivor patients, at day 1 and day 3 after diagnosis. Patient samples have been connected by lines. AR PAS on Y-axis on log2 odds scale. **(A,B)** two sided Mann–Whitney–Wilcoxon statistical tests. **(C,D)** one sided paired and unpaired *t*-test. *p*-values are indicated as $^{*}p < 0.5$, $^{***}p < 0.001$, $^{****}p < 0.0001$ or ns (not significant).

tools (Ingenuity, PANTHER, D.A.V.I.D., ToppGene) (see **Supplementary Information**). In all analyses, groups of patient samples had been compared with respect to differential gene expression. In contrast to the current study, individual samples had not been analyzed. An association between sepsis/septic shock and signal transduction pathways had been identified using Ingenuity Pathways Analysis and PANTHER. The NFκB pathway (termed NFκB or TLR) (23, 24, 26, 27), the MAPK pathway (termed "p380MAPK, PDGF") (24, 26–28), the JAK-STAT3 pathway (termed "IL6, IL10") (24, 26–28), and the PI3K-FOXO pathway (termed "Integrin signaling/Insulin signaling/IGF1 signaling") (26), were identified as associated with sepsis, but without information on activity state of these pathways. Hormonal AR and ER pathways were not mentioned and neither was the TGFβ pathway.

DISCUSSION

Using a previously reported STP assay platform, we quantified activity of the most important signal transduction pathways that determine immune cell function, using RNA expression data of whole blood samples from previously published clinical pediatric and adult sepsis studies (9–13, 16).

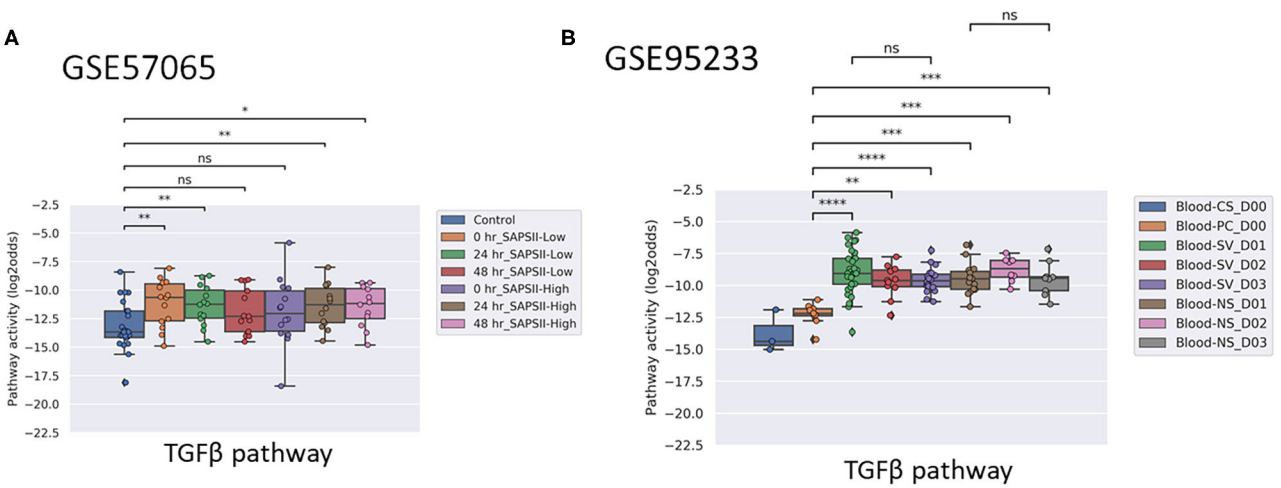

FIGURE 4 | TGFβ PAS, same datasets as in **Figures 3A,B**. Two sided Mann–Whitney–Wilcoxon statistical tests were performed; *p*-values are indicated in the figures as $^{*}p < 0.5$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ or ns (not significant). (For explanation see **Figures 3A,B** caption).

Activity of Several Signaling Pathways Is Increased in Whole Blood From Patients With Sepsis: Potential for Diagnostic Use

Activity of the AR pathway, and to a lesser extent of the TGFβ signaling pathway, was increased in sepsis, while a trend toward higher activity was observed for MAPK-AP1, ER, NFκB and JAK-STAT3 pathways, toward lower activity for the Hedgehog pathway, while activity of the JAK-STAT1/2 pathway did not differ between healthy controls and sepsis patients. Only the AR pathway assay could to some extent identify sepsis patients with a favorable prognosis. This assay showed high sensitivity and specificity for sepsis diagnosis, and high specificity for prediction of favorable (survival) prognosis in children with sepsis.

In sepsis, the immune system plays a crucial role, with both pro-inflammatory and anti-inflammatory mechanisms. A subset of patients with sepsis has been described to rapidly display signs of immunosuppression and inflammation, which is associated with worse prognosis (30, 31).

In line with our observations, for AR, ER, TGFβ, NFκB, and JAK-STAT3 pathways a number of functional roles in the immune response have been described, with a distinct immunosuppressive role for AR and TGFβ pathways and an inflammatory role for NFκB and JAK-STAT3 pathways (30–39).

The lack of increase in JAK-STAT1/2 pathway activity in sepsis patients is in line with our earlier findings that PAS scores of this signaling pathway only increase in virally, but not bacterially, infected patients, at least when measured in whole blood and Peripheral Blood Mononuclear Cell (PBMC) samples (9).

For the AR, ER, TGFβ, and NFκB pathways putative roles in sepsis have been described, generally in relation to specific immune cell types (40–44). This may explain the lack of diagnostic power of some of the measured STPs when measured in whole blood samples, since whole blood samples consist of a mix of multiple immune cell types.

Is There a Causal Relation Between AR Pathway Activity and Sepsis?

Despite the disadvantage of measuring in whole blood samples, the diagnostic performance of AR and TGFβ assays for sepsis was good. With respect to the TGFβ pathway, either decreased or elevated levels of various TGFβ pathway ligands have been reported in sepsis patients, while uncertainty remains with respect to the causal role of this pathway (44).

Multiple findings support a causal role for the AR pathway in sepsis. The AR signaling pathway is in principle activated by the presence of testosterone ligand, binding to a pocket in the AR transcription factor protein to induce RNA transcription of target genes (7). The AR protein is expressed in a wide variety of innate and adaptive immune cells including neutrophils, macrophages, mast cells, monocytes, megakaryocytes, B cells, and T cells, indicating that the AR signaling pathway can be activated in these blood cell types (42). Monocytes are one of the key cell types that play a pathogenic role in sepsis, and make up around 10% of whole blood cells (45, 46). Interestingly and in line, men have shown to be more susceptible to developing sepsis than women (47). Some studies suggest that the AR pathway may be a useful drug target in sepsis: testosterone blockade in patients with hemorrhage improved the prognosis if subsequently sepsis developed (48). Also AR pathway blockade reduced mortality in a preclinical mouse model for sepsis (38, 40). While suggestive of a role for the AR pathway in the pathophysiology of sepsis, we are not aware of any clinical studies investigating AR pathway targeted drugs in human sepsis patients.

AR Pathway Assay as a Diagnostic Assay for Sepsis

Current diagnosis of sepsis is based on assessment of multiple clinical symptoms and biomarkers, but timely diagnosis and even more so, prediction of prognosis of a sepsis patient, remain a clinical challenge. Despite the limitation of measuring in

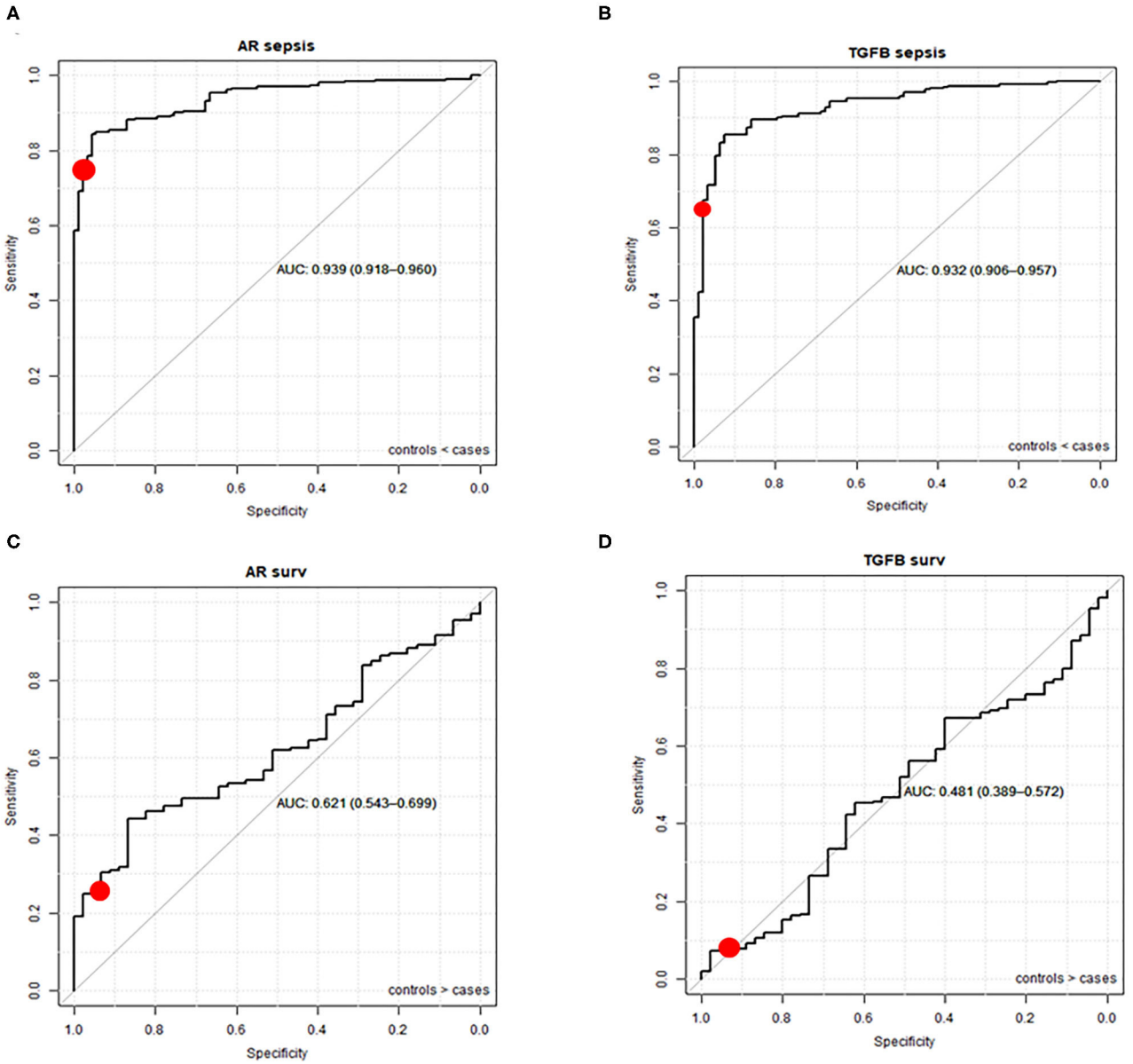

FIGURE 5 | ROC curves (and AUC) of AR and TGFβ pathway activity assays for sepsis diagnosis **(A,B)** and survival prediction **(C,D)**. Healthy controls and sepsis patients (sepsis and sepsis shock groups combined) from datasets GSE26440 (23), GSE26378 (23), GSE4607 (24), GSE13904 (25), GSE8121 (26), and GSE9692 (27) are included (duplicate samples excluded). The red dot represents sensitivity and specificity when the upper threshold is mean plus 2 SD of normal pathway activity.

whole blood samples, determination of AR PAS showed very good performance to diagnose sepsis, while as a prognostic assay, results suggest potential clinical utility to identify good prognosis patients.

The normal range in AR pathway activity differed between healthy adults and children. This may be a "real" difference, for example caused by differences in testosterone levels between adults and children. However, the normal range in AR pathway activity did not differ between men and women. Alternatively, differences may have been caused by different handling of samples between clinical centers involved in pediatric vs. adult studies (22–26, 26–28, 28, 29).

Since Affymetrix analysis of sample data takes too long to be useful in a clinical setting, STP assays have currently been adapted to qPCR for future diagnostic use (16). The time-to-result of the qPCR-based STP-assays is a few hours, and clinical implementation of such assays can be fast, following determination of normal ranges of STP activities.

TABLE 1 | Performance parameters of the AR pathway assay for diagnosis of pediatric sepsis and prediction of survival.

Sepsis	Sepsis diagnosis AR PAS above normal range $n = 492$	Sepsis survival AR PAS in normal range $n = 255$	Sepsis non survival AR PAS above normal Range $n = 255$
Sensitivity	77%	25%	93%
Specificity	97%	93%	25%
PPV	99%	95%	21%
NPV	50%	21%	95%
Fisher's exact test	$P < 2.2e{-}16$	$p = 0.005023$	$p = 0.005023$

TABLE 2 | Performance parameters of the TGFβ pathway assay for diagnosis of pediatric sepsis and prediction of survival.

Sepsis	Sepsis diagnosis TGFβ PAS above normal $n = 492$	Sepsis survival TGFβ PAS in normal range $n = 255$	Sepsis non-survival TGFβ PAS above normal $n = 255$
Sensitivity	64%	32%	69%
Specificity	98%	69%	32%
PPV	99%	83%	18%
NPV	39%	18%	83%
Fisher's exact test	$p < 2.2e{-}16$	$p = 1$	$p = 1$

In general, correlation between STP activity determined by Affymetrix-based STP analysis and qPCR-based analysis is very good (15).

Thus, while we show feasibility of determining a normal range of AR pathway activity in whole blood samples based on Affymetrix data analysis, clinical adoption of the AR pathway activity assay for diagnostic use in sepsis will require determination of normal AR pathway activity ranges for both adults and children, using the qPCR-based AR pathway assay, followed by further confirmation of clinical utility.

In addition to diagnosing sepsis, and prediction of prognosis, we hypothesize that the AR pathway activity assay may find clinical utility in prediction of risk at serious infectious complications in the period after surviving sepsis. This is of relevance since patients who survive sepsis often remain immune-compromised for longer periods of time, which is associated with increased risk at secondary infections with poor clinical outcome (3, 49). In view of the immunosuppressive role of the AR pathway, we hypothesize that this may be causally related to persistent high AR pathway activity (50).

Similarly, the assay may have value in predicting risk at developing sepsis in patients with a bacterial infection. To illustrate this clinical use case, AR pathway activity was measured in RNAseq data [GSE161731, (51)] from samples of patients presenting either with Community Acquired bacterial Pneumonia (CAP) or with viral influenza infection at the emergency ward, using an RNAseq-converted AR pathway assay (**Supplementary Figure 12**). In whole blood of CAP patients mean AR pathway activity was increased, compared to healthy individuals or patients with an influenza infection, while around one third of CAP patients had AR pathway activity in the normal range. Within the perspective of the current study, we hypothesize that the latter group with normal AR pathway activity did not have sepsis at admission and was at lower risk to develop sepsis, possibly not needing hospital admission. This is in line with data showing that at least a third of patients presenting with CAP have or develop sepsis (52, 53). Future clinical studies are necessary to further investigate these clinical use cases.

STP Assays to Guide Therapy Choices

STP activity measurements may be used to develop novel therapies for sepsis, on the premise of a causal relation with sepsis. Targeted drugs are available against the AR, TGFβ, MAPK-AP1, NFκB, and JAK-STAT3 pathways, all involved in immune responses, offering a new perspective on treating sepsis (42). It is expected that a specific drug will only be effective if the causal signaling pathway(s) is (are) abnormally active. Variation in STP PAS was high in sepsis patients, suggesting that only a subpopulation of patients may benefit from pathway-targeted therapy. Thus, investigation of potential benefit of for example androgen antagonists will require a personalized treatment approach based on measuring signal transduction pathway activity.

Interestingly, the AR pathway has been suggested to play a role in severe COVID-19 infections, and an AR inhibitor (proxalutamide) has been reported to reduce disease severity, overall mortality, and length of hospitalization (54, 55). Secondary bacterial infections frequently complicate severe COVID-19 pneumonia and we hypothesize that increased AR pathway activity may be indicative of immunosuppression and high risk at sepsis, explaining the benefit of anti-androgen therapy in this setting.

Comparison of Our Data Analysis Results With the Original Data Analysis Performed in the Clinical Studies

Since we frequently get questions regarding the difference between our STP analysis and other bioinformatics tools for Pathway Analysis, such as GSEA and Ingenuity, we compared our STP analysis results with data analysis results performed by the investigators who generated the Affymetrix data. Using a variety of bioanalytical biomarker discovery tools (Ingenuity, PANTHER, D.A.V.I.D., and ToppGene) associations between sepsis and the NFκB, MAPK, FOXO-PI3K and JAK-STAT3 pathways had been found, in agreement with our STP analysis results, but no associations with the AR, ER, and TGFβ pathways. Identification of "pathways" using these bioinformatics tools is not suited for analysis of individual sample data and is not informative on the actual (and quantitative) activation state of the signaling pathway. To our best knowledge the sepsis-associated gene signatures discovered by earlier bioinformatics analysis of these clinical studies have not been implemented in clinical practice.

STP assays were developed for diagnostic purposes and have been analytically validated for measuring signaling pathway activity prior to use on the current data. STP results are potentially clinically actionable, since many targeted drugs inhibit activity of the signal transduction pathways that were found to be overactive in sepsis and septic shock.

In summary, our current results show initial results supporting clinical validity and potential clinical utility of measuring activity of the AR and TGFβ signal transduction pathways in patients with sepsis, fulfilling the basic requirements for biomarker assays (56). In addition, good reproducibility between the different clinical studies suggests that defining a normal ("healthy") range of pathway activity in whole blood samples will be feasible. This is important for future clinical implementation. STP assays have been converted to qPCR assays, which can be easily performed in a routine hospital lab, and provide a pathway activity score within a few hours, enabling timely clinical decisions (16).

Limitations and Future Studies

Clinical studies are needed to confirm the clinical utility of measuring AR (and TGFβ) pathway activity in patients with, or at risk for developing, sepsis or septic shock. Only two studies with adult patients were available for STP analysis. Although results were comparable, causes of sepsis are far more variable in adult patients and in in future studies it would be important to include more adult patient populations.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

WB: data, results analysis, and concept and writing. WV: pathway model development and results analysis. AS: pathway model development, results analysis, and writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.767145/full#supplementary-material

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BRIEF RESEARCH REPORT
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Acute Vertigo After COVID-19 Vaccination: Case Series and Literature Review

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Objective: The aim of this study was to present some cases of acute vertigo potentially related to the coronavirus disease 2019 (COVID-19) vaccine and review the available literature about cochleovestibular dysfunction after the COVID-19 vaccination.

Methods: In the period from May to July 2021, we evaluated 33 patients (mean age 54.3 ± 14.1) with "acute vertigo" post COVID-19 vaccination. A detailed medical history was taken on comorbidities, types of vaccines received, and symptoms associated. All patients underwent otoneurological evaluation, such as head impulse test, nystagmus evaluation, test of skew (HINTS) examination. Head shaking test-induced nystagmus, hyperventilation-induced nystagmus, and parossistic positional nystagmus were studied to search for vestibular impairment.

Results: Symptoms included 16 patients (48.5%) with objective vertigo, 14 patients (42.4%) with subjective vertigo, and 3 patients (9.1%) with dizziness. Of the associated ear, nose, and throat (ENT) symptoms, the most expressed was tinnitus (18.2%). Bedside examination showed absent nystagmus in 7 patients (21.2%), 9 patients (27.3%) had horizontal or rotatory nystagmus, 17 patients (51.5%) had a vertical or oblique nystagmus, negative HST, or "central HINTS."

Discussion and Conclusions: The 9 patients had an evoked nystagmus pathognomonic for benign paroxysmal positional vertigo; in the remaining 17 cases, peripheral vestibular dysfunction could be excluded and central disorder may be suggested. Due to the prevalence of nystagmus of non-peripheral origin, a central nervous system involvement could not be excluded. However, due to the small sample size, a definite cause–effect relationship between vaccination and vertigo cannot be inferred. In light of expected third dose, large-scale and well-designed studies are needed to better define possible adverse reactions of the COVID-19 vaccine.

Keywords: HINTS examination, COVID-19, acute vertigo, dizziness, central vertigo, peripheral vertigo, tinnitus, vaccine

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INTRODUCTION

Severe acute respiratory syndrome coronavirus (SARS-COV-2) infection has led to a global pandemic and a public health crisis, resulting in over 4,806,841 deaths at the time of publication (1).

The efforts of the scientific community to prevent coronavirus disease 2019 (COVID-19) associated mortality and morbidity have resulted in multiple vaccines worldwide available and approved for use.

Severe acute respiratory syndrome coronavirus spike (S) glycoprotein is the main target for current vaccines, since antibodies directed against SARSCoV-2 spike can block the fusion between the virus and host cell membrane, inhibiting the infection (2, 3).

Currently, authorized vaccines for COVID-19 include the mRNA vaccines: BNT162b2 (Pfizer/BioNTech) and mRNA-1273 (Moderna) and the adenoviral-vectored vaccines: ChAdOx1 nCoV-19 (University of Oxford/AstraZeneca) and Ad26.COV2.S (Janssen).

Pfizer/BioNTech is currently the most widely used vaccine in the Italian vaccination campaign (71%), followed by AstraZeneca (16%), Moderna (11%), and COVID-19 Janssen vaccine (2%).

Adverse effects observed in Italy after administration of these vaccines, are recorded in the COVID-19 Vaccine Surveillance Report drawn up by the Italian Medicines Agency (AIFA) (4). As of August 2021, 91,360 reports of adverse events following vaccination have been entered in the National Pharmacovigilance Network, out of 76,509,846 vaccine doses (119/100,000 administered doses). Approximately 86.1% of adverse effects reports entered refer to non-serious events, and 13.8% to serious adverse events.

The most reported adverse events fall within general diseases as fever, injection site pain, asthenia, followed by pathologies of the nervous system, such as headache and paresthesia, by pathologies of the musculoskeletal system and of the connective tissue, mostly musculoskeletal pain, and by gastrointestinal diseases, generally nausea, vomiting, and diarrhea. Rare are psychiatric disorders, cardiac, blood, and lymphatic system disorders, eye, ear, and labyrinth disorders. Very rare are anaphylactic reactions, myocarditis/pericarditis, and facial nerve paralysis. Very rare adverse events related to Astra Zeneca include acute and subacute neuropathies (such as, Guillain–Barré's syndrome) and intracranial or atypical venous thrombosis with or without thrombocytopenia.

A recent systematic review and meta-analysis of clinical trials were conducted on the incidences of nervous and muscular adverse events (NMAEs) after COVID-19 vaccination. The incidence of NMAEs was 29.2% in the vaccine group and 21.6% in the control group, in a total of 15 randomized blinded controlled clinical trials. Systemic neurological symptoms included migraine, dizziness, vertigo, and syncope (5).

Audiovestibular side effects for the COVID-19 vaccines, as already mentioned, are generally categorized as "ear and labyrinth disorders," which include a wide range of clinical expression.

Few reports of audiovestibular symptoms after the administration of all four types of vaccine were notified by the Italian Pharmacovigilance Network (4). Recently Parrino et al. (6) published three cases of sudden unilateral tinnitus following BNT162b2 mRNA-vaccine injection, which rapidly resolved in 2 out of 3 cases.

In addition, Tseng et al. reported a single case presenting with sudden-onset tinnitus and cochleopathy after his first dosage of COVID-19 vaccine, reversible and recoverable under conservative steroid management (7).

Besides, the US Vaccine Adverse Event Reporting System (VAERS) database (8) cites possible adverse reactions involving the cochleovestibular system: 12,787 reports of tinnitus among 1,302,332 COVID-19 vaccine total adverse events, 1,627 reports of hypoacusis, 8,504 reports of vertigo, 254 of positional vertigo, and 133 of vestibular neuronitis.

It is worth noting that acute vertigo syndrome could represent an overlap between ear/labyrinth and nervous system disorders, especially if nystagmus presence/absence or peripheral/central etiopathogenesis have not been investigated.

In this work, we present some cases of acute vertigo potentially related to the vaccine, to enlarge the available literature and, if possible, suggest hypotheses about the origin of vestibular dysfunction after the COVID-19 vaccination.

MATERIALS AND METHODS

Participants

During the period from May 1 to July 30, 2021, in this observational retrospective study, we evaluated 33 patients (7 men and 26 women; mean age 54.53 ± 14 years) with "acute vertigo" after COVID-19 vaccination. These patients arrived at the vestibular clinic from the Emergency Room of our Hospital or after the request of a primary care physician. The patients reported vertigo or dizziness not more than 48 h after the COVID-19 vaccination. No patient had the COVID-19 disease before administering the vaccine. Inclusion criteria: all adult subjects (>18 years old) referred for acute vertigo after the COVID-19 vaccination were enrolled. Exclusion criteria: subjects with acute vertigo onset before COVID-19 vaccination.

For all patients, we performed: bedside examination with vestibulospinal stability tests, head impulse test, nystagmus direction, testing skew (HINTS) examination, head shaking test (HST), hyperventilation-induced nystagmus (HIN), and positional nystagmus maneuvers.

The Research Ethics Committee of Catania 1, G Rodolico-San Marco University Hospital, approved the study protocol (Permit Number: 242/2021/PO). The study was conducted in accordance with the Declaration of Helsinki and all participants provided their written consent.

Bedside Examination

The bedside examination was performed at the moment of the hospital admission. First of all, a complete medical history was taken: past and proximate medical history, paying particular attention to comorbidities and cardiovascular risk. In the proximate medical history, we asked for objective or subjective vertigo and dizziness, vertigo/dizziness length, if the patient had neurovegetative symptoms, for trigger of vertigo, visual

impairment, tinnitus or hearing loss onset, or other symptoms associated with acute vertigo.

After medical history acquisition, we evaluated equilibrium of a patient with vestibulospinal stability tests:

- Romberg test, having the patient stand in tandem or on one foot with eyes open and closed;
- Fukuda stepping test, performed by marching in place with eyes closed for 30 s and noting any excessive turning suggestive of a vestibular imbalance.
- Finger-nose-finger, heel-knee-shin, rapid alternating movements, to evaluate cerebellar function and search potential dysmetria and/or adiadochokinesia.

HINTS Examination

Head impulse test, nystagmus direction, testing skew (HINTS) examination is a triad component that we routinely perform in our clinic, and it consists of three steps: head impulse test (HI), nystagmus direction (N), testing skew (TS). HINTS was developed as a test to assess patients with acute vestibular syndrome (AVS), defined like the acute onset of vertigo, dizziness, gait instability, presence of neurovegetative symptomatology (nausea and vomiting), head movements intolerance, and presence of nystagmus (9–12).

Head impulse testing is used in both unilateral and bilateral vestibulopathy. It is to remember that a normal response to a rapid and passive eye movement during a fixation on central target (in this case, usually the nose of examiner) is an equal and opposite eye movement of the same magnitude. Moving the head of a patient toward or away from center position, vestibulo-ocular reflex (VOR) does not change; instead, if there is a peripheral vestibular damage, VOR is damaged and the acceleration signal to move eyes is impaired and resulting in gain loss. HIT is considered "positive" (or abnormal) when rapid movements of a patient's head bring to a fixation loss of the eyes and a corresponding refixation saccade: this is common in people with peripheral vertigo (for instance, in vestibular neuritis). Instead, central vertigo has a "negative" (or normal) HIT, and this is because the VOR is not damaged and the eye of a patient remains fixed on target (12).

Nystagmus direction analysis is very important to differentiate a central from a peripheral vertigo: pseudo-spontaneous nystagmus, gaze induced, direction changing nystagmus, head shaking nystagmus, pure torsional, or pure vertical nystagmus in patients with AVS are signs of possible central lesion. Instead, a spontaneous horizontal nystagmus in primary position, that is inhibited with fixation and that follows Alexander's law (the amplitude of the nystagmus increases in the gaze-direction of the primary position nystagmus fast phase) testifies for vestibular neuritis (VN). Typically, peripheral vestibular lesions have a unidirectional nystagmus that increases in the gaze direction of the fast phase (Alexander's Law) (12).

Skew deviation is a vertical ocular misalignment in primary position of gaze, and it reflects an altered otolith-ocular reflex (OOR). The physician asks the patient to fixate a central target (usually the nose of examiner), while the examiner covers the eyes of patient alternatively and observes the vertical position of the eyes. Vertical skew deviation is absent if vertigo is peripheral, while, if present, it shows a central cause (12).

If any step of HINTS indicates a central vertigo, the HINTS test is considered "central": it implies the need for further investigation, like neuroimaging (CT or RMN), referring patients to other specialists.

Signs of Vestibular Impairment

We searched for the signs of vestibular impairment under infrared binocular videonystagmoscopy through:

Head Shaking Test

Head shaking test is considered as a useful clinical tool for detecting asymmetries between the vestibular labyrinths. The test requires that the head patient is shaked rapidly at 2 Hz oscillation for approximately 20 s in the horizontal plane. A positive test HSN was defined by the presence of at least three beats of nystagmus after stopping the head shake. These movements may cause a horizontal nystagmus where the fast phase beats toward the healthy labyrinth: this finding suggests a peripheral vestibular hypofunction, and the nystagmus has a duration that can last as long as 6 s.

Instead, the presence of a vertical or oblique nystagmus after a horizontal head shaking typically suggests pathology with a central etiology; nystagmus that is downbeating has been reported as the most common direction after horizontal head shaking in patients with migrainous vertigo (13).

Hyperventilation-Induced Nystagmus

Hyperventilation-induced nystagmus is commonly used because hyperventilation induces neuro-physiological modifications able to reveal latent cerebellar or vestibular diseases, while in healthy people incidence of HIN is low.

In the cases of VN and acoustic neuroma, the HIN can evoke a paretic nystagmus (in which the fast phases beat toward the healthy side) by disrupting central compensation mechanisms, but, in these pathological conditions, it can also evoke an excitatory type of nystagmus, in which the fast phases beat, on the contrary, toward the affected side. HIN is important to test in perilymphatic fistula and in the superior canal dehiscence syndrome because it can evoke either a horizontal nystagmus, in the case of larger defects in the bony wall of the semicircular canal with associated hypofunction, or torsional nystagmus, in the case of smaller defects causing a third mobile window into the inner ear (14). In cerebellar diseases, HIN can increase or evoke a downbeat nystagmus.

Diagnosis of Benign Paroxysmal Positional Vertigo

The benign paroxysmal positional vertigo (BPPV) is derived from a dislodged otoliths from the utricle that migrate into one of the semicircular canals (most commonly the posterior canal). BPPV is suspected when a patient reports very brief episodes of objective vertigo (generally less than 1 or 2 min), and episodes of vertigo wake up the patient from sleep (10). Clinical features essential for diagnosis are the latency, direction, time course, and duration of positional nystagmus (15).

The diagnosis is confirmed reproducing symptoms and signs using canal specific maneuvers to identify a canal-specific nystagmus. The canal-specific response is diagnosed when the head-rotation on the plane of the semicircular canal evokes a positional nystagmus. These beats in the plane of the affected canals end in the expected direction for the canal excitation or inhibition, and this positional nystagmus was studied using the Dix–Hallpike test to diagnose posterior semicircular canal BPPV (pc-BPPV) and the Pagnini-McClure maneuver to diagnose horizontal semicircular canal BPPV (hc-BPPV).

In the Dix–Hallpike maneuver, the head of patient (with sitting patient) is turned 45 degrees toward the side to be tested, and then laid back quickly into a head-hanging position. Patient refers to an attack provoked by lying down or turning over in the supine position. The canalolithiasis of posterior canal had a duration attack <1 min, the positional nystagmus is elicited after a latency of few second and the nystagmus is a combination of torsional and up-beating, and typically lasting <1 min (15).

In the Pagnini-McClure maneuver, the patient lying supine and head is elevated about 30 degrees and quickly rolled to one to another side. In this case too, vertigo is provoked by lying down or turning over in the supine position and the attack has a duration <1 min. Instead, the nystagmus is elicited after a brief latency or no latency and it beats horizontally toward the undermost ear with the head turned to either side (the nystagmus changes his direction: it is geotropic) (15).

Statistical Analysis

Data collected were put into a database to be used for statistical analysis. Quantitative variables have been presented as mean ± SD or median (interquartile range, [IQR]), as appropriate. Categorical variables have been expressed as absolute numbers and percentages. We performed chi-squared test and one-way ANOVA to analyze the differences between demographics and different outcomes (vertigo, central nystagmus, and peripheric nystagmus). We used Fisher's exact test to examine the differences in type of nystagmus between dichotomous groups (Pfizer *vs*. all other vaccines, mRNA vaccines *vs*. others), calculating odds ratios (*OR*s) with 95% CIs. We considered results at two-tailed $p < 0.05$ as statistically significant. Data analysis was performed using R 4.1.0 (R Foundation, 2021).

RESULTS

Their mean age was 54.3 ± 14.1 years old, with 26 women and 7 men. We collected the general characteristics, medical history, and types of vaccines received in **Table 1** and highlighted any comorbidity in **Table 2**. Particularly, 23 patients received Pfizer, 5 patients received Astrazeneca, 4 patients received Moderna, and 1 patient received Johnson & Johnson vaccine.

Symptoms included objective vertigo (16 patients, 48.5%), subjective vertigo (14 patients, 42.4%), and dizziness (3 patients, 9.1%). Of the associated ear, nose, and throat (ENT) symptoms, the most expressed was tinnitus (18.2%).

Analyzing the results of bedside examination, HINTS examination and signs of vestibular impairment, we hypothesized the probable clinical diagnosis for each patient (**Table 3**). In particular, 7 patients (21.2%) did not show nystagmus, 9 patients (27.3%) had and horizontal or rotatory nystagmus, 17 patients (51.5%) had a vertical or oblique nystagmus, negative HST or "central HINT." No patient had HIN.

The equilibrium of a patient was evaluated with vestibulospinal stability test. Particularly, 26 patients (78.79%) presented positive Romberg Test and only 6 patients (18.18%) presented a negative Romberg Test. Moreover, 1 patient cannot execute it because of excessive instability. Of the 26 patients with positive Romberg Test, 17 patients (65.38%) presented pluridirectional oscillation, 5 patients (19.23%) presented anteroposterior oscillation, 2 patients (7.69%) presented laterolateral oscillation, and 2 patients (7.69%) showed fall tendency.

TABLE 1 | General characteristics of patients (sex and age), types of vaccines received, reported symptomatology (objective, subjective vertigo, or dizziness), numbers of patients who refer associated ear, nose, and throat (ENT) symptoms.

Total number of patients	33
Men	7 (21, 21%)
Women	26 (78, 79%)
Mean age	54.53 ± 14.14
Range	24–78
Vaccine received	**Number of patients, (%)**
MRNA vaccine Pfizer-Biontech (Tozinameran)	23 (69, 70)
Vaccine Astrazeneca (CHADOX1 NCOV-19)	5 (15, 15)
MRNA vaccine Moderna (CX-024414)	4 (12, 12)
Vaccine Janssen (AD26.COV2.S)	1 (3, 3)
Reported symptomatology	
Objective vertigo	16 (48, 5)
Subjective vertigo	14 (42, 4)
Dizziness	3 (9, 1)
Associated ENT symptoms	
Hearing loss	4 (12, 12)
Tinnitus	6 (18, 2)
Ear fullness	2 (6, 06)
Hypersensitivity to noise	1 (3, 03)

TABLE 2 | Presence of comorbidities.

Comorbidities	**Number of patients, (%)**
Cardiovascular	15 (45, 4)
Diabetes	4 (12, 1)
Neurologic	9 (27, 2)
Orthopedic	3 (9, 0)

Cardiovascular: hypertension, coronaropathy and anticoagulant antiplatelet therapy; neurologic: chronic neurovascular disease, headache, and psychiatric pathologies; orthopedic: cervical or lumbar hernia.

TABLE 3 | Analysis of nystagmus and probable clinical diagnosis.

Type of nystagmus	Number of patients, (%)
No presence of nystagmus	7 (21, 21)
Presence of horizontal or rotatory nystagmus	9 (27, 27)
Presence of positive HST/ "central HINTS" or vertical or oblique nystagmus/ "central HINTS"	17 (51, 52)
Probable clinical diagnosis	
No presence of vestibular impairment or central etiology of vertigo/dizziness	7 (21, 21)
Benign paroxysmal positional vertigo	9 (27, 27)
Probable central etiology	17 (51, 52)

Examinating Fukuda stepping test, 21 patients (63.64%) showed a positive test and 6 patients (18.18%) showed a negative test, while 6 patients (18.18%) cannot execute it due of high instability. Particularly of this 21 patients, 10 (47.61%) showed right or left deviation, 11 (52.38%) manifested fall tendency. Only 2 patients presented frenage testing dysmetria and adiadochokinesia.

Benign paroxysmal positional vertigo was diagnosed in all patients with horizontal or rotatory nystagmus, who received a therapeutic maneuver to solve the canalolithiasis. The latter 17 cases were suggestive for vertigo of central origin, were referred to the neurologist for further clinical-instrumental investigations.

Patients with no presence of vestibular impairment or sign of central etiology of symptomatology, have been sent to other specialists, such as physiatrist or cardiologist.

We have not found any statistical difference between sex and age of patients with different outcomes (vertigo, peripheric, and central nystagmus). Restricting the analysis to patients with nystagmus ($n = 26$), we have not found any difference in the type of nystagmus comparing patients subjected to Pfizer vaccination to all the other (*OR* of having central nystagmus = 0.24, 95% *CI*: 0.004–2.65; $p = 0.36$). Similarly, patients subjected to one of the two mRNA vaccines had a non-significant $OR = 0.42$ (95% *CI*: 0.007–5.33; $p = 0.63$) of having central nystagmus.

DISCUSSIONS

The cohort included in the present study revealed the incidence of audiovestibular symptoms, in particular acute vertigo, with short onset after mRNA or adenoviral-vectored SARS-CoV-2 vaccines in patients with no history of previous COVID-19 disease.

The presence of smell and taste loss, nasal congestion, rhinorrhea, sore throat, and hearing loss has been already investigated after COVID-19 vaccination.

In a large study on 3,383 healthcare workers who received the inactivated COVID-19 vaccine (CoronaVac, Sinovac Life Sciences). Otolaryngology-specific symptoms were showed as significantly more common in subjects with a history of COVID-19 infection (16). Differently from us, in this case the authors paid attention to the previous infection and postulated that vaccination may play a triggering role in the activation of symptoms in patients with the previous COVID-19 infection.

So far, very few reports on audiovestibular symptoms after the administration of all four types of vaccine have been reported in literature. Parrino and colleagues (6) have recently described three cases of sudden unilateral tinnitus no more than 1 week later Pfizer vaccine injection in patients without previous diagnosis of COVID-19. According to the definition of Guidelines for Clinical-Safety Information on Drugs, authors reported this side effect as "very rare" (17). Indeed, it is worth citing a research letter from Formeister from Johns Hopkins University School of Medicine, Baltimore, which reported that the incidence of SSNHL occurring after COVID-19 vaccination does not exceed that of the general population, and may be lower (18). Although there is no direct evidence of the association between vaccination and SSNHL, some cases of SSNHL after COVID-19 vaccination have been recently reported (19, 20).

Many works—case series and multicentric studies—in literature during pandemic have postulated a relationship between cochleovestibular deteriorations and COVID-19 infection.

A recent systematic review analyzed 28 case reports/series and 28 cross-sectional studies that fit the criteria with an overall reported prevalence of 7.6% for hearing loss, 14.8% for tinnitus, and 7.2% for rotatory vertigo (21, 22).

Seventeen case reports and one case series reported hearing loss as a potential COVID-19 related symptom; of these, nine reported sensorineural hearing loss (23–34).

Although the pathophysiology of any audio-vestibular disorder linked to COVID-19 is still unknown, myriad theories have been postulated:

- cochleitis or neuritis caused by viral involvement of the inner ear or the vestibulocochlear nerve, potentially leading to vertigo, tinnitus, and hearing loss (30, 35, 36), thus a similar neurotropism could be supposed also for Coronavirus;
- immune-mediated response such as production of proinflammatory cytokines and vasculitic events that may negatively affect the audio-vestibular system (24);
- cross-reactions of antibodies or T-cells, which may misidentify inner ear antigens as the virus, leading to accidental damage to the inner ear (30);
- vascular disorders because cochlea and semicircular canals are largely susceptible to ischaemia (37, 38) due to a lack of collateral blood supply;
- endothelial dysfunction that has been suggested as a main pathophysiological process in several viral infections, such as SARS-CoV-2. The microvascular injury affects the central and peripheral nervous systems, causing a variety of neurological symptoms, such as headache and dizziness (39).

Moreover, proneness to worry and incoming stress, together with the absence of masking sounds, have been shown as potential risk factors for tinnitus worsening during pandemic (40).

We can extend the same line of reasoning to vertigo, which was the least commonly reported audio-vestibular symptom during pandemic; in many occasions (41–47), it was not clear if the findings were referring to new or pre-existing symptoms. Moreover, the majority of studies relied on self-reported questionnaires and many studies combined the prevalence of vertigo with dizziness, being the latter not necessarily of vestibular origin (41, 44, 48, 49), and mostly a common neurological manifestation of COVID-19 (50). Moreover, in 2021 was collected a case series of six patients all over the world who had sudden, severe symptoms such as vertigo, dizziness, nausea, and vomiting, with presumptive diagnosis of vestibular neuritis (51) by excluding other possible differential diagnoses.

On the contrary, large data on incidence and mechanism potentially underlying the development of ENT- and specifically cochleovestibular-effects of vaccination are still lacking.

Recently Wichova et al. (52) in a paper about otologic manifestations after COVID-19 Vaccinations reported 25 patients (83.3%) complained of hearing loss, 15 (50%) of tinnitus, 8 (26.7%) of dizziness, 5 (16.7%) of vertigo, and 9 (30%) of aural fullness. As 36.7% of the patients had a known previous underlying inner ear disorder, this work widely focused on immunologic factors that cause possible exacerbation of pre-existing otologic symptoms, due to a spike of disease specific IgG.

A randomized, cross-sectional study was performed to investigate the side effects of the BNT162b2 vaccine among healthcare workers. Vertigo-like symptoms (2.49%, 20/803), dizziness (8.34%), tinnitus (1.99%), ear pain (0.87%), changes in hearing (0.37%), and ear discharge (0.12%) were reported by the recipients (53).

According to a recent Italian cross-sectional study on 314,671 subjects vaccinated, dizziness is recorded as one of the most frequent COVID-19 vaccination adverse effects (*n*: 296, 21%) (54).

In **Table 4**, we present a literature review on audiovestibular disorders after COVID-19 vaccination.

As far as we know, the present study is the first clinical report about acute vertigo after COVID-19 vaccination, which describes characteristics of nystagmus and related suggested peripheral/central origin. Evoked horizontal/rotatory nystagmus was pathognomonic for BPPV and led to treatment with therapeutic maneuver. In the remaining 17 cases, peripheral vestibular dysfunction could be almost excluded if spontaneous or evoked nystagmus are absent, while vertical/oblique nystagmus and central HINT are highly suggestive for central origin disorder.

However, this work has several limitations, since it evaluates a common symptom "acute vertigo" present in different diseases with multiple pathophysiological factors. Although the HINT test demonstrates excellent sensitivity and specificity in the assessment of acute vestibular syndromes, false-positive and false-negative results do exist; all tests have been used in this study in order to reach a topodiagnosis, but a specific etiology could not be identified. Moreover, the sample size included in the study was too small and heterogeneous to establish a cause–effect relationship between acute vertigo and SARS-CoV-2 vaccines.

However, it is worth noting that all reports in literature about possible vaccination side effects have small sample sizes; this phenomenon is linked to the scarce observational time elapsed since the large-scale diffusion of vaccines, as well as the variable adherence of the population to the vaccination campaign. The most extensive data on the adverse effects have been reported by the surveillance reports drawn up by medicines agencies or were collected through online questionnaires, without ever relying on a real clinical evaluation of symptoms. This exposes to multiple and worse biases, as the reports are not clinically verified.

So far, this is the first post-vaccine clinical evaluation of the complaint "acute vertigo," which has been investigated by ENT/otoneurological point of view, by means of nystagmus description, specific tests battery and symptoms characterization. Our results seem to demonstrate that after vaccination peripheral injuries are less frequent, which represents the contrary to what is expected in the general population. After all, these observations refer to a historical moment of particular attention to post-vaccine symptoms; it is reasonable to think that in other times patients with "acute vertigo" symptom may turn to the general practitioner, while ENT doctor is consulted mainly for vestibular disorders of peripheral origin.

The mechanism underlying the onset of acute vertigo of central origin remains unclear. SHNL after COVID-19 vaccination has been linked to an abnormal autoimmune response (mediated by circulating immune complexes or cytotoxic vestibule-cochlear autoantibodies) or a vasculitic event with subsequent localized damage to the cochlea (55).

Due to the prevalence of nystagmus of non-peripheral origin, a central nervous system involvement could be included. It is worth noting that a significant number of central and peripheral nervous system manifestations have been reported during pandemic, such as cerebrovascular disease, impaired consciousness, cranial nerve manifestations, and impaired vision (56, 57). Recent studies have unveiled neurotrophic and neuroinvasive characteristics possessed by the novel coronavirus, probably due to direct viral neurological injury or indirect neuroinflammatory and autoimmune mechanisms (57). This has ignited the search on the evidence available on the prevalence of audiovestibular symptoms among patients infected with SARS-CoV-2 (21, 22).

It is well known that mRNA vaccines against the SARS-CoV-2 virus provide human cells instructions to produce the Spike protein, thus inducing levels of anti-S and/or anti-RBD binding antibodies. A recent work as shown how spike protein subunit 1 (S1) of SARS-CoV-2 – in this case intravenously

TABLE 4 | Literature review on audiovestibular disorders after coronavirus disease 2019 (COVID-19) vaccination.

References	Vaccine platform	Adverse event	Events/Total (%)
Parrino et al. (6)	mRNA-vaccine BNT162b2	Tinnitus	3
Tseng et al. (7)	Adenoviral-vectored vaccine: ChAdOx1 nCoV-19	Tinnitus and cochleopathy	1
Avci et al. (16)	Inactivated COVID-19 vaccine	Ear pressure	28/1,710 (1.6%)
		Dizziness	23/1,710 (1.3%)
		Hearing loss	5/1,710 (0.3%)
Formesteir et al. (18) - data from the CDC vaccine adverse events reporting system	mRNA vaccines: BNT162b2 mRNA-1273	Sudden sensorineural hearing loss	40/86,553,330 (0.3%)
Tsetsos et al. (19)	Adenoviral-vectored vaccine: ChAdOx1 nCoV-19	Sudden sensorineural hearing loss	1
Jeong and Choi (20)	Adenoviral-vectored vaccine: ChAdOx1 nCoV-19; mRNA-vaccine BNT162b2	Sudden sensorineural hearing loss	3
Wichova et al. (52)	mRNA vaccines: BNT162b2 mRNA-1273	Hearing loss	25/30 (83.3%)
		Tinnitus	15/30 (50%)
		Dizziness	8/30 (26.7%)
		Vertigo	5/30 (16.7%)
		Aural fullness	9/30 (30%)
Kadali et al. (53)	mRNA vaccine: BNT162b2	Vertigo-like symptoms	20/803 (2.49%)
		Dizziness	67/803 (8.34%)
		Tinnitus	16/803 (1.99%)
		Ear pain	7/803 (0.87%)
		Changes in hearing	3/803 (0.37%)
		Ear discharge	1/803 (0.12%)
Gianfredi et al. (54)	mRNA vaccines: BNT162b2, mRNA-1273; adenoviral-vectored vaccines: ChAdOx1 nCoV-19, Ad26.COV2.S	Dizziness	296/314,671 (21%)

injected radio iodinated S1 (I-S1) – is capable to cross the blood–brain barrier and enter the parenchymal brain space in male mice (58). S1 is the binding protein for SARS-CoV-2; it binds to angiotensin-converting enzyme 2 (ACE2) (59) and probably other proteins as well. These mechanisms are important for understanding whether SARS-CoV-2 and S1 itself could induce responses in the brain. As ACE2 has been reported to be abundant in the brain, medulla oblongata, and temporal lobe, the hearing center becomes affected, paving the way to hearing loss.

On the other hand, an immunization anxiety-related reaction can be postulated, as anxiety has also been related to the severity and persistency of tinnitus (40, 60). It is of utmost importance to evaluate the subsequent sequelae involving not only audiovestibular system, but also connected psychological field.

Some considerations are necessary. In our cohort, the time of onset of symptoms was no longer than 48 h after vaccination. Interestingly, 9 (27.2%) patients complained of dizziness or vertigo after the first dose, while 24 (72.8%) cases had problems only after the second dose. We have to consider the frame of Immunoglobulin G (IgG) production that is at least 10–14 days after priming (61). Interesting works by Gallus et al. (62) and Dror et al. (63) about COVID-19 long-term sequelae, suggest that cochlear damage or vestibular dysfunction are mostly transitory, thus no clinically relevant impact on audiovestibular system can be found after recovery from virus. Few data about the effects of vaccination are available so far; therefore, only similarity between systems can be traced.

In conclusion, there is growing evidence from Vaccine Surveillance Reports that hearing loss, tinnitus and vertigo can be part of the clinical spectrum of COVID-19 vaccination side effects, even if available studies in literature have small sample size and do not report the difference between central or peripheral vertigo. Although, the benefits of the vaccines far outweigh the risks of possible cochleovestibular symptoms, large-scale and well-designed studies are needed to better define possible adverse reactions and long-term consequences of the COVID-19 vaccine.

In this perspective and in light of the expected third dose, our report would also be a warning to clinicians and researchers in order to point out all possible adverse events, identify possible

pathophysiological mechanisms, and enlarge systematic vaccine safety studies.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Catania 1, G Rodolico-San Marco University Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PD designed and carried out the study. PD, RA, DR, and PS collected data and contributed to the writing of the manuscript. PD and RA designed the plan of statistical analysis of the study. SC, IL, IT, SF, and AM revised the manuscript. All authors have critically reviewed and agreed this final version of the article.

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Performance of NEWS, qSOFA, and SIRS Scores for Assessing Mortality, Early Bacterial Infection, and Admission to ICU in COVID-19 Patients in the Emergency Department

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SARS-CoV-2 infection has a wide spectrum of presentations, from asymptomatic to pneumonia and sepsis. Risk scores have been used as triggers for protocols that combine several interventions for early management of sepsis. This study tested the accuracy of the score SIRS, qSOFA, and NEWS in predicting outcomes, including mortality and bacterial infection, in patients admitted to the emergency department (ED) during the COVID-19 pandemic. We described 2,473 cases of COVID-19 admitted to the ED of the largest referral hospital for severe COVID-19 in Brazil during the pandemic. SIRS, qSOFA and NEWS scores showed a poor performance as prognostic scores. However, NEWS score had a high sensitivity to predict in-hospital death (0.851), early bacterial infection (0.851), and ICU admission (0.868), suggesting that it may be a good screening tool for severe cases of COVID-19, despite its low specificity.

Keywords: COVID-19, sepsis, NEWS, qSOFA (quick sequential organ failure assessment), SIRS (for Systemic Inflammatory Response Syndrome), scores, prognosis, emergency

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INTRODUCTION

SARS-CoV-2 infection has a wide spectrum of presentations, from asymptomatic to severe cases of viral pneumonia and Acute Respiratory Distress Syndrome (1). Considering the pathophysiology and the clinical manifestations, some COVID-19 patients meet the definition of sepsis, described as an unregulated inflammatory host response to infection that results in organ failure and risk of death (2, 3).

This concept of sepsis is recent and was updated after a better understanding of pathophysiological events (4). In a consensus definition from 1991, sepsis was defined as a systemic inflammatory response (SIRS—Systemic Inflammatory Response Syndrome) caused by infection (5, 6). The diagnosis of sepsis was made in patients with suspected or confirmed infection and two of four criteria: abnormalities in body temperature, tachypnea, tachycardia and leukocytosis (6).

More recently, a new consensus, Sepsis-3, defines sepsis as organ dysfunction, represented as at least 2 points in the Sequential Organ Failure Assessment (SOFA) score in patients with suspected or confirmed infection (3). In the Emergency Department (ED), the use of a sepsis-related organ failure prediction tool (qSOFA) can help identify patients at high risk of death (3). Moreover, authors have compared the accuracy of scores based on physical examination for diagnosing sepsis in patients admitted to the ED with suspected or confirmed infection, and the NEWS (National Early Warning Score) score has been shown superior to SIRS and qSOFA (7).

These three tools have been used as triggers for protocols that combine several interventions for early management of sepsis, including the use of antibiotics (8). Although there is still controversy about how quickly antibiotics should be administered to septic patients in general (9), COVID-19 is a viral disease without indication for antibiotic treatment (10), and there is concern that the use of antibiotics may exacerbate antimicrobial resistance without a clinical benefit (11).

Thus, we designed a study to test the accuracy of the scores SIRS, qSOFA, and NEWS in predicting outcomes, including mortality and bacterial infection, in patients admitted to the ED during the COVID-19 pandemic.

METHODS

Study Design and Population

We conducted a retrospective single center cohort study from March to August 2020 at the ED in Hospital das Clínicas, in São Paulo, Brazil. This is an academic tertiary-care hospital affiliated to São Paulo University with 2,200 beds, comprising five institutes and two auxiliary hospitals. In March 2020, the main institute was converted to a COVID-19–only facility, dedicating 900 beds to the care of infected patients. Admissions to the COVID-19 Institute were centrally managed by the Regulatory Central of the State of São Paulo, and severely ill patients are preferably referred to the hospital

We included all consecutive adult patients ($\geq$ 18 years) with confirmed COVID-19, defined as at least one positive result using reverse transcriptase-polymerase chain reaction (Rt-PCR) obtained from nasopharyngeal swabs or bronchial secretions (12).

We excluded patients for whom we could not calculate scores due missing data. Patient data were collected through electronic medical records, and a database was built using REDCap software (13).

We applied risk assessment scores according to patients' admission variables. The positive qSOFA cutoff was 2 or greater (3), NEWS score was classified into low risk (1–3 points) and high risk (four or more points) of sepsis (7), and the positive cutoff for SIRS was 2 or greater (5).

Besides the SIRS, qSOFA and NEWS variables, we also collected data on demographics (age, sex), clinical history (previous diagnoses and medications, time of symptoms on admission, physical examination, supplemental oxygen), laboratory tests routinely collected on admission (complete blood count, D-dimer, C-reactive protein, urea, creatinine, fibrinogen, lactate), variables of SAPS3, treatment (antibiotics, anticoagulants and corticosteroids), and outcomes (length of hospital stay, dialyses, invasive mechanical ventilation and in-hospital mortality). We considered with severe COVID-19, patients who had SpO2 $<$ 90% on room air, clinical signs of pneumonia, or a respiratory rate $>$30 breaths/min (10).

The primary outcome was in-hospital mortality within 30 days after admission. Secondary outcomes were admission to intensive care unit (ICU) within 7 days from admission, and early bacterial infection confirmed by bacterial growth in culture.

We defined as early bacterial infection any positive culture of blood, urine or tracheal secretions in the first 7 days of hospitalization. We considered contaminants the coagulase-negative *Staphylococci, Corynebacterium species, Bacillus* spp. other than *Bacillus anthracis, Cutibacterium acnes, Micrococcus* spp., *viridans* group *streptococci, and Clostridium perfringens* (14) if isolated in only one culture of the patient. The contaminants were excluded.

All patients received standard care, according to the institutional protocol. In the emergency department, this included oxygen supplementation, dexamethasone and antibiotics.

The study protocol was approved by the Local Ethics Committee (number: 3.990.817; CAAE: 30417520.0.0000.0068), which waived the need for written informed consent. We adhered to Transparent Reporting of a Multivariable Prediction for Individual Prognosis or Diagnosis (TRIPOD) guidelines (15).

Statistical Analysis

Mean, standard deviation (SD), median, and interquartile range (IQR) were used for descriptive statistics according to variable distribution.

Model predictive performance was assessed with the area under the receiver operating characteristics curve (AUROC). Clinical utility was analyzed using sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV), positive likelihood ratio, negative likelihood ratio, and precision recall curves. Confidence intervals (95%) were calculated after 1,000 bootstrap re-samples (16–19).

SIRS, qSOFA and NEWS's variables were submitted to bivariate analysis and factors with statistical significance ($p < 0.05$) were submitted to logistic regression using multivariate analysis by calculating the Lassos lambda coefficient for the outcomes of in-hospital death, ICU admission, and early bacterial infection.

A Bonferroni correction was used to account for multiple comparisons across the pre-specified outcomes and subgroup analyses.

All statistical analyses were performed using the software R version 3.6.2.

RESULTS

A total of 3,021 patients diagnosed with COVID-19 in the Emergency Department were included in the study,

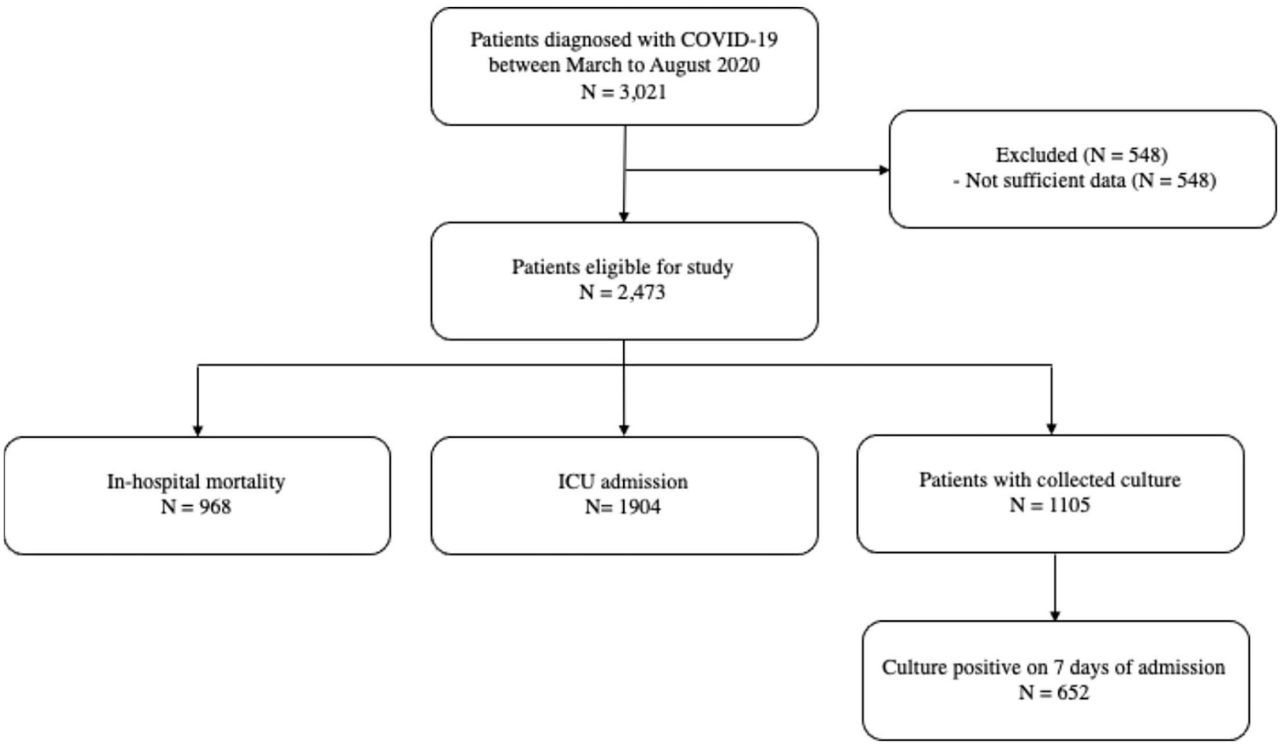

FIGURE 1 | Patients flow.

of which 2,473 patients had enough data to calculate the scores. To analyze the predictive power of the three scores, data from these 2,473 individuals were used (**Figure 1**).

The median age of patients was 61.6 years, 57% were male, and the median length of hospital stay was 14 days. The median SAPS3 was 65, and the median time between onset of symptoms of COVID-19 and hospitalization was 8 days. A total of 1,904 patients (77%) required ICU admission. In-hospital mortality was 39% (968 patients) (**Tables 1**, **2**). Cultures collected within the first 7 days of hospitalization were available for 1,190 patients, and 684 (62%) of these patients had an infectious agent isolated. The most commonly isolated agents were *Staphylococcus aureus* (112 isolates), *Candida albicans* (109 isolates), *Pseudomonas aeruginosa* (78 isolates), and *Acinetobacter baumannii* (69 isolates) (**Table 3**). The most isolated agents, considering only blood cultures, were *Staphylococcus aureus* (67 isolates), *Enterococcus* spp. (50 isolates), *Klebsiella pneumoniae* (48 isolates), *Pseudomonas aeruginosa* (34 isolates), *Acinetobacter baumannii* (30 isolates), *Candida albicans* (19 isolates), and other *Candida* species (23 isolates).

At admission, 1,364 (55%) had positive SIRS, 820 (33%) had positive qSOFA, and 2005 (81%) had high risk NEWS. In-hospital mortality frequency based on these cutoffs were: 629 (46%) for SIRS; 265 (32%) for qSOFA, and 859 (43%) for NEWS. The frequency of patients with early bacterial infection based on the cut-offs were: 423 (62%) for SIRS; 211 (66%) for qSOFA; and 582 (61%) for NEWS (**Table 4**).

Prediction of Mortality

The AUROC for each score to predict mortality was: 0.58 for SIRS, 0.55 for qSOFA, and 0.56 for NEWS. After corrections, only AUROC values for SIRS and qSOFA were considered statistically different ($p = 0.003$).

We found higher sensitivity for NEWS 0.89 (CI 95% 0.87–0.91) and its NPV was 0.77 (CI 95% 0.73–0.80). However, NEWS had a lower specificity, 0.24 (CI 95% 0.22–0.26) and lower PPV 0.43 (CI 95% 0.42–0.44) (**Table 5**, **Figure 2**).

Prediction of Early Bacterial Infection

There was no difference between the AUROC of the three scores to predict bacterial infection, with poor performance for the three. The NEWS score presented the best sensitivity [0.85 (CI 95% 0.82–0.88)], and qSOFA the best specificity [0.75 (CI 95% 0.71–0.79)] (**Table 5**, **Figure 3**).

Prediction of ICU Admission

There was also no difference between the AUROC of the three scores. The NEWS score demonstrated the best sensitivity [0.87, CI 95% (0.85; 0.88)], and SIRS [0.62, CI 95% (0.58; 0.66)] the best specificity (**Figure 4**).

Factors Associated With Mortality, Admission to the ICU, and Early Bacterial Infection

The factors associated with in-hospital death were: use of steroids, cancer, male sex, and immunosuppression. Protective

TABLE 1 | Characteristics of patients on Emergency Department admission.

	All patients (2,473)			Died in hospital (968)			Survivors (1,505)				All patients with cultures (1,105)			Patients with positive cultures (652)			Patients with negative cultures (453)			
	Median	Interquartile Interval		Median	Interquartile Interval		Median	Interquartile Interval		*P*-value	Median	Interquartile Interval		Median	Interquartile Interval		Median	Interquartile Interval		*P*-value
Age	61.6	49.1	71.3	62.5	52.5	70.1	62.5	51	70.7	0.73	62.5	51.8	70.5	62.5	52.5	70.1	62.5	51	70.7	0.73
Hospital length of stay (days)	14	8	23	25	18	37	18	11	27	<0.01	21	13	32	25	18	37	18	11	27	<0.01
Characteristics on admission																				
Duration of Symptoms on Admission (days)	8	5	11	7	4	10	8	5	12	<0.01	8	5	11	7	4	10	8	5	12	<0.01
Temperature (°C)	36.1	36	37	36.3	36	37	36.2	36	37	0.76	36.2	36	37	36.3	36	37	36.2	36	37	0.76
Heart rate (bpm)	88	77	100	89	78	100	90	78	102	0.19	90	78	101	89	78	100	90	78	102	0.19
Respiratory Rate (ipm)	24	20	28	25	20	30	24	20	30	0.22	24	20	30	25	20	30	24	20	30	0.22
Systolic blood Pressure (mmHg)	122	110	139	120	109	137	120	105	137	0.38	120	107	137	120	109	137	120	105	137	0.38
SpO2 (%)	94	91	96	93	90	96	94	91	97	0.01	94	91	96	93	90	96	94	91	97	0.01
SAPS3	65	53	77	66	54.25	77	69	58	78.5	0.01	68	56	78	66	54.25	77	69	58	78.5	0.01
BMI	26.4	23.4	31.6	25.8	22.9	30.4	26.65	23.5	32	0.04	26.2	23.4	31.3	25.8	22.9	30.4	26. 65	23.5	32	0.04
Blood tests collected up to 72 h after admission																				
Leukocytes (X $10^3/\mu L$)	9.06	6.27	12.84	9.17	5.96	13.67	10	7	15	<0.01	9.75	6.60	14.24	9.17	5.96	13.67	9.91	7.04	14.60	<0.01
Neutrophils (X 10^3 / μL)	7.48	4.85	11	7.8	5	11.85	8.49	6	12.98	0.01	8.26	5.32	12.50	7.80	4.82	11.85	8.49	5.75	12.98	0.01
Lymphocytes (X $10^3/\mu L$)	0.85	0.56	1.22	0.71	0.48	1	0.81	0.52	1	0.02	0.78	0. 50	1.14	0.71	0.48	1.08	0.81	0.52	1.19	0.02
CRP (mg/L)	128.5	63.7	236.4	168.55	88.58	271	169.2	80.8	269.3	0.61	169.2	84.2	270.8	168.55	89	271	169.2	80.8	269.3	0.61
LDH (UI/L)	436	316.5	593	495	378	631	501	376	678.5	0.40	498	377	656.5	495	378	631	501	376	678.5	0.40
D-Dimer (ng/mL)	1,631	878	5,030	1,697	940	5,286	2,954	1,198.5	7233.5	<0.01	2,241	1093.5	6,749	1,697	940	5,286	2,954	1198.5	7233.5	<0.01
Fibrinogen (mg/dL)	538	410	664	525	389	684	551	410	664	0.53	551	403	664	525	389	684	551	410	664	0.53
Lactate (mg/dL)	13	10	18	14	10	19	14	11	18	0.68	14	10.75	18	14	10	19	14	11	18	0.68

TABLE 2 | Characteristics of patients on Emergency Department admission and outcomes.

	All patients		Died in hospital (968)		Patients with positive cultures (652)	
	N (2,473)	%	N (968)	%	N (652)	%
Sex (Male) *N*, %	1,412	57%	608	43%	400	61%
Comorbidities						
Chronic kidney disease (Dialysis) *N*, %	659	27%	492	75%	282	59%
Cardiovascular disease *N*, %	460	19%	202	44%	114	57%
Hypertension *N*, %	1,445	59%	633	44%	430	62%
COPD *N*, %	166	7%	83	50%	53	64%
Asthma *N*, %	101	4%	26	26%	22	63%
Renal failure (dialysis) *N*, %	86	4%	43	50%	25	63%
Renal failure *N*, %	226	9%	111	49%	63	56%
Liver disease *N*, %	76	3%	38	50%	20	63%
Stroke *N*, %	182	7%	89	49%	51	59%
Dementia *N*, %	74	3%	42	57%	11	69%
Rheumatologic disease *N*, %	58	2%	15	26%	20	71%
Hematological disease *N*, %	176	9%	68	39%	58	60%
Psychiatric disease *N*, %	81	4%	24	30%	19	61%
Solid organ transplant *N*, %	70	9%	29	41%	14	44%
Obesity *N*, %	354	14%	95	27%	105	65%
Diabetes *N*, %	947	38%	428	45%	302	64%
Dyslipidemia *N*, %	144	18%	53	37%	32	47%
Cancer *N*, %	231	10%	134	58%	56	51%
Immunodeficiency *N*, %	44	4%	28	64%	13	42%
HIV/Aids *N*, %	21	1%	11	52%	6	50%
Hypothyroidism *N*, %	178	21%	74	42%	49	52%
Smoker *N*, %	167	7%	84	50%	56	58%
Alcoholism *N*, %	101	9%	38	38%	30	59%
Drug user *N*, %	23	3%	7	30%	7	58%
Other comorbidities *N*, %	373	24%	166	45%	104	59%
Symptoms on Admission						
Dyspnea *N*, %	1,862	75%	750	40%	532	62%
Cough	1,664	68%	630	38%	433	59%
Sputum *N*, %	119	7%	40	34%	37	73%
Tiredness *N*, %	619	25%	208	34%	167	64%
New confusion *N*, %	149	6%	66	44%	35	69%
Life support						
ICU *N*, %	1,904	77%	927	49%	637	61%
Mechanical Ventilation *N*, %	1,491	65%	878	59%	575	62%
Vasoactive drugs *N*, %	1,455	65%	881	61%	563	60%
Oxygen therapy *N*, %	2,307	95%	967	42%	669	62%
ECMO *N*, %	11	0%	9	82%	6	60%
Anticoagulant *N*, %	2,416	98%	948	39%	678	62%
Antiplaquet *N*, %	485	20%	191	39%	127	56%
Corticosteroid use *N*, %	1,695	69%	771	46%	544	62%
Use of immunosuppressants *N*, %	82	3%	31	38%	20	48%
Antibiotic *N*, %	2,291	93%	935	41%	661	61%
Antifungal *N*, %	242	10%	139	57%	118	61%
ACEi *N*, %	370	15%	74	20%	71	50%

TABLE 3 | Bacterial infections.

Isolate	Frequency
Early bacterial infection (culture positive on first 7 days of admission)	
Other non-fermenting gram negative bacilli	13
Acinetobacter baumannii complex	69
Others	9
Anaerobes	4
Other *Candida* spp.	17
Candida glabrata	39
Candida albicans	109
Candida tropicalis	46
Other Enterobacterales	15
Complexo M. tuberculosis	10
Other Enterobacterales	4
Coagulase-negative *Staphylococcus*	60
Streptococcus spp.	6
Serratia marcescens	9
Staphylococcus aureus	112
Escherichia coli	36
Klebsiella pneumoniae	45
Aspergillus spp.	2
Burkholderia spp.	2
Proteus spp.	4
Enterobacter cloacae complex	10
Pseudomonas aeruginosa	78
Stenotrophomonas maltophilia	15

TABLE 4 | Scores SIRS, qSOFA and NEWS at admission and outcomes in patients COVID-19.

	Patients	SIRS > 2		qSOFA > 2		NEWS > 4	
	N	*N*	%	*N*	%	*N*	%
Died in hospital	968	629	46%	265	32%	859	43%
Positive culture	652	423	62%	211	67%	582	61%

factors were: use of ACEi, rheumatologic disease, and hematologic disease (**Table 6**).

The factors associated with ICU admission were: dialysis, supplemental oxygen therapy, use of steroids, anticoagulation, cardiovascular disease, and immunosuppression (**Table 6**).

Precision Recall

All scores show low performance on precision-recall. They only presented a high recall value, but with small precision values. According to precision-recall, the score with the best performance is the qSOFA, which has the best specificity (**Table 4**). The scores also show a low performance to predict positive culture of patients with COVID-19. High precision values only are present with low recall (**Figures 5**, **6**).

TABLE 5 | Area under Receiver Operator Curves (AUROC) for mortality and early bacterial infection for SIRS > 2, qSOFA > 2, and NEWS > 4.

	AUC	CI 95%		*P*-value	Sensitivity	CI 95%		Specificity	CI 95%		NPV	CI 95%		PPV	CI 95%	
AUROC for mortality prediction for SIRS > 2, qSOFA > 2, and NEWS > 4																
SIRS	0.58	0.56	0.6	0.003*	0.65	0.61	0.68	0.51	0.49	0.54	0.69	0.67	0.71	0.46	0.44	0.48
qSOFA	0.55	0.53	0.57	0.08***	0.27	0.25	0.30	0.63	0.61	0.66	0.57	0.56	0.59	0.32	0.30	0.35
NEWS	0.56	0.55	0.58	0.09**	0.89	0.87	0.91	0.24	0.22	0.26	0.77	0.73	0.80	0.43	0.42	0.44
AUROC for early culture positivity prediction for SIRS > 2, qSOFA > 2, and NEWS > 4																
SIRS	0.50	0.47	0.53	0.11*	0.61	0.58	0.65	0.37	0.33	0.42	0.38	0.34	0.41	0.62	0.59	0.64
qSOFA	0.53	0.50	0.56	0.49***	0.30	0.27	0.34	0.75	0.71	0.79	0.40	0.38	0.42	0.67	0.62	0.71
NEWS	0.52	0.50	0.54	0.24**	0.85	0.82	0.88	0.11	0.79	0.14	0.31	0.24	0.38	0.61	0.60	0.62

*p-value comparison between SIRS and qSOFA.
**p-value comparison between SIRS and NEWS.
***p-value comparison between qsofa and NEWS.

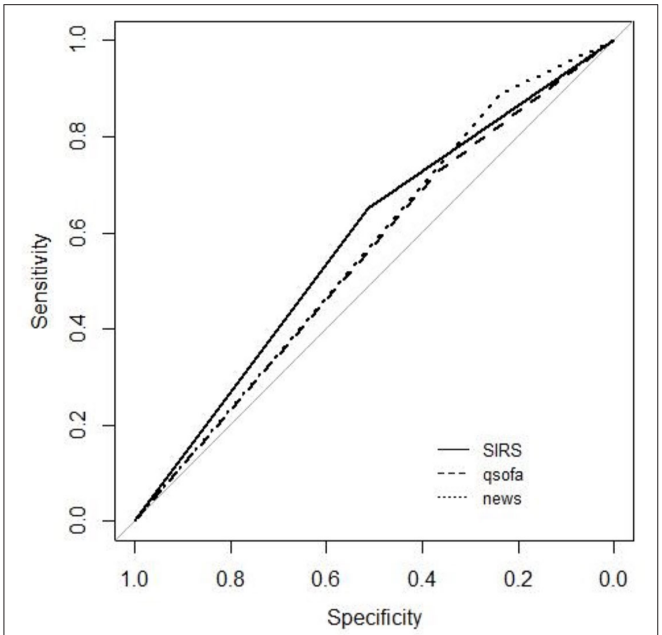

FIGURE 2 | ROC curves for mortality.

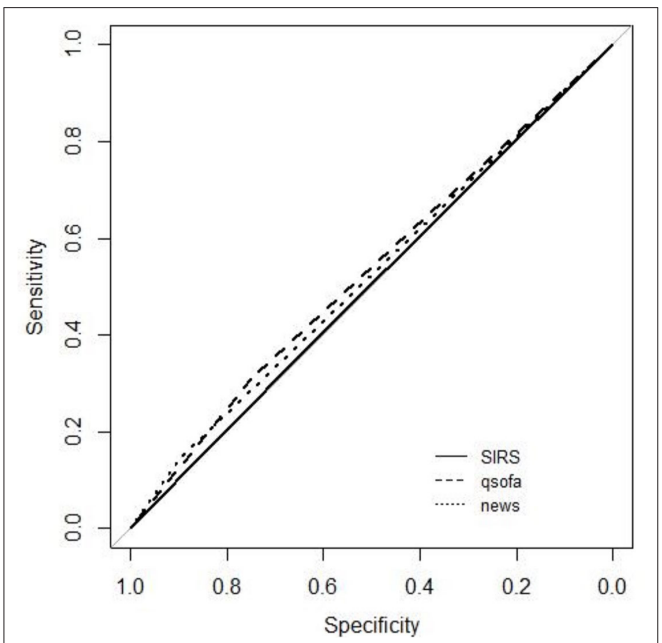

FIGURE 3 | ROC curve for culture positivity.

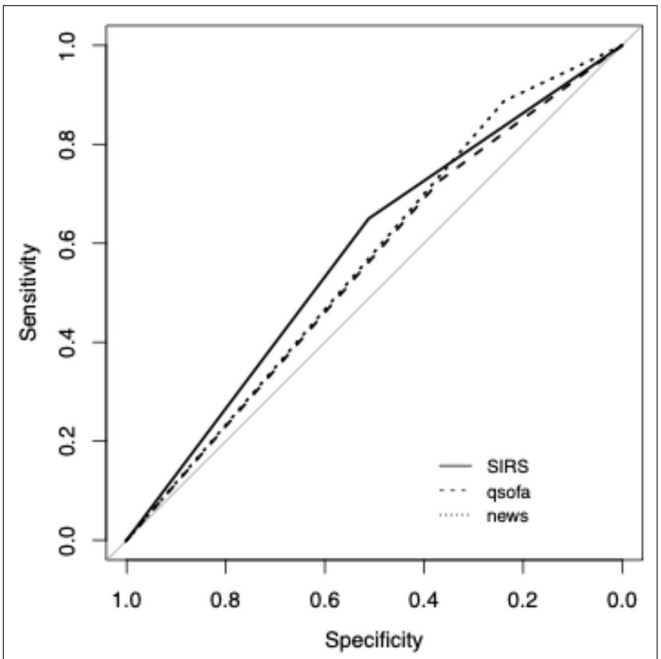

FIGURE 4 | ROC curve for ICU admission.

DISCUSSION

In this study we described 2.473 cases of COVID-19 admitted to the emergency department of a tertiary hospital during the pandemic, in order to evaluate the performance of SIRS, qSOFA and NEWS scores to predict in-hospital mortality, early bacterial infection, and ICU admission. Our findings suggest a poor performance of the 3 prognostic scores. However, they indicate a possible use of the NEWS as a screening tool for severe cases of COVID-19, given its high sensitivity to predict in-hospital death, early bacterial infection and ICU admission, despite its low specificity.

To our knowledge, this is the largest study to assess the performance of SIRS, qSOFA and NEWS scores in patients with COVID-19. Other authors have also evaluated prognostic scores to predict unfavorable outcomes for patients with COVID-19, but few have performed this assessment in the emergency department. Prognostic scores are tools that, in this context, help to make better-informed decisions (16, 17). In favor of the NEWS score, we must consider that this tool is already widely validated for the care of patients with sepsis. And, although not ideal, its high sensitivity allows NEWS to be used as a screening tool for cases that may progress badly during hospitalization. We also evaluated the performance of these tools to predict early bacterial infection, with similar results and NEWS also presented higher sensitivity than SIRS and qSOFA.

Our results are in agreement with the literature. The first study which systematically evaluated the use of NEWS2 for severe COVID-19 outcomes was carried out in five hospitals in the United Kingdom, one hospital in Norway, and two hospitals in Wuhan, China. Their results demonstrated a poor-to-moderate discrimination for 14-day ICU and death (AUC between 0.63 and 0.77 according to center) (20). Higher NEWS' cutoffs probably are better to predict COVID-19 outcomes. At Emergency Department, NEWS-2 score $\geq$ 6 at admission predicted severe disease with 80.0% sensitivity and 84.3% specificity (AUC 0.822, 95% CI 0.690–0.953), and was higher than qSOFA score $\geq$ 2 (AUC 0.624, 95% CI 0.446–0.810, $p < 0.05$) (21).

TABLE 6 | Bivariate and multivariate analysis for In-hospital mortality, early bacterial infection, and ICU hospitalization in COVID-19 patients in the Emergency Department.

	In-hospital mortality					Early Bacterial Infection					ICU hospitalization				
	RR	*P*-value	CI 95%		Lassos lambda coefficient	RR	*P*-value	CI 95%		Lassos lambda coefficient	RR	*P*-value	CI 95%		Lassos lambda coefficient
Age	1.05	<0.001	1.04	1.05	0.03	1.00	0.89	0.99	1.01		1.02	<0.001	1.01	1.02	0.01
Length of stay	0.99	0.04	0.99	1.00		0.98	<0.001	0.97	0.98		1.13	<0.001	1.12	1.15	
Time of symptoms on admission	1.00	0.73	0.98	1.01		1.05	<0.001	1.02	1.07	0.02	1.02	0.01	1.01	1.04	0.00
Temperature on admission	0.83	<0.001	0.76	0.90	−0.09	0.98	0.73	0.86	1.11		1.07	0.19	0.97	1.19	
Heart rate on admission	1.01	0.00	1.00	1.01	0.01	1.00	0.32	1.00	1.01		1.01	<0.001	1.01	1.02	0.00
Respiratory rate on admission	1.01	0.07	1.00	1.02		0.99	0.34	0.98	1.01		1.08	<0.001	1.06	1.10	0.04
Systolic blood pressure on admission	0.99	<0.001	0.99	0.99	0.00	1.00	0.44	0.99	1.00		0.99	0.00	0.99	1.00	−0.01
SpO2	0.96	<0.001	0.95	0.98	−0.02	1.02	0.04	1.00	1.04	0.01	0.96	<0.001	0.94	0.98	0.01
SAPS3	1.06	<0.001	1.05	1.07	0.05	1.01	0.01	1.00	1.02	0.01	1.10	0.05	1.01	1.21	
BMI on admission	0.98	<0.001	0.97	0.99	−0.01	1.01	0.26	1.00	1.02		1.00	0.93	0.99	1.01	
Leukocytes in the first 72 h	1.05	<0.001	1.03	1.06	0.00	1.01	0.10	1.00	1.03		1.14	<0.001	1.11	1.17	−0.01
Neutrophils in the first 72 h	1.10	<0.001	1.08	1.12	0.02	1.03	0.02	1.00	1.05	0.01	1.21	<0.001	1.18	1.25	0.12
Lymphocytes in the first 72 h	1.00	0.61	1.00	1.01		1.00	0.75	0.98	1.02		1.00	0.72	1.00	1.02	
CRP in the first 72 h	1.00	<0.001	1.00	1.01	0.00	1.00	0.94	1.00	1.00		1.01	<0.001	1.01	1.01	0.00
LDH in the first 72 h	1.00	<0.001	1.00	1.00	0.00	1.00	0.10	1.00	1.00		1.01	<0.001	1.01	1.01	0.00
D dimer in the first 72 h	1.00	<0.001	1.00	1.00	0.00	1.00	0.12	1.00	1.00		1.00	<0.001	1.00	1.00	0.00
Fibrinogen in the first 72 h	1.00	0.05	1.00	1.00		1.00	0.46	1.00	1.00		1.00	0.01	1.00	1.00	0.00
Lactate in the first 72 h	1.05	<0.001	1.03	1.06	0.01	1.00	0.60	0.99	1.02		1.04	0.00	1.02	1.06	0.02
Dialysis	8.28	<0.001	6.76	10.18		0.83	0.13	0.65	1.06		15.54	<0.001	9.92	26.00	1.89
Cardiovascular disease	1.27	0.02	1.04	1.56		0.77	0.10	0.57	1.05		1.28	0.06	1.00	1.65	0.35
Hypertension	1.61	<0.001	1.36	1.90	0.00	1.00	1.00	0.78	1.29		1.62	<0.001	1.34	1.96	0.00
COPD	1.61	0.00	1.17	2.20	0.00	1.09	0.70	0.69	1.76		1.58	0.03	1.05	2.46	0.02
Asthma	0.53	0.01	0.33	0.82	0.00	1.04	0.91	0.53	2.15		0.42	<0.001	0.28	0.63	−0.40
Renal failure (dialysis)	1.58	0.04	1.03	2.44		1.03	0.94	0.54	2.01		1.56	0.13	0.90	2.90	
Renal failure	1.57	0.00	1.19	02.06	0.00	0.75	0.16	0.51	1.12		1.09	0.62	0.79	1.53	
Liver disease	1.58	0.05	1.00	2.50	0.00	1.03	0.94	0.50	2.18		0.83	0.49	0.50	1.43	
Stroke	1.54	0.01	1.14	02.08	0.00	0.89	0.61	0.57	1.40		0.90	0.57	0.64	1.29	
Dementia	2.09	0.00	1.31	3.35	0.00	1.36	0.57	0.49	4.34		0.51	0.01	0.32	0.83	−0.21
Rheumatologic disease	0.54	0.04	0.29	0.95	−0.25	1.55	0.30	0.70	3.78		0.94	0.83	0.52	1.78	
Hematological disease	0.75	0.07	0.54	1.03	−0.22	0.94	0.79	0.62	1.46		0.37	<0.001	0.25	0.56	−0.79
Psychiatric disease	0.54	0.01	0.33	0.87	0.00	0.96	0.91	0.46	2.05		0.60	0.04	0.37	0.98	0.00
Obesity	0.52	<0.001	0.41	0.67	0.00	1.18	0.34	0.84	1.69		1.48	0.01	1.12	2.00	0.08
Diabetes	1.50	<0.001	1.28	1.78	0.00	1.18	0.19	0.92	1.51		1.48	<0.001	1.22	1.82	
Dyslipidemia	1.00	1.00	0.69	1.45		0.37	<0.001	0.22	0.65	−0.72	1.36	0.18	0.88	2.19	
Cancer	2.24	<0.001	1.71	2.96	0.26	0.62	0.02	0.41	0.92	−0.41	0.53	<0.001	0.40	0.72	−0.41
Immunodeficiency	2.45	0.01	1.33	4.69	0.18	0.44	0.03	0.21	0.91	−0.22	8.67	0.03	1.87	154.28	0.19
HIV/Aids	1.72	0.22	0.72	4.14		0.61	0.40	0.19	1.97		0.75	0.54	0.30	2.10	
Hypothyroidism	1.26	0.18	0.90	1.76		0.47	0.00	0.29	0.77	0.04	1.14	0.53	0.77	1.71	
Smoker	1.63	0.00	1.19	2.23	0.00	0.82	0.37	0.54	1.26		3.20	<0.001	1.93	5.72	0.13
Alcoholism	0.82	0.36	0.54	1.25		0.92	0.79	0.52	1.68		0.79	0.36	0.48	1.35	

(Continued)

TABLE 6 | Continued

	In-hospital mortality					Early Bacterial Infection					ICU hospitalization				
	RR	*P*-value	CI 95%		Lassos lambda coefficient	RR	*P*-value	CI 95%		Lassos lambda coefficient	RR	*P*-value	CI 95%		Lassos lambda coefficient
Drug user	0.76	0.55	0.29	1.81		0.67	0.50	0.21	2.30		0.86	0.75	0.35	2.40	
Other comorbidities	0.86	0.20	0.68	1.09		0.94	0.74	0.68	1.33		0.14	<0.001	0.09	0.21	
Dyspnea	1.21	0.05	1.00	1.47	0.00	1.10	0.53	0.82	1.46		1.88	<0.001	1.53	2.31	0.00
Cough	0.84	0.05	0.71	1.00	0.00	0.74	0.02	0.57	0.96	−0.22	1.10	0.34	0.90	1.34	
Sputum	0.97	0.86	0.65	1.42		1.68	0.11	0.91	3.27		0.64	0.02	0.44	0.95	0.00
Tiredness	0.73	0.00	0.60	0.88	−0.02	1.11	0.48	0.83	1.48		0.86	0.18	0.70	1.07	
New confusion	1.25	0.18	0.90	1.75		1.37	0.31	0.76	2.57		0.53	<0.001	0.38	0.76	−0.26
Oxygen therapy	93.09	<0.001	20.82	1639.71	0.00	0.29	0.11	0.04	1.09		31.83	<0.001	19.23	56.32	1.57
ECMO	7.05	0.01	1.81	46.30		0.92	0.90	0.26	3.63		637021.36	0.96	0.00	NA	
Antiplaquet	1.01	0.90	0.83	1.24		0.75	0.06	0.56	1.01		1.19	0.16	0.94	1.52	
Corticosteroid use	2.46	<0.001	02.04	2.97	0.48	0.93	0.62	0.68	1.25		4.31	<0.001	3.54	5.25	0.69
Use of immunossupressors	0.94	0.80	0.59	1.48		0.55	0.06	0.29	1.02		0.71	0.17	0.45	1.18	
Antibiotic	3.11	<0.001	2.14	4.65	0.00	0.35	0.03	0.12	0.85	−0.31	2.62	<0.001	1.91	3.56	0.00
Antifungal	2.28	<0.001	1.75	2.99	0.09	0.93	0.66	0.68	1.28		3.09	<0.001	2.03	4.92	0.00
ACEi	0.34	<0.001	0.26	0.44	−0.54	0.57	0.00	0.40	0.81	−0.44	0.93	0.60	0.72	1.22	

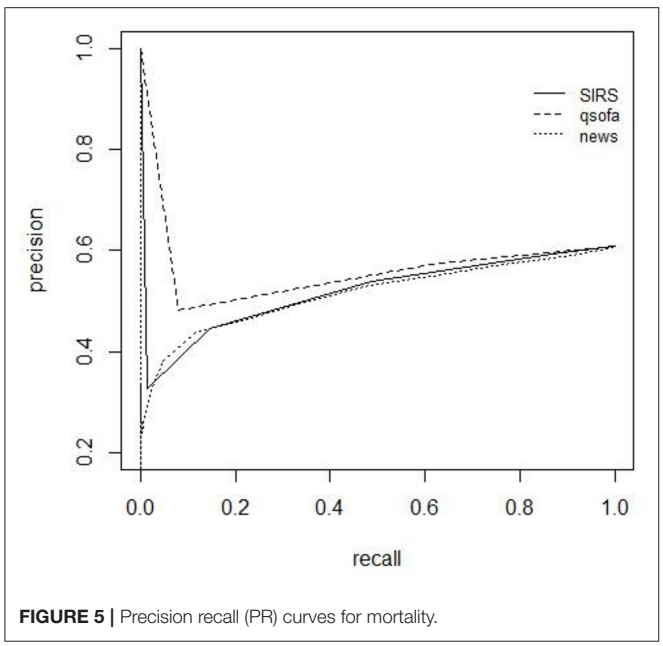

FIGURE 5 | Precision recall (PR) curves for mortality.

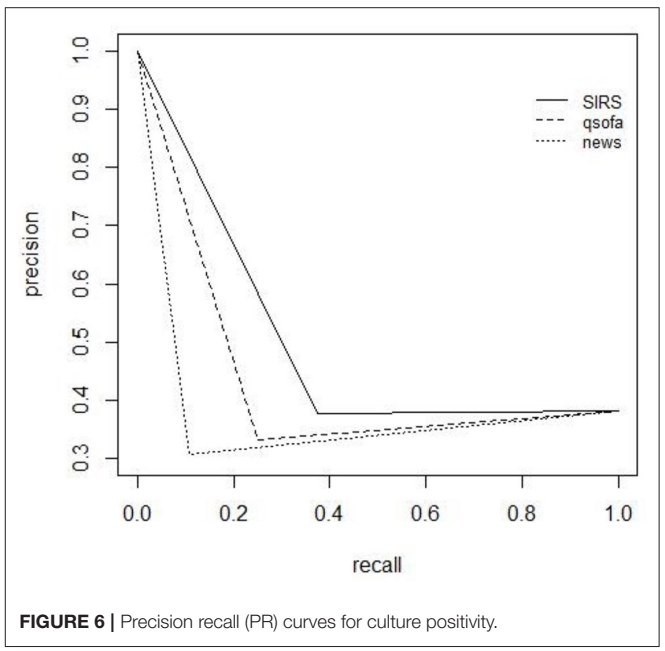

FIGURE 6 | Precision recall (PR) curves for culture positivity.

Although this study was conducted in an emergency department of a single center, this hospital was the main state referral for severe COVID-19. São Paulo has a population over 44 million, and 600 of the 6,000 critical COVID-19 care beds were located in this hospital. Because of this, our sample represents the selection of the most severe cases of the State of São Paulo, one of the world's epicenters of the pandemic at that time. This is evident when evaluating the median SAPS 3 value of 68 for patients admitted to the emergency department, which would have an expected mortality of 66.8% for patients seen in Latin America. We highlight that tools presented lower AUROCs than those found in some studies (12, 13, 16, 22, 23), mainly due to the lower specificity and PPV values. This may have happened because of the high severity of the cases. In a scenario with a higher prevalence of milder cases, there would be a better chance of detecting survivors, resulting in higher specificity and PPV values.

The incidence of early bacterial infection was high, 59% among those who collected cultures, and 26% among the 2,403 patients studied. This result is much higher than that found in other studies, 3–8% (24, 25). It would be expected that these infections had occurred later, but the median time of COVID-19 symptoms on admission was 8 days. This finding may be one of the factors related to the greater severity of our patients.

There were no factors strongly associated with early bacterial infection, but antibiotic use was associated with a reduced risk. This finding may be explained by the use of antibiotics resulting in negative cultures. Despite the high incidence of early bacterial infection, it is important to note that the use of antibiotics was not associated with lower risk of admission to the ICU or death. It was not possible to analyze the risk of developing infection by resistant bacteria in our study, but the indiscriminate use of antibiotics has been shown to be associated with the emergence of resistance, and the prescription of these drugs should be done cautiously and rationally.

Among the factors associated with in-hospital death, we found the use of steroids to be the most important factor. This may represent a bias as steroids are prescribed for severe COVID-19 as well as for comorbidities such as cancer and immunodeficiency. Paradoxically, rheumatologic disease and hematologic disease were not associated with death. The latter, it was not even a factor associated with admission to the ICU. These patients were prioritized for hospital care, which may have positively influenced the outcome, despite their potentially higher risk (26, 27). The use of ACEi was a protective factor against death in our study, as demonstrated by other authors (28, 29).

The most important factors associated with admission to the ICU admission were factors associated with the need for intensive support, such as dialysis, or the severity of COVID-19 (supplemental oxygen therapy, use of steroids and anticoagulation). The presence of cardiovascular disease and immunodeficiency were also factors associated with admission to the ICU. Factors not associated with hospitalization in ICU were: cancer, dementia, hematologic disease and asthma. Although not expected, patients with asthma had lower risk of hospitalization in ICU, as demonstrated in other studies (30–32).

This study has limitations. Data for this study were collected prospectively, but their analysis was performed later, and it was not possible to obtain retrospectively some data that were not collected initially. For instance, it was not possible to collect data on Glasgow Coma Scale for all patients, as this information was sometimes described as mental status alert, somnolent, and unconsciousness in the electronic medical record. We considered any positive culture as bacterial infection. It was not possible to evaluate the clinical features of the patients, so patients that were only colonized may have been considered as infected in our definition. We could not evaluate the antimicrobial resistance profiles in our study, so we could not analyze the impact of antibiotic use. This study was performed in a single-center, which is a limitation. However, this center was the reference hospital for severe cases of COVID-19 in the State of São Paulo, so we feel that it was broadly representative of the state which was hit hard by the pandemic. Our cases reflected the selection of the most severe cases in the state, actually representing a wider population than the study design would suggest, especially among critically ill patients in the emergency department.

In conclusion, for patients with severe COVID-19 admitted to the emergency department, SIRS or qSOFA did not perform well in predicting in-hospital mortality, early bacterial infection, or admission to the ICU. However, high sensitivity in predicting these three outcomes suggests that the NEWS score can be useful as a screening tool.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hospital of Clinics, Faculty of Medicine, University of São Paulo, Brazil. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JA and MS conceptualized the project, analyzed the results, wrote the first draft, and wrote the final manuscript. LM performed the statistical analysis. AC collected data. HP and AL conceptualized the project and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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

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Respiratory Outcomes After 6 Months of Hospital Discharge in Patients Affected by COVID-19: A Prospective Cohort

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Background: Considering millions of people affected by Coronavirus disease 2019 (COVID-19), long-lasting sequelae can significantly impact health worldwide. Data from prospective studies in lower-middle-income countries on persistent lung dysfunction secondary to COVID-19 are lacking. This work aims to determine risk factors and the impact of persistent lung dysfunctions in COVID-19 survivors.

Methods: Observational and prospective cohort of patients admitted to a tertiary hospital from June 2020 to November 2020. Persistence of chest CT scan alterations, desaturation in the six-minute walk test (6MWT), forced expiratory volume in one second (FEV1), lung carbon monoxide diffusion (DLCO), and maximum inspiratory pressure (MIP) were measured 6 months after hospital discharge. Additionally, the Barthel index (BI) and the Modified Medical Research Council (mMRC) Dyspnea Scale were used to determine the impact of lung dysfunction in activities of daily living (ADL).

Results: It was included 44 patients. Sixty percent had persistent lung CT scan abnormalities. From 18 to 43% of patients had at least one pulmonary function dysfunction, a decrease in FEV1 was the least prevalent (18%), and a reduction in DLCO and MIP was the most frequent (43%). In general, female gender, comorbidity index, and age were associated with worse lung function. Additionally, the presence of lung dysfunction could predict worse BI (r-square 0.28) and mMRC (r-square 0.32).

Conclusion: Long-term lung dysfunction is relatively common in survivors from severe COVID-19 and impacts negatively on ADL and the intensity of dyspnea, similar to studies in high-income countries.

Keywords: pulmonary function, COVID-19, activities of daily living, mMRC scale, post COVID-19 condition, long-COVID

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INTRODUCTION

In addition to the characteristic symptoms of the acute infectious process of coronavirus disease 2019 (COVID-19), such as fever, cough, and chest discomfort and, in severe cases, dyspnea and bilateral pulmonary infiltration (1, 2); "post-COVID condition" reports are increasing. Still, its prevalence, risk factors, or whether one can predict the occurrence of "post-COVID condition" is not known (3, 4). The consequences of acute lung damage drove by COVID-19 could be permanent lung damage if the patient recovers (5, 6).

Until today, there is not sufficient evidence on the long-term prognosis of patients who had pneumonia due to COVID-19 (5). The inflammatory storm that characterizes severe forms of the disease suggests that serious tissue sequelae may affect various organ systems. The most common symptoms were fatigue, cognitive problems, and new-onset dyspnea. In this context, McGroder et al. (6) evaluated patients 4 months after hospitalization. Predominantly in patients who underwent mechanical ventilation, fibrotic-type patterns were observed on CT. Elderly patients are at an even higher risk, and in this population, even less severe dysfunctions can cause increased morbidity and mortality (7). Few studies suggested that this pattern persist up to 12 months after hospitalization (8–10). Like other severe conditions (11), all these sequela would impacto on the ability to perform activities of daily living (ADL) and physical capacity (12).

Thus, lung dysfunction is a critical question in "post-COVID condition". Still, we do not have enough data on risk factors, the impact of this dysfunction on patients' daily living, mainly coming from lower-middle-income countries. In this context, we performed a prospective cohort study to identify pulmonary outcomes after 6 months of hospital discharge in patients who developed pneumonia due to COVID-19 in South Brazil. We hypothesize that 6 months after hospitalization due to COVID-19 pneumonia, lung dysfunction is frequent, and negatively affects the ADL.

METHODS

Study Design

A prospective cohort was conducted with patients admitted to a tertiary hospital from June 2020 to November 2020. The São José hospital's Institutional Review Board approved the protocol under the number 31384620.6.1001.5364. All patients or their surrogates gave written consent before inclusion in the study.

Setting

The study sample consisted of all consecutive patients admitted to the COVID-19 ward or intensive care unit (ICU) of a tertiary hospital from June 2020 to November 2020.

Abbreviations: 6MWT, 6-minute walk test; ADL, Activities of daily living; BI, Barthel index; CT, Computed tomography; CRP, c-reactive protein; FEV1, Forced expiratory volume in one second; ICU, Intensive care unit; DLCO, Lung carbon monoxide diffusion; MIP, Maximum inspiratory pressure; mMRC, Modified medical research council; SOFA, Sequential Organ Failure Assessment; SAPS, Simplified Acute Physiology Score.

Participants

The inclusion criteria were patients over 18 years old admitted to the hospital with confirmed COVID-19 diagnosis through reverse transcriptase reaction or rapid antigen test and requiring supplementary oxygen, non-invasive ventilation, or mechanical ventilation due to COVID-19 pneumonia. Exclusion criteria were patients with severe chronic diseases (chronic kidney disease under dialysis, cirrhosis child C, severe COPD, severe heart failure) or diseases capable of altering inflammatory response (such as chronic use of immunosuppressants, cancer patients without disease control, and HIV without disease control), and patients in palliative care or with life expectancy <24 h.

Procedures

Investigators daily screened all patients admitted to the hospital, and those who met the inclusion criteria were considered eligible. The patient was invited to participate in the study from hospital admission for a maximum of 120 h. All necessary information was prospectively collected directly from the patient's electronic medical record. Prehospital comorbidities were aggregated through the validated Charlson comorbidity index. The severity of critical illness at ICU admission was collected with the Simplified Acute Physiology Score (SAPS) 3. The Sequential Organ Failure Assessment (SOFA) score was used to assess organ dysfunction. A chest CT scan was performed on admission and was analyzed to determine the extent of pulmonary involvement. Approximately, 6 months after hospital discharge, patients attended the hospital's outpatient clinic. They were evaluated with chest CT scan, lung carbon monoxide diffusion (DLCO), and lung plethysmography, a six-minute walk test (6MWT), ADL, and dyspnea intensity, performed as described below. The outcome evaluation was blinded for the hospitalization variables.

Chest Computed Tomography

The extension of acute-phase ground-glass opacity was graded as <25%, 25–50%, and >50% at hospital admission, modified from Guan et al. (13). At 6-months after hospital discharge, the persistence of ground-glass and the occurrence of lung fibrosis were evaluated.

Lung Plethysmography With Carbon Monoxide Diffusion

Pulmonary function tests were done according to the American Thoracic Society (ATS)–European Respiratory Society guidelines. A variable pressure VIASYS Respiratory Care plethysmography (Vyaire, Mettawa, IL, USA) was used to determine the following parameters: FEV1, DLCO, and maximum inspiratory pressure (MIP). For FEV1, flow-volume curves were obtained and the greatest percentage of the three maneuver was used for analysis. For MIP, each participant was asked to perform five manoeuvers, with a goal of matching the highest two within 10 cm H_2O, and the largest MIP from each participant's test was used for analysis. These variables were expressed as percentages of predicted normal values. Normal values were considered those ≥80% of predicted values (6).

Six-Minute Walk Test

Each patient walked on the flat ground as fast as possible without oxygen inhalation and completed the 6MWT independently. From the 6MWT, significant oxygen desaturation was the parameter used to qualify the patient's performance, defined as a decrease of at least 4% from baseline SpO_2 (14).

The Barthel Index

The BI is a ten-item ordinal scale used to measure performance ADL (15). BI scored according to the level of physical assistance required to perform the daily task.

The Modified Medical Research Council Dyspnea Scale

The Modified Medical Research Council (mMRC) Dyspnea Scale is a self-rating tool to measure the degree of disability that breathlessness poses on day-to-day activities on a scale from 0 to 4 (16).

Outcomes

Outcomes were different aspects of lung function 6-months after hospital discharge: persistence of CT scan alterations, desaturation in the 6MWT, FEV1, DLCO, and MIP. Additionally, the BI and the mMRC were used to determine the impact of lung dysfunction in ADL.

Statistical Analysis

The collected data were analyzed in the IBM SPSS Statistics version 22.0 software (IBM Corp., Armonk, N.Y., USA). Quantitative variables were expressed as mean and standard deviation, and were compared using the Student's *t*-test. Nominal variables were expressed as frequency and percentage, and were compered using the Pearson's chi-square. The logistic binary regression was used to access the independent risk factors for the presence of lung dysfunction. Lung function parameters were dichotomized as described above, and the predictive variables were entered in the model as continuous or categorical depending on their characteristics. The model included only variables with $p < 0.20$ or $p < 0.05$ in the univariate analysis depending on the number of events observed in each outcome to not overfit the model. If variables that reached the threshold had collinearity, the variable with a lower *p*-value in the univariate analysis entered the final model. Results from univariate analysis were presented as *p*-value and logistic binary regression as relative risk and 95% CI. Linear regression was performed to determine the impact of lung dysfunction on ADL, and R squared was calculated to express the percentage of the variance in the ADL that the lung dysfunction variables explained. In all analyses, a *p*-value < 0.05 was adopted as the level for statistical significance.

RESULTS

Based on the predefined inclusion and exclusion criteria, the final sample resulted in 167 patients. From these, 56 patients died during hospitalization or follow-up. Due to the importance of a timely description of lung abnormalities, we evaluated only the first 44 consecutively included patients 6 months after hospital discharge. There were no missing cases. All patients will have a 1-year evaluation, as defined in the original protocol.

TABLE 1 | General patients' characteristics.

Variables	*N*(%)	Mean (SD)/median (25–75)
Age, years		54 (11)
Gender		
Male	31 (70)	
Female	13 (30)	
BMI		30 (5.0)
Extent of lung involvement at admission		
25–50%	16 (41)	
>50%	23 (59)	
Charlson comorbidity index		2 (1, 2)
Corticosteroids, yes	40 (91)	
SAPS III		48 (12.2)
Respiratory SOFA		
D1		2.8 (1.0)
D3		2.0 (1.4)
SOFA		
D1		3.7 (1.9)
D3		2.9 (2.6)
C-reactive protein, mg/L		
D1		118 (84)
D3		108 (96)
ICU admission		
Yes	31 (70)	
No	13 (30)	
ICU length of stay, days		12 (9.1)
Invasive mechanical ventilation		
Yes	10 (23)	
No	34 (77)	
Days on mechanical ventilation		13 (7.7)
Length of hospital stay, days		16(10.3)

SD, standard deviation; BMI, Body mass index; SAPS III, Simplified Acute Physiology Score 3; SOFA, sequential organ failure assessment score; ICU, intensive care unit; N, number of participants.

Table 1 described demographic information. Approximately, 70% of the patients were male, and the mean age was 54 ± 11 years. The mean body mass index was 30 ± 5, and the median Charlson comorbidity index was 2 (1, 2). The mean length of ICU stay was 12 ± 9.1 days and the mean length of hospital stay was 16 ± 10.3 days. The need for mechanical ventilation was 23% of the sample. The mean SAPS III score was 48 ± 12, and the mean respiratory SOFA was 2.8 ± 1 at admission (D1) and 2.0 ± 1.4 72 h after (D3). These variables were not statistically different compared to the remaining 67 patients not included in this preliminary analysis.

Lung CT scan abnormalities were ground-glass (15 from 24) and fibrosis (9 from 24). Thus, 24 (60%) of the patients had persistent lesions on CT scan. **Table 2** presented the relation of acute-phase variables and the persistence of lung abnormalities

TABLE 2 | Variables associated with the persistence of lesions on lung CT scan 6-months after hospital discharge.

Variables	Present ($n = 24$)	Absence ($n = 20$)	p-value[a]	RR (CI 95%)[b]
Age, years, mean (SD)	54 (11)	54 (11)	0.99	NA
Gender				
Male, *n* (%)	16 (66)	15 (75)	0.55	NA
BMI, mean (SD)	31 (4.8)	30 (5.4)	0.64	NA
Extent of lung involvement at admission[c] *n* (%)				
25–50%	8 (38)	7 (41)	0.85	NA
>50%	13 (62)	10 (59)		
Charlson Comorbidity Index, median (25–75)	2 (1, 2)	1 (1, 2)	0.34	NA
SAPS III, mean (SD)	47 (14.4)	48 (10.6)	0.91	NA
Respiratory SOFA				
D1, mean (SD)	2.9 (1.1)	2.7 (0.9)	0.56	NA
D3, mean (SD)	2.4 (1.3)	1.8 (1.5)	0.16*	1.04 (0.7–1.5)
SOFA				
D1, mean (SD)	3.6 (1.7)	3.9 (2.2)	0.59	NA
D3, mean (SD)	3.0 (2.5)	2.7 (2.8)	0.67	NA
C-reactive protein, mg/L				
D1, mean (SD)	99 (72)	146 (96)	0.13	NA
D3, mean (SD)	79 (54)	164 (133)	0.08*	0.98 (0.98–1.0)
ICU yes, *n* (%)	31 (55)	14 (45)	0.95	NA
ICU length of stay, days mean (SD)	12 (9.9)	11 (8.3)	0.65	NA
Mechanical ventilation, yes, *n* (%)	4 (40)	6 (60)	0.29	NA
Days on Mechanical Ventilation, mean (SD)	14 (8.2)	12 (7.9)	0.63	NA
Length of hospital stay, days, mean (SD)	17 (11.4)	15 (8.9)	0.61	NA

SD, standard deviation; RR, Relative Risk; CI, confidence interval; NA, not applied; BMI, Body mass index; SAPS III, Simplified Acute Physiology Score 3; SOFA, sequential organ failure assessment score; ICU, intensive care unit; N, number of participants.
**$p < 0.20$ variables included in the binary logistic regression.*
[a]p-value from the univariate analysis.
[b]RR form the regression analysis.
[c]for six patients, CT scan, was not performed at hospital admission.

TABLE 3 | Variables associated with desaturation in the six-minute walk test (6MWT) 6-months after hospital discharge.

Variables	Present ($n = 15$)	Absence ($n = 29$)	p-value[a]	RR (IC 95%)[b]
Age, years, mean (SD)	56 (10)	52.7 (11)	0.24	NA
Gender				
Male, *n* (%)	6 (40)	25 (86)	0.001*	0.13 (0.03–0.6)
BMI, mean (SD)	32 (6.8)	30 (3.8)	0.52	NA
Extent of lung involvement at admission[c] *n* (%)				
25–50%	4 (27)	12 (50)	0.08	NA
>50%	11 (73)	12 (50)		
Charlson Comorbidity Index, median (25–75)	1 (1, 2)	2 (1–3)	0.044*	1.5 (0.8–2.9)
SAPS III, mean (SD)	53 (15.0)	46 (10.1)	0.15	NA
Respiratory SOFA				
D1, mean (SD)	2.9 (1.2)	2.7 (0.9)	0.50	NA
D3, mean (SD)	2.6 (1.1)	1.8 (1.5)	0.045*	1.06 (0.8–1.4)
SOFA				
D1, mean (SD)	3.6 (1.7)	3.6 (1.7)	0.73	NA
D3, mean (SD)	3.6 (2.8)	2.4 (2.4)	0.15	NA
C-reactive protein, mg/L				
D1, mean (SD)	88 (45)	136 (96)	0.07	NA
D3, mean (SD)	91 (89)	122 (102)	0.40	NA
ICU, yes, *n* (%)	11 (73)	20 (69)	0.76	NA
ICU length of stay, days, mean (SD)	14 (8.0)	10 (9.6)	0.279	NA
Mechanical ventilation, yes, *n* (%)	4 (27)	6 (21)	0.65	NA
Days on mechanical ventilation, mean (SD)	15 (5.3)	11 (9.2)	0.750	NA
Length of hospital stay, days, mean (SD)	20 (10.3)	14 (9.9)	0.485	NA

SD, standard deviation; RR- Relative Risk; CI, confidence interval; NA, not applied; BMI, Body mass index; SAPS III, Simplified Acute Physiology Score 3; SOFA, sequential organ failure assessment score; ICU, intensive care unit; N, number of participants.
**$p < 0.20$ and included in the binary logistic regression.*
[a]p-value from the univariate analysis.
[b]RR form the regression analysis.
[c]For five patients, CT scan was not performed at hospital admission.

on the CT scan 6-months after hospital discharge. In the univariate analysis, no single variable was associated with CT-scan lesions. However, c-reactive protein (CRP) at D1 and D3 and respiratory SOFA at D3 reached the threshold and were included in the regression analysis. Only CRP levels were marginally, but not significantly, related to the persistence of CT-scan lesions (**Table 2**).

In the 6MWT (**Table 3**), 15 (35%) patients had a significant desaturation. In the univariate analysis, desaturation was associated with female gender, Charlson comorbidity index, and respiratory SOFA D3. Only the female gender was independently associated with desaturation in 6MWT. Of these 15 patients, 10 (66%) had persistence of CT scan alterations, being 6 (60%) ground-glass and 4 (40%) fibrotic lesions.

Only 8 (18%) of the patients had a significant (<80% of predicted value) decrease in FEV1. When analyzing the FEV1 (**Table 4**), age was significantly associated with an abnormal FEV1. Interestingly, all patients that had reduced FEV1 had more than 50% of ground-glass at hospital admission. However, due to the low number of events, every attempt to perform a binary regression resulted in an overfitted model. Of these eight patients, 2 (25%) had persistence of CT scan alterations, being 1 (50%) ground-glass and 1 (50%) fibrotic lesions.

When analyzing DLCO (**Table 5**), 19 (43%) of patients presented a significant decrease. In the univariate analysis, age, gender, and Charlson comorbidity index were significantly associated with a reduction in DLCO, but only gender was independently associated with a decrease in diffusion. Of these 19 patients, 11 (58%) had persistence of CT scan alterations, being 6 (54%) ground-glass and 5 (36%) fibrotic lesions.

Maximum inspiratory pressure assessed respiratory muscle strength and was decreased in 19 (43%) patients (**Table 6**). Age,

TABLE 4 | Variables associated with a decreased forced expiratory volume in one second 6-months after hospital discharge.

Variables	Present ($n = 8$)	Absence ($n = 36$)	p-value[a]
Age, years, mean (SD)	60 (5)	53 (11)	0.015*
Gender			
Male, *n* (%)	6 (75)	25 (10)	0.75
BMI, mean (SD)	32 (5.4)	30 (5.0)	0.35
Extent of lung involvement at admission[b] *n* (%)			
25–50%	0 (0.0)	15 (48)	0.029*
>50%	7 (100)	16 (52)	
Charlson Comorbidity Index, median (25–75)	1 (1, 2)	2 (2)	0.11
SAPS III, mean (SD)	50 (15.2)	48 (12.0)	0.71
Respiratory SOFA			
D1, mean (SD)	2.9 (1.2)	2.8 (0.9)	0.80
D3, mean (SD)	2.0 (1.4)	2.1 (1.4)	0.87
SOFA			
D1, mean (SD)	3.9 (2.8)	3.7 (1.7)	0.81
D3, mean (SD)	2.6 (2.1)	2.9 (2.8)	0.76
C-reactive protein, mg/L			
D1, mean (SD)	108 (103)	122 (80)	0.70
D3, mean (SD)	129 (172)	103 (70)	0.72
ICU yes, *n* (%)	5 (62)	26 (72)	0.59
ICU length of stay, days, mean (SD)	14 (11.6)	11 (8.7)	0.51
Mechanical ventilation, yes, *n* (%)	2 (25)	8 (22)	0.86
Days on mechanical ventilation, mean (SD)	18 (7.8)	11 (7.4)	0.25
Length of hospital stay, days, mean (SD)	19 (11.3)	15 (10.0)	0.35

SD, standard deviation; BMI, Body mass index; FEV1, forced expiratory volume in one second; SAPS III, Simplified Acute Physiology Score 3; SOFA, sequential organ failure assessment score; ICU, intensive care unit; N, number of participants.

[a] *p-value from the univariate analysis.*

[b] *For six patients CT scan was not performed at hospital admission.*

**Due to the low number of events, it was not possible to perform binary regression analysis.*

TABLE 5 | Variables associated with a decreased carbon monoxide diffusion 6-months after hospital discharge.

Variables	Present ($n = 19$)	Absence ($n = 25$)	p-value[a]	RR (IC 95%)[b]
Age, years, mean (SD)	59 (9)	50 (11)	0.008*	1.03 (0.9–1.2)
Gender				
Male. *n* (%)	9 (47)	22 (88)	0.003*	0.7 (0.006–0.7)
BMI, mean (SD)	30 (5.6)	31 (4.7)	0.73	NA
Extent of lung involvement at admission[c] *n* (%)				
25–50%	5 (36)	10 (42)	0.72	NA
>50%	9 (64)	14 (58)		
Charlson Comorbidity Index, median (25–75)	1 (1, 2)	2 (1–3)	0.02*	1.9 (0.65–5.6)
SAPS III, mean (SD)	48 (10.6)	48 (14)	0.88	NA
Respiratory SOFA				
D1, mean (SD)	2.68 (1.00)	2.88 (0.97)	0.51	NA
D3, mean (SD)	2.32 (1.25)	1.87 (1.51)	0.30	NA
SOFA				
D1, mean (SD)	3.3 (2.2)	4.0 (1.6)	0.21	NA
D3, mean (SD)	3.0 (2.0)	2.8 (3.1)	0.88	NA
C-reactive protein, mg/L				
D1, mean (SD)	96 (63)	137 (96)	0.16	NA
D3, mean (SD)	83 (51)	132 (121)	0.16*	0.99 (0.97–1.005)
ICU, yes, *n* (%)	14 (74)	17 (68)	0.68	NA
ICU length of stay, days, mean (SD)	12 (6.8)	11 (10.8)	0.68	NA
Mechanical ventilation, yes, *n* (%)	3 (16)	7 (28)	0.34	NA
Days on mechanical ventilation, mean (SD)	13 (5.5)	12 (8.8)	0.87	NA
Length of hospital stay, days, mean (SD)	17 (8.6)	15 (11.4)	0.48	NA

SD, standard deviation; RR- Relative Risk; CI, confidence interval; NA, not applied; BMI, Body mass index; SAPS III, Simplified Acute Physiology Score 3; SOFA, sequential organ failure assessment score; ICU, intensive care unit; N, number of participants.

[a] *p-value from the univariate analysis.*

[b] *RR form the regression analysis.*

[c] *For six patients, CT scan was not performed at hospital admission.*

**$p < 0.20$ variables included in the binary logistic regression.*

gender, and Charlson comorbidity index were associated with reduced MIP in the univariate analysis, but no one variable was independently associated with this outcome. It was observed a marginal, non-significant association with gender and Charlson comorbidity index. Of these 19 patients, 11 (58%) had persistence of CT scan alterations, being 7 (64%) ground-glass, and 4 (36%) fibrotic lesions.

The impact of these dysfunctions on the BI was measured. Both the presence of desaturation on 6MWT (mean BI 91 ± 25 vs. 74 ± 27) and reduced VEF1 (mean BI 89 ± 24 vs. 68 ± 32) were significantly associated with lower BI scores ($p < 0.05$). DLCO was marginally but not significantly associated with lower BI scores (mean BI 92 ± 23 vs. 76 ± 30, $p = 0.06$). There was no association between MIP and the extension of CT lesions and BI scores. When desaturation on 6MWT, FEV1, and DLCO entered in a linear regression only FEV1 ($p = 0.019$) and marginally DLCO ($p = 0.06$) were associated with lower BI (R square for the model 0.28).

Additionally, both the presence of desaturation on 6MWT (mean mMRC 0.8 ± 0.8 vs. 1.7 ± 1.4) and reduced DLCO (mean mMRC 0.8 ± 1.2 vs. 1.6 ± 1.0) were significantly associated with higher mMRC scores ($p < 0.05$). FEV1 and the extension of CT lesions were marginally, but not significantly associated with higher mMRC scores (mean mMRC 1.0 ± 1.0 vs. 1.6 ± 1.7, $p = 0.17$ for FEV1; 0.9 ± 1.1 vs. 1.3 ± 1.2, $p = 0.17$ for CT lesions). There was no association between MIP and mMRC scores. When desaturation on 6MWT, FEV1, DLCO, and CT lesions entered in a linear regression only FEV1 ($p = 0.017$) and marginally DLCO ($p = 0.12$) were associated with higher mMRC scores (R square for the model 0.32).

DISCUSSION

Confirming our hypothesis, respiratory dysfunction 6 months after hospital discharge from COVID-19 was common and negatively impacted the ADL.

TABLE 6 | Variables associated with a decreased maximum inspiratory pressure 6-months after hospital discharge.

Variables	Present ($n = 19$)	Absence ($n = 25$)	p-value[a]	RR (IC 95%)[b]
Age, years, mean (SD)	57 (9.0)	51 (11.6)	0.05*	0.96 (0.86–1.08)
Gender				
Male. *n* (%)	10 (53)	20 (80)	0.03*	0.25 (0.05–1.16)
BMI, mean (SD)	30 (6.0)	31 (4.4)	0.71	NA
Extent of lung involvement at admission[c] *n* (%)				
25–50%	7 (40)	9 (41)	0.96	NA
>50%	9 (60)	13 (59)		
Charlson Comorbidity Index, media (25–75)	1 (1, 2)	2 (1–3)	0.008*	2.6 (0.8–8.0)
SAPS III, mean (SD)	45 (10.0)	50 (13.6)	0.36	NA
Respiratory SOFA				
D1, mean (SD)	2.8 (0.8)	2.7 (1.1)	0.65	NA
D3, mean (SD)	2.3 (1.2)	1.9 (1.6)	0.36	NA
SOFA				
D1, mean (SD)	3.9 (1.6)	3.6 (2.2)	0.71	NA
D3, mean (SD)	3.0 (2.1)	2.9 (3.1)	0.78	NA
C-reactive protein, mg/L				
D1, mean (SD)	138 (70)	100 (97)	0.22	NA
D3, mean (SD)	97 (53)	125 (128)	0.45	NA
ICU, yes, *n* (%)	14 (74)	16 (64)	0.62	NA
ICU length of stay, days, mean (SD)	10 (7.4)	13 (10.7)	0.42	NA
Mechanical ventilation, yes, *n* (%)	5 (26)	5 (20)	0.67	NA
Days on mechanical ventilation, mean (SD)	9 (8.1)	16 (6.1)	0.21	NA
Length of hospital stay, days, mean (SD)	16 (8.3)	16 (11.6)	0.92	NA

SD, standard deviation; RR- Relative Risk; CI, confidence interval; NA, not applied; BMI, Body mass index; SAPS III, Simplified Acute Physiology Score 3; SOFA, sequential organ failure assessment score; ICU, intensive care unit; N, number of participants.

[a] *p-value from the univariate analysis.*

[b] *RR form the regression analysis.*

[c] *For six patients CT scan was not performed at hospital admission.*

**p < 0.20 variables included in the binary logistic regression.*

Lung CT scan were abnormal in more than half of the patients, and this is consistent with other studies (6, 8). Interestingly, even variables such as mechanical ventilation and respiratory SOFA were not a risk factor associated with abnormal CT scan at 6 months. Unlike González et al. (17) demonstrated that the persistence of lung abnormalities on CT scan was associated with the length of invasive mechanical ventilation. Dyspnea was attributed to abnormalities on lung CT scan (17), and this is similar to our results; half of the patients who presented lung dysfunction measured by the 6MWT, DLCO, and MIP had persistent CT scan abnormalities.

Results for abnormal lung function assessed by the 6MWT, FEV1, DLCO, and MIP showed that a considerable proportion (18–43%) had at least one alteration. DLCO and MIP were the most prevalent dysfunction observed; thus, intrinsic lung function and respiratory muscle strength were affected after COVID-19. A systematic review evaluated pulmonary function after COVID-19 (18). It included three hundred eighty patients in the data synthesis. In the sensitivity analysis, the study found a prevalence of 0.39 (*CI* 0.24–0.56, $p < 0.01$, $I^2 = 86\%$), 0.15 (*CI* 0.09–0.22, $p = 0.03$, $I^2 = 59\%$), and 0.07 (CI 0.04–0.11, $p = 0.31$, $I^2 = 16\%$) for altered DLCO, restrictive pattern and obstructive pattern, respectively, consistent with our findings. As previously demonstrated (9, 10), an increased risk for lung dysfunction was observed in women. In our sample, female sex was an independent risk factor for decreasing 6MWT and DLCO. Additionally, this was also true to MIP in the univariate analysis. Interestingly, confirming previous reports (19), female sex was not significantly associated with persistent CT-scan abnormalities; neither is persistent CT-scan abnormalities universally present in lung dysfunction patients, suggesting that distinct mechanisms could be related to these outcomes. Further biomarkers studies could help to understand the underlying mechanisms that drive these alterations.

Other risk factors associated with lung dysfunction at 6-months that were statistically significant in the univariate analysis were Charlson comorbidity index and age. This is an exciting finding suggesting that for "post-COVID condition" premorbid characteristics are more important as a risk factor when compared to variables associated with the COVID-19 acute phase. This is different when compared to Huang et al. findings (10). They found that impaired DLCO was most prevalent in patients with more severe illnesses. The population in Huang's study included less severe COVID-19 patients when compared to our study, and this could partially explain these differences.

These dysfunctions seem to be of clinical relevance since they are associated with a decreased BI and a higher degree of dyspnea assessed by the mMRC. It is important to note that the linear regression model explained 28 and 32% of the alterations in the ADL and mMRC, respectively. Thus, besides lung dysfunction, others factors associated with "post-COVID condition" could impact ADL impairment. Huang et al. (10) found that fatigue and muscle weakness were common 6-months after the onset of COVID-19 symptoms, and such symptoms could impact ADL and mMRC. Further studies should be performed to determine clinical factors associated with long-term limitations in ADL and dyspnea intensity.

This study has several limitations. First, due to the urgent need for data, we anticipated the evaluation for these 44 patients to 6-months after hospital discharge. The small sample size may result in false-negative results. However, the significant associations that were found, together with the prospective design with a blind analysis, strengthen the results' credibility. Initially, these patients would be followed up only by phone interview until 12-months after hospital discharge. Follow-up keeps going, and all patients will have a complete evaluation at 12-months after hospital discharge. Second, due to epidemiologic characteristics of the pandemia, it was not possible to include SARS patients of other etiologies such as influenza.

CONCLUSION

Long-term lung dysfunction is relatively common in survivors from severe COVID-19 and impacts negatively on ADL and the intensity of dyspnea, similar to studies in high-income countries. These results highlight the need to develop

strategies to prevent and treat the burden of disease associated with COVID-19.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The São José Hospital's Institutional Review Board approved the protocol under the number 31384620.6.1001.5364. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GP had full access to all of the data in the study and contributed to the study design, data collection and interpretation, and writing of manuscript. CS contributed to data collection and literature search. RW and CR contributed to the study design and to writing of the manuscript. FD-P contributed to the study design, data interpretation, and writing of manuscript. All authors contributed to the article and approved the submitted version.

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Relationship Between Endothelial and Angiogenesis Biomarkers Envisage Mortality in a Prospective Cohort of COVID-19 Patients Requiring Respiratory Support

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Purpose: Endothelial damage and angiogenesis are fundamental elements of neovascularisation and fibrosis observed in patients with coronavirus disease 2019 (COVID-19). Here, we aimed to evaluate whether early endothelial and angiogenic biomarkers detection predicts mortality and major cardiovascular events in patients with COVID-19 requiring respiratory support.

Methods: Changes in serum syndecan-1, thrombomodulin, and angiogenic factor concentrations were analysed during the first 24 h and 10 days after COVID-19 hospitalisation in patients with high-flow nasal oxygen or mechanical ventilation. Also, we performed an exploratory evaluation of the endothelial migration process induced by COVID-19 in the patients' serum using an endothelial cell culture model.

Results: In 43 patients, mean syndecan-1 concentration was 40.96 ± 106.9 ng/mL with a 33.9% increase (49.96 ± 58.1 ng/mL) at day 10. Both increases were significant compared to healthy controls (Kruskal–Wallis $p < 0.0001$). We observed an increase in thrombomodulin, Angiopoietin-2, human vascular endothelial growth factor (VEGF), and human hepatocyte growth factor (HGF) concentrations during the first 24 h, with a decrease in human tissue inhibitor of metalloproteinases-2 (TIMP-2) that remained after 10 days. An increase in human Interleukin-8 (IL-8) on the 10th day accompanied by high HGF was also noted. The incidence of myocardial injury and pulmonary thromboembolism was 55.8 and 20%, respectively. The incidence of in-hospital deaths was 16.3%. Biomarkers showed differences in severity of COVID-19. Syndecan-1, human platelet-derived growth factor (PDGF), VEGF, and Ang-2 predicted mortality. A multiple logistic regression model with TIMP-2 and PDGF had positive and negative

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predictive powers of 80.9 and 70%, respectively, for mortality. None of the biomarkers predicted myocardial injury or pulmonary thromboembolism. A proteome profiler array found changes in concentration in a large number of biomarkers of angiogenesis and chemoattractants. Finally, the serum samples from COVID-19 patients increased cell migration compared to that from healthy individuals.

Conclusion: We observed that early endothelial and angiogenic biomarkers predicted mortality in patients with COVID-19. Chemoattractants from patients with COVID-19 increase the migration of endothelial cells. Trials are needed for confirmation, as this poses a therapeutic target for SARS-CoV-2.

Keywords: angiogenesis, syndecan-1, angiopoietin-2, VEGF, COVID-19

INTRODUCTION

Aggressive and rapidly evolving symptoms characterise a subset of patients with coronavirus disease 2019 (COVID-19). Early recognition of evolution is still not possible (1–3). Long-lasting hospitalisation due to prolonged mechanical ventilation is associated with altered oxygen diffusion in lung capillaries, which may be partly due to an increase in fibrotic areas (4). Angiopoietins are critical players in vessel maturation and mediate the migration, adhesion, and survival of endothelial cells. In conjunction with vascular endothelial growth factor (VEGF), angiopoietins promote neovascularisation (5). Furthermore, neovascularisation and fibrosis are present in the lungs of patients with COVID-19 (6, 7). Endothelial damage and angiogenesis are fundamental elements of this process (8–11), where intussusceptive and sprouting angiogenesis observed in autopsies reflect rapid vascular activation and proliferation during the disease (7, 12).

Elevation of the endothelial injury biomarkers, syndecan-1 and thrombomodulin (TM) in patients with sepsis is associated with intensive care unit (ICU) mortality (13, 14). In COVID-19, reports of syndecan-1 increase are related to disease severity and have been suggested as an assessment of the clinical course of the patient (15, 16). Soluble TM plasma elevation is also related to increased mortality in patients with COVID-19, as it marks direct endothelial cell damage (17, 18).

Besides injury, endothelial angiogenic activation poses another pathophysiological feature as well as a therapeutic opportunity. It seems to be a rapid phenomenon upon severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection, as this feature is observed in lung autopsies from non-surviving patients within 10 days of hospitalisation (7). Increased angiogenic biomarkers are associated with ICU admission and patients' reduced respiratory system compliance (19). Molecules such as VEGF and angiopoietin-2 (Ang-2) are associated with angiogenesis. Hepatocyte growth factor (HGF) stimulates cell migration and branching and acts synergistically with VEGF to promote new blood vessel formation, pericyte migration, and endothelial cell migration. Tissue inhibitor of metalloproteinases-2 (TIMP-2) binds to metalloproteinases (MMPs) and decreases extracellular matrix degradation (20).

Considering the brief instauration of the endothelial and angiogenic processes, we aimed to evaluate whether there is an early increase in endothelial and angiogenic biomarkers and whether early detection of these biomarkers is associated with mortality and major cardiovascular events in patients with COVID-19.

MATERIALS AND METHODS

We performed a single-centre prospective cohort study of patients with COVID-19 hospitalised at the Hospital Clínico de la Universidad de Chile. The study was approved by the ethics committee of our centre (Ref: OAIC 1161/20), registered online at https://www.clinicaltrials.gov (Ref: NCT04609332), and conducted according to the principles of the Helsinki Declaration under monitoring by the Good Clinical Practice unit of our institution. Written informed consent was obtained from all patients who were able to sign or from a legal representative if they were unable to provide consent. We adhered to the STROBE guidelines for reporting observational studies (21). The study was performed before vaccination campaigns in our country.

We defined changes in syndecan-1 blood concentrations during hospitalisation as the primary outcome. First, serum syndecan-1 concentrations were analysed during the first day of hospitalisation. Second, in the blood samples, we determined the concentrations of TM and a set of angiogenic factors as markers of profound endothelial damage and activation. After 10 days, if the patient remained hospitalised, a second biomarker set measurement was performed in this subgroup of patients. After 6 months of follow-up, mortality and major cardiovascular events were recorded. Finally, using an endothelial cell culture model, we performed an exploratory evaluation of the endothelial migration process in the serum samples of patients with COVID-19.

Participants

We included patients aged 18 years and older with clinically suspected and laboratory reverse transcriptase-polymerase chain reaction (RT-PCR)-confirmed SARS-CoV-2 infection, hospitalised in critical patient care units with the need for high-flow nasal oxygen (HFNO) or mechanical ventilation during the first 24 h after arriving at our centre. The exclusion

criteria were symptomatic patients with a negative RT-PCR for SARS-CoV-2 and patients who were treated with anticoagulants for a pre-existing comorbidity. Recruitment was performed between December 2020 and March 2021. For serum endothelial damage and angiogenic biomarkers, 10 mL blood samples were collected during the first 24 h and on the 10th day of hospitalisation. Hospitalisation clinical data were collected, and a telephonic follow-up was performed until the 6th month after hospital admission. As for control group, we included nine blood samples from healthy volunteers who did not present COVID-19 disease. They were recruited from the hospital and research laboratory during the study period. All patients' samples were processed similarly.

Variables

For the primary outcome, increased and differences in syndecan-1 concentrations were analysed during the first 24 h and at the 10th day of hospitalisation. Blood serum concentrations of TM, human Ang-2, human HGF, human Interleukin-8 (IL-8), human platelet-derived growth factor (PDGF), human TIMP-2, and human VEGF were measured. Demographic, clinical, and laboratory data were obtained from the patients' medical charts. Major cardiovascular events were defined as death, the presence of pulmonary thromboembolism, and myocardial injury in patients with high-sensitive cardiac troponin I (Hs-cTn) elevation above 11 ng/L [99th percentile upper reference limit (URL)] (Vitros®, Ortho Clinical Diagnostics, UK) (22). Deaths outside the hospital were obtained from the National Registry of Deaths accessed online (https://www.registrocivil.cl). Surviving patients were contacted within the next 6 months after hospitalisation for clinical follow-up. As a definition of COVID-19, we analysed our cohort according to the National Institutes of Health clinical spectrum (23). We defined severity according to the need for ventilatory support and hospitalisation period as follows: severe, patients requiring HFNO ventilation who did not progress into shock or respiratory failure during the first 10 days of hospitalisation; critical, patients with the need for HFNO or mechanical ventilation due to respiratory failure, shock, or multiorgan dysfunction; and deceased patients.

Laboratory processing and outcome assessors were blinded to the patients. At study termination, the outcome assessor attained the patients' sample codes and performed the final analysis. After blinded assessments, we performed an exploratory analysis of serum from healthy patients and patients with severe COVID-19. Serum samples were used for *in vitro* endothelial migration assays.

Sample Size

By the time of the study design, we found no previous data on syndecan-1 in patients with COVID-19; therefore, we selected a sample size of 40 patients for convenience of the primary outcome. Nevertheless, considering a normal syndecan-1 value of 31.6 ng/mL, a standard deviation of 15.3 ng/mL, our sample size calculation allowed us to detect a 29.7% change in the mean concentration of syndecan-1, with an alpha of 0.05 and a power of 80% (24, 25). Considering a 10% loss of patients, 44 patients were required for the two-sided test analysis.

Statistical Methods

Categorical variables were summarised as relative frequencies. Continuous variables for primary and secondary outcomes were expressed as the mean and standard deviation (SD) or median and interquartile rank (IQR). Non-paired results were compared using the Mann–Whitney test. For group comparisons, we used the Kruskal–Wallis test with Dunn's multiple comparison test. The Wilcoxon test was used for paired data. Receiver operating characteristic (ROC) curves were generated for all biomarkers. The area under the curve (AUC) was calculated using the Youden index for cut-off values (26, 27). Multiple logistic regression was used with survivors and non-survivors as dichotomised variables as outcomes. We obtained a pseudo R2 (Tjur's R2) and used a Hosmer–Lemeshow goodness-of-fit and log-likelihood ratio test for hypothesis testing. Two-tailed P-values < 0.05, were considered significant for all analyses. Data were analysed using GraphPad Prism software (version 9.0; La Jolla, CA, USA).

Quantification of Biomarkers

All blood samples were collected in a 15 mL centrifugation tube without heparin by an anaesthesiologist and coded before delivering the samples to the processing laboratory. All blood samples were immediately incubated for 1 h at 37°C and centrifuged at 400 g for 10 min. Blood serum was stored in a −80°C freezer for the final analysis (28).

To characterise the endothelial damage, we measured one glycocalyx biomarker (syndecan-1) and one endothelial membrane biomarker (TM). For biomarker levels in blood serum, we used the following enzyme-linked immunosorbent assay (ELISA): DuoSet Human Syndecan-1 (#DY2780; R&D Technologies, MN, USA) using blood samples diluted 20 times and five times, and human TM/BDCA−3 (#DTHBDO; R&D Technologies, MN, USA) using blood samples diluted six times. All measurements were performed in duplicate in one assay. Assays and analyses were performed according to the manufacturer's instructions (25).

To quantify Ang-2, HGF, IL-8, PDGF, TIMP-2, and VEGF, we used Q-PlexTM Human Angiogenesis (#150233HU; Quansys, Cellus, Santiago, Chile). The blood samples were diluted six times. Assays and analyses were performed according to the manufacturer's instructions. Additionally, a Proteome Profiler Human Angiogenesis Array kit (#ARY007, R&D Technologies) was used to identify 55 different angiogenesis proteins in COVID-19 and in the healthy volunteers' blood serum.

Cell Migration

Cell migration was assayed using Transwell chambers (#3422; Costar, Corning Incorporated, ME, USA) with 8.0 μm-pore polycarbonate filters. 10,000 EA. hy926 cells were suspended in serum-free Dulbecco's Modified Eagle Medium (DMEM) without antibiotics and seeded in the upper compartment of the chamber. Ten samples of 10% v/v blood (five in the first 24 h and five on the 10th day of hospitalisation) from five randomly selected patients with COVID-19 and five from healthy individuals were added to the lower compartment of the chamber. Migration was allowed to occur for 24 h. Following the removal of the non-invading cells, the invading cells were fixed

TABLE 1 | Patients characteristics.

Baseline values	Total	COVID-19 severity			*p*-value
		Severe	Critical	Non-survivors	
Age—years					
Median (IQR)	62 (53–72)	55 (38–68)	62 (55–74)	73 (60–74)	<0.0001[#]
Range	55	42	49	17	
Sex—*n*/ total *n* (%)					
Female	18/43 (42)	4/43 (9)	11/43 (26)	3/43 (7)	0.9110
Male	25/43 (58)	7/43 (16)	14/43 (33)	4/43 (9)	0.9110
BMI—mean (SD)	26.4 (8.6)	28.2 (3.7)	25.7 (10.6)	26.9 (2.9)	
Comorbidities—*n*/ total *n* (%)					
Obesity	5/43 (12)	3/43 (7)	1/43 (2)	1/43 (2)	0.0751
Coronary heart disease	4/43 (9)	2/43 (4)	1/43 (2)	1/43 (2)	0.3339
Heart failure	3/43 (7)	–	1/43 (2)	2/43 (4)	0.1801
Chronic kidney failure	3/43 (7)	–	3/43 (7)	–	0.3281
Acute kidney failure	–	–	–	–	–
Stroke	–	–	–	–	–
Vascular disease	–	–	–	–	–
COPD	3/43 (7)	1/43 (2)	1/43 (2)	1/43 (2)	0.5870
Liver disease	1/43 (2)	–	–	1/43 (7)	0.0669
Diabetes	16/43 (37)	4/43 (9)	9/43 (21)	3/43 (7)	0.9402
Hypertension	22/43 (51)	5/43 (12)	14/43 (32)	3/43 (7)	0.8619
Smoking	5/43 (12)	1/43 (2)	2/43 (4)	2/43 (4)	0.2920
Dyslipidemia	4/43 (9)	1/43 (2)	3/43 (7)	–	0.9434
Laboratory—mean (SD)					
D-Dimer (ng/mL)	1,558 (1,745)	1,090 (808)	1,844 (2,168)	1,240 (585)	0.4274
C-reactive protein (mg/L)	159 (100)	96 (69)	183 (109)	169 (61)	0.0341*
ProBNP (pg/mL)	749 (914)	256 (252)	875 (1,092)	939 (943)	0.0827
Procalcitonin (ng/mL)	0.27 (0.39)	0.09 (–)	0.45 (0.5)	0.09 (0.05)	0.7000
Myocardial injury—*n* (within subgroup %)					
24 h	19 (44.2)	2 (18)	12 (46.1)	5 (71)	0.1371
Total hospitalisation	24 (55.8)	3 (27)	15 (58)	6 (85)	0.0418[&]
High-sensitive cardiac troponin I—mean (SD)					
24 h (mg/ml)	35.7 (89.8)	6.7 (6.6)	41.7 (108.9)	52.2 (72)	0.0348* 0.0318[#]
Maximum (mg/ml)	114.5 (341.1)	12.4 (20.9)	149.6 (439.8)	149.5 (119.5)	0.0362* 0.0032[#]
Thromboembolism—*n*/ total *n* (%)					
First 24 h	4/43 (9)	0/11 (0)	3/25 (7)	1/7 (14)	0.4684
During hospitalisation	9/43 (20)	1/11 (1)	5/25 (20)	3/7 (43)	0.2489
Arterial blood gases—mean (SD)					
pH	7.43 (0.04)	7.43 (0.04)	7.43 (0.05)	7.42 (0.04)	0.6802
pCO_2	32.1 (4.3)	32.8 (4.4)	31.9 (4.6)	31.5 (2.9)	0.8469
pO_2	82.6 (28.19)	96.63 (35.9)	79.42 (23.0)	72.63 (28.5)	0.2816
BE	−2.39 (3.25)	−1.98 (3.07)	−2.30 (3.40)	−3.33 (3.24)	0.6541
HCO_3	21.36 (2.99)	21.62 (3.17)	21.50 (3.04)	20.44 (2.79)	0.6171
FiO_2	49.5 (2.99)	34.2 (12.80)	55.2 (29.08)	53.7 (29.40)	0.1385
PaFi	221.0 (132.7)	316.4(151.5)	184.6 (102.7)	190.7 (133.2)	0.0265*
Use of vasopressors—*n*/ total *n* (%)					
First 24 h	4/43 (9)	0/11 (0)	2/25 (8)	2/7 (29)	0.1189
Between 24 h and 10th day	16/43 (44)	0/11 (0)	11/25 (44)	5/7 (71)	0.0052*[#]
At 10th day	7/43 (16)	0/11 (0)	6/25 (24)	0/7 (0)	0.0812

[*] *Severe vs. critical patients Kruskal-Wallis.*
[&] *Chi-square.*
[#] *Severe vs. non-survivors Kruskal-Wallis.*

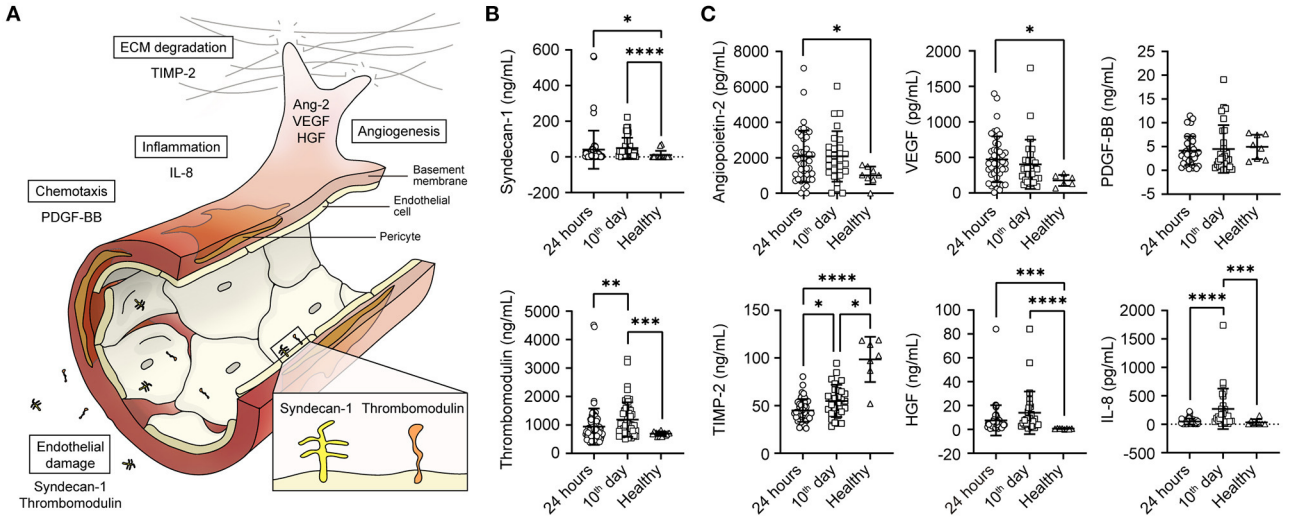

FIGURE 1 | Serum levels of endothelial damage and angiogenesis-related proteins. **(A)** Summary of key molecules involved in angiogenesis and endothelial damage. **(B)** Graph representing the mean serum of syndecan-1 and thrombomodulin during the first 24 h of hospitalisation ($n = 43$), after 10 days ($n = 28$) and in healthy individuals ($n = 9$). **(C)** Graph representing the mean serum of angiopoietin-2, TIMP-2. VEGF, PDGF-BB, HGF, and IL-8. Statistical significance (*P*-values) is obtained using two-sided Kruskal–Wallis with Dunn's multiple comparison test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. $^{****}P < 0.0001$.

and stained with 0.2% crystal violet. Cell migration was evaluated by counting five (×20) fields per chamber (29).

RESULTS

Forty-three patients were included between December 2020 and March 2021 (**Supplementary Figure 1**). The median age was 62 years (IQR 53–72); 39.5% were female and 60.5% were male. The mean body mass index was 26.4 kg/m^2. The most prevalent comorbidities were hypertension (51%), diabetes (37%), obesity (12%), and dyslipidaemia (12%) (**Table 1**; **Supplementary Table 1**). For major cardiovascular events, we found a 44.2% ($n = 19$) incidence of myocardial injury in patients presenting to the hospital (Hs-cTn above URL), which increased to 55.8% ($n = 24$) of patients during hospitalisation. The incidence of pulmonary thromboembolism (PTE) was 9.3% ($n = 4$) in the first computed tomography angiography and increased during hospitalisation to 20% ($n = 9$) (**Table 1**). The incidence of in-hospital death was 16.3%. During the 6-month follow-up period, we found no out-of-hospital mortality.

Key molecules are involved in endothelial and angiogenic processes (**Figure 1A**). The endothelial damage in patients with COVID-19 was characterised by a mean syndecan-1 concentration during the first 24 h of hospitalisation (40.96 ± 106.9 ng/mL) and, in the next 10 days, the subgroup of patients that remain hospitalised presented a 33.9% increase in serum concentrations (49.96 ± 58.1 ng/mL). Both increases were significant compared to healthy controls (Kruskal–Wallis $p <$ 0.0001) (Intra-assay Coefficient of Variation of 6.9%). The mean TM level significantly increased from 942 ± 638 ng/mL in the first 24 h to 1,189 ± 608 ng/mL on the 10th day of hospitalisation and was different from that in healthy controls (Kruskal–Wallis $p < 0.0001$) (**Figure 1B**).

The angiogenic biomarker profile was characterised by an increase in Ang-2, VEGF, and HGF concentrations during the first 24 h after arriving at the centre. We found a decrease in the MMP inhibitor MMP TIMP-2 concentrations, which remained in the subgroup of hospitalised patients after 10 days. In this subgroup of patients with COVID-19, we also observed higher IL-8 levels on the 10th day accompanied by high HGF values compared to those in healthy controls (**Figure 1C**).

For biomarkers obtained in the first 24 h, PDGF had an AUC of 0.838 (95% CI 0.69–0.99; $p = 0.005$) to predict mortality from survivors (cut-off value, 2,118 pg/mL; sensitivity, 71.43%; specificity, 81.08%). VEGF had an AUC of 0.768 (95% CI 0.56–0.98: $p = 0.0257$) to predict mortality (cut-off value, 266 pg/mL; sensitivity, 71.43%; specificity, 83.78%). On the 10th day of hospitalisation, Ang-2 had an AUC of 0.875 (95% CI 0.73–1.0; $p = 0.018$) to predict mortality from survivors (cut-off value, 2,388 pg/mL; sensitivity, 100%; specificity, 75%). Interestingly, syndecan-1 had an AUC of 0.94 (95% CI 0.84–1.0; $p = 0.005$) to predict mortality from survivors (cut-off value of 40.1 ng/mL; sensitivity of 100% and specificity of 81.82%) (**Figures 2A,B**). Finally, none of the biomarkers predicted myocardial injury or PTE.

According to severity definitions, we found that the concentrations of syndecan-1 in the first 24 h were significantly elevated in patients who developed a critical illness or died. TM levels were elevated in patients with severe and critical disease (**Figure 3A**). Patients who died from COVID-19 also presented with elevated Ang-2 and HGF levels, accompanied by low concentrations of TIMP-2. Critical disease was characterised

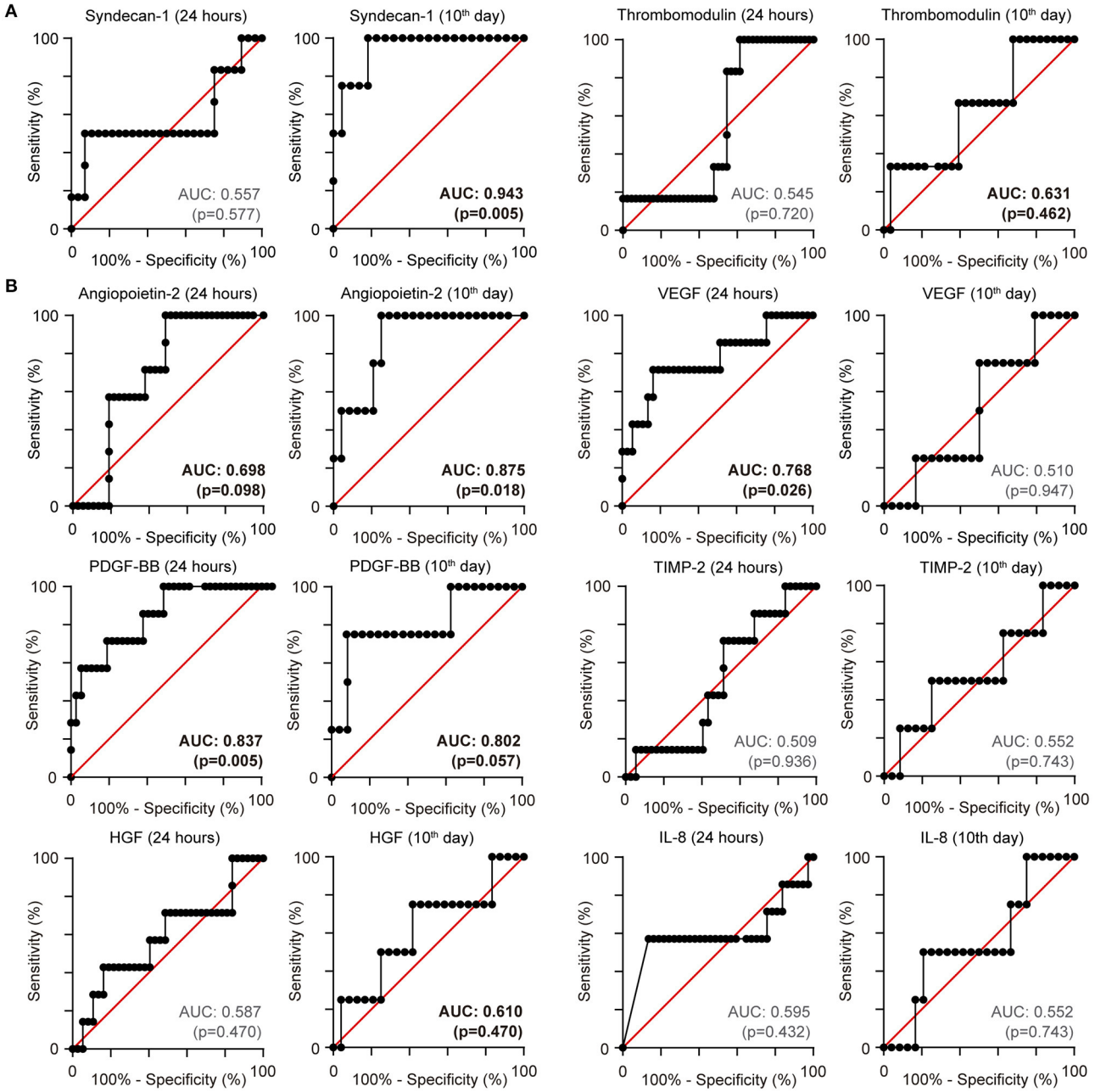

FIGURE 2 | Receiver operating characteristic (ROC) curve for mortality: **(A)** Endothelial biomarkers **(B)** Angiogenic biomarkers. ROC curves with area under the curve for mortality.

by an increase in VEGF and HGF and a decrease in TIMP-2 (**Figure 3B**).

Next, we evaluated whether a combination of the biomarkers already shown may outperform the prediction accuracy for in-hospital mortality. We performed a multiple logistic regression model using Ang-2, HGF, IL-8, PDGF, TIMP-2, VEGF, syndecan-1, and TM values obtained within 24 h of the patient arriving at the hospital. A dual combination of VDGF, PDGF, and TIMP-2 improved our model. The combination of TIMP-2 and PDGF as predictors had a positive predictive power of 80.9% and a negative predictive power of 70% for mortality, with an AUC of 0.90 (95% CI 0.816–0.997; p-value < 0.0001; Tjur's R2 of 0.43), Hosmer–Lemeshow p-value of 0.34 and a log-likelihood ratio p-value < 0.0001 (**Figure 3C**).

To further evaluate the biomarkers, we tested whether patients' serum samples were able to induce changes in endothelial function. To identify biomarkers that could be modulated by SARS-CoV-2, we used a semi-quantitative

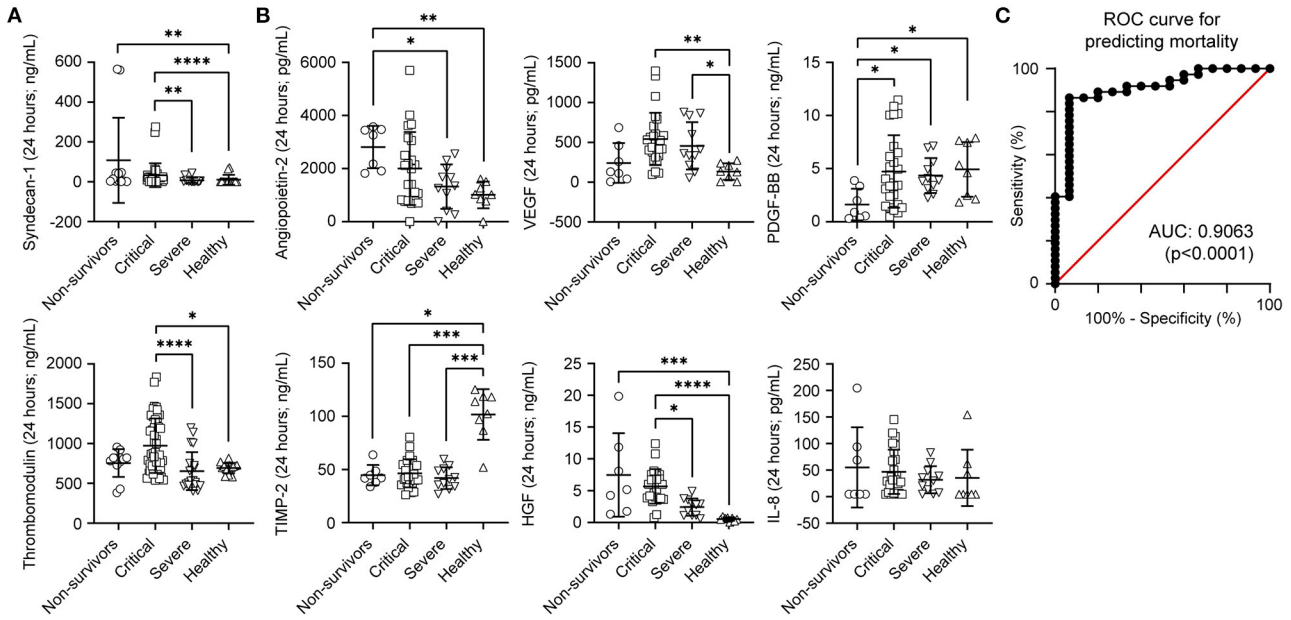

FIGURE 3 | Serum levels comparison according to coronavirus disease 2019 severity during the first 24 h of hospitalisation. **(A)** Levels of syndecan-1 and thrombomodulin. **(B)** Graph representing the mean serum of angiopoietin-2, vascular endothelial growth factor (VEGF), human platelet-derived growth factor (PDGF), human tissue inhibitor of metalloproteinases-2 (TIMP-2), human hepatocyte growth factor (HGF), and human Interleukin-8 (IL-8). Statistical significance (*P*-values) is obtained using two-sided Kruskal–Wallis test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. **(C)** Multivariate model logistic regression receiver operating characteristic curve using TIMP-2 and PDGF for predicting mortality ($n = 11$ severe, $n = 25$ critical, $n = 7$ non-survivors).

methodology, a proteome profiler array, using serum from patients with COVID-19 and healthy individuals. We found that Angiopoietin-1 (Ang-1), Ang-2, endostatin, VEGF, TIMP-1, TIMP-4, CXCL4, PDGF-AB/BB, TSP-1, EGF, CXCL16, and CD105 were modulated. Interestingly, Ang-1, Ang-2, VEGF, and CXCL-4 were involved in angiogenesis (**Figures 4A,B**) (30). Further, we evaluated whether blood serum from patients with COVID-19 and healthy individuals served as chemoattractants to endothelial EA.hy 926 cells by performing a Transwell assay (**Figures 4C–E**). We observed that blood serum from patients with COVID-19 showed increased cell migration compared to that in healthy blood serum.

DISCUSSION

COVID-19 is an aggressive disease. Here, we observed that endothelial injury and angiogenic biomarkers increased upon arrival of patients in need of high-flow nasal oxygen (HFNO) or mechanical ventilation support. An imbalance in the pro-angiogenic profile of endothelial activation is suggested in our migration and angiogenic assays using the serum samples of patients with COVID-19 as a chemotactic agent. Furthermore, an increase in biomarkers was predictive of mortality in our cohort.

The increase in syndecan-1 and TM levels confirmed substantial endothelial damage. As for angiogenesis, the sole elevation of one angiogenic protein has been described as insufficient for promoting endothelial cell survival and *in vitro* tubulogenesis, and the need for combination increases cell survival, tubulogenesis, and neovascularisation in rat corneas (20). In our cohort, we found an increase in VEGF, HGF in combination with high circulating Ang-2 levels. For PDGF, in deceased patients, we observed a decrease in the concentration in blood samples obtained upon arrival at the hospital. Also, we observed a decrease in TIMP-2 levels in the first 24 h and 10 days of hospitalisation, respectively. TIMP-2 inhibits VEGF-induced angiogenesis (31). Therefore, serum reduction reinforces the imbalance in the angiogenic profile of COVID-19 patients. Finally, IL-8 increase on the 10th day of hospitalisation adds a paracrine angiogenic factor that modulates the endothelial cell response (32, 33).

Using the proteome profiler array approximation, in patients with COVID-19 we confirmed the angiogenic and chemotactic serum profile compared to healthy volunteers of similar ages. Of the 55 molecules analysed, the observed increase in insulin-like growth factor binding proteins (IGFBPs), chemokines such as CXCL16 and Pentraxin-3, and endothelial growth factor (EGF) reaffirms the upregulation of angiogenic factors (34–39). There is compelling evidence that there is a need for crosstalk between different factors, as this has been described between HGF and VEGF, enhancing VEGF-driven angiogenesis; therefore, an increase in different molecules was expected (40). Finally, this profile was associated with *in vitro* endothelial cell migration, suggesting the ability of serum to activate endothelial functions.

SARS-CoV-2 infection through the angiotensin converting enzyme-2 (ACE-2) receptor makes COVID-19 systemic.

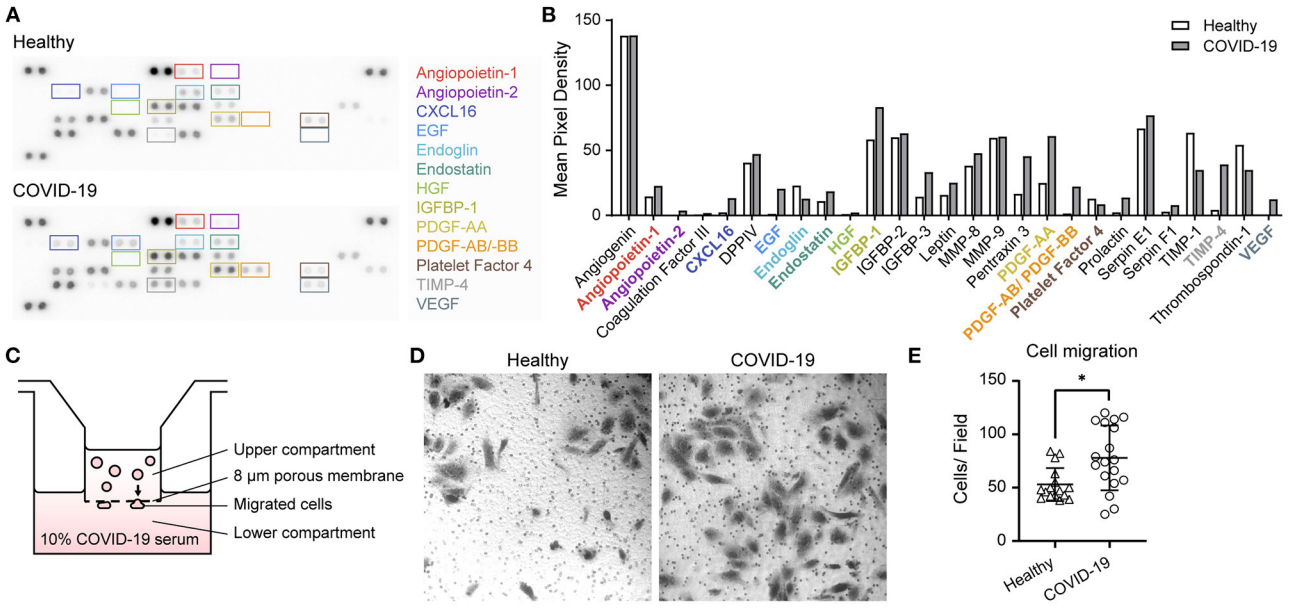

FIGURE 4 | Proteome profiler array and cell migration induced by coronavirus disease 2019 blood serum. **(A)** Representative image of the angiogenic proteome profiler array using blood serum samples from healthy participants and from those with severe COVID-19. **(B)** Quantification of pixel mean density of each angiogenesis-related protein. **(C)** Schematics of Transwell Boyden chamber assay using blood serum samples from COVID-19-infected and healthy participants in the lower compartment as chemoattractants. **(D)** Representative images of migrated endothelial EA.hy926 cells induced by blood serum samples of COVID-19-infected and healthy participants. **(E)** Quantification of migrated cells by field ($n = 5$ healthy individuals, $n = 3$ severe, $n = 1$ critical, and $n = 1$ non-surviving COVID-19 patient) using both samples in first 24 h and on the 10th day of hospitalisation. Statistical significance (P-values) is obtained using Mann–Whitney $^*P < 0.05$.

Endothelial injury is a hallmark of tissue permeability, lung oedema, and organ dysfunction. Endothelial cells control vascular tone and permeability by inducing endothelium-derived relaxation and contractile factors. Upon activation, endothelial cells secrete chemoattractants, cytokines, and adhesion molecules (41). With dysfunction, endothelial cells fail to produce nitric oxide (NO), losing the suppression effect on activated molecule release, a feature observed in patients with COVID-19 (42, 43). Other endothelial functions, such as angiogenesis and cellular migration, have received less attention in the literature. When Ackerman et al. described intussusceptive and sprouting angiogenesis as a novel feature of SARS-CoV-2 infection, compared with autopsies from influenza patients, he showed a vessel proliferation that accompanied a rapid decline in lung function and death (7). This angiogenic progression has been primarily studied in tumours. Circulating VEGF, HGF, and Ang-2 levels have been described in breast cancer, hepatocarcinoma, and melanoma (44–46).

There is increasing evidence that endothelial damage is a predictor of outcomes. Smadja et al., in an observational cohort of 40 patients, found that Ang-2 concentration at admission is a relevant factor to predict transfer to ICU with an ROC of AUC 77.2 (80.1% sensitivity and 70% specificity) (19). Vassiliou et al., using endothelial biomarkers in hospitalised patients with COVID-19 admitted to the ICU, found that elevation of sE-selectin, sP-selectin, Ang-2, and sICAM-1 levels were significantly elevated in ICU non-survivors compared to survivors, with a higher mortality probability. In addition, sE-selectin, Ang-2, and sICAM-1 from the generated ROC curves were >0.85, indicating that elevated levels of these markers upon ICU admission could predict mortality in COVID-19 (47). Recently, de Moraes et al. described that angiopoietins, their receptors, and VEGF are associated with severity of COVID-19, suggesting that targeting the Ang/Tie2 and VEGF-A pathways could be valuable strategies to modulate COVID-19 severity (48). This reinforces our findings that endothelial damage is an early phenomenon, relates to hospital admission biomarker concentrations as a predictive tool for mortality, and can be a useful strategy for patient management.

Among the limitations of our study, we find the small number of participants and controls, which may reduce the strength of the statistical analysis. Although some of the biomarkers exhibited good AUC in the ROC curves and could predict disease mortality and severity in SARS-CoV-2, a larger trial is needed to confirm our results. Additionally, the number of samples obtained on day 10 was reduced due to deceased patients. Another limitation is the variation of absolute values between assays in the literature due to the temperature of sample management. Our samples were obtained with different processing methods than other laboratories, were we aimed to maintain normothermia to avoid platelet activation and angiogenic biomarkers release (49). Hence absolute values should be interpreted carefully and compared to studies with similar sample management. In relation to our migratory assay, we did

not perform inhibitory experiments, and our findings need to be further investigated.

Finally, endothelial injury and angiogenesis biomarkers have been associated with mortality in patients with sepsis (14, 50). Here, we observed that early endothelial and angiogenic biomarkers increased the prediction of mortality, although they failed to predict myocardial injury and PET. Even though all patients in our cohort received the dexamethasone-recovery protocol (51), serum biomarkers remained altered on the 10th day of hospitalisation. The angiogenic profile associated with the known cytokine storm may be a relevant feature in COVID-19 induced organ dysfunction (52), and differences in sprouting or intussusceptive angiogenic evolution in different sepsis aetiologies may impact outcomes (7). Regarding the endothelial and angiogenic features of COVID-19, many questions still remain regarding their usefulness as biomarkers and the potential role of anti-angiogenic treatments in patients with the disease.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Hospital Clínico Universidad de Chile (Ref: OAIC 1161/20). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from all patients who were able to sign or from a legal representative when they were unable to provide consent.

AUTHOR CONTRIBUTIONS

FM and MC conceived the study, drafted the work, and performed the funding acquisition. FM, DM, CD-P, CV, CF, ML, CE, and MC performed the data collection. Data were analysed by FM, DM, CD-P, CF, NV, RGu, ÁJ, CR, OC, and MC and interpreted by FM, NV, RGo, RGu, ÁJ, CR, OC, and MC. All the authors revised the work, commented on previous versions of the manuscript, approved the version to be published, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.826218/full#supplementary-material

Supplementary Figure 1 | Strobe diagram.

Supplementary Table 1 | Healthy volunteers' characteristics.

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