ENDOCRINOLOGY AND COVID-19: A CROSS-DISCIPLINARY TOPIC

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PUBLISHED IN: Frontiers in Endocrinology

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88976-977-3
DOI 10.3389/978-2-88976-977-3

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ENDOCRINOLOGY AND COVID-19: A CROSS-DISCIPLINARY TOPIC

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Citation: Regitz-Zagrosek, V., Mauvais-jarvis, F., Hofmann, S., Holly, J. M. P., Kautzky-Willer, A., Bao, W., eds. (2022). Endocrinology and COVID-19: A Cross-Disciplinary Topic. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88976-977-3

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frontiers in Endocrinology

REVIEW
published: 22 April 2020
doi: 10.3389/fendo.2020.00161

General Adaptation in Critical Illness: Glucocorticoid Receptor-alpha Master Regulator of Homeostatic Corrections

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Specialty section:
This article was submitted to Molecular and Structural Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *23 December 2019*
Accepted: *09 March 2020*
Published: *22 April 2020*

Citation:
Meduri GU and Chrousos GP (2020) General Adaptation in Critical Illness: Glucocorticoid Receptor-alpha Master Regulator of Homeostatic Corrections. Front. Endocrinol. 11:161. doi: 10.3389/fendo.2020.00161

In critical illness, homeostatic corrections representing the culmination of hundreds of millions of years of evolution, are modulated by the activated glucocorticoid receptor alpha (GRα) and are associated with an enormous bioenergetic and metabolic cost. Appreciation of how homeostatic corrections work and how they evolved provides a conceptual framework to understand the complex pathobiology of critical illness. Emerging literature place the activated GRα at the center of all phases of disease development and resolution, including activation and re-enforcement of innate immunity, downregulation of pro-inflammatory transcription factors, and restoration of anatomy and function. By the time critically ill patients necessitate vital organ support for survival, they have reached near exhaustion or exhaustion of neuroendocrine homeostatic compensation, cell bio-energetic and adaptation functions, and reserves of vital micronutrients. We review how critical illness-related corticosteroid insufficiency, mitochondrial dysfunction/damage, and hypovitaminosis collectively interact to accelerate an anti-homeostatic active process of natural selection. Importantly, the allostatic overload imposed by these homeostatic corrections impacts negatively on both acute and long-term morbidity and mortality. Since the bioenergetic and metabolic reserves to support homeostatic corrections are time-limited, early interventions should be directed at increasing GRα and mitochondria number and function. Present understanding of the activated GC-GRα's role in immunomodulation and disease resolution should be taken into account when re-evaluating how to administer glucocorticoid treatment and co-interventions to improve cellular responsiveness. The activated GRα interdependence with functional mitochondria and three vitamin reserves (B1, C, and D) provides a rationale for co-interventions that include prolonged glucocorticoid treatment in association with rapid correction of hypovitaminosis.

Keywords: critical illness, glucocorticoid receptor-alpha, nuclear factor-κB, mitochondria, hypovitaminosis

CRITICAL ILLNESS, SPECIES EVOLUTION, AND INDIVIDUAL DEVELOPMENT

The reasons behind the evolutionary success of mammals and other multicellular organisms is their extraordinary capacity to adapt to changing environmental conditions and survive by maintaining their homeostasis (1). Homeostasis refers to the relative stability in the activity of the physiological systems of the organism that are essential to support life (2). The process of maintaining stability within a harmless range via homeostatic physiologic corrections to both predictable and unpredictable adverse forces or stressors is termed "eustasis" (2). In the course of human evolution, homeostatic corrections have emerged to increase the host's ability to cope with adverse or even catastrophic events (3). These responses are shaped by trade-offs, sometimes with benefits and disadvantages in different periods of the life cycle (4). Following the Cambrian explosion, about 450 million years ago, when multicellular organisms—originally formed in water environments—colonized the land physiological homeostatic changes emerged to allow survival. These corrective changes were essential to mammalian species evolution and emerged to solve a frequent conflict between environmental changes and preservation of the individual allowing survival and, hence, reproduction. These alterations permitted progression to future generations i.e., survival of the species through the active process of natural selection (5). These corrections involved profound neural, metabolic and immune changes mediated by a few major physiological systems (e.g., the central nervous, autonomic, cardiorespiratory, endocrine, and immune systems), and acting through integrated crosstalk pathways, that was associated with appropriate responses throughout the organism (5). Such changes have been relatively conserved across many vertebrates, including mammalian species (6, 7). They have evolved to allow coping with lack of energy, dehydration, hemorrhage, infections, toxic substances, or relatively short-lived inflammatory responses, such as those of wound healing or exposure to foreign substances (7).

When the organism is exposed to stressors that exceed the harmless stability range, individual survival is maintained at the expense of this organism's health and longevity. This condition is different from healthy homeostasis or eustasis and is called "allo-stasis" or different (allo)-stasis or even more accurately "caco (bad) –stasis. The cumulative cost of cacostasis for the organism, has been called allostatic or cacostatic burden. Excessive or prolonged cacostatic burden results in severe acute and/or chronic cacostatic pathology (2, 8). The intensive care unit (ICU) stress state represents a new and very different ecosystem from those within which humans evolved in the past. Actually, critical illness epitomizes an acute and/or chronic cacostatic burden that goes beyond an evolutionarily conserved physiological adaptive response, and if left untreated it could rapidly exhaust homeostatic compensation and lead to death of an organism (lethal cacostasis). In critically ill patients, the need for vital organ support (maintenance of arterial blood pressure, mechanical ventilation, and other support measures, which were not available until the middle of the last century), reflects near exhaustion or exhaustion of (i) neuroendocrine homeostatic compensation, also known as "critical illness-related corticosteroid insufficiency" (CIRCI) (8); (ii) cell bio-energetic and other functions; and (iii) reserves of vital micronutrients (vitamins and minerals). Even when it allows survival of the patient, homeostatic failure, ranging in acuity and severity, has a major impact on morbidity and mortality during and after hospitalization. CIRCI-associated dysregulated systemic inflammation and mitochondrial dysfunction are central to the increased morbidity and mortality of acute and/or chronic critical illness and the subject of this review.

INNATE IMMUNITY AND NUCLEAR FACTOR-κB

In multicellular organisms, the innate immune system with its recognition and signaling mechanisms is the most ancient form of host defense to infectious and non-infectious threats during evolution (9). The nuclear factor-κB (NF-κB) system, a "rapidly-acting" primary transcription factor regulated cellular response, is a central activator of innate immunity. It appears that the NF-κB was incorporated into this ancient signaling pathway more than 420 million years ago, and has been shown to play independent roles in vertebrate and insect lineages (9, 10). In most cell types, the inactive form of NF-κB, a heterodimeric protein composed of the DNA-binding proteins p65 and p50, is retained in the cytosol by association with inhibitory factors, such as IκB proteins; when activated, the latter are rapidly phosphorylated and degraded via the proteasomal pathway, liberating NF-κB and allowing it to translocate into the nucleus (11). In tumor necrosis factor-α (TNF-α)-stimulated HeLa cells, a genome-wide study identified 12,552 genome binding sites of p65 (12). Among the 1,667 distinct NF-κB target genes identified, NF-κB controlled the expression of 249 target genes, including inflammatory cytokines, chemokines, inflammatory enzymes, adhesion molecules, and immune system receptors, which are known to orchestrate the integrated inflammatory and immune responses. Interestingly, an additional 626 identified NF-κB target genes were involved in metabolic processes (13).

In critical illness, NF-κB-driven systemic inflammation, also known as a "*cytokine storm*" (14), activates a multi-system response that includes at least three major domains: (i) the stress system composed by the hypothalamic-pituitary-adrenal (HPA) axis and the locus caeruleus-norepinephrine/sympathetic nervous system activated to provide sufficient energy and hemodynamic stability to overcome the initial phase of critical illness (15); (ii) the acute-phase reaction (APR), which has several adaptive functions, including increasing the production of procoagulant factors in preparation for possible tissue damage (16); and (iii) the tissue defense response (TDR) of the target organs [**Figure 1**; (11, 17)]. The main effectors of systemic inflammation are the inflammatory cytokines, the acute-phase reactants, and the peripheral effectors of the sensory afferent nervous system. The inflammatory cytokines include TNF-α, interleukin-1β (IL-1β), IL-6, chemokines, and other mediators of inflammation. The acute-phase reactants are mostly of hepatic origin and include the C-reactive protein (CRP),

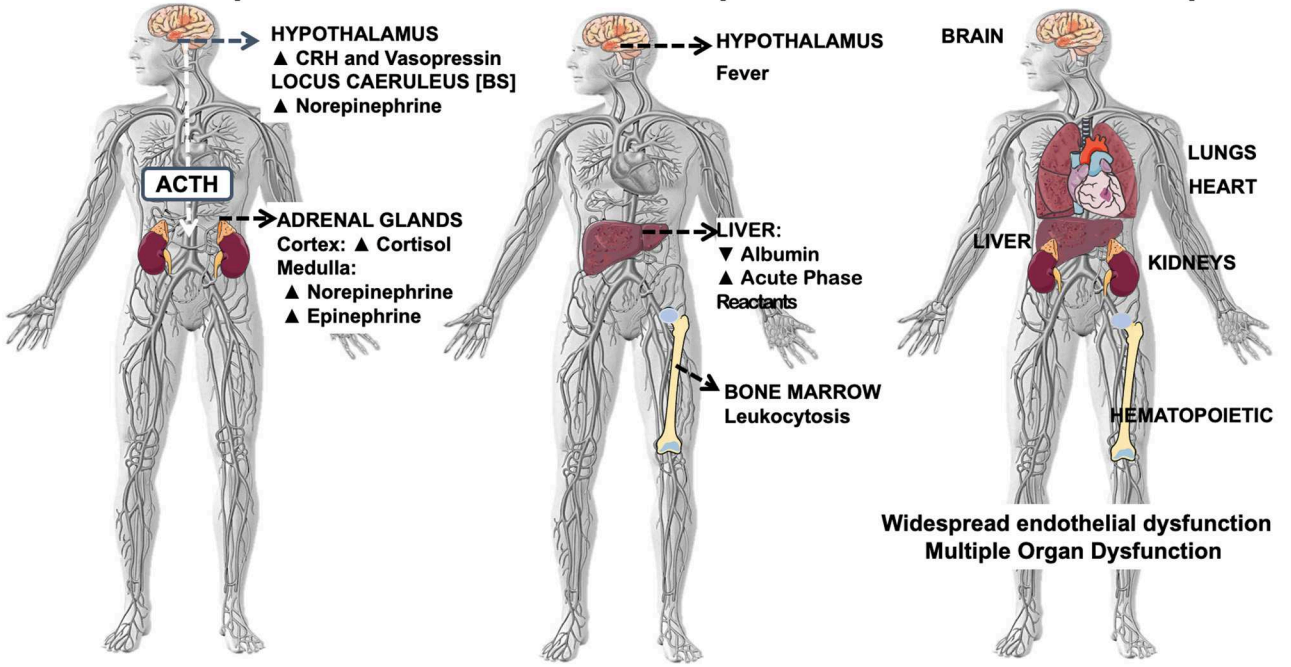

FIGURE 1 | Systemic inflammation-associated multi-system responses: stress, acute phase, tissue defense. Systemic inflammation-associated multi-system response that includes at least three major domains: (i) the stress system composed by the hypothalamic-pituitary-adrenal (HPA) axis and the locus caeruleus-norepinephrine/sympathetic nervous system; (ii) the acute-phase reaction (APR), and (iii) the tissue defense response of the target organs.

fibrinogen, and plasminogen activator inhibitor-1. During the acute-phase reaction, myelopoiesis is favored at the expense of both lymphopoiesis and hematopoiesis, explaining in part the persistent lymphopenia and anemia of critical illness (18). Substance P is an example of an effector of the sensory afferent nervous system, while hypothalamic corticotropin releasing hormone (CRH), vasopressin, cortisol, the catecholamines (norepinephrine and epinephrine), and peripheral neuronal CRH represent effectors of the stress system [reviewed in (19)].

The tissue defense response is an integrated network of three simultaneously NF-κB-activated pathways that account for much of the histological, physiological, and laboratory changes observed in vital organs during critical illness (11). These three pathways are those of tissue inflammation, hemostasis, and tissue damage repair: (i) tissue inflammation includes changes in the endothelium, such as loss of the glycocalyx, adhesion/diapedesis of activated neutrophils, endothelial injury, increased porosity with interstitial exudative edema, and loss of vascular tone, and changes in the epithelium, such as loss of integrity and cell apoptosis; (ii) the hemostasis pathway includes platelet activation and aggregation, intravascular clotting with decreased microvascular patency, extra-vascular fibrin deposition, and, lastly, inhibition of fibrinolysis, and (iii) tissue damage repair includes regenerating native parenchymal cells, fibroproliferation and deposition of extracellular matrix, resolution of granulation tissue, and clearance of apoptotic cells and debris (11).

The roles of macrophages and mitochondria in homeostatic corrections is the subject of intense research. Mononuclear phagocytic cells (MPCs), including macrophages and dendritic cells, are widely distributed throughout the tissues of the organism, where they perform essential homeostatic, surveillance, and regenerative tasks. As the neuro-endocrine-immune response progresses, macrophages change phenotype and play an essential role in both innate and adaptive immune responses, in the resolution of inflammation and in the tissue repair and regeneration (discussed further in section Glucocorticoid Receptor-Alpha and Homeostatic Corrections) (20). Mitochondria are targets of GR and critical modulators of homeostatic corrections owing to their critical role in energy production, synthesis of stress-associated steroid hormones, and their capacity to generate signals that promote cellular adaptation (see section Cellular Energetics—Mitochondrial Function) (21).

Systemic homeostatic corrections are driven by elevated levels of circulating inflammatory cytokines, and based on disease progression, can be broadly divided into either adaptive/resolving (regulated systemic inflammation) vs. maladaptive/unresolving (dysregulated systemic inflammation) (11, 14). Evidence from the literature on severe sepsis (22–30), acute respiratory distress syndrome (ARDS) (31–35), and trauma (14, 36) provides strong support that the degree of NF-κB-driven elevation in inflammatory biomarkers at ICU entry and during

ICU stay correlates with disease severity and hospital mortality (33, 37–39). In addition to elevated inflammatory markers, critically ill patients have profound activation of the coagulation system (elevated D-dimer levels, prolonged prothrombin time and activated partial thromboplastin time, and reduced levels of the anticoagulant proteins, protein C and antithrombin) and evidence of endothelial cell activation with disturbed vascular integrity that correlates with disease severity and outcome (see section Endothelium) (40–42). Evidence that hemostasis and inflammation evolved from a single-triggered mechanism can be traced back more than 450 million years ago, based on studies with the horse-shoe crab (*Limulus polyphemus*) (43).

In hospital survivors, failure to achieve homeostatic correction has a significant negative long-term impact, with experimental work suggesting that it might potentiate the peripheral and brain pro-inflammatory cytokine response to a subsequent inflammatory challenge (44). Independently of age and comorbidities, patients with elevated circulating biomarkers of inflammation and hemostasis at hospital discharge have persistent elevation over time with increased risk for cardiovascular events, re-hospitalizations, and 1-year mortality (41, 45, 46). "*Persistent Inflammation, Immunosuppression, and Catabolism Syndrome*" (PICS) has been postulated as the underlying pathophysiology of chronic critical illness (CCI) (18, 47). About 50% of sepsis patients have a debilitating condition characterized by a self-perpetuating cycle of persistent low-grade systemic inflammation mimicking chronic stress (elevated cortisol) (44, 48, 49), glucocorticoid resistance, altered hemostasis (50, 51), mitochondrial dysfunction (52, 53), and accelerated inflamm-aging (9, 54), with increased risk for chronic inflammatory systemic diseases (7, 55). The evolutionary trade-off between acutely beneficial and chronically harmful homeostatic corrections was the subject of a recent review (7).

Recently, a critical role was identified for the *FKBP5* gene, which encodes the FK506 binding protein 51, a co-chaperone of the GR along heat shock proteins (hsp), including hsp90. The expression of FK506 is stimulated by glucocorticoids and has an inhibitory effect on GR signaling, preventing the nuclear translocation of GR. If short-lived, this negative feedback mechanism to reduce GC signaling may be important to restore HPA axis homeostasis. However, aging and certain stress-related phenotypes are associated with epigenetic up-regulation of *FKBP5* via decreases in DNA methylation at selected enhancer-related FKBP5 sites, promoting NF-κB–related peripheral inflammation and chemotaxis, and heightened cardiovascular risk (56). Importantly, translational research indicates that the type of response (regulated or dysregulated) is established early in critical illness (14, 31, 57), and the previously espoused hypotheses of the second-hit model (14, 22), or the two-phase model (compensatory anti-inflammatory response syndrome) are now both considered obsolete (29, 58, 59).

Based on this pathophysiological construct, we will focus on emerging evidence indicating the central role played by the activated glucocorticoid receptor-alpha (GRα), the master regulator of NF-κB and homeostatic corrections, in the development and resolution of critical illness. This role is conditioned by its interdependence with functioning mitochondria and by presence of adequate micronutrient reserves. Additionally, we present evidence on how CIRCI, mitochondrial dysfunction/damage, and hypovitaminosis negatively interact to accelerate an anti-homeostatic process of natural selection.

GLUCOCORTICOID RECEPTOR-ALPHA

The stress system is a complex, sophisticated, and carefully regulated adaptation mechanism that has been shaped by natural selection because it offers a selective advantage (4). All vertebrates express the proopiomelanocortin protein (POMC) that gives rise to adrenocorticotropic hormone (ACTH) which then stimulates the secretion of glucocorticoids. ACTH has long been closely associated with other signaling molecules, such as CRH, vasopressin, biogenic amines (epinephrine and norepinephrine), steroids such as cortisol and aldosterone, cytokines, such as IL-1β, and nitric oxide. All of these substances are crucial to adaptation to stressors. It is remarkable that the gene DNA sequences for these molecules have not only been conserved over hundreds of millions of years but also continue to serve closely related adaptive functions (4). This is apparently a result of strong selective forces against mutations that change their sequences and functionality of their products.

Cortisol, the end-product of HPA axis activation, is synthesized from cholesterol in the mitochondria and endoplasmic reticulum of the *zona fasciculata* of the adrenal cortex. Its synthesis depends entirely on scavenger receptor class B type-I (SR-BI)-mediated cholesteryl ester selective uptake from circulating high-density (HDLs) and low-density (LDLs) lipoproteins (60). In critically ill patients, low serum HDL levels correlate negatively with circulating TNF-α and IL-6 levels (61, 62), and positively with mortality (61, 63). The combined effects of reduced HDL and SR-BI during systemic inflammation may lead to significant reductions in glucocorticoid production (64). Low HDL (65) and low total cholesterol (66) correlate with inadequate response to ACTH stimulation. In septic shock, prolonged glucocorticoid administration is associated with significant increase in total cholesterol levels within 3 days of treatment (66).

Glucocorticoids are the primary adaptive response mediators, whose signaling system interacts with other cell signaling systems, all essential for maintaining the homeostasis of many of the body's complex functions, including neural, cardiorespiratory, endocrine, metabolic, bioenergetic, and immune responses (67). Within tissues, glucocorticoids are regulated at the pre-receptor level by the isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD), which are located in the endoplasmic reticulum (ER). Glucocorticoids (GCs) bind to the ligand-binding domain of GRα to produce a biological response. Because of their lipophilic nature, GCs can readily diffuse across cellular membranes to bind to their intracellular receptor and produce a biological/pharmacological response [**Figure 2**; (15, 68, 69)]. The glucocorticoid receptor is a member of the nuclear receptor (NRs) (70) superfamily that emerged in the vertebrate lineage ∼420–440 million years

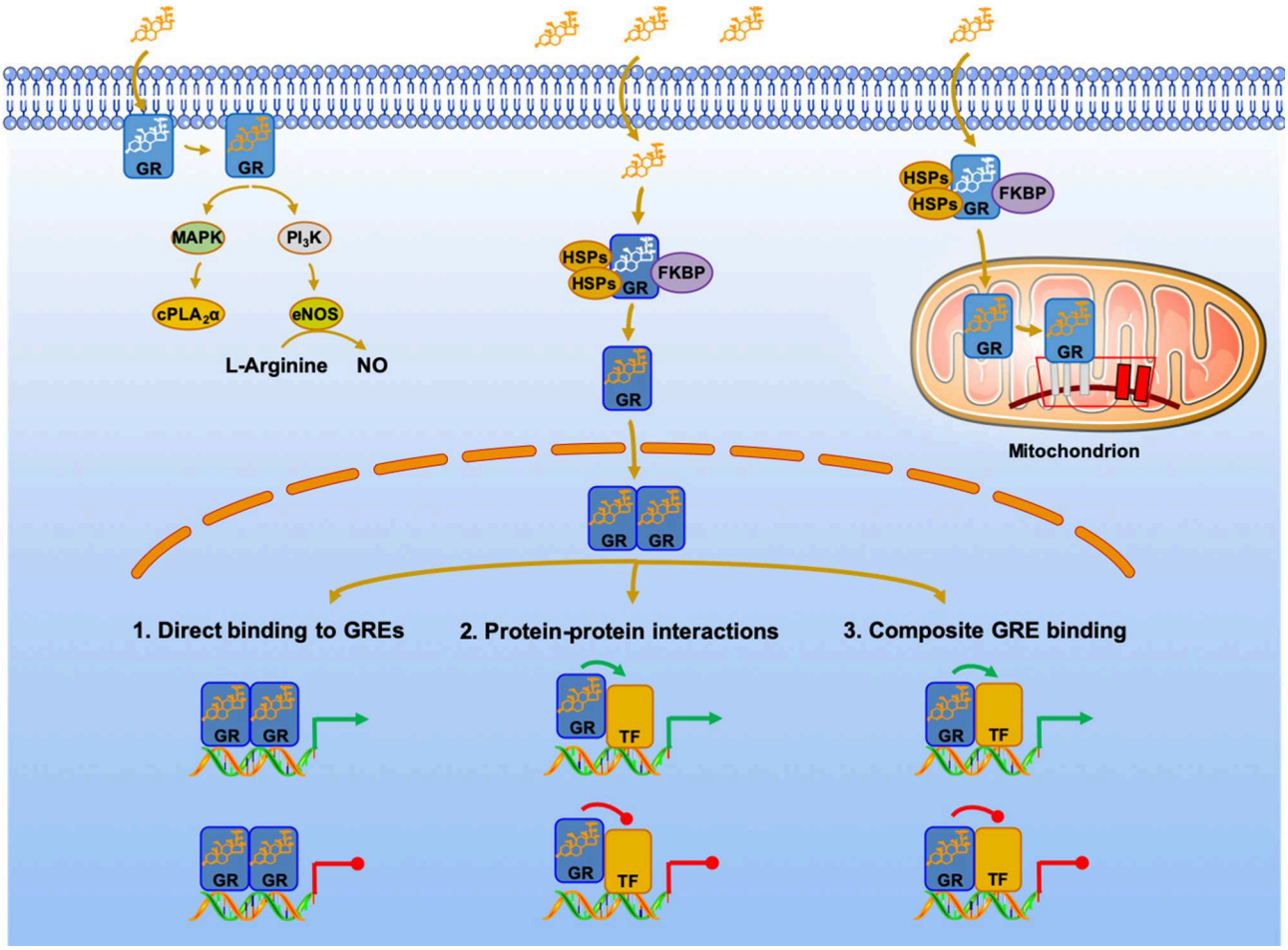

FIGURE 2 | Genomic, non-genomic, and mitochondrial glucocorticoid signaling pathways. Glucocorticoid receptor mechanisms of action. The classic actions of GRα are shown in the middle of the panel. The dormant but ligand-friendly receptor, located in the cytoplasm, is bound to co-chaperon molecules, such as heat-shock proteins and the immunophilin FKBP. Upon binding to the ligand, the activated receptor translocates into the nucleus, where it interacts with GREs and/or other transcription factors, such as NF-κB and AP1, to regulate the activity of glucocorticoid-responsive genes, which represent ∼20% of the human genome. In addition, cell membrane-associated GRα may be activated by the MAPK and PI3K kinases, as shown in the left of the panel. In addition, by as yet unknown mechanism, the GRα translocates into the mitochondria, where it interacts with the mitochondrial DNA GREs, regulating the activity of mitochondrial genes. See text for additional details cPLA2α, cytosolic phospholipase A2 alpha; eNOS, endothelial nitric oxide synthetase; FKBP, immunophilins; GR, glucocorticoid receptor; HSP, heat shock proteins; MAPK, mitogen-activated protein kinases; NO, nitric oxide; PI3 K, phosphatidylinositol 3-kinase; TF, transcription factor.

ago (similarly to NF-κB) from sequential duplications of two ancestral genes, those of the estrogen and the glucocorticoid receptors; the latter ultimately evolved into the glucocorticoid and the mineralocorticoid receptors (67, 71). Underlying its essential role in formation and regulation of multicellular life, the GR is required to establish the necessary cellular context for maintaining normal uterine biology and fertility through the regulation of uterine-specific actions (72) while GRs are vital for the structural and functional maturation of fetal organs (73, 74), affecting almost 4,000 genes (75). In late gestation of mammals, fetal glucocorticoid levels rise dramatically, an essential step for maturation in preparation for life after birth. Also, an association was found between greater maternal affection and warmth in early life and increased expression of glucocorticoid receptor genes in the offspring resulting in long-term health benefits (76).

GRs are present in the cytoplasm (68) and cell membrane (non-genomic effects) (77) in almost all cells of the body and in high concentrations (in neutrophils ∼5,000; in macrophages ∼10,000) (78). However, GR levels within the cell are not static, but are tightly controlled by numerous factors and at multiple levels. Notably, 16 variants of the human GR (hGR) have been identified to date with the potential of at least 256 combinations of homo- and hetero-dimers (68). Recent research indicates that the expression of different GR transcriptional and translational isoforms might be a significant factor determining how GCs influence the biological function and activity of specific cells and tissues (75). In contrast to GRα, the alternatively transcribed GRβ, which resides primarily in the cell nucleus, does not bind glucocorticoid, but can form homodimers with itself or heterodimers with a GRα subtype, functioning as an antagonist

of GRα. GRβ homodimers can interact with glucocorticoid response elements (GRE) in the DNA, however their binding does not activate transcription (69). Generally, GRβ is expressed at very low levels compared to GRα; however, its expression is increased in inflammatory diseases, including critical illness, and this might be associated with decreased sensitivity to GCs and CIRCI (79).

Activation of GRα is not only an essential component of the general adaptation to stress, but also contributes to the maintenance of homeostasis in stress-free conditions (15). The biological response to the GC-GRα complex is affected by cell type, tissue type, and species-specific variations in the repertoire of partnering proteins, ligand concentrations, and other contextual variables (80, 81). In stimulated HeLa and neuronal PC12 cells, genome-wide studies identified 8,306 and 2,252 GR genomic binding sites upon treatment with GC, respectively (12, 81). Of interest, the availability of these binding sites for interacting with the GC-GR complex depends on the chromatin landscape, which is tissue- and cell type-specific, explaining to some extent, why the GR has a certain effect on one tissue and a totally different effect on another (67). Thus, even though the signaling system is the same, the landscape of the landing site is not. Thus, different cells recognize these signals differently, resulting in a highly context-dependent action by glucocorticoids (67).

After GC binding takes place in the cytoplasm, the activated GC-GRα complex can either (i) bind to several pro-inflammatory transcription factors, or (ii) act as a transcription factor, after translocation into the nucleus and mitochondria (69, 82). Glucocorticoids regulation of mitochondrial transcription via activation of mtGRE was the subject of a recent review (53). In pathway (i), the activated GC-GRα complex interacts directly with activated transcription factors NF-κB and AP-1, leading to the transcriptional repression of major downstream proinflammatory factors. In pathway (ii), GC-GRα binds to positive (transactivation) or negative (transrepression) specific DNA regions, the glucocorticoid-response elements (GREs) on the nuclear and mitochondrial DNA (83), to directly regulate transcription of target genes. Finally, GC activation of membrane-bound GR rapidly induces the activity of several kinases, such as the mitogen-activated protein kinase (MAPK) or the phosphatidylinositol 3-kinase (PI3K) pathways [**Figure 2**; (69)]. The non-genomic effects of GCs clearly differ from their well-known genomic effects, with the former responding within several minutes independently of protein synthesis. Genomic studies have shown that the GC-GRα complex regulates more than 3,000 genes in unstimulated peripheral blood mononuclear cells (PBMC) from healthy donors (84), in human pulmonary type II cells (85), and several organs, including the heart (86), liver (87, 88), and uterus (72) of unstimulated mice, underscoring its essential role as a master modulator in sustaining life and restoring health. The discordance between the number of regulated genes and the GR sites (12, 81) suggests that multiple sites are involved in the regulation of a single gene and/or that binding of a transcription factor is not sufficient to drive gene expression (12).

Control of mRNA turnover is critical in regulating the levels of inflammatory- and immune-mediated gene expression. Recent studies indicate that the GR can mediate GC actions beyond transcriptional gene control; it may actually directly participate, via association with mRNA, in GC-mediated control of cytoplasmic post-transcriptional mechanisms of gene expression (89). In an experimental model (SPRET/Ei mice), increased GR levels and activity were associated with strongly reduced expression levels of cytokines and chemokines in response to LPS-induced lethal inflammation (90).

GLUCOCORTICOID RECEPTOR-ALPHA AND HOMEOSTATIC CORRECTIONS

In 1984, Munck et al. reviewed the actions of cortisol and proposed that "stress-induced increases in glucocorticoids levels protect, not against the source of stress itself, but rather against the body's normal reactions to stress, preventing those reactions from overshooting and threatening homeostasis (91)." This work and the results of the above genomic studies have led to a reevaluation of glucocorticoids' role in homeostatic corrections. Busillo and Cidlowski (92) recently reviewed the master regulatory role played the activated GC-GRα complex in the three major phases of homeostatic correction involved in disease development and resolution (**Figure 3**). While distinctions are made between the different states, variable degrees of overlap are likely. First, in the pro-inflammatory phase, GC-GRα prime the innate immune system to remove or neutralize pathogens by: (i) inducing the expression of Toll-like receptor 2, NOD-like receptor pyrin containing 3 (NLRP3) inflammasome, and purinergic receptor P2Y2R; (ii) repressing adaptive immunity (energy conservation); and (iii) cooperating with pro-inflammatory transcription factors NF-κB and activator protein 1 (AP-1) in promoting leukocyte redistribution. The GC induction of NLRP3 sensitizes the cells to extracellular ATP and significantly enhances the ATP-mediated release of proinflammatory molecules, including mature IL-1β, TNF-α, and IL-6 (93). In addition, inflammatory cytokines, particularly IL-6, nitric oxide, and GCs trigger and modulate the systemic and hepatic acute phase protein response (94). In stimulated HeLa cells, a genome-wide study identified 1,932 gene collectively regulated by the activation of NF-κB and GRα, with 43% of regulated genes responding only when both ligands are added, indicating that GRα and NF-κB crosstalk alters signaling pathways that are regulated by each factor separately (12).

During systemic inflammation, peripherally generated TNF-α, IL-1β, IL-6, and other inflammatory cytokines activate the HPA axis at multiple levels to produce GC (95–98). In addition, inflammatory cytokines increase the expression and enzymatic activity of 11β hydroxysteroid dehydrogenase type 1 (11βHSD1), which converts the inactive cortisone to the active cortisol in different cell types, as for example occurs after addition of TNF-α or IL-1β on endothelial (99) or lung epithelial cells (100). Thus, cytokines seem to amplify GC bioavailability (101). Microarray studies have shown that TNF-α and GC synergistically co-regulate 210 genes involved in inflammation

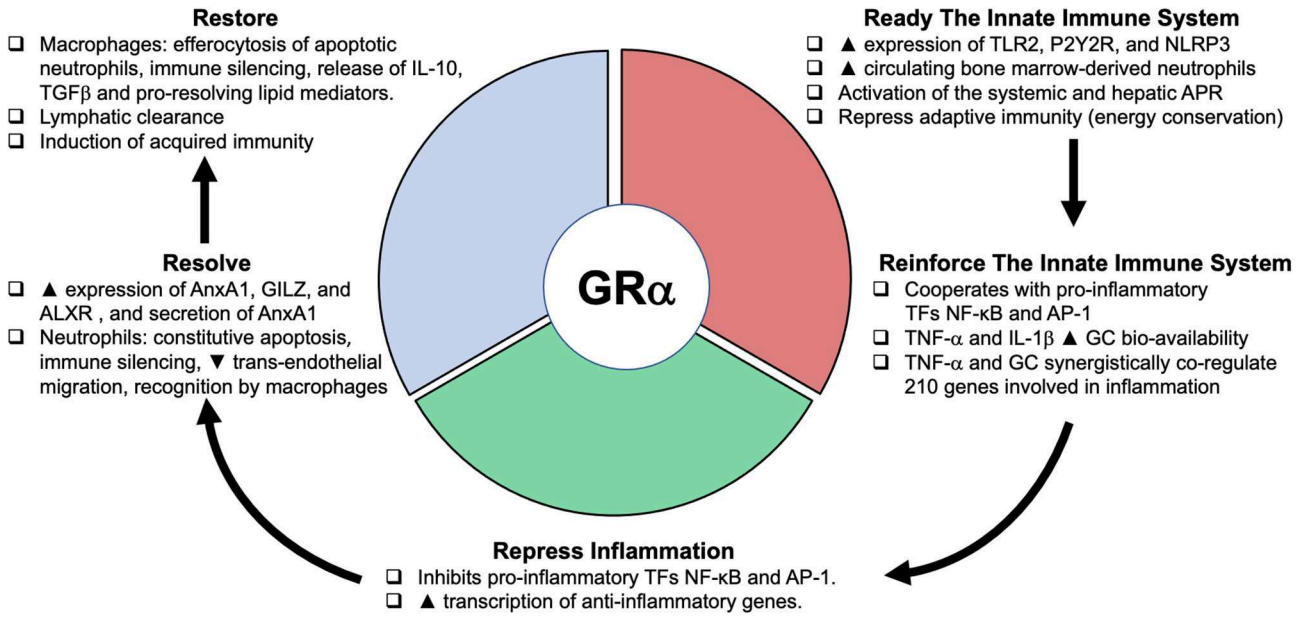

FIGURE 3 | Glucocorticoid receptorα as cellular rheostat of homeostatic corrections. The glucocorticoid receptorα (GRα) acts as a cellular rheostat to ensure that a proper response is elicited by the neuroendocrine immune system throughout the three phases of homeostatic corrections: the pro-inflammatory (ready- reinforce), the anti-inflammatory (repress), and the resolution (resolve- restore) phase. Modified with permission from Busillo and Cidlowski (92). TLR2, toll-like receptor 2; purinergic receptor P2Y2R; NLRP3, NOD-like receptor pyrin containing 3; APR, acute phase response; TF, transcription factor; NF-κB, nuclear factor-κB; AP-1, activator protein 1; AnxA1, annexinA1; ALXR, AnxA1 receptor; GILZ, glucocorticoid-induced leucine zipper; TGFβ, transforming growth factor β.

(100). In this context, the synergy between GCs and pro-inflammatory cytokines is a useful mechanism for rapidly re-enforcing initial pro-inflammatory responses. Importantly, GC-GRα is necessary to prevent excessive phagocytic cell activation and improve intracellular bacterial killing (102).

In the second phase, when regulated systemic inflammation prevails, GC-GRα exerts classic anti-inflammatory action by (i) inhibiting NF–κB, AP-1 and other signaling pathways involved in inflammation, and (ii) increasing transcription of anti-inflammatory genes and the NF–κB inhibitory protein IκB (68, 103). GC-GRα anti-inflammatory action has been extensively investigated, and we direct the reader to excellent reviews on the (11, 68, 101, 104–106). In upcoming sections, we will review selected mechanisms involved in GC-GRα failure to downregulate systemic inflammation and achieve disease resolution.

The third phase involving disease resolution, i.e., restoration of tissue anatomy/structure and function, is an active and elegantly orchestrated process associated with multiple biochemical pathways, including switching production from pro-inflammatory to pro-resolving mediators (92). As downregulation of systemic and tissue inflammation continues, the activated GC-GRα engages in a host of pro-resolution mechanisms changing, among others, the phenotype of both granulocytes and macrophages. In these immune cells, via genomic mechanisms, GC-GRα increases the expression of AnnexinA1 (AnxA1), AnxA1 receptor (ALXR), and glucocorticoid-induced leucine zipper (GILZ), while via non-genomic mechanisms it increases the secretion of AnxA1 (107–109). The coordinated action of GILZ and AnxA1 is essential to regulating resolution (109). Granulocytes undergo constitutive apoptosis, disabling their potentially injurious secretion responses, i.e., NF–κB activation and transcription of inflammatory cytokines, and decreasing trans-endothelial migration leading to their rapid recognition and internalization by macrophages (efferocytosis) (109–112).

Apoptotic cells also serve as resolution cues for macrophages, which, after phagocytosis of apoptotic granulocytes, change their phenotype toward a more resolving/restorative one. The changes in phenotype from M1 (classically) to M2 (alternatively) leads to an orchestrated series of actions leading to successful resolution of inflammation. Interestingly, GCs promote phagocytosis (112, 113) and reduce the apoptotic granulocyte ingestion requirements for generation of M2 (114). GC-mediated change to M2 phenotype is associated with (i) immune silencing, where the release of inflammatory mediators and inducible nitric oxide synthase (iNOS) are suppressed (114); (ii) an increased release of the anti-inflammatory mediators IL-10 and TGFβ and several pro-resolving lipid mediators (114); (iii) protection from apoptosis; (iv) non-phlogistic degradation; (v) production of angiogenic growth factors; (vi) increased macrophage chemokinesis (by upregulation of genes involved in cell mobility) and lymphatic clearance; and (vii) induction of acquired immunity (110, 111, 113–117). GC-mediated Annexin 1-derived peptide (Ac_{2--26}) acting through the ALXR receptor has a pivotal role in the clearance of apoptotic cells (118). In models of self-resolving inflammation, various phenotypes of macrophages may coexist. In the later phase of resolution, M2

macrophages switch to the resolution-promoting macrophage (Mres) phenotype, which display reduced phagocytosis, while producing antifibrotic and antioxidant proteins that limit tissue damage and fibrosis (resolution of granulation tissue) (119). In human monocytes, GCs induce the expression of 133 genes with upregulation of anti-oxidation, migration, phagocytosis, and anti-inflammation with consequent downregulation of adhesion, apoptosis, and oxidation (113). In agreement with microarray data, spontaneous, as well as PMA-induced production of reactive oxygen species, was significantly reduced in GC-treated cells, and GCs promoted survival of an anti-inflammatory monocytic phenotype in inflammatory reactions (113).

GLUCOCORTICOID RECEPTOR ALPHA IN CRITICAL ILLNESS

Since GRα ultimately controls GC-mediated activity, any condition that affects its concentration, binding affinity, transport to the nucleus, interactions with GREs (nuclear and mitochondrial), cofactor activity (see section Hypovitaminoses), or interaction of other relevant transcription factors (NF-κB, AP-1) and co-regulators, can eventually affect the response of cells to glucocorticoids (101, 120). The many different ways GRα function can be negatively influenced by the pro-inflammatory environment of critical illness was the subject of recent reviews (101, 121).

Critical illness-related corticosteroid insufficiency is a term used to define the central role played by the HPA-axis and the activated GRα complex in the pathogenesis of dysregulated systemic inflammation in critical illness (17). Three major pathophysiologic events account for the neuroendocrine decompensation observed in CIRCI: (i) multi-level dysregulation of the HPA-axis correlating with circulating inflammatory cytokine levels; (ii) altered cortisol metabolism [reviewed in (122)], and (iii) secondary generalized circulating and tissue specific reduction in GRα number/function with observed multifactorial tissue resistance to endogenous glucocorticoids (17). The role of mitochondrial oxidative stress in reducing GRα number/function is reviewed later (see section Oxidative Stress and CIRCI).

Experimental and clinical studies have demonstrated that critical illness is associated with a significant reduction in GRα density and transcription and an increase in GRβ-mediated dominant negative activity on GRα-induced transcription. In a human cell line, activation of NF-κB by TNF-α had a direct dose- and time-dependent effect on GR levels with a disproportionate increase in GRβ over GRα (123). Experimental sepsis is associated with decreased GRα transcription in circulating cells (124), heart (125), lymph node-spleen (124), liver (125–127), kidney (127), lung tissue (125–129), and skeletal muscle (125). Moreover, the endothelial GRα is required for protection against sepsis (see section Endothelium) (130). Importantly, the reduction of GRα expression is rapid and persists for at least 5 days (125) while it is associated with increased GRβ expression in the heart and lung but not liver (125), and increased NF-κB activation (127). Similarly, in experimental ARDS, lung tissue shows a significant reduction in GRα expression (128, 131, 132) and an increase in GRβ expression (131), leading to decreased GRα nuclear translocation (131). In transgenic mice, expression of GRα above wild-type levels leads to increased resistance to LPS-induced endotoxic shock (133).

Clinical studies, including autopsies, in patients with severe sepsis and septic shock have reported a significant reduction in GRα expression in circulating cells (79, 134–137); heart (125), liver and skeletal muscle (125, 138), and a significant increase in GRβ expression in the heart and liver (125). GRα mRNA in neutrophils correlates negatively with plasma IL-6 levels and shows gradual recovery overtime in survivors (135). In another study, neutrophil GRα mRNA levels decreased 4-fold by day 4 in the ICU and remained low for 2 weeks (79). In *ex vivo* experiments with PBMCs exposed to longitudinal plasma samples from patients with ARDS results suggested that insufficient GC-GRα-mediated activity at disease onset and over time was a central mechanism for the upregulation of NF-κB activity (**Figure 4**). Over time, patients with regulated systemic inflammation have a progressive increase in all measured GC-GRα-mediated activities, including GRα number, binding to NF-κB and to nuclear GRE, as well as increased transcription of IκBα and IL-10, and a corresponding reduction in NF-κB nuclear binding, and transcription of TNF-α and IL-1β. In contrast, patients with dysregulated systemic inflammation had only a modest longitudinal increase in GC-GRα-mediated activities and a progressive increase in NF-κB nuclear binding that was most striking in non-survivors (33). By day 3 of ARDS, no overlap was observed between groups for NF-κB and GC-GRα nuclear binding. In lung tissue obtained by open lung biopsy, histological severity correlated with increased nuclear uptake of NF-κB and a lower ratio of GRα: NF-kB uptake (33). A decrease in GRα expression in critical illness is maladaptive granted that proinflammatory pathways are not properly restrained (124).

Randomized studies (103, 129, 132) demonstrated in both circulating and tissue cells, that quantitatively adequate and prolonged glucocorticoid supplementation increased GRα number and function, reversing critical illness-associated cellular glucocorticoid resistance. In experimental ARDS, low-dose glucocorticoid treatment restored GRα number and function leading to resolution of pulmonary inflammation (129, 132). Similarly, in an *ex vivo* ARDS study, prolonged methylprednisolone treatment was associated with upregulation in all measurements of GRα activity leading to reduction in NF-κB DNA-binding and transcription of inflammatory cytokines [**Figure 4**; (103)]. Glucocorticoid treatment changes the longitudinal direction of systemic inflammation from dysregulated (NF-κB-driven, maladaptive response) to regulated (GRα-driven, adaptive response) with significant improvement in indices of alveolar-capillary membrane permeability and markers of inflammation, hemostasis, and tissue repair (11).

ENDOTHELIUM

The vascular endothelium constitutes the innermost lining of the body's circulatory system and the largest tissue in the body

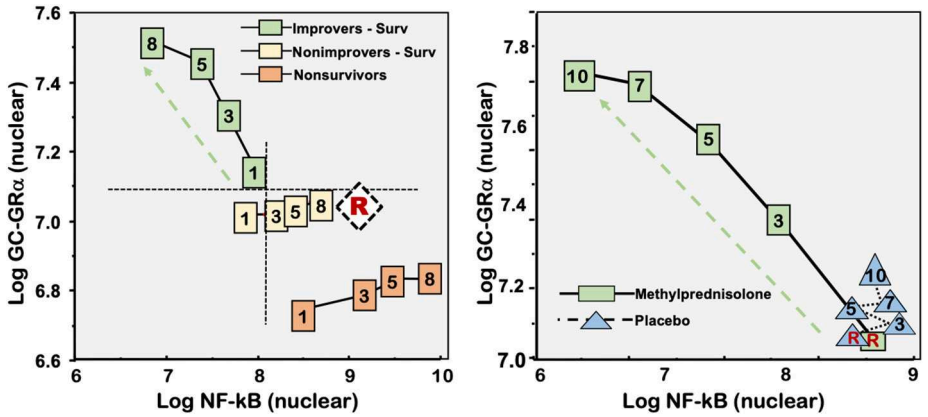

FIGURE 4 | Correlation between mean levels of nuclear NF-κB and nuclear GC-GRα during the natural progression of ARDS, and in response to prolonged glucocorticoid treatment. Mean intracellular changes of nuclear GC-activated GRα and NF-κB observed by exposing PBLs of a healthy volunteer to plasma samples collected longitudinally (days 1, 3, 5, and 8) and after randomization to methylprednisolone treatment [randomization day (R) and post-randomization days 3, 5, 7, and 10]. The mean values of nuclear NF-κB are plotted against the mean nuclear GC-GRα levels. Improvers had a pre-defined improvement in lung injury score (139) and/or gas exchange component by day 7. The left panel shows ARDS patients with adaptive and maladaptive responses. In improvers, an inverse relation was observed between these two transcription factors, with the longitudinal direction of the interaction shifting to the left (decreased NF-κB) and upward (increased GC-GRα). In contrast, in non-improvers NF-κB increased over time while GC-GRα had no significant changes. We define the first interaction as GC-GRα-driven, and the second interaction as NF-κB-driven (33). The right panel shows non-improvers-survivors randomized after day 8 of ARDS to methylprednisolone ($n = 11$) vs. placebo ($n = 6$). After natural logarithmic transformation and adjustment for repeated measurements, partial correlations among responses to plasma from the methylprednisolone group were −0.92 ($p < 0.0001$) both for nuclear NF-κB and nuclear GRα. For responses to plasma from the placebo group, no significant relation was found between nuclear NF-κB and nuclear GRα ($r = 0.11$; $p = 0.70$) (103). Reproduced with permission from Meduri et al. (11).

(close to 100,000 miles long) containing $\sim 2.5 \times 10^{12}$ endothelial cells that are typically flat and susceptible to injury, with a thin basement membrane enriched in type IV collagen and laminin (140). The endothelial lining is in continuous contact with circulating cells and soluble proteins, and the capillaries, represent the primary barrier between elements in the blood and the parenchymal cells. The space between two contiguous endothelial cells, known as the endothelial cleft (ETC), acts as an important site of regulation of endothelial (paracellular) permeability (141). Importantly, the vascular endothelium (micro- and macro-circulation) is clothed with a protective barrier, the glycocalyx, which is critical to maintain endothelial homeostasis. The endothelial glycocalyx is a negatively charged, organized mesh of membranous glycoproteins and plasma proteins that include superoxide dismutase, antithrombin III, and cell adhesion molecules, all involved in maintaining the oncotic gradient across the endothelial barrier (141). The intact glycocalyx protects endothelial cells from oxidative stress and prevents the interaction between circulating leukocytes and endothelial adhesion molecules (141). Conformational changes in glycocalyx structure lead to short bursts in the release of endothelial nitric oxide (eNO) (141), inhibiting vascular smooth muscle contraction, platelet aggregation, and leukocyte adhesion, all three processes essential for patency of microcirculation. The blood-brain barrier (BBB), composed of highly specialized endothelial cells with tight junctions that seal paracellular spaces to restrict permeability, serves as a highly restrictive interface between the systemic circulatory system and the brain (142).

Damage to the glycocalyx precedes vascular pathology. Endothelial activation with ubiquitous shedding of the glycocalyx is a major component of critical illnesses and a key pathogenic mechanism in multiple organ dysfunction. The pathways by which sepsis induces injury to the endothelium were recently reviewed (143). The "vasculo-centric view" of critical illness derives from the observation that, despite the remarkable heterogeneity of diseases, the pathobiology of multiple organ dysfunction shares near-stereotypical features that are mostly related to widespread endothelial dysfunction (144). Endothelial dysfunction manifests with a diffuse increase in paracellular permeability, expression of luminal cell adhesion molecules, recruitment of activate leukocytes, altered vasomotor tone, and microvascular thrombosis with decreased capillary density (145). Increasing evidence points to endothelial dysfunction with impairment of the BBB as a critical component of the pathobiology of delirium during critical illness (146).

Oxidative stress (see section Mitochondrial Cacostatic Load, Oxidative Stress, and Mitochondrial Damage) impairs endothelial function by interfering with eNO synthesis (147) and by participating in the degradation of the glycocalyx (141). After shedding of the glycocalyx, adhesion molecules are released in the blood and can be found in the circulation (35). Microvascular alterations, such as decreased functional capillary density and increased perfusion heterogeneity, are frequently observed in patients with sepsis and contribute to the defect in oxygen extraction by the peripheral organs and tissues of the organism (148).

Endothelial activation may also affect the HPA-axis. The adrenal gland is a highly vascularized organ, with every steroidogenic cell in close vicinity with at least one sinusoid, and a clear positive relation between adrenal blood flow

and steroidogenesis has been demonstrated (149). In critical illness, disruption of endothelial homeostasis within the adrenal gland can contribute to the HPA-axis dysfunction (150). Hypovitaminosis may also contribute to endothelial dysfunction (see section Hypovitaminoses).

In studies of circulating inflammatory cytokines, there is substantial evidence that in critically ill patients, an increase in circulating markers of endothelial integrity (angiopoietin-1; Angpt-1) (145), dysfunction [angiopoietin-2 (Angpt-2), von Willebrand factor (VVF) (150), soluble intercellular adhesion molecule-1 (sICAM-1), (35) vascular endothelial growth factor (VEGF)] (145, 151), and cell damage associated with circulating endothelial cells (152, 153) correlate with disease severity and mortality. Fittingly, a large study acquiring sublingual measurements of microcirculation in early sepsis, found that mortality strongly correlated with the severity of alterations in the proportion of perfused small vessels, i.e., the functional capillary density (154).

The endothelial GRα is a critical regulator of vascular homeostatic corrections and essential for decreasing the rolling on and adhesion of activated neutrophils to the endothelium (155). In experimental sepsis, elimination of the endothelial GRα resulted in prolonged activation of endothelial NF-κB, with increased expression of iNOS and inflammatory cytokines, both accounting for hemodynamic collapse and mortality (130). Importantly, the presence of the endothelial GR itself was necessary for GC-mediated suppression of NF-κB and for achieving survival (130). GRα also regulates the tightness of the BBB, inducing expression of the tight junction proteins occludin and claudin-5, and the adherens junction protein vascular endothelium cadherin (VE-Cadherin) (156).

GC-GRα is also strongly involved in vascular development (81). Experimental studies have shown GR-dependent upregulation of multiple mediators involved in endothelial cell homeostasis, such as sphingosine kinase 1 (SphK1) (157), angiopoietin-1 (Angpt-1) (158), serum glucocorticoid kinase-1 (SGK-1) (159, 160), GILZ (161), and eNOS (162–164). In experimental ARDS, upregulation of SphK1, an important regulator of endothelial barrier integrity, was shown to improve alveolo-capillary membrane (ACM) permeability (157). In human brain microvascular endothelial cells (HBMECs), GC treatment was associated with transcriptional activation of Angpt-1 and suppression of VEGF (158). In umbilical vein endothelial cells (HUVECs), upregulation of SGK-1 reduced oxidative stress and improved cell survival and senescence (159); meanwhile, GR-induced GILZ expression (see section Glucocorticoid Receptor-Alpha and Homeostatic Corrections) correlated negatively with vascular inflammation (161). In neuro-vascular tissue, physiological doses of hydrocortisone rapidly activated eNOS via non-genomic mechanisms (163, 164).

GRα is also a critical regulator of myocardial function. In experimental work, the GR—via Kruppel-like Factor 13—was found to play a direct role in the regulation of cardiomyocyte function and protection from hypoxia and DNA damage (86). GR inhibits cells death triggered by ischemia reperfusion, mechanical stress, or TNFα [reviewed in (86)].

The endothelial response to GCs in inflammatory diseases was extensively reviewed covering topics such as inhibition of pro-inflammatory transcription factors, restoration of endothelial barrier integrity, and induction of protective molecules (140). In experimental sepsis, low-dose glucocorticoid (hydrocortisone or dexamethasone) preserved the endothelial glycocalyx, sustained the vascular barrier and reduced interstitial edema (165, 166), and had beneficial effects on mesenteric blood flow and helped with resolution of organ injury (167). GCs play an important role in the control of vascular smooth muscle tone by their permissive effects in potentiating vasoconstrictive responses to catecholamines and other hormones, such as arginine-vasopressin, through glucocorticoid receptors (168). Finally, they inhibit the expression of inducible nitric oxide synthase and other vasodilatory agents in vascular endothelial cells (169). Additional experimental studies have shown that GR stimulates transcription of the *zonula occludens* (ZO)-1 tight junction protein, leading to reduced BBB paracellular permeability (142), while it activates eNOS increasing cerebral blood flow (163).

In patients with septic shock (170, 171) or ARDS (172, 173), prolonged glucocorticoid (hydrocortisone or methylprednisolone) treatment resulted in the following: (i) increased plasma activated protein C levels (173); (ii) reduction in markers of endothelial injury such as sICAM-1 (35); (iii) rapid and consistent improvement in capillary perfusion, independently of the cortisol response to ACTH (170); and (iv) improvement in alveolar-capillary (172) and renal (171) endothelial permeability. In addition, septic shock is associated with vascular dysfunction through NF-κB-mediated downregulation of the endothelial mineralocorticoid receptor (MR) and α1-adrenoceptor, which can be restored with mineralocorticoid (fludrocortisone) treatment (174).

CELLULAR ENERGETICS—MITOCHONDRIAL FUNCTION

A transforming event in the history of life was the evolution of photosynthetic bacteria, with biochemical pathways that allowed them to capture energy from sunlight and store it in simple sugars, a process known as photosynthesis that generates oxygen as a waste product. As a result, over the course of about one billion years, the earth's atmospheric oxygen increased from almost zero to nearly modern levels (175). The development of an ozone layer in the upper atmosphere to absorb damaging UV radiation from the sun, a derived outcome of increased atmospheric oxygen, permitted organisms to live on land for the first time (175). Some groups of organisms adapted to increased oxygen levels; the most notable adaptation was the evolution of the biochemical pathways of cellular respiration, which use oxygen to extract the energy stored in organic molecules much more efficiently.

About 2 billion years ago, complex life surfaced with two major endosymbiotic (eukaryotic cells ingesting a prokaryote bacterium that resulted in a symbiotic relationship between the engulfing and engulfed cells) events igniting the evolutionary progression to animals and plants (176, 177). First, the ancestral

eukaryotic cell engulfed an aerobic prokaryote bacterium (i.e., capable of using oxygen to produce energy) that eventually evolved into mitochondria (specialized for cellular respiration) populating the cell cytoplasm (modern heterotrophic eukaryote) to afford a selective advantage for survival (178). In the second endosymbiotic event, the early eukaryotic cell engulfed a photosynthetic prokaryote bacterium that evolved into the chloroplast (modern photosynthetic eukaryote).

Central to the integrated actions of immune and neuroendocrine responses (3, 17, 179) is cellular energetics, involving the mobilization of energy resources from GC-GRα-mediated breakdown of glucose (via glycolysis), fatty acids and amino acids for mitochondrial energy production (180). In fact, GCs were originally named by Hans Selye based on their ability to increase blood glucose concentration. Activation of the HPA axis mobilizes these energetic substrates into the circulation within minutes, underscoring the widespread role of GC-GRα in the regulation of systemic metabolism (181).

Mammalian cells, apart from erythrocytes, contain within their cytoplasm hundreds to thousands of mitochondria, the number determined by the energy demand of each cell type. Mitochondria are autonomous and highly dynamic double-membrane organelles that function as the powerhouses of the cell and utilize ∼98% of total body oxygen consumption. Oxidative phosphorylation (OXPHOS) is the metabolic pathway in the inner membrane of the mitochondria which use enzymes to oxidize ingested calories to produce adenosine triphosphate (ATP) required for normal cell functioning. Ultimately, this conversion provides energy for most cellular processes within the body intracellular reactions (gene transcription and translation, epigenetic modifications), hormonal changes in the endocrine system, structural changes in tissue, and behavioral and cognitive responses] (181), and in theory, determines the limits of an organism adaptive capacity (21).

By virtue of their origins as aerobic bacteria, mitochondria have their DNA, RNA, and protein synthesis systems. The mitochondrial DNA (mtDNA) in our proto-eukaryotic ancestors was significantly larger in genetic complement, but transfer of mtDNA encoded genes to the nucleus has occurred over the 1.5–2 billion years since the origin of the eukaryotic cell, and today the mammalian mtDNA (inherited from the mother) encodes 13 polypeptides, two rRNAs (12S and 16S) and 22 tRNAs that are essential for OXPHOS and proper cell function (178). Since the mtDNA encodes for only a handful of proteins, mitochondria depend on the cell nucleus and other cellular compartments for most of their proteins and lipids (182). In addition to being the major source of intracellular ATP, mitochondria are deeply involved in signaling pathways, elicited by perturbations in homeostasis, which promote cell adaptation (183, 184).

Mitochondria constantly generate reactive oxygen species (ROS) as a by-product of substrate oxidation and oxidative phosphorylation, a physiological process that is normally kept in check by a diversified set of antioxidant defenses (184). The introduction of oxygen to earth's early biosphere stimulated remarkable evolutionary adaptations, and in this context, ROS should be viewed as an essential consequence and driver of evolution and survival over time (185). Reactive oxygen species are required in numerous physiological cell functions, such as cellular signaling systems linked to the transcriptional machinery, maintenance of vascular tone, oxygen sensing, and host defense against pathogens (186). One of the mitochondrial oxidases, the NADPH oxidase of polymorphonuclear leukocytes (primarily neutrophils), is pivotal to the body's defense against pathogenic microorganisms (187).

Mitochondria are involved in a multitude of cellular processes, well-beyond their long-established role as the cell's powerhouse. These include processes such as intracellular calcium homeostasis (buffering of cytosolic calcium), regulation of mitochondrial metabolism, cell migration, production of biomolecules such as amino acids, lipids, hemes, purines, and steroid hormones (see section Mitochondria and HPA-Axis Cross-Talk), and activation of cell death pathways (188). Mitochondria trigger cell death pathways by two mechanisms, first via necrosis when ATP levels fall below a certain threshold, and second via apoptosis through the release of mitochondrial cytochrome c into the cytoplasm (189). Mitochondrial integrity is, therefore, essential for the function and survival of cells, and several recent publications have highlighted the critical role played by these organelles in sustaining homeostatic corrections (82, 180–182, 190–192).

MITOCHONDRIAL CACOSTATIC LOAD, OXIDATIVE STRESS, AND MITOCHONDRIAL DAMAGE

In critical illness, tissue oxygen consumption and total energy expenditure are increased, with intracellular metabolism boosted by up to 200% compared to the healthy state (193). Cells that represent the innate immune system, like neutrophils, and macrophages, are mainly responsible for the oxidative burst that takes place early in critical illness (194) along with the generation of ROS and nitrogen oxygen species (RNS) that are important for host defenses. In neutrophils of critically ill patients, oxidative activity correlates positively with the degree of intranuclear NF-κB expression (195). According to the "mitohormesis theory," when present in moderate amounts, ROS and RNS function as intracellular signaling molecules that may improve systemic defense mechanisms by inducing an adaptive response (196). However, when intracellular ROS and RNS concentrations overwhelm antioxidant defenses, cell homeostasis becomes compromised (196). For example, peroxidation of the mitochondrial lipid cardiolipin in the inner mitochondrial membrane leads to dissociation of cytochrome c, reduced production of ATP, and increased generation of ROS (197).

Increased energy and metabolic demands associated with NF-κB-driven dysregulated systemic inflammation, leads to overproduction of ROS and RNS, resulting in significant damage of lipids, proteins, and nucleic acids, both within the mitochondria and in other compartments of the cell [**Figure 5**; (197)]. Oxidative stress is a predictor of mortality in septic shock patients (198). Glutathione is one of the most important redox buffers of the cells, as it can be found in all cell compartments and acts as a cofactor of several enzymes,

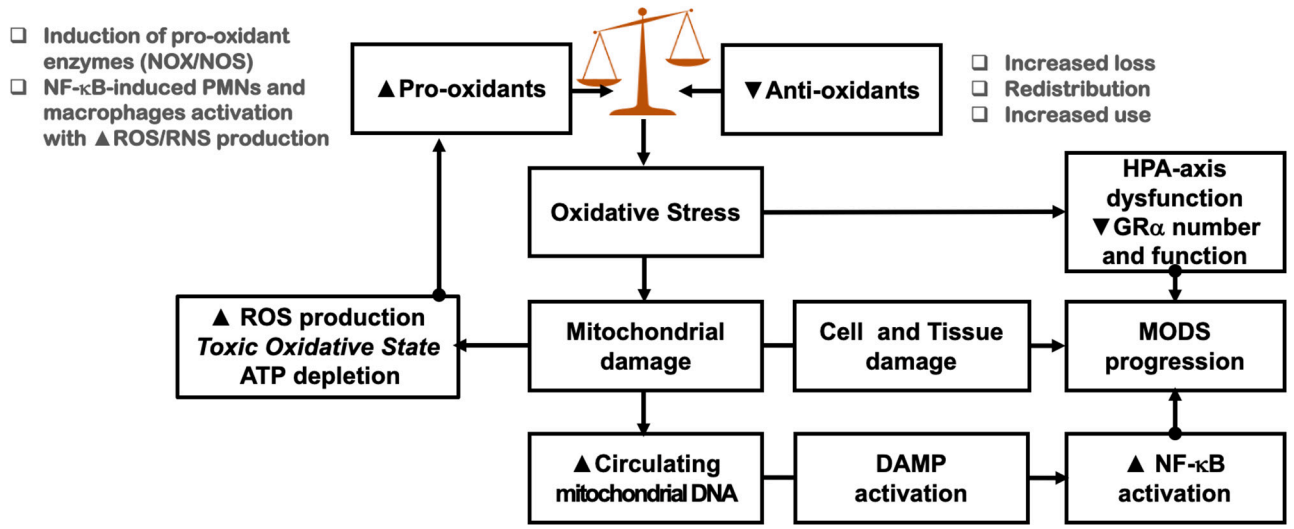

FIGURE 5 | Impact of oxidative stress on homeostatic corrections. See sections Mitochondrial Cacostatic Load, Oxidative Stress, and Mitochondrial Damage, Mitochondria and HPA-Axis Cross-Talk, and Oxidative Stress and CIRCI for explanation. PMN, polymorphonuclear cells; MODS, multiple organ dysfunction syndrome.

helps in DNA repair, scavenges ROS (e.g., hydroxyl radicals, hydrogen peroxide, and lipid peroxides), and generates other antioxidants, such as ascorbic acid (see section Hypovitaminoses) and tocopherols (194). Vitamins D and C upregulate glutathione synthesis and prevent depletion (see section Hypovitaminoses) (199, 200).

The multi-level organization of mitochondrial molecular composition, structures, functions, and signaling roles within the cell was recently reviewed (201). In laboratory models of sepsis, mitochondrial respiration is often increased in the early phase of illness, but consistently falls with protracted inflammation [reviewed in (202)]. Many clinical studies implicate mitochondrial dysfunction and bioenergetic failure as an important pathophysiological mechanism underlying dysregulated systemic inflammation-associated multiorgan dysfunction. In a study of skeletal muscle biopsies obtained in septic patients, an association was first reported between nitric oxide overproduction, antioxidant depletion, mitochondrial dysfunction, and decreased ATP concentrations, with progression of multiorgan failure and mortality (203). Human septic cardiomyopathy is accompanied by widespread down-regulation of cardiac mitochondrial genes and decrease in the expression of genes that govern cardiac myocyte contractility, analogous to the transcriptional reprogramming that occurs in myocardial hibernation (204). Early sepsis is associated with a reduction in PBMC mitochondrial copy number, and a rise in markers of mitochondrial damage, in a linear relation to proinflammatory cytokine expression (205). The PBMCs of patients with severe vs. less severe pneumonia have increased ROS density, increased DNA damage, and decreased superoxide dismutase (SOD) concentrations (206). Loss of mitochondrial function may lead to compensatory secondary metabolism, glycolysis, to produce ATP as well as lactate (207). A epithelial cell line study demonstrated that this glycolytic switch is promoted by the activation of the redox sensitive phosphoinositide 3-kinase (PI3K) pathway and subsequent inactivation of glycogen synthase kinase-3β (GSK-3β) resulting in increased production of inflammatory cytokines and reduced sensitivity to glucocorticoids (207).

Decades of laboratory and clinical studies have revealed that dysregulated systemic inflammation, including sepsis, is associated with significant macromolecular damage to mitochondria, particularly in the cells of highly metabolically active tissues, such as the liver, heart, kidneys, brain, and skeletal muscles (191, 208). In contrast to nuclear DNA, which is non-immunogenic, mitochondrial DNA resembles bacterial DNA (see section Cellular Energetics—Mitochondrial Function) and acts as a damage-associated molecular pathogen (DAMP) to activate immune responses through Toll-like receptor 9-mediated activation of NF-κB (209) and the NLRP3 inflammasome (210). Also, in comparison to nuclear DNA, mtDNA is 50-fold more sensitive to oxidative stress (191), as its close proximity to the electron transport chain and the absence of chromatin proteins makes it an easy target for oxidative damage (197).

As a consequence of increased ROS generation, mtDNA can undergo several qualitative and/or quantitative alterations. Recent studies have found that critically ill patients have decreased cellular mtDNA levels and increased circulating cell-free mtDNA levels (205, 210, 211). In septic patients, mtDNA depletion in circulating cells (mainly neutrophils) correlates with severity of illness (APACHE II scores) (212), while high TNF-α expression in neutrophil lysates correlates with increased plasma mtDNA levels (205). The exact mechanism of mtDNA delivery into the cytoplasm and then into the systemic circulation is currently unknown (189). In ICU patients with sepsis and ARDS, elevated plasma mtDNA levels (>3,200 copies/μl) are associated with dysregulation of phospholipid metabolism (211), and increased mortality (210).

The cycle of mtDNA damage with loss of function of electron transport enzymes (ATP depletion) and increased ROS generation, a state in which the antioxidant systems are overwhelmed may eventually lead to cell death, a phenomenon known as the "*toxic oxidative stress*" (213). In critical illness, impaired cell energy metabolism and mitochondrial damage augment systemic inflammation directly via NF-κB activation and indirectly by multi-level impairment of the HPA axis and GR homeostatic functions (see section Oxidative Stress and CIRCI). Effective homeostatic corrections in the adaptive response during the resolution of critical illness are associated with increased mitochondrial biogenesis, restoration of oxidative metabolism, and mitochondrial content (205). In many studies, restoration of mitochondrial homeostatic functions was observed in association with improved organ recovery and survival [reviewed in (205)]. The association between mitochondrial dysfunction, circulating cell-free mtDNA, muscle wasting, sterile inflammation, and inflamm-aging was recently reviewed (189).

Micronutrient deficiencies may also impair mitochondrial function. In septic patients, the marked early reduction in selenium levels may affect selenium-dependent anti-oxidants glutathione peroxidase and thioredoxin (214). The role of hypovitaminoses on mitochondrial function is reviewed in section Hypovitaminoses.

MITOCHONDRIA AND HPA-AXIS CROSS-TALK

Abundant mitochondria are one of the most prominent ultrastructural features of the adrenocortical cells (208) in which intracellular steroidogenic cholesterol is ultimately converted to cortisol in a tightly controlled manner (82). The central role of mitochondria in essential physiological processes has rendered these organelles a receiver and integrator hub of multiple regulatory signals (215). Mitochondria participate in the stress response, in part, by sensing levels of glucocorticoids (183). It is now accepted that mitochondria are under GC control because GRs are present in mitochondria, and GREs reside in the mitochondrial genome (82, 192). A number of studies in several tissues have observed a cytoplasmic-to-mitochondrion GR translocation or vice versa in response to GC, indicating that mitochondrial GR is dynamically regulated upon exposure to GCs (69). Lee and collaborators have classified five pathways in the functional modulation of the mitochondria by GC-GR (82). In addition to direct mitochondrial GR-mtGRE interactions, mitochondrial gene expression is regulated indirectly by nuclear GR-nuGRE interactions that result in increased transcription of the following: (i) genes encoding OXPHOS and other mitochondrial regulatory functions, (ii) transcription factors for control of nuDNA-encoded mitochondrial proteins, and (iii) several antioxidant mechanisms including uncoupling protein-2 (UCP2) (69, 82, 113, 192, 216, 217). Of interest, mitochondrial thioredoxin, an antioxidant and anti-apoptotic factor essential for cell viability and vascular homeostasis (218), interacts directly with both the ligand and the DNA-binding domains of GR, keeping the receptor in a reduced, transcriptionally active form (219, 220).

Studies have shown that fine-tuning of the response to cellular demands is coordinately regulated by the nucleus and mitochondria, making mitochondrial-nuclear interaction vital to optimal mitochondrial function (82), with GC-GR-mediated increased mtDNA gene expression augmenting the total number of mitochondria per cell, and, thus, total cellular energy production capacity (216). Altogether, there is substantial evidence that cross-talk between neuroendocrine control of the stress response and cellular antioxidant systems is essential for mammalian homeostatic regulation (220). Consistent with the cacostatic load model (21), administration of physiological doses of GCs acutely increases mitochondrial membrane potential, calcium buffering capacity, anti-oxidant capacity, and resistance to apoptotic signaling (8), whereas chronic exposure to high doses of GCs suppresses anti-oxidant capacity, decreases mitochondrial membrane potential, and sensitizes cells to apoptosis (21, 187, 190).

OXIDATIVE STRESS AND CIRCI

Oxidative stress is accompanied by multi-level impairment of the HPA axis and GR homeostatic functions (**Figure 5**). In non-survivors of septic shock, marked overexpression of iNOS in hypothalamic parvocellular neurons (PVN) was associated with decreased expression of pituitary ACTH, suggesting that the pro-apoptotic action of iNOS in the PVN may partially account for reduced activity of the HPA axis in sustained septic shock (221, 222). In experimental sepsis, adrenal cellular extracts demonstrate a pronounced increase in mRNA for iNOS and inflammatory cytokines that correlate positively with the degree of neutrophil infiltration, adrenal cell apoptosis, and mortality (213).

Changes within the adrenal gland microenvironment may also affect the HPA axis response in critical illness (149), with mitochondrial damage leading to a decreased responsiveness to ACTH (208). Importantly, iNOS expression in adrenal cells diverges at 48 h, with a significant increase observed in non-survivors vs. a reduction in survivors (223). In experimental endotoxemia, NF-κB-mediated iNOS release is associated with mitochondrial oxidative stress in adrenocortical cells with inhibition of steroidogenesis and response to ACTH (208).

Oxidative stress has a direct deleterious impact on GRs number and function. Experimental studies involving tissue cultures (220, 224–226) and murine models (227, 228) have demonstrated that oxidative stress is associated with decreased: (i) GR number (228), (ii) GC binding to GR (220, 224–227), (iii) GC-GR nuclear translocation (226, 229), (iv) binding to DNA (224), and (v) inducible gene transcription (220, 225). Nitrosylation, the covalent incorporation of a nitric oxide "nitrosyl" moiety into the critical cysteine(s) residue(s) of the GR is associated with loss of the steroid binding capacity (230).

In human monocytes, genes involved in oxidative functions were significantly overrepresented among GC down-regulated genes, while genes with antioxidant functions were upregulated

(113). A few studies have evaluated the impact of GC treatment on oxidative stress. In human monocytes, spontaneous, as well as phorbol myristyl acetate (PMA)-induced production of reactive oxygen species, is significantly reduced in GC-treated cells in comparison to controls (113). In murine macrophages, glucocorticoid treatment is associated with rapid (non-genomic) inhibition of superoxide anion production (231). In murine sepsis, GC treatment attenuated renal dysfunction by reducing mitochondrial injury with preservation of cytochrome c oxidase and suppression of pro-apoptotic protein levels (232). In clinical (233, 234) and experimental (162, 235) randomized trials, participants with severe sepsis receiving GC treatment had, in comparison to controls, a significant reduction in (i) circulating nitric oxide levels (162, 233, 235), and (ii) spontaneous release of hydrogen peroxide (H_2O_2) by neutrophils (234).

HYPOVITAMINOSES

Metabolic homeostasis is substantially disrupted in critical illness, and the degree of a vitamin deficiency can negatively impact health outcomes. Three vitamins, namely thiamine (vitamin B1), ascorbic acid (vitamin C), and vitamin D, are important for the proper function of the GR system and mitochondria, and their reserves are rapidly exhausted in critical illness (236). Vitamins B1, C, and D impact mitochondrial function, while vitamins C and D also impact GR function. A comprehensive list of suggested mechanisms for the efficacy of thiamine, ascorbic acid, and glucocorticoids in sepsis was recently reviewed (237).

Thiamine

Thiamine is a water-soluble vitamin, which is passively absorbed in the small intestine. After ingestion, free thiamine is converted to the active form thiamine pyrophosphate (TPP), commonly known as vitamin B1, by thiamine pyrophosphokinase. The majority of TPP in the body is found in erythrocytes and accounts for ∼80% of the body's total storage (238). Thiamine pyrophosphate is a key co-factor for pyruvate dehydrogenase, alpha-ketoglutarate dehydrogenase, transketolase, and branched-chain keto-acid dehydrogenase (238). Pyruvate dehydrogenase is the gatekeeper for entry into the Krebs Cycle, without which pyruvate would be converted to lactate as opposed to acetyl-coenzyme A. Alpha-ketoglutarate dehydrogenase is required for completion of the Krebs Cycle once it has begun. Transketolase is a key enzyme for the pentose phosphate pathway and for the production of NADPH with glutathione cycling, an important anti-oxidant pathway (239). There are also other proposed non-cofactor roles of thiamine within the immune system, gene regulation, oxidative stress response, cholinergic activity, chloride channel function, and neurotransmission (238).

The human adult can store around 30 mg of thiamine in muscle tissue, liver and kidneys, however, these stores can become depleted in as little as 18 days after the cessation of thiamine intake (238). A thiamine deficiency syndrome, beriberi, bears a number of similarities to sepsis, including peripheral vasodilation, cardiac dysfunction, and elevated lactate levels (237). In critical illness, the prevalence of thiamine deficiency is 10–20% upon admission (198, 240) and can increase up to 71% during ICU stay, suggesting rapid depletion of this vitamin (198). Based on limited data, no association was detected between thiamine levels, markers of oxidative stress (198) and mortality (198, 241). In one study, a significant negative correlation was reported between thiamine and lactic acid levels in patients with sepsis without liver dysfunction (240). In a pilot randomized controlled trial (RCT) of patients with septic shock ($n = 88$), the administration of thiamine (200 mg twice a day for 7 days) reduced lactate levels and improved mortality over time in a pre-defined subgroup of patients with thiamine deficiency (35% of cohort) (239). In a retrospective, single-center, matched cohort study, administration of thiamine within 24 h of septic shock ($n = 123$) was associated with improved likelihood of lactate clearance and a reduction in 28-day mortality (242). Despite some promising results, there is insufficient evidence to support or reject thiamine supplementation as a monotherapy in critically ill patients (238).

Vitamin D

Vitamin D is an ancient molecule that functions as both a nutrient and a hormone with metabolic and immunomodulatory properties; it regulates over 1,000 genes of the human genome (243, 244). The vitamin D receptor (VDR) is a member of the nuclear receptor gene family and is expressed in virtually all nucleated cells. Decreased serum levels of vitamin D have been associated with several autoimmune inflammatory diseases. Genome-and transcriptome-wide studies indicate that vitamin D signaling modulates many inflammatory responses on several levels (245), including interference with NF-κB, via upregulation of IκB expression (246). In addition, the ability of vitamin D to inhibit metabolic stress and energy expenditure in a cell microenvironment suggests that this pleiotropic hormone has a broad task as a pro-survival agent (244).

A growing body of scientific and medical literature supports the important anti-inflammatory functions of vitamin D in health and disease, including the enhancement of GC-mediated anti-inflammatory actions (247). The anti-inflammatory effect of vitamin D was consistently observed in studies of cell lines and human PBMCs, and was the subject of a comprehensive review (248). PBMCs activated with TLR ligands after incubation with 1,25(OH)D3 showed decreased release of TNF-α and IL-1β and increased anti-bacterial activity (249). In PBMCs, physiologic levels of vitamin D reduce inflammatory activities, by upregulating GC-mediated mitogen activated protein kinase (MAPK; see section Glucocorticoid Receptor-Alpha) phosphatase-1 (MKP-1) (250) to down-regulate p38 MAPK-mediated inflammatory gene expression (including TNF-α, IL-1β, IL-6, and IL-8) (251). In LPS-activated PBMCs (247) and PBMCs from patients with asthma (252), vitamin D enhanced dexamethasone-induced expression of MKP-1 (247), and this synergism was dependent on vitamin D-induced GM-CSF release (247). One study suggested that the interaction between vitamin D, glucocorticoids and their cognate receptors is related to the duration of exposure to vitamin D (253).

Beside this indirect modulation of signaling cascades, vitamin D and its receptor complex VDR/RXR can interact directly

with the GC receptor and other transcription factors (245). Of interest, Vitamin D has a high affinity binding for the GR (254), and was recently shown to increase, in a dose-dependent manner, GR concentration in T cells (255). Based on its pleiotropic functions, vitamin D is considered a "master tuner" in shifting homeostatic balance from a pro-inflammatory to a pro-resolving status (244). Several studies demonstrated a dose-dependent response of vitamin D with respect to reducing inflammation, with 1 nM and 10 nM concentrations causing the greatest effects (248). One study showed that serum 25(OH)D levels as high as 120 nmol/l may be necessary for optimal immune function (256). A small study in healthy patients with hypovitaminosis D reported that significant anti-inflammatory benefits of vitamin D supplementation were only seen by achieving serum 25(OH)D levels greater than 100 nmol/l (256).

The mitochondria also appear to be a direct target of the vitamin D endocrine system, and the two most important enzymes responsible for activation or inactivation of 25(OH)D, namely CYP27B1 (1α-hydroxylase) and CYP24A1 (24-hydroxylase), are located in the mitochondria (257). In U937 monocytes, 1,25 (OH)2 vitamin D upregulates glutamate cysteine ligase (GCLC) and glutathione reductase (GR), resulting in an increase of cellular glutathione formation, and decreased ROS and IL-8 secretion (258). Two recent studies have evaluated the impact of vitamin D on skeletal muscle mitochondrial function. Primary human skeletal muscle cells treated with 1,25(OH)D3 vs. vehicle demonstrated marked effects on mitochondrial number, morphology, physiology, and expression of key mitochondrial proteins, resulting in increased ATP production (259). In vitamin D-deficient symptomatic patients, Vitamin D supplementation was found, using phosphorus-31 magnetic resonance spectroscopy, to augment muscle mitochondrial maximal oxidative phosphorylation after exercise and improved symptoms of fatigue (260). Treatment of skeletal muscle with vitamin D is associated with a change in expression of ∼83 nuclear mRNAs encoding proteins known to localize in mitochondria (259).

Hypovitaminosis D is common in critical illness, despite parallel elevations of PTH (249) with one small study reporting a progressive drop in vitamin D levels in the first week of illness (261), while a low 25(OH)D3 status was significantly associated with all-cause and sepsis mortality (236). In early critical illness, vitamin D status is associated with a differential metabolic profile. Glutathione and glutamate metabolism, which play principal roles in redox regulation and immunomodulation, respectively, were significantly upregulated by vitamin D (199). However, evidence of a mortality benefit of vitamin D as monotherapy still remains uncertain (262, 263). A recent large RCT investigated a single dose of 540,000 international units of vitamin D_3 in critically ill patients with 1,25(OH)D3 levels <20 ng/ml (264). By day 3, the treated group achieved a level of 25-hydroxyvitamin D of 46.9 ± 23.2 ng/ml; measurements of systemic inflammation were not reported. Treatment was not associated with improvement in mortality or secondary variables (264).

Vitamin C

Ascorbic acid (vitamin C) is a potent water-soluble antioxidant and an enzymatic cofactor that plays a key role in neuro-endocrine and immune homeostatic corrections (265). Most vertebrates can synthesize ascorbic acid from glucose-6-phospate in the liver, with synthesis increasing during stress. In humans and other primates, however, ascorbic acid cannot be synthesized and has to be obtained through the diet. This is the result of a random mutation in the enzyme that catalyzes the final step of ascorbic acid biosynthesis in the common ancestor of the teleost fish some 200 million years ago (266, 267). To date, there is no satisfactory evolutionary explanation for this apparent random loss of ascorbic acid synthetic ability. Individuals from species which have lost the ability to make their own ascorbic acid were not selected against, as long as their diet contained sufficient quantities of vitamin C (266).

Ascorbic acid is actively transported into all cells of the body (except erythrocytes) by the sodium vitamin C transporter-2 (mSVCT2). Ascorbic acid is differentially accumulated by most tissues and body fluids. Studies using radiolabeled ascorbic acid predict that body stores in healthy humans are about 1,500 mg; scurvy is thought to occur when this level falls below 300 mg, with plasma ascorbic acid concentrations <11.3 μM (268). Importantly, the highest concentrations (μM) of ascorbic acid are found in critical organs involved in homeostatic corrections, such as the pituitary gland (2,300–2,800), the adrenals (1,700–2,300), the brain norepinephrine-synthesizing nuclei (800–900), and liver (600–900) (268). This vitamin-sequestering may represent an evolutionary protective or "safety" function.

Ascorbic acid is a key cellular antioxidant. As such, ascorbic acid is an electron donor that directly scavenges for free radicals, and inhibits the generation of new free radicals through its suppressive effects on the NADPH oxidase (NOX) pathway (237). Ascorbic acid also prevents the depletion of other circulatory antioxidants, such as lipid-soluble vitamin E and glutathione, although this is not the case in reverse (200). The anti-oxidant effects of ascorbic acid result in reduced endothelial permeability, improved microvascular and macrovascular function, attenuated cellular apoptosis in pathological states, and improved GR function (237).

Ascorbic acid is maintained at high levels in mature circulating leukocytes (μM amounts in lymphocytes ∼3,800; monocytes ∼3,100, and neutrophils ∼1,400) (268), suggesting an important role in many aspects of the immune response. In leukocytes, ascorbic acid content responds to variations in plasma ascorbate availability (269). Following activation, immune cells undergo dramatic metabolic reprogramming with increased aerobic glycolytic activity and fatty acid oxidation (Warburg effect) under the regulation of hypoxia-inducible factors (HIFs) (270). The result of this change is to rapidly provide ATP and metabolic intermediates for the biosynthesis of immune and inflammatory mediators. Importantly, the hydroxylase enzymes that regulate the actions of the HIFs require ascorbate for optimal activity (269). The immune-enhancing properties of ascorbic acid regulation of HIFs include increased neutrophil and macrophage bacterial killing and phagocytic capacity (269, 271). In addition,

ascorbic acid plays an important role in protecting host cells from the excessive oxidative stress caused by infections (265).

Ascorbic acid plays a crucial role in HPA axis function (**Figure 6**). In adrenocortical cells ascorbic acid is sequestered in two pools, one of which can be depleted by ACTH. In response to inflammatory cytokine-mediated ACTH release from the anterior pituitary gland, the adrenal gland rapidly secretes ascorbic acid in amounts that are sufficient to increase, by several fold, plasma ascorbic acid concentrations in the adrenal vein, without increasing systemic levels (268). More than 80 years ago Hans Selye, the pioneer of stress research, reported that the adrenal glands not only contain some of the highest concentrations of ascorbic acid in the human body, but they also employ this vitamin to synthesize cortisol in the adrenal cells (272). Today, *in vitro* and *in vivo* studies have shown that ascorbic acid is an essential cofactor required in both adrenal mitochondrial steroidogenesis and catecholamine biosynthesis (272). The level of ascorbate in the adrenals might affect their capacity to convert cholesterol into pregnenolone, the precursor from which nearly all steroid hormones, including cortisol, are made (273). Additionally, ascorbic acid, as an antioxidant, has a positive impact on GR functions (see section Oxidative Stress and CIRCI).

Oxidative conditions modulate negatively ligand-dependent and independent nuclear import of the GR, affecting GC-GRα DNA binding, and inducible gene expression (225, 229), while a phosphodiester compound of ascorbic acid reverses oxidation of the GR, thereby, restoring the cellular glucocorticoid-responsiveness in oxidant conditions (225). Finally, the cellular uptake of ascorbic acid, mediated by mSVCT2, is downregulated during inflammatory conditions. In a time and concentration-dependent manner, GCs increase the expression of mSVCT2, facilitating the uptake of vitamin C into cells (274), providing the rationale for combination treatment using GCs and ascorbic acid (275). Interestingly, there is a strong inverse correlation between the ability of an animal to endogenously produce vitamin C and the induction of a cortisol response when stressed (276).

In human cell lines and primary endothelial cells (ECV304 and HUVEC), ascorbic acid inhibits TNFα and IL-1β-induced activation of NF-κB, in a dose-dependent manner, by inhibiting phosphorylation and degradation of IκBα (277, 278), independently of its antioxidant properties (277). Preclinical studies show that high-dose vitamin C can prevent or restore microcirculatory flow impairment, reinstate vascular responsiveness to vasoconstrictors, and preserve the endothelial barrier (200). Both ascorbic acid (279) and the GR (see section Endothelium) (130) are essential for endothelial cell homeostasis, and the combination of glucocorticoids with ascorbic acid is superior to either one on its own in protecting vascular endothelium that is critical to allow recovery (280).

Many studies have demonstrated that vitamin C levels are rapidly depleted in critically ill patients, with about 40% of the septic patients having reduced serum levels, similar to those seen at scurvy diagnosis (<11.3 u/mol/l) (281, 282). As intracellular ascorbate concentrations in mononuclear leukocytes and in granulocytes are, respectively, 80 and 25 times higher than in plasma, a high production and turnover of these cells may also contribute to its depletion (200). Low plasma concentrations of vitamin C are associated with more severe organ failure and increased risk of mortality (282). Similar to thiamine, ascorbic acid deficiency syndrome (scurvy) bears a number of similarities to sepsis, including coagulation abnormalities, and breakdown of the endothelial wall (282).

In a phase I safety trial, intravenous ascorbic acid infusion was safe, well-tolerated, and associated with improvement in multiple organ dysfunction and decreased biomarkers of inflammation and endothelial injury (281). Additionally, a small RCT investigating high dose ascorbic acid administration in patients with septic shock reported a reduction in 28-day mortality (283), while a larger trial in patients with sepsis-associated ARDS reported a significant reduction in 28-day all-cause mortality

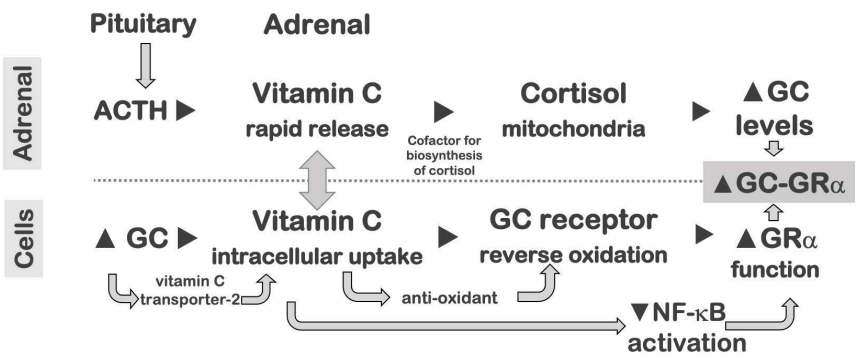

FIGURE 6 | Impact of vitamin C on activated GRα activity. **(Top)** Adrenal: The adrenal and pituitary glands have very high concentrations of ascorbic acid (vitamin C). In response to ACTH release from the corticotrophs of the anterior pituitary gland, the adrenal gland rapidly secretes ascorbic acid, an essential cofactor required for adrenal steroidogenesis in mitochondria, contributing to increased glucocorticoids synthesis. **(Bottom)** Cells: glucocorticoids, in a time- and concentration-dependent manner, increase the expression of mSVCT2, facilitating the uptake of ascorbic acid into the cell. Ascorbic acid reverses oxidation of the GR, restoring cellular glucocorticoid-responsiveness in oxidant conditions. In addition, ascorbic acid inhibits TNFα- and IL-1β-induced activation of NF-κB in a dose-dependent manner by inhibiting phosphorylation and degradation of IκBα. These combined actions result in increased glucocorticoid availability and GC-GRα activation and improved homeostatic corrections.

(secondary outcome) (284). The rationale for glucocorticoid treatment in association with high dose ascorbic acid was the subject of recent reviews (237, 267). The promising findings of a recent retrospective study in patients with severe sepsis and septic shock has spurred considerable interest in the subject (275). Randomized data to confirm or refute the observational evidence for the drug combination are needed, and several clinical trials are ongoing or planned in the near future (237).

CONCLUSIONS AND IMPLICATIONS FOR GLUCOCORTICOID TREATMENT

In critical illness, homeostatic corrections, the culmination of millions of years of evolution, are modulated by the activated GC-GRα and associated with an enormous bioenergetic and metabolic cost. We have reviewed how CIRCI, mitochondrial dysfunction/damage, and hypovitaminosis collectively interact to accelerate an anti-homeostatic active process of natural selection. Importantly, the allostatic overload imposed by homeostatic corrections impacts negatively on both acute and long-term morbidity and mortality, while the bioenergetic and metabolic reserves to support homeostatic corrections are time limited. For these reasons it is prudent to implement early interventions designed to achieve the following: (i) reinforce innate immunity, (ii) inhibit further systemic tissue damage, (iii) limit the metabolic and bioenergetic cacostatic overload imposed during vital organ support, (iv) accelerate disease resolution, and (v) prevent persistent-chronic low-grade systemic inflammation (285). This approach is supported by experimental (286) and clinical studies in patients with septic shock or ARDS (287–290).

The actions of the activated GRα cannot be categorized as merely anti-inflammatory, as it is now clear that insufficient intracellular GRα regulatory action and not relative adrenal insufficiency is the primary driver of CIRCI (17). Therefore, glucocorticoid treatment should not be viewed exclusively as anti-inflammatory or as a hormone replacement for relative adrenal insufficiency. It also is equally relevant that one should recall that full biological resolution lags weeks behind clinical resolution of an acute illness, making the clinical criteria that we frequently employ to guide duration of treatment, an inadequate reference point (291). For these reasons, glucocorticoid treatment, and other co-interventions should be directed at supporting the activated GRα regulatory function throughout all phases of homeostatic corrections, and not limited to the acute phase of organ support.

Randomized studies provide evidence that prolonged glucocorticoid administration is associated with increased GRα number and function and decreased oxidative stress (see sections Glucocorticoid Receptor Alpha in Critical Illness and Mitochondria and HPA-Axis Cross-Talk). Additionally, the activated GRα interdependence with functional mitochondria and three vitamin reserves provides a rationale for co-interventions that include rapid replacement of vitamins B1, C, and D. Recent evidence generated from a retrospective before-after clinical study in patients with severe sepsis has generated momentum for increased research in this field (275), with ongoing confirmatory randomized trials in progress (282).

Additional co-intervention with critical hormones and mediators involved in homeostatic corrections are also necessary, such as fludrocortisone (174, 292, 293) or vasopressin (294–296) in patients with septic shock. Fludrocortisone is a mineralocorticoid and glucocorticoid receptor agonist that binds to cytoplasmic receptors, activates their translocation into the nucleus and subsequently initiates the transcription of mineralocorticoid- and glucocorticoid-responsive genes (297). The inclusion or exclusion of fludrocortisone, as a co-intervention with hydrocortisone, may partly explain the differences reported in outcomes of some RCTs (see explanation for **Figure 7** below) (292, 298). Other potential co-interventions directed at increasing glucocorticoid receptor expression, such as statins (299), melatonin (300), beta-blockers (301), calcium channel blockers (301), or directed at improving mitochondrial function (194, 302, 303) have not been investigated in association with glucocorticoid treatment in acute illness or alone in chronic critical illness.

Present understanding of the activated GC-GRα's role in immunomodulation and disease resolution should be taken into account when re-evaluating how to administer glucocorticoid treatment and in monitoring treatment responses. There are many variables to consider, including the type of GC to be used, timing, dosage, mode of delivery, co-interventions, duration, and tapering. Over the last 40 years, multiple randomized trials investigating GC treatment in critical illness have clearly shown that the design of a treatment protocol has a profound impact on treatment response and outcome (304, 305). The CONSORT (306) and GRADE (307) systems, while useful in evaluating the quality of a randomized trial, unfortunately lack a position on two fundamental elements of a trial design, namely the disease pathophysiology and the pharmacological principles applicable to the investigated drug. Unfortunately, lack of these specific reference points has generated misinterpretation of the literature, fueling a non-sensical controversy that clearly is not serving the patient (308).

Based on this updated pathophysiological understanding, we offer a few observations and make recommendations for future research. Early initiation of treatment, before homeostatic corrections reach exhaustion, is critical and should be directed at approaching maximal saturation of the glucocorticoid receptor (∼100 mg of methylprednisolone equivalent) (309). An adequate initial loading bolus is necessary to achieve prompt elevation in plasma levels and to assure higher GRα saturation in the cytoplasm and on the cell membrane for genomic and non-genomic actions, respectively. In human monocytic cells activated with graded concentrations of LPS and then exposed to graded concentrations of methylprednisolone (**Figure 7**), reduction in inflammatory cytokine transcription was initially modest, then—after reaching an inflection point—followed by a rapid reduction, likely related to achieving maximal drug receptor saturation and adequate time for a measurable effect (102). To achieve optimal results, the initial loading bolus should be followed by an infusion (daily dose over 24 h) to rapidly achieve a steady state. In patients with septic shock,

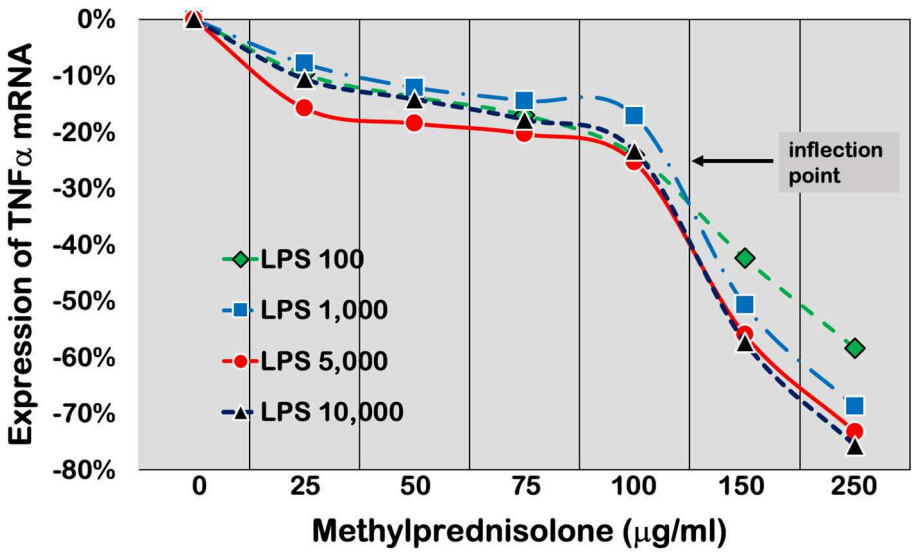

FIGURE 7 | Regression of concentration of methylprednisolone on steady-state mRNA levels of TNF-α in U937 cells primed with graded concentrations of lipopolysaccharide (LPS). In experimental simulation of severe inflammation, human monocytic cells (U937 cells) were activated with graded concentrations of LPS (100 ng/ml, 1.0 μg/ml, 5.0 μg/ml, or 10.0 μg/ml) for 6 h followed by measurement of the expression of inflammatory cytokines [TNF-α (shown), IL-1β, and IL-6]. Graded concentrations of LPS were followed by progressively higher inflammatory cytokine transcription (for TNF-α: 185, 318, 481, and 566, respectively). These cells were then exposed to graded concentrations of methylprednisolone [(μg/ml): 50, 100, 150, 200, 250] for 6 h followed by repeated measurement of inflammatory cytokine expression (see below). The steady state mRNA levels of TNF-α, IL-1β, or IL-6 in LPS-activated cells were reduced by treatment with methylprednisolone in a concentration-dependent manner. The effective dose of methylprednisolone was 175 mg, a value that appeared to be independent of the priming level of LPS and type of mRNA measured (102). Modified with permission from Meduri et al. (102).

hydrocortisone administered as an infusion vs. an intermittent bolus was associated with more rapid resolution of shock (310), and fewer hyperglycemic episodes (311, 312).

In general, synthetic glucocorticoids are more potent immunoregulators than is cortisol, because they are not subject to endogenous clearance and inhibitors of cortisol activity, including 11βHSD inactivation. Moreover, synthetic glucocorticoids bind the glucocorticoid receptors with higher affinity and remain longer in the cell nucleus, while they bind to mineralocorticoid receptors with lower affinity than do endogenous glucocorticoids, thereby minimizing mineralocorticoid-related side effects (313). Pharmacokinetics and pharmacodynamics of systemically administered glucocorticoids are reviewed in reference (314). Hydrocortisone and methylprednisolone are the two glucocorticoids most often investigated in critical care RCTs (315). In the past, different exogenous glucocorticoids were thought to be qualitatively indistinguishable from each other because they act via the same glucocorticoid receptor, however, qualitative differences have been recently discovered, and one glucocorticoid cannot be simply replaced by another (314). While hydrocortisone was initially chosen as the drug of choice for adrenal replacement, methylprednisolone may actually offer unique advantages over hydrocortisone as follows: (i) greater affinity for the glucocorticoid receptor (316); (ii) higher penetration in lung tissue (important for ARDS or pneumonia), and with longer residence time (317–319), (iii) higher potency of genomic activity especially NF-κB inhibitory activity (320); and (iv) higher potency of non-genomic activity (321). GRα binding affinity, expressed as relative receptor affinity (RRA), correlates with glucocorticoid potency. The log RRAs for selected glucocorticoids are 0.95, 1.62, and 2.0 for hydrocortisone, methylprednisolone, and dexamethasone, respectively (316). A comparison study between these three types of glucocorticoids is needed.

The suggested mode of administration for septic shock [hydrocortisone <400 mg/day for >3 days (315), or hydrocortisone 50 mg QID for 7 days without tapering] is based in part on an outdated pathophysiological model and a misconception about the risk associated with longer duration of treatment (small) and discontinuation without tapering (high). There is some evidence that a treatment duration of 3–7 days directed at reducing acuity of illness (transient reduction in systemic inflammation) might shortchange the full beneficial effects of glucocorticoid therapy (322). The impact of a longer duration of treatment on medium- and long-term mortality, as observed in RTCs of patients with *Pneumocystis jiroveci* pneumonia (323), needs to be investigated.

While glucocorticoids have an important role in supporting homeostatic corrections, this is achieved at the expense of reversible suppression of the HPA axis. In addition, the risk of glucocorticoid treatment-associated adrenal suppression in critically ill patients with dysregulated systemic inflammation is underappreciated. It has been shown that neither the total or the highest dose, nor the duration of glucocorticoid treatment is a significant predictor of HPA axis recovery (324). In the

recent "Reduction in the Use of Corticosteroids in Exacerbated COPD trial" that evaluated prednisone 40 mg daily for 5 or 14 days, adrenal suppression was detected at hospital discharge and at 30 days in 38 and 9% of patients, respectively; no differences were detected between 5 or 14 days of glucocorticoid exposure (325). Similarly to the experimental literature (326, 327), critical care RCTs have shown that abrupt glucocorticoid discontinuation after a 3-to-14 days treatment was rapidly followed by a reconstituted inflammatory response with a clinical relapse in approximately one-third of the patients (233, 322, 328, 329), and increased mortality (329). In the LaSRS trial (329), discontinuation of study drug 48 h post-extubation was associated with clinical relapse in one-quarter of methylprednisolone-treated patients. These patients were rapidly returned to mechanical ventilation (MV) without re-institution of study treatment, fared poorly, required additional days of MV and had a 9-fold increased risk of 60-day mortality ($p = 0.001$) in comparison to patients that did not return to MV (330). Gradual tapering is necessary to preserve the disease improvement achieved during glucocorticoid administration, to sustain continuous resolution and restoration of tissue homeostasis, to achieve gradual recovery of the suppressed HPA axis, to forestall disease relapse from reconstituted systemic inflammation, and finally to comply with the Food and Drug Administration package insert warnings (Reference ID: 3032293) (331).

With rapidly expanding knowledge, appreciation of how homeostatic corrections work and how they evolved provides a conceptual framework to understand and appreciate the complex pathobiology of critical illness. We have reviewed emerging literature clearly placing the activated GRα at the center of the homeostatic corrections in the general adaptation to critical illness. Future research directions should include a reassessment of the pharmacological principles that guide glucocorticoid treatment in critical illness and to devise co-interventions to improve cellular responsiveness to glucocorticoids by correcting conditions associated with a reduction in GRα and mitochondrial concentration and function.

SEARCH METHODOLOGY

In addition to the previously used sources (17), we searched the Google Scholar and PubMed databases, employing the following keywords: "glucocorticoid," "corticosteroid," "glucocorticoid receptor," "stress response," "acute phase response," "regulation," "resolution," "critical illness related corticosteroid insufficiency," "treatment," "systemic inflammation," "dysregulated" systemic inflammation," "nuclear factor kappa B," "evolution," "endothelium," "mitochondria," "reactive oxygen species," ascorbic acid, thiamine, vitamin D, melatonin, "acute," "long-term," chronic," "homeostasis," "allostasis," "cacostasis," and terms related to critical illness, sepsis, septic shock, acute respiratory distress syndrome, "cardiac events." Manual searching of articles, including reference lists of cited publications, was also performed to avoid omissions.

AUTHOR CONTRIBUTIONS

GM conceived and initiated the article and wrote the original draft. GC contributed to the conception and worked on successive drafts.

FUNDING

This material was the result of work supported with the resources and use of facilities at the Memphis VA Medical Center. Funding for the respective trials was disclosed in the original publications.

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

frontiers in Endocrinology

OPINION
published: 07 July 2020
doi: 10.3389/fendo.2020.00514

Working Hypothesis for Glucose Metabolism and SARS-CoV-2 Replication: Interplay Between the Hexosamine Pathway and Interferon RF5 Triggering Hyperinflammation. Role of BCG Vaccine?

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Keywords: HB pathway, IRF5, SARS-CoV-2, BCG, cytokine storm

OPEN ACCESS

Edited by:
Jeff M. P. Holly,
University of Bristol, United Kingdom

Reviewed by:
Antonino Belfiore,
University of Catania, Italy
Veronica Vella,
University of Catania, Italy

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Specialty section:
This article was submitted to Translational Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *02 May 2020*
Accepted: *26 June 2020*
Published: *07 July 2020*

Citation:
Laviada-Molina HA, Leal-Berumen I, Rodriguez-Ayala E and Bastarrachea RA (2020) Working Hypothesis for Glucose Metabolism and SARS-CoV-2 Replication: Interplay Between the Hexosamine Pathway and Interferon RF5 Triggering Hyperinflammation. Role of BCG Vaccine? Front. Endocrinol. 11:514. doi: 10.3389/fendo.2020.00514

INTRODUCTION

Respiratory epithelial cells, dendritic cells (DCs) and macrophages (1) secrete low levels of the antiviral factor interferons (IFNs) (2) and high levels of proinflammatory cytokines such as tumor necrosis factor alpha (TNFα), IL-6, and IL-1β, IP-10, MCP-3, characterizing the pathophysiologic features of severe acute respiratory syndrome (ARDS) induced by SARS-CoV-2 infection (3). Researchers hypothesized that after COVID-19 infects human cells, the virus utilizes an excess of glucose for a fast viral replication from the hexosamine biosynthetic pathway (HBP) hijacking substrates from the metabolic environment. This process induces overexpression of interferon IRF5, leading to a massive inflammatory gene overexpression, endoplasmic reticulum (ER) stress, and cytokine dysregulation profile. This deleterious cytokine overproduction is referred to as the cytokine storm (**Figure 1**). It leads to an increased risk of vascular hyperpermeability, multiorgan failure, and hyperinflammation (4, 5). Little is known about the molecular immunometabolic mechanism that triggers the uncontrolled surge in cytokine secretion and the cellular regulation of the hyperinflammatory cascade intertwined with the COVID-19 viral replication.

CHRONIC LOW-GRADE SUBCLINICAL INFLAMMATION: UNDERLYING MECHANISM ENHACING THE COVID-19 CYTOKINE STORM IN THE PRESENCE OF DYSGLYCEMIA

Older people, immunocompromised, diabetics and/or hypertensives are more likely to develop the cytokine storm. Dysglycemia is characterized by glucose intolerance and insulin resistance (6, 7). Additionally, aberrant expression of proinflammatory cytokines adds to the toxic milieu

of dysglycemia (8). These immunometabolic disturbances also include hyperglycemic posprandial peaks perhaps exacerbating the excess of proinflammatory cytokines in COVID-19 (9). However, a subgroup of symptom-free non-diabetic, overweight individuals with mild body fat accumulation and insulin resistance (IR) and/or prediabetes (10), and a second subgroup of symptom-free normal weight metabolically unhealthy subjects have a 3-fold higher risk of all-cause mortality and/or cardiovascular events (11). These two subgroups are also at high-risk to develop the cytokine storm. They have in common a chronic systemic low-grade subclinical proinflammatory (CLGSPI) state (12) characterized by adipose tissue dysfunction (ATdys) (13) and macrophage polarization in adipose tissue, low adiponectin levels, cytokines and circulating inflammatory C-reactive protein (CRP) that perpetuates this deleterious CLGSPI and promotes insulin resistance (14). These subgroups of individuals with underlying but undetected postprandial dysglycemia, CLGSPI and ATdys present a proinflamatory pathology that is highly underestimated and rarely diagnosed among symptom-free individuals in the practice setting. This undetected deleterious chronic low-grade proinflamatory scenario perhaps together with postprandial glucose excursions is proposed here as the underlying mechanism enhancing the hyperinflammatory state in these infected symptom-free subjects with COVID-19 triggering multi-organ failure.

GLUCOSE METABOLISM, THE HEXOSAMINE PATHWAY, IRF5, SARS-CoV-2 REPLICATION AND CYTOKINE STORM

After glucose uptake, in addition to glycolysis, glucose metabolism generates UDP-GlcNAc (uridinediphosphate-β-D-N- acetylglucosamine) via the HBP pathway. The HBP links cellular signaling and gene expression to glucose, amino acid, fatty acid and nucleotide metabolism (15, 16). It is regulated by the enzymes OGT (O-GlcNAc transferase) and OGA (O-GlcNAcase), that catalyze the addition and removal of GlcNAc on proteins (17). UDP-GlcNAc is a substrate for N-glycosylation, a process important for protein folding within the ER (18, 19) (**Figure 1**). The HBP- O-GlcNAc pathway has also been characterized as a major contributor to the deleterious effects of dysglycemic states, also influencing cellular proliferation. These dysglycemic states, mainly overt hyperglycemia, have a significant contribution in oncogenesis, tumor progression and fatal outcomes, indicating that there is a link between glucose metabolic disorders and tumor growth of cancer cells (20, 21). Similar biological characteristics are found in viruses. They rearrange the metabolic environment in the infected cells to facilitate virus replication. Indeed, virus-infected cells increase glycolytic metabolism to secure precursors for an increased biosynthesis (lipids, nucleotides) to optimize virus production and replication (22, 23).

Nuclear factor-kB (NF-kB) and IKK are associated with metabolic disorders (24), also playing important roles in inflammatory and immune responses (25). O-GlcNAcylation regulates direct modification of transcription factors such as NF-kB (26, 27). Cells use the HBP and OGT to potentiate gene expression through NF-kB as a glucose-responsive transcription factor in response to TNFα (28) (**Figure 1**). The interferon-regulatory factor (IRF) family plays a critical role in regulating the immune system, the innate immune response and the development of immune cells. IRFs are primarily implicated in antiviral responses and interferon production. IRFs also have key functions in the regulation of metabolism (29). Among the IRF family members, Interferon Regulatory Factor 5 (IRF5) is a key player in inflammation (30). IRF5 mediates induction of proinflammatory cytokines such as interleukin-6 (IL-6), IL-12, IL-23 and TNFα, is involved in the recruitment of inflammatory genes with NF-kB, and in determining the inflammatory macrophage phenotype (31). Host protection by IRF5 is achieved through its role in the nucleus triggering transcriptional activation of proinflammatory type I interferon (IFN), promotion of apoptosis-related genes and regulation of cytokines involved in cell survival, growth, proliferation, and differentiation (32). ER stress can result in the accumulation of unfolded proteins, triggering the unfolded protein response (UPR), an adaptive reaction that reduces unfolded protein load to maintain cell viability and function, capable of sensing dangerous aggressions and reverse them by influencing the immune response through interferon production (33).

Cells infected with viruses induce ER stress and stimulate strong interferon responses (34). Viral replication is inhibited by an interferon-regulated gene product, the double-stranded RNA-dependent protein kinase (PKR). Interferon employs its antiviral properties by activating PKR, thereby inhibiting viral replication. ER stress is a critical component in the response against viral infections. A prolonged ER stress triggers apoptosis. Therefore, the task of a virus is to overcome the interferon response involving PKR and manipulate the unfolded protein response (UPR) to facilitate viral replication and cause disease (34). A recent study demonstrated that influenza A virus (IAV) was able to induce a cytokine storm via interferon (IFN) regulatory factor−5 (IRF5) through glucose metabolism utilization and an increase in O-GlcNAc signaling, demonstrating that the HBP– *O*-GlcNAc signaling pathway in influenza A virus (IAV) promoted a massive inflammatory cytokine overexpression (35). SARS-CoV-2 infects human cells also leading perhaps to an excess of glucose utilization (16). Indeed, the HBP pathway activated in IAV infections generates UDP-N-Acetylglucosamine (UDP-GlcNAc), substrate for the key enzyme for protein O-GlcNAcylation O-GlcNAc transferase (OGT) (15). This enzyme has a strong binding affinity to signaling protein interferon regulatory factor 5 (IRF5) (35). Viral infections such as IAV and SARS-CoV-2 may create an excess in IRF5, leading to ER stress and rapid ubiquitination, triggering an excess of cytokine overproduction, hyperinflammation and multiorgan failure (36) (**Figure 1**). Another study identified a molecular mechanism by which HBP-mediated O-GlcNAcylation regulates mitochondrial antiviral signaling protein (MAVS) function (37) and highlighted

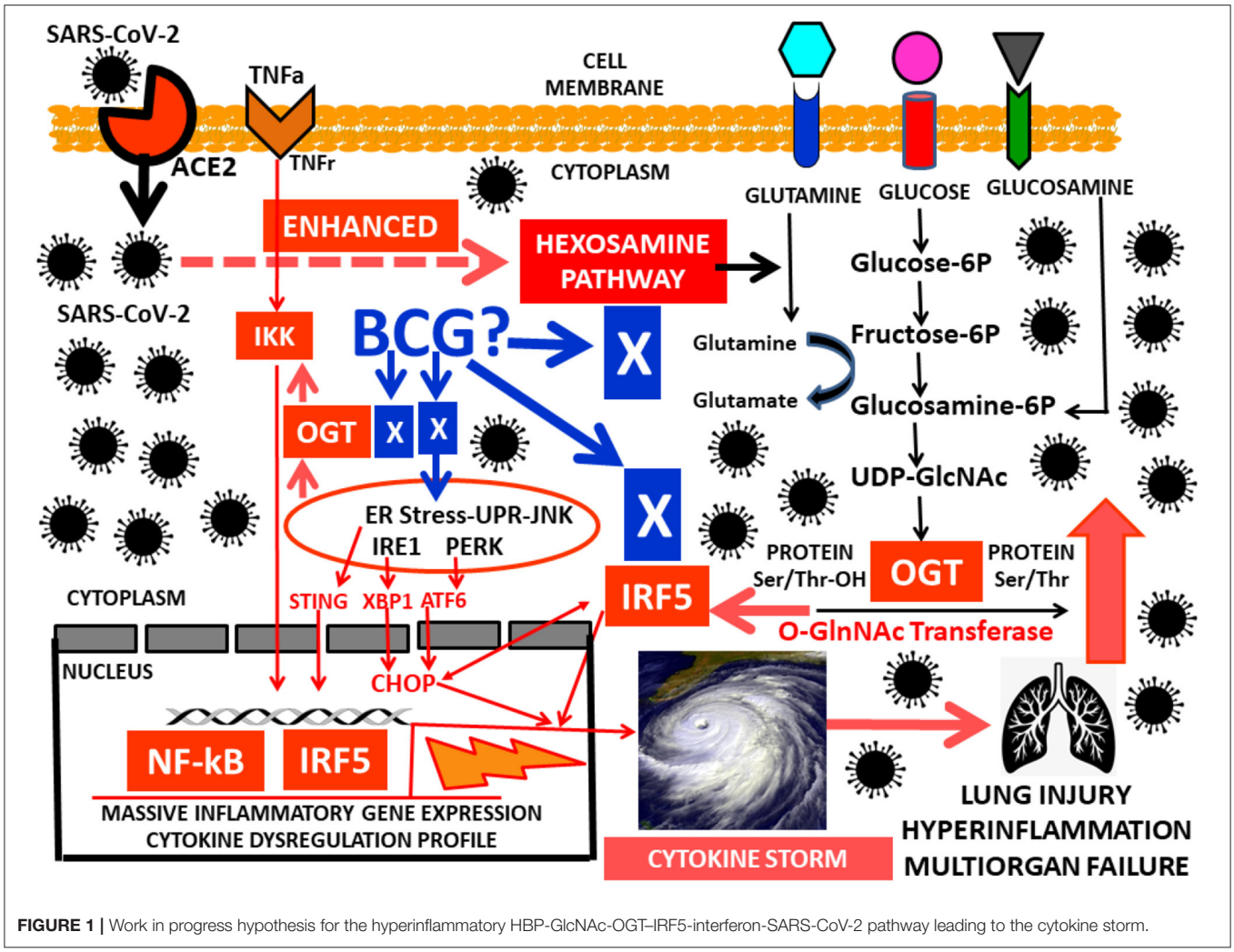

FIGURE 1 | Work in progress hypothesis for the hyperinflammatory HBP-GlcNAc-OGT–IRF5-interferon-SARS-CoV-2 pathway leading to the cytokine storm.

the importance of glucose metabolism in antiviral innate immunity (38).

COULD BCG AVOID THE HIJACKING OF THE HBP-O-GlcNAc-OGT COMPLEX BY SARS-CoV-2 TO RESTORE IMMUNITY AND BALANCE CELLULAR METABOLISM?

BCG-treated type 1 diabetes (T1D) individuals showed a durable lowering of HbA1c and glucose (39). BCG appears to switch the immune system of T1Ds from high oxidative phosphorylation to augmented glycolysis, a systemic metabolic shift that allows cells to consume large amounts of glucose to safely lower hyperglycemia (39, 40). The authors were able to reset the immune system to a state of increased glycolysis at the cellular level through turning on T regulatory (Treg) cells. The BCG effect on immune metabolism apparently accelerated glucose utilization through increased glycolysis, a high-glucose-transport process through the pentose phosphate shunt, instead of using the Krebs cycle for oxidative phosphorylation (40). The immune effects of BCG in T1D relates to the autoimmune environment comprising too few suppressive T regulatory (Treg) cells and too many cytotoxic T lymphocytes (CTLs). With BCG treatment, Treg cell expansion and augmented function occurred, and CTLs died thus restoring the immune balance toward normal at the autoimmune site (41). **Figure 1** shows multiple potential molecular targets for BCG activity in COVID-19 infected individuals.

Based on the immunomodulatory roles of mycobacteria, an effect from BCG vaccination on the spread and severity of COVID-19 dissemination in different countries could have occurred (42). Researchers classified countries into 2 groups according to presence or absence of BCG vaccination in their routine vaccine schedules and obtained confirmed numbers of COVID-19 cases and deaths from the World Health Organization (WHO). They found that the mean

of cases per population ratio was statistically significantly lower in BCG vaccinated countries when compared to BCG-non-vaccinated countries, also finding that both the mean deaths per population and the deaths per cases ratio were significantly lower in BCG-vaccinated countries. They concluded that the cessation of BCG vaccination in several countries within the last few decades should be reanalyzed given its impact regarding the immune response to hypothetical viral pandemics we might face in the future on BCG-non-vaccinated young individuals (42–44). Vaccination with BCG triggers a memory-like response in innate immune cells known as "trained immunity" associated to an epigenetic reprogramming mechanism in both humans and mice (45). However, some authors advice caution when interpreting data on Covid-19 incidence and BCG vaccination. Further research is definitively needed to prove that the BCG vaccine confers protection against COVID-19. Indeed, the current state of knowledge does not provide sufficient evidence (46). A trial for BCG vaccination to reduce the impact of COVID-19 in Australian healthcare workers (BRACE) has been set to investigate whether BCG vaccination protects against COVID-19 (mcri.edu.au/BRACE).

DISCUSSION

As depicted in **Figure 1**, we speculate as a work in progress hypothesis that after SARS-CoV-2 have entered human cells through the ACE 2 receptor, its first priority, similar to IAV, would be to enhance the HBP pathway to secure excessive glucose consumption and substrates for rapid replication. This abnormal HBP hyperactivity would lead to an excess of the OGT enzyme that would consequently trigger large amounts of IRF5 interferon. IRF5 and OGT will then coordinate efforts to exacerbate the IKK-NF-kB proinflamatory pathway triggering in the nucleus a massive inflammatory cytokine gene overexpression profile and a deleterious ER stress that ultimately result in hyperinflammation, a cytokine storm and multiorgan failure.

The detrimental consequences are not hard to imagine when this life-threatening hyperinflammatory HBP-GlcNAc-OGT–IRF5-interferon-SARS-CoV-2 pathway from the COVID-19 pandemic meets with CLGSPI found in common, complex highly prevalent pandemics such as obesity and diabetes, also found in not so old individuals with symptom-free adipose tissue dysfunction, insulin resistance, the metabolic syndrome and/or prediabetes (13, 47). These four groups of individuals present postprandial hyperglycemia which is one of the earliest abnormalities of glucose homeostasis associated with dysglycemic states (48). A recent publication highlighted the significance of angiotensin converting enzyme 2 (ACE2) and dipeptidyl peptidase-4 (DPP4), and their dual physiologic effects as transducers of metabolic signals regulating inflammation, cardiovascular physiology, and glucose homeostasis (49). This opens the door to consider glucose-lowering agents such as the DPP4 inhibitors as tools to intervene in the interaction of COVD-19, dysglycemic states and the HBP-GlcNAc-OGT–IRF5-interferon pathway, keeping in mind that DPP-4 inhibitors are not indicated in T1D individuals (50).

In summary, the HBP-GlcNAc-OGT–IRF5-interferon-SARS-CoV-2 pathway may have important implications regarding direct or indirect specific molecular targets for BCG activity (**Figure 1**). Importantly, dietary specific indications for low glycemic index diets (51, 52) instead of general dietary recommendations for COVID-19 infected individuals and/or at risk of infection may seem appropriate. This is supported by the hypothetical association between the large postprandial glucose peaks after daily meals, the HBP-GlcNAc-OGT–IRF5-interferon pathway and subjects with dysglycemia, adipose tissue dysfunction and low-grade subclinical inflammation at risk for severe COVID-19 infection.

AUTHOR CONTRIBUTIONS

RB and HL-M wrote the manuscript. IL-B edited and revised the manuscript. ER-A revised the final version. All authors have read and approved the final manuscript.

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

frontiers in Endocrinology

REVIEW
published: 10 September 2020
doi: 10.3389/fendo.2020.00622

Artificial Light at Night (ALAN): A Potential Anthropogenic Component for the COVID-19 and HCoVs Outbreak

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The origin of the coronavirus disease 2019 (COVID-19) pandemic is zoonotic. The circadian day–night is the rhythmic clue to organisms for their synchronized body functions. The "development for mankind" escalated the use of artificial light at night (ALAN). In this article, we tried to focus on the possible influence of this anthropogenic factor in human coronavirus (HCoV) outbreak. The relationship between the occurrences of coronavirus and the ascending curve of the night-light has also been delivered. The ALAN influences the physiology and behavior of bat, a known nocturnal natural reservoir of many *Coronaviridae*. The "threatened" and "endangered" status of the majority of bat species is mainly because of the destruction of their proper habit and habitat predominantly through artificial illumination. The stress exerted by ALAN leads to the impaired body functions, especially endocrine, immune, genomic integration, and overall rhythm features of different physiological variables and behaviors in nocturnal animals. Night-light disturbs "virus–host" synchronization and may lead to mutation in the genomic part of the virus and excessive virus shedding. We also proposed some future strategies to mitigate the repercussions of ALAN and for the protection of the living system in the earth as well.

Keywords: COVID-19, HCoVs, ALAN, bat, melatonin, sustainability

HIGHLIGHTS

- Increase of anthropogenic Artificial Light at Night (ALAN) in due course of the "development for mankind" may be related to Coronavirus outbreak (HCoVs).
- Bats, nocturnal natural reservoir of many Coronaviridae, are heavily affected by ALAN due to the destruction of their proper habit and habitat.
- Most of the bat species are either "threatened" or "endangered" in IUCN list.
- Night-light might disturb "virus-host" synchronization by exerting selection pressure that may lead to the mutation in the genome of the virus and excessive viral shedding.
- Proposed strategies to mitigate the repercussions of ALAN and for the protection of our planet earth as well.

OPEN ACCESS

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Specialty section:
This article was submitted to Translational Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *02 June 2020*
Accepted: *30 July 2020*
Published: *10 September 2020*

Citation:
Khan ZA, Yumnamcha T, Mondal G, Devi SD, Rajiv C, Labala RK, Sanjita Devi H and Chattoraj A (2020) Artificial Light at Night (ALAN): A Potential Anthropogenic Component for the COVID-19 and HCoVs Outbreak. Front. Endocrinol. 11:622. doi: 10.3389/fendo.2020.00622

INTRODUCTION

One of the most prevalent but least understood anthropogenic changes that impact living beings is the light pollution in the form of artificial light at night (ALAN). ALAN appears to be a massive threat to the growing human–environment conflicts, as it intervenes with all the three primary requirements (food, habitat, and health) for the sustainability of life in various animal species including humans (1–6). ALAN is one of the significant components of human-induced selection pressure, which has dramatically changed the trajectory, the rate of extinction, and speciation in this Anthropocene (7). In the case of nocturnal animals, such as bats and rodents, the threat imposed by the chief pollutants in the air, water, or soil seem to be less effective than ALAN due to its ubiquitous nature, level of influence, and diversity in biological response (1, 2). A number of studies in the last decade, by documenting the impact of ALAN on different ecological components and human health, indicated the severity and consequences of "light pollution" [**Figure 1**; (1)].

The detrimental physiological and behavioral effects resulting from the exposure to light at night are known (2, 8–10), but the influences of light pollution on the emergence and development of the infectious disease are yet to be elucidated. The appearance of new infectious diseases is increasing since the last decade and has posed a significant threat to public health worldwide. The origin of (60–80%) these emerging infectious diseases (EIDs) are reported to be from the wildlife (11). Among the EIDs, influenza, henipavirus, and coronavirus-related respiratory and neurological disorders have caused a severe threat to human health. Influenza viruses belong to the *Orthomyxoviridae* family, containing negative-single-stranded, segmented RNA genome (11). A majority of the seasonal influenza is associated with two types of influenza viruses, influenza A and influenza B (12). Influenza A in humans has originated from birds and swine (13). It is interesting to note that globally people get infected with influenza in the winter season due to the decrease in the ambient temperature and photoperiod (14). The introduction of influenza viruses in humans resulted in global pandemics ("Spanish flu" in 1918 and "swine flu" in 2009) followed by their continued circulation in human populations as seasonal flu. However, influenza B viruses have no known animal reservoir or flow within humans.

Coronaviruses (CoVs) represent a class of diverse genetic viruses found in a varied range of host species, including birds and mammals (15). CoVs are highly pathogenic single-strand RNA virus with a diameter of about 80–120 nm (16).

Graphical Abstract | Impact of artificial light at night on the physiology and behavior of the nocturnal animal, the bat.

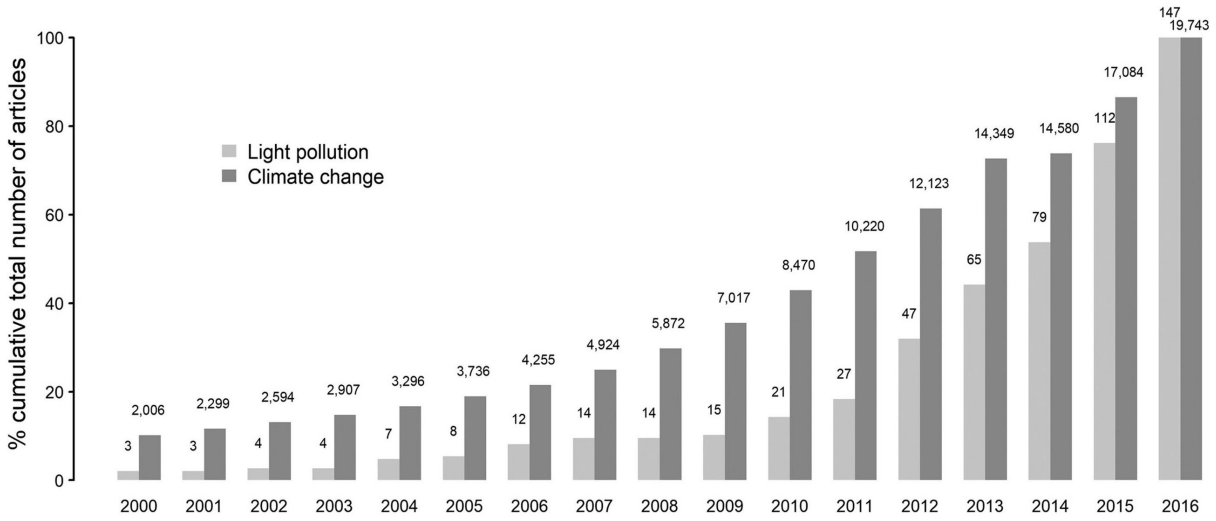

FIGURE 1 | Representation of the trend in research outputs associated with light pollution and climate change since the year 2000. Adapted with permission from (1).

TABLE 1 | Animal origins of HCoVs, Classification, natural reservoirs, outbreak species, year and country of outbreak.

Human coronavirus	Classification of virus	Animal reservoirs (high prevalence)	Outbreak species	Country of origin/Year	References
HCoV-OC43	Alpha-CoV	Mice, chickens, turkeys, swine, dogs, cats, rabbits, horses	Rodents	Russia/1890	(19–23)
HCoV-229E	Beta-CoV, lineage A	Mice, rats, chickens, turkeys, swine, dogs, cats, rabbits, horses	Bats	United Kingdom/1967	(19–21, 24)
SARS-CoV	Beta-CoV, lineage B	Masked palm civets, bats, rats, raccoon dogs, cats, swine	Bats	China/2002	(21, 25, 26)
HCoV-NL63	Alpha-CoV	Bats, mice, rats, swine	Bats	Netherlands/2004	(21, 27)
HCoV-HKU1	Beta-CoV, lineage A	Bats, mice, rats, swine	Rodents	China/2005	(26, 28)
MERS-CoV	Beta-CoV, lineage C	Cattle, chicken, bat, mice, alpacas, swine, dogs	Bats	Saudi Arabia/2012	(29–31)
SARS-CoV-2	Beta-CoV, lineage B	Bats, pangolins	Bats	China/2019	(32, 33)

They infect humans and other animal species, causing intestinal and respiratory infections. The number of confirmed cases of severe acute respiratory syndrome coronavirus-2/coronavirus disease 2019 (SARS-CoV-2/COVID-19) is much higher than that of severe acute respiratory syndrome coronavirus (SARS-CoV) in humans, due to more rapid transmission capability (17). Globally, the number of cases of COVID-19 are ascending steeply, overwhelming the governments, hospitals, and medical care, with 15,296,926 confirmed cases and 628,903 deaths and increasing as of 24 July 2020. CoVs can be divided into four types: α-coronavirus (α-COV), β-coronavirus (β-COV), γ-coronavirus (γ-COV), and δ-coronavirus (δ-COV) (18). Six CoVs were previously known to cause diseases in humans [SARS-CoV in 2002 and Middle East Respiratory Syndrome coronavirus (MERS-CoV) in 2012]. SARS-CoV-2 is the seventh member of the coronavirus [**Table 1**; (34)]. Recently, six novel CoVs have been reported from the bats in Myanmar (35). These six CoVs belong to the same family—the SARS-CoV-2—but distantly related (35). *In silico* sequence analysis gave the evidence of the emergence of SARS-CoV-2 from bats (36, 37). According to a few recent studies, weather plays a definitive role in spreading the infection of COVID-19 (38, 39), maintaining the characteristics of its ancestors, influenza. Despite understanding the mechanism of viral evolution and surveillance, new viruses continue to emerge and cause epidemics and pandemics around the world. The emergence of a novel viral strain is a result of genetic selection. The virus undergoes subtle genetic changes through mutation and major genetic changes through recombination. The main difficulties associated with the emergence of a novel viral strain are the scale of illness to human and other living organisms,

although the processes that underlie the evolutionary dynamics of viruses and the timing and nature of the emergence of new virus strain remain unpredictable.

Impacts of ALAN on animals range from constrained foraging, altered reproduction, and impaired communication (40–43) to a total swing in the trophic interactions (44–46). A recent study has reported that light pollution at night aids in the infectivity of the West Nile virus (WNV) in the house sparrow (*Passer domesticus*) (47). Moreover, light pollution at night also altered the immune defenses of animals, including human by inhibiting the secretion of melatonin, a hormone which is known to enhance viral resistance and regulate immune response (48, 49). It has been established that the rate of secretion of melatonin is reduced following the exposure of light at night (50–53). The involvement of melatonin in immunomodulation is also reported in many studies on animals (54, 55). Exposure of Siberian hamster even to dim light leads to suppressing their immune response (56). It is evident that the acute respiratory disorder after coronavirus infection to human is mainly caused by an overstated immune response (cytokine storm) through inflammation and oxidation (57, 58). Melatonin, a well-known antioxidant and anti-inflammatory agent is protective to critical care patients and is known to act through reducing the permeability of vessels and anxiety and improving sleeping quality (58). Moreover, the existence of identical biosynthesizing machinery for melatonin in several organs in animals (59–66), a subcellular component like mitochondria (67), vouches for the importance of melatonin in cellular physiology.

Bats are known to harbor a wide variety of viruses ranging from coronavirus to ebolavirus to henipavirus (68), without showing any clinical symptoms of the diseases concerned. Their long life span might be the outcome of an intricate balance between the host immune system and virus infection (68). In this communication, we reviewed the relationship between the growing use of ALAN and the ALAN linked threats to bats, as they are the primary reservoirs of CoVs. The focus is also given to the influence of ALAN on the activities of the bats and the virus–host interaction. We tried to frame some future strategies for the prevention of this type of unpredictable zoonotic virus outbreak along with some possible treatments for ALAN-induced reservoirs and infected humans.

IMPACT OF ARTIFICIAL LIGHT AT NIGHT ON THE LIVING SYSTEM—EMERGENCE OF THE IDEA IN THE GLOBAL SCENARIO

Artificial Light at Night and Melatonin—The Physiological Messenger of Environmental Darkness

The circadian and seasonal variations in the animal physiology are directly or indirectly regulated by melatonin, which is further dependent on environmental photo-thermal conditions (61, 69). Light suppresses the synthesis and release of melatonin from the pineal gland and acts as the primary zeitgeber for synchronizing internal rhythms to the temporal change of the external light and dark cycle. Under natural light–dark conditions, melatonin biosynthesis in the pineal gland of most animals including human, bats, rodents, and fish reaches its peak at midnight (70–75). Depending on the species, the biological rhythm and melatonin secretion are controlled by various organs such as the hypothalamic suprachiasmatic nucleus (SCN) and the retina (in mammals) and the brain, pineal, and retina (in fish and amphibians); nonetheless, most animals follow conserved norepinephrine and adrenergic receptor pathways (73, 76–79). The duration of melatonin biosynthesis and secretion is the pivotal parameter for the day-length signaling, which is essential for the organization of the seasonal rhythms (80). Studies following the administration of physiological concentrations of melatonin at the proper time in pinealectomized hamsters and sheep demonstrated the dose- and time-dependent roles of melatonin in the transmission of day-length signaling in animals (80). Further, the annual breeding cycle has also been found desynchronized in pinealectomized sheep (81). In ruminants, melatonin consumption (through food) in summer (before the onset of darkness) can mimic the early onset of seasonal reproductive function, which includes winter coat growth and the suppression of secretion of prolactin, characteristics of reproductive behavior of winter photoperiod (82). Studies demonstrate the importance of daily and seasonal melatonin rhythmicity profile in different animals. However, owing to ALAN, disruption in the diurnal and seasonal variations in the physiology and behavior has been reported in several animal species including humans, bats, and fish (53, 83–85). Light pollution is a global problem, a fact supported by the resolution of the American Medical Association (AMA), declaring that light at night is a source of environmental pollution as it disrupts daily rhythms and suppresses nocturnal melatonin biosynthesis (83, 86). The amount of light required to suppress the melatonin biosynthesis and secretion is dependent on both species and photoperiod (80). For instance, some laboratory-raised animals require less light than the same species raised in the wild (80). In humans, it is identified that the light spectra of between 440 and 482 nm are responsible for the peak melatonin suppression and pupillary constriction (87, 88). Furthermore, it is evident that polychromatic light enriched with short wavelength results in the suppression of melatonin (80, 89, 90). Various cross-sectional studies have pointed out that ordinary domestic light can elicit 50% of the maximum response by phase resetting the diurnal rhythm of melatonin, cortisol, and body temperature (91, 92). Animals exposed to prolonged day-light during summer are found to be partially resistant to the melatonin suppression during night-light (93). Additionally, Morita et al. found that differences in the phases of the diurnal melatonin rhythm depend on the level and pattern of exposure to light (94). The "hypothalamic light perception" may induce the suppression in the melatonin biosynthesis through ALAN even in blind patients (95). The hypothalamic light perception was studied in animals in which the retinohypothalamic projection was intact; but the primary and accessory optic tracts were surgically removed (80, 95). Cumulatively, these results demonstrate the inhibitory effect of ALAN on the diurnal and seasonal rhythmicity of animals.

Moreover, it is also pertinent that melatonin suppression by ALAN is dependent upon the level of exposure to bright light during the daytime and season (96). A comprehensive study on the seasonal or diurnal melatonin rhythm and ALAN on the bats is lacking. Nevertheless, there are several studies that demonstrate that the concentration of melatonin in bats at night ranges from 60 to 500 pg/ml while human nocturnal melatonin level ranges from 11 to 83 pg/ml (74, 97, 98). It is evident that the melatonin level in bats is higher than in humans; therefore, it should have an extremely significant contribution in maintaining bat physiology. It is also found that *Rhinolophus* bats are nocturnal and remain hidden in dark places and thereby less exposed to day-light. This behavior makes them more susceptible to melatonin suppression by ALAN than other animals that are exposed to light in the daytime (97). This suppression of melatonin secretion may be extremely high, which negatively influences the physiology of bats and possibly also on the virus residing in them.

Emergence and Evolution of Artificial Light at Night—A Chronological Country-Wise Scenario

In Russia, around 1890, when HCoV-OC43 crossed the species barrier to infect humans, a pandemic of respiratory infection was observed (23). The second episode of HCoVs named HCoV-229E originated in the 1960s in the United Kingdom (24). Genetically, HCoV-229E is closely related to bat alpha-CoVs; and between alpha-CoVs and HCoV-229E, there exists an alpaca-CoV (32). The direct transmission of previously known CoVs from bats to humans has also been reported (99). Ironically, Russia and the United Kingdom were pioneers in gas street lighting. In 1835, the company for gas lighting was established in St. Petersburg. By 1860s, most of the central streets and buildings of the capital were well-illuminated with gas street lighting. More gas works were functional in the 1870s, and by 1882, Moscow was shining with 10,000 gas lamps. In 1888, almost 210 gas works were operating in Russia, including 30 for lighting cities, 157 for factories, and 23 for railway stations (100, 101). Meanwhile, the United Kingdom became the first country in the world to be lighted by an electric bulb after the development of 16-W lightbulb by Swan/Edison in 1880 [**Table 2**; (123)]. The next significant advancement in artificial lighting was the invention of the sodium-vapor lamp by Compton and light-emitting diode (LED) by Oleg, in 1920 and 1927, respectively [**Table 2**; (124, 125)]. The night lighting quickly advanced with halogen and high-pressure sodium-vapor (HPS) lamp (126, 127), both of which were capable of emitting uninterrupted high-intensity light and became a powerful tool for street lighting. Till that time, the artificial lighting was minimal; most countries did not have electricity. Similarly, the HCoV outbreaks were limited. With massive development in electricity production (hydrothermal and nuclear), artificial light reached the untouched regions. Swiftly, artificial lighting tools became ubiquitous; after the USA and Europe, the Asian continent started to use them for lighting at night. The invention of LED, organic LED (OLED), and liquid crystal display (LCD) screens has further contributed to the artificial lighting.

In the last two decades, five incidences of the HCoV outbreaks have emerged, which is 250% more than the previous 110 years. Similarly, the use of artificial light has increased manifold in the past two decades than in the last century (1). With the beginning of the twenty-first century, another pandemic SARS-CoV surfaced in Guangdong Province of southern China. The artificial lighting was very pronounced in Guangdong Province as compared with the other parts of China (**Figure 2**). Soon after SARS-CoV, another outbreak was observed in 2004 in the Netherlands, named HCoV-NL63. The ALAN was high in the Netherlands starting on the late 20th century (**Figure 3**). Another incidence of HCoVs, the HCoV-HKU1, took place in the next year of HCoV-NL63 in Hong Kong, which is also a massively lit region of China. Subsequently, the world has seen the outbreak of MERS-CoV and SARS-CoV-2 in Saudi Arabia and China, respectively (**Table 1**). These two were rapidly developing countries, with quick urbanization and economic development; it was inevitable to control light pollution.

It may be noted that diurnal animals such as chickens, turkeys, swine, dogs, cats, rabbits, horses, and cattle are also the reservoirs of the coronavirus (25, 129). However, interestingly, all the seven

TABLE 2 | Timeline of the development of Anthropogenic Light sources on Earth.

Year	Artificial lighting technology	Inverter/Country	References
1780	Argand lamp	Aime Argand/Geneva	(102)
1792	Gas lighting	William Murdoch/England	(103)
1800-1809	Arc Lamp	Humphry Davy/England	(104)
1856	Geissler Tube	Heinrich Geissler/Saxe-Meiningen	(105)
1867	Fluorescence lamp	A. E. Becquerel/Paris	(106)
1875	Incandescent light bulb	Henry Woodward/Canada	(107)
1880	Long lasting filament	Thomas Edison/USA	(108)
1894	Gas discharge lamp	D. McFarlan Moore/USA	(109)
1901	Mercury-vapor lamp	Peter Cooper Hewitt/USA	(110)
1904	Tungsten filament	Alexander Just and Franjo Hanaman/Hungry	(111)
1910	Neon lighting	Georges Claude/France	(112)
1913	Inert gas in bulb	Irving Langmuir/USA	(113)
1920	Sodium vapor lamp	Arthur H. Compton/USA	(114)
1927	Light-emitting diode	Oleg Losev/Russia	(115)
1953	Halogen light bulb	Elmer Fridrich/USA	(116)
1962	Red light-emitting diode	Nick Holonyak Jr./USA	(117)
1963	High-pressure sodium vapor lamp	Kurt Schmidt/USA	(118)
1987	Organic light-emitting diode (OLED)	Ching W. Tang and Steven Van Slyke/USA	(119)
1995	Blue LED	Shuji Nakamura/Japan	(120)
2008	LED lighting system with helical fiber filament	G. R. Hulse/USA	(121)
2019	LED filament chips	T. Jiang/Japan	(122)

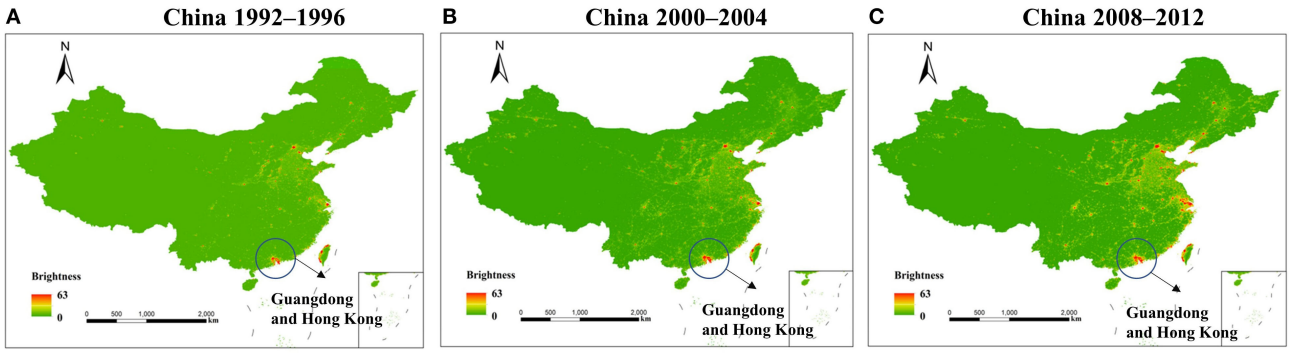

FIGURE 2 | Pixel brightness (calibrated digital number) mean value 1992–1996 **(A)**, 2000–2004 **(B)**, and 2008–2012 **(C)**; Guangdong and Hong Kong were encircled as the two centers of human coronavirus (HCoV) origin. The figure is used under the terms of the Creative Commons attribution license (128).

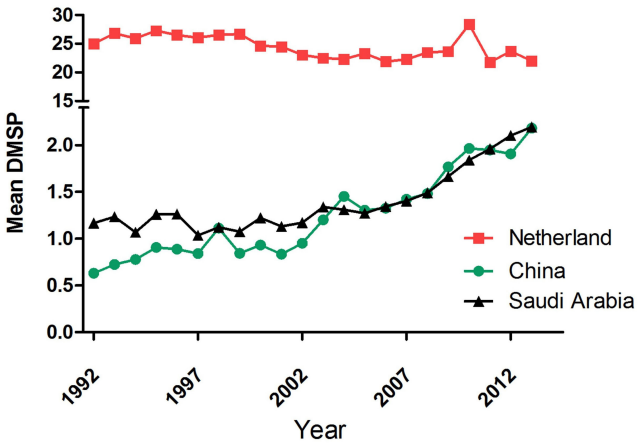

FIGURE 3 | Annual mean the defense meteorological program (DMSP) level in (—■—) the Netherlands, (—●—) China, and (—▲—) Saudi Arabia from 1992 to 2013. Data were obtained from https://www.lightpollutionmap.info.

zoonotic transfers occurred from the nocturnal animals, mostly bats (**Table 1**). Out of the seven outbreaks, three major events of HCoV outbreak happened in China. This 42% outbreak in China is probably related to the fact that these animals are kept in captivity in markets that are usually well-lit at night. The present COVID-19 outbreak is reported to be from Wuhan, China, which also witnessed a massive increase in the ALAN in the past decade (**Figure 4**). Like ALAN, the population density of human in these countries has also increased over time. It can be hypothesized that increased human density and activities, especially, deforestation may also be potential factors for HCoV outbreak, as they can also exert selection pressure on the virus residing in the bats. Considering the significant impact of ALAN on bats, it may be assumed that the population growth supplements the negative effect of ALAN on bats. The evidence from historical and technological comparative assessment might be circumstantial but is good enough to provide the basis of the belief that artificial light plays a significant role in the outbreak of HCoVs.

The Bats as the Natural Reservoirs of Human Coronaviruses

Bats are reservoirs (asymptomatic to the disease) of many viruses, including CoVs that cause severe diseases in humans and animals. The EIDs, mostly caused by the pathogen associated with wildlife species, are a critical threat to human and animal health (130–134). Since the past few decades, the fatal epidemics like acquired immunodeficiency syndrome (AIDS), SARS, filoviruses (e.g., Ebola and Marburg viruses), swine acute diarrhea syndrome (SADS), porcine epidemic diarrhea (PED), and influenza are viral diseases that originated from wildlife species (130), mostly from bats (134, 135). Among many bat-borne RNA viruses, two families of positive-sense single-stranded RNA viruses, namely *Astroviridae* and *Coronaviridae*, are important because of their high transmissibility (136). It is also reported that 100 bat species are found to be the reservoir of viruses causing the disease in animals in the Americas, Africa, Europe, Australia, and Asia (137, 138). The person-to-person transmissibility of CoVs and severe diseases associated with them have enhanced the urgency to study them, as revealed by SARS-CoV (139), MERS (140), and COVID-19 (141).

The Effects of Artificial Light at Night on the Bats

Existing literature on the effects of ALAN on bats necessitates the integration of information on their different behaviors to emphasize the possibilities of the emergence of several fatal viruses.

Behavioral Pattern

Anthropogenic encroachment of natural habitats of animals through deforestation, habitat fragmentation, urbanization (over occupying agricultural land), and bushmeat consumption are considered as the primary drivers that are promoting the interspecies transmission of pathogens from wildlife reservoirs to humans (142–145). Global urbanization and human development by anthropogenic interventions led to a dramatic increase in both the extent and intensity of artificial lighting throughout the twentieth and twenty-first centuries [**Table 2**; (146, 147)]. The use of ALAN is increasing annually by 6%

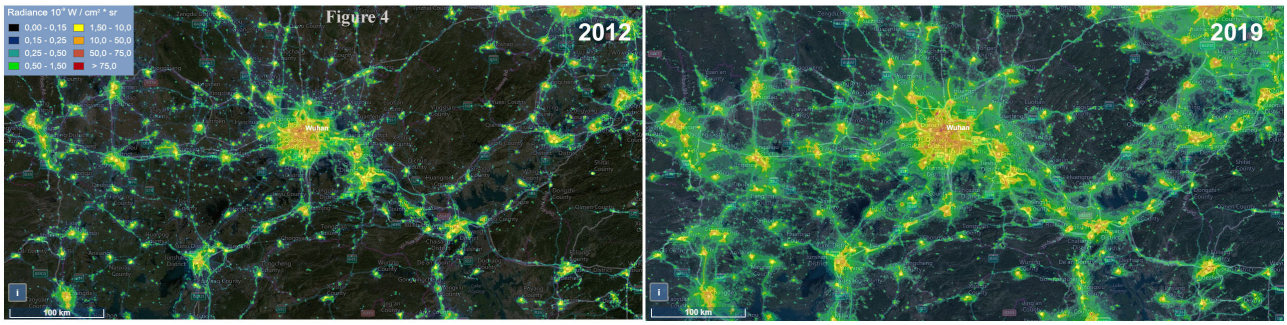

FIGURE 4 | Image showing the change in the level of artificial light at night (ALAN) in Wuhan from 2012 to 2019. Data were obtained from https://www.lightpollutionmap.info.

worldwide (148) and causes nocturnal sky brightness by 20% (9, 149, 150). Light pollution affects every inhabitant of the ecosystem. The habitats of bats are affected either through direct loss or disturbance from artificial light or human activities. Further, connectivity to roosts from foraging areas is fundamental for the survival of many bat populations and is also affected with light at night and so-called development, meant for the human activity only (151). It is evident that the light–dark cycle maintains the daily pattern of activity and behavior in bats (152). The sunset influences the timing of the nightly emergence of bats from the roost; on the other hand, moonlight (153) and the length of night affect the foraging activity and overall behavior of bats (154, 155). The significant impact of ALAN upon bat behaviors, including foraging and commuting, emergence, roosting, breeding, hibernation, and abundance have been recorded (156), but detailed information is not yet available and thereby warrants further investigation.

Commuting Behavior of Bats

The ALAN splits the bats' commuting routes or flyways between roost and foraging areas, causes avoidance behavior, and thereby, fragments the network of flyways. Many species avoid their flyways, which are illuminated with HPS and LED (157–161). As a result, bats are forced to use alternate routes to reach their foraging grounds. This alteration increases energy expenditure due to the enhancement in flight time and also the risk of predation (158). If the alternate route is not available, bat colonies are found to be isolated from their foraging areas and abandon their roost (159). Some light-tolerant bat species pay attention to the streetlights for feeding as the higher number of insects (particularly moths) get attracted toward the lamplight (162). This feeding and foraging behavior increases mortality risk due to collision with the vehicles and predation risk for the juvenile (due to their slow and less agile flight) (163), as also found in many birds (41). Light-sensitive bat species lose their foraging fields due to quick passage in the lit area (164). Further, composition and abundance of prey (insect) for bats also change in the illuminated regions (165).

Emergence, Roosting, and Breeding Behavior

The dusk period is the time for the onset of the emergence of bats due to the availability of insects in the foraging areas (166). Artificial light delays or hampers the time and duration of the emergence of the bats and, thereby, reduces the quality of foraging time and negatively affects the fitness of bats (167). Repeatedly alternating exit/entrance due to ALAN forced bats to abandon the roost and become entombed in the worst cases (168).

External and internal lighting in and around the bat roost causes reduced fitness and hinders juvenile growth rates. As a result, it makes the animals immunodeficient and susceptible to the different pathogens at a tender age and also increases the threat of predation (167). The changes in the internal physiology of the bats may also influence their ability to be the reservoir for different viruses. By virtue, viruses may mutate to find a different host for their sustainability.

Hibernation

The hibernation is a period when bats allow their body temperature to decrease below the active homeothermic level to conserve energy on a seasonal basis in response to the changes in the environmental temperature and food supply (169). The suitable microclimatic conditions allow efficient energy budgeting in bats during this hibernation to survive in winter (170). The stimulation from the artificial lighting during the hibernation of bats results in the significant energy expenditure, lowering fitness and thereby reducing the chance of survivability in the winter and subsequent spring (171). Moreover, artificial light may disrupt circadian rhythms during the hibernation in bats (171); a similar phenomenon of rhythm desynchronization is found even in non-hibernating animals (2, 9).

The anthropogenic disturbances by ALAN cause chronic stress (elevated levels of plasma glucocorticoid hormones) and disruption of homeostasis, which may be due to the desynchronization of the circadian rhythm in bats (172, 173). Stress-induced immunosuppression may increase the susceptibility of the bats to acquire and shed viruses (174). Even hot climatic conditions or long periods of high temperature stimulate rapid amplification and increase the transmission risk of WNV to vertebrates from wild birds (175).

As an obvious outcome of the above-cited studies, the bats have been logically designated as "threatened/endangered" by the International Union for Conservation of Nature (IUCN) (http://

www.batcon.org/why-bats/bats-are/bats-are-threatened). The IUCN listed 24 bat species as critically endangered, 53 others are endangered, and 104 bat species are considered vulnerable. Almost one third of the 1,296 bat species that have been assessed by the IUCN are either threatened or data deficient, indicating the need for more attention for their conservation. This status of the bats might explain the reasons for the viral shedding, the mutation, and the adaptation of these microorganisms to new hosts, and ALAN is one of the crucial factors for that.

Artificial Light at Night and Virus–Host Interaction

The appearance and subsequent circulation of influenza occur along the latitudinal belts and coincide with the changes in the photoperiod (176). This seasonal influenza is synchronized by solar elevation, day length, and solar isolation (176). Influenza mortality in elderly people is probably due to limited sun exposure (176). These studies link the viral infection with the photoperiod; however, there is a lack of information on the effect of ALAN on host–virus interaction, viral evolution, or outbreak.

It is well-accepted that rapid mutation and genetic recombination results in the emergence of novel HCoVs (177, 178). Mutation in the spike (S) protein of the virus might increase the affinity with human angiotensin-converting enzyme 2 (ACE2) receptor (129). Apart from the mechanism mentioned above, the evolution of HCoVs might also be driven by the host-associated selection pressure. However, less is known about the selection pressure exerted by the host on their reservoir community. The reservoir of the CoVs is large and mostly include bat species, and it can be easily hypothesized that CoVs are well-adapted with the anatomy and physiology of bats (179). Furthermore, asymptomatic or minimal disease symptoms were observed when bats were infected with CoVs (135), indicating that bats and HCoVs are mutually adapted.

This interaction of bats and HCoVs may seem beyond the reach for most of us, but they are not beyond our influence. Humans have indirectly disturbed the physiology and behavior of the bats (156) with inconsiderate industrialization and urbanization. Increase in the amount of ALAN has a widespread effect on wild animals, including fish, marine turtles, birds, and nocturnal animals, including bats (8, 9, 40, 180, 181). Impacts of ALAN on nocturnal animals range from constrained foraging, altered reproduction, and impaired communication to a complete shift in trophic interactions and alteration in community structure (40, 45, 182). In plants, it has been reported that appropriate lighting environment is essential for the development of a comprehensive resistance system in various plant–pathogen interactions, including viruses (183). Moreover, artificial light has enhanced the development of the disease in *Nicotiana tabacum* after inoculation with cucumber mosaic virus (CMV) (184). Similarly, in animals, seminal research pointed out that the influence of light pollution can extend the infectious-to-vector window for WNV by 41% in the house sparrow (*Passer domesticus*), an urban-dwelling avian reservoir host of WNV. This indicates that light pollution can directly aid in the virus transmission to humans (47). Light pollution at night causes the sparrow to produce more corticosterone, which alters the regulation of avian physiology via stress-response pathway (47). Similarly, ALAN-induced stress can modulate the pro-inflammatory response in bats, which can efficiently reduce the pathology triggered by CoVs, implying a direct connection between ALAN and bat–HCoV interaction (185). The experimental data on the prolonged exposure of ALAN on zebrafish also indicated a similar enhanced inflammatory response by TNF-α and NF-κβ pathways (9). Moreover, light pollution at night also altered the immune defenses by inhibiting the secretion of melatonin, a chronobiotic hormone that enhances viral resistance, regulates immune response, maintains the level of reactive oxygen species (ROS), and acts as a mediator between the environment and epigenome (83, 186, 187). The high degree of ROS could suppress the replication of CoV and could alter proofreading activity of exoribonuclease (179). Cumulatively, ALAN can change the level of corticosterone, melatonin, viral resistance, immune response, and epigenome along with the high level of ROS. These factors are more than adequate to disturb the natural balance between bats and CoVs and exert a selection pressure on the virus to find a novel host through the mutation in the genetic structure of virus. Therefore, ALAN should be considered as a potential factor that is causing the emergence of the present COVID-19 and previous CoVs. The emergence of five novel CoVs in two ALAN-driven decades should not be considered as a matter of chance or a laboratory construct.

Efficacy of Melatonin in the Reduction of Oxidative Stress and Immune Defense

The antioxidant property of melatonin is mediated by its inherent free radical scavenger activity, up-regulating anti-oxidative enzymes, and down-regulating pro-oxidative enzymes (e.g., nitric oxide synthase) (188, 189). Along with antioxidant property, melatonin has high bioavailability as it can penetrate the blood–brain barrier and placenta (190, 191). Melatonin reduces molecules or particles, which cause oxidative stress along with an increase in anti-oxidative enzymes, such as superoxide dismutase, glutathione peroxidase, and catalase activity (191–194). Viral infection causes oxidative stress by elevating levels of ROS and/or nitrogen species (RNS) (195). Similarly, the high expression of oxidative stress-sensitive gene Group IID secretory phospholipase A2 (PLA2G2D) reduces anti-viral immunity of the organisms (97). Oxidative stress reduces the number and activity of protective immune cells by stimulating immunosuppressive mechanisms, thus producing a pro-inflammatory response (196). Like the neuroendocrine system, the immune system has its circadian rhythm. The production of granulocyte, macrophage, and its phagocytic activity correlates with the nocturnal peak of melatonin (197, 198). Any change in the circadian system can desynchronize the immune system. Melatonin also regulates the immune system and enhances the immune response by improving proliferation and maturation of natural killer cells, T and B lymphocytes, granulocytes, and monocytes in both bone marrow and other tissues (199). Recently, melatonin is considered as a potential adjuvant for improving clinical outcomes of COVID-19 patients (97, 200, 201), though a detailed study regarding the efficacy of this indoleamine in host–virus interaction in both bats and humans is warranted (202, 203).

THE FUTURE STRATEGIES TO MITIGATE THE REPERCUSSIONS OF ARTIFICIAL LIGHT AT NIGHT AND TO MINIMIZE VIRUS OUTBREAK FROM THE BATS

Multiple cross-sectional studies have proved that there is no substitute for natural darkness, and any change in the lighting might have severe implications as observed in several animals (9, 204, 205). On the basis of the research, we want to put forward some strategies to minimize the effect of ALAN on virus outbreak from the bats, like HCoVs.

Evasion

The easiest and effective means to reduce the impact of lighting on bats is to define the bat zones and avoid illuminating them. The use of part-night lighting (PNL: switch off the lights between midnight and 05:30 a.m.) can be imposed in the illuminated bat zones. The PNL will help in limiting the adverse effects of light on bats and other nocturnal animals (206). If it is unavoidable to use the lights in the bat zones, a physical barrier should be built to reduce the area of illumination. In the newly developing sites, with a little bit of research, light exclusion zones (dark regions) can be created to allow movement of bats.

The banning of the bats as food item will reduce the captivity stress and exposure of light on them in the market. This strategy will also help to maintain the bat population.

Use of Artificial Intelligence

Artificial intelligence (AI) coupled with high-resolution infrared cameras may be utilized to develop an artificial system that can distinguish between the human and wild animals at night. Lin et al. have developed a framework consisting of optical deep learning methods, through neural networks using multiple layers of diffractive surfaces programmed to execute subjective task that resulted in the statistical learning of the network through the computer (207). This method can be implemented to create a camera system that can capture and analyze any fast-moving object or an animal, such as the bats, and can classify them based on machine learning and deep neural network. The camera system should be off at all the time unless it detects a human, or the program will turn off the lights of the zone in which bats are flying. This strategy can be modified as per the need of the area to be monitored.

Variable Lighting Regimes/Planned Positioning of Artificial Lights

As avoidance is not possible in all the scenarios and AI can be expensive, alternatively, a careful study should be conducted to develop variable lighting regimes (VLRs), which will be compatible for both human being and the wildlife. It has been suggested that tree cover can help in mitigating the adverse effects of ALAN on the bats by shielding the light (208). A careful study of tree cover and other shades before installing artificial lighting might help to shield the bats from ALAN. Moreover, adding trees in already lit areas will help in reducing the repercussions of ALAN on the bats. Horizontal or upward emission contributes substantially to light pollution by generating skyglow; this can be significantly reduced by using directional lighting (209). As poorly designed luminaires cause most of the light pollution and skyglow, effective luminaire design, installation of shielding fixtures, and correct column height can reduce the skyglow. Hedgerows, the vegetation canopies, can also be used to decrease light exposure, because many bat species use linear features as traveling routes (210). Even though developing a tree fence is very promising and might help the environment in multiple ways, including the reduction of the effect of ALAN, the negative impact of ALAN on the plants should also be considered. Therefore, utmost care should be given in the selection of plant species for developing the tree canopy.

Changing the Type and the Intensity of the Artificial Light

Several studies have shown that bats are equally active in red light and darkness (211). Therefore, careful selection of lighting wavelength is paramount to reduce the stress of ALAN on the bats and other nocturnal animals. The red light should be used in places where it is unavoidable to limit the timing of illumination. Similarly, some bats and insects species thrive better in low-intensity lighting (212). So lower-intensity lights can be utilized to mitigate the negative impact of ALAN on bats. These two strategies will inevitably be a compromise among human necessities. However, these minor changes do not appear to be a bad deal if they can help in avoiding the outbreak of pandemics and protect species from getting extinct.

The Use of Melatonin Spray as Reversal Therapy for the Treatment of Artificial Light at Night-Exposed Animals

Exposure of light on the bats can decrease their level of melatonin (74). Melatonin has potent antioxidant activity and anti-inflammatory activity, maintains biological rhythm, and protects against lipid peroxidation (213, 214); thereby, the reduction in the level of melatonin causes severe consequences on animal physiology. Recently, researchers have developed melatonin-loaded nano-capsule, spray-dried powders, and hydrogels to improve their stability even in the aqueous solution (215, 216). These nano-capsules can be sprayed on the animals that are already exposed to ALAN. This melatonin spray will decrease the inflammation in the bats and might also help in minimizing the level of selection pressure on the HCoVs residing in the bats.

Exploratory Research

Previous studies demonstrated that CoV genomes display a high degree of plasticity in terms of gene content and recombination (32). Furthermore, relatively large CoV genome increases the probabilities for adaptive mutations, making it easier for the viral spike protein to exploit cell surface receptors of other species for virus attachment and entry (32, 33, 217). Therefore, exploratory research is warranted to understand the factors determining the emergence and evolution of the novel pathogens like COVID-19. Further, emphasis should also be given to the factors (including anthropogenic stress like light pollution) that increase the rate of selection pressure, transmission, and infection. It is of note that the deadly HCoVs come mainly from their nocturnal reservoir host. More research is required to find out the other host of CoVs

in the wild and potential factors that are causing the evolution and origin of HCoVs.

Transdisciplinary Approach

Multiple disciplines should collaborate to study the origin and evolution of HCoVs and to develop rules and guidelines to minimize such pandemic outbreaks. Scientists, policymakers, and engineers need to work together to implement strategies to reduce the impact of artificial light on bats. Finally, it is imperative to expand awareness of light pollution and its ecological impacts.

CONCLUSION

The present review is a meaningful attempt to correlate the development of ALAN and emergence of HCoVs. The influence of ALAN on bats including inappropriate foraging and commuting, untimely emergence, roosting, breeding, and impaired physiology have put them under the threatened status in the IUCN list. ALAN-induced behavioral and physiological stress might have exerted an immense amount of selection pressure on the diverse form of CoVs residing in the bats. Except for the study on WNV by Kernbach et al. (47), most evidence of the impact of ALAN and virus infectivity is correlational; therefore, more studies are required to confirm the role and potential of ALAN in virus outbreaks from wildlife. Moreover, the effect of ALAN on farmed and domesticated animals is mostly unknown. The future research should be focused on these animals to prevent any outbreak of anonymous zoonotic transmission, influenced by ALAN. Given the fact that bats carry a variety of viruses with the capability to infect human and other organisms, it is essential to monitor wild animal species for any novel zoonosis. A global surveillance network involving veterinarians and animal biologists is urgently needed to monitor, and possibly to predict, potential sources for the emergence of other highly pathogenic CoVs. Besides, as the entire human population goes under lockdown, there is a surge in the use of ALAN; the classes, meetings, and cinema are all online. Presently, we are exposed to ALAN more than ever in the history of mankind and also in the companionship of arrhythmic lifestyle. Strict measures should be taken to minimize this exposure. Otherwise, we will be facing a huge group of human beings with lifestyle disorders.

Scientists are furnishing data since the last decades about the detrimental effect of ALAN and are recommending various strategies to reduce the effect (https://www.anses.fr/en/content/leds-anses%E2%80%99s-recommendations-limiting-exposure-blue-light). The earth is rhythmic in both circadian and circannual patterns, the human physiology is synchronized with the "natural light," and any desynchronization in these processes may lead to an unmatched pandemic, to which we are fighting.

AUTHOR'S NOTE

This paper reviewed the potentiality of the artificial light at night (ALAN) in the outbreak of the HCoVs.

AUTHOR CONTRIBUTIONS

ZK, TY, GM, SD, CR, RL, and HS drafted the original of the respective subsections. ZK and CR prepared the figures. ZK and GM reviewed the manuscript. AC developed the concept with thorough literature searching and discussion in the group, made the outlines, analyzed the subsections through review, and critically edited the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by a core grant from IBSD, Imphal, Unit of Excellence grant of Department of Biotechnology (DBT), (BT/407/NE/U-Excel/2013), Department of Science and Technology (SR/FT/LS-179/2010), Ministry of Science and Technology, Government of India to AC. The CSIR-JRF Programme was granted to CR [(09/1044(0002)/2012-EMR-I(14/11/2012)], IBSD PhD-JRF Programme to HS, DBT-JRF Programme to ZK (DBT/JRF/14/AL/819), and CSIR-JRF Programme to GM [09/1044(0003)/2016-EMR-I(20/12/2016)].

ACKNOWLEDGMENTS

We are grateful to the Directors, IBSD, India, along with all the scientists, technicians, administrative persons, for their support. We are appreciative to Mr. L. Umakanta Singh, Mr. S. Surjit Singh, Mr. H. Bishorjit, and Mr. W. Rahul for their constant support. We are also grateful to the security personnel of the Institute for their assistance. We are indebted to the reviewers for valuable suggestions that improved the manuscript. Our sincere gratitude to all Indian taxpayers for their contribution toward the funding for research.

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

frontiers in Endocrinology

OPINION
published: 30 September 2020
doi: 10.3389/fendo.2020.567824

Vitamin D and Sex Differences in COVID-19

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Keywords: coronavirus disease 2019, vitamin D, estrogen, inflammation, sex/gender differences

INTRODUCTION

Hypovitaminosis D is implicated in various inflammatory, infectious and autoimmune diseases and recent lines of evidence suggest that it may represent a risk factor also for the ongoing epidemic of coronavirus disease 2019 (COVID-19) (1–3). In fact, the outcome of COVID-19 appears to be influenced by vitamin D status of populations (4, 5).

Several studies have clearly shown that 1,25(OH)2 vitamin D(3) (Vitamin D3, the active metabolite of vitamin D), besides its classical function in calcium dependent bone homeostasis, is actively involved in the regulation of innate and adaptive immune responses (6). In particular, it plays a key role in the control of the cytokine storm, i.e., the sudden acute increase in circulating levels of different pro-inflammatory cytokines, induced in several inflammatory conditions and also in COVID-19 (7). This activity of Vitamin D3 is carried out by inhibiting the production of the pro-inflammatory cytokines such as tumor necrosis factor- α (TNF-α) and interferon-γ (IFN-γ) but, also, by increasing the expression of the anti-inflammatory cytokine interleukin-10 (IL-10). Moreover, Vitamin D3 enhances the production of antimicrobial peptides such as human cathelicidin, LL-37 and defensins in several infections (8). A further important feature of Vitamin D3 is its capacity to reduce the risk of viral infections maintaining the integrity of the epithelium by the upregulation of genes which encode proteins required for tight, gap and adherens junctions (6). Further studies will be necessary to clarify whether all these anti-microbial effects could also occur against SARS-CoV-2, assigning to Vitamin D3 a protective role against COVID-19. Notably, Vitamin D3 enhances the expression of human angiotensin-converting enzyme 2 (ACE2), the functional receptor for SARS-CoV-2 (9, 10). ACE2 plays a protective role in acute respiratory distress syndrome and higher levels of ACE2 seem to be associated with better outcomes for lung diseases and, in particular, for COVID-19 (11–13).

Based on these considerations, in COVID-19 the inter-individual variability in circulating levels of 25-hydroxyvitamin D (25(OH)D), the biomarker of vitamin D status could be involved in the different severity of pulmonary inflammation and viral pathogenicity among individuals (14). Supporting the important protective role of Vitamin D3 in COVID-19 outbreak, negative correlations between mean levels of Vitamin D3 of European countries and the number of COVID-19 cases were observed (1, 13). Moreover, the lethality rate increased with age and with chronic disease comorbidity, both of which are associated with decreased vitamin D3 levels (15).

OPEN ACCESS

Edited by:
Alexandra Kautzky-Willer, Medical University of Vienna, Austria

Reviewed by:
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Specialty section:
This article was submitted to Translational Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *30 May 2020*
Accepted: *14 September 2020*
Published: *30 September 2020*

Citation:
Pagano MT, Peruzzu D, Ruggieri A, Ortona E and Gagliardi MC (2020) Vitamin D and Sex Differences in COVID-19. Front. Endocrinol. 11:567824. doi: 10.3389/fendo.2020.567824

COVID-19 AND SEX DIFFERENCES

To note, epidemiological data indicate that COVID-19 has a significantly higher lethality in men than in women (ratios up to 3:1), suggesting the presence of sex-dependent biological factors underlying these differences in disease outcome (16, 17). It is known that, in general, innate and acquired immune responses are more intense in females than in males (18). This can provide women with a more effective defense to fight new and infective pathogens, favoring viral clearance. Another significant explanation for sex differences in COVID-19 lethality is the sex–dependent modulation of cellular receptors and co-receptors used by SARS-CoV-2 to enter human host cells. In particular, ACE2 is encoded on X-chromosome, in sites commonly escaping the inactivation of one X-chromosome in mammalian XX cells (XCI), and could therefore be overexpressed in women (19). Moreover, estrogen induces an increase of ACE2 expression that, as reported above, could play a protective role in acute respiratory distress (11, 20), whereas androgen can increase the expression and activation of transmembrane serine-protease 2, (TMPRSS2), that facilitates virus-cell membrane fusion, thus favoring the infection (21).

Moreover, a large number of COVID-19 patients exhibit severe cardiovascular damage and those with pre-existing cardiovascular diseases appear to have an increased risk of death (22). To note, estrogen has known protective effects on the cardiovascular system mediated by estrogen receptors, resulting in the activation of endothelial nitric oxide synthase. Moreover, estrogen modulates serum lipoprotein and triglyceride levels and influences the expression of coagulant and fibrinolytic proteins. These estrogen-mediated actions could represent a further reason for the sex-specific differences in the outcome of COVID-19 (23, 24).

COVID-19 AND SEX DIFFERENCES: A ROLE FOR VITAMIN D3

A further interesting point is represented by the potential differences in serum level of 25(OH)D among men and women. Sanghera and co-workers (15) observed a significantly reduced level of 25(OH)D in both men and women with obesity that represents a further important risk factor for COVID-19. In this study, 25(OH)D level remains consistently lower in obese men than in obese women (15). On the contrary, in another study, Mucogiuri and co-workers (25) stratifying the sample population according to sex and body mass index (BMI), found that 25(OH)D concentrations were significantly higher in males compared to females in all BMI classes and decreased along with the increase of BMI values. Although these contrasting data seem to not assign to 25(OH)D a clear role in determining sex differences in obese COVID-19 patients, we think that attention could be paid to 25(OH)D levels in the context of this comorbidity.

Interestingly, sex differences have been observed in the immunomodulatory and anti-inflammatory effects of Vitamin D3 in some autoimmune diseases. In particular, a study of Correale and co-workers (26) showed that Vitamin D3 induces a stronger inhibition of the production of pro-inflammatory cytokines and a higher increase of anti-inflammatory cytokines in lymphocytes from multiple sclerosis female patients in comparison to those from male patients. Interestingly, Spanier and co-workers (27) suggested that Vitamin D3 acts in an estrogen-dependent manner in controlling T regulatory cell differentiation. Moreover, estrogen seems to increase the expression of the nuclear vitamin D receptor (VDR) gene in CD4+ T cells (28) and to decrease the expression of CYP24A1, the cytochrome P450 component of the 25-hydroxyvitamin D(3)-24-hydroxylase enzyme which inactivates Vitamin D3. In turn, Vitamin D3 exerts tissue-specific effects on peripheral estrogen metabolism (29). Hence, the sex-related immunomodulatory effects of Vitamin D3 suggest that it is possible to speculate that also in COVID-19, Vitamin D3 could play a role in the outcome and lethality.

CONCLUSIONS

In conclusion, the outcome of COVID-19 appears to be influenced by the interaction among genetic, hormonal and environmental factors. The low levels of 25(OH)D could represent a risk factor for development of disease. In particular, it is tempting to hypothesize that the synergy between Vitamin D3 and estrogen could affect the sex differences in the outcome of patients with COVID-19. However, further studies will be mandatory in order to investigate the efficacy of Vitamin D3 supplements, in combination or not with estrogen agonists, as a valid adjuvant for prevention and/or treatment of this severe infectious disease.

AUTHOR CONTRIBUTIONS

MP and DP: study conception and design, and manuscript drafting. AR: critical revision. EO and MG: study conception and design, and critical revision. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We thank Simona Anticoli, Maria Bellenghi, Camilla Cittadini, Katia Fecchi, Elisabetta Iessi, and Giada Pontecorvi for their precious support and constructive discussions.

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

frontiers in Endocrinology

REVIEW
published: 30 September 2020
doi: 10.3389/fendo.2020.583006

Stabilizing Cellular Barriers: Raising the Shields Against COVID-19

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The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and its clinical manifestation (COVID-19; coronavirus disease 2019) have caused a worldwide health crisis. Disruption of epithelial and endothelial barriers is a key clinical turning point that differentiates patients who are likely to develop severe COVID-19 outcomes: it marks a significant escalation in respiratory symptoms, loss of viral containment and a progression toward multi-organ dysfunction. These barrier mechanisms are independently compromised by known COVID-19 risk factors, including diabetes, obesity and aging: thus, a synergism between these underlying conditions and SARS-CoV-2 mechanisms may explain why these risk factors correlate with more severe outcomes. This review examines the key cellular mechanisms that SARS-CoV-2 and its underlying risk factors utilize to disrupt barrier function. As an outlook, we propose that glucagon-like peptide 1 (GLP-1) may be a therapeutic intervention that can slow COVID-19 progression and improve clinical outcome following SARS-CoV-2 infection. GLP-1 signaling activates barrier-promoting processes that directly oppose the pro-inflammatory mechanisms commandeered by SARS-CoV-2 and its underlying risk factors.

Keywords: glucagon like peptide 1 (GLP-1), enteroendocrine, lung, immune cells, tumor necrosis factor (TNF), tumor necrosis factor converting enzyme (TACE), endothelial barrier disruption, acute respiratory and circulatory disruption

OPEN ACCESS

Edited by:
Pieter de Lange, University of Campania Luigi Vanvitelli, Italy

Reviewed by:
Stuart Maudsley, University of Antwerp, Belgium
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Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *13 July 2020*
Accepted: *24 August 2020*
Published: *30 September 2020*

Citation:
Hanchard J, Capó-Vélez CM, Deusch K, Lidington D and Bolz S-S (2020) Stabilizing Cellular Barriers: Raising the Shields Against COVID-19. Front. Endocrinol. 11:583006. doi: 10.3389/fendo.2020.583006

INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreak, first reported in December 2019 in Wuhan, China (1), rapidly evolved into a global pandemic. Comprehensive lock-down measures in most affected jurisdictions have slowed down the spread of the virus, however, in the face of the massive collateral societal and economic damage, these measures are clearly unsustainable. The core issue at hand is the lack of specific treatments for SARS-CoV-2: several antiviral strategies are undergoing clinical study, but thus far, efficacy is limited (2, 3); vaccines are in development, but these will likely not be ready in time to mitigate the current wave or prevent a second global wave. Thus, it is incumbent that we identify interventions that increase the resilience to SARS-CoV-2 infection, particularly in populations at risk of severe responses. To do so, a comprehensive understanding of the disease pathology and the risk factors that increase susceptibility for severe disease progression is required.

SARS-CoV-2 infection can be symptomless in some individuals, while on the other end of the continuum, severe cases elicit terminal multi-organ failure (4, 5). The majority of cases are mild; ∼20% of cases require clinical intervention, with ∼5% progressing to critically ill stages where mortality is high (5.8% global case fatality ratio, ranging from 0.1 to 16% by country) (1, 6, 7). Overall susceptibility, the likelihood of developing severe symptoms, and mortality all correlate with several known risk factors, including high BMI (>30) (8), diabetes (9–11), hypertension (8, 10) and age [corrected for comorbidities (8–10)]. In all likelihood, these risk factors compromise immune responses and/or permit systemic viral entry and replication. With regard to the latter, the SARS-CoV-2 virus is capable of compromising two critical barriers: the epithelial-endothelial barrier in lung alveoli and the vascular endothelial barrier in the systemic circulation. Indeed, alveolar-endothelial barrier failure is likely the key turning point differentiating patients who will quickly worsen into severe cases, as it marks a significant escalation in respiratory symptoms, the loss of viral containment and a progression toward multi-organ dysfunction (12–14).

This review will focus on the key cellular mechanisms that SARS-CoV-2 utilizes to disrupt epithelial and endothelial barriers. These barrier mechanisms are independently compromised by known coronavirus disease 2019 (COVID-19) risk factors; a combination effect may explain why these risk factors correlate with more severe outcomes. As an outlook, we propose a therapeutic intervention that may slow COVID-19 progression and improve clinical outcome following SARS-CoV-2 infection. In this regard, glucagon-like peptide 1 (GLP-1) signaling activates barrier-promoting processes that directly oppose the pro-inflammatory mechanisms commandeered by SARS-CoV-2 and its underlying risk factors. Thus, medications that stimulate GLP-1 signaling, e.g., exendin-4, may have unappreciated utility for COVID-19 treatment.

INFECTION MECHANISM

The mechanisms mediating SARS-CoV-2 infection and viral replication are already defined and will not be described in detail in this review (15–17). Briefly, host cells must express two components that are critical for SARS-CoV-2 infection: (i) angiotensin converting enzyme 2 (ACE2), the surface receptor that mediates viral attachment to the host cell, and (ii) the transmembrane serine protease TMPRSS2, which cleaves the viral spike protein, thereby priming viral fusion to the host cell's membrane (16, 18). All barrier forming cells, including lung epithelial cells (19–21), enteric epithelial cells (22, 23) and vascular endothelial cells (22) express ACE2 and TMPRSS2 in high abundance and therefore, are targeted by the SARS-CoV-2 virus. An additional element, named tumor necrosis factor converting enzyme (TACE; ADAM17), may facilitate viral entry, although the molecular mechanisms mediating the enhanced entry have not been defined (24, 25).

In the absence of an effective vaccine, intervention strategies have primarily focussed on reducing (i) SARS-CoV-2 fusion/entry, (ii) SARS-CoV-2 replication and (iii) excessive inflammation (2, 3). Obviously, preventing SARS-CoV-2 infection is more desirable than reacting to infection: thus, targeting ACE2/SARS-CoV-2 binding and TMPRSS2 activity, the crucial host proteins involved in viral entry, are highly attractive therapeutic strategies. In this regard, a clinical-grade recombinant ACE2 decoy receptor (26) and the clinically available TMPRSS2 inhibitor camostat (18) have displayed positive results *in vitro*; however, these strategies have yet to be assessed in clinical trials and are currently a long way from the patient's bedside. In fact, after months of intensive study, most medications repurposed to combat COVID-19, including the notable candidates hydroxychloroquine (27) and lopinavir-ritonavir (28), have failed to demonstrate benefit in randomized placebo-controlled clinical trials. At present, remdesivir, an adenosine nucleotide analog that hampers viral replication (29), is the only candidate (30, 31) with an active FDA emergency use authorization (EUA) at present. However, remdesivir is clearly not a *magic bullet intervention* (31) and may yet fail to demonstrate benefit in properly powered randomized placebo-controlled clinical trials.

Since targeting SARS-CoV-2 viral entry and replication has not been successful to date, "containing" the virus to the respiratory tract is of paramount importance. Since SARS-CoV-2 is predominantly transmitted through the inhalation of airborne droplets and aerosols, epithelial cells within the upper and lower respiratory tract are the first barriers to be attacked. If the virus breaches this barrier and enters the cardiovascular system, the virus will have the opportunity to infect every organ in the body via the microcirculation (32). Indeed, pronounced vascular injury in association with diffuse alveolar damage is a key feature of SARS-CoV, a relative of SARS-CoV-2 that also targets ACE2 (33).

POSSIBLE ROUTES TO THE SYSTEMIC CIRCULATION

In most cases, SARS-CoV-2 remains confined to the upper respiratory tract, favoring mild symptoms. Epithelial cell infection in the upper airways is associated with copious viral shedding, high person-to-person transmissibility, occasional loss of olfaction, sore throat, fever, and a characteristic dry cough. The nasal mucosa potentially provides a highly vascularized entry point to the systemic circulation if the virus can alter the properties of the restrictive tight junctions in the nasopharyngeal epithelium and underlying microvascular endothelium (34).

From the upper respiratory tract, the SARS-CoV-2 may descend down the trachea and infect cells in the lower respiratory tract and alveoli. At the bronchiolar level, SARS-CoV-2 can infect epithelial goblet cells (35), resulting in airway inflammation and mucous secretion. The inflammatory response subsequently impairs mucociliary clearance, which hampers the clearance of the viral particles, and elicits complications such as bronchiectasis and bronchial wall thickening (36). At the alveolar level, infection and subsequent disruption of the "blood-air barrier," which comprises alveolar epithelial cells and pulmonary microvascular endothelial cells, is a central event in disease progression: in essence, it is a transition point from relatively

moderate symptoms to the severe respiratory symptoms and lung injury observed in severe COVID-19 cases. The compromised barrier becomes leaky, permitting alveolar fluid accumulation (edema), and the development of pneumonia and inflammatory cell infiltration. The resulting hypoxia and damage unleashes a "cytokine storm" in a subset of patients that perpetuates a vicious cycle of progressive lung injury, as the inflammatory response further damages pulmonary cells and compromises endothelial function and barrier integrity (37). In addition to driving severe lung injury, the breach of the blood-air barrier permits viral entry into the systemic circulation, where the virus can then cause widespread multi-organ damage (12, 14).

TACE IS A KEY DRIVER OF SARS-COV-2 SEVERITY

Not all coronavirus infections elicit severe respiratory system injury and multiorgan damage: for example, HNL63-CoV, a coronavirus that also binds to ACE2 (38), generally causes relatively mild common cold symptoms (39). Although HNL63-CoV and SARS-CoV-2 bind to the same surface receptor, a key difference between the two viruses resides in the activation of TACE: SARS-CoV strongly activates TACE sheddase activity (24, 25), while HNL63-CoV does not (24). This suggests that TACE activation is a key underlying aspect of the SARS-CoV-2 disease severity.

TACE has more than 80 known substrates, including growth factors, cytokines, cell surface receptors and adhesion molecules (40) and hence, plays complex roles in many regulatory processes (40): thus, it is not surprising that perturbing normal TACE function yields a broad spectrum of deleterious effects. In the context of the COVID-19 pathology, three particular TACE substrates stand out: ACE2 (41), tumor necrosis factor (TNF) (4, 42), and the endothelial protein C receptor (EPCR) (43). All three of these proteins play important anti-inflammatory and barrier-stabilization roles: TACE-dependent shedding of these cell surface proteins, therefore, shifts a delicate balance in favor of inflammation and reduced barrier integrity (**Figure 1**).

The physiological functions of ACE2 and the implications of ACE2 shedding in COVID-19 have been extensively reviewed by Gheblawi et al. (41). ACE2 is a central element in the Renin-Angiotensin-Aldosterone System (RAAS) and therefore, has wide ranging effects that intersect with virtually every endocrine and inflammatory mechanism (44). At the molecular level, ACE2 converts angiotensin II into angiotensin (1-7): angiotensin II activates the pro-inflammatory angiotensin II receptor subtype 1 (AT1R) (45), while angiotensin (1-7) preferentially activates the anti-inflammatory angiotensin II receptor subtype 2 (AT2R) (46) and Mas receptors (46–48). Thus, TACE-dependent ACE2 shedding (41, 49) in COVID-19 shifts RAAS signaling in favor of the pro-inflammatory AT1 receptors (50), which favors immune cell adhesion (51), cellular damage (51), and increased vascular permeability (via the modulation of VE-cadherin function) (52, 53).

Although TNF is best known as a pro-inflammatory cytokine, TNF serves many developmental, homeostatic and reparative functions (54–56). In pathological settings, TNF is a critical initiating factor in the immune response; importantly, if the initial pathogenic insult and/or tissue damage is severe, the immune response can spiral out-of-control into the highly damaging "cytokine storm" (54–58). At the level of the endothelium, TNF stimulates two key homeostatic changes: (i) the expression of immune cell adhesion molecules (e.g., VCAM-1 and ICAM1) (59, 60), and (ii) an increase in barrier permeability, via cytoskeletal rearrangement (61, 62) and the regulation of cell-to-cell adhesion junctions (63, 64). These changes allow immune cells to bind to the endothelium at the site of injury/infection and transmigrate into the tissue through the paracellular junctions (54, 58). Among other notable acute effects, TNF also simulates endothelial reactive oxygen species generation and impairs nitric oxide production (54), which can have significant effects on tissue damage and vascular control mechanisms (65, 66). In COVID-19, TACE-dependent TNF shedding favors the rapid breakdown of the endothelial-alveolar barrier, resulting in lung edema, immune cell infiltration, and ultimately, lung tissue damage. This barrier breakdown also opens the gateway to the systemic circulation: once distal endothelial cells are infected; the same inflammatory mechanism provides a means for the virus to escape the systemic circulation and into organ parenchymal cells.

The endothelial protein C receptor (EPCR) (67, 68) is best known for its anti-coagulatory functions (69); however, EPCR signaling also plays an important role in moderating inflammation (70–72), maintaining endothelial barrier function (73, 74) and conferring cytoprotection (75–77). EPCR binds the zymogen protein C and cleaves it into an active protease: this activated form of protein C remains bound to the EPCR and subsequently (i) cleaves protease-activated receptor 1 (PAR1) and (ii) transactivates the sphingosine-1-phosphate receptor 1 subtype ($S1PR_1$) (67, 68). The activation of these receptors inhibits nuclear factor-κB (NF-κB) translocation/signaling, thereby reducing proinflammatory gene expression, the release of cytokines, and the expression of adhesion molecules (67, 68). In addition, activated protein C shifts PAR1 signals from RhoA-dependent, barrier permeabilizing actions to Rac1-dependent, barrier stabilizing actions (67, 68). In the context of COVID-19, TACE-dependent EPCR shedding removes an important molecular brake that dampens immune cell infiltration, edema and tissue damage; by eliminating a barrier stabilization mechanism, the loss of EPCR signaling also undermines viral containment. Finally, deficient EPCR signaling, a key brake element in the coagulation pathway, likely also contributes to the high incidence of thrombotic complications observed in serious COVID-19 cases, including widespread microthrombi, pulmonary embolism, stroke and disseminated intravascular coagulation (12, 78, 79).

In summary, the activation of TACE may significantly augment the inflammatory response following SARS-CoV-2 infection. As a component of the inflammation, endothelial permeability may become severely compromised, permitting the SARS-CoV-2 virus with access to the systemic circulation. Given the remarkably large surface area of the microvascular endothelium (3,000-4,000 m^2) and its presence in every tissue,

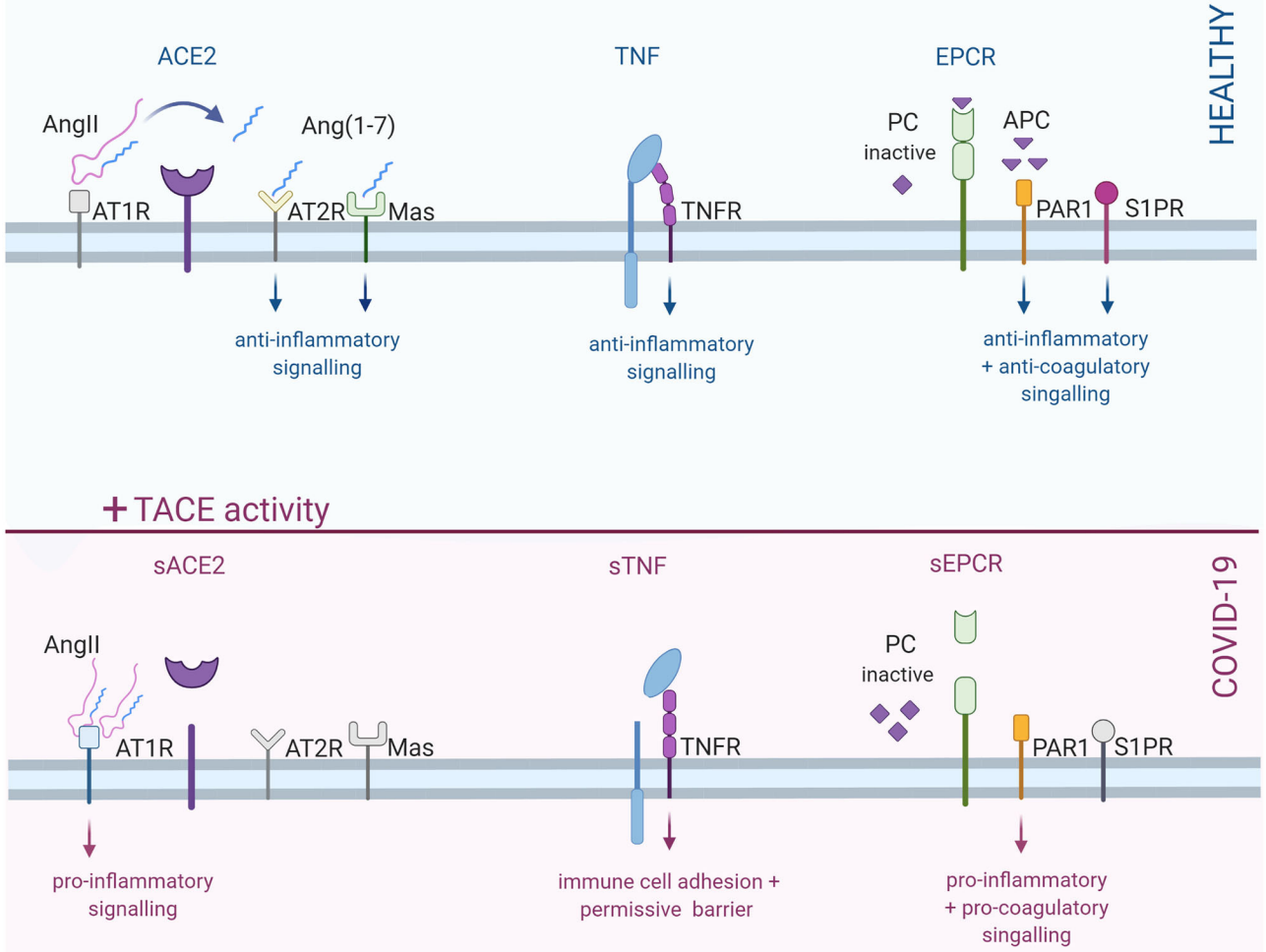

FIGURE 1 | Increased TACE activity in COVID-19 shifts barrier cells toward a pro-inflammatory, pro-coagulatory and permissive state. In healthy cells, TACE is predominantly inactive, thereby permitting anti-inflammatory signals via TACE substrates, including ACE2, TNF, and EPCR. ACE2, a key player in the RAAS system, converts angiotensin II (AngII) into angiotensin-1-7 [Ang (1–7)]: the subsequent activation of AT2R and Mas receptors stimulates anti-inflammatory signals. When ACE2 is cleaved, angiotensin signaling shifts from AT2R/Mas receptors to the pro-inflammatory AT1R. TNF receptors stimulate differential signals, depending on whether the receptor is activated by the membrane-bound or soluble form of TNF: membrane bound TNF elicits important anti-inflammatory signals; in contrast, soluble TNF mediates pro-inflammatory signals. Finally, EPCR plays a critical role in regulating coagulation by converting inactive protein C (PC) into activated protein C (APC). Activated protein C subsequently inactivates pro-coagulatory factors; it also cleaves PAR1 and transactivates S1PR, resulting anti-inflammatory and barrier-stabilizing signals. TACE-dependent EPCR cleavage prevents the activation of PC, leading to reduced capacity to inhibit coagulation; RhoA-dependent signaling via uncleaved PAR1 is barrier destabilizing. TACE, tumor necrosis factor alpha converting enzyme; ACE2, angiotensin converting enzyme 2; TNF, tumor necrosis factor; EPCR, endothelial protein C receptor; RAAS, rennin-angiotensin-aldosterone system; AT2R, angiotensin receptor 2; AT1R, angiotensin receptor 1; PAR1, protease activated receptor 1; S1PR, sphingosine-1-phosphate receptor; TNFR, TNF receptor. Created with BioRender.com.

the failure to maintain this barrier eliminates the last line of defense against multi-organ damage and failure.

COVID-19 RISK FACTORS THAT COMPROMISE BARRIER FUNCTION

Several cardiovascular risk factors, including hypertension, obesity, type 2 diabetes mellitus (T2D) and cardiovascular disease, are common underlying conditions in COVID-19 patients (80, 81). Although these risk factors tend to associate with more severe cases, the individual contribution of each risk factor to COVID-19 disease severity/outcome is difficult to define, due to the small sample size of most studies, combined with the fact that these risk factors largely overlap [e.g., 52% of patients with T2D are obese; and 60% of obese patients have metabolic syndrome including hypertension as a leading symptom (82–85)]. To add a further challenge, age is a significant COVID-19 risk factor (86) that has generally not been taken into account in most risk factor studies (81). According to Tadic et al.

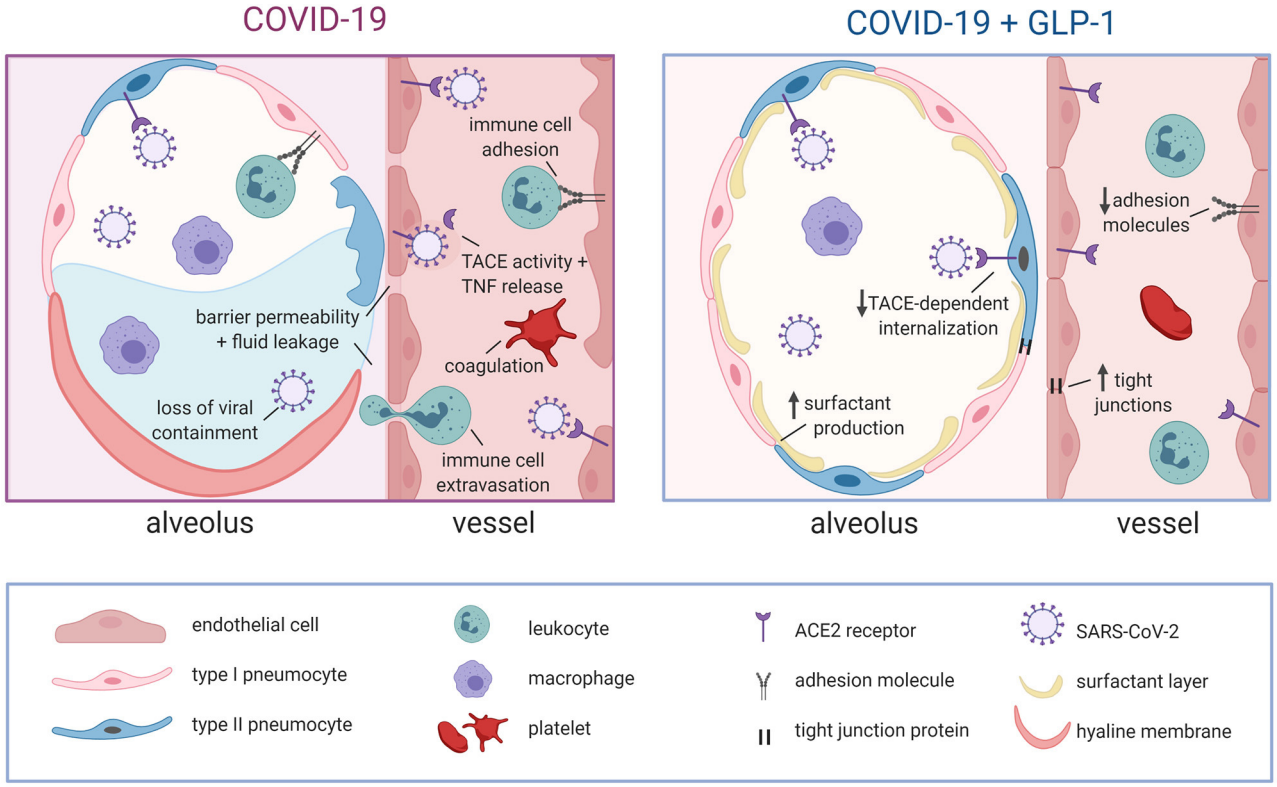

FIGURE 2 | Proposed mechanisms for GLP-1-mediated stabilization of alveolar epithelial and endothelial barriers in COVID-19. In COVID-19, alveolar epithelial cells are severely damaged by direct viral attack and the immune response, leading to hyaline membrane formation, increased barrier permeability, alveolar fluid accumulation and a loss of viral containment. Viral attack on the underlying endothelial cells leads to TACE activation that: augments pro-inflammatory signaling, increases endothelial barrier permeability, promotes immune cell adhesion/extravasation and compromises EPCR-dependent regulation of coagulation. Notably, the loss of endothelial barrier integrity further exacerbates alveolar fluid accumulation. GLP-1 receptor signaling directly opposes these pathological mechanisms by: attenuating TACE expression/activity, initiating counteracting anti-inflammatory signals, promoting barrier function via increased tight junction expression and stimulating surfactant secretion (reduces alveolar fluid accumulation by decreasing surface tension). The GLP-1 mediated attenuation of TACE activity/expression is also expected to hinder viral entry into cells. GLP-1, glucagon like peptide 1; ACE2, angiotensin converting enzyme 2; TACE, tumor necrosis factor alpha converting enzyme; TNF, tumor necrosis factor; EPCR, endothelial protein C receptor. Created with BioRender.com.

(81), most risk assessment studies are too small, inconsistent, and fail to account for several confounding factors, most notably age and obesity: consequently, they are ill-equipped to discern important interactions between comorbidities and outcomes. Thus, the data are more epidemiological than analytical and should be viewed cautiously (81).

Although the precise relationships between COVID-19 risk factors and disease severity require clarification, there are obvious mechanistic commonalities across these risk factors that permits speculation as to why certain common underlying conditions appear to cluster with more severe outcomes. In this sense, we can predict that the outcomes should be more severe, based on deleterious mechanisms that are already activated prior to infection. As a prime example, metabolic conditions such as obesity and T2D down-regulate tissue inhibitor of metalloproteinase 3 (TIMP3), an important endogenous TACE inhibitor that critically regulates TACE and the release of TNF in metabolic tissues (87, 88). The resulting increase in TACE activity induces a proinflammatory state (e.g., cytokine production, immune cell adhesion molecule expression, and hyperpermeability) and pronounced endothelial dysfunction (e.g., reactive oxygen species generation and impaired autacoid release) (89–92) observed in obese and T2D patients. Aging is a double-hit: in addition to inducing pro-inflammatory endothelial dysfunction (93, 94), it also diminishes infection defense ("immune senescence") (95, 96).

A common thread across these risk factors is chronic low-level inflammation and endothelial dysfunction: these risk factors facilitate infection, prime an exaggerated immune response and/or compromise viral containment. Thus, it is entirely reasonable to expect that these underlying conditions would synergize with SARS-CoV-2 infection mechanisms to more profoundly erode barrier function and drive more severe disease progression. These underlying conditions also profoundly impact the patient's immunological status, which is an obvious determinant of COVID-19 severity (97). Indeed, deep immune profiling reveals that hospitalized patients fall across a spectrum of immune response patterns (98),

with underlying conditions contributing to the diversity in host responses.

GLP-1 AGONISTS: A POTENTIAL INTERVENTION FOR MITIGATING COVID-19

Glucagon-like-peptide 1 (GLP-1) is an enteroendocrine hormone, originally characterized in the orchestration of insulin release in response to ingested nutritional stimuli (99, 100). However, GLP-1 signaling plays a vital role in energy metabolism and cell viability in several tissues: thus, targeting GLP-1 receptors can potentially elicit systems-level effects (101). In this regard, GLP-1 has emerged as an important homeostatic element within the cardiovascular system, where it possesses significant endothelial-protective functions (102, 103). It is also interesting to note that all of the barrier-forming cells in the lung and vascular system express GLP-1 receptors (104–108).

In the lung, GLP-1 tightens barriers via the upregulation of tight junction proteins in barrier-forming cells (108, 109); in alveolar type 2 pneumocytes, GLP-1 stimulates the production of surfactant that, by reducing surface tension, helps to minimize fluid accumulation within alveolar spaces (110). In endothelial cells, GLP-1 inhibits TACE expression and activity (111): it therefore directly opposes key mechanisms that SARS-CoV-2 commandeers to augment inflammation and compromise barrier function. Accordingly, GLP-1 signaling attenuates TACE-dependent EPCR shedding (111); GLP-1 signaling also increases deficient ACE2 levels in pathological settings (112), presumably via reduced ACE2 shedding. Consistent with this anti-inflammatory role, GLP-1 receptor agonists possess several desirable actions, including (i) antagonizing inflammatory NF-κB signaling (103), (ii) reducing immune cell adhesion molecule expression on endothelial cell surfaces (e.g., ICAM-1 and VCAM-1) (103, 113), (iii) reducing immune cells cytokine production (113), and (iv) attenuating endothelial cell oxidative stress (114).

With regard to the risk factors associated with severe COVID-19 cases, it is remarkable to note that GLP-1 receptor agonists (e.g., exendin-4, liraglutide, semaglutide) are either FDA-approved (diabetes) (115) or proposed (obesity, age-related decline) (116, 117) as an intervention for the underlying conditions. GLP-1 receptor agonists were specifically developed to harness GLP-1's potent hypoglycemic effect in the treatment of diabetes (115): the improved glycemic control results in weight loss in T2D patients and consequently, clinical trials are currently assessing their utility as an anti-obesity drug for patients without T2D (116). In addition to its functions as a metabolic hormone, GLP-1 possesses significant anti-inflammatory effects that are independent of glucose homeostasis (118). Consequently, GLP-1 receptor agonists are now being considered for age-related pathologies, including Alzheimer's disease, Parkinson's disease, and cognitive decline, all of which possess a strong inflammatory component (117, 119). Since GLP-1 signaling exerts clear beneficial effects in obesity, T2D and aging, it must potently ameliorate a common pathological thread across these conditions: chronic low-level inflammation and endothelial dysfunction. Since (i) both the risk factors for severe COVID-19 (e.g., obesity, diabetes, age) and the SARS-CoV-2 virus harness similar pro-inflammatory mechanisms and (ii) GLP-1 signaling is anti-inflammatory and has demonstrable benefits in patients with underlying conditions (118), it stands to reason that GLP-1 signaling should directly oppose the inflammatory mechanisms activated in COVID-19. In this context, GLP-1 agonists would be a useful mechanism-based treatment strategy (**Figure 2**).

GLP-1 SECRETAGOGUES: A NOVEL OPPORTUNITY FOR PROPHYLAXIS

Given that GLP-1 signaling confers many positive benefits, especially in individuals with COVID-19 risk factors (118, 120, 121), it is intriguing to hypothesize that the benefits of GLP-1 can be harnessed prophylactically. In essence, the objective would be to activate the endogenous barrier-promoting and anti-inflammatory actions of GLP-1 signaling prior to SARS-CoV-2 infection, with the prospect that this would increase resilience in the event of infection. Based on the rapid and dramatic metabolic effects observed in Roux-en-Y Gastric Bypass (RYGB) patients (122, 123) there is no doubt that the intestinal tract has the enteroendocrine capacity to drive significant beneficial effects on the systemic level. Further, extreme measures are not necessary to elicit systemic effects: even normal, post-prandial GLP-1 secretion is sufficient to stimulate nitric oxide production in the forearm, thereby increasing blood flow and oxygen uptake (124, 125). The fact that "normal" enteroendocrine GLP-1 signaling activates the endothelium is important, because it suggests that the full repertoire of positive GLP-1 effects may be in play. Thus, if endogenous GLP-1 signaling could be stably or perpetually activated, it may be possible to increase endothelial and epithelial "resilience" to SARS-CoV-2 infection.

Eliciting prophylactic GLP-1 release from the intestine is likely mechanistically simple, extremely safe, and in all probability, very low cost. Virtually any carbohydrate, lipid or protein macronutrient could be used as a candidate secretagogue, as these nutrients clearly mobilize GLP-1 secretion mechanisms (126–128). Assuming that secretagogues stimulate sufficient GLP-1 release to positively influence inflammatory and barrier-promoting mechanisms at the systemic level, there would be little argument that these stimuli would: (i) be safe to ingest, as they are basic food components, (ii) not cause the adverse effects associated with the supra-physiological levels of GLP-1 signaling elicited by GLP-1 agonists, such as headache, vomiting or diarrhea (129), (iii) be cost effective to manufacture and purchase and (iv) have immediate worldwide availability. This strategy is obviously speculative and not currently available for use against COVID-19; it is nevertheless worth pursuing, as it could have broad implications for patients with obesity, T2D, cardiovascular disease and other pathologies that target the endothelium, inflammatory mechanisms or barrier function.

SUMMARY AND OUTLOOK

In summary, the breakdown of cellular barriers is a key driver of severe SARS-CoV-2 disease progression. The underlying pro-inflammatory mechanisms that disrupt barrier function in COVID-19 are well-characterized and substantially overlap with the disease mechanisms operating in diabetes, obesity and aging, all putative COVID-19 risk factors. Since anti-inflammatory interventions that strongly supress immune function (e.g., anti-TNF therapeutics) are not recommended for treating COVID-19, we need to deploy other options that interfere with these barrier-disrupting mechanisms. GLP-1 is an intriguing candidate, because it possesses both anti-inflammatory and barrier-promoting properties. Indeed, GLP-1 signaling is currently proposed as an intervention for the very risk factors that also drive aggravated COVID-19 severity. It is, therefore, tempting to speculate that GLP-1 signaling could be harnessed to fight COVID-19 on two levels: secretagogues could prophylactically increase the global population's resilience to the infection, and in acute COVID-19, GLP-1 receptor agonists may be useful in supporting acute therapeutic interventions.

AUTHOR CONTRIBUTIONS

JH, CC-V, and S-SB conceived this review. JH and CC-V contributed equally to the literature research and figure preparation. JH, CC-V, KD, DL, and S-SB all significantly contributed to writing and revising the manuscript.

FUNDING

S-SB receives stipend support from a Heart and Stroke Foundation of Ontario Mid-Career Investigator award. Aphaia Pharma AG provided funding for publication charges, and had no other involvement in the study.

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Conflict of Interest: S-SB and KD are executive board members of Aphaia Pharma AG, JH and CC-V are employees of Aphaia Pharma AG, and DL is a paid consultant for Aphaia Pharma AG.

frontiers in Endocrinology

SYSTEMATIC REVIEW
published: 11 November 2020
doi: 10.3389/fendo.2020.557333

Understanding the Clinical Features of Coronavirus Disease 2019 From the Perspective of Aging: A Systematic Review and Meta-Analysis

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Problems: An outbreak of novel coronavirus (2019-nCov) infection is now widespread in multiple countries. Compared with adult patients, elderly patients have not received enough attention. The aim of the meta-analysis was to assess the clinical characteristics of elder patients with COVID-19.

Methods: A deep literature search was performed in the databases through August 21, 2020. Risk ratio (OR) and 95% confidence intervals (CIs) were pooled using analysis models.

Results: Three studies including 2046 infected patients were precisely evaluated, and the results show that the elderly group has a higher risk of hypertension, diabetes, and cardiovascular disease than the younger patients. Their total white blood cells are higher than that of the younger patients, and their lymphocytes are relatively reduced compared with the younger patients.

Conclusion: We comprehensively assessed the clinical characteristics of patients of different ages with COVID-19 and found that elder patients had a high risk of chronic cardiovascular and metabolism comorbidities. The characteristic clinical manifestations and laboratory examinations of elderly patients support their excessive inflammation and weak immune defenses against 2019-nCoV. All these findings provide important information for understanding the general clinical characterization of the aging immune defense against the virus and enhancing the public awareness of the prevention and treatment of elder patients in the COVID-19 pandemic.

Keywords: 2019 novel coronavirus, 2019 coronavirus disease, aging patients, meta-analysis, immune senescence, clinical characteristics

OPEN ACCESS

Edited by:
Alexandra Kautzky-Willer, Medical University of Vienna, Austria

Reviewed by:
Ma. Cecilia Opazo, Andres Bello University, Chile
Peter Klimek, Medical University of Vienna, Austria

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Specialty section:
This article was submitted to Translational Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *21 May 2020*
Accepted: *07 September 2020*
Published: *11 November 2020*

Citation:
Yifan C and Jun P (2020) Understanding the Clinical Features of Coronavirus Disease 2019 From the Perspective of Aging: A Systematic Review and Meta-Analysis. Front. Endocrinol. 11:557333. doi: 10.3389/fendo.2020.557333

INTRODUCTION

Since a group of unexplained pneumonia related to the Huanan seafood market was reported in Wuhan, China, on December 31, 2019 (1), this new coronavirus, named 2019 novel coronavirus (2019-nCoV) or severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which was soon confirmed by Chinese scientists as a new betacoronavirus (2), is now causing the 2019 coronavirus disease (COVID-19) pandemic. It has been undoubtedly confirmed that the number of incidence cases, the total death toll, the duration of the epidemic, and the property damage to the international community caused by this virus pandemic are far superior to other coronavirus infectious diseases, including the severe acute respiratory syndrome (SARS) caused by the coronavirus (SARS-CoV) from 2002 to 2003 (3) and the middle east respiratory syndrome (MERS) caused by the coronavirus (MERS-CoV) since 2012 (4). Although the global fatality rate of this disease has yet to be determined, it has caused more than 22,054,300 infections and 779,443 deaths worldwide until August 19, 2020 (5), and has brought huge short- and long-term losses to the globe economy without specific therapies.

It is a huge challenge for the global health system, especially for developed communities with a high proportion of elderly people. Interpersonal transmission of this new virus has even occurred in many nursing facilities around the world (6). In past research, aging is considered to be a complex multilevel change, characterized by a progressive physiological dysfunction and disability to stabilize the homeostasis, leading to an increased incidence of degenerative diseases and deaths (7). At the same time, aging shows significant modulations at the cellular and system level, including the formation of senescent cells and the imbalance of cytokine regulation. Among them, the most obvious phenomenon is the disorder of the immune system, including immune decline caused by changes in the number and proportion of immune cells and chronic inflammation caused by high expression of inflammatory factors, which was also called "inflammaging" or "immune senescence" (8). The decline of the immune system weakens the immune function of the elderly population against pathogens such as the coronavirus, and the state of chronic inflammation increases the risk of cytokine storms.

Although some information about the epidemiology of older patients with COVID-19 has been accumulated, there is a lack of relevant comprehensive reports, and the related protective and diagnostic measures are not perfect. Therefore, it is urgent to pay attention to the prevention and control of infectious diseases in elderly people in aging society today because people of different ages are generally susceptible to this virus. Meanwhile, comparing the differences of clinical features between two patients of different ages can help doctors to carry out targeted individual treatments all over the world to save unnecessary waste of medical resources and maximize medical efficiency. Here, we have systematically reviewed the single-center or multicenter observational studies of older patients with COVID-19 and comprehensively dissected the true impact of age as a complex variable on COVID-19 disease.

METHODS

Search Strategy and Selection Criteria

A literature search was performed on studies published from December 1, 2019, to August 21, 2020, in PubMed, EMBASE, Cochrane Library, Scopus, and Web of Science databases without restriction to regions or languages. The following terms —"Coronavirus" OR "Coronavirus infections" OR "betacoronavirus 1" OR "betacoronavirus" OR "SARS-CoV-2" OR "COVID-19" OR "2019-nCoV" OR "new coronary pneumonia" OR "novel coronavirus" OR "coronavirus disease" OR "coronavirus disease 2019" OR "2019 novel coronavirus infection" combined with "descriptive study" OR "retrospective study" OR "cross-sectional study" OR "case-control study" OR "cohort study"—were used for searching. We also searched the preprint platform medRxiv, bioRxiv, and the reference list of each selected study to make sure not to miss relevant papers. All retrieved publications were managed by EndNote X9.0 software.

The studies were selected based on the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement. Eligibility criteria are as follows: (a) research types: descriptive studies including case-control studies, retrospective cross-sectional studies, cohort studies, and case series; (b) research subjects: patients with laboratory-confirmed COVID-19; (c) studies comparing clinical characteristics, laboratory findings, and outcomes for the elderly and young. Exclusive criteria are as follows: (a) study types: reviews, editorials, opinions, letters, case reports, consensus documents, meta-analyses, and clinical trials; (b) studies, including duplicate data; (c) studies about nonhuman SARS-CoV-2 infection; (d) studies without young patients as controls.

Data Collection and Quality Assessment

Two independent investigators reviewed titles and abstracts of all retrieved studies to include eligible studies and then extracted data from these proper studies using a predefined data extraction form. Disagreements in the process between two investigators were resolved by discussion or consensus with a third investigator. Extracted data included the following: study name, first author name, date of publication, country, sample size, baseline features (i.e., mean age, sex, clinical symptoms, comorbidities), lung computed tomography (CT), therapies, complications and prognosis. The risk of bias of selected studies was assessed using the Newcastle-Ottawa Scale (NOS) standard, which consists of three factors: patient selection, comparability of the study groups, and assessment of outcome. A score of <6 means a low-quality study and would be excluded from meta-analysis.

Statistical Analysis

Categorical variables were reported as the number of cases and percentage, and continuous variables were reported as mean ± standard deviation (SD). We used the data conversion method proposed to estimate mean and standard deviation when the studies only include median and interquartile range (IQR) (9). All statistical analysis was performed by STATA MP, version

16.0. For meta-analysis, the standard mean difference (SMD) and odds ratio (OR) were respectively used to compare continuous and categorical outcomes. All results were reported with 95% confidence intervals (CIs), and were presented as Forest plots. I-square (I^2) value was used to evaluate heterogeneity across studies, with $I^2 \leq 50\%$ representing statistically homogeneous and $I^2>50\%$ representing statistically heterogeneous. If I^2 is negative, we set it to 0.00%. We performed a fixed-effects model using the Mantel Haenszel statistical method to evaluate average effects when $I^2 \leq 50\%$ and a random-effects model with REML method when $I^2>50\%$. The significance level of the meta-analysis was set to $\alpha = 0.05$. Because the number of included studies is small, we didn't assess the publication bias by both funnel plots and Egger's linear regression test.

RESULTS

Research Flow and the Baseline Data of the Aging Patients With COVID-19

A preliminary search included a total of 9228 publications. After removing duplicates, a total number of 7076 documents could be initially identified. By overlapping articles and excluding case reports, reviews, editorials, opinions, and randomized controlled trials (RCTs) by the title and abstract, 7004 documents were left. Then, 62 studies were excluded because they were not designed to group by age, and 7 studies were excluded because they provided incomplete clinical baseline data after reviewing the full text. One additional study was included from the reference list of included articles. Therefore, four descriptive researches were included in the final pooled analysis (**Figure 1**), meeting the predetermined inclusion and exclusion criteria with a sample of 2047 patients confirmed with COVID-19 (736 aging patients, 35.96% of the whole) (10–13).

As the baseline presented in **Table 1 (A)**, 2047 patients confirmed with COVID-19 from different areas in China were included in the meta-analysis. The average ages of the older group are 67.67 ± 3.62, 68.28 ± 7.31, 76.08 ± 4.73, and 76.67 ± 19.79 years and 12 (66.67%), 58 (42.65%), 250 (47.44%), and 34 (61.81%) of them are men, and the average ages of the younger group are 44.67 ± 11.94, 41.15 ± 11.38, 44.33 ± 14.13, and 43.33 ± 32.94 years and 19 (50.00%), 349 (53.53%), 216 (45.67%), and 74 (50.00%) are men.

Higher Risk of Cardiovascular and Metabolic Comorbidities of the Aging Patients With COVID-19

Chronic basic comorbidities of the aging patients with COVID-19 in **Table 1 (B)** include hypertension, diabetes, chronic kidney disease, liver disease, heart diseases, chronic obstructive pulmonary disease (COPD), immune dysfunction, cancers, and other diseases. The result of this meta-analysis shows a

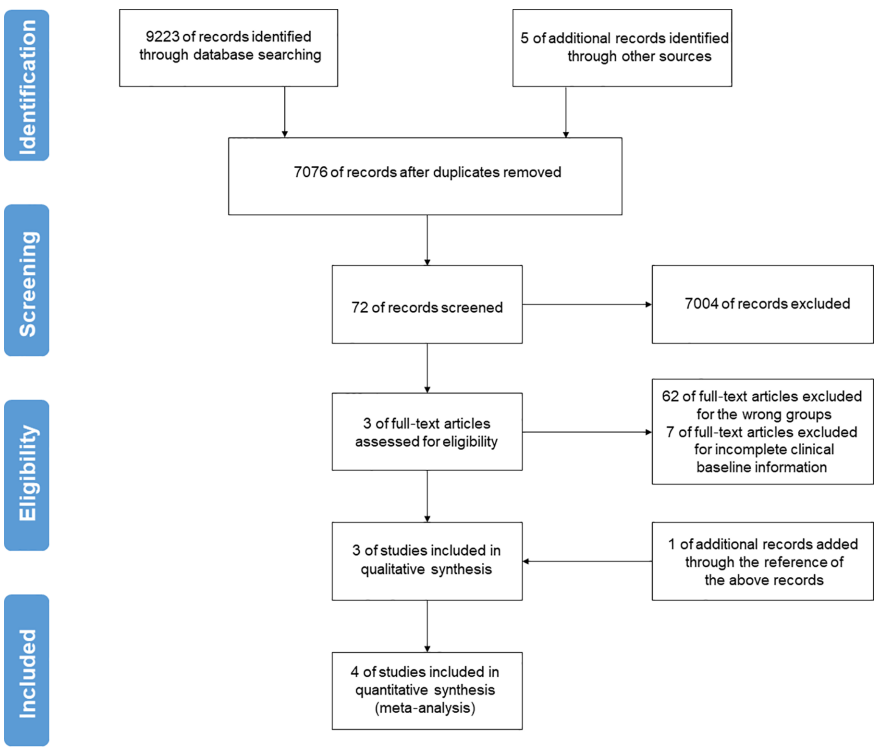

FIGURE 1 | Flow-chart of the literature search and selection strategy in this meta-analysis. After applying the inclusion and exclusion criteria, a total of 4 studies were included in the final pooled analysis.

TABLE 1 | Baseline characteristics and chronic comorbidities of the included studies in this meta-analysis.

(A) Baseline characteristics of the aging patients with COVID-19.

Studies	Years	Countries	Provinces	Centers	No. of patients			Age		Sex (Male/Female)		
					Total	Older	Younger	Older	Younger	Total	Older	Younger
Liu et al. (12)	2020	China	Hainan	Single-center	56	18	38	67.67 ± 3.62	44.67 ± 11.94	31/25	12/6	19/19
Lian et al. (10)	2020	China	Zhejiang	Multi-center	788	136	652	68.28 ± 7.31	41.15 ± 11.38	407/381	58/78	349/303
Zhao et al. (13)	2020	China	Hubei	Single-center	1000	527	473	76.08 ± 4.73	44.33 ± 14.13	466/534	250/277	216/257
Chen et al. (11)	2020	China	Hubei	Single-center	203	55	148	76.67 ± 19.79	43.33 ± 32.94	108/95	34/21	74/74

(B) Chronic comorbidities of the aging patients with COVID-19.

Study	Hypertension			Diabetes			Chronic liver disease			Chronic renal disease		
	Total	Younger	Older	Total	Younger	Older	Total	Younger	Older	Total	Younger	Older
Zhao et al.	282	62	220	118	34	84	29	12	17	24	7	17
Liu et al.	10	5	5	4	1	3	1	0	1	1	1	0
Chen et al.	43	22	21	16	4	12	8	6	2	8	5	3
Lian et al.	126	73	53	57	33	24	31	25	6	7	5	2

Study	Heart disease			COPD			Immune dysfunction			Cancers		
	Total	Younger	Older	Total	Younger	Older	Total	Younger	Older	Total	Younger	Older
Zhao et al.	60	6	54	23	0	23	13	3	10	28	7	21
Liu et al.	3	0	3	NA	NA	NA	NA	NA	NA	NA	NA	NA
Chen et al.	16	5	11	8	1	7	6	5	1	7	2	5
Lian et al.	11	5	6	3	0	3	1	0	1	6	3	3

significant difference between the elderly group and the young group (**Figure 2**). Compared with the youth group, the elderly group has the higher risk of hypertension, diabetes, and cardiovascular disease (OR 4.60, 95% CI: 3.63–5.84), (OR 4.02, 95% CI: 2.15–7.52), and (OR 8.24, 95% CI: 4.54–14.26) and with a lower heterogeneity (I^2 = 0.00%) in the fixed-effects models and obvious statistical significance (p <0.01) except the analysis of diabetes, which has a slightly larger heterogeneity (I^2 = 58.45%)

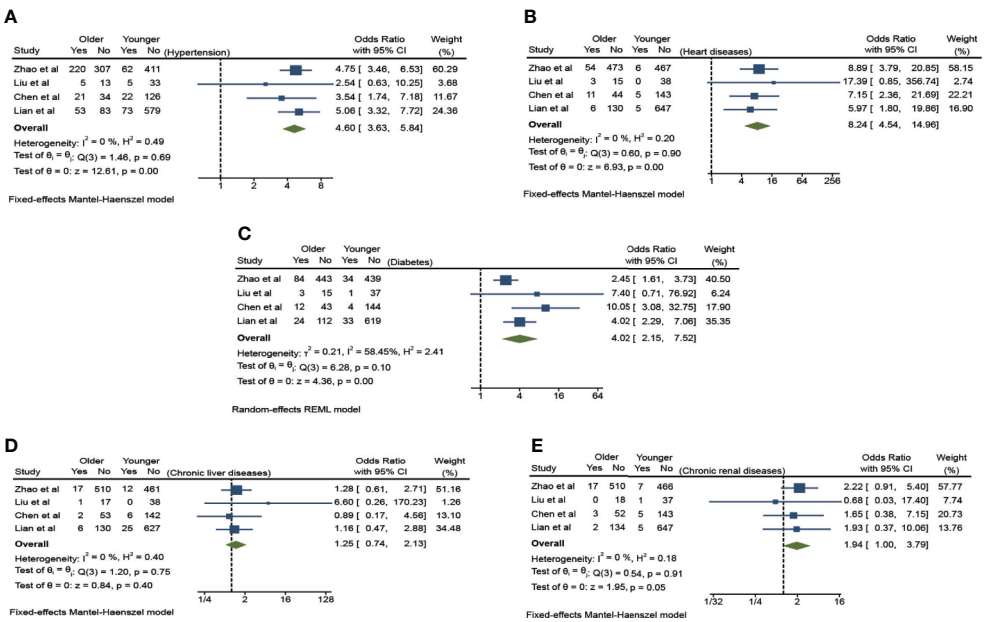

FIGURE 2 | The effect of chronic comorbidities on the risk of elderly patients with COVID-19 compared to younger patients. **(A)** Hypertension, **(B)** Diabetes, **(C)** Heart diseases, **(D)** Chronic liver diseases, **(E)** Chronic renal diseases.

in the random-effects model. Although the risk of chronic kidney disease and liver disease is slightly increased (OR 1.94, 95% CI: 1.00–3.79), (OR 1.25, 95% CI: 0.74–2.13), there is no significant statistical difference ($p > 0.05$).

Differences of Clinical Symptoms in Aging Patients With COVID-19

In addition to comorbidities, the clinical manifestations of elderly patients are also worthy of attention. Here, we have classified and summarized the common clinical manifestations between the two groups in **Table 2** according to general symptoms, respiratory symptoms and extrarespiratory symptoms, which makes it more concise and clearer to show the symptomatic differences. Because different studies have different statistical standards on symptomology, it is difficult to make quantitative analysis of catarrhal symptoms, sore throat, cough, expectoration, chest pain, chest tightness, dyspnea, gastrointestinal symptoms, and neuromuscular symptoms. The meta-analysis results here (**Figure 3**) show that the risk of fever is slightly increased in the elderly group among the common symptoms (OR 1.19, 95% CI: 0.93–1.51), and the risk of fatigue in aging patients is slightly decreased (OR 0.85, 95% CI: 0.68–1.06), but there are no statistically significant differences between the two groups ($p > 0.05$).

High Inflammatory State and Low Immune Defense of the Aging Patients With COVID-19

Laboratory tests of elderly patients show great heterogeneity according to the differences between the elderly group and the young group in **Table 3**. Here, blood routines are accounted to analyze the changes in the proportion of peripheral blood lymphocytes in elderly patients. Statistical analysis is performed on the C-reactive protein, procalcitonin, and interleukin-6 to infer the inflammatory and immune response of elderly patients, and the platelets and D-dimer are counted to characterize the coagulation function of aging patients. The total number of white blood cells in elderly patients is higher than that in young patients (SMD 0.30, 95% CI: 0.20–0.39, $p < 0.01$) (**Figure 4A**). At the same time, the risk of an increase in the total number of white blood cells in elderly patients is higher than that in the youth group (OR 3.37, 95% CI: 1.53–7.43, $p < 0.01$) (**Figure 4B**). Meanwhile, the risk of a decreased white blood cell count in elderly patients was also higher than that in the younger group (OR 0.77, 95% CI: 0.60–0.98, $p = 0.04$) (**Figure 4C**) with absolutely no heterogeneity ($I^2 = 0\%$). These changes depict a high variability of white blood cells in aging patients, which means a fragile unsteady state caused by COVID-19 in the elderly population.

When it comes to lymphocytes, the situation is opposite. The total number of lymphocytes in elderly patients is lower than that in young patients (SMD -0.47, 95% CI: -0.72 to -0.22, $p < 0.01$) (**Figure 4D**), and the risk of lymphocyte decline is higher than that in young patients (OR 2.73, 95% CI: 2.22–3.36, $p < 0.01$), and there is no significant heterogeneity ($I^2 = 0.00\%$) (**Figure 4E**). All these findings support the high inflammatory state in elderly patients because the total white blood cells increase in aging patients while the lymphocytes decrease inversely. The decline of lymphocytes also shows a fragile immune defense of the elderly population.

In terms of measuring the coagulation function, the platelet number of elderly patients is lower than that of the young group (SMD -0.22, 95% CI: -0.32 to -0.13, $p < 0.01$), and there is no significant heterogeneity ($I^2 = 0.00\%$) (**Figure 4F**). In terms of inflammation-related measures, the C-reactive protein (CRP) of elderly patients is higher than that of young people (SMD 0.65, 95% CI: 0.23–1.07, $p < 0.01$) (**Figure 4G**) with a high heterogeneity ($I^2 = 91.92\%$) in a random-effects model, which also reflects the excessive inflammation in the elderly patients.

DISCUSSION

With the intensification of global aging, how to deal with the medical challenges brought about by the aging society is a topic of widespread concern. Aging is considered to be closely related to the increasing incidence of a series of chronic diseases (14, 15). Changes in the composition of multiple immune cells and the decline of immune function constitute "immune aging" in the natural aging process (16). Meanwhile, the global novel coronavirus epidemic reminds us that the prevention and control strategy of new infectious diseases in the elderly population is a more complex and comprehensive issue. Recent studies show that the incidence of COVID-19, the incidence of critical illness, and the mortality rate in the elderly population have increased significantly compared with the younger population (17). In addition, in past studies, the elderly population is also considered to have low responsiveness toward vaccine, which means that vaccines against COVID-19 may not provide adequate immune defense to the elderly population who need them most. However, there is still a lack of comprehensive systemic understanding of age-related changes in immunity. Moreover, the mechanism of how the aging immune disorders cause the incidence and mortality of COVID-19 increase is still unclear. What is more, age was always simply regarded as a confounding factor in many studies, which means that we have never treated age as a major factor to discuss its impact on emerging infectious diseases.

Here, we summarize three observational studies of new coronary pneumonia directly grouped by age and comprehensively analyze the true impact of age as a complex variable on COVID-19 disease. The main limitation of this meta-analysis is the limited number of studies included. Due to the small number of studies, we did not analyze the risk of bias either. What is more, the existing studies did not convey sufficient laboratory tests, such as myocardial enzymes, etc. In fact, we have found that various observational studies on COVID-19 do not contain a unified clinical symptom collection standard, which will bring challenges to subsequent systematic analysis of new diseases. At the same time, the laboratory examinations selected by different centers are not uniform, and some studies do not even list the normal range of indicators. Here, we propose that clinical manifestations and laboratory tests can be classified by different modules. In addition, our statistical analysis

TABLE 2 | Clinical manifestations of the aging patients with COVID-19 included studies in this meta-analysis.

(A) General symptoms and respiratory symptoms of the aging patients with COVID-19.

Studies	General symptoms (Older/Younger)						Respiratory symptoms (Older/Younger)											
	Fever		Fatigue		Catarrhal symptoms		Sore throat		Cough		Expectoration		Chest pain		Chest tightness		Dyspnea	
	Older	Younger	Older	Younger	Older	Younger	Older	Younger	Older	Younger	Older	Younger	Older	Younger	Older	Younger	Older	Younger
Zhao et al.	401 (76.09%)	353 (74.63%)	163 (30.93%)	172 (36.36%)	8 (1.52%)	12 (2.54%)	12 (2.28%)	31 (6.55%)	307 (58.25%)	290 (61.31%)	109 (20.68%)	81 (17.12%)	10 (1.90%)	9 (1.90%)	131 (24.86%)	78 (16.49%)	151 (28.65%)	104 (21.99%)
Liu et al.	14 (77.78%)	30 (78.95%)	2 (11.11%)	3 (7.89%)	1 (5.56%)	2 (5.26%)	NA	NA	15 (39.47%)/6 (33.33%)				NA	NA	2 (11.11%)/2 (5.26%)			
Chen et al.	52 (94.55%)	129 (87.16%)	5 (9.09%)	11 (7.43%)	NA	NA	NA	NA	38 (69.09%)	84 (56.76%)	NA	NA	1 (1.82%)	3 (2.03%)	35 (63.64%)	37 (25.00%)	1 (1.82%)	2 (1.35%)
Lian et al.	115 (84.56%)	521 (79.91%)	24 (17.65%)	115 (17.64%)	2 (1.47%)	45 (6.90%)	17 (12.50%)	94 (14.42%)	85 (62.50%)	421 (64.57%)	49 (36.03%)	216 (33.13%)	NA	NA	NA	NA	17 (12.50%)	20 (3.07%)

(B) Extra-espiratory symptoms of the aging patients with COVID-19.

Studies	Gastrointestinal symptoms (Older/Younger)										Neuromuscular symptoms (Older/Younger)							
	Anorexia		Nausea		Diarrhea		Abdominal pain		Vomiting		Lethargy		Headache		Dizziness		Muscle ache	
	Older	Younger	Older	Younger	Older	Younger	Older	Younger	Older	Younger	Older	Younger	Older	Younger	Older	Younger	Older	Younger
Zhao et al.	73 (13.85%)	61 (12.90%)	13 (2.47%)	8 (1.69%)	52 (9.87%)	48 (10.15%)	4 (0.76%)	3 (0.63%)	15 (2.85%)	11 (2.33%)	4 (0.76%)	6 (1.27%)	13 (2.47%)	19 (4.02%)	18 (3.42%)	15 (3.17%)	24 (4.55%)	42 (8.88%)
Liu et al.	NA	NA	NA	NA	NA	NA	NA	NA	3 (16.67%)/7 (18.42%)				NA	NA	NA	NA	NA	NA
Chen et al.	5 (9.09%)	1 (0.68%)	1 (1.82%)	2 (1.35%)	7 (12.73%)	3 (2.03%)	3 (5.45%)	1 (0.68%)	1 (1.82%)	2 (1.35%)	NA	NA	3 (5.45%)	7 (4.73%)	1 (1.92%)	3 (2.03%)	11 (20.00%)	43 (29.05%)
Lian et al.					11 (8.09%)/77 (11.81%)						NA	NA	8 (5.88%)	67 (10.28%)	NA	NA	20 (14.71%)	71 (10.89%)

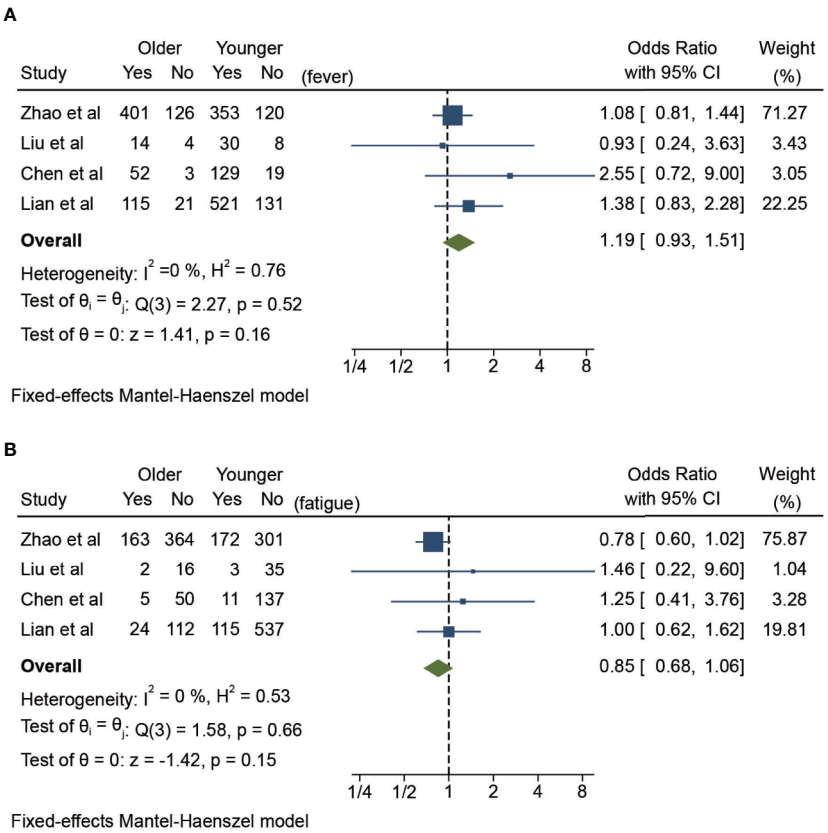

FIGURE 3 | Symptomatic differences between elderly and younger patients with COVID-19 in fixed-effects Mantel-Haenszel model. **(A)** Fever, **(B)** Fatigue.

TABLE 3 | Laboratory findings of aging patients with COVID-19 of the included studies in this meta-analysis.

(A) Blood routine findings of the aging patients with COVID-19.

Study	White blood cell count (× 10 ^{9}/L)		Total white blood cells increased, n (%)		Total white blood cells decreased, n (%)		Lymphocyte count (× 10 ^{9}/L)		Lymphocyte ratio, %		Lymphocyte ratio decreased, n (%)	
	Older	Younger	Older	Younger	Older	Younger	Older	Younger	Older	Younger	Older	Younger
Zhao et al.	6.14 ± 2.57	5.26 ± 1.99	69 (13.09%)	37 (8.1%)	59 (11.20%)	77 (16.8%)	0.87 ± 0.52	1.23 ± 0.54	NA	NA	338 (64.14%)	197 (43.10%)
Liu et al.	5.28 ± 1.45	4.70 ± 1.65	2 (11.11%)	2 (5.26%)	3 (16.67%)	8 (21.05%)	NA	NA	18.89 ± 13.15	28.99 ± 7.03	9 (50.00%)	8 (21.05%)
Chen et al.	11.27 ± 18.04	7.60 ± 11.98	10 (18.18%)	4 (2.70%)	20 (36.36%)	59 (39.86%)	2.30 ± 4.57	7.00 ± 14.97	NA	NA	45 (81.82%)	72 (48.65%)
Lian et al.	5.03 ± 1.87	4.83 ± 1.56	9 (6.62%)	9 (1.38)	38 (27.94%)	196 (30.06%)	1.07 ± 0.52	1.23 ± 0.52	NA	NA	42 (30.88%)	92 (14.11%)

(B) Inflammatory response and coagulation function of the aging patients with COVID-19.

Study	C-reactive protein (mg/L)		Interleukin-6 increased, n (%)		Procalcitonin increased, n (%)		Platelets (× 10 ^{9}/L)		D-dimer increased, n (%)	
	Older	Younger	Older	Younger	Older	Younger	Older	Younger	Older	Younger
Zhao et al.	50.37 ± 55.31	21.13 ± 31.22	NA	NA	49 (9.30%)	13 (2.75%)	196.60 ± 84.14	213.00 ± 72.87	NA	NA
Liu et al.	23.63 ± 17.29	8.45 ± 12.59	NA	NA	NA	NA	217.67 ± 102.97	227.33 ± 82.43	NA	NA
Chen et al.	123.77 ± 214.68	111.33 ± 219.35	22 (40.00%)	22 (14.86%)	5 (9.09%)	2 (1.35%)	199.33 ± 298.42	234.00 ± 395.29	19 (34.55%)	7 (4.73%)
Lian et al.	23.10 ± 29.30	8.55 ± 11.07	NA	NA	NA	NA	169.67 ± 56.57	186.33 ± 52.75	NA	NA

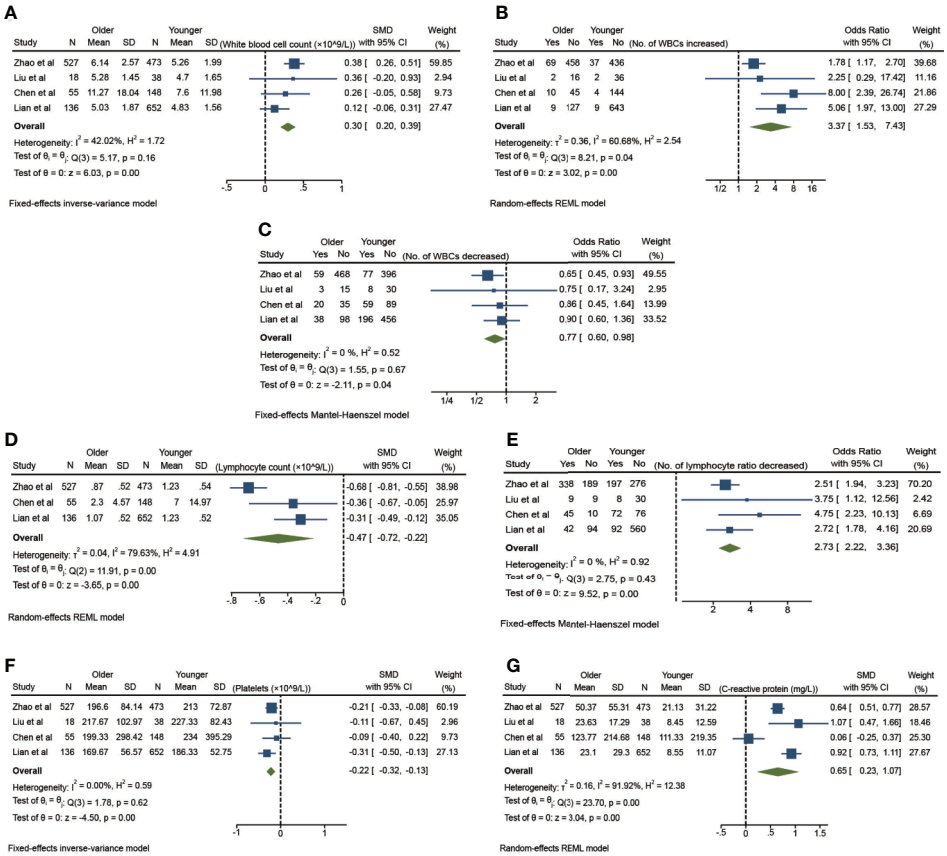

FIGURE 4 | Laboratory tests on the risk of elderly patients with COVID-19 compared to younger patients. **(A)** The total white blood cells, **(B)** Number of increases in the total white blood cells, **(C)** Number of decreases in the total white blood cells, **(D)** Lymphocytes, **(E)** Number of decreases in lymphocytes, **(F)** Platelets, **(G)** C-reactive protein.

shows that, sometimes, the number of abnormal values of indicators is more attractive than direct statistics on numerical variation. Notably, we have also found several observational studies on elderly patients did not contain younger patients as controls (18, 19). Although some experiments have carried out a stratified analysis within the elderly patients (20), not all trials provide enough data on age. Therefore, our analysis is not yet able to do a systematic hierarchical analysis of elderly patients, which could be updated later.

However, we think that this preliminary summary analysis may provide a little help in specifying risk stratification according to age and establishing a multidisciplinary comprehensive treatment strategy model in this stage of the pandemic.

Our meta-analysis finds that the risk of hypertension, diabetes, and other cardiovascular diseases in elderly patients is significantly higher than that in young people. This means poor basic conditions of blood vessels and metabolic systems in te elderly patients, which might be related to the higher incidence of COVID-19, especially the critical illness, the poorer prognosis, and the higher mortality rate in the elderly population (21). This virus infects the human body through ACE2 receptors, which are widely expressed in the vascular endothelium (22). At the same time, this novel virus has also been detected in other organs of the human body (23). These evidences indicate that COVID-19 may be a systemic multiple organ damage disease (24). Poor vascular and metabolic conditions may also cause some elderly patients with COVID-19 to be more likely to have multiple organ damage, except the lungs, and increase the risk of death (25). In addition, this high risk also means that elderly patients need to add treatment for their basic chronic diseases as well as antiviral treatment because viral infection may, in turn, aggravate their own comorbidities. Therefore, the influence of preexisting conditions on the prognosis of infection is also crucial in understanding COVID-19. Recent studies show that smoking is likely to be related to the negative progress and adverse outcomes of COVID-19 (26), and smoking is proven to increase the gene expression of ACE2 (a protein that binds to SARS-CoV-2), which may promote COVID-19 infection (27). Similarly, there are many studies with a strong interest in basic comorbidities. Some studies initially discuss the diabetes management of patients with COVID-19 (28), and some studies from China discuss in detail the impact of comorbidities on the 2019-nCoV infection (29). It is revealed that patients with any comorbidities are worse than those without comorbidities in COVID-19. However, existing research did not pay special attention to the elderly. In this

meta-analysis, our findings may further suggest that such management also needs to be treated differently according to age.

The elderly patients with basic chronic diseases need multidisciplinary standardized management. This means that more cardiovascular and metabolic disease doctors will be included in the MDT team to face the epidemic challenges jointly. The management of basic chronic diseases, such as hypertension, is often inseparable from ACEI/ARB drugs, which has attracted widespread attention in the prevention and control of new coronavirus epidemics (30). In fact, calcium antagonists are more widely used in elderly hypertensive patients for their better adaptability to relieve microvascular spasm and increase cerebral blood supply (31). This reminds us that dose adjustment and standardized use of calcium antagonists are a more important issue for the management of hypertension in elderly patients with COVID-19.

Meanwhile, we also analyzed the symptomatic differences between elderly patients and young infected persons. Although the data obtained are not enough, the going analysis suggests that there is no significant difference between these two groups. But this may not be a negligible negative result. Traditionally, it is believed that the elderly lack immunity, and their symptoms are severe after having a cold or flu, which might be a good sign for a doctor to screen. However, the initial features of elderly patients infected by 2019-nCov is relatively nonspecific, which is a new challenge for community screening in aging society.

In addition, changes of age-related molecules in the blood are considered as new entry points for understanding aging-related diseases (32). We also compared the blood routine and some serological indexes between the elderly patients and the young infected. The results suggest that elderly patients have more white blood cells and a higher risk of increased white blood cells, which can attribute the aging immune mobilization. The total number of lymphocytes in elderly patients is lower than that in young patients, and the risk of their lymphocyte decline is higher. Both results suggest immune aging and lack of defense against viruses. In addition, CRP, the inflammation-related index, has a higher risk in the elderly group, which might be related to the chronic inflammation caused by aging. This pro-inflammatory state may induce an acute exacerbation of older patients with COVID-19. All these results may lay a foundation for building an elderly targeted, anti-inflammatory therapy and interferon therapy to improve immunity.

In summary, our meta-analysis shows that elderly patients with COVID-19 have a higher risk of having chronic comorbidities, including hypertension, diabetes, and other cardiovascular diseases. At the same time, our results show that the clinical manifestations of elderly patients are not significantly different from those of younger patients. Their risk of fever is higher than that of younger patients, but their weakness is less severe than that of younger patients. This is obviously unexpected from what we thought in the past that the elderly would suffer severe symptoms once they became ill. Indifference in initial symptoms and subsequent high mortality make us boldly speculate that the indifference in symptoms of elderly patients with COVID-19 may cause a kind of confusion that they are not very serious, thereby delaying the treatment of the elderly. In addition, there is a certain significant difference between the blood routine and some serological indicators, which may be used as a guide for subsequent treatment selection and continuous monitoring. In addition, we look forward to studies in the future, including larger samples and longer-term follow-up, to evaluate the effect of age on the end-of-life events of 2019-nCov infection. Meanwhile, we encourage more cardiovascular and metabolic doctors to participate in the intensive treatment of elderly patients with COVID-19.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

PJ and CY conceived the need for the article. CY drafted the initial version. PJ helped perform the final version with constructive discussions. PJ provided expert critical input regarding the content. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the National Science Fund for Distinguished Young Scholars (81625002), the National Natural Science Foundation of China (81930007, 81470389, 81500221, and 81800307), the Shanghai Outstanding Academic Leaders Program (18XD1402400), Innovative research team of high-level local universities in Shanghai, and Shanghai Municipal Education Commission Gaofeng Clinical Medicine Grant Support (20152209).

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

frontiers in Endocrinology

MINI REVIEW
published: 10 December 2020
doi: 10.3389/fendo.2020.569241

Activation of Melanocortin Receptors as a Potential Strategy to Reduce Local and Systemic Reactions Induced by Respiratory Viruses

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The clinical hallmarks of infections caused by critical respiratory viruses consist of pneumonia, which can progress to acute lung injury (ALI), and systemic manifestations including hypercoagulopathy, vascular dysfunction, and endotheliitis. The disease outcome largely depends on the immune response produced by the host. The biomolecular mechanisms underlying certain dire consequences of the infection partly arise from an aberrant production of inflammatory molecules, an event denoted as "cytokine storm". Therefore, in addition to antiviral therapies, molecules able to prevent the injury caused by cytokine excess are under investigation. In this perspective, taking advantage of melanocortin peptides and their receptors, components of an endogenous modulatory system that exerts marked anti-inflammatory and immunomodulatory influences, could be an effective therapeutic strategy to control disease evolution. Exploiting the melanocortin system using natural or synthetic ligands can form a realistic basis to counteract certain deleterious effects of respiratory virus infections. The central and peripheral protective actions exerted following melanocortin receptor activation could allow dampening the harmful events that trigger the cytokine storm and endothelial dysfunction while sustaining the beneficial signals required to elicit repair mechanisms. The long standing evidence for melanocortin safety encourages this approach.

Keywords: respiratory viruses, SARS-CoV-2, acute lung injury, cytokine storm, endothelial dysfunction, melanocortin receptors, adenocorticotropin, alpha melanocyte stimulating hormone

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Translational Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *03 June 2020*
Accepted: *10 November 2020*
Published: *10 December 2020*

Citation:
Lonati C, Gatti S and Catania A (2020) Activation of Melanocortin Receptors as a Potential Strategy to Reduce Local and Systemic Reactions Induced by Respiratory Viruses. Front. Endocrinol. 11:569241. doi: 10.3389/fendo.2020.569241

INTRODUCTION

The present pandemic caused by Severe Acute Respiratory Syndrome (SARS)-Coronavirus (CoV)-2 follows outbreaks of other highly pathogenic respiratory viruses that emerged over the past two decades, namely SARS-CoV-1, Middle East respiratory syndrome (MERS)-CoV, and influenza A virus (IAV) H5N1, H1N1, and H7N9.

Clinical hallmarks of severe diseases are pneumonia with marked leukocyte infiltration into the lungs, diffuse alveolar damage (DAD), hyaline membrane formation, and edema (1–4). Pulmonary disease can progress to acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and, in the most severe cases, to fibrosis (5). In addition, systemic manifestations are a major component of the clinical picture. Among these, endotheliitis with lymphocyte infiltration into vessel walls of the

lung, heart, liver, and kidney (3, 6–8), deranged coagulation (2, 7, 9–11), and lymphopenia (4, 7, 12–14) significantly contribute to disease deterioration. There is also evidence for a viral spreading into the central nervous system (CNS) (7, 15, 16).

The bio-molecular mechanisms underlying clinical pathology partly arise from an aberrant release of inflammatory molecules, an event denoted as "cytokine storm". Therefore, in addition to antiviral therapies, molecules able to prevent the consequences of the cytokine storm are under investigation (17–19). Use of corticosteroids was initially controversial due to the immunodepressive potential side effect (10, 14, 20–23), but their administration is now recommended in selected patients (24). In this perspective, taking advantage of melanocortin peptides and their receptors, components of an endogenous modulatory system, could be an effective and safe therapeutic strategy.

Melanocortins exert marked anti-inflammatory and immunomodulatory influences. Physiological role and effectiveness in treatment of acute, chronic, and systemic inflammatory disorders are well-documented (25–28). Exploiting the melanocortin system could form a realistic basis to counteract certain deleterious effects of respiratory viruses not only for the present but also for possible future pandemics.

PATHOLOGICAL CONSEQUENCES OF VIRUS/HOST INTERACTIONS

The outcome of CoVs and IAVs-induced disease largely depends on the immune response mounted by the host in response to infection (29–32). The term "cytokine storm" was firstly used to denote a hypercytokinemic state observed in H5N1-infected patients (33). Lung inflammation appears to be the primary "driver" (1–4) triggering excessive systemic production of interferons (IFN), interleukins (IL), chemokines, colony stimulating factors (CSF), and tumor necrosis factors (TNF) (19, 29, 30). There is now compelling evidence that the cytokine storm significantly contributes to both pulmonary and systemic damage associated with respiratory viral infections (19, 29, 30, 34, 35).

Cytokine storm arises from a virus-induced transient impairment of the physiological antiviral response. The inhibitory effects exerted by CoVs and IAVs on the host immunity are mediated by common mechanisms (14, 29, 36). Indeed, virus-encoded non-structural proteins are able to prevent immune recognition and IFN-dependent antiviral responses (19, 29, 31, 32, 37–39). Besides inhibitory effects, the virus also triggers host inflammatory signals (31, 39) through both activation of the nuclear factor (NF)-κB and inflammasome formation (40, 41). The initial deficiency of the host immune response allows rapid viral replication and spreading in pulmonary cells. At later stages, deranged activation of the host immunity causes aberrant pulmonary secretion of IL-1ß, TNF-α, IL-6, IL-8, MCP-1, MIP-1α, Granulocyte macrophage (GM)-CSF, and Granulocyte (G)-CSF as well as massive recruitment of monocytes/macrophages and neutrophils into the lungs (14, 30–32, 36–38, 42).

When locally-produced inflammatory mediators reach the circulation, the clinical picture turns into systemic inflammation mainly due to activation of endothelial cells (43). Increased expression of adhesion molecules mediates leukocyte–endothelial adhesion leading to massive immune cell migration from the blood circulation into the extravascular connective tissue (34). Sustained inflammatory activation of endothelial cells can result in endothelial dysfunction (8) marked by exposure of tissue factor, aberrant release of Von Willebrand factor multimers, and decreased production of endogenous anticoagulants such as tissue factor pathway inhibitor (TFPI) and antithrombin (44). Hypercytokinemia likewise activates the hepatic acute phase response (34). In addition, increased blood concentration of CSFs stimulates the hematopoietic system to release both leukocytes and platelets in the circulation (34). These biological events further boost production of pro-inflammatory molecules contributing to amplify inflammation. At later stages, high TNF-α concentration induces leukocyte apoptosis, while sustained vascular inflammation elicits endothelial cell death (8). Apoptosis of cells of the respiratory tract and extra-pulmonary tissues is also directly activated by viral proteins (14, 31, 45, 46).

Unbalanced immune responses give rise to many of the pathologic hallmarks observed in respiratory virus-induced disease (5, 14). Apoptosis of epithelial and endothelial cells compromises alveolar-epithelial barrier leading to gas exchange impairment and fluid accumulation (30). Cytokine-induced uncontrolled epithelial cell replication, aberrant matrix remodeling, and fibrosis deposition perturb parenchyma architecture with further deterioration of respiratory function. Systemic manifestations are likewise dire components of the picture. Endothelial dysfunction significantly affects the physiological regulation of coagulation, shifting vascular equilibrium towards vasoconstriction, hypercoagulability, and immunothrombosis (9, 11). In addition, endotheliitis and consequent vascular leakage mediate translocation of inflammatory molecules as well as recruitment of activated neutrophils and cytotoxic T cells into peripheral tissue. Therefore, microvascular endothelial dysfunction, widespread immunothrombosis, and diffuse endothelial injury are significant contributing factors in development of tissue injury in extra-pulmonary sites (29, 30, 35, 43). Endothelial cell damage is likewise associated with unbalanced vasoactive molecule production leading to impaired blood pressure control. CD4+ and CD8+ T-cell lymphopenia is likely consequent to both virus-related direct effects (32) and hypercytokinemia-dependent induction of leukocyte death (4, 7, 14, 30). Finally, enhanced expression of IL-1 and/or other cytokines within the CNS could participate in T cell exhaustion and natural killer and B cell suppression observed in COVID-19 (32), leading to development of systemic immunosuppression (47).

This being said, it is important to mention that cytokines also contribute to the biological events required for a successful resolution of infection, including extracellular matrix remodeling and tissue repair (19). Therefore, their production should be modulated but not suppressed. In this view there is a

clear therapeutic challenge aimed at reducing inflammation without canceling its defensive properties. Indeed, a balanced control of the deleterious virus/host interactions, is crucial to reduce damage and promote resolution; this strategy should complement direct anti-viral procedures.

THE MELANOCORTIN SYSTEM: A POTENT ENDOGENOUS MODULATORY PATHWAY

Melanocortin Peptides and Their Receptors

Melanocortins are a family of endogenous peptides produced by CNS and peripheral cells (25, 48, 49). These peptides derive from post-translational processing of the precursor proopiomelanocortin (POMC) and include the adrenocorticotropic hormone (ACTH) and the melanotropins, α, β, and γ-melanocyte-stimulating hormone (MSH).

Although α-MSH was initially described as a melanogenic hormone and activity of ACTH was confined to its steroidogenetic effect, it is now well established that melanocortins exert multiple actions on the host physiology. Indeed, over four decades, research has demonstrated that melanocortins have the remarkable ability to restore normal pathways when a certain stimulus perturbs the host homeostasis. Heterogeneous stimuli such as pathogens or their components, ischemia/reperfusion (I/R) injury, or irritants elicit production of melanocortin peptides that, in turn, act to restore equilibrium (25, 49, 50). Blockade of the natural peptides increases the expression of proinflammatory mediators in the blood, lungs, and liver of endotoxemic mice (51). These observations suggest that when endogenous production is not sufficient to face the challenge, supplementary administration could reach the target.

Melanocortin effects are exerted through recognition of five melanocortin receptors (MCRs) that are broadly distributed in peripheral cells and in brain regions. MCRs are G-protein-coupled receptors associated with adenylyl cyclase and mediate their effects primarily by activating the cAMP-PKA-CREB-dependent signaling pathway but also through activation of mitogen-activated-protein-kinase (MAPK), calcium-inositol triphosphate-PI3K, and JAK-STAT pathways (25, 52–54). With the exception of the MC2R, which is selectively activated by ACTH, the other MCR subtypes, MC1R, MC3R, MC4R, and MC5R, are recognized by all the natural melanocortins, although with different affinity (55). MC1R is widely expressed in body cells including fibroblasts, neutrophils, monocytes, B and T lymphocytes, dendritic cells, alveolar macrophages, glial cells, epithelial cells, and endothelial cells (25, 56–59). Binding affinity of endogenous agonists for this receptor is α-MSH=ACTH>β-MSH<γ-MSH (55). A large number of in vitro and in vivo studies demonstrate a primary role of MC1R in immunomodulation (25, 48, 49) as well as in regulation of endothelial cell function (57, 59). Of note, mice bearing a non-functional MC1R ($Mc1r^{e/e}$) show exacerbated inflammatory responses relative to wild-type animals (60) and selective MC1R silencing in macrophages is associated with loss of α-MSH-mediated suppression of inflammation in vitro (61). Unlike the other MCRs, MC2R exclusively binds to ACTH that selectively induces glucocorticoid production in the adrenal cortex. MC3R is expressed in hypothalamus cells, macrophages, monocytes, dendritic cells, CD4+ T cells, and B lymphocytes and its binding ability to natural melanocortins is γ-MSH>ACTH=α-MSH=β-MSH (55). MC3R activation appears to be associated with broad modulation of inflammation in response to acute pro-inflammatory stimuli (62). MC4R [α-MSH=ACTH>β-MSH>γ-MSH (55),] is mainly expressed in the CNS and participates in immunodulation through activation of the cholinergic anti-inflammatory pathway (63). MC5R is widely expressed in both the CNS and in peripheral tissues, including the lung, kidney, lymph nodes, bone marrow, thymus, and immune competent cells such as B and T lymphocytes, mast cells, and antigen presenting cells (APC) (53, 64). Recent evidence suggests that MC5R is deeply involved in regulation of immune reactions and inflammatory responses (53, 65). Natural melanocortin order of potency in activating this receptor is: α-MSH>ACTH=β-MSH>>γ-MSH (55).

Synthetic Ligands of MCRs

Development of synthetic derivatives of melanocortins may allow a better exploiting of the remarkable properties of the natural peptides due to their enhanced chemical stability, resistance to enzymatic degradation, and, for certain of them, more selective receptor recognition (66). A significant breakthrough in this approach was the synthesis of the α-MSH analog Nle4,DPhe7-α-MSH (NDP-α-MSH) marked by prolonged and increased biological activity compared to the endogenous peptide (67). NDP-α-MSH (afamelanotide) is clinically used to prevent phototoxicity in erythropoietic protoporphyria and could represent an excellent candidate for other melanocortin-based therapies. Additional α-MSH analogs include AP214 (modimelanotide) (68) and STY39 (69) that show higher affinity for MC1R/MC3R and for MC1R/MC5R, respectively. Of particular relevance with regard to clinical use, ACTH-related sequences form a solid basis for MCR-based effective therapies. Indeed, the extra-adrenal effects of ACTH are definitely recognized and these molecules have already been used to treat human inflammatory disorders (70–72).

Selective ligands for individual MCR subtypes are likewise being designed (66), including BMS-470539 (MC1R agonist) (73), [D-Trp8]-γ-MSH (MC3R) (74), RO27-3225 (MC4R) (75), PG-901 (MC5R) (54), and the N-terminally "capped" tetrapeptide 3,3,3-triphenylpropionyl-His-D-Phe-Arg-Trp-NH (2) (MC5R) (76).

Protective Effects of Melanocortins in Systemic Inflammation and Secondary Organ Damage

Activation of the melanocortin system with natural or synthetic ligands exerts beneficial effects in acute, chronic, and systemic inflammatory disorders (25–27, 48, 49, 77). Moreover, different clinical studies have investigated efficacy

of ACTH and NDP-α-MSH therapies in systemic inflammatory diseases, including ARDS, rheumatoid arthritis, multiple sclerosis (MS), lupus erythematosus (SLE), kidney diseases, and nephrotic syndromes (70–72).

Table 1 reports the beneficial effects exerted by melanocortins in preclinical models of systemic inflammatory diseases, including sepsis, MODS, I/R injury, hemorrhage shock, and vasculitis.

The marked protection in systemic inflammation was demonstrated by studies conducted in rabbits (50) and mice (51, 77–79). NDP-α-MSH administration in murine MODS not only increases survival rate but also reduces pulmonary leukocyte infiltration, vascular congestion, and interstitial edema (80). In a porcine model of systemic inflammatory response syndrome, treatment with the α-MSH analog AP214 prevented the LPS-induced increase in pulmonary pressure (81). α-MSH or STY39 administration in mice with endotoxemia is associated with increased concentration and mRNA pulmonary expression of TFPI (82).

In a model of renal I/R injury α-MSH administration prevented activation of NF-*k*B and AP-1 in the lungs and reduced the expression of stress response genes, intracellular adhesion molecule (ICAM)-1, and TNF-α (83). In addition, α-MSH significantly decreased leukocyte infiltration and lung edema.

Treatment with NDP-α-MSH or with the selective MC4 agonists RO27-3225 and PG-931 leads to restoration of cardiovascular and respiratory functions, improved survival, and reduced circulating free radicals in a rat model of severe hemorrhagic shock (84). Moreover, RO27-3225 prevents the hemorrhage-induced immunopathological changes in peripheral organs. Treatment with RO27-3225 normalizes hemogasanalysis parameters after hemorrhagic challenge (85).

Injury to vascular endothelium significantly contributes to convert a local inflammation into a systemic disease. In this perspective, the observation that α-MSH administration to endothelial cell challenged with pro-inflammatory stimuli is associated with reduced adhesion molecule expression (56, 87) and leukocyte adhesion (56, 87) has particular relevance. Further, melanocortin treatment reduced endothelial cell damage and barrier permeability in an *in vitro* model of blood-brain barrier inflammation (59). With regard to *in vivo* studies, $Mc1r^{e/e}$ mice exposed to high-sodium diet or LPS challenge show increased susceptibility to inflammation-dependent vascular dysfunction (57). In leukocytoclastic vasculitis in mice, α-MSH significantly suppresses vascular damage and hemorrhage by inhibiting the early LPS-induced expression of E-selectin and VCAM-1 (86, 87). Activation of MC1R was associated with inhibition of cell adhesion/emigration and reduced tissue expression of CXCL1 and CCL2 in a murine model of vascular inflammation induced by I/R injury (88).

Protective Effects of Melanocortins in Primary Inflammatory Disorders of the Lung

Pulmonary cells express both MC1R (89, 90) and MC3R (89, 91). Expression of MC1R mRNA was documented in rat native lungs and a significant up-regulation of this receptor was observed in lungs subjected to *ex vivo* perfusion (92). As the procedure involves removal of non-resident cells from the lungs, this observation documents that MC1R is expressed by pulmonary tissue and is further induced during I/R injury.

In *in vitro* studies, α-MSH suppressed IL-1-induced PGE production by fetal human lung fibroblasts (93) and prevented LPS-induced activation of NF-*k*B in human pulmonary epithelial cells (94). The inhibitory effect is mediated by preservation of the IkBα protein (94). Similarly, γ-MSH treatment suppresses NF-*k*B signaling and exerts several protective effects in a human epithelial cell line challenged with different inflammatory stimuli (91).

The effectiveness of melanocortin treatment in control of primary lung inflammatory diseases is well documented (**Table 2**): this property could be very beneficial in the protection of organs that are a primary target of respiratory viruses.

In a preclinical model of ARDS based on intratracheal infusion of endotoxin, treatment with α-MSH was associated with reduced leukocyte count in BAL fluids (77). Similar salutary effects were observed in mice treated with α-MSH or a synthetic MC3R agonist after LPS administration (89). In an ARDS model based on induction of hemorrhagic shock followed by intratracheal LPS administration, α-MSH-treated rats showed reduced leukocyte infiltration and endothelial cell apoptosis (95). In LPS-induced ALI in mice, MC1R activation with the selective agonist BMS-470539 was associated with improved pulmonary edema, reduced inflammation and neutrophil infiltration (96).

Experiments based on intratracheal instillation of bleomycin are particularly interesting in that this model is widely used to reproduce human ALI and fibroproliferative disorders. A research based on bleomycin instillation in rats, showed that NDP-α-MSH administration markedly improves the clinical and molecular picture of ALI (97). In particular, NDP-α-MSH treatment was associated with a significant reduction of interstitial edema. At a molecular level, melanocortin treatment prevented bleomycin-induced increase in plasma NO concentration and modulated the expression of genes involved in stress response, inflammation, fluid homeostasis, and fibrosis development. Consistent with the observations that endogenous α-MSH participates in responses to host challenge, bleomycin caused a significant increase in circulating α-MSH relative to controls. The synthetic α-MSH analog STY39 (98), increased survival and improved the lung edema index in the bleomycin model. An interesting observation in this research is that STY39, in addition to inflammation, prevents bleomycin-induced pulmonary expression of pro-fibrotic factors and improves MMP-1/TIMP-1 mRNAs ratio.

A protective influence of melanocortins on altered cytokine production was likewise documented in a mouse model of allergic airway inflammation (90). Endogenous production of α-MSH in BAL after airway allergen challenge was observed. Treatment with α-MSH provided further benefit, including reduction in BAL eosinophils and a decrease in serum allergen-specific IgE, IL-4, and IL-5. Lower concentration of BAL eosinophils and lymphocytes was likewise reported by Getting and coworkers in allergic mice treated with [D-TRP8]-γ-MSH (89).

TABLE 1 | Protective effects exerted by melanocortin treatment in preclinical models of systemic inflammation and secondary organ damage.

MCR	Compound	Experimental model	Melanocortin treatment	Melanocortin effect	Ref.
Systemic inflammation					
MC1R, MC3R, MC4R, MC5R	α-MSH	rabbit, LPS i.p.; blood collection and body temperature assessment at 0, 1, and 3 h	i.v. immediately before LPS administration	↓ fever	(50)
		mice septic shock (cecal ligation and puncture); survival at 12 and 24 h	i.p. at 0 and 3 h	improved survival rate	(77)
		mouse, LPS i.p.; blood collection at 1 and 6 h, liver and lung biopsy at 6 h	i.c.v. 15 min before LPS	↓ plasma TNF-α and nitrate ↓ iNOS activity and iNOS mRNA in the lungs and liver ↓ lung myeloperoxidase activity	(51)
		mouse peritonitis (monosodium urate crystals i.p.); peritoneal lavage at 4 and 6 h	s.c. 30 min before inflammatory challenge	↓ neutrophil migration ↓ IL-1β and CXCL-1 in peritoneal lavage fluids	(78)
	NDP-α-MSH	mouse peritonitis (zymosan i.p or monosodium urate crystals i.p.); peritoneal lavage at 4 and 6 h	s.c. 30 min before inflammatory challenge	↓ neutrophil migration ↓ IL-1 β and CXCL-1 in peritoneal lavage fluids	(79)
		mouse, LPS i.p. + zymosan i.p. after 6 d; lung biopsy and histology at 7 d, survival at 16 d	i.p. daily from 0 to 16 d	improved survival rate ↓ inflammatory cells infiltrate into the lungs ↓ vascular congestion and interstitial edema in the lung tissue ↓ plasma TNF- α ↓ TNF- α and ↑ IL-10 mRNAs in the lungs	(80)
	AP214	pig endotoxemia (LPS i.v.); cardiovascular monitoring from 20 to 180 min	i.v. at 0 h	↓ increase in pulmonary pressure and vascular resistance	(81)
	α-MSH, STY39	mouse, LPS i.p. + D-galactosamine; blood collection at time intervals, lung biopsy at 8 h	i.p. at 1, 2 or 3 h following LPS administration	↑ plasma TFPI ↑ TFPI mRNA in the lungs	(82)
MC3R	γ2-MSH	mouse peritonitis (monosodium urate crystals i.p.); peritoneal lavage at 4 and 6 h	s.c. 30 min before inflammatory challenge	↓ neutrophil migration ↓ IL-1 β in peritoneal lavage fluids	(78)
I/R injury					
MC1R, MC3R, MC4R, MC5R	NDP-α-MSH	mouse bilateral renal I/R (40 min ischemia + 4 8 or 24 h reperfusion); lung histology at 4 h after ischemia, lung biopsy at 0, 5, 4, and 8 h after ischemia	i.v. immediately before clamp removal and at 8 and 16 h post ischemia	↓ lung edema ↓ leukocyte infiltration ↓ TNF- α and ICAM-1 mRNAs in the lungs ↓ lung myeloperoxidase activity ↓ IkBα, p38, and c-Jun phosphorylation in the lung tissue ↓ NF-kB and AP-1 DNA-binding activity in the lung tissue	(83)
Hemorrhagic shock					
MC1R, MC3R, MC4R, MC5R	NDP-α-MSH	rat, acute hemorrhagic shock; lung histology at 25 min and at 24 h, survival at 24 h	i.v. 5 min after termination of bleeding	improved survival rate improved cardiovascular parameters ↓ blood free radicals	(84)
MC4R	RO27-3225, PG-931	rat, acute hemorrhagic shock; lung histology at 25 min and 24 h, survival at 24 h	i.v. 5 min after termination of bleeding	improved survival rate improved cardiovascular parameters ↓ histological damage in the lungs ↓ blood free radicals	(84)
	RO27-3225	rat, acute hemorrhagic shock; hemogasanalysis at 5, 15, and 60 min	i.v. 5 min after termination of bleeding	improved pH, pO_2, pCO_2, HCO_3-, BE, SO_2, and lactate	(85)
Vasculitis					
MC1R, MC3R, MC4R, MC5R	α-MSH	mouse leukocytoclastic vasculitis (s.c LPS priming + i.p LPS after 24 h); ear histology at 24 h after LPS priming and at 2, 3, 5, 6 h after LPS	i.p. at 3 h after LPS priming	↓ vascular hemorrhage score ↓ E-selectin and VCAM-1-positive vessels	(86)
		mouse leukocytoclastic vasculitis (s.c. LPS priming + i.p. LPS after 24 h); ear histology at 2, 3, 5, 6 h after LPS	i.p. at 3 h after LPS priming	↓ vascular hemorrhage score ↓ E-selectin and VCAM-1-positive vessels	(87)
MC1R	BMS-470539	mouse vasculitis (35 min mesenteric ischemia + 90 min reperfusion); intravital microscopy, mesentery tissue biopsy	i.v. before ischemia	↓ leukocyte adhesion and migration ↓ CXCL1 and CCL2 mesenteric tissue expression	(88)

BE, base excess; CXCL-1, chemokine (C-X-C motif) ligand 1; d, day; h, hour; HCO_3-, bicarbonate; ICAM-1, intercellular adhesion molecule 1; IL-1β, interleukin-1β; IL-10, interleukin-10; IkBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; iNOS, inducible nitric oxide synthase; i.p., intraperitoneal; I/R, ischemia/reperfusion; i.v., intravenous; LPS, lipopolysaccharide; min, minutes; pO_2, partial pressure of oxygen; pCO_2, partial pressure of carbon dioxide; s.c., subcutaneous; SO_2, oxygen saturation; TNF-α, tumor necrosis factor α; TFPI, tissue factor protein inhibitor; VCAM-1, Vascular cell adhesion protein.

TABLE 2 | Protective effects exerted by melanocortin treatment in preclinical models of primary pulmonary inflammation.

MCR	Compound	Experimental model	Melanocortin treatment	Melanocortin effect	Ref.
ALI/ARDS					
MC1R, MC3R, MC4R, MC5R	α-MSH	rat, LPS i.t.; BAL at 6 h	i.p. at 0, 2, and 4 h	↓ BAL WBCs	(77)
		rat ARDS (acute hemorrhagic shock + LPS i.t after 3 h); lung histology at 9 h post LPS	i.v. at 0, 3, and 6 h after LPS administration	↓ infiltration of inflammatory cells into alveoli ↓ apoptosis of pulmonary endothelial cells	(95)
		mouse, LPS i.n.; BAL at 4 h	i.p. 1 h before LPS administration	↓ BAL neutrophils ↓ BAL TNF-α	(89)
MC1R	BMS-470539	mouse, LPS i.t.; BAL and lung biopsy at 18 h	s.c. 1 h before LPS administration	↓ lung W/D ratio ↓ BAL leukocytes and PMNs ↓ BAL TNF-α ↓ lung myeloperoxidase activity	(96)
MC3R	[D-TRP8]-γ-MSH	mouse, LPS i.n.; BAL at 4 h	i.p. 1 h before LPS administration	↓ BAL neutrophils; ↓ BAL TNF-α	(89)
ALI and fibroproliferative disorder					
MC1R, MC3R, MC4R, MC5R	NDP-α-MSH	rat, bleomycin i.t.; blood collection and lung biopsy at 8 and 24 h, lung W/D at 24 h	i.p. immediately before bleomycin instillation and at 12 h	↓ lung W/D ratio ↓ expression of genes involved in stress response, fluid homeostasis, inflammation, fibrosis ↓ plasma nitrate	(97)
	STY39	mouse, bleomycin i.t.; daily monitoring, lung index at 7 and 14 d, BAL at 7 d, lung biopsy at 9 d, lung histology at 14 d	i.p. from 1 to 14 d after bleomycin instillation	improved survival rate improved cyanosis, tachypnea ↓ BAL TNF-α, IL-6, MIP-2, and TGF-β1 ↓ BAL macrophages, neutrophils, and lymphocytes ↓ type I and III procollagen pulmonary expression improved MMP-1/TIMP-1 mRNA pulmonary ratio ↓ hydroxyproline and myofibroblast proliferation in the lung tissue	(98)
Airway allergic inflammation					
MC1R, MC3R, MC4R, MC5R	α-MSH	mouse OVA-induced allergic inflammation (i.p allergic sensitization at 1,14, and 21 d; aerosol challenge at 26 and 27 d); BAL and histology at 48 h	i.v. 30 min before each sensitization and each allergen challenge	↓ perivascular inflammation ↓ peribronchial inflammatory cell infiltrate ↓ BAL eosinophils ↓ BAL IL-4, IL-13 and ↑ IL-10 ↓ blood allergen specific IgE	(90)
MC3R	[D-TRP8]-γ-MSH	mouse OVA-induced allergic inflammation (i.p. allergic sensitization at 0 and 7 d, aerosol challenge at 14, 15, and 16 d); BAL at 24 h	i.p. 5 min before each allergen challenge	↓ BAL eosinophils and lymphocytes	(89)

BAL, bronchoalveolar lavage; d, days; h, hour; IL-4, interleukin-4; IL-6, interleukin 6; IL-10, interleukin-10; IL-13, interleukin-13; i.n., intranasal; i.p., intraperitoneal; i.t., intratracheal; i.v., intravenous; LPS, lipopolysaccharide; min, minutes; MIP-2, macrophage inflammatory protein 2; MMP-1, matrix metalloproteinase 1; OVA, ovoalbumin; PMNs, polymorphonuclear leukocytes; TGF-β1, transforming growth factor β1; TIMP-1, metallopeptidase inhibitor 1; TNF-α, tumor necrosis factor α; WBC, white blood cells; W/D, wet-to-dry.

Main Protective Pathways Influenced by Melanocortins

The anti-inflammatory effects of melanocortins are mainly exerted through PKA-mediated prevention of IκBα degradation and consequent inhibition of the NF-*k*B signaling in leukocytes (48, 99), endothelial cells (56, 59, 87), and other peripheral cells (48, 94). As NF-*k*B induces the expression of hundreds of genes relevant to inflammation including cytokines, cytokine receptors, chemokines, growth factors, and adhesion molecules, its modulation enables a wide-range regulation of inflammatory responses.

Besides the direct antiphlogistic effects, melanocortins have the peculiar feature to stimulate pro-resolving endogenous circuits (68, 70, 100–102). In fact, MCR activation is associated with induction of the expression of inhibitors of inflammation and resolutive factors, including IL-10 (103), IL-1 receptor-associated kinase (IRAK)-M (104), suppressor of cytokine signaling (Socs) 1 and 3 (101, 102), IkBα (102), cyclooxygenase (COX) 2 (102), Chemokine (C-C motif) ligand (CCL) 20 (101, 102), Interleukin 1 receptor antagonist (IL-1ra) (101, 102), Dual specificity phosphatase 1 (DUSP1) (105), and IL-1 receptor-like 2 (IL-1rl2) (105). MCR-induced resolving activities likewise include pro-efferocytic effects mediating clearance of apoptotic neutrophils (68).

Moreover, melanocortin peptides can activate immune regulatory mechanisms through induction of T cell differentiation toward a tolerogenic phenotype (106). In particular, Taylor and coworkers reported α-MSH-induced Treg

cells are CD25+CD4+ and express CTLA4, CD44, CD62L, and latency-associated peptide (LAP) (65). Activation of regulatory activity in effector T cells and APCs was documented in a model of ocular inflammatory disease (64, 65, 106, 107). The modulatory effect on immunity could represent an additional mechanism through which melanocortin peptides promote resolution of inflammation (106).

There is increasing evidence that the diverse immunomodulating effects of melanocortins are carried out through specific MCR targeting. In fact, while resolution of inflammation is mainly achieved through activation of MC1R (25, 48, 49, 61) and MC3R (62, 78), immune regulation appears to be mediated by MC5R (64, 65, 108). As an instance, among the receptors expressed by macrophages, MC1R (61) and MC3R (68, 78) are involved in inflammatory response inhibition, whereas MC5R engagement promotes monocyte differentiation toward either myeloid suppressor cell or tolerogenic APCs with subsequent reduction of effector T cell activation (109).

A peculiar effect of melanocortin peptides consists of indirect influences exerted on peripheral cells through stimulation of MC3R/MC4R within the brain and activation of descending anti-inflammatory neural pathways (25, 48, 49, 110). Lipton and colleagues showed that α-MSH given centrally inhibits inflammation in the skin through activation of adrenergic pathways (110, 111). A further protective mechanism is based on MC4R signaling and subsequent stimulation of the cholinergic anti-inflammatory pathway (63) that inhibits inflammation in tissue macrophages and lymphoid organs.

Of note, due to its unique peptide sequence, ACTH elicits MC2R-mediated steroidogenesis in the adrenal gland. Therefore, in addition to the protective influences exerted through activation of the other MCRs, ACTH induces glucocorticoid production (70). This additional effect can provide a remarkable clinical advantage as glucocorticoids and melanocortins exert distinct effects as immune modulators (112). Consequently, ACTH can provide combined beneficial effects based on two different pathways. In fact, glucocorticoids can be exploited to induce a general suppression of immune cell activity, inhibit pro-inflammatory mediator release, promote leukocyte apoptosis, and prevent cell recruitment into damaged tissue. On the other hand, melanocortin peptides, including ACTH, are able to modulate rather than dampen the release of inflammatory mediators, elicit production of resolutive factors, induce leukocyte differentiation towards protective phenotype, and exert a considerable anti-microbial activity (113, 114). The distinctive features of glucocorticoids- and melanocortins-based therapies are particularly manifest in the treatment of ocular inflammatory disorders in which α-MSH administration not only suppresses eye inflammation, but also induces immune tolerance and promotes retinal cell survival (115).

Finally, another potentially beneficial effect exerted by melanocortins is protection against apoptotic cell death. This action was observed in pulmonary vascular endothelial cells in a model of ARDS (95), in macrophages exposed to serum starvation *in vitro* (116), and in a rat model of prolonged myocardial I/R (117).

DISCUSSION: EXPLOITING MELANOCORTIN PATHWAYS TO COUNTERACT RESPIRATORY VIRUS-INDUCED DETRIMENTAL EVENTS

As stated above, respiratory viruses can alter inflammatory reactions built up by the host to combat the infection. In this scenario, a potentially beneficial cytokine production can turn into a dysregulated harmful event that eventually contributes to development of the severe clinical manifestations observed in CoVs and IAVs-induced diseases. Therefore, modulation of the host aberrant inflammatory responses appears as crucial as controlling viral replication to prevent disease progression.

Melanocortins could reinforce endogenous signals providing protection *versus* respiratory virus-induced detrimental events. In particular, disruption of the dire and self-amplificating vicious-cycle triggered by mediator release/leukocyte recruitment can significantly reduce pulmonary tissue damage. Through modulation of NF-*k*B signaling, melanocortin peptides do not influence a single inflammatory mediator but they rather exert control on the whole inflammatory cascade. This effect could be of key importance during respiratory virus infection. Evidence obtained in SARS-CoV-1-infected mice clearly demonstrated that chemical blockade of NF-*k*B improves lung pathology, reduces inflammation, and increases survival (40). Moreover, melanocortin-induced expression of negative regulators of inflammation as well as the activation of immune regulation mechanisms can counteract pro-inflammatory signals and trigger repair mechanisms crucial to restore homeostasis.

The possibility to induce glucocorticoid production is another peculiar feature inherent in therapeutic activation of melanocortin pathways. Availability of ligands with different affinities to MCRs provides the unique opportunity to either take advantage of or avoid adrenal stimulation, depending on the specific patient clinical situation. In fact, similar to steroid treatment, overproduction of endogenous cortisol can be associated with adverse side effects including increased susceptibility to infections, hypertension, diabetes mellitus, electrolyte disturbances, gastric ulceration, and impaired wound healing (118). On the other hand, based on the most recent guidelines on the use corticosteroid-based therapy in COVID-19, severe and critical patients could significantly benefit from glucocorticoid production (24). Therefore, the use of ACTH requires a preventive assessment to determine the balance between benefits and harms of enhancing corticosteroids (118). Further, it is important to consider that ACTH-induced steroidogenesis produces glucocorticoid plasma concentrations that could be insufficient when high dose steroid therapy is needed (115). Conversely, α-MSH and its analogs can be safely administered when adrenal stimulation should be avoided.

In addition to immunomodulation, melanocortin treatment could ameliorate pulmonary edema and fibrosis. Of note, experiments in rodent liver fibrosis demonstrated that melanocortin therapy not only can prevent the biological events leading to fibrosis development but it can also reverse

established fibrosis and exert a collagenolytic effect (119). Melanocortin-mediated inhibition of pulmonary endothelial cell apoptosis could likewise contribute to preserve alveolar-epithelial barrier integrity. Moreover, pharmacological activation of MCRs can exert a protective effect against endothelial inflammation and aberrant activation of coagulation cascades that substantially contribute to disease progression in respiratory virus infections.

Finally, targeting melanocortin system allows exploiting the beneficial effects exerted by the cholinergic anti-inflammatory pathway on systemic inflammation and deranged coagulation (120). In addition, the central neurogenic influences of melanocortins and their effects on descending anti-inflammatory neural pathways could be of paramount importance in view of a potential immunosuppression elicited by virus-induced inflammatory mediator release within the CNS (121).

Therefore, the central and peripheral protective actions exerted following MCR activation could collectively allow dampening the harmful events that trigger the cytokine storm and endothelial dysfunction while sustaining the beneficial signals required to elicit resolution and repair mechanisms.

CONCLUDING REMARKS

Enhancing endogenous protective responses to viral infection while inhibiting harmful signals is a key approach to prevent disease progression to a critical life-threatening state, particularly in the absence of specific antiviral drugs. We deem that translational use of melanocortin molecules could be an exploitable strategy in this setting. The key to the unique beneficial modulatory effects induced by melanocortins resides in their capacity to reduce the aberrant responses to infection without impairing the host defense mechanisms. Despite several overlapping influences, this represents a major distinction relative to the other potent, endogenous anti-inflammatory system formed by glucocorticoids. Capacity to take advantage of a protective endogenous system, deeply explored over the years, could help to face present and future emergencies marked by severe pathogen/host interactions.

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

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 08 January 2021
doi: 10.3389/fendo.2020.593179

The Adrenal Cortex, an Underestimated Site of SARS-CoV-2 Infection

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OPEN ACCESS

Edited by:
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University Hospital Würzburg, Germany

Reviewed by:
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Specialty section:
This article was submitted to Translational Endocrinology, a section of the journal Frontiers in Endocrinology

Received: 10 August 2020
Accepted: 01 December 2020
Published: 08 January 2021

Citation:
Mao Y, Xu B, Guan W, Xu D, Li F, Ren R, Zhu X, Gao Y and Jiang L (2021) The Adrenal Cortex, an Underestimated Site of SARS-CoV-2 Infection. Front. Endocrinol. 11:593179. doi: 10.3389/fendo.2020.593179

Background: The majority of the critically ill patients may have critical illness-related corticosteroid insufficiency (CIRCI). The therapeutic effect of dexamethasone may be related to its ability to improve cortical function. Recent study showed that dexamethasone can reduce COVID-19 deaths by up to one third in critically ill patients. The aim of this article is to investigate whether SARS-CoV-2 can attack the adrenal cortex to aggravate the relative adrenal insufficiency.

Methods: We summarized the clinical features of COVID-19 reported in currently available observational studies. ACE2 and TMPRSS2 expression was examined in human adrenal glands by immunohistochemical staining. We retrospectively analyzed serum cortisol levels in critically ill patients with or without COVID-19.

Results: High percentage of critically ill patients with SARS-COV-2 infection in the study were treated with vasopressors. ACE2 receptor and TMPRSS2 serine protease were colocalized in adrenocortical cells in zona fasciculata and zona reticularis. We collected plasma cortisol concentrations in nine critically ill patients with COVID-19. The cortisol levels of critically ill patients with COVID-19 were lower than those in non-COVID-19 critically ill group. Six of the nine COVID-19 critically ill patients had random plasma cortisol concentrations below 10 μg/dl, which met the criteria for the diagnosis of CIRCI.

Conclusion: We demonstrate that ACE2 and TMPRSS2 are colocalized in adrenocortical cells, and that the cortisol levels are lower in critically ill patients with COVID-19 as compared to those of non-COVID-19 critically ill patients. Based on our findings, we recommend measuring plasma cortisol level to guide hormonal therapy.

Keywords: severe acute respiratory syndrome coronavirus 2, coronavirus disease 2019, critically ill patients, adrenal cortex, adrenal insufficiency

INTRODUCTION

In December 2019, Wuhan, the capital of Hubei province in China, became the centre of an outbreak of severe respiratory illness caused by a novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which was later designated coronavirus disease 2019 (COVID-19) by WHO (1). COVID-19 that has a high sequence similarity (~80%) with SARS-CoV, triggered a global pandemic of novel COVID-19-infected pneumonia (NCIP) (2, 3). As of May 10, 2020, there are more than 3,917,366 confirmed cases of COVID-19 and 274,361 deaths worldwide (4). The clinical spectrum of COVID-19 infection appears to be wide. In addition to the typical symptoms of severe viral pneumonia, critically ill patients also suffer from acute kidney injury, acute cardiac injury, as well as multiple organ failure (5, 6). It has been known that COVID-19 invades respiratory epithelial cells *via* the receptor angiotensin converting enzyme II (ACE2) (7, 8). However, ACE2 expression is not limited to the lung, and extrapulmonary expression of ACE2 is also found in heart, vessels, kidney and digestive systems, which may contribute to the non-respiratory symptoms observed in NCIP patients (9).

The epidemiological and clinical characteristics of COVID-19 have been widely reported recently (10). Sepsis is the most frequently observed complication in patients with COVID-19 (11, 12). Notably, another common complication of severe COVID-19 illness, shock, is observed in 23~31% of COVID-19 patients in ICU (5, 13). Moreover, in a recent retrospective cohort study, septic shock was observed in 70% of non-survival patients with COVID-19 (38 of 54 patients) (6).

Prolonged critical illness is associated with adrenocortical dysfunction (14). The term "critical illness-related corticosteroid insufficiency" (CIRCI) is defined as aberrant synthesis and secretion of cortisol, and cellular corticosteroid activity that is inadequate for the severity of the patient's critical illness (15). CIRCI is thought to occur in several critical conditions, including sepsis and septic shock, severe pneumonia and acute respiratory distress syndrome (ARDS) (15). CIRCI is associated with an increase in circulating levels of biological markers of inflammation and coagulation over time (16), which is seen frequently in patients with severe COVID-19. However, it remains unknown whether adrenocortical dysfunction occurs in COVID-19 patients.

The present study summarized the clinical features of COVID-19 reported in currently available observational studies, and examined ACE2 expression in human adrenal glands. Serum cortisol levels were measured in critically ill patients with or without COVID-19. The main goal of this study was to provide theoretical rationale for the use of corticosteroids in critical ill patients with COVID-19.

MATERIALS AND METHODS

Reviewing the Reports of Vasopressors Use in COVID-19 Critically Ill Patients

We searched for clinical studies on critically ill patients with SARS-COV-2 infection published from January 1st, 2020 to May 1st, 2020. The searched databases included "pubmed", "Scopus" and "Web of Sciences". The search terms included: "Novel coronavirus", "Coronavirus disease", "COVID-19", "2019-nCoV", "SARS-COV-2", "Wuhan coronavirus", and "Wuhan pneumonia". Only clinical observational research studies and clinical studies were included. The language was limited to English.

The inclusion criteria were as follows: (1) age ≥18 years old; (2) SARS-CoV-2 in respiratory tract specimens, detected by quantitative reverse-transcription PCR (q-RT-PCR); (3) PaO_2/FiO_2 ≤300 mmHg; (4) presence of least one subgroup reporting critically ill patients receiving vasopressors. The exclusion criteria were as follows: (1) neuroendocrine system diseases (including disorders of hypothalamus, pituitary gland, or adrenal gland); (2) long-term hormone use; (3) mental diseases; (4) pregnancy. The retrieval of the data was done independently by two researchers. Disagreements were resolved by discussion with the third researcher. The following patient information was recorded: number of patients, the male to female ratio, acute physiology and chronic health evaluation II (APACHE II) score, sepsis-related organ failure assessment (SOFA) score, number of patients with cardiovascular disease, endocrine disease, cardiovascular complications due to SARS-CoV-2 infection, including shock, myocardial injury, arrhythmia, use of vasopressors or extracorporeal membrane oxygenation (ECMO) support, and mortality.

Ethics and Clinical Registration

The study was reviewed and approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiaotong University, School of Medicine and Shanghai Public Health Center (Number XHEC-D-2020-073). The Ethics Committee evaluated the design and implementation process of the study. The researchers strictly followed the medical ethics guidelines of the "Helsinki Declaration" and "International Ethical Guidelines for Human Health Related Research". All the patients had signed the "Extensive Informed Consent" when they were admitted to the hospital. The study was registered at the Chinese Clinical Trial Registry (http://www.chictr.org.cn) with registration number ChiCTR-ORC-16008623.

Immunohistochemical and Immunofluorescent Analysis

Healthy tissue specimens around tumors surgically resected from inpatients in Xinhua Hospital affiliated to Shanghai Jiaotong University School of Medicine from Jan 2020 to Apr 2020, were collected. Specimens included three samples of lung tissues and the same number of small intestines tissues, thyroid tissues, adrenal tissues, and adrenal glands surgically removed from pheochromocytoma patients for histological examination and immunohistochemical analysis.

The tissues were then fixed in 4% paraformaldehyde, and processed for standard (4-mm) paraffin sectioning. Fully automated immunohistochemistry (IHC) stainer (LEICA Bond RX) was used for the staining. Briefly, sections were incubated with primary antibodies against ACE2(Item No.:ab108209,

Abcam, USA)at a dilution of 1: 100 or transmembrane protease serine 2 (TMPRSS2) at a dilution of 1:1,000 for 30 min and the bound antibodies were detected by DAB (3, 3'-diaminobenzidine) staining system. The same concentration of normal IgG served as a negative control. In the adrenal tissue sections, hematoxylin-eosin (H&E) staining was also performed for histological localization. Co-staining of anti-ACE2 and anti-TMPRSS2 or anti-CYP11B1 (Item No.:MABS502, Sigma-Aldrich, USA) antibodies at a dilution of 1:100 was performed for immunofluorescence analysis.

Retrospective Analysis of Serum Cortisol Level in COVID-19 and Non-COVID-19 Critically Ill Patients

This study included COVID-19 critically ill patients in the intensive care unit (ICU) of the Shanghai Public Health Center from March 1st to May 1st, 2020. The inclusion criteria were as follows: (1) age ≥18 years old; (2) SARS-CoV-2 positive result from respiratory tract specimens as confirmed by quantitative reverse-transcription PCR (q-RT-PCR) detection; (3) PaO2/FiO2 ≤200 mmHg; (4) total plasma cortisol concentration measured in ICU. Exclusion criteria for the study were as follows: neuroendocrine system diseases (including disorders of hypothalamus, pituitary gland or adrenal gland), long-term hormone use (more than 7 days), basic pulmonary diseases, mental diseases, and pregnancy.

The control group of non-COVID-19 critically ill patients came from the ICU of Xinhua Hospital, affiliated to Shanghai Jiaotong University, School of Medicine. The inclusion and exclusion criteria were consistent with the group of COVID-19 critically ill patients. The total plasma cortisol was measured at 8am by the assay from Beijing North Institute of Biotechnology Co., Ltd and its reference range was 6.7-22.6 ug/fl. We selected the first measure of the total plasma cortisol for data analysis. We collected all the basic information and factors affecting serum cortisol level, including age, sex, APACHE II score, diagnosed cardiovascular and endocrine system diseases, use of hormones, administration of etomidate or azole antifungals during treatment, use of vasopressors as well as ventilators or ECMO support.

Statistical Methods

SPSS 26.0 software was used to analyze all the data in this study. Continuous variables with normal distribution were expressed as Mean ± SD, while non-normally distributed data were expressed as median ± interquartile range. For data comparison between COVID-19 group and non- COVID-19 group, Student's t test or Mann Whitney U test was used to evaluate continuous variables. Chi-square test or Fisher's exact test was used to assess categorical variables. P values ≤ 0.05 was considered statistically significant.

RESULTS

Critically Ill Patients With SARS-COV-2 Infection Have a High Incidence of Vasopressors Use

A total of 438 studies were initially selected based on the search strategy. After screening the title and abstract, 54 articles were selected for a full text evaluation. Of them, 26 were excluded due to lack of molecular diagnostic information (3), age of the patients (<18) (17) or pregnancy status (7). 23 additional articles were excluded due to lack of reports of vasopressors or shock. Finally, five clinical studies on critically ill patients with SARS-COV-2 infection were analyzed (5, 13, 17–19) (**Supplement Figure 1**). **Table 1** shows the main characteristics of the included studies and the cardiovascular complications of patients during treatment.

Of these 5 clinical studies on critically ill COVID-19 patients, 2 articles reported both critically ill patients and mildly ill patients, and the patients were subgrouped according to the severity of their condition (5, 13). We selected the critically ill patients' data. Three articles reported that 22%–67% of COVID-19 patients used vasopressors (17–19).

Nonetheless, the incidence of hypotension may still be underestimated. One possible reason is that many patients were still in hospital at the end of the included studies, thus

TABLE 1 | Cardiovascular complications in patients with SARS-CoV-2 infections.

Cohort	Cao et al.	Wang et al.	Arentz et al.	Huang et al.	Yang et al.
No. of patients	199	36	21	13	52
Age, years	58.0 (49.0–68.0)	66 (57–78)	70(43–92)	49.0(41–61)	59.7-13.3
Sex ratio (F:M)	79:120	14:22	10:11	2:11	17:35
APACHE II	-	17 (10–22)	-	-	17(14-19)
SOFA	-	5 (3–6)	-	-	-
Cardiovascular disease	-	21 (58%)	9 (43%)	3 (13%)	5 (10%)
Hypertension	-	9 (25%)	-	2 (15%)	-
Endocrine disease	23 (12%)	8 (22%)	7 (33%)	1(8%)	9 (17%)
Cardiovascular complications					
Use of vasopressors	44 (22%)	-	14(67%)		18 (35%)
Shock	4 (2%)	11 (31%)	-	3 (23%)	-
Myocardial injury	-	8 (22%)	7 (33%)	4 (31%)	12 (23%)
Arrhythmia	1(0.5%)	16 (44%)	-	-	-
Acute heart failure	1(0.5%)	-	4(19.1)	-	-
Use of ECMO	4 (2%)	4 (11%)	-	2 (15%)	6 (12%)
Mortality	44 (22%)	11 (31%)	11(52%)	6 (46%)	32 (62%)

still potentially at risk of developing hypotension. Cao et al. reported that the proportion of patients receiving vasopressor therapy was much higher than the proportion of patients with shock (17). Overall, the incidence of shock was between 4-31%, and lower than the incidence of patients that received vasopressor therapy. In addition, the incidence of myocardial injury patients was between 22%–33%, and the mortality rate was between 22%–62%.

Immunohistochemical and Immunofluorescent Analysis

As shown in **Figures 1A–C**, in healthy lung tissue sections ACE2 was widely expressed in pulmonary vascular endothelial cells, alveolar epithelial cells, bronchial mucosal epithelial cells, and on the apical surface of ciliated columnar epithelial cells. In human normal small intestine sections, ACE2 was mainly expressed on the surface of villi epithelial cells (**Figures 1D, E**). However, ACE2 immunoreactivity was not observed in normal human thyroid tissue sections (**Figures 1F, G**).

As shown in the **Figures 2B, E**, immunohistochemical staining of ACE2 in human adrenal gland sections showed no obvious immunoreactivity in the zona glomerulosa, which is the unique source of the mineralocorticoid aldosterone (19). In contrast, ACE2 was widely distributed in the zona fasciculata/reticularis, which produces the glucocorticoids and the androgens. Transmembrane Serine Protease 2 (TMPRSS2) appears to prime the viral spike (S) protein to enhance ACE2-mediated SARS-CoV-2 entry (7). As shown in **Figures 2C, F**, we found that TMPRSS2 was widely expressed in all three zones of the adrenal cortex. **Figures 2A, D** showed the observation place with HE staining.

Steroid 11-hydroxylase (CYP11B1), an enzyme catalyzing the terminal steps of cortisol synthesis, is mainly expressed in the human adrenal zona fasciculata/reticularis, and determines the functional differentiation of adrenocortical cells (20). As shown in **Figure 3**, double immunofluorescence staining clearly demonstrated the colocalization of CYP11B1/ACE2, CYP11B1/TMPRSS2 and ACE2 /TMPRSS2 in the adrenal cortex.

The expression of ACE2 was also detected in the adrenal medulla. In the healthy adrenal tissues (**Figures 4B, F**), chromaffin cells (indicated by black arrows) could only be distinguished in the adrenal medulla under high magnification (×400), and exhibited no obvious ACE2 immunoreactivity. To further confirm these findings, sections obtained from pheochromocytoma tissues were stained with ACE2 antibody. As shown in **Figures 4D, H**, ACE2 immunoreactivity was not observed in pheochromocytoma cells (indicated by yellow arrows). **Figures 4A, E** showed the observation place in adrenal medulla. **Figures 4C, G** showed the observation place in pheochromocytoma tissues.

The Cortisol Levels of Critically Ill Patients With COVID-19 Are Lower Than Those in Non-COVID-19 Critically Ill Patients

Since the epidemic continued to decline in China, there were only nine critically ill patients with COVID-19 in the ICU of Shanghai Public Health Center by the end of this study. All the nine patients met the inclusion criteria without exclusion criteria, and were recruited in this study.

Of the 15 non-COVID-19 critically ill patients, 3 patients did not meet the inclusion criteria and were excluded. In the end, nine COVID-19 and 12 non-COVID-19 patients were included in this study (**Figure 5**). During treatment, 88.9% of COVID-19 critically ill patients used vasopressors, while 100% non-COVID-19 critically ill patients used vasopressors. Thus, there was no significant difference between the two groups (P = 0.429). 88.9% of COVID-19 critically ill patients and 100% of non-COVID-19 critically ill patients needed ventilator support (P = 0.429). 77.7% of COVID-19 patients received Vein-Artery ECMO support, and none of non-COVID-19 patients required ECMO support (P = 0.000).

We compared the differences in plasma cortisol levels between the two groups. Concentrations of cortisol in COVID-19 patients were considerably lower than those in non-COVID-19 critically ill patients (P = 0.000) (**Figure 6**). It was worth noting that random plasma cortisol elevated beyond the normal range in 50% non-COVID-19 critically ill patients. In contrast,

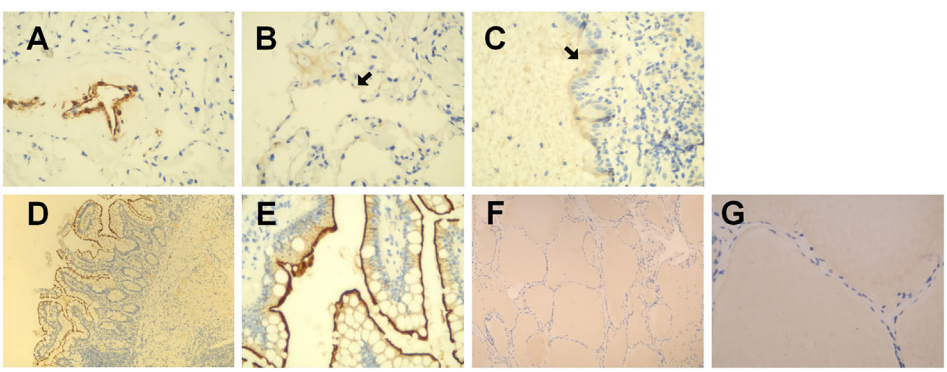

FIGURE 1 | Immunohistochemical staining of ACE2 in human healthy lung tissues. small intestines and thyroid tissues. **(A–C)** in healthy lung tissue sections, ACE2 was widely expressed in pulmonary vascular endothelial cells, alveolar epithelial cells, bronchial mucosal epithelial cells, and on the apical surface of ciliated columnar epithelial cells. **(D, E)** in human normal small intestine sections, ACE2 was mainly expressed on the surface of villi epithelial cells. **(F, G)** ACE2 immunoreactivity was not observed in human normal thyroid tissue sections. Original magnification: **(A–C, E, G)**: ×400; D&F:×100.

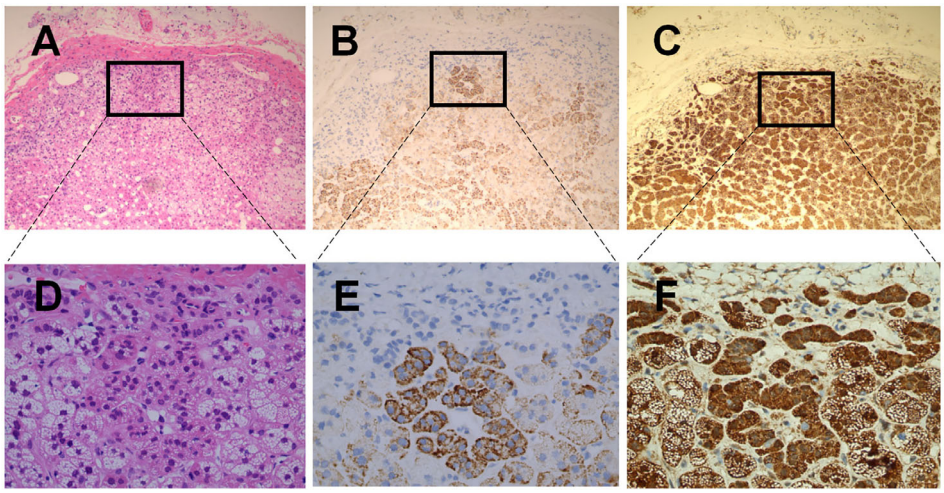

FIGURE 2 | Haematoxilin-Eosin staining and immunohistochemical staining of ACE2 & TMPSS2 in human healthy adrenal tissues. **(A–C)** were the serial sections of the same adrenal cortex. Panel A showed Haematoxilin-Eosin (HE) staining. **(B, C)** were stained with antibodies aganist ACE2 **(B)** and TMPSS2 **(C)**, respectively. Original magnification: ×100. Areas in black boxes in **(A–C)** were shown enlarged in **(D–F)** (×400), respectively.

six of the nine COVID-19 critically ill patients had plasma cortisol concentrations below 10 μg/dl, which met the criteria for the diagnosis of CIRCI (21). Plasma cortisol levels are known to be affected by several factors, such as use of etomidate, glucocorticoids or azole antifungals, and bile acids level in blood (22). Therefore, we evaluated the differences in these

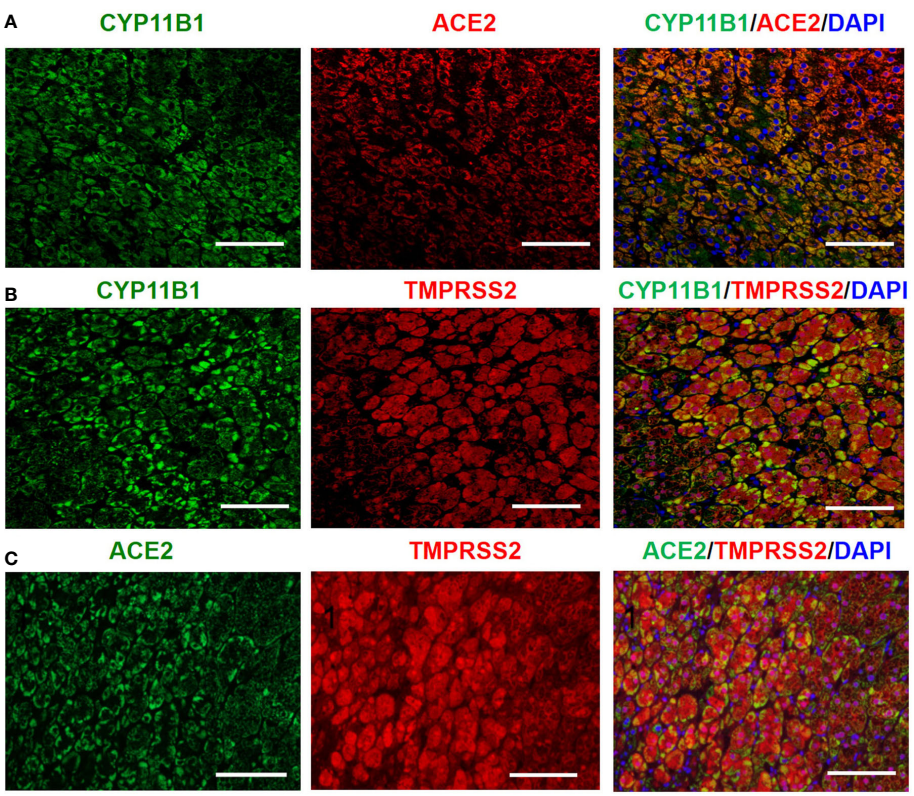

FIGURE 3 | Double immunofluorescence staining demonstrates the colocalization of CYP11B1/ACE2 **(A)**, CYP11B1/TMPRSS2 **(B)** and ACE2/TMPRSS2 **(C)** in human healthy adrenal tissues. Original magnification: ×200. Scale bars correspond to 50 μm.

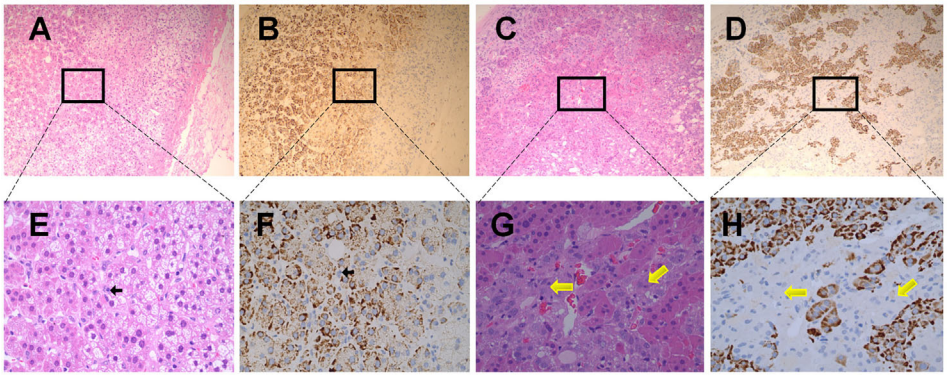

FIGURE 4 | Immunohistochemical staining of ACE2 in human normal adrenal medulla and pheochromocytoma tissues. **(A, B)** were the serial sections of the same adrenal medulla. **(C, D)** were the serial sections of the same pheochromocytoma tissues. **(A, C)** showed Haematoxilin-Eosin (HE) staining. **(B, D)** were stained with antibodies aganist ACE2. Original magnification: ×100. Areas in black boxes in **(A–D)** were shown enlarged in **(E–H)** (×400), respectively. In the healthy adrenal tissues, c cells (pointed by black arrows) could only be distinguished in the adrenal medulla under high magnification **(E, F)**, and exhibited no obvious ACE2 immunoreactivity. In sections obtained from pheochromocytoma tissues, ACE2 immunoreactivity was also not observed in pheochromocytoma cells (pointed by yellow arrows).

factors between the two groups (**Table 2**). We did not detect significant difference in the composition ratio of using glucocorticoids (P = 0.063), or using the azole antifungals (P = 0.063) between the two groups. The level of bile acid was similar between the two groups (P = 0.310). None of the patients in the two groups used etomidate.

DISCUSSION

To date, the ongoing COVID-19 pandemic has affected more than 210 countries and territories around the world (1). The epidemiological and clinical features of COVID-19 have been widely reported (2, 3, 5, 6). However, mechanisms underlying the pathogenesis of COVID-19 still need to be fully elucidated. Previous studies have identified the main path for SARS-CoV-2 entry into the cell – namely *via* the viral spike (S) protein attaching to ACE2 and employing the cellular serine protease (TMPRSS2) for S protein priming in human lung cells (7, 23). Thus, COVID-19 patients display characteristic respiratory symptoms including dyspnea, low oxygen saturation, and rapid progress of chest radiological abnormality (24). In addition, several studies have provided bioinformatic evidence of ACE2 expression in cardiovascular, digestive, urinary, and reproductive systems, implying that these organs are all potential routes of SARS-CoV-2 infection (9, 25, 26). Consistent with these studies, our data confirmed that ACE2 protein expression was enriched in pulmonary epithelial/endothelial cells and the enterocytes of small intestine tissues.

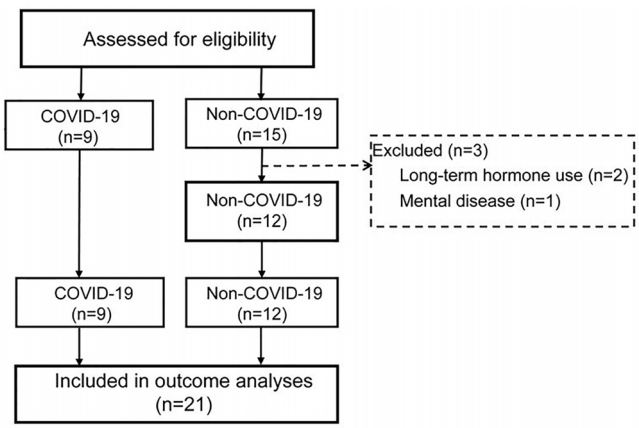

FIGURE 5 | Flowchart of trial procedures. All the COVID-19 patients met the inclusion criteria: 1. Age ≥18 years, 2. Positive results of sars-cov-2 by qRT-PCR, 3. PaO2/FiO2 ≤ 200mmHg, 4. Total plasma cortisol was measured. Patients did not receive prolonged hormonal therapy, and do not have hypothalamus, pituitary gland, adrenal gland disease or mental illness; Female patients were not pregnant.

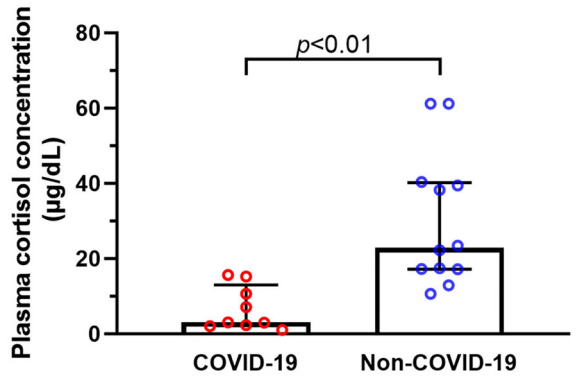

FIGURE 6 | Plasma cortisol concentrations of critically ill patients with or without COVID-19. We collected data on plasma cortisol concentrations in nine critically ill patients with COVID-19, and the results showed that the cortisol levels of COVID-19 critically ill patients were considerably lower than those in non-COVID-19 critically ill patients.

Based on the systematic literature review (17–19), we found that 22%~67% of patients infected with SARS-CoV-2 received vasopressors therapy, due to septic shock induced by SARS-CoV-2 or secondary infections including bacteria and fungi. On the other hand, given that SARS-CoV-2 pandemic seems to be particularly deleterious to patients with underlying cardiovascular diseases (CVD), including congestive heart failure (27), vasopressors may also be used in patients with cardiogenic shock. Notably, a recent bioinformatic study indicates medium-level expression of ACE2 in adrenal glands (28). The hormones produced by the adrenal cortex and adrenal medulla, specifically steroid hormones and catecholamines, play critical roles in the regulation of vascular reactivity (29, 30). We investigated the presence and localization of ACE2 protein in human adrenal glands by immunohistochemistry. ACE2 immunostaining was not observed in adrenal medulla obtained from normal adrenal glands or from adrenal glands with pheochromocytoma. However, both ACE2-positive and TMPRSS2-positive immunostained cells were widely observed in zona fasciculata and zona reticularis of adrenal cortex that are mainly responsible for synthesis and secretion of glucocorticoids. This finding was further confirmed by the colocalization of ACE2 or TMPRSS2 with CYP11B1, a marker for the functional differentiation of cells in the zona fasciculata and reticularis. These results suggest that SARS-CoV-2 may potentially directly target zona fasciculata/reticularis of adrenal cortex, thereby influencing circulating glucocorticoid levels.

The importance of glucocorticoids has been illustrated in studies that demonstrated the crucial role of the adrenal glands for survival under conditions of stress (31, 32). Glucocorticoids have profound metabolic, cardiovascular and immunological roles in adequate stress response that allows to maintain and restore homeostasis in the human body (33). Previous studies showed that elevated plasma concentrations of cortisol in critically ill patients are evoked by the activation of the HPA axis and systemic hyperinflammation (34). Consistent with this notion, we found that random plasma cortisol levels were elevated beyond the normal range in 50% of non-COVID-19 critically ill patients. On the other hand, random plasma cortisol levels in COVID-19 critically ill patients were considerably lower than those in non-COVID-19 ones. It's worth noting that 67% of critically ill patients with COVID-19 had random plasma cortisol concentrations below 10 μg/dl, which met the criteria for the diagnosis of CIRCI (21). By mediating and enhancing the action of angiotensin II and catecholamines, cortisol maintains cardiac contractility, vascular tone, and blood pressure, which are crucial for critically ill patients (35). Therefore, the cardiovascular symptoms of CIRCI include hypotension refractory to fluid resuscitation and decreased sensitivity to catecholamines (21). This was also supported by our findings obtained from the systematic literature review, which showed that vasopressors were required in 22%~67% of COVID-19 patients.

Previous studies have identified dysregulation of the HPA axis and impaired steroidogenesis as major pathophysiologic events that account for the development of CIRCI (21). For example, circulating bile acids can pass the blood–brain barrier and bind to the hypothalamic glucocorticoid receptor, causing central

TABLE 2 | Clinical characteristics of the critically ill patients and complication in ICU.

Characteristic	COVID-19 group (n=9)	Non-COVID-19 group (n=12)	*p*
Age, mean (SD), year	71.7 (8.1)	68.9(20.2)	0.706
Sex ratio (F:M)	8:1	8:4	0.338
APACHE II, mean (SD)	25.4(2.3)	24.3(2.6)	0.330
Coexisting conditions, no.			
Cardiovascular disease	1/9	2/12	1
Endocrine disease	1/9	1/12	1
Treatment in ICU			
Glucocorticoid(in 7 day)	3/9	0/12	0.063
Etomidate	0/9	0/12	>0.05
Azole antifungals	3/9	0/12	0.063
Complication in ICU			
Bile acid (umol/l), median (IQR)	5.96 (4.10-9.75)	4.2 (1.82-10.35)	0.345
Use of vasopressors	8/9	12/12	0.429
Use of ventilator	8/9	12/12	0.429
Use of V-A ECMO	7/9	0/12	0.000

HPA suppression, and consequent decrease in ACTH release and adrenocortical steroidogenesis (36). Another possible cause of ICU-acquired CIRCI is the administration of drugs that interfere with steroidogenesis, such as etomidate, azole antifungals, and exogenous glucocorticoid therapy (37). In this study, we found no significant difference in circulating levels of bile acids, utilization of etomidate, azole antifungals, and glucocorticoids between non-COVID-19 and COVID-19 critically ill patients. Our results suggest therefore that these factors do not contribute to the considerably lower cortisol levels in COVID-19 critically ill patients. Components of various pathogens, including bacteria and viruses, have been found to directly impact adrenocortical steroidogenesis (38–40). The present study found the colocalization of ACE2 and TMPRSS2 in the zona fasciculata and zona reticularis of adrenal cortex. Our findings suggest that adrenocortical cells in zona fasciculata and zona reticularis may be potential targets of SARS-CoV-2 infection, thereby providing a possible cause for the reduction of cortisol levels in COVID-19 critical ill patients. Just on June 16, 2020, a study published in "Nature" found that a cheap, widely available steroid drug-dexamethasone can reduce COVID-19 deaths by up to one third in critically ill patients. However, the steroid had no effect on people with mild cases of COVID-19 (41). The authors speculate that the therapeutic effect of dexamethasone on critically ill COVID-19 patients may be related to its anti-inflammatory response. However, it should be noted that most critically ill patients may have CIRCI, and the therapeutic effect of dexamethasone may also be related to its improvement of cortical function. Our research was focused on the latter and could partly explain why dexamethasone treatment reduced mortality in severely ill patients with COVID-19.

The reproductive implications of coronavirus infection recently have attracted much attention. Several studies clearly demonstrated that ACE2 is highly expressed in spermatogonia, leydig and sertoli cells, as well as in cells of the seminiferous tubules in the human testis (42–44). Thus, the binding of the virus to these ACE2-positive cells may cause severe alteration of testicular tissue, and therefore have a serious impact on fertility. In addition, the effect of SARS-CoV-2 infection on male sex hormones has recently been investigated in a single center-based study (45). In this study, Ma et al. found that serum luteinizing hormone (LH) was significantly increased, but the ratio of testosterone (T) to LH and the ratio of follicle stimulating hormone (FSH) to LH were dramatically decreased in reproductive-aged males with COVID-19 compared to age-matched healthy men. The zona fasciculata and zona reticularis of adrenal cortex are recognized as the main source of extragonadal production of androgens (dehydroepiandrosterone [DHEA] and DHEA sulfate [DHEAS]) (46). The adrenal androgens are converted into androstenedione and then into testosterone in the peripheral tissues. The importance of the adrenal-derived androgens to the overall production of sex steroid hormones is highlighted by the fact that approximately 50% of total androgens in the prostate of adult men are derived from adrenal steroid precursors (19). In the present study, we found that both ACE2 and TMPRSS2 proteins were expressed in the zona fasciculata/reticularis of the human adrenal cortex. These results may indicate that SARS-CoV-2 infection might impact extragonadal androgen production by targeting adrenocortical steroidogenic cells.

Our study has several limitations. First, the results of this study need to be interpreted with caution because of its retrospective nature and the small sample size. Retrospective study with small sample size always increases the likelihood of a Type II error and decreases statistical power. In the retrospective study, there is no way to control when and how often the total cortisol measured, which would also result in some degree of research bias. Second, this study used the APACHE II score to evaluate the severity of the patients' illness. Notably, seven of the nine COVID-19 critically ill patients received ECMO therapy to improve gas exchange. Since several important parameters related to gas exchange were included in the APACHE II scoring system, the severity of disease might be underestimated in COVID-19 critically ill patients requiring ECMO support. Third, vasopressors were used in 20 of the 21 critically ill patients recruited in this study, thereby making it difficult to assess the relationship between plasma cortisol level and haemodynamic instability. Although intravenous glucocorticoids are commonly used in patients with severe SARS or MERS pneumonia, lack of their effect on the overall survival made their use to treat SARS-CoV-2 infection questionable (47). WHO guidance on management of COVID-19 advises against corticosteroids, unless indicated for other reasons, such as adrenal insufficiency (47). A growing body of evidence reveals that dysfunction of the HPA axis occurs frequently in patients with serious infections (48, 49). The colocalization of ACE2 and TMPRSS2 in zona fasciculata and zona reticularis of adrenal cortex suggests that SARS-CoV-2 may directly interfere with the process of cortisol synthesis, thereby further increasing the incidence of CIRCI in COVID-19 critical ill patients.

Our findings suggest that the adrenal cortex may be the target organ of SARS-CoV-2. In comparison with non-COVID-19 critically ill patients, a lower random plasma cortisol level was observed in COVID-19 critically ill patients, among whom 67% met the criteria for the diagnosis of CIRCI. Since CIRCI is characterized by hypotension unresponsive to fluid resuscitation, and requires vasopressor therapy, it is necessary to carefully consider whether to add an appropriate dose of corticosteroids to maintain the stability of the circulatory system. Our findings recommend measuring plasma cortisol levels to guide hormonal therapy.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Xinhua Hospital Affiliated to

Shanghai Jiaotong University, School of Medicine and Shanghai Public Health Center (Number XHEC-D-2020-073). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LJ, YG, and XZ conceived and performed experiments and wrote the manuscript. YM, BX, and WG performed experiments. FL and RR provided data analysis. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We thank assistant Professor Xi Zhang from the Clinical Research Center of Xinhua Hospital for her suggestion on statistical methods.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.593179/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | The PRISMA diagram of literature review.

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

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 25 February 2021
doi: 10.3389/fendo.2021.638621

Vitamin D Association With Macrophage-Derived Cytokines in Polycystic Ovary Syndrome: An Enhanced Risk of COVID-19 Infection?

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OPEN ACCESS

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Specialty section:
This article was submitted to Translational Endocrinology, a section of the journal Frontiers in Endocrinology

Received: 07 December 2020
Accepted: 25 January 2021
Published: 25 February 2021

Citation:
Moin ASM, Sathyapalan T, Butler AE and Atkin SL (2021) Vitamin D Association With Macrophage-Derived Cytokines in Polycystic Ovary Syndrome: An Enhanced Risk of COVID-19 Infection? Front. Endocrinol. 12:638621. doi: 10.3389/fendo.2021.638621

Background: Women with polycystic ovary syndrome (PCOS) often have vitamin D deficiency, a known risk factor for severe COVID-19 disease. Alveolar macrophage-derived cytokines contribute to the inflammation underlying pulmonary disease in COVID-19. We sought to determine if basal macrophage activation, as a risk factor for COVID-19 infection, was present in PCOS and, if so, was further enhanced by vitamin D deficiency.

Methods: A cross-sectional study in 99 PCOS and 68 control women who presented sequentially. Plasma levels of a macrophage-derived cytokine panel were determined by Slow Off-rate Modified Aptamer (SOMA)-scan plasma protein measurement. Vitamin D was measured by tandem mass spectroscopy.

Results: Vitamin D was lower in PCOS women ($p<0.0001$) and correlated negatively with body mass index (BMI) in PCOS ($r=0.28$, $p=0.0046$). Basal macrophage activation markers CXCL5, CD163 and MMP9 were elevated, whilst protective CD200 was decreased ($p<0.05$); changes in these variables were related to, and fully accounted for, by BMI. PCOS and control women were then stratified according to vitamin D concentration. Vitamin D deficiency was associated with decreased CD80 and IFN-γ in PCOS and IL-12 in both groups ($p<0.05$). These factors, important in initiating and maintaining the immune response, were again accounted for by BMI.

Conclusion: Basal macrophage activation was higher in PCOS with macrophage changes related with increased infection risk associating with vitamin D; all changes were BMI dependent, suggesting that obese PCOS with vitamin D deficiency may be at greater risk of more severe COVID-19 infection, but that it is obesity-related rather than an independent PCOS factor.

Keywords: COVID-19 risk factors, polycystic ovary disease, vitamin D, macrophage, cytokines

BACKGROUND

Polycystic ovary syndrome (PCOS) is considered to be a cardiometabolic condition with consequences that include obesity and insulin resistance that drive the excess prevalence of type 2 diabetes, hypertension, and cardiovascular diseases in later life (1). It has been suggested that these features of PCOS put subjects at a higher risk for severe COVID-19 infection (2, 3). Those with PCOS are more commonly affected by vitamin D deficiency than those without PCOS (4), deficiency occurring in over 60% of subjects. Controversially, vitamin D deficiency has been suggested to increase the risk and severity of COVID-19 disease, with an inverse correlation of COVID-19 incidence and mortality to vitamin D levels (5, 6); however, others have reported that there is no link between vitamin D and mortality (7).

In severe COVID-19 disease, acute respiratory distress syndrome (ARDS) results, caused by an unconstrained systemic inflammation to which differing populations of macrophages (resident alveolar macrophages (AMs), and recruited macrophages from the circulation) contribute (8). Macrophages are key players in inflammation and, upon activation, two polarized states result in an activated phenotype M1, macrophages that are pro-inflammatory and cytotoxic, and an activated phenotype M2, macrophages that are involved in tissue remodeling and matrix deposition (9, 10). Inflammation has been suggested to underlie insulin resistance and obesity in PCOS caused by macrophage stimulation (11); therefore, we hypothesized that there would be an increase in activated macrophages in those subjects with PCOS that would be further increased by vitamin D deficiency, predisposing these women to increased risk for severe COVID-19 disease.

METHODS

Study Population

99 PCOS and 68 control women "who presented sequentially to the Department of Endocrinology, Hull and East Yorkshire Hospitals NHS Trust were recruited to the local PCOS biobank (ISRCTN70196169) from January 2014 to December 2016. The Newcastle & North Tyneside Ethics committee approved this study; all patients gave written informed consent (2).

PCOS diagnosis was based on the Rotterdam consensus diagnostic criteria, namely clinical or biochemical evidence of hyperandrogenism (Ferriman-Gallwey score >8; free androgen index >4.5 respectively), self-reported oligomenorrhea (≤ 9 menses per year) or amenorrhea (no menses for 3 months or more) and polycystic ovaries on transvaginal ultrasound (≥12 antral follicles in at least one ovary or ovarian volume of ≥10 cm^3) (12). Study participants had no concurrent illness, were not on any medication for the preceding 9 months and were not planning to conceive. All PCOS women fulfilled the NIH criteria for diagnosis of PCOS (2)."

Collection and Analysis of Blood Samples

Blood samples were collected and were measured in the Chemistry Laboratory, Hull Royal Infirmary, UK as previously described (13). "Insulin, C-reactive protein (CRP) and sex hormone binding globulin (SHBG) were measured by an immunometric assay with fluorescence detection on the DPC Immulite 2000 analyzer using the manufacturer's recommended protocol, as previously described (13). Testosterone was measured by isotope dilution liquid chromatography-tandem mass spectrometry (Waters Corporation, Manchester, UK) as previously described (14).

The free androgen index (FAI) was calculated as the total testosterone x 100/SHBG. Serum insulin was assayed using a competitive chemiluminescent immunoassay performed on the manufacturer's DPC Immulite 2000 analyzer (Euro/DPC, Llanberis, UK). The analytical sensitivity of the insulin assay was 2 μU/ml, the coefficient of variation was 6%, and there was no stated cross-reactivity with proinsulin. Plasma glucose was measured using a Synchron LX 20 analyzer (Beckman-Coulter), using the manufacturer's recommended protocol. The coefficient of variation for the assay was 1.2% at a mean glucose value of 5.3 mmol/L during the study period. The insulin resistance was calculated using the HOMA method [HOMA-IR= (insulin x glucose)/22.5]." All analyses were undertaken according to current guidelines, regulations and quality control. Serum vitamin D levels and testosterone were quantified using isotope-dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) (15): vitamin D sufficiency was defined as >70 ng/ml, insufficiency as 50–69 ng/ml and deficiency as <50 ng/ml (16).

SOMA-Scan Assay

Plasma levels of macrophage-related proteins were determined by Slow Off-rate Modified Aptamer (SOMA)-scan plasma protein measurement as has been previously described (17, 18). The macrophage panel included measurement of M1 macrophage activation biomarkers (cytokines TNF-α, IL-6, IL-1β, IL-12, CD80 and chemokines CXCL1, CXCL2/CXCL3, CXCL5, CXCL8, CXCL9, CXCL10, CCL5, TLR4); activated M2 macrophage biomarkers (LBP, CD163, TFGβ-1, CD200, CD200R1, MMP7, MMP9, and CD36); conventional mediators of both M1 and M2 macrophage activation markers (IFN-γ, IL-4, IL-13). "The SOMAscan assay used to quantify proteins was

Abbreviations: PCOS, polycystic ovary syndrome; SOMA, Slow Off-rate Modified Aptamer; RFU, Relative Fluorescent Units; CRP, C-reactive protein; SHBG, sex hormone binding globulin; BMI, body mass index; LC-MS/MS, liquid chromatography tandem mass spectrometry; ARDS, acute respiratory distress syndrome; AM, alveolar macrophages; COVID-19, coronavirus disease of 2019; MMPs, Matrix metalloproteinases; TNF-α, Tumor Necrosis Factor alpha; IL-6, Interleukin-6; IL-1β, Interleukin-1 beta; IL-12, Interleukin-12; CD80, Cluster of differentiation 80; CXCL1, chemokine (C-X-C motif) ligand 1; CXCL2/CXCL3, chemokine (C-X-C motif) ligand 2/chemokine (C-X-C motif) ligand 3; CXCL5, chemokine (C-X-C motif) ligand 5; CXCL8, chemokine (C-X-C motif) ligand 8; CXCL9, chemokine (C-X-C motif) ligand 9; CXCL10, chemokine (C-X-C motif) ligand 10; CCL5, Chemokine (C-C motif) ligand 5; TLR4, Toll-like receptor 4; LBP, Lipopolysaccharide-binding protein; CD163, Cluster of Differentiation 163; TFGβ-1, transforming growth factor-β1; CD200, Cluster of Differentiation 200; CD200R1, Cluster of Differentiation 200 receptor 1; MMP7, Matrix metalloproteinase-7; MMP9, Matrix metalloproteinase-9; CD36, Cluster of Differentiation 36; IFN-γ, Interferon gamma; IL-4, Interleukin-4; IL-13, Interleukin-13.

performed on an in-house Tecan Freedom EVO liquid handling system (Tecan Group, Maennedorf, Switzerland) utilizing buffers and SOMAmers from the SOMAscan HTS Assay 1.3K plasma kit (SomaLogic, Boulder, CO) according to manufacturer's instructions and as described previously (19, 20). The assay was performed in 96-well plates containing up to 85 plasma samples, three quality control and five calibrator plasma samples. Briefly, EDTA plasma samples were diluted into bins of 40%, 1%, and 0.05% and incubated with streptavidin-coated beads immobilized with dilution-specific SOMAmers *via* a photocleavable linker and biotin. After washing bound proteins were first biotinylated and then released from beads by photocleaving the SOMAmer-bead linker. The released SOMAmer-protein complex was treated with a polyanionic competitor to disrupt unspecific interactions and recaptured on the second set of streptavidin-coated beads. Thorough washing was performed before 5' Cy3 fluorophore labelled SOMAmers were released under denaturing conditions, hybridized on microarray chips with SOMAmer-complementary sequences, and scanned using the SureScan G2565 Microarray Scanner (Agilent, Santa Clara, CA) (17).

Data Processing and Statistics

As previously described (17) "initial Relative Fluorescent Units (RFUs) were obtained from microarray intensity images using the Agilent Feature Extraction Software (Agilent, Santa Clara, CA). Raw RFUs were normalized and calibrated using the software pipeline provided by SomaLogic." Comparisons were performed using Student's t-test where a p-value <0.05 was taken as significant (GraphPad Prism 8.0, San Diego, CA, USA). No power analysis could be performed for this study because no data available relating the effect of vitamin D upon macrophage proteins in PCOS is available.

RESULTS

The PCOS women were older (p=0.03) with elevated BMI (p<0.001), weight (p<0.0001), waist and hip circumference (p<0.0001), systolic (p<0.001), and diastolic (p=0.03) blood pressure (**Table 1**). Biochemically, the PCOS women had elevated anti-Mullerian hormone (AMH) (p<0.0001), CRP (p<0.0001), testosterone (p=0.001) and free androgen index (p<0.0001). Vitamin D was significantly lower in the PCOS group (p<0.0001) and correlated negatively with BMI in PCOS (r=0.28, p=0.0046) (**Table 1**).

Macrophage Proteins in PCOS

Baseline macrophage proteins are shown in **Figure 1**. Basal macrophage activation markers CXCL5, CD163 and MMP9 were elevated, whilst the protective CD200 was decreased (p<0.05); their correlation with BMI is shown in **Figure 2** and the changes in these variables were related to and fully accounted by BMI. The additional macrophage proteins that did not differ between controls and PCOS are shown in **Supplementary Figure 1** (10.6084/m9.figshare.13090652).

Vitamin D Stratified Groups

The PCOS and control women were then stratified according to vitamin D status. Vitamin D status was stratified into sufficient, insufficient and deficient. Of the 99 PCOS women, 16 (16%) were sufficient, 11 (11%) were insufficient and 72 (73%) were deficient. Of the 68 control women, 26 (38%) were sufficient, 22 (32%) insufficient, and 20 (29%) deficient (**Supplementary Table 1**). Vitamin D deficiency was associated with decreased CD80 and IFN-γ in PCOS (both p<0.05) and IL-12 (p<0.05) in both PCOS and controls, as shown in **Figure 3**. Those proteins that did not differ within groups stratified for vitamin D are shown in **Supplementary Figure 2** (10.6084/m9.figshare.13090652). As noted above, vitamin D correlated negatively with BMI in PCOS (r=0.28, p=0.0046) and the changes in CD80, IL-12 and, IFN-γ did not differ when BMI was adjusted for.

BMI Stratified Groups

The macrophage-related proteins that were significantly different between PCOS and control groups were then divided into lean (BMI less than or equal to 25 kg/m^2) and obese (BMI 26 kg/m^2 or above).

In PCOS women, CD163 (p<0.005) and MMP9 (p<0.005) were elevated while CD200 (p<0.05) was reduced in the obese relative to the lean group. No difference was seen for CXCL5

TABLE 1 | Demographic and biochemical characteristics of the PCOS and control women. Data are presented as mean (SD).

	CONTROL (n = 68)	PCOS (n = 99)	P value
Age (years)	27.5 (0.6)	29.8 (0.9)	0.03
BMI (kg/m2)	26.6 (0.8)	34.6 (0.8)	<0.0001
Weight (kg)	73.7 (2.1)	97.8 (2.3)	<0.0001
Waist circumference (cm)	81 (2)	102 (2)	<0.0001
Hip circumference (cm)	101 (1)	119 (2)	<0.0001
Systolic blood pressure (mmHg)	115 (1)	122 (2)	0.0008
Diastolic blood pressure (mmHg)	74 (1)	77 (1)	0.03
AMH (pmol/l)	22.3 (2.1)	46.2 (3.2)	<0.0001
Sex Hormone Binding Globulin (SHBG) (nmol/l)	72.6 (9.5)	42.9 (4.5)	0.002
CRP (mmol/l)	2.1 (0.5)	4.8 (0.6)	<0.0001
Testosterone (nmol/l)	1.1 (0.1)	1.5 (0.1)	0.001
FAI	2.1 (0.2)	5.7 (0.6)	<0.0001
HOMA-IR	1.64 (1.60)	3.92 (6.22)	0.0052
Total vitamin D (ng/ml)	62 (3)	43 (3)	<0.0001

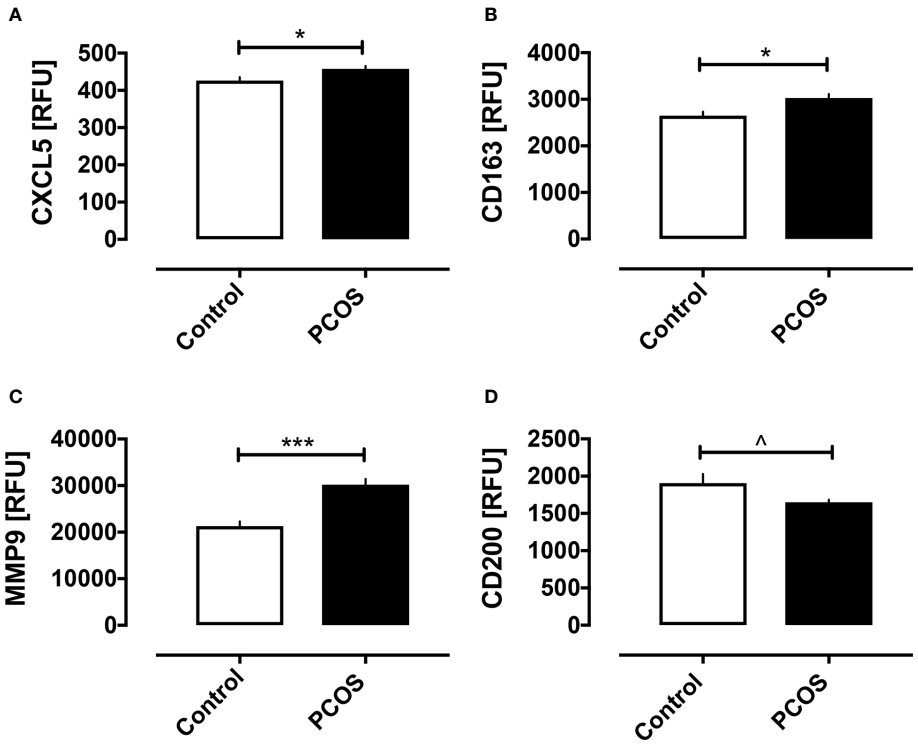

FIGURE 1 | Macrophage-related proteins in women with and without PCOS. Baseline macrophage proteins are shown for the proteins that differed between PCOS and controls: CXCL5 **(A)**, CD163 **(B)**, MMP9 **(C),** and CD200 **(D)**. *p < 0.01, ***p < 0.0001, ^p < 0.05. RFU, relative fluorescent units.

between lean and obese PCOS women. There were no differences in these protein levels seen between lean and obese control women.

DISCUSSION

Macrophage-derived cytokines promote the inflammation in ARDS, and post-mortem lung histopathology in COVID-19 disease reveals inflammatory infiltrates of macrophages in the alveolar lumina (21). Systemic cytokine profiles of macrophage activation syndrome resemble that seen in patients with severe COVID-19 disease (22).

Low grade inflammation in PCOS is the key mediator of the insulin resistance and metabolic effects seen and are promoted by cytokines derived from macrophages (11). This study shows that basal macrophage-derived biomarkers associated with inflammation, CXCL5, CD163, and MMP9, were elevated in subjects with PCOS while CD200 was decreased. CXCL5 is a proinflammatory chemokine that promotes insulin resistance and is secreted from white adipose tissue in excess in obesity. CXCL5 correlated with BMI and, when the data were corrected for BMI, it was no longer significant, in accord with the serum levels reported to being no different in normal weight PCOS versus controls (23).

Soluble CD163 is a biomarker of macrophage activation that is associated with the development of diabetes (24). Elevated serum levels of CD163 have been reported in PCOS (25) though the mRNA levels in adipose tissue of PCOS and overweight individuals did not differ (26). This is in accord with the data reported here, where CD163 levels correlated with BMI and were no longer significant when the data were adjusted for BMI.

Matrix metalloproteinases (MMPs) are macrophage M2 markers that have been suggested to be important in the pathogenesis of PCOS, with reports differing with regard to serum MMP9 elevation or not (27). In this study, MMP9 was elevated basally in PCOS, in accord with previous reports (27); however, MMP9 correlated with BMI, and when the data were adjusted for BMI, significance was lost. Thus, this would explain the differing reports on MMP9 serum levels if BMI was not taken into account.

CD200 expression has been associated with a shift away from proinflammatory macrophages and therefore its reduction would promote the inflammatory process (28); this is in accord with our findings where basal levels of CD200 were reduced in PCOS women in this study. CD200 correlated with BMI and, when the data was adjusted for BMI, then CD200 was no longer significantly reduced in PCOS.

Overall, it can be seen that the proinflammatory expression of macrophage-derived proteins seen in PCOS were all driven by obesity and were therefore not independent markers of inflammation in PCOS.

Vitamin D deficiency has been shown to be related to the expression of proinflammatory macrophage cytokines and fibrosis (29), factors that may contribute to its association with a poor outcome in COVID-19 disease (5, 6). The data here show that vitamin D deficiency was associated with decreased CD80

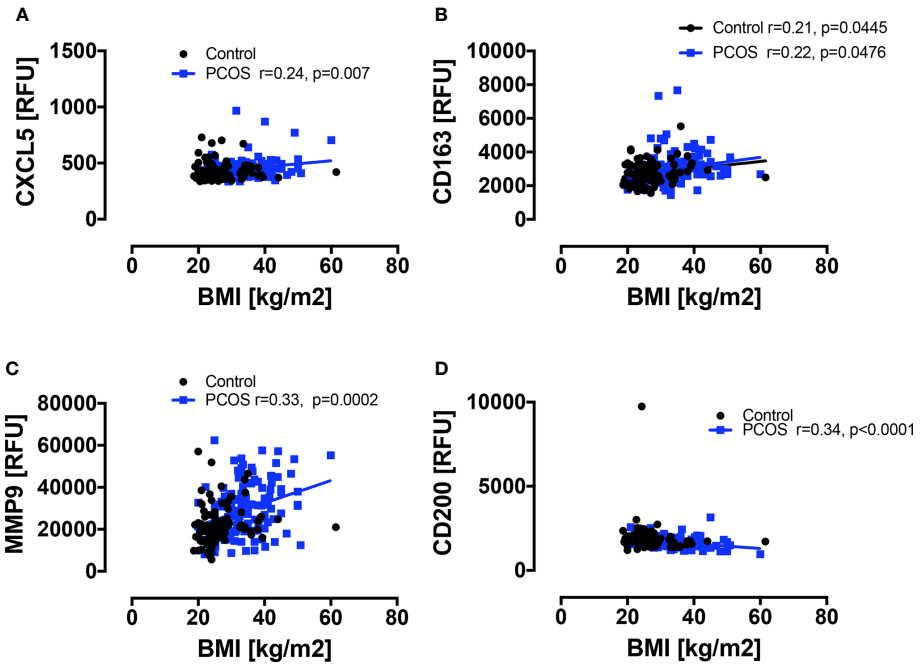

FIGURE 2 | Correlations of macrophage-related proteins with BMI in PCOS and control women. A positive correlation with BMI was seen in PCOS women only for CXCL5 **(A)** and MMP9 **(C)**, and for CD163 **(B)** in both PCOS and control women; a negative correlation with BMI was seen in PCOS women only for CD200 **(D)**.

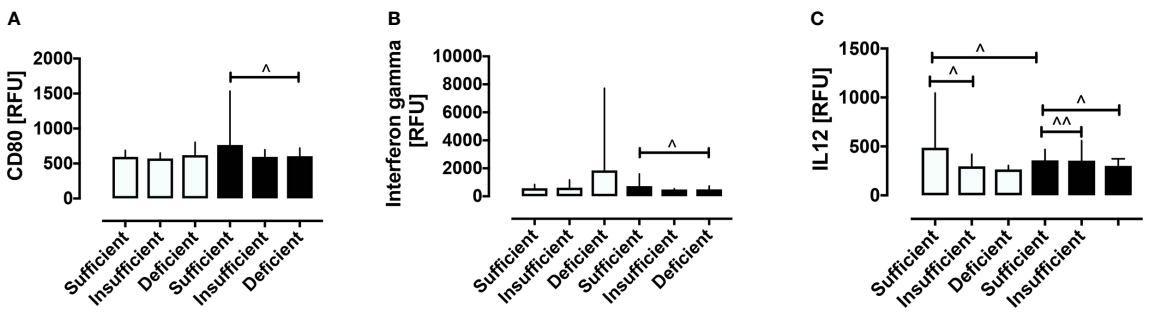

FIGURE 3 | Stratification according to vitamin D status of macrophage-related proteins in women with and without PCOS. Stratification according to vitamin D status revealed that vitamin D deficiency was associated with decreased CD80 **(A)**, IFN-γ **(B)** in PCOS, and decreased IL-12 **(C)** in both PCOS and control women. ^p < 0.05, ^^p < 0.005. RFU, relative fluorescent units.

and IFN-γ in PCOS (both p<0.05), and IL-12 (p<0.05) in both PCOS and controls. CD80 is a costimulatory molecule produced by macrophages that is important in maintaining T cell activation (30), while IL-12 activates natural killer and cytotoxic T lymphocytes that are important as mediators of inflammation-induced apoptosis (31). However, in all cases, after adjustment for BMI, none of these proteins remained significantly altered. This suggests that if vitamin D deficiency is a risk factor for increased severity of COVID-19 disease in PCOS, then the mechanism is not through macrophage cytokine mediation independent of obesity.

After ingestion or production in the skin, the fat-soluble prohormone vitamin D affects many physiological functions, including the regulation of both innate and adaptive immunity (32–34). Activation of vitamin D can occur through canonical and non-canonical pathways. In the classic pathway, vitamin D is first metabolized to 25-hydroxyvitamin D_3 by CYP2R1 and CYP27A1 in the liver, then in the kidney and other organs, such as the skin and the immune system, to the active 1,25-dihydroxyvitamin D_3 by CYP27B1 (35–37). In the alternative pathway, vitamin D is activated by CYP11A1, resulting in the production of over 10 different metabolites (36, 38–43); this includes activation of lumisterol, a photoproduct of pro-vitamin D (44).

Both 1,25-dihydroxyvitamin D_3 and the CYP11A1-derived metabolites can affect immune functions (39), and much evidence supports their potent anti-inflammatory and antioxidative activities (45). While the possible association between vitamin D deficiency/

insufficiency and more severe COVID-19 disease remains speculative, there is increasing evidence in support of the potential role of classical and alternative forms of vitamin D in damping down the production of pro-inflammatory cytokines (cytokine storm) and oxidative stress induced by COVID-19 infection and thus mitigating their harmful effects (45).

Limitations of this study include that it was only a moderately sized cross-sectional study and that only total vitamin D was measured and not the active 1,25 dihydroxyvitamin D or its metabolites that may also be active. In addition, no functional assays were undertaken, and only circulatory levels of macrophage-related proteins were measured that may not reflect concentrations at the tissue level.

CONCLUSIONS

In conclusion, obese subjects with PCOS show a basal proinflammatory macrophage-derived protein profile together with vitamin D deficiency that was also associated with reduced T cell regulatory proteins compared to controls. However, all of these features could be accounted for by BMI suggesting that obese, but not lean, PCOS subjects may be at risk for more severe COVID-19 disease and that vitamin D effects on macrophage-related proteins is not independent of obesity.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Newcastle & North Tyneside Ethics committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AM and AB analyzed the data and wrote the manuscript. TS supervised clinical studies and edited the manuscript. SA contributed to study design, data interpretation and the writing of the manuscript. All authors contributed to the article and approved the submitted version. AB is the guarantor of this work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.638621/full#supplementary-material

Supplementary Figure 1 | Macrophage-related proteins where no difference was seen between PCOS and control women.

Supplementary Figure 2 | Macrophage-related proteins stratified according to vitamin D status where no difference was seen between stratified groups for either PCOS or control women.

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

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 08 March 2021
doi: 10.3389/fendo.2021.604100

Association and Interaction Between Serum Interleukin-6 Levels and Metabolic Dysfunction-Associated Fatty Liver Disease in Patients With Severe Coronavirus Disease 2019

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Specialty section:
This article was submitted to Translational Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *08 September 2020*
Accepted: *11 January 2021*
Published: *08 March 2021*

Citation:
Gao F, Zheng KI, Yan H-D, Sun Q-F, Pan K-H, Wang T-Y, Chen Y-P, Targher G, Byrne CD, George J and Zheng M-H (2021) Association and Interaction Between Serum Interleukin-6 Levels and Metabolic Dysfunction-Associated Fatty Liver Disease in Patients With Severe Coronavirus Disease 2019. Front. Endocrinol. 12:604100. doi: 10.3389/fendo.2021.604100

Background and Aim: Circulating levels of interleukin (IL)-6, a well-known inflammatory cytokine, are often elevated in coronavirus disease-2019 (COVID-19). Elevated IL-6 levels are also observed in patients with metabolic dysfunction-associated fatty liver disease (MAFLD). Our study aimed to describe the association between circulating IL-6 levels and MAFLD at hospital admission with risk of severe COVID-19.

Methods: A total of 167 patients with laboratory-confirmed COVID-19 from three Chinese hospitals were enrolled. Circulating levels of IL-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ were measured at admission. All patients were screened for fatty liver by computed tomography. Forty-six patients were diagnosed as MAFLD.

Results: Patients with MAFLD (n = 46) had higher serum IL-6 levels (median 7.1 [interquartile range, 4.3–20.0] vs. 4.8 [2.6–11.6] pg/mL, $p = 0.030$) compared to their counterparts without MAFLD (n = 121). After adjustment for age and sex, patients with MAFLD had a ~2.6-fold higher risk of having severe COVID-19 than those without MAFLD. After adjustment for age, sex and metabolic co-morbidities, increased serum IL-6 levels remained associated with higher risk of severe COVID-19, especially among infected patients with MAFLD (adjusted-odds ratio 1.14, 95% CI 1.05–1.23; $p = 0.002$). There was a significant interaction effect between serum IL-6 levels and MAFLD for risk of severe COVID-19 (p for interaction = 0.008).

Conclusions: Patients with MAFLD and elevated serum IL-6 levels at admission are at higher risk for severe illness from COVID-19.

Keywords: COVID-19, SARS-CoV-2, MAFLD, cytokine, IL-6, NAFLD

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic continues to attract worldwide attention. The occurrence of a virus-induced cytokine 'storm' is associated with greater disease severity and unfavorable in-hospital outcomes (1, 2). Elevated levels of serum interleukin-6 (IL-6) are a hallmark inflammatory signature commonly seen in patients with severe COVID-19 and the use of IL-6-receptor blocking antibodies has recently been approved in China for treatment of COVID-19 patients with serious pulmonary damage and elevated serum IL-6 levels (3).

Metabolic dysfunction-associated fatty liver disease (MAFLD), the newly proposed name for non-alcoholic fatty liver disease (NAFLD) (4), has reached epidemic proportions, affecting up to a quarter of the world's adult population (5). MAFLD is known to be associated with various hepatic and extra-hepatic complications, such as cirrhosis, liver cancer, cardiovascular disease, type 2 diabetes and chronic kidney disease (6). We recently showed that younger patients with MAFLD have higher odds of severe COVID-19 (7).

Patients with MAFLD were characterized by impaired hepatic immunity (8, 9). Hepatic macrophages are associated with fatty liver disease through their effects on chronic inflammation, including cytokine and adipokine secretion (9). Previous studies reported that the status of inflammation associated with MAFLD might contribute to the cytokine 'storm', which further exacerbates the infection in patients with COVID-19 (10–12). The angiotensin-converting enzyme (ACE) 2 is the receptor for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). An exploratory analysis found that ACE 2 mRNA expression in visceral fat (VF) positively correlated with BMI, and the engagement of SARS-CoV-2 on ACE 2 in the VF would impair the enzymatic activity of ACE 2 and enhance the production of inflammatory cytokines and their release into the systemic circulation (13). Therefore, we postulated the presence of MAFLD might exacerbate the virus-induced cytokine 'storm' associated with COVID-19, possibly through the hepatic release of multiple pro-inflammatory cytokines.

In this study, we investigated the association between peripheral blood IL-6 levels and MAFLD at admission and risk of having more severe illness in hospitalized patients with COVID-19.

MATERIALS AND METHODS

Patients and Study Design

We enrolled 214 patients with laboratory-confirmed COVID-19 from three Chinese hospitals (the First Affiliated Hospital of Wenzhou Medical University, the Ningbo No.2 Hospital, and the Ruian People's Hospital) between 17th January and 11th February 2020. As detailed in **Figure 1**, 47 subjects were excluded for the following reasons (1): younger than 18 years or older than 70 years; (2) having a history of active cancers, chronic obstructive or restrictive pulmonary diseases, or other end-stage diseases; (3) incomplete clinical/biochemical data. As a result of these exclusions, a sample of 167 patients was included in the final analysis. Some of these patients have been reported in a previous study (14). All patients had received standard treatments according to the Chinese COVID-19 management guidance.

The local ethics committees of the hospitals approved the study protocol (2020-002, 5 February 2020). All procedures performed involving the participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration. The requirement for written informed consent was waived due to the retrospective and anonymous nature of the study design.

Laboratory and Clinical Data

Demographic and laboratory data, including age, sex, serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl-transpeptidase (GGT) and various cytokines were collected on the first day of hospital admission. In particular, circulating levels of IL-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ were measured using a flow cytometer (FACSCanto^{TM} plus, America) and cytometric bead array techniques. The neutrophil-to-lymphocyte ratio (NLR), i.e., an established marker of systemic inflammation, was also calculated by dividing the absolute number of neutrophils by the absolute number of lymphocytes. FIB-4 score was calculated from the published formula (15).

Body mass index (BMI) was calculated using the formula weight (kilograms) divided by height (meters) squared. Overweight and obesity were defined, respectively, as BMI between 23 and 24.9 kg/m^2 and BMI ≥ 25 kg/m^2 in this Asian population. Type 2 diabetes mellitus (T2DM) was diagnosed as either self-reported history of disease, fasting glucose levels ≥7.0 mmol/L, hemoglobin A1c ≥6.5% (≥48 mmol/mol) or use of any anti-hyperglycemic drugs. Hypertension and dyslipidemia were diagnosed according to the consensus criteria (16).

In the whole cohort of patients, COVID-19 was diagnosed as a positive result by high-throughput sequencing or real-time reverse transcriptase-polymerase chain reaction assay of oropharyngeal swab specimens. Severity of COVID-19 was assessed during hospitalization and classified as two subtypes (severe and non-severe) based on the Chinese guidelines for management of COVID-19 (see **Supplementary Table 1**) (17).

Liver Imaging

All patients were screened for fatty liver by computed tomography (CT). Liver and spleen measurements were performed using a CT

Abbreviations: CI, confidence intervals; COVID-19, coronavirus disease-2019; IL, interleukin; OR, odds ratio; MAFLD, metabolic dysfunction-associated fatty liver disease; TBIL, total bilirubin.

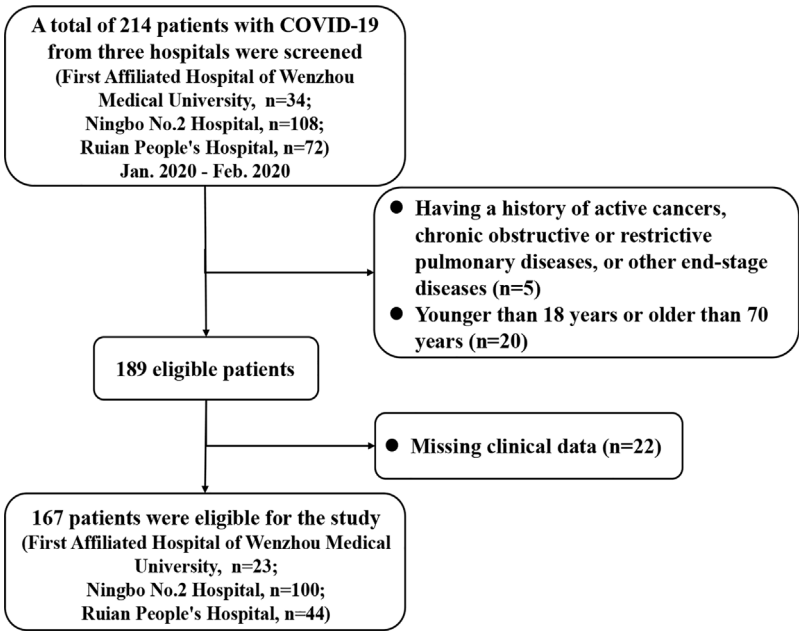

FIGURE 1 | The flow chart for the study.

post-processing workstation, and only one image (level of portal vein into the liver) of each patient was selected to complete attenuation measurements of liver and spleen. Three regions of interest in the liver were selected to avoid blood vessels, bile ducts and calcification. Two regions of interest in the spleen were selected at the same level. The respective means of the three attenuation values of the liver and two attenuation values of the spleen were calculated. Fatty liver was diagnosed by widely accepted CT characteristics, namely attenuation value of liver parenchyma (CTLP) <48 HU, or attenuation ratio of liver and spleen (LS ratio) <1.0, or attenuation difference of liver and spleen (LS diff.) <5 HU, respectively.

MAFLD Definition

MAFLD was diagnosed as the presence of hepatic steatosis on CT scan and one of the following criteria: 1) overweight or obesity as defined by a BMI ≥23.0 kg/m^2; 2) established T2DM; or 3) presence of at least two metabolic risk abnormalities (18). These metabolic risk abnormalities were defined as follows: 1) waist circumference ≥90/80 cm in men and women; 2) blood pressure ≥130/85 mmHg or drug treatment; 3) plasma triglycerides ≥1.70 mmol/L or drug treatment; 4) plasma HDL-cholesterol <1.03 mmol/L for men and <1.29 mmol/L for women or drug treatment; and 5) prediabetes (i.e., fasting glucose levels 5.6 to 6.9 mmol/L, or HbA1c 5.7% to 6.4%) (18).

Statistical Analysis

Continuous variables are expressed as means ± SD or medians (inter-quartile ranges [IQR]) and compared using either the unpaired Student's *t*-test or the Mann-Whitney test as appropriate. Categorical variables are expressed as numbers (percentages) and compared using the chi-squared test or the Fisher's exact test as appropriate. The odds ratios (OR) and 95% confidence intervals (CIs) for the risk of having severe COVID-19 (as the outcome) associated with serum IL-6 levels and MAFLD at hospital admission were estimated using binary logistic regression analyses with adjustment for age, sex and metabolic co-morbidities (overweight/obesity, T2DM and hypertension). Interaction tests were used to evaluate associations between exposure variables and the outcome. Statistical analyses were two-sided and significance was set at $p < 0.05$. All statistical tests were performed using SPSS version 23.0 (SPSS Inc., Chicago, USA).

RESULTS

A cohort of 167 (42.5% men; mean age 49 years) hospitalized patients with laboratory-confirmed COVID-19 was included in the study. The median time from symptom onset to hospital admission was 9.0 (IQR 4.0–13.0) days. Forty-six (27.5%) of these patients had imaging-defined MAFLD and 32 (19.2%) patients were classified as having severe COVID-19. Among MAFLD patients, patients with severe COVID-19 had significantly higher FIB-4 score than those with non-severe COVID-19 (1.64 IQR [1.23–2.57] vs. 1.03 IQR [0.71–1.61], $p = 0.047$). The data about the cytokine levels is presented in the **Supplementary Table 2**.

As shown in **Table 1**, patients with imaging-defined MAFLD had significantly higher levels at admission of serum IL-6 (median 7.1 [IQR, 4.3–20.0] vs. 4.8 [2.6–11.6] pg/mL, $p = 0.030$; **Figure 2**), liver enzymes and a higher proportion of overweight/obesity and dyslipidemia compared to those without MAFLD. After adjustment for age and sex, patients with MAFLD had an approximate 2.6-fold higher risk of severe

TABLE 1 | Baseline characteristics of COVID-19 patients, stratified by MAFLD status at hospital admission.

	Without MAFLD (n = 121)	With MAFLD (n = 46)	*P* value
Demographics			
Age, years	49.9 ± 13.2	47.7 ± 13.9	0.343
Male sex, n (%)	52 (43.0%)	19 (41.3%)	0.845
Coexisting disorders			
Type 2 diabetes, n (%)	15 (12.4%)	10 (21.7%)	0.131
Hypertension, n (%)	22 (18.2%)	13 (28.3%)	0.153
BMI ≥23 kg/m^2, n (%)	75 (62.5%)	40 (87.0%)	**0.002**
Dyslipidemia, n (%)	85 (70.2%)	43 (93.5%)	**0.002**
Laboratory parameters			
WBC, x10^9/L	4.8 (3.9–6.5)	5.0 (4.3–6.7)	0.458
Lymphocyte count, x10^9/L	1.1 (0.8–1.4)	1.2 (0.9–1.6)	0.208
NLR	2.8 (1.8–4.9)	2.5 (1.7–3.6)	0.282
C-reactive protein, mg/L	11.5 (2.3–29.8)	17.2 (4.2–41.0)	0.122
Procalcitonin, ng/mL	0.03 (0.01–0.05)	0.03 (0.01–0.06)	0.474
D-dimer, mg/L	0.28 (0.14–0.66)	0.36 (0.20–0.59)	0.702
ALT, U/L	21.0 (15.0–31.0)	26.0 (20.0–39.8)	**0.024**
AST, U/L	22.0 (17.0–29.0)	27.0 (20.2–34.8)	**0.028**
GGT, U/L	24.0 (16.0–39.0)	31.0 (21.2–50.0)	**0.037**
TBIL, μmol/L	10.0 (6.6–14.0)	10.2 (7.5–14.0)	0.395
Cytokines			
IL-2, pg/mL	0.9 (0.6–1.4)	0.8 (0.5–1.4)	0.670
IL-4, pg/mL	1.3 (1.0–2.1)	1.0 (1.0–2.0)	0.518
IL-6, pg/mL	4.8 (2.6–11.6)	7.1 (4.3–20.0)	**0.030**
IL-10, pg/mL	2.6 (1.0–4.5)	3.6 (1.0–5.5)	0.251
TNF-α, pg/mL	1.1 (0.9–1.5)	1.0 (0.9–1.5)	0.306
IFN-γ, pg/mL	1.0 (1.0–1.6)	1.0 (0.9–1.9)	0.790
Time from symptom onset to hospital admission, days	9.0 (4.5–13.0)	7.0 (4.0–11.3)	0.267
Severe COVID-19, n (%)	19 (15.7%)	13 (28.3%)	0.065

Data are expressed as means ± SD, medians (IQRs) and number (percentages).
ALT, alanine aminotransferase; AST, aspartate transaminase; BMI, body mass index; GGT, gamma glutamyl-transpeptidase; IFN-γ, interferon gamma; IL, interleukin; MAFLD, metabolic dysfunction-associated fatty liver disease; NLR, Neutrophil-to-lymphocyte ratio; TBIL, total bilirubin; TNF-α, tumor necrosis factor-α; WBC, white blood cell.
Bold values represent statistical significant P values.

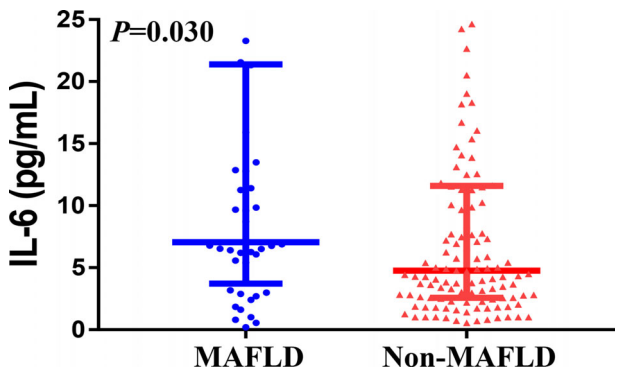

FIGURE 2 | The association between IL-6 levels and MAFLD status among COVID-19 patients.

COVID-19 than those without MAFLD (odds ratio 2.61, 95% CI 1.10–6.23, p = 0.030)).

As shown in **Table 2**, compared to those with non-severe COVID-19, patients with severe COVID-19 had significantly higher circulating levels of neutrophil-to-lymphocyte ratio, C-reactive protein, procalcitonin, D-dimer, IL-6 and IL-10 both in MAFLD and in non-MAFLD patients. Among non-MAFLD patients, those with severe COVID-19 were older, had significantly higher levels of WBC, liver enzymes (ALT, AST, GGT, and TBIL), and also had a higher proportion of T2DM, hypertension, and overweight/obesity than those with non-severe COVID-19. However, the significant differences in the aforementioned parameters were not observed among MAFLD patients, after stratification by severe COVID-19.

As shown in **Table 3**, in the whole cohort of COVID-19 patients, there was a significant association between higher serum IL-6 levels and risk of having severe COVID-19 (unadjusted model: OR 1.06 [95% CI 1.03–1.09], p<0.001). Notably, this association remained significant even after adjustment for age, sex, overweight/obesity, T2DM, and hypertension (adjusted model 2: OR 1.04 [95% CI 1.02–1.09], p = 0.002). We also performed subgroup analyses and interaction tests to evaluate the existence of significant differences in serum IL-6 levels between patients with and without MAFLD at hospital admission, and to investigate interactions between exposure variables and the outcome. In the unadjusted model, elevated serum IL-6 levels were significantly associated with a higher risk of severe COVID-19 in both groups of patients. However, after adjustment for age and sex (adjusted model 1) and other metabolic co-morbidities (adjusted model 2), the significant association between elevated serum IL-6 levels and higher risk of severe COVID-19 persisted only in patients with MAFLD (adjusted model 2: OR 1.14 [95% CI 1.05–1.23], p = 0.002). The interaction test between IL-6 and MAFLD

TABLE 2 | Baseline characteristics of COVID-19 patients, stratified by both COVID-19 severity and MAFLD status.

	With MAFLD (n = 46)			Without MAFLD (n = 121)		
	Non-severe COVID-19 (n = 33)	SevereCOVID-19 (n = 13)	*P* value	Non-severe COVID-19 (n = 102)	SevereCOVID-19 (n = 19)	*P* value
Demographics						
Age, years	46.8 ± 14.1	50.2 ± 13.8	0.453	48.3 ± 13.3	58.2 ± 8.6	**0.003**
Male sex, n (%)	12 (36.4%)	7 (53.8%)	0.278	39 (38.2%)	13 (68.4%)	**0.015**
Coexisting disorders						
Type 2 diabetes, n (%)	9 (27.3%)	4 (30.8%)	0.813	10 (9.8%)	10 (52.6%)	**<0.001**
Hypertension, n (%)	8 (24.2%)	5 (38.5%)	0.335	15 (14.7%)	7 (36.8%)	**0.022**
BMI ≥23 kg/m^2, n (%)	29 (87.9%)	11 (84.6%)	0.767	60 (58.8%)	15 (83.3%)	**0.048**
Dyslipidemia, n (%)	31 (93.9%)	12 (92.3%)	0.840	74 (72.5%)	11 (57.9%)	0.200
Laboratory parameters						
WBC, x10^9/L	5.0 (3.9–6.4)	5.4 (4.8–7.2)	0.183	4.7 (3.7–5.9)	7.4 (5.4–10.2)	**<0.001**
Lymphocyte count, x10^9/L	1.3 (0.9–1.7)	1.1 (0.9–1.2)	0.094	1.2 (0.9–1.6)	0.7 (0.5–0.8)	**<0.001**
NLR	2.5 (1.6–3.7)	10.2 (7.1–16.8)	**<0.001**	2.1 (1.6–3.1)	3.7 (3.2–4.4)	**0.012**
C-reactive protein, mg/L	9.7 (3.1–24.5)	47.6 (18.9–74.0)	**0.001**	9.2 (2.0–24.9)	32.0 (14.8–67.8)	**<0.001**
Procalcitonin, ng/mL	0.01 (0.01–0.04)	0.06 (0.04–0.08)	**0.013**	0.01 (0.01–0.04)	0.05 (0.04–0.07)	**<0.001**
D-dimer, mg/L	0.23 (0.12–0.34)	0.59 (0.47–1.21)	**0.002**	0.22 (0.13–0.30)	0.68 (0.55–0.83)	**<0.001**
ALT, U/L	30.0 (20.0–49.0)	22.0 (20.0–24.0)	0.241	20.0 (15.0–27.8)	26.0 (21.5–41.5)	**0.009**
AST, U/L	27.0 (19.0–35.0)	28.0 (25.0–34.0)	0.329	22.0 (17.0–28.0)	32.0 (21.5–40.5)	**0.003**
GGT, U/L	30.0 (20.0–51.0)	31.0 (24.0–47.0)	0.413	22.0 (15.2–32.8)	43.0 (27.5–84.5)	**<0.001**
TBIL, μmol/L	10.0 (7.1–13.6)	13.2 (9.3–14.0)	0.121	9.4 (6.4–13.6)	12.0 (9.2–18.5)	**0.033**
Cytokines						
IL-2, pg/mL	0.9 (0.5–1.6)	0.6 (0.5–1.1)	0.508	0.9 (0.5–1.5)	0.9 (0.7–1.0)	0.568
IL-4, pg/mL	1.0 (1.0–2.0)	1.0 (0.7–1.7)	0.267	1.4 (1.0–2.1)	1.2 (0.6–1.7)	0.058
IL-6, pg/mL	6.4 (2.9–9.8)	26.3 (12.9–45.4)	**<0.001**	4.6 (2.3–10.2)	4.9 (3.3–31.9)	**0.043**
IL-10, pg/mL	2.2 (1.0–4.2)	6.5 (4.3–12.5)	**<0.001**	2.3 (1.0–4.0)	4.8 (3.2–7.0)	**<0.001**
TNF-α, pg/mL	1.0 (1.0–1.5)	0.7 (0.2–1.4)	0.120	1.2 (1.0–1.6)	0.6 (0.2–1.5)	**0.007**
IFN-γ, pg/mL	1.0 (1.0–1.6)	1.4 (0.6–2.3)	0.834	1.0 (1.0–1.5)	1.3 (0.8–1.8)	0.439
Time from symptom onset to hospital admission, days	8.0 (4.0–12.0)	7.0 (5.5–9.5)	0.922	9.0 (4.0–13.0)	8.0 (5.0–10.0)	0.778

Data are expressed as means ± SD, medians (IQR) and number (percentages).
ALT, alanine aminotransferase; AST, aspartate transaminase; BMI, body mass index; GGT, gamma glutamyl-transpeptidase; IFN-γ, interferon gamma; IL, interleukin; MAFLD, metabolic associated fatty liver disease; NLR, Neutrophil-to-lymphocyte ratio; TBIL, total bilirubin; TNF-α, tumor necrosis factor-α; WBC, white blood cell.
Bold values represent statistical significant P values.

TABLE 3 | Association between serum IL-6 levels (as exposure) and severity of COVID-19 (as the outcome) in infected patients with and without MAFLD at hospital admission.

	Total (n = 167)		Without MAFLD (n = 121)		With MAFLD (n = 46)		*P* for interaction
	OR (95% CI)	*P value*	OR (95% CI)	*P value*	OR (95% CI)	*P value*	
Unadjusted Model	1.06 (1.03–1.09)	**<0.001**	1.04 (1.00–1.07)	**0.028**	1.11 (1.04–1.19)	**0.003**	0.055
Adjusted Model 1	1.05 (1.02–1.09)	**<0.001**	1.03 (0.99–1.06)	0.086	1.12 (1.04–1.21)	**0.004**	**0.022**
Adjusted Model 2	1.04 (1.02–1.09)	**0.002**	1.02 (0.99–1.05)	0.091	1.14 (1.05–1.23)	**0.002**	**0.008**

Data are expressed as odds ratio (OR) and 95% confidence intervals as tested by univariable (unadjusted) and multivariable (adjusted) logistic regression analysis.
Adjusted Model 1: adjusted for age and sex
Adjusted Model 2: adjusted for age, sex, overweight/obesity, type 2 diabetes, and hypertension.
Bold values represent statistical significant P values.

status on risk of severe COVID-19 was found to be statistically significant (p-value for interaction = 0.008). As shown in **Figure 3**, there was a clear dose-effect relationship between increasing values of IL-6 and the proportion of patients with severe COVID-19 in both MAFLD and non-MAFLD patients. Notably, elevated IL-6 levels had higher odds of severe COVID-19 among MAFLD patients than non-MAFLD patients.

As shown in **Figure 4A**, patients with MAFLD and elevated IL-6 levels had a significantly higher proportion of severe COVID-19 than those with elevated IL-6 levels but without MAFLD (47.83% vs. 19.57%, p = 0.015). Among patients with normal IL-6 levels (**Figure 4B**), there was no significant difference in the percentage of severe COVID-19 between MAFLD and non-MAFLD patients (8.70% vs. 13.33%, p = 0.553). As shown in **Figure 4C**, among non-MAFLD patients, patients with elevated IL-6 levels appeared to have a higher proportion of severe COVID-19 than those with normal IL-6 levels but the effect was not statistically significant (19.57% vs. 13.33%, p = 0.360). There was a significantly lower proportion of patients with severe COVID-19 in MAFLD patients with normal IL-6 levels, than in those with elevated IL-6 levels (8.70% vs. 47.83%, p = 0.003; **Figure 4D**).

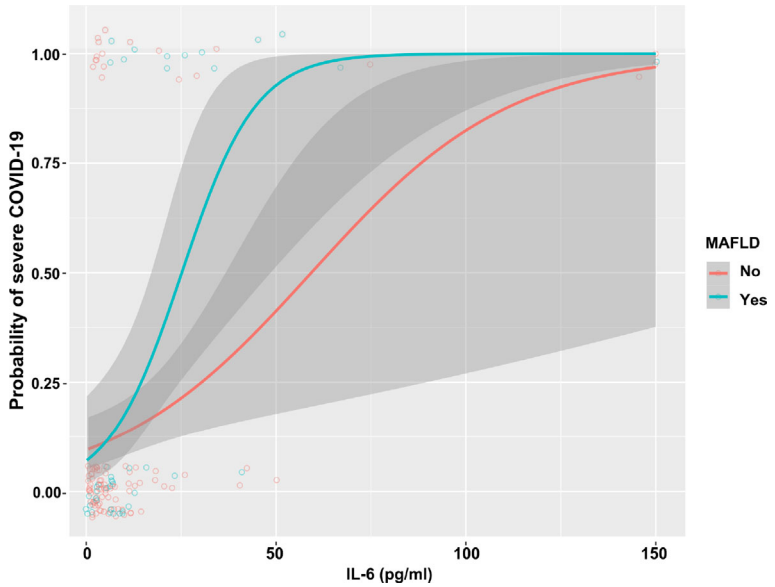

FIGURE 3 | The association between increasing IL-6 levels and COVID-19 severity, stratified by MAFLD status.

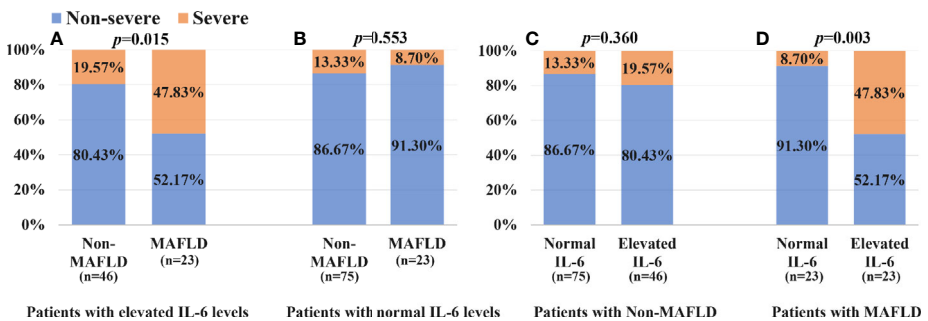

FIGURE 4 | Association between IL-6 levels, MAFLD status and COVID-19 severity: **(A)** patients with elevated IL-6 levels; **(B)** patients with normal IL-6 levels; **(C)** patients with Non-MAFLD; **(D)** patients with MAFLD.

DISCUSSION

Our study shows for the first time that patients with laboratory-confirmed COVID-19 and imaging-defined MAFLD at hospital admission have significantly higher circulating IL-6 levels than their counterparts without MAFLD; increased IL-6 levels are also associated with higher odds of severe COVID-19; elevated IL-6 levels had higher odds of severe COVID-19 among MAFLD patients than non-MAFLD patients; and there is a significant interaction between elevated IL-6 levels and MAFLD with severe COVID-19. Notably, these significant associations persisted after adjusting for age, sex and coexisting metabolic co-morbidities. These results remind health care professionals caring for COVID-19 patients should be cognizant of the increased likelihood of severe COVID-19 in patients with elevated IL-6 levels, especially those with MAFLD.

It is known that serum IL-6 levels are elevated in metabolic syndrome, cardiovascular diseases and chronic inflammatory airways diseases (19). Wang et al. reported that fatty liver was independently associated with elevated IL-6 levels (20). However, the association between MAFLD and elevated IL-6 levels is currently uncertain. Our study showed that patients with MAFLD had higher levels of IL-6 than those without MAFLD.

Previous studies have shown that the inflammatory response plays an important role in COVID-19 severity (21, 22). The clinical deterioration observed in some infected patients has been related to the occurrence of a virus-induced cytokine 'storm' (23). Liu et al. have reported that the serum levels of IL-6 and C-reactive protein may effectively assess disease severity and predict adverse in-hospital outcomes in patients with COVID-19 (24). Besides, tocilizumab, i.e., a recombinant humanized monoclonal antibody IL-6 receptor inhibitor, has recently emerged as an

alternative treatment for COVID-19 patients at risk of cytokine 'storm' (25). Accordingly, the results of the present study demonstrated that patients with severe COVID-19 had higher levels of several inflammatory biomarkers (e.g., NLR, CRP, procalcitonin, PCT, IL-6, and IL-10 levels).

Among COVID-19 patients, risk of severe infection and mortality are increased by co-morbidities such as obesity, T2DM, hypertension, cardiovascular disease, and cancer (26). Our previous studies showed that the presence of MAFLD in COVID-19 patients is associated with increased odds of severe COVID-19 (27, 28). A recent study by Petersen et al. also found that greater visceral adipose tissue accumulation was associated with a higher risk of ICU admission in COVID-19 patients (29). Since the diagnosis of MAFLD is based on the evidence of hepatic steatosis in addition to one or more metabolic disorders (namely overweight/obesity, presence of T2DM, or evidence of metabolic dysregulation), it is reasonable to hypothesize that the aggregation of these metabolic risk abnormalities in MAFLD may further aggravate the severity of COVID-19 (30, 31).

We do not know the exact underlying mechanisms by which MAFLD influences the association between elevated IL-6 levels and greater COVID-19 severity, but it is possible that the presence of MAFLD exacerbates the virus-induced cytokine 'storm', possibly through the hepatic release of multiple pro-inflammatory cytokines (including IL-6). Moreover, it is also possible to hypothesize that COVID-19 increases the risk of gut-derived endotoxemia, which may lead to macrophage activation and increased secretion of IL-6, thus further contributing to the virus-induced cytokine 'storm', especially in infected patients with coexisting abnormal liver function (9, 32). Thus, our findings further highlight the need for clinicians to pay close attention to COVID-19 patients with elevated serum IL-6 levels at hospital admission, especially those with coexisting MAFLD. Moreover, similar to liver, it is commonly to see COVID-19 affects the skeletal muscle such as loss of muscle mass, strength, and physical function (33). Skeletal muscle is also an essential source of IL-6 (34). Therefore, the COVID-19-muscle-IL-6 triangle is also worth investigating.

Our study has some limitations, including the relatively small sample size, the Asian ancestry of the cohort, and the lack of serial monitoring of circulating IL-6 levels during the hospital stay. Therefore, the results of our study will require further validation in larger cohorts of Asian and non-Asian patients with COVID-19. We excluded patients under the age of 18 years and over the age of 70 years, whether our research conclusions are applicable to these patients still needs to be verified. Moreover, sex hormone might affect the severity of MAFLD through the MyD88-dependent IL-6 signaling pathway (35), and recent studies highlighted the role of bio-immunological sex disparities underlying differences in the susceptibility to develop SARS-CoV-2 infection and its sequelae between females and males (36–38). Considering the MAFLD and COVID-19 are both sexually dimorphic and, therefore, additional and more extensive studies are needed to process data separately by sex (39). Finally, relying on CT imaging to diagnose fatty liver might misclassify mild MAFLD cases, and patients were recruited from three different Chinese hospitals, which might have introduced inter-individual variability in the assessment of MAFLD.

In conclusion, the results of this multicenter study show for the first time that there is an independent association and an interaction between serum IL-6 levels and MAFLD in hospitalized patients with severe COVID-19.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committees of the First Affiliated Hospital of Wenzhou Medical University, Ningbo No.2 Hospital, and Ruian People's Hospital. The ethics committees of the First Affiliated Hospital of Wenzhou Medical University, Ningbo No.2 Hospital, and Ruian People's Hospital waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

Study concept and design: FG, KZ, and M-HZ. Acquisition of data: H-DY, Q-FS, K-HP, T-YW, and Y-PC. Analysis and interpretation of data: FG and KZ. Drafting of the manuscript: FG and KZ. Critical revision of the manuscript for important intellectual content: GT, CB, and JG. Study supervision: M-HZ. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the National Natural Science Foundation of China (82070588), Medical Health Science and Technology Project of Zhejiang Provincial Health Commission (2018ZD039), Ruian Science and Technology Bureau (2020023), High Level Creative Talents from Department of Public Health in Zhejiang Province and Project of New Century 551 Talent Nurturing in Wenzhou. GT is supported in part by grants from the University School of Medicine of Verona, Verona, Italy. CB is supported in part by the Southampton NIHR Biomedical Research Centre (IS-BRC-20004), UK.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.604100/full#supplementary-material

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

frontiers in Endocrinology

MINI REVIEW
published: 16 March 2021
doi: 10.3389/fendo.2021.607179

The Double Edge Sword of Testosterone's Role in the COVID-19 Pandemic

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COVID-19 is a complex disease with a multifaceted set of disturbances involving several mechanisms of health and disease in the human body. Sex hormones, estrogen, and testosterone, seem to play a major role in its pathogenesis, development, spread, severity, and mortalities. Examination of factors such as age, gender, ethnic background, genetic prevalence, and existing co-morbidities, may disclose the mechanisms underlying SARS-CoV-2 infection, morbidity, and mortality, paving the way for COVID-19 amelioration and substantial flattening of the infection curve. In this mini-review, we focus on the role of testosterone through a discussion of the intricate mechanisms of disease development and deterioration. Accumulated evidence suggests that there are links between high level (normal male level) as well as low level (age-related hypogonadism) testosterone in disease progression and expansion, supporting its role as a double-edged sword. Unresolved questions point to the essential need for further targeted studies to substantiate these contrasting mechanisms.

Keywords: COVID-19, age-related, hypogonadism, SARS-CoV-2, testosterone, ACE2, MPRSS2

OPEN ACCESS

Edited by:
Alexandra Kautzky-Willer, Medical University of Vienna, Austria

Reviewed by:
Giuseppe Novelli, University of Rome Tor Vergata, Italy
Rosaria Meccariello, University of Naples Parthenope, Italy

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Specialty section:
This article was submitted to Translational Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *17 September 2020*
Accepted: *19 January 2021*
Published: *16 March 2021*

Citation:
Younis JS, Skorecki K and Abassi Z (2021) The Double Edge Sword of Testosterone's Role in the COVID-19 Pandemic. Front. Endocrinol. 12:607179. doi: 10.3389/fendo.2021.607179

INTRODUCTION

Almost one year has elapsed since the outbreak of the coronavirus disease 2019 (COVID-19) pandemic, caused by the novel severe acute respiratory syndrome coronavirus (SARS-CoV-2). The cumulative number of infected cases and death toll around the world continues to rise. As of January 1, 2021, the number of confirmed global cases of SARS-CoV-2 is 81,658,440 and the number of established human deaths is 1,802,206 cases, reported to WHO, while the numbers continue to evolve. The availability of effective vaccines brings hope for an end to the pandemic, though limitations of distribution might require a year or more to achieve global control. Accumulating evidence suggests that male infection is predominant (1, 2), especially in cases above 60 years of age and specifically in critically ill adults (2–5). Furthermore, intensive care unit admissions and mortality rates are far higher for male than female patients, independent of age (2–5).

Accumulating data from around the globe also shows that the incidence of COVID-19 is strikingly variable in different populations and various ethnic backgrounds, with diverse heterogeneity in virulence (6, 7). Evidence emerging from the United States and England shows that COVID19 mortality is disproportionately high amongst African Americans, Black, Asian, and

other ethnic minority communities. Estimates suggest that American counties where Black residents are in the majority have almost six times the rate of death due to COVID19 compared to counties with predominantly white residents (7–9). Socioeconomic and lifestyle factors seem also to be implicated in COVID-19 severity and gender differences (10–13). The likelihood of SARS-CoV-2 infection is significantly higher among minority ethnic communities even after adjustment for important socio-demographic and co-morbidity factors (14).

Furthermore, the incidence of COVID-19 before puberty is particularly low, and even when presenting it is generally mild (1, 15). Contrary to adults, there is no significant gender difference in young patients (16).

Taken together, epidemiological data continue to accumulate since the outbreak of the novel COVID-19 pandemic that suggest the possibility that sex hormone differences between males and females, specifically testosterone levels, normal male and age-related hypogonadism, in addition to genetic factors between different ethnic communities, may play a crucial role in the occurrence, pathogenesis, severity, and subsequent mortality of COVID-19.

SARS-COV-2 PATHOGENESIS OF INFECTION

The entire cell cycle of SARS-CoV-2 infection has been lately elucidated and clarified at the molecular level (17). It is currently well accepted that the port of entry of SARS-CoV-2 to the lungs, as to other vital organs of the body, is *via* the angiotensin-converting enzyme 2 (ACE2) receptor. This receptor is a key element of the renin-angiotensin-aldosterone system (RAAS), a cardinal endocrine/metabolic axis that regulates blood pressure and fluid balance. ACE2 is responsible for the generation of angiotensin 1-7 from angiotensin II. The angiotensin 1-7-Mas receptor axis provokes beneficial balancing and salutary actions to counterpart the adverse actions of the ACE/angiotensin II/AT1R pathway in vital organs, such as the lung, heart, and kidney (18, 19). Therefore, being a receptor for SARS-CoV-2 penetration of host cells, ACE2 integrity plays a crucial protective role against lung and other vital organ injuries (20). Coronavirus mediated ACE2 receptor down-regulation may escalate the counter-part impact of the renin-angiotensin I-angiotensin II-AT1R axis and contribute to the deleterious hyper-inflammatory response of COVID-19 in the lungs (21, 22). Yet, it has been shown that this effect does not hold in all parts of the body (23, 24).

SARS-CoV-2 is enveloped with a single-stranded positive sense RNA genome. The viral envelope bears transmembrane spike proteins (S) as well as other proteins (25). SARS-CoV-2 and SARS-CoV employ the same receptor-binding domain, *via* their surface S glycoproteins, to attach to the ACE2 receptor (26, 27). The S proteins of both viruses have been shown to have a high rate of homology, possess almost identical 3-D structures, and share 76.5% identity in amino acid sequences (28), although the affinity of SARS-CoV-2 to ACE2 has been revealed to be 10 to 20- fold higher than that of SARS-CoV (29). The S proteins have two fused binding subunits, S1 and S2 respectively. The first, S1, is responsible for the virus surface attachment to the host cell, and the second, S2, for the fusion of viral and cellular membranes and viral internalization into the host cell (17). Viral infection and entry to host cells require S protein priming by cellular proteases, which entails S protein cleavage at the S1/S2 and the S2 sites (17). Spike protein priming and cleavage are triggered by the host cellular transmembrane protease serine 2 (TMPRSS2) (17, 30–32). SARS-CoV-2 host cell entry was shown to be blocked by the clinically validated inhibitor of TMPRSS2 - Camostate (17). The priming process by TMPRSS2 seems to be vital for the entry of SARS-CoV-2 into human host cells, and thus plays an integral role in COVID-19 infection and disease progression. Moreover, TMPRSS2 may also cleave ACE2 thus augmenting viral entry (33).

More recently, it has been demonstrated that the S protein of SARS-CoV-2 infects lung host cells by a two-step activation mechanism. A pre-cleavage of the S proteins at the S1/S2 site by furin proteases is essential for subsequent S protein priming and activation (at the S2 site) by TMPRSS2 lung cells (33, 34). This mechanism explains the fusion of infected cells with non-infected cells, which might allow the virus to spread in the body without leaving the host cell. Furin, encoded in humans by the *FURIN* gene, is an enzyme that belongs to the subtilisin-like proprotein convertase family. The latter consists of a family of nine serine secretory proteases that regulate various biological processes in both healthy and disease states (35, 36). Furin is a calcium-dependent serine endoprotease that can efficiently cleave precursor proteins at their paired basic amino acid processing sites.

HIGH TESTOSTERONE IMPACT ON COVID-19 SEVERITY—THE TMPRSS2 CONNECTION

TMPRSS2 is a cell-surface protein expressed by the epithelial cells of specific tissues including those in the aero-digestive tract. It is a member of the type II Transmembrane Serine Proteases (TTSPs) family that are involved in multiple physiological and pathological processes, including viral infections and cancer, although its exact physiological role is still under investigation. *TMPRSS2* transcription is regulated by the androgen receptor (AR) (37). Specifically, AR activity is considered a requirement for the transcription of the *TMPRSS2* gene because no other known *TMPRSS2* gene promoter has been described in humans to date (37).

The human AR, located on the X chromosome, functions as a steroid hormone–activated transcription factor, which signals through classical and non-classical signaling pathways (ligand-dependent and independent actions) (38, 39). Androgens can work along three known paths, by intracellular conversion of serum testosterone into dihydrotestosterone (DHT), by testosterone itself, or by intracellular conversion of testosterone

to estradiol through aromatization. The AR has widespread expression in many cells and tissues with a diverse range of biological actions involving the development and maintenance of the reproductive, musculoskeletal, cardiovascular, immune, neural, and haemopoietic systems (39, 40).

Androgen receptor expression is low prior to pubertal maturation and may contribute to the low incidence of severe COVID-19 infection in children (41, 42). In addition, the lower rate of severe COVID-19 infection in female patients may be attributed to lower AR expression (43, 44). AR contains two polymorphic nucleotide repeats, GGN and CAG, encoding for glycine and glutamine, respectively (45). Several mutations or polymorphisms have been described in the gene encoding the AR in a variety of diseases or among various ethnic groups. Some of these mutations/polymorphisms are associated with functional changes in the AR expression and mutations in or around the receptor (46–48). Testosterone's biological action is dependent on the length of the CAG repeat of the androgen receptor gene (49).

Androgen mediated expression of ACE2 and TMPRSS2 may explain the gender difference in COVID-19 disease severity and mortality (50). Furthermore, the frequency of genetic variations in the AR differs by ethnicity, which may suggest a possible explanation for the wide differences in COVID-19 severity and mortality rates between countries and between different ethnic backgrounds in the same country (51, 52).

Various experimental data in mammalian animal models, as well as in numerous, unrelated clinical manifestations, in diverse in-vivo as well as human clinical settings, support the interplay between SARS-CoV-2 and sex hormones, specifically testosterone and AR, most likely *via* the cell host TMPRSSE2.

In animal models, ACE and ACE2 activity in cardiac cells were significantly higher in male compared to female rats, whereas orchiectomy decreased the activity of these enzymes and ovariectomy increased ACE2 but did not change ACE activity (53). In addition, androgen administration to a lung adenocarcinoma cell line up regulated the TMPRSS2 transcript more than two-fold, accompanied by an androgen dependent loading of the AR protein onto the TMPRSS2 enhancer (54). Furthermore, TMPRSS2 inhibition or knock down has been shown to reduce SARS-CoV infection *in vitro* (33).

Just recently, in-vitro studies employing human embryonic stem cell-derived cardiac cells and lung organoids have substantiated that testosterone regulates SARS-CoV-2 development, intensifying its severity in men. Furthermore, the pharmacological dampening of testosterone activity by inhibitors of 5 alpha reductases can reduce ACE2 levels in the target cells, leading to the decay of SARS CoV-2 infectivity (55).

In the clinical setting, recent preliminary studies suggest a high incidence of androgenic (androgenetic) alopecia among male and female patients hospitalized due to severe COVID-19 (56, 57). Androgenic alopecia, often referred to as male pattern (scalp) hair loss, is the most common form of hair loss among men and is associated with AR polymorphism (58). In one small-scale study, clinically significant androgenic alopecia was shown to complicate 71% of males with COVID-19 as compared to 31-57% in literature controls (57).

The androgen-dependency of TMPRSS2 activity is normally expressed at its highest level in the prostate epithelium, as evidenced by several fold abundance compared to all other body tissues (59, 60). While the physiological role of TMPRSS2 is still under investigation, it is significantly up regulated in men with a prostatic disease, including those with prostate cancers (37). Elevated free testosterone was recently shown in a large scale study to be associated with COVID-19 complications in this subgroup of men (55).

Men with metastatic prostatic cancer are usually treated with androgen-deprivation therapies to control the disease. It is noteworthy that these therapies substantially decrease the levels of TMPRSS2. While cancer patients have an increased risk of SARS-CoV-2 compared to non-cancer patients, it has been recently shown by two independent preliminary studies that prostate cancer patients receiving androgen-deprivation therapies are partially protected from SARS-CoV-2 infections (61, 62), supporting further the deleterious role of androgens in the pathogenesis of COVID-19. The active controversy surrounding this topic in contemporary literature calls for well-designed targeted studies to substantiate the potential protective effects of androgen-deprivation therapy.

A more recent prospective longitudinal study of hospitalized males with COVID-19 suggested that longer AR CAG repeats are associated with a more severe form of the disease, supporting the active role of testosterone in the pathogenesis of the complicated disease (63).

Furthermore, there is evidence that AR has an impact on furin and other members of the convertase proprotein family in prostate cancer, which may support an alternative role for testosterone in the pathogenesis of COVID-19 (64, 65). This two-pronged position of testosterone to employ either TMPRSS2 or furin to intensify the virulence of SARS-CoV-2 warrants investigation in targeted studies.

Further to the AR genetic disparities among various ethnic populations, other natural candidate genetic polymorphisms related to *ACE2, TMPRSS2, or FURIN* genes, as well as other host invasion genes such as *DPP4 or PCSK3*, which have been shown to differ among different population ancestries, may also provide a supplementary explanation for COVID-19 pandemic spread and progression (66–68). It is possible that the presence of different *ACE2, TMPRSS2, FURIN*, *DPP4*, and *PCSK3* gene variants, the main machinery for orchestrating SARS-CoV-2 cellular host access, may modulate viral infectivity among humans, making some people less or more vulnerable than others.

Taken together, epidemiological data emerging from the COVID-19 pandemic, backed by animal studies and further by preliminary clinical studies in diverse clinical settings, support the notion that high (male) testosterone levels acting *via* the AR modulate TMPRSS2 function positively to further prime SARS-CoV-2 S proteins and eventually increase COVID-19 infectivity and severity. Additionally, as in various ethnic backgrounds, *AR* mutations or other gene polymorphisms along the pathway of SARS-Co-2 pathogenesis may further lead to COVID-19 expansion and deterioration. This concept ought to be further explored in properly performed targeted studies.

LOW TESTOSTERONE IMPACT ON COVID-19 SEVERITY—THE ACE2 CONNECTION

Serum testosterone levels decline with aging among men (69, 70) and the presence of comorbidities such as obesity, diabetes mellitus, cardiovascular disease, and chronic obstructive pulmonary disease, may further accentuate testosterone decrease among these men (71–74). Functional hypogonadism, which was previously referred to as "late-onset" hypogonadism, is a condition in which the endogenous secretion of testosterone is either insufficient or inadequate to maintain serum testosterone levels within the normal range, and may manifest as a variety of signs and symptoms. In addition to reduced sexual function, age-related hypogonadal men may have impaired energy, muscle mass and performance, cognitive function, bone mass with increased fracture risk, and anemia (74).

Age-related hypogonadism in men is due to a combination of primary hypogonadism (testicular insufficiency) and secondary hypogonadism (hypothalamic-pituitary insufficiency). While the first is the result of a reduced number of Leydig cells in the testis and less responsiveness of these cells to LH stimulation, the latter is the result of decreased GnRH production by the hypothalamus causing a decrease in LH secretion (75, 76). In these cases, serum T levels are low (below 10.5 nmol/L), however in some cases hypogonadism may be compensated, apparent by normal serum T level and high LH level.

Functional hypogonadism in adult men is often underdiagnosed and therefore undertreated. This has been explained as related symptoms are easily attributed to aging or other medical causes or ignored by patients and physicians. More than 60% of men over age 65 have free testosterone levels below the normal values of men aged 30 to 35. The community prevalence estimates of potentially functional hypogonadism in middle-aged and older men vary from 2.1% to 12.3%, with wide geographic and racial variation (74, 77). However, in men with comorbidities the prevalence may be much higher, reaching a rate of 22 to 69% in men with chronic obstructive pulmonary disease (71).

There are remarkable sex differences between the physiological mechanisms regulating arterial pressure, renal and vascular functions in humans (78–80). Accumulating evidence suggests that several components of the RAAS are regulated by sex hormones, as well as influenced by hormone replacement therapies (80). This is attributed to the differential balance in the pressor and the counterpart depressor arms of the RAAS in related organs. Mounting evidence suggests that sex hormones, androgens and estrogens, and modulation of the ACE2 expression may also take place in the lungs (81, 82). The *ACE2* gene is located on the X chromosome, with females generally having higher ACE2 activity than males (83), yet ACE2 expression levels in the lungs as well as in the myocardium have recently been demonstrated to be higher in males (53, 82). Furthermore, while *ACE2* gene expression decreases with age, it has been shown to have a negative correlation with COVID-19 severity and mortality (84). ACE2 is the receptor entry of SARS-CoV-2 infection and progression, but then again is a guardian against lung injury. It is, therefore, reasonable to speculate that in men with functional hypogonadism, low testosterone levels may aggravate COVID-19 infection and exacerbate morbidity and mortality.

RAAS components, and specifically ACE2, have also been shown to be involved in normal testosterone production, steroidogenesis, and spermatogenesis in mammalian animal models as well as in humans (85). Recently, ACE2 expression patterns were found to predominate spermatogonia, Leydig, and Sertoli cells in the adult human testis (86), offering evidence that the reproductive system in men is a potential target of COVID-19. There is some preliminary evidence to show that acute SARS-CoV-2 infection has the potential to infect the testes causing orchitis, and leading to a reduced ratio of serum testosterone to LH levels (87–89). This may potentially enhance the susceptibility of men with functional or borderline hypogonadism to COVID-19 infection and deterioration.

Moreover, testosterone is implicated in physiological processes in adult males other than reproduction and sexuality. Among these functions, testosterone has anti-inflammatory and immune-modulatory protective effects, achieved by regulating the differentiation of T lymphocytes (90–92). Androgens seem to be essential to mounting an anti-viral response and combating infection in males. Accumulating evidence suggests that in cases with severe SARS-CoV-2 infection, there is an acute disruption of the immune response. Specifically, secondary cytokine storm syndrome has been shown to complicate severe COVID-19 cases, leading to multiple-organ failure and mortality (93).

Furthermore, testosterone seems to have a modulatory influence on vascular integrity in aging men. Although most of the literature on sex differences has focused on the effects of estrogen deficiency associated with menopause and the protective effect of hormone replacement therapy, little attention has been paid to testosterone and its contribution to vascular aging. Accumulating data suggest that testosterone deficiency in aging men is related to endothelial dysfunction, arterial stiffness, and thrombocyte malfunction, predisposing men with COVID-19 to increased risk of venous and arterial thrombo-embolic phenomenon causing mortality (94, 95).

Indeed, two recent preliminary, unrelated cohort studies targeted patients with severe COVID-19, admitted to intensive care units, with a high rate of comorbidities. Both studies independently showed that low testosterone and dihydrotestosterone levels were correlated with COVID-19 severity and mortality (96, 97). Furthermore, low testosterone levels were found to correlate with high levels of inflammatory cytokines (96).

Taken together, low testosterone levels, a pathognomonic biomarker of aging males with functional hypogonadism, seems to be a substantial factor for poor prognosis and mortality in SARS-CoV-2 infected men. This may be substantially aggravated in men with co-morbidities admitted to intensive care units. Further studies are needed to substantiate this notion.

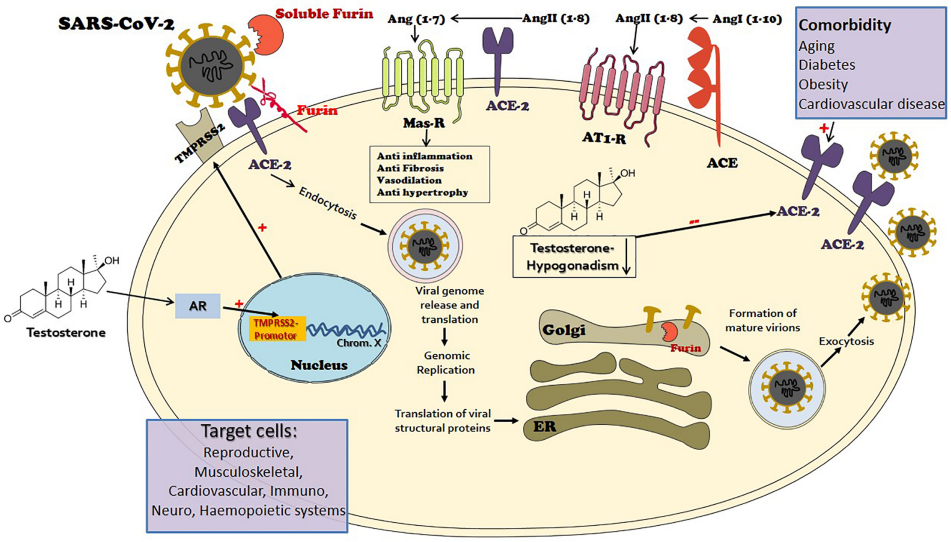

FIGURE 1 | The port of entry of the novel mutant virus Severe Acute Respiratory Syndrome (SARS)-CoV-2 to target cells is *via* the angiotensin-converting enzyme 2 (ACE2), a key element of the renin-angiotensin-aldosterone system (RAAS). ACE2 is widely expressed in the human body and is largely responsible for the generation of angiotensin 1-7 from angiotensin II. The angiotensin 1-7-Mas receptor axis provokes beneficial balancing and salutary actions to counterpart the adverse branch of renin-angiotensin I-angiotensin II-AT1R axis in the RAAS, in vital organs such as the lung, heart, and kidney. The viral envelope bears transmembrane spike (S) glycoproteins applied to ACE2 attachment. Following ACE2 binding, cleavage of the viral spike protein (S) by proteases including transmembrane protease serine 2 (MPRSS2) and furin is considered as an essential step to effectuate host cell membrane fusion and virus infection. The priming process by TMPRSS2 seems to be vital for the entry of SARS-CoV-2 into human host cells. TMPRSS2 transcription is exclusively regulated by the androgen receptor (AR). The AR has a widespread expression in many tissues with a diverse range of biological actions, including the cardiovascular, reproductive, musculoskeletal, immune, neural, and haemopoietic systems. Male level testosterone seems to play a vital role in COVID-19 pathogenesis and severity, *via* the TMPRSS2 connection. While functional hypogonadism, a prevalent occurrence in aging men that is more widespread in men with comorbidities, also has an adversative role, *via* the ACE2 connection.

DISCUSSION

This mini review illustrates that COVID-19, an amply complex disease generated by the novel mutated SARS-CoV-2, has paved the way to explore numerous mechanisms of health and disease in the human body. Sex hormones, specifically testosterone seem to be a key factor in the development and spread of the disease. It seems that testosterone may be considered a double-edged sword in the pathogenesis of COVID-19 morbidity and mortality. In turn, the edges may correspond to countervailing connectors and pathways: TMPRSS2 promoting the contribution of testosterone excess and ACE-2 promoting the role of age-related testosterone deficiency (**Figure 1**). Epidemiological data, animal models and in-vitro cell experiments, and clinical studies support our conclusion. Unfolding the mechanisms and pathways of COVID-19 development and spread related to testosterone may open new horizons for disease containment, treatment, and eradication. This paper paves the way for several future directions of clinical, translational, and basic science investigations. **Table 1** summarizes the outstanding and unresolved questions that warrant examination in future targeted studies.

TABLE 1 | Unresolved questions relating to the "double-edged" role of testosterone in COVID-19.

What is the relation between serum testosterone levels and biomarkers of severe COVID-19 such as: lymphocyte count, CRP, D-dimers, ferritin and IL-6 (98–100)?
What is the relation between testosterone levels, androgen receptor mutations/ polymorphisms and TMPRSSE2 function in priming SARS-CoV-2 spike proteins, and in turn COVID-19 morbidity and mortality?
What is the relation between testosterone levels in men with age-related functional hypogonadism, COVID-19 and ACE2 expression, and in turn to disease severity and mortality, in men with co-morbidities or patients admitted to intensive care units?
Can precision guidance be used to consider whether testosterone replacement therapy or, conversely, testosterone deprivation drugs, in the appropriate settings, for management of patients with COVID-19?

AUTHOR CONTRIBUTIONS

JY and ZA created and developed the concept. JY drafted the manuscript. ZA and KS edited and revised the manuscript for important intellectual content. 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.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 22 March 2021
doi: 10.3389/fendo.2021.642755

The Impact of the COVID-19 Pandemic on Women's Reproductive Health

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Background: The COVID-19 pandemic has profoundly affected the lives of the global population. It is known that periods of stress and psychological distress can affect women's menstrual cycles. We therefore performed an observational study of women's reproductive health over the course of the pandemic thus far.

Materials and Methods: An anonymous digital survey was shared by the authors *via* social media in September 2020. All women of reproductive age were invited to complete the survey.

Results: 1031 women completed the survey. Mean age was 36.7 ± 6.6 years (range, 15–54). 693/70% reported recording their cycles using an app or diary. 233/23% were using hormonal contraception. 441/46% reported a change in their menstrual cycle since the beginning of the pandemic. 483/53% reported worsening premenstrual symptoms, 100/18% reported new menorrhagia ($p = 0.003$) and 173/30% new dysmenorrhea ($p < 0.0001$) compared to before the pandemic. 72/9% reported missed periods who not previously missed periods ($p = 0.003$) and the median number of missed periods was 2 (1–3). 17/21% of those who "occasionally" missed periods pre-pandemic missed periods "often" during pandemic. 467/45% reported a reduced libido. There was no change in the median cycle length (28 days) or days of bleeding (5) but there was a wider variability of cycle length ($p = 0.01$) and a 1 day median decrease in the minimum ($p < 0.0001$) and maximum ($p = 0.009$) cycle length. Women reported a median 2 kg increase in self-reported weight and a 30-min increase in median weekly exercise. 517/50% of women stated that their diet was worse and 232/23% that it was better than before the pandemic. 407/40% reported working more and 169/16% were working less. Women related a significant increase in low mood ($p < 0.0001$), poor appetite ($p < 0.0001$), binge eating ($p < 0.0001$), poor concentration ($p < 0.0001$), anxiety ($p < 0.0001$), poor sleep ($p < 0.0001$), loneliness ($p < 0.0001$) and excess alcohol use ($p < 0.0001$). Specific stressors reported included work stress (499/48%), difficulty accessing healthcare (254/25%), change in financial (201/19%) situation, difficulties with home schooling (191/19%) or childcare (99/10%), family or partner conflict (170/16%), family illness or bereavement (156/15%).

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *16 December 2020*
Accepted: *15 February 2021*
Published: *22 March 2021*

Citation:
Phelan N, Behan LA and Owens L (2021) The Impact of the COVID-19 Pandemic on Women's Reproductive Health. Front. Endocrinol. 12:642755. doi: 10.3389/fendo.2021.642755

Conclusions: The COVID-19 pandemic has significantly impacted the reproductive health of women. The long term health implications of this are yet to be determined and future studies should address this.

Keywords: menstrual abnormalities, COVID-19 pandemic, psychological distress, oligomenorrhea/amenorrhea, libido, dysmenorhea, menorrhagia

INTRODUCTION

The COVID-19 pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1, 2), has caused over 106 million infections and 2.3 million deaths worldwide, as of February 2021, according to the WHO COVID-19 Dashboard (3). The virus itself, as well as the measures taken to reduce its spread, have profoundly affected the lives of the global population. The pandemic has significantly impacted the mental health of many people within the population, resulting in loneliness, social isolation, financial strain, as well as the anxiety and fear of contracting the virus, and uncertainty for the future. Analysis of a national, longitudinal cohort study found that by late April 2020, mental health in the UK had deteriorated compared with before the COVID-19 pandemic (4). A US study in April 2020 found higher rates of psychological distress among adults, compared with 2018 (5). In this study the increase in psychological distress was greatest in women and young people aged 18–24 years. On the other hand, there is theoretically a group who have experienced reduced stress and improved mental well-being, for example those with financial security, those who have spent more time with their families and less time commuting since the outbreak of the pandemic.

It is known that periods of stress and psychological distress can affect women's menstrual health. Stressors can activate the hypothalamic-pituitary-gonadal (HPG) axis and can alter the neuro-modulatory cascade that drives gonadotropin releasing hormone (GnRH) regulation (6). This can result in functional hypothalamic amenorrhoea (FHA), chronic anovulation which is not due to an underlying organic cause (7, 8). Behavioural modification such as cognitive behavioural therapy can reverse this amenorrhoea (9). FHA also occurs secondary to excessive exercise, dieting and caloric restriction and disordered eating (10, 11).

Psychological distress is not only associated with missed periods but also worsening of symptoms associated with menstruation and psychosexual health. Dysmenorrhoea has been shown to be associated with high stress levels (12), emotional instability and depression (13). Pre-menstrual symptoms (PMS) and menorrhagia are also associated with high psychological distress (14, 15). Higher perceived stress is also associated with lower libido in women (16).

Our objective therefore was to survey the general female population of reproductive age with regards to their menstrual cycle, libido and changes in their lifestyle over the course of the pandemic thus far. We performed an anonymous observational study by circulating a survey *via* social media and text message.

MATERIALS AND METHODS

Study Design

This was an anonymous observational study. A digital survey was created using the Typeform platform (www.typeform.com). A link to the survey was shared by the authors *via* social media (Facebook, Twitter) and all women of reproductive age were invited to participate. The survey contained 50 questions on demographic information, menstrual cycle and mental health symptoms, diet, exercise and working patterns from before and since the beginning of the pandemic. It took between 5 and 10 min to complete. Ethical approval was granted for the study by the St James's and Tallaght University Hospital Research Ethics Committee (JREC 2020-10-CA-10). Written consent was not required as all data was collected anonymously. The survey was circulated over a 2-week period in the second half of September 2020. The study was conducted and reported according to published best practice guidelines for reporting observational studies (17). A full list of survey questions can be found in **Supplementary Table 1**.

Participants

In total 1031 women completed the survey. All women of reproductive age were invited to participate *via* digital link. Menstrual cycle and BMI data were excluded from women who became pregnant or delivered a baby during the pandemic. Menstrual cycle data was also excluded from women who stated they were amenorrhoeic for any reason (i.e. due to an intrauterine system, intrauterine device or implant, menopause, breastfeeding).

Statistical Analysis

Data was analysed using Graphpad Prism version 8.4.3. Parametric data is reported as mean and standard deviation (SD). Non-parametric data is reported as median and interquartile range (IQR). A p-value < 0.05 was considered statistically significant. A paired t-test was used to compare parametric data for data comparing before and during the pandemic (e.g. comparing dysmenorrhoea experienced before and since the beginning of the pandemic). A Wilcoxon matched pairs rank test was used to compare non-parametric paired data.

RESULTS

Patient Demographics

A total of 1031 women completed the survey. The participants demographic information is summarized in **Table 1**. The mean age of the women was 36.7 ± 6.6 years (range, 15–54 years). The mean self-

TABLE 1 | Participant demographics/medical history.

Demographics	
Age (mean ± SD), years	36.7 ± 6.6 (range, 15–54)
BMI (mean ± SD), kg/m^2	25.8 ± 5.5 (range, 16.6–65.5)
Ethnicity	1000/97% White (White Irish, White British) 23/2% Asian 3/0.3% Black 5/0.5% Other
Location	862/84% Ireland 145/14% UK 24/2% other countries
Marital status	Single 249/25% Married 567/57% Cohabiting 150/15% Separated/Divorced 25/3% Widowed 1/0.1%
Occupation	301/29% Healthcare workers (HCW) 175/17% Doctors, 57/6% nurses, 69/7% other HCWs
Work status and location during pandemic	Full time in the workplace 392/38% Full time from home 311/30% Part time in the workplace 130/13% Part time from home 64/6% Maternity leave 47/5% Unemployed before pandemic 56/5% Made redundant/unemployed during pandemic 23/2%
Participants with children (yes/no)	Yes 593/58% No 435/42%
Currently breastfeeding	74/7.2%
Pre-existing medical conditions	Polycystic Ovary Syndrome 68/7% Excess unwanted hair 308/30% Hypothalamic amenorrhoea 3/0.3% Endometriosis 65/6% Premature ovarian insufficiency 22/2% Osteopenia/Osteoporosis 13/1% Acne 115/11% Thyroid disorder 91/9%
Covid-19 history	Yes and tested positive 35/3.4%
"Did you have Covid-19?"	Yes, had symptoms but did not get tested 63/6.1% No 870/83% No, but had contact with a confirmed case 63/6%

reported body mass index (BMI) was 25.8 ± 5.5 kg/m^2. (range, 16.6–65.5 kg/m^2). 1000/97% of the women were of white ethnicity and 1007/98% were based in Ireland or the United Kingdom. 717/72% were married or cohabiting, 249/25% were single and 25/3% were separated or divorced. 593/58% stated that they had children and 74/7.2% were currently breastfeeding. 897/88% of women were working during the pandemic, 522/51% in the workplace and 375/37% from home. 326/59% of women who had children, home-schooled them when schools were closed and 369/66% had to provide childcare while also trying to work. 301/29% were healthcare workers (HCW); 175/17% Doctors, 57/6% nurses and 69/7% were other HCWs. 35/3.4% stated that they had contracted COVID-19 and tested positive, 63/6.1% had symptoms of COVID-19 but did not get tested.

Menstrual History

834/81% of women stated they usually had regular periods. 693/70% of those who had periods recorded them using an app, diary, smartphone or other recording method (**Table 2**). 747/72% of women were not using any form of contraception and 231/22% were using hormonal contraception (**Table 2**). The median cycle length was 28 days (27–30) with a 5 day bleed (4–6) and the minimum usual cycle length was 27 (25–28) days and the maximum usual cycle length was 30 days (28–32) (**Table 2**). 131/13% of women reported missing periods, 82/8% reported missing them occasionally and 49/5% often. 420/42% reported heavy periods and 416/42% painful periods (**Table 2**).

TABLE 2 | Menstrual history and contraceptive use.

Parameter	Result
Contraception use	None—747/72% Combined contraceptive pill/patch—110/11% Intrauterine system—77/7% Progesterone only pill—31/3% Intrauterine device—24/2% Implant—13/1% Depot—2/0.2% Other—27/3% (Barrier, tubal ligation, vasectomy)
Cycles recorded using app/diary/ smartphone/other	Yes 693/70% No 297/30%
Regular periods under normal circumstances	Yes 834/81% No 163/16% N/A 32/3%
Median cycle length (days)	28 (27–30)
Median no. of days of bleeding	5 (4–6)
Minimum length of cycle (days) (median)	27 (25–28)
Maximum length of cycle (days) (median)	30 (28–32)
Missed periods	131/13% (occasionally 82/8%, often 49/5%)
Heavy periods	420/42%
Painful periods	416/42%

Menstrual Cycle Changes During the COVID-19 Pandemic

441/46% of women who got periods reported an overall change in their menstrual cycle during the COVID-19 pandemic. See **Table 3** for menstrual cycle changes. 483/53% reported a worsening in premenstrual symptoms (PMS), whereas 60/7% felt that their PMS improved. The median cycle length was 28 days, similar to before the pandemic but with a significantly wider range (25–30) ($p = 0.01$). 255/29% noted a reduced cycle length and the median reduction was 3 days (2–6) and 28% reported a longer cycle with a median increase of 3 days (2–6). The median number of days of bleeding was 5 (4–6) and was unchanged compared to before the pandemic ($p = 0.3$). The minimum length of the cycle was 26 days (22–28), a median of 1 day shorter than before the pandemic ($p < 0.0001$). The maximum length of the cycle was 29 days (22–28), also a median of 1 day shorter than before the pandemic ($p = 0.009$).

158/17% had missed periods during the pandemic, 4%/27 more than pre-pandemic ($p = 0.0003$). 72/9% reported new missed periods, of which 56/7% were "occasional" and 16/2% were "often." The median number of missed periods was 2 (1–3). 17/21% who "occasionally" missed periods pre-pandemic missed periods "often" during pandemic. 40/31% of those who had

TABLE 3 | Menstrual cycle changes during the Covid-19 pandemic.

Change	Result, n/%	p-value
Overall change in menstrual cycle	Yes—441/46% No—523/54%	
Change in libido/sex drive	No change—429/42% Increased libido—131/13% Decreased libido—467/45%	
Change in premenstrual symptoms (PMS)	PMS unchanged—366/40% PMS better—60/7% PMS worse—483/53%	
Median cycle length (days)	28 (25-30) 255/29% reduced cycle length –3 days (2–6, range 1–55) 239/28% increased cycle length +3 days (2–6, range 1–103)	0.01
No. of days of bleeding (median, IQR)	5 (4–6)	P = 0.3
Minimum length of cycle (days) (median, IQR)	26 (22–28)	p < 0.0001
Maximum length of cycle (days) (median, IQR)	29 (28–32)	p = 0.009
Missed periods	–158/17% (+4%/27) (occasional—93/10%, often—65/7%) –72/9% new missed periods (occasional—56/7%, often—16/2%) -Median missed periods= 2 (1–3) -17/21% who 'occasionally' missed periods pre-pandemic missed periods 'often' during pandemic -40/31% who had missed periods previously had no missed periods during pandemic	p = 0.0003
Heavy periods	447/47% 18%/100—new heavy periods 15%/63—less heavy periods	P = 0.003
Painful periods	469/49% 173/30%—new painful periods 49/12%—previously painful periods improved	p < 0.0001
Conceived during pandemic	84/8%	

missed periods previously had no missed periods during pandemic. 467/45% of women reported a decrease in their libido and 131/13% reported an increase in their libido. 447/47% of women reported heavy periods, 27/5% more than before the pandemic (p = 0.003). 469/49% reported painful periods, 53/7% more than before the pandemic (p < 0.0001). 173/30% reported new painful periods and 49/12% reported that previously painful periods improved during the pandemic.

Lifestyle and Mental Health Change During the COVID-19 Pandemic

The overall median change in self-reported weight was an increase of 2 kg (0–4 kg) (p < 0.0001) (**Table 4**). 622/65% of women gained weight, and the median weight gain was 3 kg (2–5 kg) (**Table 4**). 158/16% lost weight, and the median weight loss was also 3 kg (2–5 kg) (**Table 4**). Women carried out on average 150 (40–270) min of exercise per week during the pandemic, 30 min (60–220) more than before the pandemic (p = 0.02) (**Table 4**). 517/50% of women felt that overall their diet was worse during the pandemic. 232/23% felt that their diet overall had improved (**Table 4**). 127/12% reported drinking excess alcohol compared to 72/7% before the pandemic (**Table 5**). 407/40% reported working more than before the pandemic and 169/16% were working less (**Table 4**).

Women reported a significant increase in suffering from mental health symptoms (**Table 5**). 868/84% of women reported suffering from at least one symptom, including low mood (519/50%, p < 0.0001), anxiety (514/50%, p < 0.0001), poor sleep (509/49%, p < 0.0001), significant stress (373/36%, p < 0.0001), binge eating (373/36%, p < 0.0001), poor concentration (373/36%, p < 0.0001), loneliness (373/36%, p < 0.0001), poor appetite (373/36%, p < 0.0001) and excess alcohol use (373/36%, p < 0.0001). There was no change in illicit drug use (1%, p = 0.34). Women reported experiencing a number of stressors as outlined in **Table 6**, the most prevalent of which was work stress (499/48%), followed by difficulties accessing healthcare (254/25%) (**Table 6**).

TABLE 4 | Changes in lifestyle during the COVID-19 pandemic.

Question	Result	P-value
Change in weight (n = 964) (median, IQR)	Median +2 kg (0–4) 184/19% no change in weight 158/16% lost weight, median –3 kg (2–5 kg) 622/65% gained weight, median +3 kg (2–5 kg)	<0.0001
Change in minutes of exercise/ week (median, IQR)	+ 30 (40–270)	0.02
Type of exercise	Running—350/34% Yoga/pilates—300/29% HiiT—202/20% Strength training—209/20% Walking—193/19% Other—51/5% None—65/6%	
Diet	Overall diet is unchanged—281/27% Overall diet is better—232/23% Overall diet is worse—517/50%	
Change in work practices	No change—453/44% Working more—407/40% Working less—169/16%	

TABLE 5 | Mental health symptoms.

	Before pandemic	During pandemic	P-value
Low mood	359/34%	519/50%	<0.0001
Anxiety	382/37%	514/50%	<0.0001
Poor sleep	341/33%	509/49%	<0.0001
Significant stress	268/26%	373/36%	<0.0001
Binge eating	236/23%	335/32%	<0.0001
Poor concentration	172/17%	360/35%	<0.0001
Loneliness	136/13%	299/29%	<0.0001
Poor appetite	64/6%	129/12%	<0.0001
Excess alcohol use	72/7%	127/12%	<0.0001
Illicit drug use	11/1%	7/1%	0.34

TABLE 6 | Stressors. "Have you had any of the following stressors over the course of the pandemic?"

Stressor	n/%
Work stress or change in employment status	499/48%
Difficulties accessing healthcare	254/25%
Change in financial situation	201/19%
Difficulties with home schooling	191/19%
Family illness or bereavement	169/16%
Change in living situation	156/15%
Family or partner conflict	170/16%
Difficulties providing or arranging childcare	99/10%

Women who reported experiencing one or more of low mood, anxiety, or significant stress were significantly more likely to report an overall change in their menstrual cycles since the beginning of the pandemic (50% versus 34%, $p < 0.0001$). These women with mental health symptoms were also more likely to report painful periods (54% versus 36%, $p < 0.0001$), worsening pre-menstrual symptoms (62% versus 32%, $p < 0.0001$), as well as decreased libido (51% versus 31%, $p < 0.0001$). 18% of women who experienced low mood, anxiety, and/or significant stress reported missing periods since the beginning of the pandemic, whereas 13% of those who did not experience these mental health symptoms reported missed periods, however this difference was not significant ($p = 0.08$). Those women who reported an overall change in their menstrual cycles were more likely to have reported poor sleep (41%) than those who did not report a change in their menstrual cycles (28%), $p < 0.0001$.

The final question of the study asked "do you have any other comments related to the impact of COVID-19 pandemic on your life?" 420/41% of respondents added a comment. Selections of the responses are provided in **Supplementary Table 2**. We labelled the comments as "positive," "negative," "mixed" or "neutral" depending on what impact was described by the respondents. 322/77% of comments described a negative impact of the pandemic on their lives, 41/10% described an overall positive impact of the pandemic, 27/6% commented that the pandemic had both positive and negative impacts on their lives, and 30/7% of comments were neutral.

DISCUSSION

This large, anonymous observational study has demonstrated that a large proportion of the female population have experienced reproductive health disturbance as a result of the COVID-19 pandemic. These disturbances are associated with a significant increase in suffering from mental health symptoms, as well as weight gain, longer working hours and an unhealthier diet. A minority of women have described improvement in their reproductive health and lifestyle over the course of the pandemic.

Women reported disturbances in their menstrual cycles that are known to be associated with psychological distress. Stress has an inhibitory effect on the hypothalamic pituitary gonadal axis (HPG). Stress and stress hormones inhibit GnRH release from the hypothalamus, and glucocorticoids inhibit luteinising hormone (LH) release and oestrogen and progesterone production by the ovary (18, 19). Stress regulates HPG axis through activation of hypothalamic sympathetic neural pathways that result in norepinephrine release in the ovary (20).

Functional hypothalamic amenorrhoea (FHA), chronic anovulation which is not due to an underlying organic cause, is associated with vigorous excess and an energy deficit, as well as stress, anxiety and mood disorders (7, 8, 21, 22). FHA has long-term health consequences including subfertility, osteoporosis, increased risk of cardiovascular disease and psychiatric disease (23). There was a significant increase in missed periods, likely as a result of psychological distress and an increase in the amount of exercise being carried out. Whether these missed periods will ultimately progress to chronic anovulation is as yet unknown. Women who missed periods occasionally before the pandemic reported missing them often during the pandemic. Given that many women gained weight and reported that their diet had worsened, this amenorrhoea is likely related to not only stress related amenorrhoea, but also overweight/obesity and worsening of PCOS symptoms, both known to be affected by incremental increases in weight (24, 25).

Over half of respondents reported worsening symptoms of pre-menstrual syndrome (PMS). Studies have demonstrated a higher prevalence of PMS among women with a high psychosocial stress level (15). PMS can have a significant impact on women's health and is associated with impairment of activities of daily living and mental health disorders such as anxiety disorders, postnatal and perimenopausal depression (26). Almost half of women reported periods that were heavy and painful, a significant increase compared to before the pandemic. Again this is largely unsurprising as both have been shown to be associated with stress, psychological distress and low mood (12–15). 45% of women also reported a reduction in their libido. Hypoactive sexual desire disorder, when symptoms persist for over 6 months and are accompanied by distress, affects 6% to 13% of the European adult female population (27, 28). Self-reported reduced sexual desire has been shown to be associated with lower quality of life, poor self-esteem and hopelessness, as well as depression and anxiety (27).

Women reported no change in the median length of their cycle but there was a significantly wider range in the length of their cycle and a shortening of the minimum and maximum cycle length recorded. It is known that for those trying to conceive, shorter or longer menstrual cycles are less likely to be ovulatory and be followed by conception and are more likely to end in spontaneous abortion (29).

There was a significant increase in reported acute mental health suffering since the outbreak of the pandemic. Approximately half reported low mood and anxiety and approximately one third reported stress, binge eating, poor concentration and loneliness. Those who experienced low mood, anxiety and/or significant stress were more likely to report an overall change in their menstrual cycle, as well as worsening pre-menstrual symptoms, more painful periods, and reduced libido. Almost half of women reported poor sleep, and those who reported poor sleep were more likely to have experienced alteration in their menstrual cycle. It is known that sex hormones influence circadian rhythm, and vice versa (30, 31). PMS, which increased during the pandemic, is also associated with sleep disturbance (32). Sleep disturbance may actually affect fertility, as it has found to be more prevalent in those with infertility and those with diminished ovarian reserve (33).

Women also recorded lifestyle changes which may have impacted their menstrual cycles. There was an overall increase in the amount of exercise being undertaken, by half an hour per week. Despite this women gained weight, a median of 2 kg, likely because half of the women reported that their diet was worse and almost one third reported binge eating since the beginning of the pandemic. 40% of women were also working more during the pandemic, which would limit the time available for preparing healthy meals.

While a significant proportion of women described the negative impact of the pandemic on their menstrual cycle and lifestyle, there was a minority who described a positive effect. Some women had noted more regular periods, and periods that were less heavy and painful with less PMS. Some women reported an increase in their libido. There was an increase in average exercise per week, and 1 in 6 women lost weight. When they were invited to comment about the impact of the COVID-19 pandemic one in ten women described positive aspects of the pandemic; including a slower pace of life, less commuting and getting to spend more time with their families. Some women (6%) recognised both a positive and negative impact of the pandemic on their lives. It is as yet impossible to know if any positive effects will endure as the pandemic continues and progresses.

The major strength of this study is the large number of women surveyed, as well as the novel nature of the data. Another strength of the study is the fact that the majority (70%) of women were recording their menstrual cycle pattern using diary or smartphone app, therefore the menstrual cycle data is likely to be largely unbiased. In addition, the survey was anonymous, reducing the effect of social desirability bias, where people are more likely to report experiences that are considered to be socially acceptable.

There are several limitations to this observational study. The first is that the study recorded self-reported data, which is subject to bias. Self-reporting of menstrual cycle has been shown to have measurement error (34). However the majority of women in our study were recording their cycles using an app or a diary, reducing recall bias. Self-reported weight can also be inaccurate, however a study showed that when young people self-report weight they under-estimate by an average of only 0.4 kg (35), a modest under-estimate. In addition the mean BMI was 25.8 kg/m^2, similar to previous large studies in women of reproductive age (36). Another potential limitation is that there may have been sampling bias, where those who opted to complete the survey were those who were more likely to have suffered menstrual disturbance. While we acknowledge that self-reported data collection is subject to bias, using digital surveys is a safe way to perform research during the pandemic, and given the anonymous nature of the study, the results have merit and provide a valuable insight into reproductive disturbances women have experienced as a result of the pandemic.

Another limitation is the population of women that completed the survey. The majority were of white ethnic background, so the data may not be representative of all women. The pandemic has disproportionately affected certain sectors of society, such as women of black, Asian or minority ethnic (BAME) background, who are more likely to be severely affected if they contract COVID-19 (37) and more likely to be from lower socio-economic backgrounds (38). 88% of respondents were in employment, which is higher than the European Union average for women of 66.5% (39). The average age was 36, and it is known younger women are more likely to develop hypothalamic amenorrhoea (40), so it is possible that a younger cohort of women may have worse symptoms. There was also a high number of healthcare workers surveyed, who may be more significantly affected by the pandemic due to enhanced workplace stressors and fear of exposure to COVID-19. Lastly, the reported mental health symptoms were intended only to provide a snapshot of how women were feeling subjectively, and are not an objective, qualitative measure of symptoms using validated mental health questionnaires. The main focus of this study was on the reproductive symptoms women were experiencing.

While it is clear from this study that women have suffered significant reproductive health disruption since the beginning of the pandemic, the medium and long term impacts of this are, as yet, unknown. This study captured the first approximately 6 months of the pandemic (February/March 2020 to September 2020). The longer term health implications are likely to also depend on the duration of the pandemic and other uncertainties such as the economic impact and the distribution of and access to COVID-19 vaccines. Future work should include continued multi-modal assessment at different intervals over the course of the pandemic, including ongoing validated assessment of mental health and measurements of BMI, hormone levels and ovulation.

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 St James's and Tallaght University Hospitals REC.

Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

LO is the primary investigator and completed data analysis and wrote the manuscript. NP wrote the manuscript and was involved in setting up and completing the study. LB wrote the manuscript and was involved in setting up and completing the study. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors would like to acknowledge the women who kindly gave of their time and completed the survey, and the PMGUK Facebook group who completed and shared the survey.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.642755/full#supplementary-material

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

frontiers in Endocrinology

REVIEW
published: 31 March 2021
doi: 10.3389/fendo.2021.644298

New Roles for Vitamin D Superagonists: From COVID to Cancer

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Vitamin D is a potent steroid hormone that induces widespread changes in gene expression and controls key biological pathways. Here we review pathophysiology of vitamin D with particular reference to COVID-19 and pancreatic cancer. Utility as a therapeutic agent is limited by hypercalcemic effects and attempts to circumvent this problem have used vitamin D superagonists, with increased efficacy and reduced calcemic effect. A further caveat is that vitamin D mediates multiple diverse effects. Some of these (anti-fibrosis) are likely beneficial in patients with COVID-19 and pancreatic cancer, whereas others (reduced immunity), may be beneficial through attenuation of the cytokine storm in patients with advanced COVID-19, but detrimental in pancreatic cancer. Vitamin D superagonists represent an untapped resource for development of effective therapeutic agents. However, to be successful this approach will require agonists with high cell-tissue specificity.

Keywords: COVID-19, pancreatic cancer, pancreatic stellate cell, superagonist, vitamin D, paricalcitol

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *20 December 2020*
Accepted: *10 March 2021*
Published: *31 March 2021*

Citation:
Easty DJ, Farr CJ and Hennessy BT (2021) New Roles for Vitamin D Superagonists: From COVID to Cancer. Front. Endocrinol. 12:644298. doi: 10.3389/fendo.2021.644298

INTRODUCTION

Vitamin D is a steroid hormone with well-characterized effects on bone metabolism and calcium homeostasis. More recently, attention has focused upon non-classical effects, including important roles in regulation of the immune response and lung function and less well described effects in multiple additional tissues such as the cardiovascular system (1–3).

Vitamin D deficiency appears to be widespread globally, although significant data gaps exist for low-income countries (4, 5). Deficiency has been defined as serum calcidiol (25 OH vitamin D) less than 50nmol/L in the USA. Insufficiency occurs between 50-75 nmol/L. To reduce the risk of infectious disease, the US Endocrine Society recommended serum calcidiol should be above 75nmol/L (6). Approximately 40% of Europeans live with moderate deficiency (below the 50nmol/L cut off) and 5% of the US population have severe deficiency with serum levels less than 30nmol/L (5, 7). Serum calcidiol levels show seasonal variation and at the end of winter, 36% of young adults in the USA have insufficient (70 ± 25nmol/L) vitamin D (8).

In developed countries, deficiency appears more common within specific populations. In the UK this includes BAME (Black, Asian and Minority Ethnic), elderly and obese individuals (9, 10).

Low serum calcidiol has been linked to increased susceptibility to a variety of diseases, including renal, dermatological, cardiovascular and autoimmune disease, infections and cancer (11, 12).

Evidence suggests that vitamin D may protect against COVID-19 infection and decrease severity of symptoms in patients hospitalized with severe viral pneumonia. This might involve vitamin D inhibition of the cytokine storm and anti-fibrotic effects in the acute respiratory distress syndrome (ARDS) responsible for much mortality in COVID-19 (3, 13, 14).

Here we briefly review the physiology of vitamin D and regulation of target gene transcription. We describe activated vitamin D receptor (VDR) binding to DNA elements within promoters of target genes in association with coactivators and corepressors. Next, we discuss vitamin D superagonist (VDSA) interaction with the normal process of transcription to supercharge target gene expression. Then, in the main part of this review, we describe the pathophysiology of vitamin D with particular reference to COVID-19 and pancreatic cancer. Finally, we discuss approaches for the development of therapeutically useful VDSAs in some common human diseases.

VITAMIN D PHYSIOLOGY

The physiology of vitamin D has been extensively reviewed (3, 15) and is only briefly considered here. Cholecalciferol is obtained from ultraviolet light-induced cutaneous synthesis and to a lesser extent from diet. Within the skin, cholesterol precursor 7-dehydrocholesterol is converted to vitamin D3, which is hydroxylated to 25(OH)-D3 (calcidiol) in the liver and then to the active metabolite, 1,25(OH)2-D3 (1α, 25-dihydroxycholecalciferol, (calcitriol) in the kidney. Importantly, conversion of calcidiol into calcitriol also occurs within multiple tissues including normal and malignant epithelial cells and activated macrophages, suggesting an autocrine/paracrine function in non-calcium regulating cells (15, 16).

Calcitriol binds to the vitamin D receptor (VDR), a nuclear hormone receptor and transcription factor, present in most human tissues (17). The VDR contains a ligand binding domain (LBD) comprising one β-sheet and 13 α-helical structures. Helix H-12 contains an activation function-2 (AF-2) domain, which forms a hydrophobic binding pocket for vitamin D and its analogues. Vitamin D binds the ligand-binding pocket (LBP) of VDR-LBD and triggers a conformational change resulting in dimerization, and co-activator and co-repressor binding. Activated VDRs bind to vitamin D receptor elements (VDRE) within promoter and enhancer regions of target genes to regulate gene expression. VDR-binding pattern within the genome and the identity and number of genes targeted is dependent upon specific cell type, with between 200-1000 vitamin D target genes per cell) (17, 18).

VDRs bind to VDREs as either homodimers or a heterodimer with the retinoid X receptor (RXR). Basal transcription factors also bind together with transcriptional coactivators, including: steroid receptor coactivator-1 (SRC-1), glucocorticoid receptor interacting protein-1 (GRIP1), steroid receptor coactivator 1 (SRC-1), amplified in breast cancer 1 (AIB-1) and glucocorticoid receptor interacting protein (GRIP), see below). The VDR/RXR/VDRE complex modulates the epigenome *via* recruitment of coactivators that increase histone acetylation and upregulate transcription. VDR/RXR complexes also bind to VDRE in association with co-repressors to suppress transcription of vitamin D target genes. The VDR also interacts with chromatin modifying proteins including BRD7 and KDM6B (19). Vitamin D signaling results in up or down-regulation of gene expression involved in common cellular functions. This includes proliferation, differentiation, cell adhesion, apoptosis and autophagy and affects multiple key biological processes, such as fibrosis, inflammation and immunity which are commonly disrupted in disease (3, 20).

Vitamin D and the Immune System

Vitamin D has multiple effects upon cellular and humoral immunity. On the one hand, vitamin D stimulates the innate immune system *via* modulating activity of Toll-Like Receptors (TLR). Calcitriol binds to VDRs in macrophages resulting in increased production of antimicrobial secreted peptides such as defensin and cathelicidin and CD14, a glycoprotein, co-receptor for TLRs (21). Here, Th1 cytokines promote a pro-inflammatory response. On the other hand, vitamin D down-regulates the adaptive immune response, which may have beneficial effects, safeguarding against autoimmunity (22) (see below). Calcitriol inhibits B-cell activation and promotes Treg cell activity leading to cytokine mediated activation of Th2 and suppression of Th1 cells (23). T cell activation requires induction of VDR expression which occurs downstream of T cell receptor (TCR) signaling and activation of the p38 map kinase pathway (24). Vitamin D influences neutrophil and macrophage function. Neutrophil activity is decreased (25), likely resulting in an over-all beneficial effect in patients with COVID-19 and PDAC. However, vitamin D increases formation of neutrophil extracellular traps (NET), meshes composed of DNA fibers, histones and proteolytic enzymes. It is postulated that NETs may result in detrimental effects in patients with COVID-19 and PDAC) (26, 27) (see below). Alternatively, another group report vitamin D reduced expression of proinflammatory cytokines and inhibited NET formation, an effect seen only at low dose (28). Divergent findings between these studies may reflect the animal model used and differences between doses. Vitamin D also influences macrophage function; an initial pro-inflammatory, anti-tumor M1 phenotype is converted into immune-suppressive, pro-tumor M2 macrophages.

Vitamin D signaling is controlled through a feedback mechanism. VDR activation up-regulates *CYP24A1* expression, encoding vitamin D3 24-hydroxylase an enzyme that inactivates calcidiol and calcitriol, in a reaction dependent upon cytochrome P450. This reaction occurs in the liver and kidneys and prevents toxicity at physiological levels of vitamin D (20, 29). VDRs also localize in the membrane and mediate non-genomic actions, with ligand-dependent effects on signal transduction, affecting kinase and phosphatase activity. Calcitriol interacts with membrane VDR (mVDR, also known as membrane-associated rapid response steroid-binding proteins (1,25-MARRS). mVDR signals *via* mitogen-activated protein kinase (MAPK) and cyclic AMP (cAMP). Nevertheless, many of these effects only occur at

supra-physiological concentrations of calcitriol, and the significance of these pathways remains uncertain (18, 29).

Vitamin D and Physical Activity

Many factors combine to regulate vitamin D levels (see below), and these may include several less well-known mechanisms. Data from the ARIC study (10,342 participants) suggest physical exercise is associated with increased serum calcidiol. Vitamin D deficiency (<20 ng/mL) was significantly reduced in individuals meeting levels of physical activity recommended by the American Heart Association (30).

Recent data suggest physical exercise increases immunity and decreases the risk of cancer. Leisure-time physical activity was associated with decreased rates in 13 out of 26 cancers studied, although, melanoma and prostate cancer were exceptions with increased risk (31). The effect in infectious disease is less certain, although, physical activity has also been suggested to offer benefit against COVID-19 (32, 33). The benefits of physical activity are likely multifactorial, but may include increased levels of vitamin D, however, it is difficult to rule out effects of reverse causation in such studies.

VITAMIN D SUPERAGONISTS

More than 3000 vitamin D analogues (VAD) have been synthetized, most directly derived from calcitriol and containing a variety of modifications. The aim has been to produce analogues with enhanced VDR binding and increased stability to metabolism. The structure of these compounds has been described previously and a further detailed description is beyond the scope of this review (34). Structural modifications within vitamin D have typically been made in four sites: the side chain, A-ring, CD-ring and triene system. More recently non-steroidal vitamin D mimics have also been described (34). To date, there have been few comparative studies of vitamin D and VDSAs in clinical trials. Most data derive from *in vitro* studies and animal models. Vitamin D analogues have been characterized using *in vitro* assays, including VDR binding affinity, reporter gene assays, and cellular assays for anti-proliferative effects and enhanced differentiation. However, since reporter gene assays may be influenced by analogue uptake and metabolism, further analysis of VDAs in yeast or cell-free transcription may be required to demonstrate increased activity (35).

Vitamin D superagonists (VDSA) demonstrate a significantly increased activity compared to calcitriol. Increased physiological effects are seen in cellular assays for anti-proliferative effects and enhanced differentiation. VDSAs also increase transcriptional activity in assays using reporter gene inserted downstream of promoters containing VDRE. Mechanisms for superagonist properties may include: (1) enhanced VDR-RXR dimerization, (2) increased co-activator recruitment and (3) reduced sensitivity to metabolism (36, 37).

Various VDR coactivators have been described. These include VDR Interacting Protein (DRIP205), also known as MED1 (mediator of RNA polymerase II transcription subunit 1), which directly interacts with the receptor. Additional interactions occurring with coactivators: SRC-1, AIB-1 and GRIP, have been described (38). Interestingly, many analogues displaying selective co-activator recruitment also demonstrate an enhanced tissue selectivity for activity. Here, tissue specific effects may arise secondary to preferential VDA VDR/RXR complex binding to VDREs in promoters upstream of target gene, as has been suggested for the IP9 type of VDRE (37, 39).

X-ray crystallography studies of VDAs complexed with the VDR yielded mechanistic information useful for ligand design. Orientation within the LBP influences agonist activity, and compounds with 20-epi side chain modifications sit inside this pocket to promote coactivator binding, mediating transcription at 100-fold lower concentration than calcitriol (40). Various additional side chain modifications have also generated ligands with superagonist activity (34). Removal of C19 from within the A-ring resulted in 19-nor vitamin D compounds (including paricalcitol) with increased pro-differentiation and anti-proliferative activity on cancer cells and decreased calcemic activity.

Finally, C/D ring modified derivatives such as the 14-epi-analogs of calcitriol, including TX527 and TX522 (inecalcitol) also show markedly increased anti-proliferative effects in *in vitro* assays and lower calcemic effects compared with calcitriol. Increased activity was associated with a tighter association of these VDAs with coactivators SRC-1 and DRIP205, where a 10-fold lower dose of inecalcitol was required for VDR-coactivator interaction compared to calcitriol (40).

On the other hand, vitamin D signaling may also be enhanced by decreasing interaction of the VDR with corepressors, resulting in increased transcription. However, to date, VDAs inhibiting corepressor interactions have not been described (41).

Several VDAs with enhanced efficacy have been described. Structures containing fluorinated side chains are resistant to degradation. These compounds, including CD578, mediate increased VDR-coactivator binding and stronger pro-differentiation activity compared to vitamin D *in vitro*. Fluorination stabilizes H12 resulting in enhanced binding to SRC-1, for VDR/CD578 compared to VDR/calcitriol (42).

More recently, Corcoran et al. (43) also describe double-point modified VDAs, derived from calcitriol with superagonist activity. Compared to calcitriol, these compounds are less calcemic with lower toxicity (resulting in less weight loss in experimental animals) and mediate more than ten-fold increased pro-differentiation effects in keratinocyte (HaCat) and acute myeloid leukemia (HL60) cell lines.

Finally, a group in China reported the synthesis of novel VDR ligands with non-secosteroidal structures based upon a phenyl pyrrolyl pentane backbone. Preclinical studies suggest some of these (including compound I5) may be useful for treatment of patients with pancreatic cancer [(44); discussed below].

VDAs have also been identified using high throughput screening of chemical compound libraries and in silico screening methods (45, 46) assayed a set of 21 potential VDAs previously identified by high throughput screening of a 10K chemical compound library (the Tox21 qHTS data set).

Interestingly, they found a wide range of structurally diverse chemicals displayed VDA activity. Most of these compounds induced VDR signaling *via* effects upon heterodimerization with RXRα and coactivator and corepressor recruitment (46).

PATHOLOGY OF VITAMIN D

Traditionally recognized for its role in childhood rickets and adult osteomalacia, there is a growing recognition that vitamin D deficiency may confer increased risk in multiple additional conditions, ranging from COVID-19 to cancer (20, 47, 48). Several extra-skeletal roles for vitamin D are recognized, but this has resulted in only a small number of therapeutic options using vitamin D agonists. Patients with chronic renal failure and secondary hyperparathyroidism have been treated with paricalcitol (49) and plaque psoriasis with topical calcipotriol (50). Epidemiological data and preclinical studies suggest that vitamin D deficiency may play a role in multiple common diseases such as cancer, coronary artery disease, fibrosis and infectious and autoimmune diseases (12, 20). Hence there is much current interest regarding possible beneficial effects of vitamin D supplementation. In addition, a new idea is emerging that VDAs may offer new therapeutic avenues for several common diseases.

Autoimmunity

Consistent with its role in inhibition of the acquired immune response, vitamin D seems likely to decrease risk of autoimmune diseases (including diabetes mellitus type 1 (51), multiple sclerosis (MS) and systemic lupus erythematosus) and immune mediated diseases such as inflammatory bowel disease (IBD). Epidemiological and preclinical data support a role for vitamin D in MS. Interestingly, calcitriol reduced demyelination in experimental autoimmune encephalomyelitis (EAM) a mouse model of MS. This was associated with increased Treg activity, activation of Th2 and suppression of Th1 cells (52). However, a therapeutic role in IBD and autoimmune diseases including MS has been limited due to hypercalcemia of vitamin D at doses required for treatment (20, 53).

Musculoskeletal Conditions

Vitamin D protects against fracture risk through several mechanisms, including effects upon bone, muscle strength and immunoregulation. Multiple studies find a positive association between serum calcidiol and bone mineral density. Moreover, vitamin D directly affects muscle function to decrease risk of falls. Finally, osteoporosis appears to be initiated by pro-inflammatory cytokines, driving increased bone metabolism and vitamin D may down-regulate inflammation through effects upon the immune system thereby decreasing risk of fracture (54, 55).

Numerous epidemiological studies have analyzed serum calcidiol and risk of osteoporotic fractures. The data is conflicting with some studies showing support for a protective effect (56, 57). Consistent with this idea, several interventional trials of vitamin D indicate a reduced risk of fracture (54). Furthermore, a meta-analysis of supplementation (vitamin D plus calcium) versus fracture risk, found a significant reduction in total fractures and concluded vitamin D to be a useful preventative intervention for fracture risk reduction (58).

Finally, a recent meta-analysis of 41,738 patients studied the correlation between serum calcidiol and risk of senile osteoporotic fractures. High serum levels were associated with a reduced risk of hip fractures in elderly patients, but not with reduction in total fracture risk. The authors suggested that disparate results seen in earlier studies may have arisen due to selection of fracture sites studied and analysis of both perimenopausal and senile osteoporotic fractures (59).

Fibrosis

Pathological fibrosis occurs in multiple diseases. Following repetitive injury there is a replacement of parenchyma by scar like tissue, resembling an unhealed wound. Occurring in tissues such as the liver (associated with alcohol and chronic viral infections) and kidney, fibrosis is driven by release of TGF-β from macrophages or damaged parenchymal cells, together with growth factors (such as CTGF and PDGF). These mediators activate signal transduction pathways in stromal cells and increase production of the extracellular matrix (ECM). TGF-β signaling mediates phosphorylation of SMAD2 and SMAD3. Then a SMAD2/3/4 complex translocates into the nucleus, binds to SMAD-binding elements and drives expression of pro-fibrotic genes (60).

The VDR directly interacts with SMAD3 and inhibits TGF-β-SMAD signal transduction, an effect that is independent of VDR-mediated transcription (60, 61) found calcitriol inhibited TGF-β upregulation of pro-fibrotic genes in mouse kidney epithelial cells, and reduced plasminogen activator inhibitor–1 and α-SMA expression. These same authors went on to synthesize two VDAs that inhibited TGF-β without activation of classical VDR gene expression. They found 1,25-lactone and two synthetic derivatives of 1,25-lactone (DLAMs) inhibited pro-fibrotic signals without hypercalcemia. X-ray crystallography found 1,25-lactone and DLAMs interaction with the H12 helix differed from calcitriol, providing a mechanistic explanation for their properties and a template for further attempts to design therapeutically useful new VDAs. The authors suggested that selective VDAs may prove useful for anti-fibrosis treatments (60, 61).

Work from Evans and colleagues found that quiescent hepatic stellate cells (HSC) rapidly expanded following tissue injury, resulting in fibrosis in murine liver. This response was inhibited by VDA calcipotriol (62). Interestingly, vitamin D receptor knock out (VDRKO) mice developed spontaneous liver fibrosis. Moreover, activation of VDR signaling inhibited TGFβ-SMAD-dependent transcription of pro-fibrotic genes in HSCs. The authors suggested VDR ligands might be a potential therapy in liver fibrosis (62). A similar effect was seen for pancreatic stellate cells (PSCs) with calcipotriol repression of chronic pancreatitis in a mouse model (63). Furthermore, a number of other groups have found VDA inhibition of fibrosis in additional tissues using animal models in heart, kidney and skin [reviewed in (13) and references within].

Pathological fibrosis occurs in multiple diseases including renal disease, colon and pancreatic cancer (associated with cancer associated fibroblasts (CAF) and in the lungs of patients with COVID-19 that develop ARDS (discussed below). Vitamin D inhibition of the TGF-β-SMAD signaling pathway may offer a useful therapeutic approach in such patients.

Infectious Disease

It has been suggested that low vitamin D status confers an increased risk of viral respiratory infections, including influenza (20). Some preclinical studies support this idea. Incubation of primary human bronchial epithelial cells with calcitriol *in vitro* increased secretion of pro-inflammatory cytokines CXCL8 and CXCL10. Such cytokines might be expected to recruit macrophages and play a role in antiviral responses (64). A protective role for vitamin D supplementation in patients with respiratory infections still remains controversial. A meta-analysis found reduced risk of acute upper and lower respiratory tract infections after supplementation (65). A recent randomly controlled clinical trial found a positive effect for vitamin D in patients with influenza (66). However, some other studies found no significant effect, and in one clinical trial the duration of symptoms was increased compared to placebo controls [(67); reviewed in (68)]. Vitamin D inhibited lung fibrosis in several mouse models and may have therapeutic potential in idiopathic pulmonary fibrosis (2). Much current interest concerns the putative protective role of vitamin D in pathogenesis of COVID-19.

COVID-19

COVID-19 (coronavirus disease 2019) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The virus is closely related to several bat coronaviruses and it seems likely COVID-19 began as a zoonotic disease, with subsequent development of transmission between humans. Following initial isolation of SARS-CoV-2 in Wuhan (China), COVID-19 has now become pandemic (69). Globally, on the 30th October, 2020, there were 44,592,789 confirmed cases of COVID-19, including 1,175,553 deaths (The world health organization (WHO) interactive Dashboard COVID-19 web site. Available from: www.WHO.INT, 2020); meanwhile, interactive web-based methods continue to track the progress of COVID-19 (70).

The SARS-CoV-2 receptor is angiotensin-converting enzyme 2 (ACE2), mediating viral entry together with TMPRSS2 protease activity (71). Alveolar type II epithelial cells and enterocytes are primary targets for infection, and damage to heart, lung, liver and kidney (organs expressing ACE2) is largely responsible for mortality in patients with COVID-19 (72). The severity of COVID-19 corresponds to the degree of host immune response against the virus. Infection results in effects ranging from: asymptomatic to mild respiratory symptoms (most commonly), severe lung injury (viral pneumonia and ARDS), followed by septic shock, and multiple organ failure. Among patients presenting with COVID-19 in Wuhan (China), ARDS occurred in 42% of those with severe pneumonia, and in 61–81% of cases admitted into intensive care (73, 74); a separate Chinese study reported deaths of around 65% of patients with ARDS (75).

Evidence for a Protective Role for Vitamin D in COVID-19

Evidence from multiple sources is accumulating to suggest a protective role for vitamin D against COVID-19 (48). Indirect evidence suggests populations predicted to be vitamin D insufficient/deficient have higher rates of infection and severity of COVID-19. This includes individuals with diabetes, hypertension and obesity, all associated with low vitamin D status and increased COVID-19 mortality (76). Serum calcidiol concentration is dependent upon solar irradiation. Hence mortality is increased in people of color, with more melanized skin and proportionally reduced rates of vitamin D synthesis. This includes BAME individuals in the UK and African Americans in the USA (9, 10). Consistent with this, a small study of 392 health workers in Birmingham, (UK), found increased rates of seroconversion in patients with BAME ethnicity was associated with deficiency (< 30 nmol/l) of vitamin D (77). Nevertheless, other possible explanations cannot be excluded, perhaps including differences in socioeconomic factors (78–80).

Also consistent with this idea, latitude has been associated with COVID-19 mortality, likely related to rate of UV mediated cutaneous synthesis of vitamin D (76). Moreover, a comparative study across 20 European nations found a significant negative correlation between mean vitamin D level and COVID-19 related mortality comparing countries (81). However, such ecological approaches have been criticized, since confounding factors (including local screening methods and detection of COVID-19 cases) will make analysis difficult (82).

Studies, linking vitamin D status and outcome in patients with COVID-19 are summarized in **Table 1**. Most studies have been retrospective with small patient numbers. Serum vitamin D was correlated with outcome (biochemical, imaging and clinical, depending upon the study) in patients with COVID-19. Both the time of testing of serum vitamin D (sometimes years before COVID-19 infection; see below) and the cut off for vitamin D insufficiency/deficiency varied between studies.

Most studies found low vitamin D status was associated with increased disease severity and risk of mortality; this was confirmed by a recent meta-analysis (97). On the other hand, some reports have found no evidence for a protective role for vitamin D (48, 86), and a large UK Biobank study (93).

Grant and McDonnell questioned whether the multivariate analysis in the UK Biobank study was over adjusted for confounding variables. Furthermore, they posited that protective effects may not have been seen in the study due to low serum vitamin D in the majority of participants (98). Finally, in this study, vitamin D was assayed 10-14 years prior to the COVID-19 pandemic. Hence it was questioned whether serum levels would remain unchanged and be a useful indicator for

TABLE 1 | Observational studies, linking vitamin D status with outcome (severity of disease and mortality) in patients with COVID-19.

Study	N	Design	Effect	Reference
Patients diagnosed with COVID-19 were investigated for serum calcidiol and CT Thorax	73	Retrospective, observational	Higher VD[a] associated with reduced lung involvement and better outcome. VDD[b] associated with increased risk of mortality.	(83)
Patients ≥65 years, COVID-19 positive. Groups: VDD (≤30 nmol/L) versus VD replete. Assessed for in-hospital mortality, requirement for NIV. Biochemistry and CT Thorax.	105	Retrospective, observational	COVID-19-positive arm had lower serum calcidiol compared with COVID-19-negative arm. Patient with VDD had increased incidence of NIV[c] and high dependency unit admission.	(84)
Serum calcidiol versus positive SARS-CoV-2 result.	107	Retrospective, observational	Serum VD is significantly lower in SARS-CoV-2 positive patients	(85)
Serum calcidiol measured in patients on day of admission and 8 weeks post PCR diagnosis of COVID-19. Results compared to symptoms, CT Thorax, biochemistry.	109	Prospective, observational cohort study	VDD was common, and not an indicator of pathology seen in CT-scans, or severity of symptoms.	(86)
Serum calcidiol in patients hospitalized with COVID-19 versus disease severity	134	Retrospective, observational	VDD is associated with greater disease severity	(87)
Patients diagnosed with COVID-19, investigated for serum calcidiol at first presentation versus severe disease (IMV[d] or death).	185	Retrospective, observational	VDD (≤30 nmol/L) was associated with higher risk of severe disease.	(88)
Serum calcidiol concentration versus clinical outcome and mortality due to SARS-CoV-2 infection. Where VD < 75 nmol/L is insufficient	235	Cross-sectional analysis	Significant association between VD insufficiency and increased mortality.	(89)
Serum calcidiol in COVID-19 positive and negative group	347	Retrospective, observational	No significant difference between groups.	(90)
Serum calcidiol or calcitriol measured in the year prior to COVID-19 testing versus risk of positive test.	489	Retrospective, observational	VDD status was associated with increased COVID-19 risk.	(91)
A previous serum calcidiol level was compared to risk of SARS-CoV-2 infection and severity of disease. Where VD < 75 nmol/L is suboptimal.	7807	Retrospective, observational	Low Serum VD was associated with increased likelihood of COVID-19 infection and hospitalization.	(92)
Serum calcidiol concentration versus SARS-CoV-2 positivity.	190,000	Retrospective, observational	Serum VD concentration is inversely associated with SARS-CoV-2 positivity.	(78)
Baseline serum calcidiol versus COVID-19 mortality.	341,484	Retrospective, observational. UK Biobank study.	No association between VD concentration and risk of severe infection and mortality.	(93)
VDD patients diagnosed with COVID-19 received: standard dose cholecalciferol or high dose ergocalciferol for 5 days	4	Case series	high dose VD supplementation shortened length of stay, lowered oxygen requirement, and reduced inflammatory marker.	(94)
Serum calcidiol tested in controls versus patients diagnosed with COVID-19	145	Case control study	VDD increases risk for COVID-19, most clearly seen in severe infections.	(95)
Frail elderly patients, with COVID-19 infection. Received VD in 3 groups: (1) preceding and (2) post diagnosis or (3) no supplementation.	77	Quasi-experimental	Survival was increased in Group 1 with regular supplementation over the preceding year	(96)

[a]VD, vitamin D; [b]VDD, VD deficiency; [c]NIV, Non-invasive ventilation; [d]IMV, invasive mechanical ventilation.

levels at the time of infection (99, 100). A further caveat is that acute inflammatory diseases (likely including COVID-19) may affect serum calcidiol levels. Hence measurements at time of diagnosis may be less reliable than seasonally adjusted samples (101).

A quasi-experimental study found that survival was increased in frail elderly patients where there had been regular supplementation of vitamin D in the year preceding COVID-19 infection (96). Finally, Entrenas Castillo and colleagues report a small, pilot clinical trial in patients with COVID-19, where high dose oral calcidiol reduced the requirement for admission into intensive care (102).

Supplementation with vitamin D appears to protects against COVID-19. Counterintuitively, vitamin D increases ACE2 and facilitates SARS-CoV-2 entry. However, protective effects may be part explained by vitamin D stimulation of the innate immune system, with possible additional effects upon the renin–angiotensin system (RAS). SARS-CoV-2 binding to ACE2 drives an increase in ACE activity and angiotensin II production, resulting in vasoconstriction, pulmonary edema and increased severity of COVID-19. Calcitriol may play a protective role here *via* induction of ACE2 expression and inhibition of renin activity (103–105). Interactions between RAS and vitamin D in advanced COVID-19 are further discussed [see below and shown in **Figure 1** (function B)].

Taken together, the data strongly suggest vitamin D plays a protective role in patients with COVID infection and may decrease severity of ARDS. However, this hypothesis remains unconfirmed and data from a number of on-going clinical trials of vitamin D supplementation is eagerly awaited in order to better answer this question [**Table 2** and reviewed in (21)].

ACUTE RESPIRATORY DISTRESS SYNDROME

For the purpose of description ARDS may be separated into acute and late phases (106). Vitamin D may be detrimental in

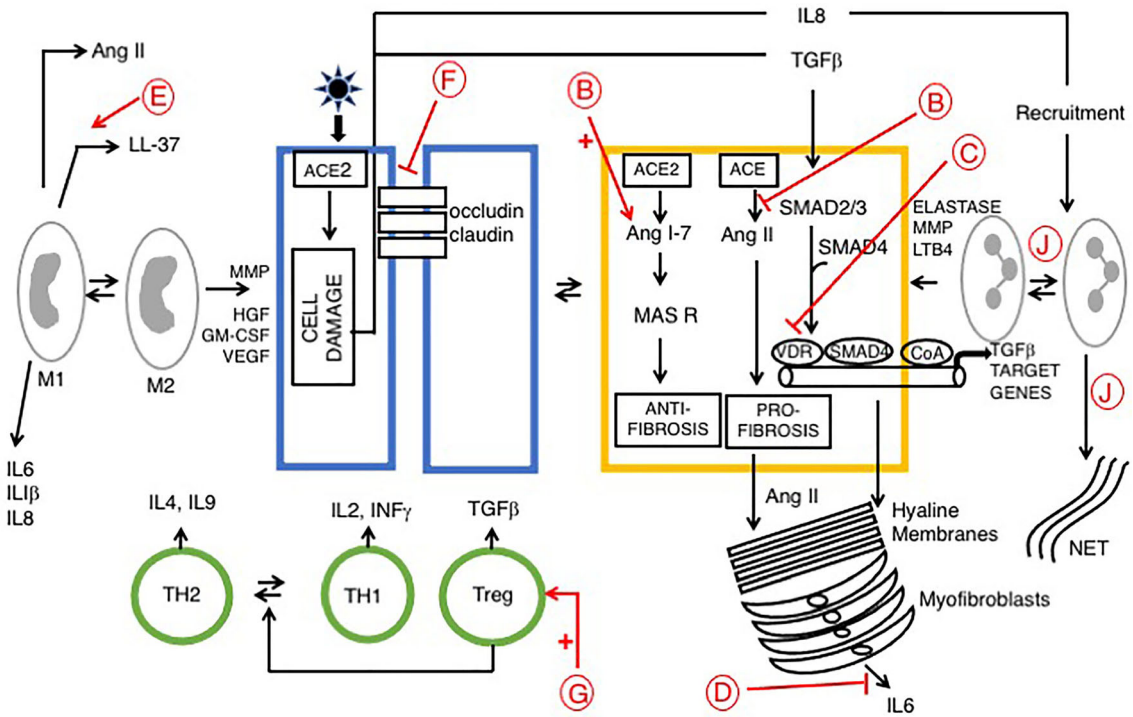

FIGURE 1 | Signaling pathways in late phase ARDS in patients with COVID-19; role of vitamin D Disease progression is influenced by stromal/inflammatory infiltrate. Epithelial cells are colored blue, lung stellate cells (yellow), lymphocytes (green), and myeloid cells: macrophages M1, M2 and neutrophils (grey). SARS-CoV-2 infected alveolar type II epithelial (ATE2) cells release IL8, recruiting inflammatory cells, with neutrophil secretion of IL-6 contributing to the cytokine storm. Lung fibrosis occurs *via* two pathways. Macrophages and lung stellate cells secrete angiotensin II, promoting myofibroblast formation and increasing ECM formation with hyaline membrane disease. ATE2 cells secrete TGF-β, which drives the myofibroblast phenotype. Predicted effects of vitamin D are labeled in red A–J as described in **Table 6**, where labels indicate: B: Increased ACE/Ang II/AT1R signaling; C: Down-regulation of TGF-β-SMAD-dependent transcription; D: Inhibition of pro-inflammatory chemokine release; E: Increased antimicrobial peptides, defensins and cathelicidin; F: Maintains epithelial cell tight-junction integrity; G: Increased Treg activity; J: Decreased Neutrophil activity and increased NET formation. VDA effects D and E are likely more relevant in early stage COVID-19 respiratory tract infections.

early COVID-19 due to attenuation of the antibody response. Conversely, it may be beneficial in patients with advanced COVID-19. Attenuation of the immune response likely prevents the cytokine storm and may decrease the severity of ARDS (13, 14). Here we consider predicted protective roles for vitamin D within each stage.

Acute Phase

SARS-CoV-2 grows rapidly and damages lung tissue. During the first few days there is an influx of neutrophils and macrophages into the alveolar space resulting in alveolitis and loss of an intact alveolar epithelial barrier. This leads to intra alveolar edema and hyaline membranes are deposited onto basement membranes resulting in diffuse alveolar damage (DAD) characteristic of ARDS (107). Here, vitamin D delays pathology by decreasing neutrophil activity and maintains integrity of airway epithelial cell tight-junctions by up-regulation of occludin and claudin-5 in tight junctions. The inflammatory cell infiltrate produces proinflammatory cytokines and chemokines, constituting a cytokine storm that drives development of ARDS. Vitamin D protects against development of ARDS by inhibiting release of cytokines (including IL-6, IFN-γ, IL-1β) and chemokines such as CXCL8 and CXCL10 (21, 106). On the other hand, vitamin D increases NET formation and this has been suggested to contribute to severity of pulmonary inflammation (108).

Late Phase

The late phase of ARDS (7 to 10 days after initial injury) is dominated by a fibro-proliferative process that fills the alveoli with granulation tissue, with increased ECM production and proliferation of myofibroblasts and type II alveolar cells. This is followed by irreversible pulmonary fibrosis (**Figure 1**). The renin-angiotensin system (RAS) plays a role in development of ARDS. Two signaling pathways downstream of ACE and ACE2 play competing roles. The ACE2/Ang 1-7/MasR axis signals are anti-inflammatory and anti-fibrotic, whereas the ACE/Ang II/AT1R axis drives inflammation, fibrosis and vasoconstriction. ARDS pathology is driven by the second pathway, *via* unregulated Ang II, largely derived from lung fibroblasts and activated macrophages. Ang II then stimulates activation of lung myofibroblasts (109, 110). Vitamin D is protective, it reduces Ang II production and increases ACE2/Ang 1-7/MasR signaling, which may prevent the cytokine storm and inhibit development

of ARDS (21). Evidence suggests that vitamin D deficiency may aggravate ARDS (111).

As discussed previously, VDAs inhibit TGFβ-SMAD-dependent transcription of pro-fibrotic and pro-inflammatory genes by transcriptional interference with Smad2/3 in the liver [(62); see above] and pancreas (63, 112) (see below). This same mechanism likely also operates in pulmonary disease, particularly given the recent identification of lung stellate cells, suggesting that anti-fibrotic agents such as paricalcitol may be beneficial in patients with ARDS (13).

Current clinical trials of vitamin D in patients with COVID-19 are summarized in **Table 2**. Most trials utilize oral vitamin D. Doses of vitamin D range from a single dose of 25,000 IU (NCT04334005) to 50,000 IU of vitamin D, once weekly for 2 weeks (NCT04363840). Liu et al. (113) suggested that a single large dose of vitamin D (300,000 IU) may be used for treatment of COVID-19. Finally, Evans and Lippman suggest that paricalcitol might be an effective treatment for patients with COVID-19 (13).

CANCER

Vitamin D influences carcinogenesis *via* several mechanisms, including: (a) promotion of differentiation and inhibition of the epithelial mesenchymal transition, (b) regulation of cancer stem cells, (c) reprogramming of gene expression and induction of quiescence in cancer associated fibroblasts (CAFs), and (d) modulation of immune response (63, 114, 115). Observational studies suggest low vitamin D status is associated with an increased risk of cancer incidence and mortality (12, 116). Consistent with this idea, earlier studies found a significant inverse correlation between UVB irradiation and mortality rates for several cancers (117, 118).

Cancer and Vitamin D Supplementation Studies

A large vitamin D intervention, random control clinical trial (VITAL) did not find a significant reduction in the primary end point of cancer mortality (119). Subsequent analysis found evidence for a protective effect; a second analysis of the data suggested decreased cancer mortality in some subgroups of enrolled individuals receiving vitamin D (18, 120). Very recently, a further analysis of the VITAL data suggests that vitamin D reduces the risk of metastatic or fatal cancer (121). Apparently consistent with this idea, a meta-analysis of 10 clinical trials (81362 pooled participants) found vitamin D supplementation was associated with a significantly lower risk of cancer mortality (47). Finally, a meta-analyses of clinical trials, testing high dose vitamin D supplementation (6537 participants), found a significant decrease in cancer mortality, although, there was no reduction in cancer incidence (122). Interestingly, several large scale studies, collectively indicate a greater effect upon progression rather than protection against carcinogenesis, consistent with previous reports (123).

Studies focusing on specific malignancies may provide a more homogenous data set for analysis. Here we describe vitamin D

TABLE 2 | Representative interventional clinical trials investigating Vitamin D in patients with COVID-19.

Study identifier:	Study design	[a]Dosage/Regimen/Route	N	Participants	Status
NCT04483635	Phase 3 Placebo-Controlled	[b]VD oral loading dose of 100,000 IU + 10000 IU VD weekly. Endpoint incidence of COVID-19 infection.	2414	[c]HCW caring for patients with COVID-19	Not yet recruiting
NCT04535791	Phase 3 Placebo-Controlled	VD, 4,000 IU orally daily for 30 days. Endpoint: COVID-19 infection status.	400	HCW caring for patients with COVID-19	Recruiting
NCT04536298	Phase 3 Placebo-Controlled	Daily VD for 4 weeks. Endpoints: hospitalization and/or death, risk of infection in household member.	2700	Newly diagnosed with COVID-19.	Not yet recruiting
NCT04386850	Phase 2/ Phase 3 Placebo-Controlled	Calcidiol, 25 mcg once daily for 2 months. Endpoints: ARM 1 incidence of infection, severity of disease, hospitalization and death. ARM 2 severity and death.	1500	ARM 1 Prevention in HCWs. and ARM 2 Treatment of COVID-19 infected patients.	Recruiting
NCT04334005	Not Applicable	VD, 25000 UI, orally, daily. For 10 weeks. Endpoints: requirement for [d]IAV, [e]NIV and [f]ICU admission.	200	Non-severe symptomatic, patients infected with COVID-19. Excludes patients presenting with ARDS.	Not yet recruiting
NCT04363840	Phase 2	VD, 50,000 IU, orally once weekly for 14 days + Aspirin 81mg, each day. Endpoint: Hospitalization.	1080	[g]VDD patients with new (24h) COVID-19 infection.	Not yet recruiting
NCT04385940	Phase 3	VD, 50,000 IU, orally, Determine low VD (<50 nmol/L), Endpoint: disease severity, Hospitalization.	64	VDD in inpatients/outpatients with COVID-19 infection.	Not yet recruiting
NCT04525820	Not Applicable Placebo-Controlled	Single high dose VD (140,000 IU) plus 800 IU of VD per day versus 800 IU of VD per day. Endpoint: Length of hospitalization until discharge or fatality	80	Hospitalized Patient Ongoing COVID-19 VDD	Not yet recruiting

[a]Dosage/Regimen in intervention group; [b]VD, vitamin D; [c]Health care workers (HCW); [d]Invasive assisted ventilation (IAV); [e]Non-invasive assisted ventilation (NAV); [f]Intensive care unit (ICU). [g]VDD, VD deficiency.

supplementation in preventative studies (breast and colon cancers and melanoma) and clinical studies in patients with advanced colon and pancreatic cancers.

A meta-analysis of vitamin D supplementation in breast cancer (72,275 participants) found relative risk reduction was below 30%, which lays within the futility boundary for the trial (124). On the other hand, two additional studies provide evidence suggesting vitamin D supplementation in healthy individuals inhibits carcinogenesis and mediates a reduction in breast cancer incidence (125, 126).

Epidemiological studies and clinical trials have investigated the role of serum calcidiol and vitamin D supplementation on the risk of colorectal cancer. Multiple prospective studies suggest vitamin D deficiency is a risk factor for colorectal cancer (127). On the other hand, vitamin D supplementation studies have yielded mixed results. No reduction of risk was observed in the Women's Health Initiative, a trial in which 18,176 women received vitamin D and 18,106 received a placebo for an average of 7 years (128). However, the design of this study has been questioned regarding the low dose of vitamin D (400 IU/day) used for supplementation (129).

The role of vitamin D in melanoma is complex. A recent meta-analysis described a positive correlation between circulating calcidiol and risk of melanoma. Here ultraviolet B irradiation is a major risk factor and analysis of the relationship between serum calcidiol and melanoma is confounded by sun exposure. Importantly, no increased risk of melanoma occurred with vitamin D supplementation (130). Interestingly, several recent preclinical studies have suggested 7-dehydrocholesterol (a precursor of vitamin D) exerts anti-tumor activity in melanoma (131).

Extensive preclinical data from cell lines grown *in vitro* supports vitamin D anti-proliferative and pro-differentiation activity in multiple cancers, including prostate cancer and melanoma (132, 133); these effects occurred at supraphysiological concentrations of calcitriol (see below) (28).

Studies following the effect of vitamin D supplementation have also been conducted in patients with cancer: during surveillance of indolent prostate cancer and adjuvant treatment of colon and HER2+ breast cancer (134–137).

Interestingly, in the prostate study a second biopsy (performed after one year of vitamin D supplementation) found a decrease in number of positive cores or Gleason score in 55% of subjects; however, there was no significant change in PSA (134).

A small number of studies have evaluated vitamin D supplementation in patients with advanced cancer. The SUNSHINE, phase II clinical trial enrolled 139, untreated patients with colorectal cancer. Two groups received vitamin D (either 400 or 4,000 IU per day) in combination with best standard of care chemotherapy. This resulted in a median progression free survival of 13 months in the high-dose group, versus 11 months in the low-dose group (138). More definitive data are expected from on-going clinical trials.

Finally, vitamin D levels were measured in 1267 patients with PDAC. Pre-treatment serum, and OS were compared for patients with sufficient (>50 nmol/L), versus deficient (<25 nmol/L) levels. Survival was increased in patients with sufficient versus deficient serums in early stage PDAC, but not in patients with advanced stage PDAC (139). This interesting observational study requires further clinical trials to determine whether vitamin D supplementation might improve survival in patients with pancreatic cancer (see below).

The analysis of clinical studies of nutrients like vitamin D is complex and may require a different approach, compared to methods commonly used to follow pharmacological responses. Heaney focuses upon the importance of choosing a plausible dose range centered around where inadequate to adequate status occurs and suggests general guidelines for nutrient studies (140).

More recently, Boucher further explores Heaney's ideas and additional factors complicating analysis of vitamin D supplementation studies (141); further confounding issues are also discussed elsewhere (18, 120, 121).

HYPERCALCEMIC EFFECT OF VDR LIGANDS

Calcitriol has anti-tumor effects in animal models of various cancer types, and further, promotes the antitumor activities of various chemotherapeutic agents including 5FU, Gemcitabine and Paclitaxel (**Table 3**). However, this has not always been translated into clinical trials, and is thought to be largely due to hypercalcemic properties of calcitriol, limiting doses that can be delivered (154), but may also partly reflect deficiencies in trial design (155).

Physiological levels of calcitriol in humans, range between 0.05–0.16nM. In animal models, anti-tumor activity of vitamin D requires supraphysiologic levels (156), and the therapeutic mechanism here is distinct from a simple correction of vitamin D deficiency. Supraphysiologic levels of calcitriol result in a limiting toxicity of hypercalcemia. This reduces dosage that can be administered to patients and is one possible explanation for the less marked anti-tumor effects seen in clinical trials (**Table 3**), as compared to the preclinical data (**Table 4**) (166).

Administration of calcitriol orally (1.5-2.5mcg/day or 10.5-17.5mcg/week) was associated with a rate of hypercalcemia of 20-30% in prostate cancer patients (156, 167). VDSAs such as inecalcitol, which display up to 100-fold less hypercalcemic activity, have been developed in an attempt to circumvent this problem.

Paricalcitol demonstrates preclinical anti-tumor effects in various cancer types and clear anti-fibrotic activity. Reiter and colleagues found paricalcitol (but not calcitriol) inhibited fibrosis *in vivo* in the mouse CCl_4 model, a finding likely of particular significance in PDAC (168).

Pancreatic Ductal Adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) has one of the poorest prognoses of all solid tumors. This likely reflects multiple factors, including desmoplasia. Desmoplasia is an increase in stromal cell proliferation of alpha smooth muscle (α-SMA) positive fibroblasts

TABLE 3 | Preclinical studies of VDA (Vitamin D Analogues) in animal models of various cancer types.

Tumor	Delivery/Dose	VD agonist	Effect	Reference
Prostate Ca, Dunning rat model.	s.c., 1mcg, 3x/week x3 weeks.	Calcitriol	Inhibition of tumor growth	(142)
Metastatic lung disease	s.c., continuous osmotic minipump rate 1 µg/kg/24 h x18 days 2.5 µl/h.	Calcitriol	Prevents met lung disease	(143)
Ovarian cancer, xenograft	gavage v placebo, 0.3 or 1.0 µg/kg body weight in a volume of 20µL, OD	Calcitriol	Suppression of growth	(144)
Breast cancer, nitrosomethylurea-induced rat mammary tumor model.	0.25 and 1.25 mcg/kg	Calcitriol	Inhibition of tumor growth, Hypercalcemia	(145)
Bladder cancer, xenograft	s.c., µg/mouse/d, x3 days GEM (6 mg/mouse/d, cisplatin (0.12 mg/mouse/d).	Calcitriol	Enhances activity of GEM and cisplatin	(146)
Pancreatic cancer xenograft	i.p., 2.5 and 5 mcg kg3×/week x28days	Calcitriol	Inhibition of tumor growth	(147)
Squamous cell Ca Xenograft	i.p., 80/160/320µg/mouse/day	Inecalcitol	Inhibition of tumor growth, increased apoptosis, decreased proliferation	(148)
Prostate Ca, xenograft	i.p.,1300µg/kg3×/week x42days	Inecalcitol	50% decrease tumor weight	(149)
Prostate Ca, xenograft	i.p.,0.5µg/kg every other day x45days	Seocalcitol	Reversal of growth stimulatory effects of PTHrP	(150)
Pancreatic cancer xenograft	s.c., 2.5µg/kg3×/week	Paricalcitol	Inhibition of tumor growth	(151)
Pancreatic cancer xenograft	i.p., 0.3µg/kg2×/week, x3weeks	MART-10	Inhibition of tumor growth	(152)
Breast cancer, nitrosomethylurea-induced rat mammary tumor model.	i.p.	Calcipotriol	Inhibition of tumor growth, No Hypercalcemia	(145)
UV-induced non-melanoma skin cancer	Topical application	Calcipotriol	Decrease in number and area of tumors combined with diclofenac	(153)
Pancreatic cancer, orthotopic model	i.p., 60 mg/kg QDX20, +/- GEM.	Calcipotriol	Induced stromal remodeling, increased intratumoral GEM, reduced tumor volume, increased survival.	(63)

TABLE 4 | Completed Clinical trials of VDAs in various cancer types.

Tumor, Sample size	Delivery/Dose	Drug	Outcome	Reference
Prostate cancer n = 37	Rocaltrol (0.5mcg/kg) on day 1 + docetaxel (36 mg/m(2)) on day 2, repeated weekly for 6 weeks.	Calcitriol	30/37 (81%) achieved PSA response. 22 had 75% reduction in PSA.	(156)
Prostate cancer n = 250	DN-101, PO formulation, Weekly docetaxel 36 mg/m^2 iv for 3 weeks of a 4-week cycle combined with either 45mcg DN-101 v placebo PO 1 day before docetaxel.	Calcitriol	Overall, PSA response rates were 63% (DN-101) and 52% (placebo), P = 0.07.	(157)
Prostate cancer n = 34	PO Dexamethasone, 1mg OD + 0.5mcg calcitriol at the start of week 5. Carboplatin (Area Under the Curve (AUC) = 2) started Week 7	Calcitriol	PSA response in 13 of 34 patients and had an acceptable side-effect profile	(158)
Prostate cancer n = 30	calcitriol 0.5 µg/kg PO, 4 divided doses over 4 h on day 1 + docetaxel 36 mg/m(2) i.v. on day 2 of each treatment week and zoledronic acid 4 mg i.v. on day 2 on the 1st and 5th week.	Calcitriol	PSA response in 47.8%	(159)
Metastatic NSCLC Phase I/II n = 34	Escalating doses: 30, 45, 60, and 80 mcg/m(2), calcitriol iv q21, prior to docetaxel 75 mg/m(2) and cisplatin 75 mg/m(2)	Calcitriol	Pre-specified endpoint 50% RR was not met	(160)
Pancreatic Cancer, non resectable n = 25	Calcitriol 0.5 u/kg on day 1, docetaxel 36 mg/m(2) IV on day 2, administered weekly for three consecutive weeks, and 1 week without treatment.	Calcitriol	Modest increase in TTP, 3/25 PR, 7/25 stable disease. Median TTP 15 weeks, and median OS 24 weeks.	(161)
Hepatocellular carcinoma n = 33	PO, 10µg/day, up to 1 year	Seocalcitol	2 complete response (CR), 12 stable disease (SD), 19 progressive disease (PD).	(162)
Pancreatic cancer n = 36	PO, 10–15µg/day x 8 week	Seocalcitol	No OR	(163)
Prostate cancer n = 54	PO, MTD 4mg/d + Docetaxel, max 18/52	Inecalcitol	85% response rate. As per PSA decline of 30%	(164)
Cutaneous metastatic breast cancer n = 19	Topical100µg/d, 6weeks	Calcipotriol	3 patients, 50% reduction in diameter of treated lesions	(165)
Metastatic Breast cancer n = 24	PO, 4–7 µg/day for 8 weeks + taxane	Paricalcitol	Most women tolerated 2–3mcg/d, (up to 7 µg per day without hypercalcemia	(151)

with increased extracellular matrix formation. Desmoplastic stroma restricts vasculature, impeding delivery of chemotherapy and inhibiting an immune response. In addition, reciprocal signaling pathways occur between PDAC and PSCs and most studies find activated PSCs facilitate PDAC growth (169, 170). Much interest surrounds targeting PSCs and HSCs in hepatic metastases from PDAC, the cells responsible for desmoplasia (171). Cancers arising within the pancreatic head compress the biliary outlet, decreasing absorption of fat soluble vitamins. The VDR is expressed by PSCs and PDAC cells. VDAs reduce PSC activation and are potential stromal targeting agents; there are also putative therapeutic effects upon malignant cells (172). Consistent with this, a genome-wide synthetic lethal screen identified VDR as one out of 27 validated genes that sensitized pancreatic cancer cells line (Panc1) to gemcitabine (173).

The VDR is expressed in PSCs, HSCs, and in cancer associated fibroblasts (CAFs) derived from these cells. VDR activation suppresses pro-fibrotic and pro-tumorigenic properties of cancer associated fibroblasts (CAFs). Moreover, spontaneous pancreatic fibrosis occurs in VDR knockout mice, consistent with a role for VDR inhibition of PSC activation (62). Importantly, calcitriol, the naturally occurring product of vitamin D metabolism and synthetic VDAs, inhibit tumor growth in xenografts of pancreatic cancer (63, 151). In addition, in preclinical studies, calcitriol potentiated cytotoxic activity of gemcitabine in human pancreatic cancer cell line (Capan-1) xenografts, with promotion of caspase dependent apoptosis (**Table 3**). Taken together, the data suggest that calcitriol exerts anti-fibrotic effects upon CAFs in pancreatic cancers. However, results from clinical trials with calcitriol have not generally demonstrated the activity expected from preclinical studies, in part because of dose limiting hypercalcemia (161).

A combination of Calcitriol and Docetaxel in PDAC resulted in a modest increase in time to progression, with 3/25 partial responses (PR) and 7/25 patients with stable disease (SD), (**Table 4**) (161). Recently, several new VDAs have emerged and clinical trial data suggests they may have anti-tumor effects in some specific cancer types.

VDSAs such as calcipotriol and inecalcitol (which display up to 100-200 fold less hypercalcemic activity) have been developed to circumvent the problem of dose limiting hypercalcemia (164, 174). Calcipotriol induced morphological changes in PSCs, with lipid droplet formation and decreased α-SMA expression. Calcipotriol mediated genome-wide changes in specific gene expression (with 664 increases and 1616 decreases), including down-regulation of IL-6, Stromal derived factor 1 (SDF-1) and collagen. Taken together, the data suggest that VDAs promote PSC differentiation and quiescence. In an orthotopic mouse model of PDAC, calcipotriol reduced desmoplasia and enhanced anti-tumor efficacy of gemcitabine resulting in a 58% increase in survival compared to gemcitabine alone (63). This important study became the basis for several clinical trials testing VDAs for anti-stromal activity in patients with PDAC (13).

A very recent study supports the data published by Evans's group in 2014. Using a novel VDSA they replicate some of the original findings in an animal model of PDAC. Kang and colleagues synthesized 57 new non-secosteroidal VDR ligands (see above). Three of these compounds inhibited activation of PSCs, and in combination with gemcitabine, one of these (compound I5) demonstrated anti-tumor activity. In animal studies I5 activity displayed similar activity to calcipotriol with minor calcemic effects (44).

We previously planned a study testing inecalcitol (an orally bioavailable VDSA) in combination with standard of care gemcitabine/Nab-paclitaxel chemotherapy in patients with advanced PDAC. Unfortunately, inecalcitol became unavailable shortly prior to opening our Phase II clinical trial when Hybrigenics' discontinued production in 2018. Most studies of VDAs in PDAC have utilized paricalcitol, which is currently the subject of several on-going clinical trials (**Table 5**), including our own recently opened Phase II study in patients with metastatic PDAC, (*ClinicalTrials.gov* identifier: NCT04617067).

TABLE 5 | Completed and ongoing clinical trials involving Paricalcitol in patients with PDAC.

Study identifier:	Study design	Dosage/Regimen/Route	N	Patients	Status
NCT02030860	Phase I.	Paricalcitol (25mcg, IV), 3x weekly, with Nab-Pac and GEM D1, 8, 15, q 28 days.	15	Neoadjuvant and post-operatively.	Completed
NCT03520790	Phase I. Run-in safety study and Phase II Formulations (IV or Oral).	Paricalcitol (25mcg, IV), 3x per week, or PO OD, with Nab-Pac and GEM D1, 8, 15, q 28 days.	112	Metastatic	Recruiting
NCT03883919	Phase I.	Paricalcitol, 75 mcg IV on D1 and D8 combined with liposomal Irinotecan and 5-FU/LV.	20	Advanced PDAC, Progressed on GEM.	Recruiting
NCT03519308	Phase I.	Paricalcitol, and Nivolumab with Nab-Pac and GEM D1, 8, 15, q 28 days.	20	Resectable PDAC	Recruiting
NCT04054362	Phase II. PINBALL	Nab-Pac and GEM plus or minus cisplatin, followed by Paricalcitol, 25mcg PO (days 1, 3, 5, 8, 10, 12, 15 in a 28 day cycle)	14	Metastatic	Recruiting
NCT03331562	Phase II.	Pembrolizumab with paricalcitol (25mcg IV 3 xs per week) versus placebo.	24	Advanced PDAC	Recruiting
NCT03415854	Phase II.	Nab-Pac, GEM and Cisplatin. Plus paricalcitol Upon Disease Progression.	14	Metastatic	Recruiting
NCT03138720	Phase II	Paricalcitol with Nab Pac, GEM and cisplatin,	24	Neoadjuvant	Recruiting
NCT03300921	Phase Ib Pharmacodynamic Study.	Arm A: 50mcg IV weekly; Arm B: 12mcg PO once daily	20	Neoadjuvant	Recruiting
NCT02930902	Phase Ib	Arm A: Paricalcitol IV over D1, 8, and 15 and pembrolizumab IV d1, or Arm B: above, plus GEM and Nab-paclitaxel IV D1, 8, and 15.	23	Neoadjuvant	Recruiting

Taken together, the data suggest that VDAs are likely to be useful stromal targeting agents in PDAC but this issue is complicated by stromal heterogeneity (175). Two subtypes of PSCs have been described. Inflammatory CAFs (iCAF) are α-SMA low/IL-6 high and express chemokines (CXC11 and CXC12), whereas myofibroblast CAFs (MyCAF) are contractile, stroma remodeling cells, with α-SMA high/IL-6 low. Reciprocal signaling pathways between PDACs and stromal cells (myoCAFs and iCAFs) are described in **Figures 2** and **3** respectively. Within unmanipulated surgical specimens iCAFs are located distantly from PDAC cells, whereas myCAFs were closely juxtaposed (181). Inhibition of iCAFs reduced PDAC volume in animal studies, *via* the IL1-α pathway (IL-1R, JAK/STAT, IL-6), suggesting these stromal cells are a useful target for stromal therapy (180). Blockade of IL-6 signaling from inflammatory CAFs may be a useful therapeutic avenue and results are awaited from a phase II study (NCT02767557), testing effects of Nab-paclitaxel and gemcitabine with Tocilizumab (an anti IL-6 receptor monoclonal antibody) in patients with PDAC.

Gene expression studies describe subtypes of PDAC, displaying distinct biological and clinical behaviors. Classical PDACs are well differentiated, respond better to 5FU based chemotherapy, and have a better prognosis than the poorly differentiated, basal-like subtype (182). Single cell sequencing studies of organoids established from PDAC biopsies found mixtures of classical and basal subtype cells coexisting within a single tumor. Where this is the case, chemotherapy might select for resistant subpopulations of PDAC cells and a shift in subtype may be seen during treatment (183).

Induction of differentiation of myCAFs together with inhibition of cytokine signaling in iCAFs might increase therapeutic effects of chemotherapy. A combination of VDA and a neutralizing antibody targeting signals from iCAFGs (anti IL-6R or LIF) might improve response to current best standard of care chemotherapy.

VDAs such as paricalcitol and calcipotriol mediate global changes in gene expression and affect multiple pathways (summarized in **Table 6**), including: (1) anti-stromal effects, (2) direct anti-proliferative and pro-differentiation effects on PDAC cells, and (3) effects upon the immune system.

Vitamin D results in multiple effects, some of which may be detrimental in patients with advanced PDAC. A more recent

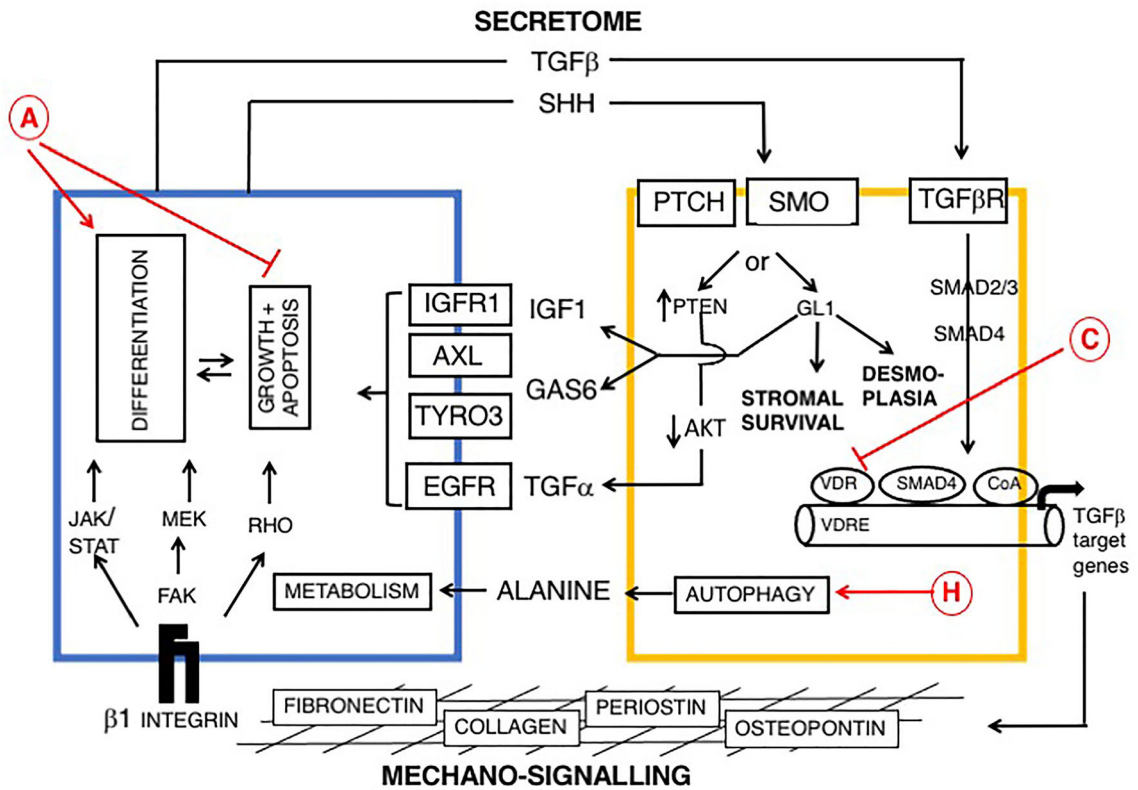

FIGURE 2 | Reciprocal signaling pathways between PDAC and myofibroblasts (myCAFs) and role of vitamin D, modified from (169, 176, 177). Cells are colored as in **Figure 1**. Epithelial (PDAC) cells are blue and stromal cells, (PSCs) are yellow. Two broad pathways are shown: a PDAC-derived secretome, including TGF-β and Sonic hedgehog (SHH), upper and mechano-signaling *via* ECM/β-1 integrin and FAK (lower part of figure). TGF-β drives a myofibroblast phenotype. SHH signals *via* GLI to promote dysplasia. Ligands derived from myCAFs (including IGF1, AXL, TYRO3 and TGFα) support tumor growth and inhibit apoptosis. Predicted effects of vitamin D are labeled A-J as described in **Table 6**, where labels indicate: A Pro-Differentiation and Anti-Proliferation; C Inhibition of fibrosis and down-regulation of TGF-β-SMAD-dependent transcription of pro-fibrotic genes and H Promotion of autophagy in PDAC cells and CAFs with provision of alanine to PDAC cells (178, 179).

study found that calcipotriol inhibited CAF proliferation and reduced secretion of pro-tumorigenic factors PGE2, leukemia inhibitory factor (LIF) and IL-6, consistent with the known anti-tumor effects of VDAs (187). This was also consistent with earlier studies that had shown LIF is secreted by PSCs, drives tumor progression and may be a useful therapeutic target in patients with PDAC (170). However, a new finding was that calcipotriol reduced CD8+T cell proliferation, decreased IFN-γ and IL-17, and increased IL-10 secretion, indicating an immunosuppressive effect (187). Furthermore, the authors discussed the role of vitamin D inducible peptide cathelicidin and its active cleavage product (LL-37) in PDAC. Previous work has shown that LL-37 is associated with cancer stem cell growth and survival in PDAC (188). Finally, vitamin D increases NET formation and recent work suggests this enhances hepatic micrometastasis in patients with PDAC (192).

Paricalcitol mediates beneficial effects upon the immune system. A pilot study of neoadjuvant paricalcitol in patients with resectable PDAC found 10-100 fold increased T cell migration into tumors after 28 days of treatment (193). Furthermore, a number of reviews suggest utilization of VDAs to inhibit immune-suppressive activity of stromal cells and the hope is that this will improve response to checkpoint inhibitors in patients with PDAC (194, 195).

Clinical trials using paricalcitol will likely benefit most from anti-stromal effects and subsequent improvement in delivery of chemotherapy. Taken together, the data suggest that overall, VDAs like calcipotriol decrease pro-inflammatory activity in patients with PDAC (187). Thus, VDSAs in clinical trials are predicted to result in significant benefits derived from decreased inflammation and desmoplasia but also with detrimental effects upon the immune system. It may be that selective agonists with reduced activation of Treg cells, while retaining strong inhibition of cytokine (IL-6) release will be particularly beneficial in patients with pancreatic cancer (**Table 6**; **Figure 3**).

PDAC subtype is influenced by intrinsic (genetics and epigenetics) and extrinsic factors, including chemotherapy and the stromal/inflammatory infiltrate. Inhibition of TAM and neutrophil activities result in a shift from basal to classical subtype. Finally,

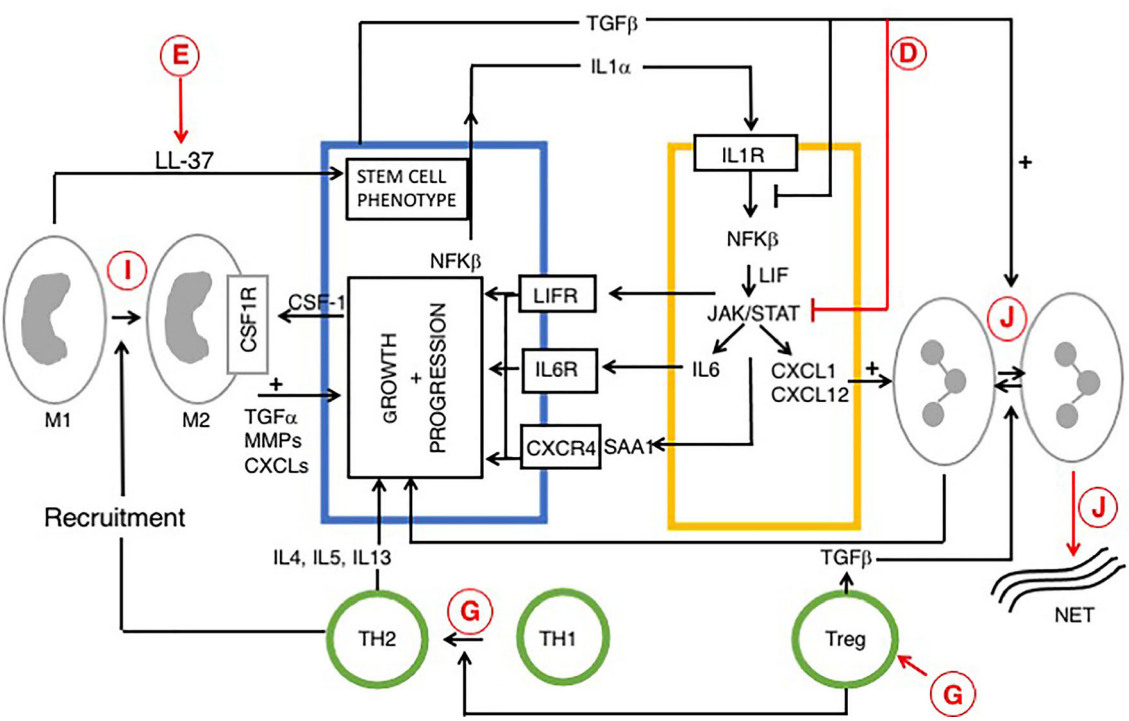

FIGURE 3 | Reciprocal signaling pathways within PDAC and iCAFs, modified from (170, 180) and role of vitamin D. Tumor cell interactions with immune cells (tumor associated macrophages (TAM), neutrophils and T cells are also indicated. Cells are colored as in **Figures 1** and **2**. Stromal/inflammatory signaling pathways influence PDAC progression. Growth of PDAC cells is driven by cytokines and growth factors: LIF, IL-6 and SAA1 (derived from iCAFs), IL-4 (from TH2) and TGFα from TAMs. PDAC cells secrete IL-1α or TGF to drive an iCAF or myo-CAF phenotype. VDAs have direct effects upon cancer, stromal and immune cells and indirect effects upon intercellular signaling pathways. Predicted effects of vitamin D are labeled A-J as described in **Table 6**, where labels indicate: D Inhibition of cytokine and pro-inflammatory chemokine release; E Increased macrophage production of cathelicidin and cleavage product (LL-37), associated with a cancer stem cell phenotype; G Suppression of immunity: increased Treg activity, activation of Th2 and suppression of Th1 cells; I Anti-tumor M1 phenotype conversion into immune-suppressive, pro-tumor M2 macrophages, and J Decreased neutrophil activity and increased formation of NETs. The role of vitamin D in NET formation remains controversial and is discussed above.

TABLE 6 | Predicted effects of VDAs in PDAC and COVID-19.

	Vitamin D/VDA effect	PDAC	COVID-19 (ARDS)	Reference
A	Pro-Differentiation/ Anti-Proliferation	Beneficial[a]	Uncertain	(172, 184)
B	Increases ACE2/Ang 1-7/MasR axis, inhibits ACE/Ang II signaling.	Uncertain	Beneficial	(109, 110, 185)
C	Inhibition of Fibrosis. Down-regulates TGFβ-SMAD-dependent transcription of pro-fibrotic genes.	Beneficial	Beneficial	(62, 63)
D	Inhibition of cytokine and pro-inflammatory chemokine release.	Beneficial	Beneficial	(63, 186)
E	Increases macrophage production of antimicrobial peptides, defensins and cathelicidin	Detrimental[b]	Beneficial	(187, 188)
F	Maintains integrity of epithelial cell tight-junctions	Uncertain	Beneficial	(21)
G	Suppresses immunity: increases Treg activity, activation of Th2 and suppression of Th1 cells	Detrimental	Beneficial	(52, 187)
H	Promotes Autophagy	Uncertain[c]	Uncertain	(178, 179, 189)
I	Pro-inflammatory, anti-tumor M1 phenotype is converted into immune-suppressive, pro-tumor M2 macrophages	Uncertain[d]	Uncertain	(75, 190)
J	Decreased Neutrophil activity	Beneficial[e]	Beneficial[e]	(21, 191)

[a]VDAs promote cancer cell and PSC differentiation. [b]Cathelicidin active cleavage product (LL-37) is associated with PDAC stem cell growth and survival. [c]Autophagy occurs in CAFs and resultant amino acids (including alanine) are made available to neighboring PDAC cells. Autophagy is likely a pro-survival mechanism in PDAC cells; [d]M2 polarization may contribute to fibroproliferative phase of ARDS. [e]Decreased neutrophil activation is expected to be beneficial; however, the role of vitamin D in NET formation remains controversial.

current interest surrounds the role of super enhancers in PDAC. In preclinical studies inhibition of BET family members resulted in a shift from basal to classical subtype, likely involving down-regulation of P63 (196, 197). It may be that BET inhibitors will sensitize basal subtype tumors to combined anti-stromal therapy and chemotherapy, suggesting new therapeutic approaches.

DISCUSSION AND PERSPECTIVES

There is a growing recognition for the importance of extra-skeletal roles of vitamin D. Several key biological processes controlled by vitamin D are disrupted in common diseases, and it has long been anticipated this might provide new therapeutic targets. Efforts to target vitamin D signaling pathways have focused upon (a) correction of deficiency as a prophylactic measure to decrease disease severity, and (b) use of VDAs as therapeutic agents in advanced disease. It is instructive to compare the role of vitamin D signaling agents in patients with PDAC or COVID-19 (**Table 6**). Most current trials in COVID-19 have addressed early disease, whereas studies in patients with PDAC have focused on the neoadjuvant or metastatic setting and premalignant disease has not been targeted.

Epidemiological data suggest that vitamin D deficiency is common and may play a role in multiple diseases including cardiovascular and autoimmune disease (11); Fan, 2020 #276}. Multiple studies have addressed the role of vitamin D supplementation in extra-skeletal disease. Some, but not all, studies found positive effects for supplementation in respiratory infections including influenza (66, 68). Interpretation in such studies, is complicated by disagreement concerning serum levels of calcidiol required for sufficiency (198), multiple confounding factors (18, 120, 121) and the application of methods designed for testing drugs rather than nutrients (140, 141).

Several on-going clinical trials in patients with COVID-19 address early-stage disease and prevention of infection by vitamin D (NCT04483635 and NCT04535791), other studies have started patients onto vitamin D supplementation immediately after COVID-19 diagnosis (NCT04536298 and NCT04536298). No current study begins intervention in patients with COVID-19 following development of ARDs. Although most of these clinical trials have utilized vitamin D; one Iranian study (NCT04386850) used calcidiol (reviewed in (21)). Abnormal liver function tests (LFTs) are seen in 14–53% of patients with COVID-19, perhaps reflecting expression of ACE2 in cholangiocytes (199). Calcidiol seems a good choice for supplementation, since it does not require activation in the liver; calcitriol might also be an effective agent. Finally, Evans and Lippman suggest that paricalcitol might be a useful agent for future clinical trials in COVID-19 patients (13).

In the face of new emerging viral pathogens there will inevitably be periods without effective treatment in the time before vaccines can be prepared. The availability of VDSAs with anti-fibrotic activity may provide a useful approach in patients with ARDS secondary to influenza, COVID-19, or some as yet unknown, new viral respiratory tract infection.

Clinical trials of VDAs in pancreatic cancer have focused on advanced stage disease. Premalignant lesions are commonly found in patients prior to the development of PDAC. This includes pancreatic intraepithelial neoplasia (PanIN) seen early during PDAC development in both human pancreas and genetically engineered mouse models. Premalignant lesions are commonly present in patients with chronic pancreatitis with a 15-fold increased risk of PDAC (when present for more than 5 years) (200) and hereditary pancreatitis syndrome, where risk is increased by >25-fold (201). Stromal activation and ECM deposition occurs surrounding PanINs and it may be that prophylactic treatment with vitamin D or VDAs would reduce the incidence of pancreatic cancer development within these high-risk patients.

New tools have recently become available for interpreting the effects of stromal targeted therapy in PDAC. Firstly, gene expression studies have identified subtypes of PDAC and stromal cells displaying distinct biological and clinical

behaviors, and secondly, organoids, a game-changing new technology which allow analysis of reciprocal signaling pathways between cancer and stromal cells. It may be that a combination of VDSA and Tocilizumab (an IL-6 receptor monoclonal antibody) targeting MyCAF and iCAFs will provide a useful adjuvant to current best standard of care chemotherapy in patients with PDAC.

Results from clinical trials testing vitamin D have often found decreased activity compared to prior expectations based upon preclinical studies. This has stimulated attempts to discover VDSAs with increased efficacy and reduced calcemic effect. A second aim has been to uncover agonists with high cell-tissue specificity. The search for new VDSAs has used high throughput screening of chemical libraries and computational modeling based methods. High throughput screens are often dependent on the promoters used to drive reporter gene readout and the cell lines used for the assay. Hirschfeld and colleagues placed a partial promoter sequence from the α-SMA gene upstream of a green fluorescent reporter protein, to specify myofibroblast function (202). The reporter gene was expressed within a rat hepatic stellate cell line and this system might provide a valuable assay for a high throughput screening to find VDAs with anti-fibrotic activity. Vitamin D likely promotes a mixture of beneficial and detrimental effects in any particular disease and consideration of **Table 6** suggests that selective agonists driving specific pathways may be most useful for treatment. Finally, selection of VDAs displaying decreased up-regulation of CYP24A1 expression may result in increased activity.

A large body of evidence suggests that vitamin D and VDAs likely possess therapeutic potential in several common diseases. We might soon anticipate a better understanding of their role in cancer and infectious disease. Two sets of clinical trials, comprising 42 and 11 studies in patients with COVID-19 and PDAC respectively, will deliver data within the next 2-3 years. The hope is that, where such studies yield positive results, this will act as a springboard to encourage isolation of further effective VDSAs. Selective VDAs represent an untapped resource for development of effective therapeutic agents. They may be useful in multiple diseases ranging from ARDS in patients with COVID-19 to tumor growth and metastasis in patients with pancreatic cancer.

AUTHOR CONTRIBUTIONS

DE and CF developed the idea for the review. All authors contributed to writing the manuscript 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.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 06 May 2021
doi: 10.3389/fendo.2021.633767

Serum Uric Acid Concentrations and Risk of Adverse Outcomes in Patients With COVID-19

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Specialty section:
This article was submitted to Translational Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *26 November 2020*
Accepted: *07 April 2021*
Published: *06 May 2021*

Citation:
Chen B, Lu C, Gu HQ, Li Y, Zhang G, Lio J, Luo X, Zhang L, Hu Y, Lan X, Chen Z, Xie Q and Pan H (2021) Serum Uric Acid Concentrations and Risk of Adverse Outcomes in Patients With COVID-19. Front. Endocrinol. 12:633767. doi: 10.3389/fendo.2021.633767

Background: Although hyperuricemia frequently associates with respiratory diseases, patients with severe coronavirus disease 2019 (COVID-19) and severe acute respiratory syndrome (SARS) can show marked hypouricemia. Previous studies on the association of serum uric acid with risk of adverse outcomes related to COVID-19 have produced contradictory results. The precise relationship between admission serum uric acid and adverse outcomes in hospitalized patients is unknown.

Methods: Data of patients affected by laboratory-confirmed COVID-19 and admitted to Leishenshan Hospital were retrospectively analyzed. The primary outcome was composite and comprised events, such as intensive care unit (ICU) admission, mechanical ventilation, or mortality. Logistic regression analysis was performed to explore the association between serum concentrations of uric acid and the composite outcome, as well as each of its components. To determine the association between serum uric acid and in-hospital adverse outcomes, serum uric acid was also categorized by restricted cubic spline, and the 95% confidence interval (CI) was used to estimate odds ratios (OR).

Results: The study cohort included 1854 patients (mean age, 58 years; 52% women). The overall mean ± SD of serum levels of uric acid was 308 ± 96 μmol/L. Among them, 95 patients were admitted to ICU, 75 patients received mechanical ventilation, and 38 died. In total, 114 patients reached composite end-points (have either ICU admission, mechanical ventilation or death) during hospitalization. Compared with a reference group with estimated baseline serum uric acid of 279-422 μmol/L, serum uric acid values ≥ 423 μmol/L were associated with an increased risk of composite outcome (OR, 2.60; 95% CI, 1.07- 6.29) and mechanical ventilation (OR, 3.01; 95% CI, 1.06- 8.51). Serum uric acid ≤ 278 μmol/L was associated with an increased risk of the composite outcome (OR, 2.07;

95% CI, 1.18- 3.65), ICU admission (OR, 2.18; 95% CI, 1.17- 4.05]), and mechanical ventilation (OR, 2.13; 95% CI, 1.06- 4.28), as assessed by multivariate analysis.

Conclusions: This study shows that the association between admission serum uric acid and composite outcome of COVID-19 patients was U-shaped. In particular, we found that compared with baseline serum uric acid levels of 279-422 μmol/L, values ≥ 423 μmol/L were associated with an increased risk of composite outcome and mechanical ventilation, whereas levels ≤ 278 μmol/L associated with increased risk of composite outcome, ICU admission and mechanical ventilation.

Keywords: COVID-19, uric acid, relationship, U-shape, adverse outcome

INTRODUCTION

In December 2019, a cluster of patients with pneumonia, which was later identified as COVID-19, were identified in Wuhan. Thereafter, COVID-19 rapidly spread around the world (1), and, in November 25, 2020, the World Health Organization reported a total of 46,166,182 confirmed cases globally, with an average mortality being of 2.4% (2).

Patients with COVID-19 present with a variety of signs and symptoms as well as different prognoses, including recovery, admission to the intensive care unit (ICU), the need for mechanical ventilation, and death. Mild cases manifest fever and cough, whereas critical cases may manifest acute respiratory distress syndrome (ARDS), sepsis, or septic shock. Early detection of patients who are likely to develop critical disease is fundamental to identifying high-risk patients and allocating limited resources.

A high incidence of renal abnormalities and gastrointestinal symptoms has been reported in patients with COVID-19, and kidney diseases are frequently associated with mortality in these patients (3–6). The kidneys and gut are both targets of SARS-CoV-2 and the primary sites of uric acid excretion. Therefore, it is likely that infection with SARS-CoV-2 could affect regulation of uric acid metabolism and levels in the serum. Indeed, studies have shown that serum uric acid concentrations were markedly lower in patients with severe COVID-19 disease (7–9), which may be caused by decreased net renal tubular reabsorption of uric acid due to inflammation. In SARS-CoV-2-affected patients, hypouricemia was also found to be strongly associated with a poor prognosis (10). However, hyperuricemia is known to be associated with hypoxia and systemic inflammation in respiratory diseases (11). Angiotensin converting enzyme 2 (ACE2), the receptor for the entry of SARS-CoV-2, is strongly expressed in the kidney (12, 13), and SARS-CoV-2 can be detected in COVID-19 patients' urine (14, 15). COVID-19-associated nephritis, which manifests as leukocyturia, albuminuria, and hematuria, is considered an early indicator of disease severity (16). Furthermore, a single-cell analysis showed enriched expression of ACE2 in all subtypes of proximal tubular cells of the kidney (13), which are the most important regulators of serum urate (17). A recent study also observed that uric acid significantly increases in children with severe COVID-19 compared with non-severe children on admission (18).

To date, no evidence on the precise association between serum concentrations of uric acid in COVID-19 patients on admission and in-hospital adverse outcomes exists. In this study, we investigated in detail the relationship between admission serum uric acid and the adverse outcomes in hospitalized patients.

METHODS

Patients

This retrospective cohort study included 1854 adult patients (≥18 years old) admitted to Leishenshan Hospital, a hospital specifically built for COVID-19 treatment during disease outbreak, between February 16 and April 14, 2020, when the COVID-19 epidemic occurred in Wuhan (China). The diagnosis of COVID-19 was confirmed according to the WHO interim guidance. SARS-CoV-2 positivity was diagnosed by real-time reverse-transcriptase polymerase-chain-reaction (RT-PCR) assay conducted on nasal and pharyngeal swab specimens. Patients with incomplete or missing serum uric acid values within 24 hours after admission were excluded (n = 14). Patients with chronic kidney disease, gout, chronic liver disease with severe liver dysfunction, or malignancies (n = 50) were also excluded from the study. The flowchart of participants' progress through the study is shown in **Supplementary Figure S1**.

Clinical data of all COVID-19 patients were collected. Due to the outbreak of the COVID-19 epidemic, written informed consent could not be collected from patients. We only made use of the deidentified retrospective data in this study. In addition, our study has been approved by the Ethics Committee of the Zhongnan Hospital of Wuhan University.

Baseline Measurements and Definition

Relevant patients' characteristics were recorded from electronic medical records, including age, sex, and comorbidities. Clinical and laboratory data were obtained within the first 24 hours of admission. They included vital signs, long-term use of medications, mode of respiratory support, complete blood count, coagulation profile, serum uric acid, creatinine, electrolytes, renal and liver function, lactate dehydrogenase, and D-dimer concentrations. Outcome data were also collected from electronic medical records. The Leishenshan hospital was closed on April 14, enabling complete extraction of outcome

data. The primary outcome was a composite outcome of three different components, such as mortality, occurrence of mechanical ventilation and admission to the ICU. The following variables were associated with serum uric acid levels: age, sex, hypertension, diabetes, chronic lung disease, lymphocyte and platelet counts, aspartate aminotransferase, total bilirubin, albumin, creatine, C-reactive protein and D-dimer. An overnight fasting blood sample was collected to measure serum uric acid, aspartate aminotransferase, total bilirubin, albumin and creatinine.

Fever was defined as stated history or presence of axillary temperature of at least ≥37.5°C. The diagnosis of hypertension was based on a systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm, or by previous diagnosis of hypertension. Hypoproteinemia was defined as a serum albumin below 25 g/L.

Outcomes

Complete outcome data were collected from electronic medical records. The primary outcome was a composite outcome defined as mortality or occurrence of mechanical ventilation or admission to the ICU. In our study, all participants were hospitalized patients with laboratory-confirmed COVID-19 and had a definite outcome (dead or discharged).

Statistical Analysis

Participants were classified into three groups according to admission serum uric acid concentrations. The cut-off values of serum uric acid concentrations were based on the results of logistic regression with restricted cubic spline analysis. Continuous variables were reported as means ± standard deviation (SD) and categorical variables as frequencies expressed as percentages based on data distribution. Baseline characteristics among groups were compared using the χ^2 test for categorical variables and analysis of variance or Kruskal–Wallis tests where appropriate.

Odds ratios for the association between plasma uric acid and composite outcome, ICU admission, mechanical ventilation, and mortality were estimated by modeling serum uric acid as a categorical variable using multivariable logistic regression. The covariates for poor outcome of COVID-19 were selected based on previous studies (19, 20), and were adjusted in multivariable logistic regression analyses. Serum uric acid concentrations were excluded at the 1% and 99% points. Three models were used: 1) age and sex were adjusted in multivariable models (Model 1); 2) comorbidities (hypertension, diabetes, and chronic lung disease) were added to the multivariable models (Model 2) laboratory tests (lymphocyte and platelet counts, aspartate aminotransferase, total bilirubin, albumin, creatine, C-reactive protein and D-dimer) were included in the multivariable models (Model 3).

Possible nonlinear relationships between serum uric acid and the composite outcome, ICU admission, mechanical ventilation, and death were examined with restricted cubic splines (21). Based on initial analysis of relationships between serum uric acid and primary outcome measurement, which revealed a nonlinear association, serum uric acid was analyzed as a continuous variable, fitting a restricted cubic spline function with three knots (located at the 5th, 50th, and 95th percentiles). Considering the restricted cubic spline analysis for serum uric acid and the composite outcome, 279–422 μmol/L was selected as the reference category. This range was associated with the lowest risk of all events, as multivariable cubic spline plots revealed significance thresholds at 279 μmol/L and 422 μmol/L.

R software (version 3.6.2; http://www.R-project.org), EmpowerStats and the IBM SPSS 25.0 software (IBM Corp., Armonk, NY, USA) were used for the statistical analyses. $P < 0.05$ was considered statistically significant in all analyses.

RESULTS

Patient Characteristics

A total of 1854 participants (mean age, 58 years; 52% women) were included in this analysis (**Supplementary Figure S1**).

The mean age of patients was 58.1 ± 14.7 years and 48% were male. The mean SBP was 131.4 ± 15.7 mmHg, and 310 (16.7%) patients reported dyspnea. 478 (25.8%) patients had a history of hypertension, 222 (12%) had diabetes, 47 (2.5%) had chronic lung diseases, and 164 (8.8%) cardiovascular diseases. The overall mean serum uric acid level was 308.4 ± 95.9 μmol/L. Participants with higher serum uric acid concentrations were more likely younger, male, and showed higher SBP, and higher creatinine levels, as well as lower D-dimer. On the other hand, participants with lower uric acid concentrations were older, female, and showed lower SBP and creatine levels, as well as higher D-dimer levels (**Table 1**).

Association Between Serum Uric Acid and In-Hospital Outcomes

During hospitalization, 95 subjects (5.1%) were admitted to ICU, 75 (4.0%) received mechanical ventilation, 38 (2.1%) undergone death, and 114 (6.1%) participants had composite outcome consisting of ICU admission, mechanical ventilation and death. Among the patients with uric acid ≤278 μmol/L (n = 789), 73 (9.3%) received composite outcome, 63 (8%) had ICU admission, 48 (6%) with mechanical ventilation, and 18 (2.3%) died. In contrast, among the patients with uric acid ≥423 μmol/L (n = 225), 14 (6.2%) subjects had composite outcome, 10 (4.4%) had ICU admission, 10 (4.4%) received mechanical ventilation, and 8 (3.6%) died (**Table 2**, **Figure 1**).

Restricted multivariable cubic spline analysis was performed to analyze the relationship between serum uric acid and adverse outcomes of COVID-19 (**Table 2**, **Figure 2**).

Composite Outcome

Associations between serum uric acid and composite outcome were U-shaped. The composite outcome occurred in 114 (6.1%) subjects. Statistical models were fully adjusted for several factors, including age, sex, hypertension, diabetes, chronic lung disease, lymphocyte and platelet counts, aspartate aminotransferase, total bilirubin, albumin, creatine, C-reactive protein and D-dimer levels. These models showed that compared with baseline serum uric acid of 279 to 422 μmol/L (n = 840; 3.2% with the

TABLE 1 | Baseline patient characteristics by serum uric acid concentrations.

Characteristics	Overall (N = 1854)	Admission Serum Uric Acid Level, μmol/L			
		≤199 μmol/L (n = 200)	200-399 μmol/L (n = 1360)	≥400 μmol/L (n = 294)	*P*-value
Baseline and demographic					
Age	58.1 (14.7)	61.6 (14.9)	58.5 (14.2)	53.8 (15.9)	<0.001
Male	890 (48.0)	67 (33.5)	587 (43.2)	236 (80.3)	<0.001
SBP, mmHg	131.4 (15.7)	129.7 (15.4)	131.3 (15.8)	132.8 (15.4)	0.035
DBP, mmHg	81.1 (11.1)	78.0 (10.9)	81.1 (10.9)	83.2 (11.5)	<0.001
Comorbidities					
Hypertension	478 (25.8)	43 (21.5)	367 (27.0)	68 (23.1)	0.134
Diabetes	222 (12.0)	31 (15.5)	164 (12.1)	27 (9.2)	0.103
Chronic lung disease	47 (2.5)	6 (3.0)	33 (2.4)	8 (2.7)	0.869
Cardiovascular disease	164 (8.8)	24 (12.0)	108 (7.9)	32 (10.9)	0.068
Lab test					
Platelet, 10^9/L	238.51 ± 80.52	237.47 ± 83.11	235.07 ± 71.04	240.41 ± 80.48	0.604
Lymphocyte, 10^9/L					<0.001
<1	282 (14.9)	72 (36.0)	177 (13.0)	28 (9.5)	
≥1	1577 (85.1)	128 (64.0)	1183 (87.0)	266 (90.5)	
CRP, mg/L					0.004
<30	1736 (93.6)	722 (91.5)	212 (94.2)	802 (95.5)	
≥30	118 (6.4)	67 (8.5)	13 (5.8)	38 (4.5)	
AST, U/L					0.005
<40	1688 (91.0)	707 (89.6)	197 (87.6)	784 (93.3)	
≥40	166 (9.0)	82 (10.4)	28 (12.4)	56 (6.7)	
Total bilirubin, μmol/L	10.37 ± 5.34	10.35 ± 5.47	11.27 ± 6.34	10.15 ± 4.89	0.020
Albumin, g/L	37.11 ± 4.07	36.15 ± 4.07	38.86 ± 3.99	37.54 ± 3.85	<0.001
Creatinine, μmol/L					<0.001
<64.3	927 (50.0)	149 (74.5)	732 (53.8)	46 (15.6)	
≥64.3	927 (50.0)	51 (25.5)	628 (46.2)	248 (84.4)	
D-dimer, mg/L					<0.001
<1	1432 (77.2)	122 (61.0)	1064 (78.2)	246 (83.7)	
≥1	422 (22.8)	78 (39.0)	296 (21.8)	48 (16.3)	

Data are shown as mean (with SD) or n (%). P values comparing across three ranges of serum uric acid concentrations using χ^2 tests for categorical variables and analysis of variance and Kruskal–Wallis tests for normally and nonnormally distributed continuous variables, respectively.

TABLE 2 | Association Between Outcomes and Serum Uric Acid by Categories at Admission.

Outcome	No.of patients	Event (%)	Model 1		Model 2		Model 3	
			OR (95% CI)	*P* value	OR (95% CI)	P value	OR (95% CI)	*P* value
Composite Outcome		114 (6.1)						
UA ≤ 278 μmol/L	789	73	3.05 (1.92- 4.86)	<0.001	3.02 (1.89- 4.82)	<0.001	2.07 (1.18- 3.65)	0.011
UA 279- 422 μmol/L	840	27	1 [Reference]		1 [Reference]		1 [Reference]	
UA ≥423 μmol/L	225	14	2.14 (1.07- 4.25)	0.031	2.10 (1.05- 4.21)	0.036	2.60 (1.07- 6.29)	0.035
ICU admission		95 (5.1)						
UA ≤ 278 μmol/L	789	63	3.19 (1.92- 5.30)	<0.001	3.13 (1.88- 5.21)	<0.001	2.18 (1.17- 4.05)	0.014
UA 279- 422 μmol/L	840	22	1 [Reference]		1 [Reference]		1 [Reference]	
UA ≥423 μmol/L	225	10	1.81 (0.83- 3.98)	0.0172	1.74 (0.79- 3.86)	0.267	1.75 (0.63- 4.85)	0.281
Mechanical Ventilation		75 (4.0)						
UA ≤ 278 μmol/L	789	48	3.04 (1.71- 5.38)	<0.001	3.01 (1.70- 5.36)	<0.001	2.13 (1.06- 4.28)	0.033
UA 279- 422 μmol/L	840	17	1 [Reference]		1 [Reference]		1 [Reference]	
UA ≥423 μmol/L	225	10	2.41 (1.06- 5.46)	0.036	2.42 (1.06- 5.50)	0.048	3.01 (1.06- 8.51)	0.038
Death		38 (2.1)						
UA ≤ 278 μmol/L	789	18	1.40 (0.66- 2.97)	0.383	1.40 (0.66- 2.97)	0.388	0.92 (0.33- 2.60)	0.876
UA 279- 422 μmol/L	840	12	1 [Reference]		1 [Reference]		1 [Reference]	
UA ≥423 μmol/L	225	8	3.01 (1.16- 7.81)	0.023	3.05 (1.16- 7.98)	0.026	3.94 (0.99- 15.8)	0.052

Model 1: Adjusted for age, sex. Model 2: Model 1 plus hypertension, diabetes, and chronic lung disease. Model 3: Model 2 plus adjustment for lymphocyte, platelet, aspartate aminotransferase, total bilirubin, albumin, Creatinine, C-reactive protein and D-dimer.
ICU, intensive care unit; MV, mechanical ventilation; UA, uric acid.

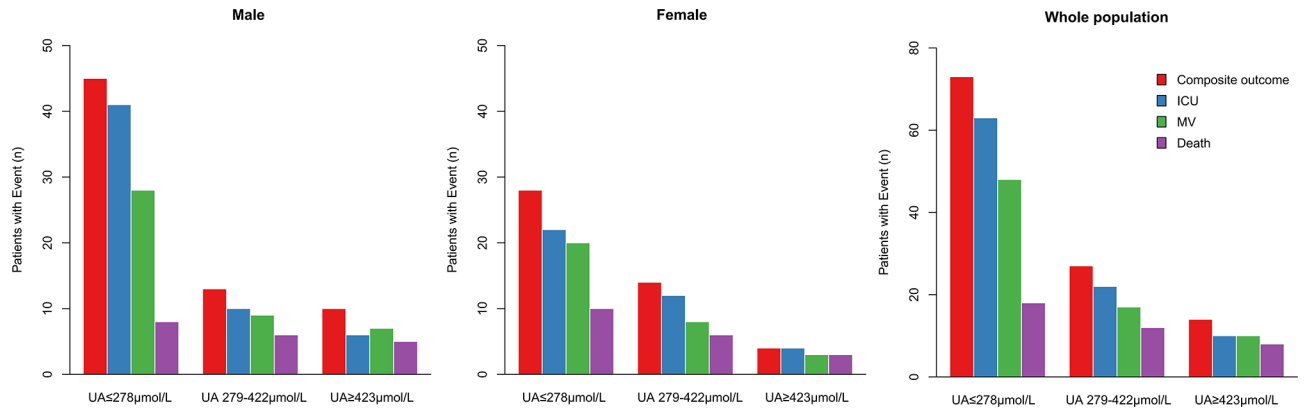

FIGURE 1 | Distribution of males, females and whole population of COVID-19 patients reaching the composite outcome, ICU admission, mechanical ventilation, or death relatively to uric acid concentrations. Distribution of patients' outcomes in the three ranges of serum uric acid concentrations from low to high (UA ≤ 278 μmol/L, UA 279–422 μmol/L, ≥ 423 μmol/L). ICU, intensive care unit; MV, mechanical ventilation.

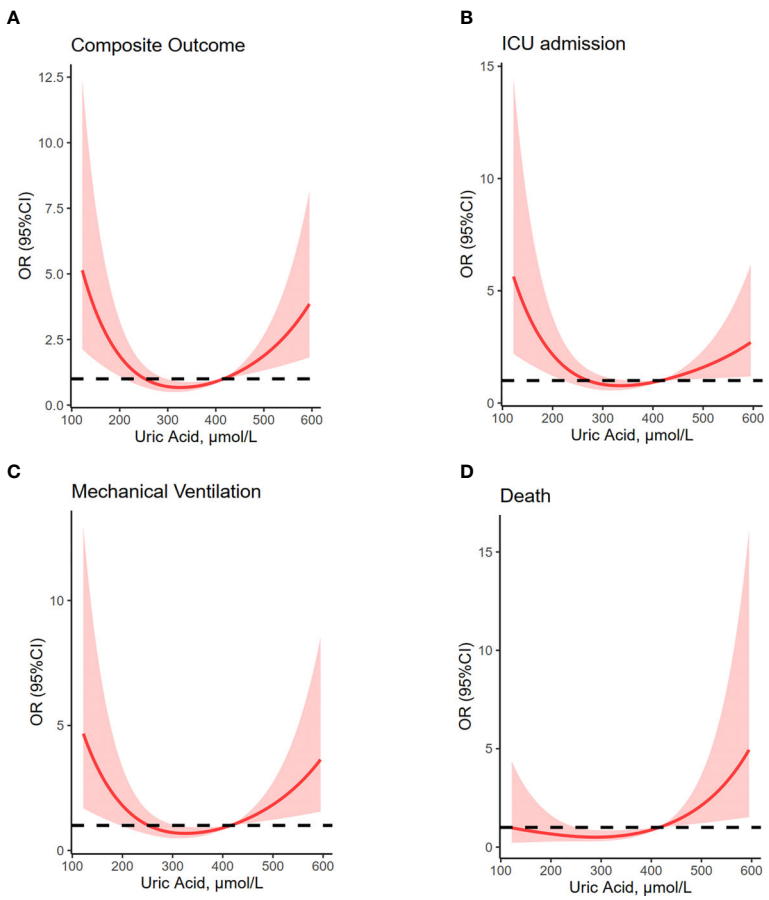

FIGURE 2 | U-shaped association between uric acid concentrations and composite outcome, ICU admission, mechanical ventilation and death. Restricted multivariable cubic spline plots show U-shaped associations between admission serum uric acid and composite outcome **(A)**, ICU admission **(B)**, mechanical ventilation **(C)**, and death **(D)**. OR were adjusted for age, sex, hypertension, diabetes, lung disease, creatine, lymphocyte and platelet counts, aspartate aminotransferase, total bilirubin, albumin, creatine, C-reactive protein and D-dimer values. Median uric acid concentrations (279–422 μmol/L) have been considered as reference. Shaded areas are 95% CI derived from restricted cubic spline regressions with three spaced knots at the 5th, 50th, and 95th percentiles. The dashed line indicates OR equal to 1. ICU, intensive care unit; MV, mechanical ventilation.

composite outcome), both higher and lower serum uric acid levels (OR, 2.60; 95% CI, 1.07- 6.29 for ≥423 μmol/L and OR, 2.07; 95% CI, 1.18- 3.65 for ≤278 μmol/L, respectively) were associated with an increased risk of composite outcome consisting of ICU admission, mechanical ventilation, and death.

ICU Admission

A total of 95 (5.1%) COVID-19 patients were admitted to ICU. Compared with baseline serum uric acid concentrations (279-422 μmol/L) shown by the 2.6% of subjects admitted to ICU, lower uric acid levels were significantly associated with increased risk of ICU admission (OR, 2.18; 95% CI, 1.17- 4.05 for ≤278 μmol/L).

Mechanical Ventilation

A total of 75 patients received mechanical ventilation. Among them the 2.0% showed serum uric acid baseline concentrations. Levels of serum uric acid higher and lower than 279-422 μmol/L were associated with an increased risk of receiving mechanical ventilation (OR, 3.01; 95% CI, 1.06- 8.51 for ≥ 423 μmol/L and OR, 2.13; 95% CI, 1.06- 4.28 for ≤278 μmol/L, respectively).

Death

Overall, 38 patients of the study cohort died. Among them, the 1.4% had baseline uric acid levels. Levels of serum uric acid higher than 279-422 μmol/L was associated with increased risk of death (OR, 3.94; 95% CI, 0.99- 15.8 for ≥ 423 μmol/L).

DISCUSSION

In this study, we report that serum concentrations of uric acid and adverse clinical outcomes of COVID-19 patients are U-shaped associated, thus suggesting that uric acid values on admission can be an independent predictor of prognosis. In fact, higher baseline serum uric acid levels were associated with an increased risk of composite outcome and mechanical ventilation. Similarly, lower baseline serum uric acid levels were associated with increased risk of composite outcome, ICU admission, and mechanical ventilation. Threshold effect analysis showed that serum uric acid levels between 279 and 422 μmol/L can be considered generally safe with respect to poor outcomes. To our knowledge, this is the first study describing serum uric acid, a widely available and low-cost diagnostic biomarker, as a predictor of adverse outcomes in COVID-19 patients. Among others used, this parameter might be useful for the identification of high-risk COVID-19 patients who could benefit from intensive management.

Hyperuricemia is frequently found in patients affected by chronic obstructive pulmonary disease (COPD) or other respiratory diseases (22–24). High level of uric acid can also be detected in children with severe COVID-19 on admission (18). However, patients with severe coronavirus disease 2019 (COVID-19) and severe acute respiratory syndrome (SARS) have been shown to develop marked hypouricemia (7–9). Thus, the association between uric acid and outcomes of COVID-19 is still under debate. The present study found that both high and low uric acid were associated with increased risk of adverse clinical outcomes. A smooth curve was fitted to show the U-shaped relationship between serum uric acid and composite outcome, as well as each of its components.

Angiotensin converting enzyme 2 (ACE2), the receptor for the entry of SARS-CoV-2, is strongly expressed in the kidney (12, 13), and SARS-CoV-2 can be detected in COVID-19 patients' urine (14, 15). COVID-19-associated nephritis, which manifests as leukocyturia, albuminuria, and hematuria, is considered an early indicator of disease severity (16). Furthermore, a single-cell analysis showed enriched expression of ACE2 in all subtypes of proximal tubular cells of the kidney (13), which are the most important regulators of serum urate (17). SARS-CoV-2 infection is likely to cause uric acid dysregulation, resulting in abnormal serum uric acid concentrations. Moreover, increased uric acid excretion has been observed in patients with respiratory failure (25). It has been shown that proinflammatory cytokines can influence uric acid excretion or serum uric acid level (10, 26). For example, serum IL-8 level positively correlated with fraction excretion of uric acid while negatively correlated with serum uric acid level in SARS patients (10); whereas IL-6 level during gouty attach was correlated with serum uric acid change (26). Although little is known about the effects of cytokines on uric acid transporting, it is speculated that some cytokines modulate activities of specific channels (such as calcium channels and sodium channels) or transporters (such as SGLT2) by various mechanisms (27–29), and thus may affect functions of urate transporters including URAT1 and GLUT9 (30, 31). Therefore, the cytokine storm syndrome initiated by SARS-CoV-2 infection may play a role in the uric acid disruption in COVID-19 patients.

Uric acid is an important antioxidant, scavenging reactive oxygen species and free radicals. A prospective randomized controlled clinical trial showed that a nucleotide-supplemented diet significantly reduces complications and shortens the hospital stay of ICU patients (32), suggesting serum uric acid as a possible surrogate of antioxidant capacity. Moreover, uric acid crystals can be sensed by Clec12A, a regulator of type-I interferon responses, which is a pivotal defensive mechanism against viral infection (33). Uric acid strongly enhances T-cell immune responses to viruses (34), and low uric acid concentrations increase the risk of infection with viruses, such as Epstein–Barr virus (35). However, few studies on associations between serum uric acid concentrations and infections have been previously reported. Hypouricemia is a poor prognostic indicator in patients with intra-abdominal sepsis (36) or radiating pneumonitis (37). Another study showed that hypouricemic patients with SARS had poorer outcomes, especially in terms of respiratory failure (10). Consistent with this, we observed that COVID-19 patients with hypouricemia had higher risks of admission to ICU and mechanical ventilation. It remains unclear whether poor outcomes in hypouricemic patients with COVID-19 are in part due to a shortage of antioxidants.

Nevertheless, high uric acid concentrations can have direct pathophysiological effects, including increased oxidative stress, inflammation, endothelial dysfunction, activation of the renin–angiotensin–aldosterone system, and insulin resistance (17).

Hyperuricemia has been found to be associated with various diseases, including coronary heart disease (38), hypertension (39), kidney failure (40), and chronic obstructive pulmonary disease exacerbations (22). It is likely that we have identified a phenomenon that reflects complex interactions between uric acid and other risk factors.

The present study had some limitations. First, the observation design did not permit to establish causality between serum uric acid concentrations and mortalities, ICU admission, and requirement for mechanical ventilation. In addition, residual confounding may persist, even after multivariable adjustment. We only analyzed serum concentrations of uric acid on admission and did not have access to follow-up measurements over time, and some vital variables such as fraction excretion of uric acid were unavailable.

Nevertheless, our findings could be useful to predict whether ICU or mechanical ventilation will be needed and, thus, to optimize patient allocation for special therapies and initiation of strategies to save lives.

In conclusion, the present study has shown a U-shaped association between serum uric acid levels and composite outcome, thus suggesting a prognostic role of serum uric acid for COVID-19 patients.

Our findings suggest that more attention should be paid to serum uric acid levels in the evaluation of COVID-19 disease status. This study also suggests a possible role for serum uric acid in the identification of COVID-19 patients at high risk of adverse clinical outcomes that need early intensive management. More studies are needed to establish a safe range values of serum uric acid in patients with COVID-19, which is urgent for patients who are taking uric acid-lowing drugs, and to clarify the molecular basis of this relationship.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the research ethics board of Zhongnan Hospital of Wuhan University, Wuhan, China. Written informed consent from the patients was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

HP, GZ, XYL, LZ, ZL, YH, XML, and ZC collected the data. BC and HG performed the statistical analysis. HP and QX supervised the project. BC, CL, and YLwrote the manuscript. HG, JL, and QX revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Scientific and Technological Project of COVID-19, West China Hospital, Sichuan University (HX-2019-nCoV-032) and National Natural Science Foundation of China (Grant no. 81700493).

ACKNOWLEDGMENTS

We are grateful to all physicians and nursing staff taking care of COVID-19 patients and making clinical records.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.633767/full#supplementary-material

Supplementary Figure 1 | Flowchart summarizing the selection of study participants.

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

frontiers in Endocrinology

REVIEW
published: 18 June 2021
doi: 10.3389/fendo.2021.649405

Could Exogenous Insulin Ameliorate the Metabolic Dysfunction Induced by Glucocorticoids and COVID-19?

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The finding that high-dose dexamethasone improves survival in those requiring critical care due to COVID-19 will mean much greater usage of glucocorticoids in the subsequent waves of coronavirus infection. Furthermore, the consistent finding of adverse outcomes from COVID-19 in individuals with obesity, hypertension and diabetes has focussed attention on the metabolic dysfunction that may arise with critical illness. The SARS coronavirus itself may promote relative insulin deficiency, ketogenesis and hyperglycaemia in susceptible individuals. In conjunction with prolonged critical care, these components will promote a catabolic state. Insulin infusion is the mainstay of therapy for treatment of hyperglycaemia in acute illness but what is the effect of insulin on the admixture of glucocorticoids and COVID-19? This article reviews the evidence for the effect of insulin on clinical outcomes and intermediary metabolism in critical illness.

Keywords: coronavirus – COVID-19, insulin, glucocorticoid, critical-illness, dexamethasone

OPEN ACCESS

Edited by:
Jeff M. P. Holly,
University of Bristol, United Kingdom

Reviewed by:
Meng Zhang,
Capital Medical University, China
Stephen Smith,
Smith Center for Infectious Diseases and Urban Health, United States

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Specialty section:
This article was submitted to Translational Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *04 January 2021*
Accepted: *02 June 2021*
Published: *18 June 2021*

Citation:
Whyte MB, Vas PRJ and Umpleby AM (2021) Could Exogenous Insulin Ameliorate the Metabolic Dysfunction Induced by Glucocorticoids and COVID-19? Front. Endocrinol. 12:649405. doi: 10.3389/fendo.2021.649405

TRIALS OF GLUCOCORTICOIDS IN COVID-19

The RECOVERY Trial

At the time of writing, no pharmacological intervention for COVID-19 has been as successful as steroids for treating the acute illness. The Randomized Evaluation of COVID-19 Therapy (RECOVERY) trial showed that dexamethasone 6mg daily for 10 days reduced the mortality of mechanically ventilated patients by 29% (1). This was despite 8% of the usual care group receiving Dexamethasone in RECOVERY, which would bias results towards the null, raising the possibility of even greater benefit. However, mortality was measured at 28 days and longer-term data will be informative as the adverse impacts of steroid administration (in other acute conditions) may be seen up to 90 days (2).

i) Dose: It is not entirely clear how the dose of 6 mg was decided upon. Immediately prior to the pandemic, the Dexamethasone treatment for the acute respiratory distress syndrome: a multicentre, randomised controlled trial' (DEXA-ARDS) reported on outcomes in ARDS of dexamethasone at a starting dose of 20mg daily (3). This dose is consistent with prior studies of ARDS (which used methylprednisolone regimens dosed at 1-2 mg/kg/day initially) (4).

A common pattern evolving from five retrospective trials early in the course of the pandemic was for greater benefit with low dose steroids compared to the high dose steroids (5). It is likely that the

6mg dose was a trade-off between the beneficial effects of resolving pulmonary and systemic inflammation and supporting blood pressure; and the adverse effects of inhibiting immune response, reduce pathogen clearance, and provoking viral replication (6).

ii) Pharmacokinetics: In RECOVERY, the trial drug could be given orally or intravenously and surprisingly, the route of administration was not recorded in the study documentation. It is conceivable that for patients receiving mechanical ventilation, the route of administration was more likely to be intravenous, whereas it was probably given orally outside of this subgroup. The bioavailability of oral dexamethasone is between 70% and 78%, and therefore dexamethasone in tablet form may not have an equivalent therapeutic effect (7).

Meta-Analysis of Glucocorticoids in COVID-19

Following release of RECOVERY outcomes, several ongoing hydrocortisone trials were stopped as it was considered ethically imperative to use dexamethasone. This reduced the numbers of participants and hard end points were not achieved.

A meta-analysis was undertaken by WHO Rapid Evidence Appraisal for COVID-19 Therapies (REACT) Working Group (8). This incorporated data from seven trials (RECOVERY, REMAP-CAP, CoDEX, CAPE COVID, and three additional trials) totalling 1703 patients (678 had been randomized to corticosteroids and 1025 to usual care or placebo), hospitalized with COVID-19 critical illness.

The 28-day mortality was lower in patients randomised to corticosteroids: 222 deaths among 678 patients randomized to corticosteroids compared with 425 deaths among 1025 patients randomised to usual care or placebo (odds ratio [OR], 0.66 [95% CI, 0.53-0.82]; P < 0.001). The RECOVERY trial provided 59% of the patients (8). In the analysis that excluded patients recruited to the RECOVERY trial, the OR was 0.77 (95% CI, 0.56-1.07) for all-cause mortality comparing corticosteroids with usual care or placebo. The point-estimate for reduced mortality was similar between dexamethasone and hydrocortisone: OR for mortality reduction was 0.64 (95% CI 0.50 to 0.82) with dexamethasone and 0.69 (0.43 to 1.12; P=0.13) with hydrocortisone). Of note, the only trial that assessed methylprednisolone (Steroids-SARI) was underpowered and OR for effect was 0.91 with wide confidence interval (0.29 to 2.87). Outcomes were also similar with lower- *vs* higher-dose corticosteroid regimens (demarcation between low and high-dose was pre-specified at 15 mg/d of dexamethasone, 400 mg/d of hydrocortisone, and 1 mg/kg/d of methylprednisolone).

Since publication of the WHO meta-analysis, a more recent randomised, placebo-controlled, double-blind study of 0.5 mg/kg of methylprednisolone conducted in Brazil in 393 patients found no difference in 28-day mortality and patients on steroids required more insulin therapy (9). However, the Brazilian cohort were on average about ten years younger than in RECOVERY, had less heart disease (7% *vs* 28%), and there was a greater proportion on mechanical ventilation at enrolment – suggesting more severe disease (33.8% on mechanical ventilation *vs* 15.5% without). This runs counter to the idea that greater benefit is seen in more severely unwell patients - the majority of the studies in the WHO meta-analysis were conducted in patients with serious or critically unwell patients, particularly those who required high flow nasal oxygen or ventilation (8). In the RECOVERY trial itself, there was no benefit among those who were receiving no respiratory support at randomization (17.8% dexamethasone *vs*. 14.0% control; rate ratio, 1.19; 95% CI, 0.91-1.55).

IMPLICATIONS OF COVID-19, AND THE RECOVERY TRIAL PROTOCOL, ON DIABETES

Diabetes was present in 24% of dexamethasone group *vs* 22% of usual care of the RECOVERY trial. The study investigators did not adjust for multiplicity in the study, between treatment arms or for any of the pre-specified endpoints, meaning there is a potential inflation of the type I error rate. This would be more of an issue for some of the secondary endpoints. Even so, it is surprising that data for patients with diabetes was not reported. Two serious adverse events (SAEs) for hyperglycaemia, requiring a longer admission for stabilisation, were recorded in the dexamethasone group (10). Six milligrams of dexamethasone OD is, in effect, five- to six-fold greater than the therapeutic glucocorticoid replacement dose and therefore metabolic perturbation is to be anticipated but the extent of this is uncertain.

Prior to the COVID-19 pandemic, few papers examined the acute effects of steroids on glucose homeostasis, when newly administered to general medical inpatients. In these studies (11–14), up to 50–70% of hospitalized patients (without known diabetes) prescribed moderate-to-high glucocorticoid doses, developed hyperglycaemia.

New hyperglycaemia (capillary glucose ≥11 mmol/L after initiation of glucocorticoid therapy) was found in 14% of general medical admissions treated with the equivalent of 30mg prednisolone (~4.5mg of dexamethasone daily), over a short period of time (median 2.5 days; interquartile range [IQR] 1-4 days) (11). At higher doses of prednisolone (~40mg daily) over four weeks - and including a subgroup receiving pulsed methylprednisolone 500-1000mg per day - two-thirds of patients developed steroid-induced diabetes (14). In these studies of individuals without diabetes, SID was more likely with older age, higher HbA_{1c} level, lower estimated glomerular filtration rate (eGFR) and greater illness severity (11–14).

In a meta-analysis of two randomised controlled trials, single-dose 8mg dexamethasone, administered pre-operatively, led to a mean 0.39mmol/L higher blood glucose than control, after 24 hours (95% CI: 0.04 - 0.74 mmol/L, P=0.03) (15–17). Extended data, past 24 hours, is unavailable. Given the long half-life of dexamethasone (36-54 hours), a prolonged effect might be anticipated. Continuous day-curves of glucose sampling after dexamethasone are not reported but after a single pre-operative

dose 10mg dexamethasone in people without diabetes, peak glucose was 2.5 mmol/L higher at 4 hours compared to control (18), and significant increment, within 2 hours may be seen after intravenous dosing (19).

COVID-SPECIFIC EFFECTS ON GLUCOSE HANDLING

There may be a bidirectional relationship between diabetes and COVID-19 whereby COVID-19 can worsen, or precipitate diabetes and the presence of diabetes may worsen the severity of the COVID-19 illness (20). A positive feedback loop is thus engendered.

COVID-19 Causing Hyperglycaemia and Diabetic Ketoacidosis

Acute hyperglycaemia has been seen in individuals infected with SARS-CoV-2 but without known diabetes (21–26). In these patients, the degree of admission hyperglycemia predicts mortality and disease severity. The risk of COVID-19-related hospitalisation and mortality has also been shown to be greater in individuals with long-term hyperglycaemia (represented by higher HbA_{1c}) (27–29).

Hyperglycaemia in COVID-19 may represent an effect on insulin resistance but it has also been questioned whether insulin production might also be affected. A decade ago, it was hypothesized that SARS coronavirus may directly damage islet cells (30). More recently, *in vitro* studies suggest that SARS-CoV-2 infection of pancreatic endocrine cells results in robust chemokine induction and upregulation of markers of cell death (31). Observational data of clinical outcomes provides support for a direct pancreatic insult: diabetic ketoacidosis (DKA) has been associated with COVID-19 disease (32–34). Reports from China, early in the pandemic, suggested ketosis was a relatively frequent occurrence: of 658 patients, 42 (6.4%) presented with positive urine or serum ketones, and, of these, three (7%) patients met the American Diabetes Association (ADA) criteria for DKA (33). Those with ketosis were about twice as likely to have diabetes at baseline, and the 3 individuals who developed DKA had underlying diabetes (one with type 1 diabetes, two with type 2 diabetes). A marked increase in DKA was also observed in children and adolescents in Germany and Australia during the COVID-19 pandemic (35, 36). However, other groups have found no increased incidence of new-onset type 1 diabetes during this COVID-19 pandemic, compared to historical rates (37). Furthermore, antibody positivity to SARS-CoV-2 has not been associated with greater risk of type 1 diabetes in children (38).

SARS-CoV-2 enters human cells *via* co-expression of its cell entry factors, angiotensin-converting enzyme 2 (ACE2) and its obligate co-factor, transmembrane serine protease 2 (TMPRSS2). However, analysis of six transcriptional datasets of primary human islet cells found that *ACE2* and *TMPRSS2* were not co-expressed in single β cells (39), suggesting that direct viral entry is not the means of pancreatic damage with COVID-19.

There have been small case series of individuals with COVID-19 with mildly raised lipase and/or amylase but not meeting the criteria for pancreatitis (40). Therefore there is no convincing evidence for more diffuse pancreatic injury as a mechanism for insulinopaenia.

Black individuals have been particularly affected by COVID and are over-represented in series with ketosis (41). It is possible that the clinical picture of ketosis in COVID relates, in part, to unmasking ketosis-prone type 2 diabetes (KPDM) – which has been linked with Black ethnicity (42). Alternatively, increase in the prevalence of severe DKA in COVID-19 positive patients might relate to delayed hospital admission and/or accessing medical advice.

In summary, ketosis is associated with length of hospital admission and overall mortality (33). The data appear to show that Covid-19 causes DKA more often than other respiratory tract viral infections.

Diabetes Predisposing to Infection With COVID-19

Elevated glucose levels can directly induce SARS-CoV-2 viral replication in human monocytes. Glycolysis appears to sustain SARS-CoV-2 replication *via* the production of mitochondrial reactive oxygen species and activation of hypoxia-inducible factor 1α (HIF1α) (43). HIF1α, in turn, upregulates glycolytic genes and IL-1β expression. Therefore, acute hyperglycaemia might directly support viral proliferation. Furthermore, people with diabetes have a number of pathophysiological changes that may underlie a more severe clinical response to COVID-19, these include: greater proinflammatory cytokine release, compromised host immune responses, endothelial dysfunction, and a greater propensity for development of coagulation-related complications (44, 45). Taken together, diabetes leads to greater viral replication and more severe COVID-19 disease, leading to greater hyperglycaemia.

EFFECT OF CRITICAL ILLNESS ON GLUCOSE, FAT AND PROTEIN METABOLISM

Endogenous Hypercortisolaemia

Under non-stressed conditions, the adrenal cortex produces approximately 20 mg of cortisol during the day. This then increases within 4-6 hours of acute stress, from a baseline of approximately 400 nmol/l, to a peak of more than 1500 nmol/L (depending on the severity of illness) (46). Estimates for equivalency of hydrocortisone dosing have ranged from 60 to 200 mg cortisol per day (47, 48).

Cortisol production is at least partially ACTH-dependent. There is a stimulatory effect on the hypothalamus by inflammatory mediators such as TNFα and IL-1 for the release of CRH (49). Cytokines can also have an effect downstream on the pituitary; for instance, IL-6 appears to directly stimulate the release of ACTH (49). However, the concept of vastly increased corticosteroid production in critical illness has been challenged.

Using stable isotope tracers, the rate of appearance of cortisol, was only 1.8-fold higher in critically ill patients than in healthy matched controls in the presence of low morning plasma ACTH values (47). Therefore impaired cortisol clearance likely also contributes to hypercortisolaemia. Hypoperfusion of cortisol-metabolizing organs could, theoretically, reduce cortisol breakdown but there is evidence for reduced hepatic expression and activity of cortisol-metabolizing enzymes 5α- and 5β-reductase and renal 11β-hydroxysteroid dehydrogenase-2 **(Figure 1)** (47). Cortisol-binding globulin (CBG) decreases in the context of physiological stress (50). The concentration of CBG being negatively associated with mortality in septic shock (51). The net effect of an elevation in total cortisol and a reduction in cortisol-binding globulin will be to increase free cortisol levels (50). Greater cortisol concentration is associated with increased mortality in COVID-19 (52).

Secretion of cortisol can be driven by factors outside the HPA axis in critical illness (53). This is supported by reduced adrenocorticotropic hormone (ACTH) and the increased irregularity and asynchrony of the ACTH and cortisol time series during critical illness (54). A biphasic response to critical illness has been proposed whereby an initial ACTH-dependent process gives way to later non-ACTH pathway (55). Within the adrenal gland, macrophages, and lymphocytes, physiologically widely infiltrating the adrenal cortex, and adrenocortical, and chromaffin cells produce cytokines, as IL-1, IL-6, TNFα, leukaemia inhibitory factor (LIF), and IL-18 which have a key role in the immune-adreno-cortical communication (56).

Insulin Resistance

Hypercortisolaemia will increase the rate of hepatic gluconeogenesis and inhibit glucose uptake and utilisation by peripheral tissues (57, 58). Unlike in health, where glucocorticoids promote hepatic glycogen storage, acute illness is characterised by markedly reduced glycogen synthesis (59).

The action of glucocorticoids will be compounded by elevated circulating catecholamines, which can antagonise the actions of insulin by several mechanisms: they can stimulate glucagon by a β-adrenergic effect, increase hepatic glucose production by direct stimulation of glycogenolysis and gluconeogenesis and decrease glucose uptake (60). A β2 receptor mediated increase in lipolysis could also exacerbate insulin resistance through ectopic fat distribution, release of adipokines or promoting macrophage infiltration of adipose tissue (61). Critical illness is associated with markedly elevated levels of glucagon which increases hepatic amino acid catabolism, contributing to the illness-induced hypoaminoacidaemia (62). In COVID-19, the profound viral induced inflammation, in particular IL-6 mediated, will further increase insulin resistance (63). The severity of pneumonitis correlates with the insulin requirement, but there does not appear to be a specific effect of COVID-19 on insulin resistance (64).

Catabolism Induced by Insulin Resistance

The surge in proinflammatory mediators and counter-regulatory hormones, favours the shift to catabolism marked by insulin resistance - with insulin sensitivity reduced by 70% **(Figure 2)** (65). Indeed, in the presence of critical-illness, hepatic glucose production increases at least twofold compared to healthy controls, to rates approaching 15 – 25 μmol/kg/min (66, 67). Hyperglycaemia is also the result of diminished insulin-mediated glucose uptake by skeletal muscle (59, 68). Critically ill patients have significantly lower, and more variable insulin sensitivity, on day 1 than later in their intensive care unit (ICU) stay (69, 70), although insulin resistance may persist for months (71). The acute effect is likely due to the acute counter-regulatory response to critical illness as described above. Catabolism, insulin resistance and stress hyperglycaemia are evolutionarily responses designed to allow the host to survive during periods of severe stress. Glucose can be utilized by tissues that are central to the recovery process. These include the central and peripheral nervous system, bone marrow, leucocytes and erythrocytes and the reticuloendothelial system. Glucose uptake to these tissues is non-insulin dependent - hence greater glucose concentration

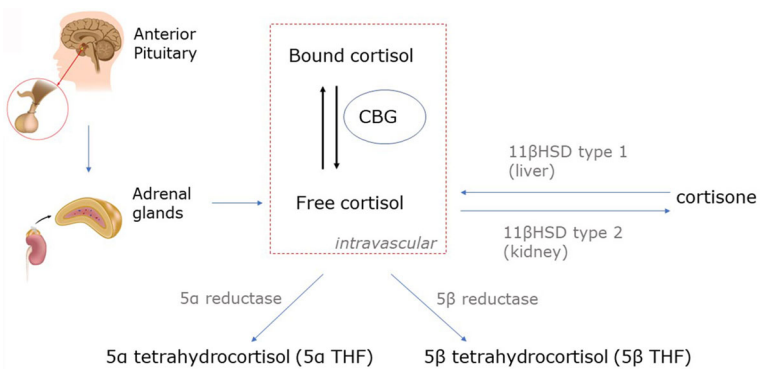

FIGURE 1 | Cortisol metabolism. Cortisol is converted in peripheral tissues to cortisone by 11β-hydroxysteroid dehydrogenase (11β-HSD). Cortisone has marginally reduced glucocorticoid activity compared to cortisol (80-90%), and thus, cortisone can be considered an active metabolite of cortisol. Unbound cortisol is biologically active, but the majority of circulating cortisol is bound to corticosteroid-binding globulin (CBG) and albumin. Cortisol is metabolized by 5α- and 5β reductases to form 5α- and 5β-tetrahydrocortisol (5α- and 5β-THF).

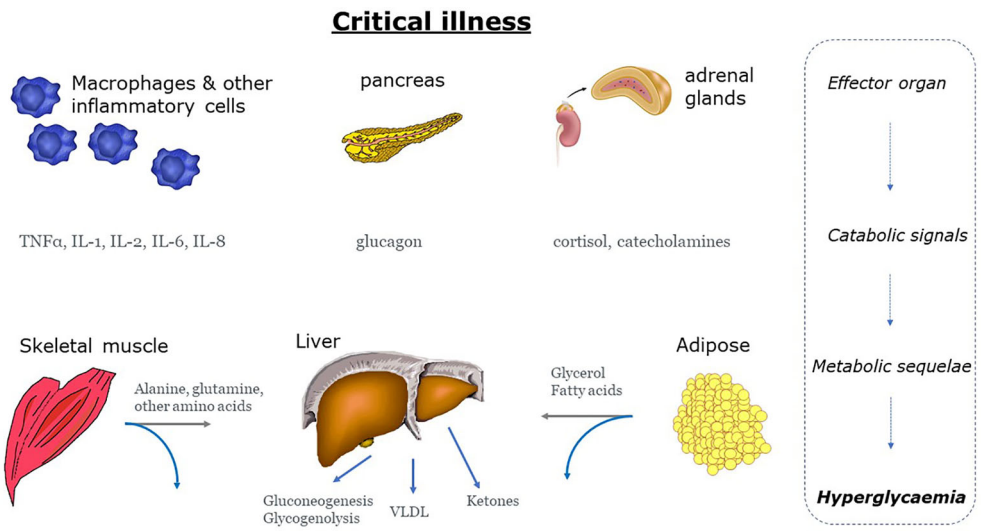

FIGURE 2 | Mechanisms of hyperglycaemia during states of stress and inflammation. Stress hyperglycemia is the end result of a neurohumeral and inflammatory process characterized by excessive gluconeogenesis and glycogenolysis and impaired insulin-mediated glucose uptake. Grey arrows: gluconeogenic precursors IL, interleukin; TNF-α, tumour necrosis factor-α; VLDL, very low-density lipoprotein.

facilitates uptake (72). This evolutionary paradigm - of either survival or rapid deterioration - has been superseded by the ability to 'suspend' critical illness for days or weeks with modern critical care. In the modern era, prolonged or severe hyperglycaemia is associated with increased risk of critical illness polyneuropathy and prolonged mechanical ventilation. Loss of lean body mass is associated with poor ICU survival, or delayed recovery in survivors (73).

Metabolomic and lipidomic approaches have shown that circulating triglyceride and fatty acid concentrations correlate with disease severity in COVID-19 (74). This mirrors data from septic patients in the first days of hospital admission (75). Microdialysis catheters have been used in femoral adipose tissue in patients with systemic inflammatory response syndrome/severe sepsis or shock. On day 1 of ICU admission 56% of patients had increased interstitial levels of glycerol and FFA, the two products of lipolysis, with glycerol concentrations being higher in those receiving glucocorticoids (76). Increased very-low density lipoprotein (VLDL) production by the liver also contributes to the elevation of plasma triglyceride concentration in sepsis (77). By contrast, the absorption of lipid from the small intestine is diminished in critical illness (78).

Protein Catabolism

Negative nitrogen balance has been linked to detrimental clinical outcomes. The survival of critically-ill patients, their duration of ICU admission, and the duration to recovery of normal physiological function, are all inversely correlated with loss of lean body mass during hospitalisation (79). As the largest protein pool, it is unsurprising that the major site of protein loss is from skeletal muscle. Muscle biopsy studies in the critically-ill have shown rapid decreases in myosin heavy-chain mRNA and protein expression by the fifth day of ICU admission (80), with average of 2% loss per day over the first 10 days (80–82). The duration of corticosteroid treatment, independent of duration of intensive care unit stay or other risk factors, is a dominant risk factor for a low myosin/actin ratio (81). Long-term outcomes from ICU-acquired weakness are significant and include lower one-year survival, and reduced walk and exercise ability five-years later (83).

The predominant defect appears to be an accelerated rate of proteolysis that cannot be compensated for by a moderate rise in the rate of protein synthesis (84, 85). There are multiple stimuli for the increase in muscle catabolism, including hormonal and cytokine but regression analysis found that plasma cortisol concentration was the most significant predictor of protein breakdown (where it explained nearly 40% of the variance) (84). These data are consistent with earlier studies in normal subjects, whereby artificial elevation of plasma cortisol - to levels observed after trauma - resulted in a 15% increase in whole body protein breakdown (86). The possibility of hyperglycaemia, itself, acting as a spur for proteolysis has been explored in normal subjects with the use of combined insulin and somatostatin administration (87). Using stable isotopic tracer methodology, hyperglycaemia (~ 10.5mmol/L) was associated with a three-fold increase in proteolysis, without alteration of whole-body protein synthesis or protein oxidation compared to normoglycaemia (~5.2mmol/L). A retrospective review of burned patients suggested a correlation between the extent of proteolysis and prevailing glycaemia, with maximal proteolysis occurring in patients with plasma glucose above 12.8 mmol/L and least catabolism in those with plasma glucose below 8.6 mmol/L (88). However, both hyperglycaemia and protein degradation may have merely represented the disease severity and by extension, the degree of insulin resistance. Sepsis can significantly increase protein catabolism and exacerbate muscle

protein loss in already hypercatabolic patients (89), suggesting a significant role for cytokines as catabolic factors. Cytokines and stress hormones increase protein turnover *via* a common mechanism involving the activation of muscle-specific ubiquitin-ligases (82).

Proteolysis and Secondary Infection

A catabolic state may compromise the immune response by mechanisms such as poor wound healing, altered mucosal barrier, tissue oedema due to low albumin and reduced muscle strength (and vital capacity) leading to pneumonia. Loss of respiratory muscular power will prolong ventilation and adversely affect the patient's ability to clear the airways with sufficient cough and thus increase the risk of pneumonia (79). Skeletal muscle contributes in a bidirectional role in systemic inflammatory signalling and the modulation of the inflammatory response including by release of heat shock proteins (HSP) (90). Skeletal muscle provides a key nutrient to the immune system in the form of glutamine (91), which is a constitutively essential amino acid during catabolic situations. Glutamine acts as an energy substrate for leucocytes and is necessary for tissue repair and intracellular pathways associated with pathogen recognition (92). Deficiency of a skeletal muscle amino acid reservoir would render a patient more susceptible to death from multiple organ failure following a 'second-hit' episode of sepsis as there would be inadequate substrate supply for immune function.

The proportion of patients with COVID-19 plus secondary bacterial infections ranges from 5% to 30% (93) and the incidence rate of bacterial blood-stream infections among patients with COVID-19 admitted to the ICU appears to be higher than in historical cohorts (93, 94). Rates of bacterial secondary infection in severe COVID-19 will be skewed by prescription of antibiotics - to cover for bacterial superinfection (as with during influenza pandemics) – as advocated by several guidelines (95).

THE METABOLIC EFFECTS OF A SHORT-COURSE OF GLUCOCORTICOIDS

Administration of even relatively low doses of prednisolone (6-7.5 mg daily) over one to two weeks acutely increases basal hepatic glucose production and reduces insulin mediated suppression of hepatic glucose production and stimulation of peripheral glucose disposal (57, 58). Glucocorticoids will inhibit the conversion of pyruvic acid to acetyl-coenzyme A, leading to an accumulation of pyruvic acid and resulting in glucose re-synthesis (96). Induction of gluconeogenic enzymes, such as glucose-6-phosphatase, fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase, add to this effect (97). In the liver, glucocorticoids increase glycogen storage, which can be observed from three to twenty-four hours after the administration of glucocorticoids (96), whereas in skeletal muscle they play a permissive role for catecholamine-induced glycogenolysis and/or inhibit insulin-stimulated glycogen synthesis (98). A negative effect on first- and second-phase insulin release is also seen with glucocorticoids, possibly mediated *via* a reduced insulinotropic effect of glucagon-like peptide-1 (GLP-1) (99, 100).

Acutely, over 5-7 days, glucocorticoids in therapeutic doses can induce protein catabolism, in healthy subjects, by increasing the rate of protein degradation by the ubiquitin-proteasome system and autophagy lysosome system (101) and by increasing whole-body protein oxidation (102). Protein synthesis is also suppressed at the level of translational initiation, preventing the production of new myofibrillar protein (101). A dose-response gradient with worsening whole body protein metabolism at increased steroid doses, has been measured with isotopic techniques (103).

ANTICIPATED OUTCOMES OF INSULIN USE IN HOSPITALISED PATIENTS RECEIVING DEXAMETHASONE

Clinical Outcomes

The historic paradigm that hyperglycaemia in critically-ill patients was an adaptive response that provided glucose for the brain, red cells, and wound healing meant that the approach to treatment was to treat the blood glucose only once high enough to cause an osmotic drag and produce a diuresis (approximately 11-12mmol/L). This approach was reconsidered following the publication of two randomised controlled trials from Leuven of insulin use in critically-ill patients (104, 105). The first study involved adults admitted to a surgical ICU with glucose targets in the intervention group of 4.5 - 6.1 mmol/L, compared with a comparatively high ceiling for the control group of 10.0 – 11.mmol/L) (105). Tight control reduced ICU mortality from 8% to 4.6%. Only 13% of the patients had diabetes. Most benefit was amongst patients with multiple organ failure and sepsis. Of importance, 62% of admissions were due to cardiac surgery and an effect of glucose/insulin on the myocardium was postulated. The insulin infusion rate was (mean) 0.04 iU/kg/hr; consuming 9g glucose/hr (105). In contrast, studies using a fixed glucose-insulin-potassium (GIK) regime, with acute myocardial infarction, to promote a switch away from myocardial fatty acid metabolism to glucose metabolism, were approximately 0.1 - 1 iU/kg/hr; 30 - 80 g glucose/hr) (106). Expectation that cardio-metabolic modulation with high-dose insulin could improve outcomes were diminished after the neutral results seen in the large Clinical Trial of Reviparin and Metabolic Modulation in Acute Myocardial Infarction Treatment and Evaluation-Estudios Clinicos Latino America (CREATE-ECLA) (107). Furthermore, *post-hoc* analysis of the Leuven surgical study (105) suggested that the benefit accrued from normoglycaemia, rather than from hyperinsulinaemia (108).

The second Leuven study was in medical ICU patients, where no mortality benefit was seen, except in those requiring ICU stays of three or more days (104). These data suggest that insulin may protect against the development of organ failure (particularly from sepsis), rather than reversing pathological processes once established. Three other studies also did not

show benefit in mixed medical and surgical ICUs. The Volume Substitution and Insulin Therapy in Severe Sepsis (VISEP) study enrolled 480 severe sepsis patients who were randomized to tight glycaemic control or standard glucose control (109). VISEP was suspended early for increased rates of hypoglycaemia in the intensive control arm (17.6% *vs* 4.5%) and no difference in 28-day or 90-day mortality. The Glucontrol study was also suspended after enrolment of 1101 patients due to safety and protocol concerns (110). There was no difference in mortality, but rates of hypoglycaemia were approximately 4 times higher in the intensive insulin group (9.8% *vs* 2.7%). The Normoglycemia in Intensive Care Evaluation–Survival Using Glucose Algorithm Regulation (NICE-SUGAR) study randomised 6104 patients to a target of 4.5 - 6.0 mmol/L or to < 10 mmol/L (111). There was a greater risk of mortality in the intensive glycaemic control group (odds-ratio 1.14) with no difference in the length of ICU or hospital stay. Once again, the risk of hypoglycaemia was significantly higher in the intensively treated group than conventionally treated (6.8% *vs* 0.5%). Thereafter, glycaemic targets in ICU have been pragmatically orientated at 8 - 10 mmol/l (112).

By contrast, there has been little direct evidence that treating hyperglycaemia reduces morbidity or mortality on a general medical or surgical ward. New hyperglycaemia in hospitalized patients, of any aetiology, is associated with a much greater risk of mortality than chronic hyperglycaemia (113). Acute hyperglycaemia affects the innate and adaptive immune responses at multiple levels: it reduces neutrophil degranulation, chemotaxis, and phagocytic activity; impairs complement activation; and inhibits lymphocyte proliferative response (114). However, the pathogenesis of hyperglycaemia is important for the interpretation of clinical outcome data as in those without pre-existing diabetes it has worse prognosis. In these cases, it may be that hyperglycaemia is a surrogate for illness severity.

Historically, the effect of hyperglycaemia on viral outcomes has been less clear (44). However, given the unique interplay between hyperglycaemia and SARS-CoV-2 replication, an *a priori* case can be made for glycaemic control to reduce the severity of COVID-19. Retrospective reports have shown that glucose control preceding admission impacts illness severity and mortality (27, 29). Few data exist for post-admission glycaemic control. In a small study of 25 patients with hyperglycaemia and hospitalised with COVID-19, use of intravenous insulin to achieve a mean glucose of 7.69 ± 1.85 mmol/L (*vs* 10.65 ± 0.84 mmol/L in the no insulin infusion group) was associated with reduced IL-6 and D-dimer levels and improved composite end-point (admission to an ICU, the use of mechanical ventilation, or death) (23).

Anti-Catabolic Action

Hepatic Glucose Production and Peripheral Glucose Uptake

Glucose infusion at 4mg/kg/min, raising blood glucose to 10mmol/L and endogenous plasma insulin to ~400pmol/L failed to suppress lipolysis following elective colorectal surgery (115). By contrast, normalisation of blood glucose (to 5.9 ± 0.3 mmol/L) with exogenous insulin can significantly reduce plasma triglycerides within 24 hours (116), through suppression of lipolysis (68). Therefore, infusion of glucose, without concomitant insulin, is unable to suppress lipolysis in critical illness.

Normalisation of blood glucose is associated with an increase of peripheral glucose uptake (68, 116), but it has been suggested that exogenous insulin administration is unable to overcome hepatic glucose production in critically-ill patients (117). Insulin regulates hepatic gluconeogenesis *via* phosphoenolpyruvate carboxylase (PEPCK) which decarboxylates oxaloacetate to phosphoenolpyruvate in the gluconeogenic pathway. Uncontrolled expression of PEPCK was associated with poor prognosis in critically-ill patients (117), which led the authors to conclude that hepatic insulin resistance could not be overcome and that normalisation of blood glucose with insulin in critically-ill patients must instead be attributable to increasing glucose disposal. However, these data came from post-mortem studies and so the lack of an hepatic effect of insulin might simply represent the degree of metabolic derangement associated with illness severity: for instance glucocorticoids can independently up-regulate PEPCK gene expression (97). Patients in this study had an ICU stay greater than 5 days. This is pertinent as it has been proposed that the site of insulin-resistance could change with time; within 24 hours postoperatively it is mainly the peripheral tissues that are affected (118), whereas by the third postoperative day, the liver appears to be most resistant to insulin (119). Our group has shown that variable dose intravenous insulin administered to medical ICU patients for 48 hours (started within 36 hours of admission), to maintain blood glucose between 7 – 9 mmol/l is sufficient to limit hepatic glucose production rate (68).

Protein Turnover

Glucose intolerance seen in critical-illness is but one manifestation of insulin resistance – a process that could also manifest in terms of muscle protein catabolism.

Insulin's effect on protein metabolism in the healthy adult has been contentious but it appears primarily to act *via* the inhibition of proteolysis (120–122), although increased protein synthesis has also been suggested (123). Interpreting the mechanism of action of insulin on protein anabolism is complicated by its other physiological action – that of causing hypoaminoacidaemia. Models of protein turnover involving the measurement of blood-flow across a limb combined with muscle biopsies have been used, predominantly in burned subjects, to examine the effect of insulin on protein turnover. It has been considered that critical-illness leads to impaired amino acid uptake by myocytes, resulting in reduced protein synthesis. In two papers it was suggested that resistance to amino acid uptake may be overcome by a combination of high-dose insulin infusion (to achieve plasma insulin concentrations in the range of 2000 to 5000 pmol/L) plus amino acid provision (124, 125). This had the effect of increasing protein synthesis by approximately 350%, due to a six-fold increase in amino acid transport into the cells. As amino

acids by themselves were unable to fully support protein synthesis, it was suggested that insulin may have an independent role in protein synthesis. However, two groups have used the amino acid clamp technique to show that in the presence of adequate amino acid availability, increasing the insulin concentration had no further effect on protein synthesis (126, 127). One small study showed decreased whole-body protein breakdown and synthesis in cardiac surgery patients when administering glucose and insulin under maintenance of normoglycemia (128). However, other studies of ICU patients randomized to tight blood glucose control (4.4-6.1 mmol/L) with conventional, low-dose, insulin infusion protocols have shown no amelioration of muscle loss (81, 129), or whole-body protein turnover (68). None of these studies delivered supplemental amino acids although 0.13 to 0.26 g of nitrogen per kilogram per 24 hours was the standard approach, within 24 hours of ICU admission (68, 81).

What if a hyperinsulinaemic approach was used, rather than conventional low-dose insulin? Exogenous provision of glucose has several theoretical benefits in terms of protein sparing. Firstly, it would be expected to shift substrate utilisation towards increased oxidation of glucose instead of protein. Secondly, exogenous glucose might decrease hepatic glucose production and thereby act indirectly to reduce the need for gluconeogenic precursors. Thirdly, it might drive the accompanying need for insulin and the benefits on protein sparing that might ensue. We have previously reported that despite the infusion of high-dose insulin, causing a six-fold rise in plasma insulin (to ~1500 pmol/L) over the conventional insulin infusion rate, proteolysis was unaffected and remained significantly higher than in the control subjects (68). Such a finding is consistent with previous observations, in both normal subjects and surgical patients, that glucose administration (≥ 4mg/kg/min, causing a doubling of insulin concentration) does not influence the degradation of peripheral protein (130, 131). Our group has also shown that insulin and glucose administration was capable of full suppression of glucose rate of appearance despite ongoing proteolysis (68), suggesting that the function of proteolysis is not to provide gluconeogenic precursors.

FUTURE RESEARCH QUESTIONS

Work is needed to further understand the interplay between diabetes and COVID-19. Mechanistic studies are needed to determine the effect of COVID-19 on tissue-specific insulin resistance, the impact on pancreatic B-cell dysfunction, and pulmonary perfusion in the presence of hyperglycaemia (44, 45). The international CoviDiab registry is expected to address a number of these questions (20, 44). Knowledge by which SARS-CoV-2 impacts upon glucose metabolism will be critical for understanding disease pathogenesis and development or choice of therapies.

It would be unrealistic to expect a prospective randomised controlled trial of glucose normalisation on COVID-19 outcomes, but effort must be made for retrospective analyses of propensity-matched subjects. Attention must also be paid to the long-term metabolic sequalae of COVID – given the catabolic processes outlined in this review. Further data are needed on COVID-19 survivors for nutritional status and measures of functional independence in the months after critical care for COVID-19. Early rehabilitation programs are already being evaluated in ongoing clinical studies (132).

CONCLUSION

Following the RECOVERY trial results, the use of short-courses of glucocorticoid therapy will be widespread in the remaining time of the COVID-19 pandemic. Based upon the evidence reviewed, the ten-day course of the RECOVERY protocol will be expected to increase both hepatic and peripheral insulin resistance and lead to skeletal muscle loss. Current evidence suggests exogenous insulin should be able to overcome the hepatic and peripheral insulin resistance of glucose metabolism but is unlikely to impact upon skeletal muscle loss engendered by glucocorticoids. Strategies to achieve glycaemic normalisation might have a direct disease modifying effect on the SARS-CoV-2 virus. Further work is needed to develop strategies to limit muscle loss. Even so, we may see a long-term effect on functional capacity from the critical-illness induced by COVID-19.

AUTHOR CONTRIBUTIONS

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.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 18 June 2021
doi: 10.3389/fendo.2021.694083

Testosterone Deficiency Is a Risk Factor for Severe COVID-19

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Background: Male sex is related to increased COVID-19 severity and fatality although confirmed infections are similarly distributed between men and women. The aim of this retrospective analysis was to investigate the impact of sex hormones on disease progression and immune activation in men with COVID-19.

Patients and Methods: We studied for effects of sex hormones on disease severity and immune activation in 377 patients (230 men, 147 women) with PCR-confirmed SARS-CoV-2 infections hospitalized at the Innsbruck University Hospital between February and December 2020.

Results: Men had more severe COVID-19 with concomitant higher immune system activation upon hospital admission when compared to women. Men with a severe course of infection had lower serum total testosterone (tT) levels whereas luteinizing hormone (LH) and estradiol (E_2) levels were within the normal range. tT deficiency was associated with elevated CRP (rs = - 0.567, $p < 0.001$), IL-6 levels (rs = - 0.563, $p < 0.001$), lower cholesterol levels (rs = 0.407, $p < 0.001$) and an increased morbidity and mortality. Men with tT levels < 100 ng/dL had a more than eighteen-fold higher in-hospital mortality risk (OR 18.243 [95%CI 2.301 – 144.639], $p = 0.006$) compared to men with tT levels > 230 ng/dL. Moreover, while morbidity and mortality showed a positive correlation with E_2 levels at admission, we detected a negative correlation with the tT/E_2 ratio upon hospital admission.

Conclusion: Hospitalized men with COVID-19 present with rather low testosterone levels linked to more advanced immune activation, severe clinical manifestations translating into an increased risk for ICU admission or death. The underlying mechanisms remain elusive but may include infection driven hypogonadism as well as inflammation mediated cholesterol reduction causing gonadotropin suppression and impaired androgen formation. Finally, in elderly late onset hypogonadism might also contribute to lower testosterone levels.

Keywords: testosterone, estradiol, inflammation, COVID-19, SARS-CoV-2, disease severity, outcome

OPEN ACCESS

Edited by:
Alexandra Kautzky-Willer, Medical University of Vienna, Austria

Reviewed by:
Hao Chen, Nantong University, China
Johnny S. Younis, The Baruch Padeh Medical Center, Poriya, Israel

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *12 April 2021*
Accepted: *01 June 2021*
Published: *18 June 2021*

Citation:
Lanser L, Burkert FR, Thommes L, Egger A, Hoermann G, Kaser S, Pinggera GM, Anliker M, Griesmacher A, Weiss G and Bellmann-Weiler R (2021) Testosterone Deficiency Is a Risk Factor for Severe COVID-19. Front. Endocrinol. 12:694083. doi: 10.3389/fendo.2021.694083

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is still influencing the daily life of people all over the world. Most patients infected with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have developed mild symptoms, while up to 20% need hospitalization due to severe disease course characterized by shortness of breath and hypoxia (1). Several comorbidities including hypertension, obesity, diabetes, cardiovascular disease, and chronic pulmonary disease as well as age were shown to be related to more severe COVID-19 courses (2–5). Additionally, male sex is related to more severe COVID-19 manifestation, even though there are no sex differences in the absolute number of confirmed COVID-19 cases (6). Currently, up to 60% of hospitalized patients and even up to 82% of patients treated in the intensive care unit (ICU) are men (3, 4, 6). Accordingly, the case fatality rate (CFR) is 1.7 times higher in men compared to women (6–8). Inflammatory biomarkers were shown to be associated with COVID-19 morbidity and mortality in men and women: interleukin 6 (IL-6) as main inducer of C-reactive protein (CRP) in the liver (9), neopterin reflecting macrophage activation and thus T-helper cell type I (Th1) immune response (10) as well as other acute phase proteins including procalcitonin (PCT) and ferritin (11).

Several reviews have elucidated potential mechanisms underlying these sex differences in COVID-19 patients and will be only shortly summarized hereafter (6, 8, 12). Biological sex affects immune responses to invading pathogens through hormonal regulation, gene expression and environmental factors (8). Generally, women have a stronger innate and adaptive immune response than men resulting in faster pathogen clearance (13). Many genes located on the sex chromosomes regulate innate immune function by encoding for pattern recognition receptors, cytokine receptors or transcriptional factors (14). On the other hand, androgen response elements and estrogen (E_2) response elements are found in promoters of several innate immunity genes thereby affecting their expression (15, 16). These mechanisms are suggested to contribute to faster containment and clearance of SARS-CoV-2 in female patients (8). Moreover, sex-associated differences in expression and activity of the virus entry receptor angiotensin-converting 2 (ACE2) and its co-receptor transmembrane protease serine subtype 2 (TMPRSS2) are suggested to contribute to a higher mortality in men (17, 18). The ACE2 gene is located on the X chromosome and estrogens were shown to upregulate its expression (19), while TMPRSS2 was shown to be regulated by androgen receptor signalling (20–22). Preclinical studies of ACE2 tissue expression have shown different results depending on the tissue type (23): ACE2 expression seems to be higher in the lungs, heart or kidney of male (24–26), while pancreatic ACE2 expression seems to be higher in female (27). However, the relationship of tissue ACE2 expression and circulating ACE2 activity is still not well understood and data from the literature is partial contradictory (23): in human, the ACE2 activity was shown to be higher in healthy men and men with heart failure compared to matched women (28, 29) while other studies showed no sex-related differences in serum ACE2 activity (30, 31). Interestingly, ACE2 activity does not differ between young and old men but is significantly higher in older compared to younger women (31).

However, whether these mechanisms actually affect disease severity and clinical outcome of COVID-19 patients or explain the sex differences remains unknown. Therefore, we analyzed different sex hormones and their interactions with inflammatory markers to assess the impact on disease severity and outcome in men: the androgen testosterone is the primary male sex hormone playing a key role in the male reproductivity and produced by testicular Leydig cells upon stimulation by luteinizing hormone (LH) released by the pituitary gland and regulated by the gonadotropin-releasing hormone (GnRH) released by the hypothalamus (32). Estradiol (E_2) is the primary female sex hormone but also involved in male reproduction and synthesized from androgens by aromatase (33). Sex hormones are transported in the human body unbound in the serum or bound to the sex hormone binding protein (SHBG) (32).

MATERIAL AND METHODS

Study Population

We analyzed 377 patients with polymerase chain reaction (PCR)-proven COVID-19 hospitalized at our department at the Medical University of Innsbruck, Austria, between February and December 2020. Information on past medical history, concomitant medication, clinical characteristics and laboratory parameters were obtained from the local clinical electronic data management. The severity of the disease was categorized according to the score by the WHO Working Group on the Clinical Characterization and Management of COVID-19 infection (34). Events during hospital stay including death, ICU admission and need for mechanical ventilation were recorded for outcome analysis.

The study was conformed to the principles outlined in the Declaration of Helsinki and was approved by the ethics committee of the Innsbruck Medical University and patients had given informed consent (ID of ethical vote: 1167/2020).

Laboratory Measurements

All analyses were conducted at the ISO 15189 accredited Central Institute of Clinical and Chemical Laboratory Diagnostics (Medical University of Innsbruck, Austria) according to the manufacturers' procedures. Blood samples for detection of sex hormones were taken guideline conform till 11 a.m. within the first three days after hospital admission. A fully automated analyzer (Cobas 8000) by Roche Diagnostics GmbH (Mannheim, Germany) comprising an indirect potentiometric unit (ISE module), a chemistry unit (module 702), and an immunological unit (module e602) was used to determine the following parameters: creatinine (CREP2, enzymatic), aspartate transaminase (AST; ASTPM), alanine aminotransferase (ALT; ALTPM), alkaline phosphatase (ALP; ALP2), interleukin 6 (IL-6; Elecsys IL-6), cholesterol (CHOL2), high-density lipoprotein (HDL; HDLC4), low-density lipoprotein (LDL; LDLC3),

triglycerides (TRIGL), estradiol (E_2; Elecsys Estradiol III), procalcitonin (PCT; Elecsys BRAHMS PCT), C-reactive protein (CRP; CRP4), and ferritin (FERR4). Glycated hemoglobin (HbA1c) was measured by liquid chromatography on a TOSOH G8 instrument (Tosoh Corporation, Shiba, Minato-ku, Japan). Fibrinogen was determined on a Siemens analyzer (BCS-XP) using reagents from Siemens (Multifibren U). Androstenedione, dehydroepiandrosterone (as sulfate), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and sexual hormone-binding globulin (SHBG) were quantified using reagents from Siemens on an IMMULITE ® 2000 XP analyzer. Neopterin was measured using the ELISA from IBL International (Hamburg, Germany) on a Dynex DS2 automated ELISA system (Dynex Technologies, Chantilly, USA) and total testosterone (tT) was determined using high-pressure liquid chromatography hyphenated with tandem mass spectrometry *via* an in-house developed method. Free testosterone (fT) was measured *via* a CLIA assay obtained from IDS iSYS (Immunodiagnostic Systems GmbH, Frankfurt am Main, Germany) on an IDS-iSYS Multi-Discipline Automated System. All hematological parameters (thrombocytes, leucocytes, lymphocytes, hemoglobin, and hematocrit) were measured on a Sysmex automated hematology analyzer (XN series).

We calculated the luteinizing hormone to total testosterone (LH/tT) ratio to specify the hypothalamic-pituitary-gonadal axis (35) and the total testosterone to estradiol (tT/E_2) ratio to reflect aromatase activity (36). Reference ranges for total testosterone (tT) levels were based on the guideline of the investigation, treatment and monitoring of functional hypogonadism in males by the European Academy of Andrology (EAA): serum total testosterone (tT) levels were classified to be reduced when tT levels ≤ 230 ng/dL, borderline when tT levels between 231 – 350 ng/dL and normal when tT levels > 350 ng/dL (37).

Statistical Analyses

Parameters are reported as n (%) or medians (25th, 75th percentile) since the data were not normally distributed (Shapiro-Wilk test). To test for differences between men and women we used the Mann-Whitney-U test or Pearson chi-square tests. Kruskal-Wallis test was performed to test for significant differences between more than two groups. Analysis of the effect of risk factors on the probability of death or ICU admission during the in-hospital stay was performed with logistic regression analysis (not normally distributed parameters were logarithmized with the natural logarithm). All tests were two-tailed and p-values < 0.05 were regarded as statistically significant. Statistical analysis was performed using SPSS Statistics Version 27 (IBM Corporation, Armonk, NY, USA).

RESULTS

Sex Differences in Baseline Characteristics

Within this retrospective analysis we investigated clinical, hormonal and inflammatory parameters in 230 men (61.0%) and 147 women (39.0%) with PCR-confirmed COVID-19 disease and a median age of 67 years in men and 70 years in women (p = 0.540). Upon initial hospital admission men presented with significantly lower serum cholesterol (121 *vs.* 141 mg/dL, p < 0.001), LDL (72 *vs.* 83 mg/dL, p = 0.001) and HDL levels (31 *vs.* 40 mg/dL, p < 0.001) as well as significantly higher immune activation markers, namely C-reactive protein (CRP; 6.01 mg/dL *vs.* 3.70 mg/dL, p < 0.001), interleukin 6 (IL-6; 44.5 *vs.* 24.0 ng/L, p < 0.001), procalcitonin (PCT; 0.13 *vs.* 0.07 ng/mL, p < 0.001), neopterin (51.2 *vs.* 41.3 nmol/L, p = 0.002), fibrinogen (511 *vs.* 439 G/L, p = 0.002) and ferritin levels (662 *vs.* 265 µg/L, p < 0.001) as well as higher leukocytes counts (5.80 *vs.* 5.00 G/L, p = 0.003) compared to women. The prevalence of cardiovascular disease (53.2 % *vs.* 49.0 %, p = 0.007) and diabetes mellitus (35.7 % *vs.* 25.9 %, p = 0.046) was also significantly higher in men than women. Finally, men were at higher risk to die during hospital stay (16.2 % *vs.* 6.8 %, p = 0.008) with significantly longer hospitalizations compared to women (11 *vs.* 8 days, p = 0.002). We will focus on sex hormones in SARS-CoV-2 infected men in the following analysis. Baseline characteristics for men upon hospital admission are depicted in **Table 1**.

Baseline Sex Hormones and Lipids in Men With COVID-19

Sex hormones were available from 267 patients (155 men, 112 women) in the first three days after hospital admission. Most hospitalized men with available sex hormones upon initial hospital admission (n = 155) presented with reduced total testosterone (tT) levels ≤ 230 ng/dL (n = 107, 69.0 %), while 22 men (14.2 %) presented with borderline tT levels between 231 – 350 ng/dL and 24 men (15.5 %) with normal tT levels > 350 ng/dL. When differentiating men according to their age, men over the age of 60 had a significantly higher prevalence of reduced tT levels (81.5% *vs.* 52.5 %) and a lower prevalence of borderline tT levels (9.8 % *vs.* 21.3 %) when compared to men under the age of 60 (p < 0.001). Irrespective of the median age of 67 years, only 15 men out of 155 (9.7 %) were on pharmacological therapy for their benign prostate enlargement with a 5-alpha-reductase inhibitor, generally accepted to not influence the tT concentrations; indeed, these men showed median tT levels of 191 ng/dL (65 – 419 ng/dL) and median free testosterone (fT) levels of 3.94 ng/L (2.05 – 5.65 ng/L) as compared to men without 5-alpha reductase inhibitor therapy with tT levels of 148 ng/dL (70 – 262 ng/dL, p = 0.494) and fT levels of 4.19 ng/L (2.49 – 5.89 ng/L, p = 0.510). Interestingly, although tT levels were rather low in the majority of hospitalized men, levels of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) as well as the steroid hormone estradiol (E_2) were within the upper normal range (**Table 1**). However, when differentiating men again according to their age, men over the age of 60 presented with slightly elevated LH levels (7.6 U/L [4.8 – 12.0]) which were also significantly higher compared to men under the age of 60 (4.8 [3.5 – 6.1], p < 0.001). Conversely, tT levels were significantly lower in men over the age of 60 compared to men under the age of 60 (130 ng/dL [50 – 198] *vs.* 219 ng/dL [120 – 359], p < 0.001),

TABLE 1 | Baseline characteristics of men with corresponding reference ranges.

	All men	Men with available sex hormones	Reference
	n = 230	n = 155	
	Median (IQR) or n (%)	Median (IQR) or n (%)	
Clinical characteristics			
age [years]	67 (54 – 78)	66 (53 – 78)	
BMI [kg/m^2]	26.4 (24.1 – 29.4)	26.4 (24.2 – 29.3)	
Temperature [°C]	37.8 (36.8 – 38.6)	37.9 (37.0 – 38.7)	
SpO_2 [%]	91.5 (88.0 – 94.0)	92.0 (88.0 – 94.0)	
O_2 requirement [L]	2.0 (0.0 – 4.0)	2.0 (0.0 – 4.0)	
WHO score	4 (4 – 5)	4 (4 – 5)	
Hospitalization, days‡	11 (7 – 16)	9 (6 – 13)	
ICU admission#	20.5%	20.5%	
Death during hospital stay	16.1%	12.9%	
Duration symptoms till hospitalization, days	6 (3 – 10)	9 (6 – 12)	
Comorbidities and risk factors			
Cardiovascular disease	63.2%	61.0%	
Arterial hypertension	46.5%	46.8%	
Diabetes Mellitus	35.7%	27.7%	
Chronic Kidney Disease	13.5%	15.5%	
Malignancies	13.6%	13.0%	
COPD	8.3%	6.5%	
Bronchial asthma	5.2%	6.5%	
Nicotine abuse	20.5%	21.9%	
Concomitant Medication			
Therapy for BPH	10.1%	9.7%	
Lipid lowering drugs	29.1%	29.0%	
General laboratory parameters			
eGFR [mL/min/1.73m^2]	77.66 (52.51 – 95.17)	76.54 (56.05 – 95.17)	>60
AST [U/L]	37 (28 – 59)	36 (28 – 59)	10 – 50
ALT [U/L]	28 (19 – 46)	27 (18 – 46)	10 – 50
ALP [U/L]	65 (51 – 86)	67 (54 – 80)	40 – 129
HbA1c [%]	6.2 (5.7 – 6.8)	6.1 (5.7 – 6.8)	4.8 – 5.7
Thrombocytes [G/L]	182 (140 – 243)	178 (137 – 237)	150 – 380
Hemoglobin [g/L]	137 (124 – 150)	136 (124 – 149)	130 – 177
Hematocrit [L/L]	0.396 (0.358 – 0.437)	0.396 (0.359 – 0.429)	0.400 – 0.520
Cholesterol [mg/dL]	121 (97 – 148)	122 (97 – 147)	130 – 200
LDL [mg/dL]	72 (50 – 95)	72 (50 – 94)	0 – 116
HDL [mg/dL]	31 (24 – 39)	32 (24 – 39)	>40
Triglycerides [mg/dL]	104 (84 – 135)	106 (85 – 138)	40 – 150
Inflammatory biomarkers			
CRP [mg/dL]	6.01 (2.09 – 12.29)	5.25 (1.57 – 11.10)	0.00 – 0.50
IL-6 [ng/L]	44.5 (17.3 – 91.4)	38.3 (14.4 – 81.1)	0.00 – 0.50
IL-10 [pg/mL]	5.60 (2.90 – 10.20)	5.60 (2.90 – 10.20)	0.00 – 3.50
PCT [ng/mL]	0.13 (0.07 – 0.33)	0.11 (0.07 – 0.32)	0.00 – 0.50
Neopterin [nmol/L]	51.2 (31.5 – 81.3)	50.3 (31.4 – 78.2)	0.0 – 10.0
Fibrinogen [G/L]	511 (398 – 592)	498 (392 – 586)	210 – 400
Ferritin [µg/L]	662 (348 – 1.371)	535 (313 – 1140)	30 – 400
Leukocytes [G/L]	5.80 (4.40 – 8.10)	5.70 (4.30 – 7.50)	4.0 – 10.0
Lymphocytes absolute [G/L]	0.97 (0.65 – 1.39)	1.01 (0.71 – 1.40)	0.80 – 4.00
Hormones			
Total testosterone [ng/dL]	148 (70 – 270)	148 (70 – 270)	>350
Free testosterone [ng/L]	4.01 (2.47 – 5.87)	4.01 (2.47 – 5.87)	4.81 – 22.42
DHEA [mg/L]	0.70 (0.33 – 1.09)	0.70 (0.33 – 1.09)	1.29 – 4.09
Androstenedione [µg/L]	1.3 (0.6 – 1.9)	1.3 (0.6 – 1.9)	0.6 – 3.1
Estradiol [ng/L]	28 (24 – 38)	28 (24 – 38)	11 – 43
SHBG [nmol/L]	31.0 (21.2 – 41.5)	31.0 (21.2 – 41.5)	10.0 – 57.0
LH [U/L]	6.0 (4.0 – 9.4)	6.0 (4.0 – 9.4)	0.8 – 7.6
FSH [U/L]	5.3 (3.0 – 11.1)	5.3 (3.0 – 11.1)	1.6 – 20.4
TSH [mU/L]	1.00 (0.68 – 1.68)	1.02 (0.70 – 1.65)	0.35 – 3.50
FT4 [pmol/L]	15.4 (13.4 – 17.7)	15.1 (13.3 – 17.4)	10.3 – 21.9
FT3 [pmol/L]	3.23 (2.60 – 3.95)	3.26 (2.72 – 3.99)	2.50 – 6.70

‡without patients who died during hospital stay, #only patients over the age of 80 (n = 127).

IQR, interquartile range; BMI, body mass index; SpO2, peripheral capillary oxygen saturation; O2, oxygen; WHO, World Health Organization; ICU, intensive care unit; COPD, Chronic obstructive pulmonary disease; BPH, benign prostatic hyperplasia; eGFR, estimated glomerular filtration rate; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; CRP, C-reactive protein; IL-6, interleukin 6; IL-10, interleukin 10; PCT, procalcitonin; HbA1c, glycated hemoglobin; HDL, high density lipoprotein; LDL, low density lipoprotein; TSH, thyroid stimulating hormone; FT4, free thyroxine; FT3, free triiodothyronine; LH, luteinizing hormone; FSH, follicle-stimulating hormone; DHEA, dehydroepiandrosterone; SHBG, sexual hormone binding globulin.

Cardiovascular disease, arterial hypertension, diabetes mellitus and hypercholesterinemia were frequently encountered in men within our study. Interestingly, serum low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were quite low even in men without *vs.* with lipid-lowering therapy (LDL: 81 mg/dL *vs.* 57 mg/dL; HDL: 32 mg/dL *vs.* 30 mg/dL). Low serum cholesterol, LDL and HDL levels were associated with older age, higher immune activation, WHO-score and temperature and lower SpO_2 with concomitant higher O_2 requirements (**Table 2**). Moreover, low tT levels as well as a lower tT/E_2 and higher LH/tT ratio were associated with lower cholesterol, LDL and HDL levels (**Table 3**).

Sex Hormones, Disease Severity and Immune Activation

In men (n = 155) lower tT and fT levels upon hospital admission were correlated with older age, higher WHO score and temperature, as well as lower SpO_2 with concomitant higher O_2 requirement and longer hospital stay (**Table 3**). Also, lower dehydroepiandrosterone (DHEA) levels were significantly correlated with an older age, higher WHO score and lower SpO_2, again with higher O_2 requirement and longer hospital stay. SHBG was positively correlated with age and hospitalization duration and negatively with BMI and O_2 requirement. Androstenedione (ASD), E_2, LH and FSH were not related to disease severity. Finally, a higher LH/tT and a lower tT/E_2 ratio were associated with an older age, higher WHO score and temperature as well as lower SpO_2 with accordingly higher O_2 requirements and longer hospital stay (**Table 3**).

When analyzing relations of sex hormones with markers of immune activation, tT and fT levels negatively correlated with CRP, IL-6, IL-10, neopterin, PCT, fibrinogen and ferritin levels as well as with leukocyte counts and positively correlated with absolute lymphocyte numbers. Also, other sex hormones

TABLE 2 | Correlations of lipoproteins and thyroid hormones with clinical characteristics and laboratory parameters in men.*

	Cholesterol [mg/dL]	LDL [mg/dL]	HDL [mg/dL]	Triglycerides [mg/dL]	TSH [mU/L]	FT4 [pmol/L]	FT3 [pmol/L]
Clinical characteristics							
Age	- 0.124	**- 0.194**	**0.159**	- 0.132	- 0.043	- 0.062	**- 0.473**
[years]	p = 0.128	**p = 0.016**	**p = 0.050**	p = 0.108	p = 0.595	p = 0.456	**p < 0.001**
BMI	- 0.126	- 0.063	**- 0.248**	0.194	0.050	0.088	0.087
[kg/m^2]	p = 0.142	p = 0.466	**p = 0.003**	p = 0.023	p = 0.554	p = 0.314	p = 0.324
Temp.	**- 0.189**	**- 0.186**	- 0.124	- 0.016	- 0.094	- 0.059	**- 0.192**
[°C]	**p = 0.021**	**p = 0.023**	p = 0.130	p = 0.845	p = 0.250	p = 0.482	**p = 0.022**
SpO_2	**0.171**	**0.202**	**0.214**	- 0.119	0.046	0.074	**0.315**
[%]	**p = 0.038**	**p = 0.013**	**p = 0.009**	p = 0.151	p = 0.571	p = 0.380	**p < 0.001**
O_2 req.	**- 0.275**	**- 0.274**	**- 0.360**	0.184	- 0.076	- 0.131	**- 0.358**
[L]	**p < 0.001**	**p < 0.001**	**p < 0.001**	p = 0.025	p = 0.355	p = 0.119	**p < 0.001**
WHO	**- 0.264**	**- 0.348**	**- 0.263**	0.155	- 0.151	- 0.082	**- 0.401**
score	**p = 0.001**	**p < 0.001**	**p = 0.001**	p = 0.060	p = 0.062	p = 0.329	**p < 0.001**
Hosp.	**- 0.218**	**- 0.219**	- 0.047	- 0.037	- 0.029	- 0.007	**- 0.256**
[days] ‡	**p = 0.013**	**p = 0.013**	p = 0.598	p = 0.676	p = 0.744	p = 0.943	**p = 0.005**
Inflammatory biomarkers							
CRP	**- 0.347**	**- 0.382**	**- 0.369**	0.136	- 0.129	- 0.142	**- 0.452**
[mg/dL]	**p < 0.001**	**p < 0.001**	**p < 0.001**	p = 0.096	p = 0.112	p = 0.088	**p < 0.001**
IL-6	**- 0.384**	**- 0.384**	**- 0.246**	- 0.044	- 0.054	- 0.136	**- 0.427**
[ng/L]	**p < 0.001**	**p < 0.001**	**p = 0.002**	p = 0.589	p = 0.508	p = 0.103	**p < 0.001**
IL-10	**- 0.213**	**- 0.228**	- 0.025	0.063	0.057	- 0.006	**- 0.224**
[pg/mL]	**p = 0.030**	**p = 0.019**	p = 0.802	p = 0.522	p = 0.562	p = 0.953	**p = 0.022**
PCT	**- 0.346**	**- 0.402**	**- 0.273**	0.109	- 0.076	**- 0.270**	**- 0.470**
[ng/mL]	**p < 0.001**	**p < 0.001**	**p < 0.001**	p = 0.184	p = 0.350	**p = 0.001**	**p < 0.001**
Neopterin	**- 0.242**	**- 0.322**	- 0.086	0.039	- 0.079	**- 0.215**	**- 0.493**
[nmol/L]	**p = 0.003**	**p < 0.001**	p = 0.290	p = 0.634	p = 0.329	**p = 0.009**	**p < 0.001**
Fibrinogen	**- 0.224**	**- 0.221**	**- 0.304**	0.145	- 0.027	- 0.057	**- 0.258**
[G/L]	**p = 0.007**	**p = 0.007**	**p < 0.001**	p = 0.082	p = 0.742	p = 0.504	**p = 0.002**
Ferritin	**- 0.199**	**- 0.259**	**- 0.270**	**0.238**	**- 0.202**	- 0.065	**- 0.236**
[µg/L]	**p = 0.014**	**p = 0.001**	**p < 0.001**	**p = 0.003**	**p = 0.012**	p = 0.439	**p = 0.004**
Leukocytes	0.025	- 0.002	- 0.071	0.098	- 0.116	0.095	- 0.022
[G/L]	p = 0.764	p = 0.979	p = 0.388	p = 0.235	p = 0.152	p = 0.256	p = 0.793
Lymph.	**0.216**	**0.223**	0.124	0.016	**0.177**	0.149	**0.333**
abs. [G/L]	**p = 0.008**	**p = 0.006**	p = 0.129	p = 0.844	**p = 0.028**	p = 0.075	**p < 0.001**

**Spearman-rank correlation coefficient with according p-Value, ‡without patients who died during hospital stay. Statistically significant correlations are marked in bold. LDL, low density lipoprotein; HDL, high density lipoprotein; TSH, thyroid stimulating hormone; FT4, free thyroxine; FT3, free triiodothyronine; tT, total testosterone; fT, free testosterone; DHEA, dehydroepiandrosterone; ASD, Androstenedione; E_2, estradiol; SHBG, sexual hormone binding globulin; LH = luteinizing hormone; FSH = follicle-stimulating hormone; BMI, body mass index; Temp., temperature; SpO2, peripheral capillary oxygen saturation; O_2 req., oxygen requirement; WHO, World Health Organization; Hosp., hospitalization duration; CRP, C-reactive protein; IL-6, interleukin 6; IL-10, interleukin 10; PCT, procalcitonin; Lymph. abs., lymphocytes absolute.*

TABLE 3 | Correlations of sex hormones with clinical characteristics and laboratory parameters in men.*

	tT [ng/dL]	fT [ng/L]	DHEA [mg/dL]	ASD [µg/L]	E_2 [ng/L]	SHBG [nmol/L]	LH [U/L]	FSH [U/L]	LH/tT ratio	tT/E_2 ratio
Clinical characteristics										
Age	**- 0.361**	**- 0.417**	**- 0.568**	0.116	0.095	**0.455**	**0.434**	**0.402**	**0.485**	**- 0.379**
[years]	**p < 0.001**	**p < 0.001**	**p < 0.001**	p = 0.154	p = 0.243	**p <0.001**	**p < 0.001**	**p < 0.001**	**p < 0.001**	**p < 0.001**
BMI	- 0.135	- 0.046	0.090	- 0.082	0.042	**- 0.279**	- 0.064	0.018	0.063	- 0.153
[kg/m^2]	p = 0.114	p = 0.589	p = 0.291	p = 0.335	p = 0.624	**p = 0.002**	p = 0.473	p = 0.842	p = 0.482	p = 0.072
Temp.	**- 0.245**	**0.237**	0.002	0.070	- 0.012	- 0.115	0.067	0.067	**0.213**	**- 0.241**
[°C]	**p = 0.002**	**p = 0.003**	p = 0.978	p = 0.394	p = 0.883	p = 0.191	p = 0.436	p = 0.434	**p = 0.012**	**p = 0.003**
SpO_2	**0.500**	**0.237**	**0.190**	- 0.074	**- 0.172**	0.065	- 0.160	- 0.006	**- 0.472**	**0.530**
[%]	**p < 0.001**	**p = 0.003**	**p = 0.020**	p = 0.365	**p = 0.035**	p = 0.456	p = 0.062	p = 0.946	**p < 0.001**	**p < 0.001**
O_2 req.	**- 0.512**	**- 0.197**	**- 0.187**	- 0.055	0.157	**- 0.208**	0.082	- 0.077	**0.454**	**- 0.557**
[L]	**p < 0.001**	**p = 0.015**	**p = 0.022**	p = 0.503	p = 0.055	**p = 0.017**	p = 0.339	p = 0.370	**p < 0.001**	**p < 0.001**
WHO	**- 0.552**	**- 0.384**	**- 0.273**	0.048	0.093	- 0.005	0.079	- 0.032	**0.488**	**- 0.569**
score	**p < 0.001**	**p < 0.001**	**p = 0.001**	p = 0.561	p = 0.256	ü = 0.953	p = 0.357	p = 0.708	**p < 0.001**	**p < 0.001**
Hosp.	**- 0.256**	**- 0.305**	**- 0.226**	0.004	0.013	**0.232**	**0.146**	**0.087**	**0.267**	**- 0.262**
[days] ‡	**p = 0.003**	**p < 0.001**	**p = 0.010**	p = 0.966	p = 0.887	**p = 0.014**	**p = 0.119**	**p = 0.354**	**p = 0.004**	**p = 0.003**
Inflammatory biomarkers										
CRP	**- 0.567**	**- 0.252**	- 0.091	0.076	**0.163**	- 0.156	0.017	**- 0.178**	**0.435**	**- 0.596**
[mg/dL]	**p < 0.001**	**p = 0.002**	p = 0.262	p = 0.349	**p = 0.045**	p = 0.072	p = 0.846	**p = 0.036**	**p < 0.001**	**p < 0.001**
IL-6	**- 0.563**	**- 0.364**	**- 0.237**	**0.198**	**0.165**	- 0.035	0.126	0.004	**0.536**	**- 0.595**
[ng/L]	**p < 0.001**	**p < 0.001**	**p = 0.003**	**p = 0.014**	**p = 0.041**	p = 0.688	p = 0.139	p = 0.959	**p < 0.001**	**p < 0.001**
IL-10	**- 0.376**	**- 0.332**	**- 0.206**	**- 0.062**	- 0.123	**- 0.204**	0.050	- 0.002	**0.362**	**- 0.339**
[pg/mL]	**p < 0.001**	**p < 0.001**	**p = 0.034**	**p = 0.531**	p = 0.210	**p = 0.047**	p = 0.621	p = 0.983	**p < 0.001**	**p < 0.001**
PCT	**- 0.542**	**- 0.233**	- 0.133	0.152	**0.257**	- 0.020	0.056	- 0.054	**0.445**	**- 0.616**
[ng/mL]	**p < 0.001**	**p = 0.004**	p = 0.102	p = 0.061	**p = 0.001**	p = 0.819	p = 0.514	p = 0.531	**p < 0.001**	**p < 0.001**
Neopterin	**- 0.392**	**- 0.319**	**- 0.375**	0.099	0.114	0.167	**0.347**	**0.252**	**0.532**	**- 0.439**
[nmol/L]	**p < 0.001**	**< 0.001**	**p < 0.001**	ü = 0.223	p = 0.159	p = 0.054	**p < 0.001**	**p = 0.003**	**p < 0.001**	**p < 0.001**
Fibrinogen	**- 0.420**	- 0.139	**0.038**	0.059	0.132	**- 0.231**	- 0.003	**- 0.212**	**0.305**	**- 0.540**
[G/L]	**p < 0.001**	p = 0.090	**p = 0.643**	p = 0.473	p = 0.110	**p = 0.008**	p = 0.973	**p = 0.013**	**p < 0.001**	**p < 0.001**
Ferritin	**- 0.272**	- 0.001	0.070	0.022	**0.326**	- 0.012	- 0.033	**- 0.178**	**0.194**	**- 0.387**
[µg/L]	**p = 0.001**	p = 0.988	p = 0.391	p = 0.785	**p < 0.001**	p = 0.888	p = 0.697	**p = 0.036**	**p = 0.023**	**p < 0.001**
Leukocytes	**- 0.166**	0.006	0.000	**0.159**	0.146	- 0.025	- 0.054	- 0.152	0.164	**- 0.204**
[G/L]	**p = 0.040**	p = 0.940	p = 1.000	**p = 0.049**	p = 0.071	p = 0.772	p = 0.528	p = 0.073	p = 0.055	**p = 0.011**
Lymph.	**0.357**	**0.302**	**0.169**	0.101	- 0.079	- 0.027	- 0.137	0.019	**- 0.299**	**0.358**
abs. [G/L]	**p < 0.001**	**p < 0.001**	**p = 0.037**	p = 0.215	p = 0.331	p = 0.760	p = 0.109	p = 0.827	**p < 0.001**	**p < 0.001**
Lipoproteins and thyroid hormones										
Cholesterol	**0.407**	0.147	0.142	0.127	- 0.107	0.124	- 0.112	0.056	**- 0.361**	**0.447**
[mg/dL]	**p < 0.001**	p = 0.073	p = 0.083	p = 0.123	p = 0.196	p = 0.159	p = 0.195	p = 0.516	**p < 0.001**	**p < 0.001**
LDL	**0.430**	**0.183**	**0.197**	0.063	- 0.081	0.061	- 0.160	0.013	**- 0.407**	**0.461**
[mg/dL]	**p < 0.001**	**p = 0.025**	**p = 0.015**	p = 0.446	p = 0.327	p = 0.491	p = 0.061	p = 0.884	**p < 0.001**	**p < 0.001**
HDL	**0.345**	0.019	0.068	0.101	0.001	**0.296**	0.086	**0.188**	**- 0.232**	**0.346**
[mg/dL]	**p < 0.001**	p = 0.813	p = 0.409	p = 0.221	p = 0.986	**p < 0.001**	p = 0.316	**p = 0.028**	**p = 0.007**	**p < 0.001**
Triglycerides	- 0.082	0.072	- 0.050	0.035	0.012	**- 0.224**	- 0.024	- 0.037	0.114	- 0.079
[mg/dL]	p = 0.323	p = 0.386	p = 0.549	p = 0.677	p = 0.890	**p = 0.010**	p = 0.782	p = 0.669	p = 0.187	p = 0.341
TSH	0.015	- 0.005	- 0.015	0.097	- 0.067	**- 0.198**	- 0.042	0.047	- 0.039	0.038
[mU/L]	p = 0.858	p = 0.955	p = 0.851	p = 0.235	p = 0.410	**p = 0.022**	p = 0.623	p = 0.581	p = 0.652	p = 0.640
FT4	**0.171**	0.126	0.049	- 0.034	- 0.081	0.067	- 0.030	- 0.078	- 0.111	**0.169**
[pmol/L]	**p = 0.041**	p = 0.130	p = 0.559	p = 0.688	p = 0.337	p = 0.450	p = 0.727	p = 0.367	p = 0.202	**p = 0.043**
FT3	**0.505**	**0.426**	**0.381**	- 0.074	- 0.019	**- 0.173**	- 0.144	- 0.139	**- 0.452**	**0.486**
[pmol/L]	**p < 0.001**	**p < 0.001**	**p < 0.001**	p = 0.376	p = 0.823	**p = 0.049**	p = 0.096	p = 0.107	**p < 0.001**	**p < 0.001**

**Spearman-rank correlation coefficient with according p-Value, ‡without patients who died during hospital stay. Statistically significant correlations are marked in bold.*
tT, total testosterone; fT, free testosterone; DHEA, dehydroepiandrosterone; ASD, Androstenedione; E_2, estradiol; SHBG, sexual hormone binding globulin; LH = luteinizing hormone; FSH = follicle-stimulating hormone; BMI, body mass index; Temp., temperature; SpO2, peripheral capillary oxygen saturation; O_2 req., oxygen requirement; WHO, World Health Organization; Hosp., hospitalization duration; CRP, C-reactive protein; IL-6, interleukin 6; IL-10, interleukin 10; PCT, procalcitonin; Lymph. abs., lymphocytes absolute; LDL, low density lipoprotein; HDL, high density lipoprotein; TSH, thyroid stimulating hormone; FT4, free thyroxine; FT3, free triiodothyronine.

correlated widely with different biomarkers of immune activation depicted in **Table 3**. Interestingly, a higher LH/tT and lower tT/E_2 ratio was associated with higher CRP, IL-6, IL-10, neopterin, PCT, fibrinogen and ferritin levels as well as lower absolute lymphocyte counts. (**Table 3**)

Testosterone and Estradiol Levels Predict the Outcome in Men With COVID-19

Univariate logistic regression analysis showed that lower tT, fT and DHEA levels, but higher ASD and E_2 levels, as well as a lower tT/E_2 and higher LH/tT ratio, were associated with an increased risk to die

TABLE 4 | Logistic regression analysis of sex hormones and the risk do die or admission to ICU during hospital stay in men.

	Univariate Model				Multivariate Model *			
	Wald	**HR**	**95% CI**	**p-Value**	**Wald**	**HR**	**95% CI**	**p-Value**
Risk to die during hospital stay								
tT [ng/dL] _Ln	**16.184**	**0.340**	**0.201 - 0.575**	**<0.001**	2.095	0.574	0.270 - 1.217	0.148
fT [ng/L] _Ln	**5.145**	**0.512**	**0.287 - 0.913**	**0.023**	0.073	0.887	0.372 - 2.117	0.787
DHEA [mg/L] _Ln	**5.786**	**0.365**	**0.160 - 0.830**	**0.016**	0.038	1.133	0.323 - 3.970	0.845
ASD [µg/L] _Ln	**4.584**	**2.076**	**1.064 - 4.051**	**0.032**	0.004	1.028	0.439 - 2.406	0.949
E_2, [ng/L] _Ln	**11.612**	**5.243**	**2.022 - 13.598**	**<0.001**	**4.807**	**6.554**	**1.221 - 35.187**	**0.028**
SHBG [nmol/L] _Ln	1.580	1.841	0.711 - 4.768	0.209	0.521	0.527	0.093 - 3.000	0.470
LH [U/L] _Ln	0.382	1.235	0.633 - 2.411	0.536	1.220	0.601	0.243 - 1.483	0.269
FSH [U/L] _Ln	0.155	1.108	0.665 - 1.845	0.694	2.456	0.519	0.229 - 1.179	0.117
LH/tT ratio _Ln	**11.334**	**2.045**	**1.348 - 3.100**	**<0.001**	0.088	1.093	0.608 - 1.965	0.767
tT/E_2 ratio _Ln	**21.680**	**0.313**	**0.192 - 0.510**	**<0.001**	**4.616**	**0.440**	**0.208 - 0.931**	**0.032**
Risk for ICU admission during hospital stay †,‡								
tT [ng/dL] _Ln	**18.978**	**0.247**	**0.132 - 0.463**	**<0.001**	**9.398**	**0.314**	**0.150 - 0.658**	**0.002**
fT [ng/L] _Ln	**15.933**	**0.189**	**0.083 - 0.428**	**<0.001**	**7.376**	**0.269**	**0.105 - 0.694**	**0.007**
DHEA [mg/L] _Ln	2.279	0.601	0.310 - 1.164	0.131	0.000	0.993	0.428 - 2.304	0.987
ASD [µg/L] _Ln	2.054	0.642	0.351 - 1.177	0.152	1.974	0.599	0.293 - 1.224	0.160
E_2, [ng/L] _Ln	1.274	1.932	0.616 - 6.062	0.259	0.186	1.338	0.356 - 5.028	0.667
SHBG [nmol/L] _Ln	0.118	0.849	0.333 - 2.166	0.732	0.134	0.794	0.231 - 2.729	0.715
LH [U/L] _Ln	0.026	1.064	0.499 - 2.269	0.871	0.072	0.883	0.356 - 2.189	0.788
FSH [U/L] _Ln	3.119	0.588	0.326 - 1.060	0.077	**3.984**	**0.486**	**0.240 - 0.987**	**0.046**
LH/tT ratio _Ln	**12.106**	**2.447**	**1.478 - 4.052**	**<0.001**	**6.260**	**2.242**	**1.191 - 4.221**	**0.012**
tT/E_2 ratio _Ln	**19.583**	**0.280**	**0.159 - 0.492**	**<0.001**	**9.043**	**0.348**	**0.175 - 0.692**	**0.003**

**adjusted for age, BMI, LDL, the time of symptom onset and concomitant antiandrogen therapy, †only patients under the age of 80 (n = 127), ‡without patients who died during hospital stay. Statistically significant results are marked in bold.*

tT, total testosterone; fT, free testosterone; DHEA, dehydroepiandrosterone; ASD, Androstenedione; E_2, estradiol; SHBG, sexual hormone binding globulin; LH, luteinizing hormone; FSH, follicle-stimulating hormone; BMI, body mass index; LDL, low density lipoprotein.

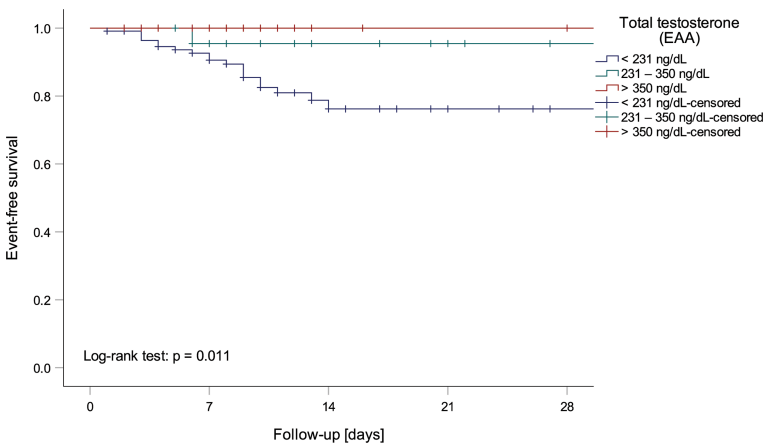

FIGURE 1 | Kaplan-Meier curve depicting mortality of men within testosterone normality ranges by the European Academy of Andrology (EAA) (37).

during hospital stay (**Table 4**). Men with tT levels < 100 ng/dL (n = 52) had a more than eighteen-fold higher risk to die during hospital stay when compared to men with tT levels > 230 ng/dL (n = 46; OR 18.243 [95%CI 2.301 – 144.639], p = 0.006, **Figure 1**). Actually, 19 out of 20 men who died had reduced tT levels ≤ 230 ng/dL; yet one man who died had a tT level of 244 ng/dL.

Moreover, men with a tT/E_2 ratio < 3.30 (n = 52) had a more than 22-fold higher mortality risk when compared to men with a tT/E_2 ratio > 7.30 (n = 51; OR 22.222 [95%CI 2.818 – 175.265], p = 0.003), while those with a LH/tT ratio > 6.95 (n = 46) had a more than 15-fold higher mortality risk when compared to men with a LH/tT ratio < 2.85 (n = 45; OR 15.529 [95%CI 1.924 – 125.367], p = 0.010). However, in multivariate logistic regression analysis adjusted for age, BMI, LDL, and the time of symptom onset only E_2 and the tT/E_2 ratio were significantly predicting mortality (**Table 4**).

Since men over the age of 80 were less probably transferred to the ICU because of their age, we only included men below the age of 80 (n = 127) in the following analyses. Increased risk for ICU admission during hospital stay was found in men with lower baseline tT or fT levels as well as in those with a lower tT/E_2 ratio

and higher LH/tT ratio. These findings were independent of age, BMI, LDL and the time of symptom onset in multivariate Cox regression analysis (**Table 4**). All 26 men who subsequently needed be transferred to the ICU had median tT baseline levels of only 80 ng/dL (range: 41 – 122 ng/dL), thus were hypogonadal per definition. Further subdivision in tertiles showed that men with tT levels < 100 ng/dL (n = 37) had a 12-fold higher risk for ICU admission compared to men with tT levels ≥ 100 ng/dL (n = 88; OR 12.214 [95%CI 4.467 – 33.398], p < 0.001). Additionally, men with a tT/E_2 ratio < 3.30 (n = 37) had a more than 39-fold higher risk for ICU admission compared to men with a tT/E_2 > 7.30 (n = 47; OR 39.100 [95%CI 4.865 – 314.225], p < 0.001), while men with a LH/tT ratio > 6.95 (n = 32) had a 24-fold higher risk for ICU admission compared to men with a LH/tT ratio < 2.85 (n = 41; OR 24.000 [95%CI 2.911 – 197.845], p = 0.003).

DISCUSSION

In the present study we found that low serum total and free testosterone levels upon hospital admission are associated with disease severity indicated by lower oxygen saturation, higher WHO score and increased risk for ICU admission or death during the hospital stay in men with SARS-CoV-2 infection. These data are in line with recent published small cohort studies suggesting that low testosterone levels predict clinical adverse outcome (38, 39). Our results strongly suggest that SARS-CoV-2 infected men with the need for hospitalization present with distinct low testosterone levels that are further not compensated by hypothalamic-hypophyseal feedbacks especially in younger men, since LH levels were within the normal range. Testosterone itself was shown to be associated with a reduced cytokine response following cellular immune activation in men but not in women (40). This might be due to already rather low testosterone levels in women who are therefore not further strongly affected by inflammation (41). In addition, androgen receptor expression in male immune cells is more distinctive than in female ones (18, 42). Thus testosterone is suggested to be partly responsible for the lower prevalence of autoimmune disease but also for the higher incidence of cancer in men compared to women (43). Interestingly, it was also shown that androgens can reduce lung pathology in influenza-infected mice (44) suggesting that testosterone prevents inappropriate overwhelming immune activation following infection (45). In the case of low testosterone levels, such suppressive mechanism may be reduced, which might result in an imbalance between immunosuppressive and pro-inflammatory regulating mechanism in men (46). It's generally assumed that this can lead to enhanced pro-inflammatory cytokine response following a SARS-CoV-2 infection, also known as a cytokine storm. Therefore, a reduced innate immune response toward viral infections in men compared to women results in a higher and longer persisting viral load (47, 48) with a subsequently more pronounced cellular immune response (49). Such situation is mainly present in men with low serum levels of the immunosuppressive acting testosterone. Accordingly, serum testosterone levels were negatively correlated with all investigated inflammatory biomarkers including IL-6, IL-10, neopterin (Th1 immune response), PCT, CRP, fibrinogen and ferritin which all were associated with a poorer clinical course and higher risk for ICU admission or death in SARS-CoV-2 infected patients (10, 48, 50, 51). Vice versa, it was shown in previous studies, that the testosterone production in Leydig cells is also downregulated by immune activation itself (52). The findings of our study, that rather low than high testosterone levels predict mortality in men, contrast with recent findings showing that androgen stimulates the expression of the ACE2 coreceptor TMPRSS2 (53), which further provides access of SARS-CoV-2 to cells (54), suggesting that higher testosterone levels with consequently higher receptor density provides more docking sites for SARS-CoV-2. However, high mortality in COVID-19 is suggested to be caused by hyperinflammation and not actually by higher viral loads (55). Thus, results of our study suggest that low levels of testosterone might be even more immunosuppressive than high testosterone levels with consequently higher SARS-CoV-2 receptor expression.

However, other risk factors for severe COVID-19 disease also interact with hormonal status. Particularly, lower serum testosterone levels are associated with older age, higher BMI and lower cholesterol levels. Several studies have shown that viral and bacterial infections cause decreased cholesterol levels (56) and that the alterations in lipid levels correlate with the severity of the underlying infection (57, 58). Since cholesterol is the precursor of testosterone, its deficiency also causes testosterone deficiency (59). Actually, a highly significant positive correlation of testosterone with lipoproteins was also found in our cohort. Interestingly, statin therapy, which was frequently encountered in our patients, was found to cause lower total testosterone levels in men with diabetes mellitus (60). However, testosterone levels did not significantly differ between men with or without lipid lowering therapy and also the predictive value of testosterone was independent of concomitant use of lipid lowering drugs.

Testosterone levels are typically decreased in older men (61) due to several factors, including a decline in ability of Leydig cells to produce adequate testosterone in response to LH stimulation (62), which might also partly contribute to the higher COVID-19 related morbidity and mortality within this vulnerable group. Actually, the prevalence of hypogonadism in geriatric hospitalized men over the age of 65 was found to be 53.3 % (63). This is supported by the finding that higher LH/tT ratio was also related to morbidity and mortality. LH levels were within the normal range suggesting the presence of normogonadotropic hypogonadism (64). The absence of compensatory LH secretion in case of low testosterone levels (65) most probably might be caused by altered GnRH and consecutive LH suppression by cytokines as found in patients with immune activation (66, 67). Actually, men were hospitalized in median one week after symptom onset providing a long period for suppression of gonadotropins. The finding that older men over the age of 60 had again lower tT levels compared to young men with concurrent slightly elevated (but still low) LH levels suggests that in older men also the occurrence of late onset hypogonadism might contribute to this rather low tT levels. This is further supported by the positive correlation of the LH/tT ratio with age. Unfortunately, a differentiation of whether these men had age-related Leydig cell

dysfunction before COVID-19 or whether low testosterone levels are primarily due to inflammation-related Leydig cell dysfunction or impaired testicular steroid-biosynthesis, cannot be provided by our results.

Testosterone expression can also be suppressed by cortisol which is produced in the adrenal cortex upon stimulation by the adrenocorticotropic hormone (ACTH) secreted by the pituitary gland upon stress-related corticotropin-releasing hormone (CRH) stimulation (68, 69). Activation of the hypothalamic-pituitary-adrenal axis (HPA) in patients with COVID-19 reflected by elevated total serum cortisol levels was shown to be associated with an increased mortality (70) and might also contribute to low testosterone levels in these patients. However, testosterone was also shown to suppress CRH-stimulated cortisol production in men (71) which is why loss of this suppressive mechanism might promote stress-induced HPA activation. Unfortunately, we do not have detected serum hormonal levels of the hypothalamic-pituitary-adrenal axis. Interestingly, hydroxysteroid dehydrogenases, which catalyse steroid biosynthesis (e.g. DHEA to androstenedione, androstenedione to testosterone), is also suggested to be suppressed by immune activation (40).

On the other hand, obesity, linked to disease severity and outcome in SARS-CoV-2 infected patients, is considered to be stringently linked to testosterone deficiency (72). Testosterone can be aromatized to estradiol by the aromatase which is found in abundance in the visceral fatty tissue but also in male gonads (typically in Leydig cells (73), placenta, brain, muscle, bone and vascular tissues (74), and stimulated by cytokines such as IL-6 (75) or Tumor necrosis factor alpha (TNF-α) (76). This is supported by the highly significant negative correlation of inflammatory markers with the tT/E_2 ratio. Interestingly, estradiol levels were within the normal range thus representing a disturbed relation of testosterone to estradiol primarily caused by quite low testosterone levels rather than increased aromatase activity. Finally, the median BMI was normal. This would also explain the contrary findings in airway epithelial cells in which estradiol was demonstrated to downregulate ACE2 expression (77). Also, recent study results suggest that estrogen receptor signaling is actually protective in mice infected with SARS-CoV-2 (78) by suppressing ACE2 expression as well as pro-inflammatory pathways (79). Th1 immune activation might have stronger impacts on ACE2 expression than estradiol receptor signaling (80). Moreover, lower levels of cardiovascular- and lung-protective ACE2 might also aggravate existing co-morbidities (81). However, higher estradiol level (but within normal range) as well as a lower tT/E_2 ratio were associated with mortality and morbidity suggesting that this might primarily reflect inflammation-induced aromatase activity, which only slightly increase estradiol levels in men with distinctive testosterone deficiency.

Finally, low DHEA levels upon hospital admission were (similarly to testosterone) also related to higher morbidity and outcome. In vivo experiments showed that DHEA increases macrophage function and promotes a shift of Th1/Th2 balance toward Th1 immunity (82) thus enhancing immunity against viral infection (83). At the same time, DHEA suppresses the expression of various pro-inflammatory cytokines thus preventing overwhelming immune activation (83), suggesting that its deficiency enables overwhelming inflammation with reduced immunity.

Limitations

This was a retrospective explorative analysis of continuous COVID-19 patients hospitalized at our department. Additionally, sex hormones were not available of all men initially included in the study, which might be an unmeasured bias although baseline characteristics of all men and only those with available sex hormones were almost the same (**Table 1**). This represents a risk for possible type I and II errors. We further have no information about the hormonal status of the patients before their COVID-19 infection which does not allow any conclusion whether these rather low testosterone levels represent hypogonadal hypogonadism or decreased following SARS-CoV-2 related immune activation.

Conclusion

Our results indicate that men hospitalized due to COVID-19 present with lower testosterone levels showing advanced immune activation and having high risk for an adverse clinical course and a poor prognosis. The origin of testosterone deficiency in these patients might be primarily caused by altered cholesterol biosynthesis in the case of SARS-CoV-2 infection as well as being due to inflammation-induced gonadotrophin suppression. The impact of enzymatic aromatase activation on hypogonadism pathogenesis might be negligible. The latter is supported by the finding that high estradiol levels (but within the normal range) were associated with more severe SARS-CoV-2 infections. Whether these men already had hypogonadotropic hypogonadism before COVID-19 cannot be clarified by our study. Effects of testosterone supplementation on outcome in men with severe SARS-CoV-2 infections should be evaluated in further studies.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics committee of the Innsbruck Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization and methodology, AG and RB-W. Software and formal analysis, LL. Investigation and data curation, AE, FB, GH, LL, LT and RB-W. Resources, AG, AE, FB, GH, GW, MA and RB-W. Writing – original draft preparation, LL. Writing – review and editing, AG, AE, FB, GH, GW, LT, MA, RB-W, G-MP and SK. Supervision, GW. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: Author GH was employed by company MLL Munich Leukemia Laboratory.

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

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 02 July 2021
doi: 10.3389/fendo.2021.645787

Spectrum of Endocrine Dysfunction and Association With Disease Severity in Patients With COVID-19: Insights From a Cross-Sectional, Observational Study

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Edited by:
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University of Bristol, United Kingdom

Reviewed by:
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University of Udine, Italy
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Specialty section:
This article was submitted to Translational Endocrinology, a section of the journal Frontiers in Endocrinology

Received: 24 December 2020
Accepted: 21 June 2021
Published: 02 July 2021

Citation:
Das L, Dutta P, Walia R, Mukherjee S, Suri V, Puri GD, Mahajan V, Malhotra P, Chaudhary S, Gupta R, Jayant SS, Agrawal K, Kumar V, Sachdeva N, Rastogi A, Bhadada SK, Ram S and Bhansali A (2021) Spectrum of Endocrine Dysfunction and Association With Disease Severity in Patients With COVID-19: Insights From a Cross-Sectional, Observational Study. Front. Endocrinol. 12:645787. doi: 10.3389/fendo.2021.645787

Introduction: Evidence on new-onset endocrine dysfunction and identifying whether the degree of this dysfunction is associated with the severity of disease in patients with COVID-19 is scarce.

Patients and Methods: Consecutive patients enrolled at PGIMER Chandigarh were stratified on the basis of disease severity as group I (moderate-to-severe disease including oxygen saturation <94% on room air or those with comorbidities) (n= 35) and group II (mild disease, with oxygen saturation >94% and without comorbidities) (n=49). Hypothalamo-pituitary-adrenal, thyroid, gonadal axes, and lactotroph function were evaluated. Inflammatory and cell-injury markers were also analysed.

Results: Patients in group I had higher prevalence of hypocortisolism (38.5 *vs* 6.8%, p=0.012), lower ACTH (16.3 *vs* 32.1pg/ml, p=0.234) and DHEAS (86.29 *vs* 117.8µg/dl, p= 0.086) as compared to group II. Low T3 syndrome was a universal finding, irrespective of disease severity. Sick euthyroid syndrome (apart from low T3 syndrome) (80.9 *vs* 73.1%, p= 0.046) and atypical thyroiditis (low T3, high T4, low or normal TSH) (14.3 *vs* 2.4%, p= 0.046) were more frequent in group I than group II. Male hypogonadism was also more prevalent in group I (75.6% *vs* 20.6%, p=0.006) than group II, with higher prevalence of both secondary (56.8 *vs* 15.3%, p=0.006) and primary (18.8 *vs* 5.3%, p=0.006) hypogonadism. Hyperprolactinemia was observed in 42.4% of patients without significant difference between both groups.

Conclusion: COVID-19 can involve multiple endocrine organs and axes, with a greater prevalence and degree of endocrine dysfunction in those with more severe disease.

Keywords: COVID-19, endocrinology, hormones, central hypoadrenalism, mixed thyroid dysfunction, hypogonadism

INTRODUCTION

The pandemic of COVID-19, caused by SARS-CoV-2, has established itself as one of the greatest challenges to healthcare globally. Despite the lower case fatality rate as compared to other coronaviruses, COVID-19 has underscored the vulnerability of certain subsets of populations to emerging diseases, particularly with respect to gender, age, and co-morbidities (especially diabetes, hypertension, and obesity) (1–3). The hormonal basis of most of these underlying risk factors is undeniable, highlighting a crucial link between pre-existing metabolic conditions and predisposition to adverse outcomes with COVID-19 (4–7). On the other hand, the fact that SARS-CoV-2 can cause new-onset endocrine dysfunction is being increasingly recognized, with connotations extending to mortality (association with high cortisol) and possible therapeutic interventions (dexamethasone use in the RECOVERY trial) (8, 9).

The basis of endocrine dysfunction in COVID-19 can be attributable to organ-specific ACE2 mediated viral entry and damage, direct viral toxicity, and a 'cytokine storm' mediated by various cytokines (IL-6, IL-10, IL-1β, IL-17, and TNF-α) (10). ACE2, the entry receptor for SARS-CoV-2, has been identified in several endocrine organs like the thyroid, testes, pancreatic islets, and adipose tissue (11). Interestingly, the thyroid, testes, and adipose tissue have higher demonstrable levels of ACE2 than in the lungs. Others, like the hypothalamus, pituitary, and adrenals, have been found to harbor low expression of this receptor. Further, the transmembrane serine protease TMPRSS2, which acts as a co-receptor in viral entry by priming the spike protein of SARS-CoV-2, is also present in various endocrine organs, having the highest levels in the thyroid followed by the pancreas, pituitary, and testes (11).

However, overall clinical evidence of endocrine involvement in COVID-19 patients is limited (8, 12–20), mostly confined to case reports and small patient series (15–18, 20). Moreover, some of the patients in these reports had other disorders that could explain their presentation (including adrenal hemorrhage in a COVID-19 patient with APLA syndrome, death in a COVID-19 patient with myxoedema coma) (18, 20). The other available body of evidence is extrapolated from studies involving SARS patients, their autopsy data, and retrospective studies in SARS survivors (21–23). Though there is a genomic concordance between SARS-CoV and SARS-CoV-2 of almost 80% and ACE2 is the common host viral entry receptor for both, there is an inherent difference between infectivity and binding affinity to ACE2 between both viruses. In this context, caution needs to be exercised in the generalisability of clinical parameters and outcomes, and evidence pertaining to COVID-19 needs to be explored so as to improve patient management. Further, whether there is an association between disease severity and endocrine function in COVID-19 needs investigation.

This was a single-center, cross-sectional study to evaluate endocrine function in patients with COVID-19 and to investigate whether the hormonal function is similar or different between patients with mild (symptomatic or asymptomatic) and moderate-to-severe disease.

MATERIAL AND METHODS

This was an observational study of consecutive patients with COVID-19 admitted at a single dedicated COVID care center at the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India (n=74). Patients were diagnosed on the basis of RT-PCR positivity for SARS-CoV-2 in nasopharyngeal and/or throat swab samples. Their clinical, biochemical, and hormonal parameters were recorded. Clinical parameters included symptomatology type and duration and prior co-morbidities. Biochemical profiles included complete hemogram, renal and liver function tests, and inflammatory markers (CRP, procalcitonin, D-dimer, LDH, and ferritin). The neutrophil-lymphocyte ratio (NLR) and platelet-lymphocyte ratios (PLR) were calculated from the differential cell count. Patients were divided into two groups depending on disease severity and comorbidities: Group I [consisting of patients with moderate or severe disease defined as hypoxia with an inability to maintain oxygen saturation to at least 94% on room air (requiring high flow or conventional oxygen therapy or mechanical ventilation) or with chronic comorbidities, including diabetes, hypertension, malignancy, chronic liver or kidney disease, and chronic pulmonary disease] (n=35) and Group II (consisting of mild disease defined as the ability to maintain oxygen saturation to 94% or above at room air and without any chronic disease conditions) (n=49). Patients whose clinical data were not available were excluded from the study. The severity of disease for each patient was calculated as per CSS (scoring system of COVID-19), developed and validated previously for admitted patients with COVID-19 (24). This score has been found to be useful for predicting in-hospital complications, with good discriminatory ability (AUC 0.919), which is comparable to the APACHE-II and higher than the SOFA and CURB-65 scores in patients with COVID-19.

Hormonal parameters were assessed within 24 to 48 hours of admission in a sample taken at 0800 to 0900h and sent under appropriate cold-chain conditions. Serum cortisol, ACTH, DHEAS, free T3, free T4, TSH, Prolactin, LH, FSH, testosterone, and estradiol were estimated. For assessment of the hypothalamo-pituitary-adrenal (HPA) axis, patients who had been initiated on systemic glucocorticoid therapy for COVID-19 or on chronic glucocorticoid therapy for autoimmune or other conditions were excluded. Further, those with serum albumin<3g/dl were also excluded in order to eliminate falsely low cortisol concentrations since 10% of circulating cortisol is bound to albumin. For assessment of the hypothalamo-pituitary-thyroid (HPT) axis, patients already on thyroid hormone replacement or anti-thyroid drugs were excluded. There was no instance of exposure to iodinated contrast for CT scan and exposure to heparin was nil/minimal as samples were withdrawn within 24 hours (maximum 48 hours of admission). Hemogram, electrolytes, and liver and renal function tests were estimated by Beckman Coulter. All hormones and procalcitonin were estimated by electro-chemiluminescence assay (ECLIA, COBAS 8000, Roche Diagnostics, Germany) with a co-efficient of variation (CV) of 6% to 8%. HbA1c was measured by HPLC (Biorad variant II. CV 0.5 to 1%). CRP was measured by

immunoturbidimetry, LDH by spectrophotometry, and ferritin by ECLIA.

The HPA axis status was defined based on the disease severity categorization. Cortisol was estimated in all patients by ECLIA, with the normal range of the assay being 170 to 550nmol/L (6 to 20μg/dl). Patients in Group I (with moderate-to-severe disease) were considered to be hypocortisolic if they had serum cortisol below 15μg/dl (414nmol/L) and eucortisolic with a level exceeding 15μg/dl (414nmol/L) (25). Patients in Group II (with mild disease or even asymptomatic) were considered hypocortisolic if they had serum cortisol levels below 6μg/dl (170nmol/L, lower limit of normal for the assay) and eucortisolic if their levels exceeded 6μg/dl (170nmol/L). Hypercortisolism was defined as cortisol levels exceeding 30μg/dl (900nmol/l) for group I and 20μg/dl (550nmol/l, upper limit of normal [ULN] of assay) for group II. Further subclassification was done depending on low/inappropriately normal ACTH (<65pg/ml, ULN) or high ACTH (>65pg/ml). DHEAS levels were classified on the basis of age- and gender-matched range. Regarding thyroid function tests, low T3 syndrome was defined as free T3 less than 2pg/ml (lower limit of normal [LLN] of the assay) (2pg/ml), and low T4 syndrome was defined as free T4 less than 0.9ng/dl (LLN), both irrespective of TSH. Sick euthyroid syndrome (SES) was defined either in the presence of low T3 or low T4 syndromes with suppressed or normal TSH as described above, or normal free T4 with low TSH. Thyrotoxicosis associated with typical thyroiditis was defined as elevated free T4 (>1.71ng/dl ULN), elevated free T3 (>4.4pg/ml, ULN), with a suppressed TSH (<0.4mIU/L). Hypothyroidism was defined as subclinical in the presence of normal free T4 with a concurrently raised TSH (4.2 and above) and overt in the presence of low free T4 with TSH exceeding 10mIU/L. Secondary hypogonadism was defined as testosterone (T) below the lower limit of normal (9nmol/l) with low or inappropriately normal gonadotropins (LH, FSH). Primary hypogonadism was defined as low testosterone with elevated gonadotropins. Gonadal status was evaluated and classified using standard criteria, as mentioned above, in male patients. In females, menopausal status was determined in those with levels exceeding the standard cutoff of 40mIU/ml. Hyperprolactinemia was defined as prolactin levels exceeding 20ng/ml in males and 25ng/ml in females, and hypoprolactinemia was defined as serum prolactin levels below 5ng/ml.

All study protocols were carried out in accordance with relevant guidelines and recommendations. The study was approved by the Institutional Ethics Committee, PGIMER (IEC/NK/6515/study/854).

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) 22.0 software program (IBM Statistics 22.0). Qualitative variables were compared between the groups using the Pearson $\chi 2$ test or Fisher's exact test. Quantitative variables were checked for normality using the Kolmogorov-Smirnov test and classified as parametric and non-parametric. The Student T-test was used to compare the means of two groups for parametric data and the Mann-Whitney U test for non-parametric data. Spearman correlation was used to determine the association between hormonal and biochemical parameters, with 0.3 to 0.5 being defined as moderate and >0.5 defined as strong correlation. A p-value <0.05 was considered significant.

RESULTS

There were a total of 84 patients enrolled in the study, of which 35 (41.7%) had moderate-to-severe disease (Group I) and 49 (58.3%) had mild disease (Group II). In Group I, 34.2% had hypoxia (defined as the inability to maintain O2 saturation of 94% at FiO2 of 0.2) and 77.1% had chronic comorbidities. Of these, hypertension was the most common (45.7%) followed by diabetes mellitus (33.3%), chronic airway disease (18.5%, including COPD, interstitial lung disease, chronic pulmonary aspergillosis, and cryptococcosis), chronic liver disease (18.5%), and CAD (11.1%). A single comorbidity was present in 51.8% of patients, and the rest had two or more chronic disease conditions; both hypertension and diabetes were present in 22.8% of patients. Diabetes mellitus was present in 33.3% of patients and the details of the medication of these patients were present in all 10 subjects. Only two patients were on insulin, and the rest were on oral antidiabetic agents, the most common being metformin followed by DPP-IV inhibitors (teneligliptin and linagliptin) and glimepiride, at the time of admission. CSS>2 was present in a significantly greater proportion of patients in group I. Baseline clinical and biochemical parameters were analyzed for all patients. Among hormonal axes, the HPA axis was analyzed in all except those who were initiated on systemic glucocorticoid therapy (n=7) and those with albumin below 3g/dl (n=11). The HPT axis was analyzed in all except four patients, who were already on thyroid hormone replacement for hypothyroidism. All four patients were on levothyroxine replacement (in doses varying from 50 to 100μg). Further, there were two patients with non-alcoholic fatty liver disease, one of whom presented with hematemesis and decompensated liver disease.

Baseline clinical parameters are summarised in **Table 1**. There was an equal number of males and females, but more males in group I (p=0.076). The mean age of the patients was significantly higher in group I (moderate to severe disease). In group I, fever was the most common symptom (80%), followed by cough (31.4%), shortness of breath (28.6%), and sore throat (8.5%). Chest pain and GI symptoms were present in 5.7% each. One patient (old treated case of pulmonary tuberculosis) had recurrent hemoptysis at presentation and another (known case of alcohol-related chronic liver disease) had altered sensorium due to hepatic encephalopathy. In group II, 67.3% were asymptomatic. Among those with symptoms, sore throat was the most common (52.9%) followed by cough (29.4%). Biochemical analysis revealed significantly higher leucocytosis with lymphopenia, hypokalemia, renal function parameters, and transaminases (**Table 2**). A significantly higher proportion of patients had hyperferritinemia, elevated D-dimer, and higher LDH, CRP, and procalcitonin in group I.

TABLE 1 | Clinical parameters of disease severity in patients with COVID-19.

Parameter	Moderate to severe disease (n=35)	Mild disease (n=49)	p value
Age (years)	**53.5 ± 14**	**31.9 ± 13**	**0.000**
Gender (Males) (%)	62.9	40.8	0.076
Fever (%)	**75.9**	**31.4**	**0.000**
Asymptomatic (%)	**20**	**67.3**	**0.000**
Duration of symptoms (days)	**6 (3-9)**	**2 (1-7)**	**0.019**
DM (%)	**33.3**	**–**	**0.000**
HT (%)	**45.7**	**–**	**0.000**
PR (/min)	88.2 ± 11.8	87.5 ± 12.2	0.817
SBP (mm Hg)	**128.4 ± 19.0**	**114.7 ± 13.3**	**0.002**
DBP (mm Hg)	**80.3 ± 12.1**	**73.0 ± 9.0**	**0.008**
PP (mm Hg)	**48.1 ± 10.4**	**41.6 ± 9.3**	**0.013**
MAP (mm Hg)	**74.9 ± 12.6**	**66.0 ± 9.9**	**0.003**
RR (/min)	**22.2 ± 5.1**	**18.8 ± 2.3**	**0.001**
FiO2	**0.33 ± 0.17**	**0.20 ± 0.00**	**0.000**

Data are expressed as mean ± SD or median (q25-q75), as appropriate.
DBP, Diastolic blood pressure; DM, Diabetes mellitus; FiO2, Fraction of inspired oxygen; HT, Hypertension; MAP, Mean arterial pressure; PR, Pulse rate;, PP, Pulse pressure; RR, Respiratory rate; SBP, Systolic blood pressure.
The bold values refer to the parameters that were significantly different between both groups.

Parameters of endocrine dysfunction are summarized in **Table 3**. In group I, 38.5% of patients had hypocortisolism and the rest were eucortisolic among those who had paired hormone (cortisol and ACTH) values available (n=13), excluding patients like those mentioned above (**Figure 1A**). All hypocortisolic patients had low or inappropriately normal ACTH. Among those who were eucortisolic, 85.7% had normal or low ACTH, and 14.3% had high ACTH (>65pg/ml). In group II, 86.4% were eucortisolic, and 6.8% each had hypocortisolism and hypercortisolism. Among those who were eucortisolic in group II (n=40), 92.5% had normal ACTH, with only three having high ACTH. The lone patient with hypocortisolism in this subgroup had normal ACTH (38pg/ml). Among those with cortisol values exceeding 550nmol/l (n=3), only one had a high ACTH of 71 pg/ml. The majority of patients had normal DHEAS (78.3 *vs* 85.7%, p=0.49), whether eucortisolic or hypocortisolic.

With respect to the assessment of thyroid function, all patients (with entire thyroid function test values available, n=62) had low T3 syndrome in both groups. Low T4 syndrome was present in 9.5% of patients in group I, while none in group II had this syndrome. The absolute levels of free T3 were lower in group I (p=0.05) while there was no difference between free T4 levels in both groups (p=0.38). Overall, SES (apart from low T3 syndrome) was present in 80.9% of patients in group I and 73.1% in group II (**Figure 1B**). Typical thyroiditis was not observed in any patient in either of the groups. Atypical thyroiditis (defined as low free T3, high free T4, and normal/low TSH) was present more in group I than group II (14.3% *vs* 2.4%) (p= 0.046).

In group I, ACTH and T showed a moderate negative correlation with NLR (-0.447, p=0.042; -0.456, p=0.043). TSH showed a strong negative correlation with inflammatory parameters CRP (-0.547, p=0.004) and moderate correlation with hyperferritinemia (serum ferritin -0.460, p=0.014; ferritin ULN -0.465, p=0.015). In both groups, serum cortisol showed a

TABLE 2 | Biochemical parameters of disease severity in patients with COVID-19.

Parameter (Reference Range)	Moderate to severe disease (n=35)	Mild disease (n=49)	p value
Hemoglobin (g/dl) (12-14g/dl)	11.6 ± 3.1	12.5 ± 1.9	0.171
TLC (x 10^9/L) ($4.0-11.0 \times 10^9$/L)	**9.5 ± 5.5**	**7.0 ± 2.9**	0.017
Neutrophil (%) (41-72%)	**72.0 ± 15.5**	**55.5 ± 11.8**	**0.000**
ANC (x 10^9/L) ($1.8-7.7 \times 10^9$/L)	**7.3 ± 5.3**	**4.0 ± 2.3**	**0.005**
Lymphocyte (%) (15-45%)	**16.7 ± 11.0**	**32.9 ± 10.4**	**<0.001**
ALC (x 10^9/L) ($1-4.8 \times 10^9$/L)	**1.2 ± 0.7**	**2.2 ± 0.9**	**<0.001**
Platelet count (x 10^9/L) ($150-450 \times 10^9$/L)	182.2 ± 108.2	197.2 ± 83.1	0.563
NLR	**7.4 ± 5.9**	**2.0 ± 1.3**	**0.000**
PLR	**18.4 ± 12.4**	**9.9 ± 4.8**	**0.010**
Na (mmol/L) (135-145mmol/L)	141.6 ± 9.2	143.3 ± 3.6	0.484
K (mmol/L) (3.5-5mmol/L)	4.1 ± 0.6	4.3 ± 0.4	0.057
Hypokalemia (%) (<3.5mmol/L)	**17.2%**	**–**	**0.011**
Urea (mg/dl) (10-50mg/dl)	**40.2 (31.0-90.9)**	**22.6 (19.2-24.5)**	**0.004**
Creatinine (mg/dl) (0.5-1.2mg/dl)	**0.88 (0.78-1.15)**	**0.69 (0.55-0.84)**	**0.000**
BUN/Cr	22.7 (14.6-34.9)	18.9 (14.6-24.1)	0.347
Bilirubin (mg/dl) (0.2-1.2mg/dl)	0.51 (0.24-0.90)	0.39 (0.28-0.58)	0.522
AST(U/L) (2-40U/L)	**56.7 (45.7-85.7)**	**23.9 (19.3-44.4)**	**0.000**
ALT(U/L) (2-41U/L)	**61.6 (31.3-117)**	**29 (18.1-49.8)**	**0.002**
ALP (U/L) (42-128U/L)	92.5 (69.7-121.2)	96 (80-151.5)	0.512
Albumin (g/dl) (3.4-4.8g/dl)	**3.26 (2.76-3.80)**	**4.42 (4.23-4.64)**	**0.000**
D-dimer (ng/ml) (0-240ng/ml)	**794.5 (361.9-1954.2)**	**253.2 (148.4-470.7)**	**0.000**
Elevated D-dimer (%) (>240ng/ml)	**91.7**	**55**	**0.002**
D-dimer ULN	**3.31 (1.50-8.14)**	**1.06 (0.63-1.98)**	**0.000**
Ferritin (ng/ml) (30-400ng/ml)	**425.5 (171.0-813.9)**	**81.0 (33.7-155.5)**	**0.000**
Hyperferritinemia (%) (>400ng/ml)	**66.7**	**33.3**	**0.000**
Ferritin ULN	**2.91 (1.24-5.60)**	**0.54 (0.22-1.03)**	**0.000**
LDH (U/L) (135-225U/L)	**278 (186-398)**	**212 (172-271)**	**0.032**
CRP (mg/L) (0-5mg/L)	**27.4 (6.08-77.09)**	**1.71 (0.47-5.79)**	**0.000**
CRP ULN	**5.48 (1.21-15.42)**	**0.34 (0.09-1.14)**	**0.000**
Procalcitonin (ng/ml) (0.01-0.50ng/ml)	**0.12 (0.06-0.29)**	**0.03 (0.02-0.04)**	**0.000**
HbA1c (%) (<5.7% normal)	**6.4 (5.5-7.1)**	**5.3 (5.0-6.0)**	**0.012**
CSS Category 2 or less	**57.7**	**95**	**< 0.001**
CSS category > 2	**42.3**	**5**	

Data are expressed as mean ± SD or median (q25-q75), as appropriate.
- Nil (None of the patients with mild disease had hypokalemia).
The CSS (Scoring system of COVID-19) is a severity score developed and validated for patients with COVID-19. It includes variables like age, coronary heart disease, lymphocytes <8%, procalcitonin >0.15ng/ml, and D-Dimer >0.5µg/ml, with a cut-off exceeding 2 as associated with poor prognosis.
ALC, Absolute lymphocyte count; ALT, Alanine transaminase; ALP, Alkaline phosphatase; ANC, Absolute neutrophil count; AST, Aspartate transaminase; BUN, Blood urea nitrogen; CRP, C- reactive protein; CSS, Scoring system of COVID-19; K, Potassium; LDH, Lactate dehydrogenase; Na, Sodium; NLR, Neutrophil lymphocyte ratio; PLR, Platelet lymphocyte ratio; TLC, Total leucocyte count; ULN, Upper limit of normal.
The bold values refer to the parameters that were significantly different between both groups.

moderate correlation with PLR (0.394, p=0.069 and 0.433, p=0.021). Both serum free T3 and free T4 showed moderate negative correlation with procalcitonin (-0.541, p=0.001; -0.435, p=0.009) in group II.

Patients in group I showed higher levels of LH and FSH as compared to group II (p>0.05). Testosterone levels were lower in group I, with moderate-to-severe disease (2.93 *vs* 7.50nmol/l, p=0.07). A significantly higher number of males in group II had normal gonadal function (78.9 *vs* 25%, p=0.006) (**Figure 1C**). Among those with gonadal dysfunction in group I, central hypogonadism was the more common pattern but primary hypogonadism was present in 18.8% of patients in group I and 5.3% of patients in group II. The prevalence of postmenopausal status was 15.4% in group I and 6.9% in group II. Serum prolactin was lower in group I, with 35.7% of patients having hyperprolactinemia and 10.3% of patients having hypoprolactinemia (**Figure 1D**).

TABLE 3 | Comparative analysis of hormone levels between those with moderate to severe and mild COVID-19.

Parameter (Reference Range)	Moderate to severe disease (n=35)	Mild disease (n=49)	p value
Cortisol (nmol/l) (170-550nmol/l)	433 (353-571)	370 (279-454)	0.053
ACTH (pg/ml) (5-65pg/ml)	16.3 (11.3-53.2)	32.1 (21.7-44.8)	0.234
DHEAS (µg/dl) (age and gender specific)	86.2 ± 80.4	117.4 ± 62.0	0.086
Free T3 (pg/ml) (2-4.4pg/ml)	0.26 (0.21-0.38)	0.33 (0.30-0.35)	0.057
Total T3 (ng/ml) (0.8-2ng/ml)	0.63 (0.52-0.78)	1.11 (0.817-3.695)	0.119
Free T4 (ng/dl) (0.9-1.7ng/dl)	1.41 (1.14-1.56)	1.29 (1.17-1.45)	0.380
Low free T4 (%) (<0.9ng/dl)	**9.5**	**–**	**0.020**
Normal free T4 (%) (0.9-1.7ng/dl)	**76.2**	**97.6**	
High free T4 (%) (>1.7ng/dl)	**14.3**	**2.4**	
Total T4 (µg/dl) (4-12 µg/dl)	5.14 (4.50-6.84)	4.98 (3.54-5.64)	0.913
TSH (µIU/ml) (0.2-4.2 µIU/ml)	**1.48 (0.69-2.70)**	**2.64 (1.68-4.15)**	**0.003**
Low TSH (%) (<0.2 µIU/ml)	**15.6**	**–**	**0.024**
Normal TSH (%) (0.2-4.2 µIU/ml)	**75**	**77.8**	
LH (mIU/mL) (males) (1.7-8.6mIU/mL in males 2.4-12.6mIU/mL in females)	8.7 (1.3-22.7)	6.3 (2.9-19.2)	0.136
FSH (mIU/mL) (males) (1.5-12.4mIU/mL in males 3.5-12.5mIU/mL in females)	2.65 (1.99-20.50)	3.8 (1.76-6.20)	0.474
Testosterone (nmol/l) (9-27nmol/l)	2.93 (0.31-6.28)	7.50 (0.35-13.10)	0.079
LH (mIU/mL) (females)	18.7 (0.5-29.9)	7.4 (1.1-9.2)	0.124
FSH (mIU/mL) (females)	19.5 (1.8-26.6)	4.4 (1.5-6.2)	0.131
Estradiol (pg/ml)	36.8 (10.1-116)	68.6 (18.4-115)	0.455
Prolactin (ng/ml) (5-20ng/ml in males 5-25ng/ml in females)	15.4 (8.7-26.6)	19.7 (14.6-36.6)	0.057

Data are expressed as mean ± SD or median (q25-q75), as appropriate.
- Nil (None of the patients with mild disease had low free T4 or low TSH).
ACTH, Adrenocorticotrophic hormone; DHEAS, Dehydroepiandosterone sulphate; FSH, Follicle-stimulating hormone; LH, Luteinising hormone; T3, Tri-iodothyronine; T4, Tetra-iodothyonine; TSH, Thyroid-stimulating hormone.
The bold values refer to the parameters that were significantly different between both groups.

DISCUSSION

The current study is the first, to the best of our knowledge, to comprehensively explore the prevalence, nature, and degree of endocrine dysfunction stratified based on disease severity at a dedicated COVID care center. We observed a higher prevalence of hypocortisolism, low ACTH, and DHEAS in patients with moderate-to-severe disease. Low T3 syndrome was a universal finding irrespective of disease severity. SES (apart from the low T3 syndrome) was more common in those with moderate-to-severe disease. A unique, less-frequently described pattern of atypical thyroiditis was present in 6.4% of the entire population, with greater prevalence in those with moderate-to-severe disease. Male hypogonadism was observed in a significant majority of those with moderate-to-severe disease, with secondary hypogonadism being more prevalent. Lactotroph function analysis showed a similar prevalence of hyperprolactinemia in both groups. These findings suggest that endocrine dysfunction is common and more severe in patients with severe COVID-19.

The dysregulated systemic inflammation characteristic of COVID-19 leads to severe multisystem pathology (26). We observed significantly greater renal and hepatic dysfunction, elevated ferritin, D-dimer, LDH, inflammatory markers, as well as a greater proportion of high CSS in those with moderate-to-severe disease. Patients with moderate-to-severe disease were significantly older and showed a male gender predilection.

Lessons from past experience with SARS, have taught us that multiple endocrine axes can be affected, manifesting as delayed central hypocortisolism and hypothyroidism and thyroid follicular cell injury (21–23). Due to the etiopathogenetic similarity between SARS-CoV and SARS-CoV-2, endocrine involvement in COVID-19 is likely to be present. But, it is imperative to acknowledge the multiplicity of involvement within a single endocrine axis. For example, thyroid function can be influenced directly by the virus, SES, HPT dysfunction, or any combination of these, resulting in a varying clinical picture. Similarly, the HPA axis may be involved at the central level (hypothalamus and pituitary) or at the level of adrenals due to hemorrhage/infarction and the HPG axis may be involved at the central level or direct viral-mediated cytotoxicity to the gonads owing to high expression of ACE2. Therefore, studies aimed to explore the integrity of the entire endocrine axis are essential, so as to improve understanding, with possible potential ramifications for therapy in the overall management of a COVID-19 patient.

HPA Axis Function Assessment

In the current study, we observed a higher prevalence of hypocortisolism (38.5%) in those with moderate-to-severe disease, whereas nearly 86.4% of patients with mild disease

	Low DHEAS (%)	Hypercortisolism (%)	Eucortisolism (%)	Hypocortisolism (%)
Mild disease (n=49)	14.2	6.8	86.4	6.8
Moderate to severe disease (n=35)	21.7		61.5	38.5

	Hypothyroidism (%)	Atypical thyroiditis (%)	SES (apart from low T3 syndrome) (%)	Secondary hypothyroidism (%)
Mild disease (n=49)	24.5	2.4	73.1	0
Moderate to severe disease (n=35)	4.8	14.3	52.4	28.5

	Normal gonadal function in males (%)	Secondary hypogonadism (%)	Primary hypogonadism (%)
Mild disease (n=49)	79.4	15.3	5.3
Moderate to severe disease (n=35)	24.4	56.8	18.8

	Hypoprolactinemia (%)	Hyperprolactinemia (%)
Mild disease (n=49)	0	46.7
Moderate to severe disease (n=35)	10.7	35.7

FIGURE 1 | **(A)** Hypothalamo-pituitary-adrenal (HPA) axis dysfunction in patients with COVID-19 stratified on the basis of disease severity. **(B)** Hypothalamo-pituitary-thyroid (HPT) axis dysfunction in patients with COVID-19 stratified on the basis of disease severity. **(C)** Hypothalamo-pituitary-gonadal (HPG) axis dysfunction in male patients with COVID-19 stratified on the basis of disease severity. **(D)** Lactotroph dysfunction in patients with COVID-19 stratified on the basis of disease severity.

were eucortisolic. Paired cortisol and ACTH, DHEAS analysis suggested that the predominant pattern of hypocortisolism was central hypoadrenalism. Further, in eucortisolic patients, the predominant pattern was a low or inappropriately normal ACTH, indicating insult to the corticotropes.

The basis of adrenal dysfunction stems from the observation of hypocortisolism in 39.3% of patients (n=61) in a cohort of SARS survivors (21). Concurrent thyroid dysfunction was present in 10% of patients, suggesting hypophysitis or hypothalamic dysfunction. Further, molecular mimicry between SARS-Co-V and peptide fragments of ACTH points towards the role of anti-ACTH antibodies (27). A similar strategy may be utilized by SARS-CoV-2, and exploring this possibility in the sera of affected patients can be useful. However, in an earlier study reporting cortisol assessment in COVID-19 patients, the median levels of cortisol were higher (619nmol/l) than in patients without COVID-19 (519nmol/l), but both groups were eucortisolic in terms of HPA axis evaluation during stress (8). Lack of reporting of concurrent ACTH and DHEAS levels and comparison with non-COVID patients was performed without information on disease severity, preventing analysis of the entire axis integrity. Our observation of secondary hypoadrenalism especially in those with moderate-to-severe disease suggests the possibility of hypophysitis as an underlying etiopathogenic mechanism in these patients. Alternatively, a relative increase in cortisol due to reduced clearance and acquired glucocorticoid resistance at GR-α during the initial stages of the neuroendocrine stress response will inhibit ACTH, which mimics a profile of functional central hypoadrenalism (28, 29).

The atypical finding in the current study was that DHEAS levels were normal, as opposed to expected lower levels in accordance with the progression of adrenal insufficiency (30). Usually, there is a dissociation between the rates of cortisol and DHEAS production, such that cortisol output is maintained till later during the course of illness by diverting the intermediates from adrenal androgen synthesis to the formation of glucocorticoids including cortisol. However, there is a small proportion of patients reported with normal DHEAS in presence of secondary adrenal insufficiency (21, 31). Additionally, DHEAS levels have been found to increase as an adaptive response in otherwise normal individuals exposed to acute stress (32, 33). This may be a plausible reason for our observation of normal DHEAS in the current study, as DHEAS estimation was performed within 24 to 48 hours of hospital admission.

HPT Axis Function Assessment

We noted a range of patterns varying from new-onset hypo to hyperfunctioning of the HPT axis in COVID-19. Nearly three-fourths of patients with mild disease were euthyroid as compared to only half of patients with moderate-to-severe disease. Low T3 syndrome was not discriminatory as it was present in all patients. SES (apart from low T3 syndrome) was equally prevalent in both groups. However, the exceptional pattern of thyroid dysfunction that emerged in the current study included atypical thyroiditis in 6.4% of the entire cohort, with higher prevalence in those with moderate-to-severe disease.

The major pattern of HPT axis function in acute illness includes cytokine-induced SES (low T3 in mild illness, low T4 with increasing illness severity) with low or normal TSH (34). The other pattern of SES can be normal T4 with low TSH. Rarely, SES with high total T4 has been described, but mostly in patients with an underlying increased cause for thyroid-binding globulin (TBG) excess (35). Other factors that could influence the HPT axis in acute illness include alteration of TBG, use of drugs that can displace circulating T4 from TBG leading to high free T4 (e.g., heparin), and use of iodinated contrast for diagnostic imaging. In the current study, the issue of TBG alteration was circumvented by measuring free hormones and there was no/minimal exposure to contrast agents and heparin respectively. SARS-CoV-2 has been proposed to cause direct thyroid dysfunction owing to ACE2 mediated viral entry and destructive thyroiditis (15, 16). Though rarely, triggering of autoimmunity and Graves' disease and myxoedema coma have also been reported in COVID-19 patients (18, 36).

In the current study, we observed a higher prevalence of thyroid dysfunction than reported in the THYRCOV study, possibly due to the inclusion of participants with moderate-to-severe disease (12). We noted a good negative correlation of TSH with CRP and ferritin. But, low free T3 was not observed in that study, while in another study assessing thyroid function in COVID-19 patients, lower total T3 and TSH were documented as compared to non-COVID-19 pneumonia and normal patients, without a significant difference in total T4 levels (13). We observed a similar state of low T3 syndrome in accordance with the fact that T3 is reduced even in mild disease severity, with increased conversion of T4 to reverse T3.

However, the most intriguing aspect of HPT axis assessment in our study was the observation of a subset of patients exhibiting a mixed pattern of thyroid dysfunction (atypical thyroiditis), possibly due to combined systemic inflammation at the level of the gland (destructive thyroiditis) as well as the pituitary (a component of SES). This is only the second report in the available literature to explore and validate this unique pattern of HPT involvement in COVID-19 (14).

HPG Axis and Lactotroph Function Assessment

The gonadal axis in the current study, evaluated only in males, revealed a significantly higher prevalence of hypogonadism and lower levels of testosterone in those with moderate-to-severe disease. Further, testosterone levels were negatively correlated with NLR, suggesting an association between disease severity. More patients had secondary hypogonadism despite no greater prevalence of hyperprolactinemia than those with mild disease, suggesting a direct HPG axis suppression owing to the virus or cytokines. Interestingly, a three times higher prevalence of primary hypogonadism was also observed in those with moderate-to-severe disease with higher LH than FSH, suggesting a non-negligible proportion of patients with primary injury at the level of the gonad (especially Leydig cells) itself.

Gonadal function has been evaluated in patients with COVID-19 in a prior study which showed a primary testicular failure with significantly higher LH and a lower testosterone:LH ratio but no significant difference in testosterone levels (19). Our

findings are similar to this study and suggest that primary testicular failure, especially Leydig cell insult, should be borne in mind in managing male patients with COVID-19.

Gonadal function in COVID-19 can be influenced by gonadal ACE2 expression leading to viral orchitis, hypogonadism due to cytokines inhibiting the HPG axis, use of glucocorticoids, or orchitis. Data on female gonadal function in COVID-19 are scarce. In the current study, we did not analyze detailed gonadal function since data on menstrual cycle details was not available in the majority of patients.

The strengths of the study include a comprehensive evaluation of all the components of various endocrine axes and the determination of the association of hormonal dysfunction with disease severity in COVID-19. Further, the exclusion of patients with variables that could affect HPA or HPT axes and estimation of free T3, T4 made the analysis more robust. The study has limitations, including a small patient number, cross-sectional nature, single time estimation, lack of a control group (including non-COVID pneumonia or patients undergoing major surgery with negative RT-PCR status), non-availability of adiposity measures or menopausal status, non-performance of parameters like cytokine profile and hormone-binding globulins, and non-evaluation of final clinical outcomes in enrolled patients. Though previous hormonal parameters were not available, patients with a known endocrine condition on treatment for the same were excluded from the analysis. Non-assessment of the short synacthen test (SST), anti-hypothalamic and anti-pituitary antibodies, MRI sella, nuclear scintigraphy of the thyroid, reverse T3 (which could have helped us to differentiate between sick euthyroid syndrome and secondary hypothyroidism) and thyroid autoantibodies, and evaluation of diabetes insipidus are other perceived limitations. The SST currently holds low accuracy in diagnosing hypocortisolism in acute stress, but we plan to follow-up patients for re-evaluation of hormonal axes and antibodies in sera in the future. This can aid in delineating whether it is the viral cytopathic effect or immunoinflammatory effect that is the predominant cause of endocrine involvement. Non-inclusion of a control group with non-COVID pneumonia or acute respiratory distress syndrome of similar severity to assess the COVID-specific involvement of the endocrine system is another limitation of the study. However, due to the limited sensitivity of PCR for diagnosing SARS-CoV-2 and the possibility that secondary bacterial infection in a given patient of COVID-19, rather than the viral disease per se, can cause endocrine dysfunction as well as COVID being the major focus of healthcare with resource constraints for other illness conditions, a control group was not included.

CONCLUSION

Multiple endocrine organs and axes are potentially involved by the novel coronavirus SARS-CoV-2, with a greater prevalence of endocrine dysfunction in more severe disease. The involvement of multiple axes, particularly at the hypothalamo-pituitary level, suggests the possibility of hypophysitis as an underlying etiology. Follow-up surveillance of these patients at periodic intervals should be considered to unfold the etiology as well as the reversibility of dysfunction.

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 Institute Ethics Committee, PGIMER. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the study and approved the final manuscript.

ACKNOWLEDGMENTS

We wish to acknowledge the patients and all healthcare workers involved in the management of the current cohort as well as worldwide for their tireless service against this immense health challenge.

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

frontiers in Endocrinology

MINI REVIEW
published: 22 July 2021
doi: 10.3389/fendo.2021.587801

Metformin and Covid-19: Focused Review of Mechanisms and Current Literature Suggesting Benefit

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OPEN ACCESS

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Specialty section:
This article was submitted to Translational Endocrinology, a section of the journal Frontiers in Endocrinology

Received: 28 July 2020
Accepted: 11 May 2021
Published: 22 July 2021

Citation:
Ibrahim S, Lowe JR, Bramante CT, Shah S, Klatt NR, Sherwood N, Aronne L, Puskarich M, Tamariz L, Palacio A, Bomberg E, Usher M, King S, Benson B, Vojta D, Tignanelli C and Ingraham N (2021) Metformin and Covid-19: Focused Review of Mechanisms and Current Literature Suggesting Benefit. Front. Endocrinol. 12:587801. doi: 10.3389/fendo.2021.587801

Metformin is the first-line medication for type 2 diabetes, but it also has a long history of improved outcomes in infectious diseases, such as influenza, hepatitis C, and *in-vitro* assays of zika. In the current Covid-19 pandemic, which has rapidly spread throughout the world, 4 observational studies have been published showing reduced mortality among individuals with home metformin use. There are several potential overlapping mechanisms by which metformin may reduce mortality from Covid-19. Metformin's past anti-infectious benefits have been both against the infectious agent directly, as well as by improving the underlying health of the human host. It is unknown if the lower mortality suggested by observational studies in patients infected with Covid-19 who are on home metformin is due to direct activity against the virus itself, improved host substrate, or both.

Keywords: metformin, COVID-19, mechanisms of action, obesity, microbiome

INTRODUCTION

Metformin was discovered in the 1920s (1). In the 1940s-50s, metformin showed benefit when used in influenza infection, and was noted to lower glucose, but not below physiologic levels (2). Other biguanide medications then had safety issues, so by association metformin then fell out of favor until the 1990s (1). Currently, metformin has Food and Drug Administration (FDA) approval as a first-line medication for type 2 diabetes, on and off-label indications for diabetes prevention in prediabetes; and off label for polycystic ovarian syndrome; anti-psychotic associated weight gain; weight loss; gestational diabetes; and fertility enhancement (3, 4). There is also mounting evidence supporting the potential effects of metformin beyond glucose control in the aging population (5). It appears that increased biologic aging may be a key underlying risk factor for poor outcomes from

severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) disease, Covid-19, as Covid-19 has disproportionately affected older individuals (6, 7).

Several observational cohort studies have been published in peer review journals showing associations with reduced mortality from Covid-19 among patients already who were already on metformin (**Table 1**) (8, 9, 11–13). It appears that metformin may be associated with less severe Covid-19 disease, however there are no prospective studies published to date. It must be highlighted that observational results are not conclusive because of the inherent challenge of eliminating residual confounding. Comorbidities associated with Covid-19 outcomes (i.e. hypertension, coronary artery disease, obesity) are commonly present in groups exposed and not exposed to metformin in retrospective cohorts, which make it challenging to know if observational data would be replicated in randomized, prospective trials (7, 28).

Here, we present a targeted review of the literature on the mechanistic reasons why metformin might improve outcomes from Covid-19, and a review of the observational data showing the potential benefit from metformin use in Covid-19. Furthermore, given the known sex-specific differences in Covid-19 outcomes (29) and sex-specific associations between metformin and mortality from Covid-19, we also highlight potential sex-specific effects of metformin in this review.

PRIMARY MECHANISM OF ACTIONS

Currently and prior to the Covid-19 pandemic, the molecular mechanisms by which metformin works have been a topic of debate and responses to metformin have often been variable (30, 31). In patients with diabetes mellitus, metformin improves glycemia largely by decreasing hepatic gluconeogenesis, primarily through phosphorylation of AMP-activated protein kinase (AMPK) (32). Activation of AMPK enhances hepatic insulin sensitivity and gut utilization of glucose, promoting glucagon-like peptide-1 (GLP-1) secretion, and favorably altering the gut microbiome (33). Metformin has been found to have cytokine-reducing effects in both patients with and without diabetes mellitus, which may be of primary import in Covid-19 (34).

The effect of metformin on cytokines has been thought to be mediated by blocking the AMPK cytokine receptor pathway and thus decreasing in proinflammatory genes, though some literature has suggested AMPK-independent means exist as well (34–36).

Cytokine storm leads to severe disease in COVID 19, and hence contributes significantly to morbidity and mortality (37). By the virtue of dampening the cytokine storm, metformin may reduce the morbidity in COVID 19. The many possible mechanisms by which metformin may be protective against Covid-19 have been the topic of several recent papers (38–41). A summary of mechanisms by which metformin has been theorized to convey benefit from Covid-19 is found in **Table 2**. We also summarize published literature evaluating the relationship between Covid-19 and metformin use.

THEORIZED MECHANISMS TO SUPPORT THE USE OF METFORMIN IN COVID-19

High Glucose Is Associated With Worse Covid-19 Outcomes

Hyperglycemia was found to portend worse outcomes in severe acute respiratory syndrome (SARS) infection in 2003 (72), and is associated with increased length of stay and mortality in patients hospitalized with Covid-19 (58). Worse glucose control has also been associated with higher mortality and end-organ complications in patients with Covid-19 (15). A recent study by Crouse et al. found that metformin use prior to the diagnosis of COVID-19 was associated with a ~70% decrease in mortality in persons with diabetes (10), though this is a larger effect size than what is seen in most studies (10).

Metformin Could Decrease Endothelial Injury and Its Complications

Metformin has been shown to improve microvascular endothelial function in women, perhaps due to a significant increase in response to acetylcholine, decrease in insulin resistance, and non-significant decrease in tissue plasminogen activating factor, as was shown in a randomized control study of 8 weeks of metformin *versus* placebo in women with angina and normal coronary arteries (67). Endothelial dysfunction may be an important mechanism and therapeutic target in mitigating Covid-19 sequelae. Metformin decreases thrombosis in long-term follow-up, possibly by inhibiting platelet activation factor and mitochondrial DNA (mtDNA) release (63, 64), and may mediate improved cardiovascular outcomes *via* mechanisms beyond glucose control. COVID associated coagulopathy and thrombosis is a unique attribute of this disease, and the pathophysiology is poorly understood. Widespread micro and macrovascular thrombosis has been reported in autopsies of patients with Covid-19 (65, 66).

Metformin Has Been Associated With Beneficial Effects on the Lungs

In vivo models have demonstrated that metformin overcomes anabolic metabolism promoting AMPK-dependent resolution of lung tissue damage, indicating a potential role in addressing inflammation induced pulmonary fibrosis as a result of severe infections (73). Pulmonary vascular endothelitis has been observed in the lungs of patients with Covid-19 (68). Earlier studies also demonstrated that metformin inhibits AGEs-induced inflammatory response in murine macrophages partly through AMPK activation and RAGE/NFκB pathway suppression, felt to be important in Covid-19 related lung-centric inflammation and endothelitis (74). Further, metformin has been associated with reduced pulmonary fibrosis through reduced TGF-beta and VEGF, and resolution of pulmonary

TABLE 1 | Overview of papers in 2020 with findings related to metformin and Covid-19.

Author	Population	Methods	Finding	Mechanism or other findings
Outcomes in Covid-19				
Luo et al. (8) *Am J Trop Med Hyg*	283 adults with T2DM hospitalized with Covid-19 in Wuhan.	Retrospective cohort, 104 adults on metformin with 179 not on metformin. Metformin use appears to be home metformin use.	- Hospital mortality, metformin *vs* no metformin: 2.9% *vs* 12.3% (p=0.01) - No difference in length of stay - No associations with other T2DM meds - OR for survival: 4.36 (1.22-15.59, p=0.02)	Lower glucose levels in the metformin group, 9.19 *vs* 7.36 (p<0.01), no difference in neutrophils or lymphocytes.
Cariou et al (9), "Coronado study" *Diabetalgia*	1,317 adults with T2DM in France, with or without home metformin use	Multi-center observational study. Main outcome: mortality or intubation; Secondary outcome was mortality	- HbA1C not associated with main outcome (p=0.28) or death (p=0.91) - Preadmission metformin use associated with lower mortality OR 0.59 (0.42, 0.84), - No association with other T2DM meds - OR mortality with insulin 1.71 (1.20, 2.43)	CRP 1.99, (1.69, 2.43), lymphocyte count (OR 0.69, 0.60-0.80), fibrinogen OR 1.32 (1.09, 1.58) and AST (OR 2.23, 1.70-2.93) predicted mortality.
Crouse et al. (10), *Frontiers in Endocrinology*	25,326 subjects tested for Covid-19 between 2/25/20 and 6/22/20 in Alabama.	Retrospective electronic health records study assessing mortality in Covid-19	- Association between prior metformin use and a reduction in mortality (OR 0.33, 95% CI 0.13-0.84; p=0.02) compared to those with T2DM not on metformin.	Glucose levels similar between both groups, Metformin mechanism may reside outside of its glycemia.
Bramante et al (11), *Lancet Health and Longevity*	6,256 adults with T2DM or obesity hospitalized for Covid-19 in the US	Retrospective review of USA UnitedHealth Group claims data; 2,333 in metformin group, 3,923 in no-metformin group	- Metformin associated with reduced mortality in females: OR 0.759 (0.601, 0.960) by propensity matching; OR 0.780 (0.631, 0.965) by mixed effects; OR 0.785 (0.650, 0.951) by Cox proportionalhazards.	In same sample, TNFα inhibitors were associated with decreased mortality (only 38 patients), suggesting TNFα a possible pathway.
Lalau et al (12), *Diabetes & Metabolism*	2449 adults with T2DM with or without previous metformin use	Multi-center observational study. Main outcome: mortality or intubation within 7 days and 28 days of admission	- Mortality rate in metformin users *vs* non: day 7 (8.2 *vs* 16.1%, $P < 0.0001$); day 28 (16.0 *vs* 28.6%, $P < 0.0001$) - Mortality by propensity score weighting, metformin users *vs* non: day 7 OR 0.67 (0.47 −1.01); day 28 0.71 (0.54–0.94).	Metformin users presented greater case severity on admission regarding clinical, radiological, and biological features, compared with non-users.
Lukito et al (13), *Diabetes & Metab Synd: Clin Res& Rev*	Meta-analysis of 10,233 adults across 9 studies	The mean NOS of the included studies was 8.55 ± 0.52, indicating high-quality studies.	- Metformin use associated with lower mortality in pooled non-adjusted model, OR 0.45 (0.25, 0.81), p=0.008; and adjusted (OR 0.64 (0.43, 0.97), p = 0.035.	SARS-CoV-2 damages β-cells. Optimal control of T2DM, for chronic and transient cases, may help in treating COVID-19
Mechanistic, diabetes, and safety findings in Covid-19				
Chen et al. (14) *Diabetes Care*	904 patients with Covid-19, 136 of whom had T2DM	Characteristics and outcomes of patients with T2DM and Covid-19. No results reporte for use of GLP-1 receptor agonists.	- Metformin users *vs* non-users: No significant difference in likelihood of 'poor prognosis': 30% *vs*. 50%, p=0.688 - In PCR-confirmed cases, no difference in in-hospital death (18.2% *vs* 26.1%, p=0.77). No associations with DDP-4i's	Metformin users had lower IL-6 (4.07 *vs* 11.1, p=0.02). In PCR-confirmed cases, IL-6 was also lower in metformin users than non-metformin users (4.77 *vs* 11.1, p=0.024).
Zhu et al. (15) *Cell metabolism*	952 adults with T2DM Covid-19 and in Hubei, China	Retrospective review. Metformin was given in hospital to 278 patients.	- Metformin was more likely to be given to those with poor glucose control. - No metformin specific results reported.	Worse glucose control associated with mortality and end-organ complications.
Montastruc et al. (16)	10,771 ICSRs involving hydroxychloroquine	Retrospective review, outcomes of mortality	- Hydroxychloroquine + metformin associated with a ROR of 57.7 (23.9-139.3) compared to hydroxychloroquine - Hydroxychloroquine + metformin was associated with a ROR value of 6.0 (2.6-13.8) compared to metformin alone	More autophagosomes in heart, liver, kidneys of mice treated with both. Synergism of inhibition of mitochondrial complex I, and autophagy from hydroxychloroquine (17).
Huh et al (18), medrxiv.org	65,149 adults, claims data in S Korea.	Case control study, metformin (n=219) *vs* control (n=3604)	- Risk of Infection, crude OR 0.69 (0.60-0.80), aOR: 0.95 (0.81-1.11).	Covariates: sex, age, region, comorbidities, meds, utilization
Nafakhi et al, *Diabetes & Metab Synd: Clin Research & Reviews*	192 patients with COVID-19 pneumonia, of whom 67 patients had T2DM	Retrospective cohort of patients with newly diagnosed COVID-19 pneumonia; August 20, to October 5, 2020 in Iraq	- Metformin use associated with lower ICU days, OR 0.30 (0.20-0.40, p=0.03); hospital days, OR 0.40 (0.20-0.30,p=0.02); and in-hospital mortality OR 0.10 (0.1–0.6), p = .025.	Insulin use was associated with extensive lung injury and post-acute COVID-19 pneumonia partial recovery
Cheng et al. (19)	1,213 patients with Covid-19 and T2DM	Retrospective cohort of individuals hospitalized	HR Acidosis 2.73 (1.04-7.13,p=0.04); lactic acidosis 4.46 (1.11,18.00,p=0.04)	Appears to be for use of 2-3g/day during hospitalization.

(Continued)

TABLE 1 | Continued

Author	Population	Methods	Finding	Mechanism or other findings
Evidence that metformin does reduce TNF-alpha in both males and females.				
Author	Population		Finding	
Krysiak et al. (20)	Humans, 36% female, did not compare men *vs* women.		After 12 weeks of treatment, metformin "reduced plasma C-reactive protein levels and monocyte release of TNFα and IL-6, as well as tended to reduce monocyte release of IL-1β and monocyte chemoattractant protein-1, which was accompanied by an improvement in insulin sensitivity.	
Andrews et al. (21)	Humans, men only with obesity and diabetes, ave age 55 years.		Those "treated with metformin had lower levels of hsCRP expression of TNFα and TLR 2/4, than their counterparts not receiving the drug."	
Hyun et al. (22)	Mice, male only		Metformin suppresses scavenger receptors in macrophages, down-regulates TNFα	
Metformin and sex-specific findings.				
Author	Population		Finding	
Park, J, et al. (23)	Patients with colorectal cancer		Interaction test between metformin and sex after adjustment for relevant factors revealed that female CRC patients taking metformin exhibited a significantly lower CRC-specific mortality rate than male CRC patients taking metformin (HR = 0.369, 95%CI: 0.155-0.881, P = 0.025). Subgroup analysis revealed significant differences in CRC-specific mortality between the metformin and non-metformin groups in female patients (HR = 0.501, 95%CI: 0.286-0.879, P = 0.013) but not male patients (HR = 0.848, 95%CI: 0.594-1.211, P = 0.365).	
DPP (24)	Adults with overweight & preDM		Metformin reduced CRP by 7% in men and 14% in women	
Quan, H, et al. (25)	105 human patients		Combined exenatide and metformin showed better effects on female than male patients for improving insulin sensitivity and serum lipid profile, reducing insulin resistance, increasing adiponectin levels, and decreasing the levels of HbA1c, BMI, resistin, TNF-alpha, CRP ($p<0.05$).	
Naffaa et al (26),	113, 749 patients who started metformin from 1998-2014.		Adherence assessed by the mean proportion of follow-up days covered (PDC) with metformin. Adherence with was associated with a reduced risk of developing RA in women, not men.	
Jiang et al. (27)	328 patients with T2D and Covid, 100 of which were on metformin while hospitalized		In the mixed-effected model, metformin use was associated with the lower incidence of ARDS. Metformin may have potential benefits in reducing the incidence of ARDS in patients with COVID-19 and type 2 diabetes. However, this benefit differs significantly by gender as confirmed by subgroup analysis, metformin use was associated with the lower incidence of ARDS in females.	

T2DM, Type 2 diabetes mellitus; PCR, Polymerase chain reaction; GLP-1, glucadon-like-peptide 1; DDP-4, Dipeptidyl peptidase-4; WBC, White blood cells, HbA1C, Hemoglobin A1c; CRP, C-reactive protein, AST, Aspartate aminotransferase; OR, odds ratio; ROR, Risk Odd Ratio; AMPK, adenosine monophosphate protein kinase; mTOR, mammalian target of rapamycin; NFK, nuclear factor kappa light enhancer of activated B cells (NFKB); TLR, Toll Like Receptor. DPP, Diabetes Prevention Research Group; CRC, Colorectal cancer; BMI, Body mass index; RA, Rheumatoid arthritis.

fibrosis *via* activation of lung myofibroblasts (41, 69). Pulmonary fibrosis has been noted in persons with Covid-19, specifically in those with elevated IL-6 levels (70).

Metformin Has Immune-Modulatory Effects

In patients with and without diabetes, metformin has been shown to favorably alter inflammatory mediators, including interleukin 6 (IL-6), TNFα, to possibly boost interleukin 10 (IL-10), and suppress the C-C motif chemokine ligand (34, 44–46). Metformin's activation of the AMPK/mTor/Stat3 pathway appears to steer macrophages away from the pro-inflammatory classical activation that produces TNF/IL6/IL1b, cytokines that contribute to morbidity in Covid-19 (37, 49, 50). Possible evidence of this effect was seen in a retrospective study by Chen et al: 904 patients with Covid-19 which showed that metformin users had lower IL-6 levels compared to non-metformin users (**Table 2**) (56). Metformin also inhibits toll-like-receptor 7 (TLR7) signaling and interferon production, which appears important to Covid-19 pathophysiology (47, 48). Metformin also inhibits IgE- and aryl hydrocarbon-mediated mast cell activation (59). Mast cell activation has been implicated as an early indicator of inflammatory response to SARS-CoV-2. and possibly an indicator of impending cytokine storm (61, 62). Mast cells from female rats have been found to cause a greater increase in tumor necrosis factor alpha (TNF-alpha) than mast cells in male rats, which may explain the observational findings of reduced mortality in women on metformin, but not among men on metformin (**Table 1**) (60).

Metformin decreases neutrophil-extracellular traps (NETs), and the neutrophil to lymphocyte ratio (34, 51, 52). NETs are microbiocidal compounds containing DNA, histones, and proteins (51, 52). Sera from patients with Covid-19 demonstrate elevated levels of these histones and DNA components (52). It has been hypothesized that excessive NET formation leads to cytokine storm and microthrombus (possibly independent of tissue factor), and ultimately acute respiratory distress syndrome (ARDS) in Covid-19 (54). Lymphopenia and neutrophil infiltration in pulmonary capillaries have been an important feature of severe Covid-19 disease (7, 34, 56, 57). Metformin's inhibition of NET release could therefore mitigate the development of downstream lung injury.

TABLE 2 | Overview of mechanisms of action of metformin and their relationship to SARS-CoV-2 infection.

Pathways	Metformin's overlapping mechanisms of action	Theorized Covid-19 relationship
I. Viral entry and lifecycle	• Activates AMPK, which can lead to conformational changes to ACE2 (39, 42, 43). • inhibits mTOR reducing -viral protein complexes central to viral replication (39).	Decreased SARS-CoV-2 entry *via* the ACE2, and replication through Orf9c and Nsp7 (38, 39, 42, 43).
II. Immune modulation includes	• Decreases IL-6, TNFα, and suppresses c-c motif chemokine ligand (34, 44–46); Inhibits TLR-7 signaling (40, 47, 48). Possibly boosts IL-10 as well (IL-10 is hard to interpret because it may be elevated in a response to reduce TNFα.) (34)	These cytokines contribute to morbidity in Covid-19 (37, 49, 50). Chen et al. found lower IL-6 with metformin use (14).
III. Neutrophil-extracellular traps	• Decreases neutrophil-extracellular traps (NETs), which are released from neutrophils and contain DNA, histones, and proteins that are microbiocidal (51, 52). • Neutrophil count dropped by >1,000 cells/mm (3) in 3 months of metformin (53). • Patients with Covid-19 have had elevated levels of histones and DNA components from NETs (54). A byproduct of NETs may accelerate viral entry (55)	Excessive NET formation leads to cytokine storm and microthrombus (possibly independent of tissue factor), and ARDS in Covid-19 (54). Neutrophil infiltration in pulmonary capillaries have been an important feature of severe Covid disease (7, 34, 56, 57).
IV. Decreased glycemia	• Phosphorylates AMPK (32), improving hepatic insulin sensitivity, gut utilization of glucose, GLP-1 secretion and favorably altering the gut microbiome (33).	Glycemia is associated with increased length of stay and mortality in patients with Covid-19 (58).
V. Mast cell stabilization	• Inhibits IgE- and aryl hydrocarbon- mediated mast cell activation (59). Mast cells in female rats cause greater increase in TNFα than mast cells in male rats, which may explain a larger benefit from metformin in women than men with Covid (60).	Mast cell activation has been cited as an early indicator of inflammatory response to SARS-CoV2 and cytokine storm (61, 62).
VI. Decreased thrombosis	• Decreases thrombosis in longterm follow-up, felt to be by inhibiting platelet activation factor and mtDNA release (63, 64).	Thrombosis is an important component of Covid-19 pathology (65, 66).
VII. Endothelial function	• Significantly decreases HOMA-IR and non-significant decrease in tissue plasminogen activating factor 8 weeks after randomization to metformin (67).	Pulmonary vascular endothelitis has been found in lungs of patients with Covid-19 (68).
VIII. Pulmonary fibrosis	• Increase resolution of fibrosis *via* AMPK activation of lung myofibroblasts (69) • Reduced pulmonary fibrosis (through NFK, reduced TGF-beta, VEGF) (41).	Fibrosis occurs after Covid-infection, especially in patients with high IL-6 (70).
IX. Endosomal pH	Increasing pH *via* action on vacuolar ATPase, endosomal Na+/H+ exchangers (71)	High endosomal pH inhibits viral replication.

Proposed assays to assess these pathways in patients with Covid-19, with and without metformin use:

- Flow cytometry; mean platelet volume
- ELISA assays for glycemia and inflammatory markers
- MPO/dsDNA and citrullinated-histone H3 to assess NETs
- Cytokine 45-plex assay
- IFABP and LPS limulus assay to assess gut-epithelial integrity and microbiotal translocation
- M30-apoptosis ELISA (for CK-18) to assess the influence of hepatic steatosis
- Stool microbiome, and in-vitro assays with metformin and SARS-CoV-2
- Viral load

NETs, neutrophil-extracellular traps; ACE2 angiotensin-converting enzyme 2; AMPK, AMP-activated protein kinase; GLP=1, glucagon-like peptide-1; IL, interleukin; mTOR, mammalian target of rapamycin.

Metformin Could Decrease the Viral Cycle

There is some evidence that metformin increases endosomal pH *via* action on vacuolar ATPase and/or endosomal Na+/H+ exchangers (71), thus reducing viral replication. Metformin also leads indirectly to alteration of the mammalian target of rapamycin (mTOR) pathway (38), which could decrease the viral lifecycle through effects on proteins including Orf9c and Nsp7 (38). Orf9c may enable immune evasion (75), and Nsp7 is necessary for RNA polymerase activity (76). Other studies suggest a link between elevated levels of IL-6 and AMPK/mTOR signaling pathway and their role in exacerbating diabetes-induced complications and insulin resistance (77). This has led to mTOR inhibitors being suggested as potential therapeutics in treatment of Covid-19 (78). Gordon et al. did find *in-vitro* efficacy of metformin against SARS-CoV-2, but did not elucidate the mechanism of viral inhibition (79).

Metformin Could Decrease Entry of the SARS-CoV-2 Into Cells

Activation of AMPK by metformin increases phosphorylation and expression of angiotensin-converting enzyme 2 (ACE2) (80). Phosphorylation of the ACE2 receptor may alter the conformation of the extracellular domain of ACE2 and decrease SARS-Cov-2 entry into cells (6, 39, 42, 43, 81).

Li and colleagues found that expression of ACE2 is equal in male and female human lungs (82), which differs from one prior study showing lower levels of ACE2 receptor in the lungs of middle-aged male rats, however no sex difference in young and

old-age rats (83). Li and colleagues did find that the immune response to SARS-CoV-2 in the lungs differs between men and women, with differing cytokine responses (82). This pathologic finding, with our observational findings that metformin conveyed greater protection in women than men, may support that anti-inflammatory effects may be the primary ways in which metformin is protective in Covid-19 (11).

Metformin Also Reduces Body Weight

Obesity is a known risk factor for poor outcomes in Covid-19 infection, second only to increased age (31). Several studies have shown that metformin use is associated with reduced body weight in patients with and without T2DM (3, 84), and growth/differentiating factor 15 (GDF 15) appears to be an important mechanism of action by which metformin causes weight loss (85). An increase in GDF15 has been associated with decreased food intake and lowered body weight (85). It is unknown if weight loss in the weeks, months, or years prior to a Covid-19 infection would improve outcomes from Covid-19.

BY IMPROVING THE MICROBIOME, METFORMIN MAY LIMIT SYSTEMIC INFLAMMATION

In recent years, it has become increasingly clear that healthy microbial communities that make up the microbiome are critical to human health. Obesity, diabetes mellitus, and other metabolic disorders are all associated with imbalanced microbial communities, known as "microbial dysbiosis." (86) Dysbiosis of the microbiome can result in several deleterious effects that can lead to poor health outcomes, including mucosal and systemic inflammation, microbial translocation, and damage to the tight epithelial barrier of mucosal sites such as the gut. Furthermore, novel studies have demonstrated that Covid-19 disease is associated with microbial dysbiosis, which may be a potential mechanism underlying overt inflammation and dysregulated immunity in Covid-19 disease (87, 88).

Given the above, another mechanism by which metformin may limit disease severity in Covid-19 is by enhancing the microbiome to promote anti-inflammatory effects. Indeed, several studies have demonstrated that metformin can alter the microbiome in a potentially beneficial manner. This includes: (i.) increased "probiotic" strains (i.e. beneficial, anti-inflammatory bacteria such as *Lactobacillus*) (89). (ii.) increased bacteria strains such as *Bifidobacteria, Megasphera, Ruminococcus*, and *Butyrivibrio* that produce short-chain fatty acids (89, 90) which are essential for epithelial barrier function and regulation of inflammation; (iii.) increased bacteria strains such as *Bacteroides* spp. that produce bile acids, which are essential in cholesterol homeostasis and metabolic health (91); (iv.) improvement of microbial communities associated with decreased overall inflammation through lowered TLR-4 signaling, microbial translocation and barrier dysfunction (86, 89); (v.) increased levels of bacteria such as *Akkermansia* species that degrade mucins, which can prevent biofilms from forming that can promote inflammatory bacteria species; and (vi.) reduced bacteria known to be associated with barrier damage and inflammation including *Prevotella.* While there are some conflicting reports regarding microbial alterations after metformin treatment (92), this is likely due to confounders and study design. Overall, it appears that metformin improves the microbiome and can contribute to better mucosal health and overall lowered inflammation. Thus, a potential mechanism by which metformin may improve prognosis in Covid-19 may be through improvement of the microbiome and downstream lowered inflammation.

METFORMIN AND SEX-SPECIFIC INFLAMMATORY AND MORTALITY FINDINGS

As mentioned, observational data suggest a mortality benefit only among female adult patients with diabetes or obesity hospitalized with Covid-19 (**Table 1**) (11). Another study found reduced incidence of ARDS in patients with Covid-19 taking metformin *vs* those who were not; however, this association was only found among females (27). Additionally, metformin use has been associated with a sex-specific mortality benefit in women compared to men with colorectal cancer (23). Possible reasons for sex-specific effects of metformin include the influence of sex hormones and epigenetic changes on the Y chromosome (93). While metformin has been associated with decreased TNF-alpha use after starting metformin in both men and women, these benefits have been shown to be greater in females *versus* males in several studies (see **Table 1**) (23, 25, 34, 94, 95). These sex-specific findings may be related to c-src modulation of sex steroids (96).

The National Institutes of Health started to seriously promote sex as a biologic variable in 2014 (97). Much of the mechanistic research into the basic science behind metformin was done before 2014, which may limit the understanding of sex-specific effects of metformin. This remains an important area for future investigation (98).

METFORMIN'S HISTORY OF ANTI-INFECTIOUS PROPERTIES

Metformin was found to have antiviral activity before SARS-CoV-2. In the 1940s and 1950s, metformin was used against influenza (as "Flumamine"), and was found to be effective against parainfluenza and cowpox (1, 99). Metformin has also been associated with improved affect against tuberculosis and is being assessed in HIV (100). With the Zika virus, another RNA virus, activation of AMPK by metformin resulted in restricted viral replication by potentiating innate antiviral responses and decreasing glycolysis, with PKA Inhibitor PKI leading to decreased viral infection and replication (101, 102). Metformin was only assessed *in-vitro* against Zika, not *in-vivo*. In patients

with hepatitis C, metformin has been associated with improved virologic response to antivirals and decreased insulin resistance (103). Metformin's past anti-infectious benefits have been both against the infectious agent directly, as well as by improving the underlying health of the human host. In May 2020, Gordon et al. did find that metformin both reduced SARS-CoV-2 virus and improved cell viability during *in-vitro* assays (79). It is unknown if the lower mortality observed in patients infected with Covid-19 who are on metformin is due to direct activity against the virus itself, improved host substrate (i.e. lower inflammation pre-infection, or lower biologic aging), or both.

SAFETY

Metformin is overall a safe medication that has been widely used for decades (104). It is well-tolerated in most individuals, and there is flexibility in the timing of administration which can improve side-effects and adherence (105). Metformin use without other glucose-lowering medications does not lead to glucose reduction below physiologic levels. For this reason, it is considered a safe medication among older adults (106). The most common safety concern is the possibility of lactic acidosis, but this adverse side effect is rare (104) A review found that, even in patients with advanced liver disease, the risk for lactic acidosis is low (107). Recent evidence from Cheng et al. also found that, *inpatient* metformin use of 2 to 3 g/day was significantly associated with an increased incidence of developing lactic acidosis (OR, 22.57; 95% CI, 1.99–256.71; p = 0.012) and acidosis (OR, 12.79; 95% CI, 1.24–132.14; p = 0.032), neither low-dose (<1 g/day) nor moderate-dose (1 to 2 g/day) was significantly associated with the acidosis or lactic acidosis. Additionally, the incidence of heart failure was significantly lower in the metformin group compared to the non-metformin group (adjusted HR, 0.61; 95% CI, 0.43–0.87; p = 0.006) (19). An analysis of individual case safety reports of persons with Covid-19 on hydroxychloroquine suggested an increased risk of mortality associated with hydroxychloroquine + metformin use (ROR value of 57.7 (23.9-139.3) compared to hydroxychloroquine use alone (16). The authors found autophagosomes in mice treated with both, and hypothesized that the excess mortality was from a synergistic inhibition of autophagy (16). Caution should be considered for co-administration in humans.

There is currently a voluntary FDA recall of some long-acting metformin. While some manufacturers of long-acting metformin are currently under voluntary recall because of elevated nitrosodimethylamine (NDMA), a water treatment chemical, no elevated levels of NDMA have been found in the short acting formulation (108).

Additionally, while metformin does cross the placenta, it appears to be safe and has been used off-label in pregnancy. In studies randomizing pregnant women to glucose-lowering therapy, metformin was associated with lower gestational weight gain and a lower risk of pre-eclampsia compared with insulin. Further, other randomized controlled trials have found that metformin is associated with reduced risk of hypertensive disorders of pregnancy in women with obesity or diabetes mellitus (109, 110). Most medications being considered for Covid-19 treatment and prevention are safe during pregnancy.

CONCLUSION

The goal of this targeted review was to provide a high-level overview of initial data around metformin and Covid-19, and mechanistic theories and data that pre-dated Covid-19. Observational studies have suggested associations with decreased risk of mortality in patients who were on metformin before being hospitalized with Covid-19. Observational studies are significantly limited by confounding by indication and contraindication. While many laboratory studies suggest plausible mechanistic reasons why metformin would be protective in Covid-19, and metformin has been shown to inhibit SARS-CoV-2 *in vitro*, the data so far are not conclusive. Given the above-mentioned observational findings, plausible mechanisms of metformin's effect in reducing Covid-19 related morbidity/mortality, acceptable safety profile, low cost, and the devastating nature of the global Covid-19 pandemic, metformin should be prospectively assessed as a potential Covid-19 treatment. Additionally, observational studies should assess outcomes in individuals who are chronically on hydroxychloroquine and metformin, as there may be a safety issue in this combination.

While vaccine development for SARS-CoV-2 has been promising and is the most important approach to preventing severe COVID-19, there may be reduced willingness among the public to receive a vaccine developed so quickly. Additionally, it will be many months before vaccines are available world-wide, some individuals will still get Covid-19 even after vaccination, and at times viral variants may evade vaccine effectiveness until new vaccines can be distributed (111). Because medical practice should not change without being informed by rigorous data, randomized clinical trials should be done to prospectively assess safe and readily available medications such as metformin for reducing the risks associated to Covid-19.

AUTHOR CONTRIBUTIONS

SI contributed to the planning and execution of the significant revisions. JL contributed to the updating of the manuscript and content. CB wrote the initial draft of the manuscript. NI contributed to critical review and making the figure. SS contributed to critical review and making the figure. NK wrote parts of the article and contributed to critical review. NS contributed to critical review. LA contributed to critical review. MP contributed to critical review and designing the article. LT contributed to critical review and making the figure. AP contributed to critical review. EB contributed to critical review. MU contributed to critical review and making the figure; BB contributed to critical review. DV contributed to critical review. CT contributed to critical review and conceptualizing the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

1. This research was supported by the Agency for Healthcare Research and Quality (AHRQ) and Patient-Centered Outcomes Research Institute (PCORI), grant K12HS026379 (CJT).
2. This research was supposed by the Minnesota Learning Health System Mentored Training Program (MH-LHS), M Health Fairview Institutional Funds (CTB, SS).
3. This research was supported by the National Center for Advancing Translational Sciences, grants KL2TR002492 and UL1TR002494 (CTB).
4. This research was supported by the National Heart, Lung, Blood Institute T32HL07741 (NEI).
5. This research was supported by the National Cancer Institute (NCI), grant P30CA077598.
6. This research was supported by COVID-19 Rapid response grant UM 2020-2231.

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Conflict of Interest: DV is employed by the company UnitedHealth Group.

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