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REVIEWS

Pathogenesis of COVID-19 described through the lens of an undersulfated and degraded epithelial and endothelial glycocalyx

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Abstract

The glycocalyx surrounds every eukaryotic cell and is a complex mesh of proteins and carbohydrates. It consists of proteoglycans with glycosaminoglycan side chains, which are highly sulfated under normal physiological conditions. The degree of sulfation and the position of the sulfate groups mainly determine biological function. The intact highly sulfated glycocalyx of the epithelium may repel severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) through electrostatic forces. However, if the glycocalyx is undersulfated and 3-*O*-sulfotransferase 3B (3OST-3B) is overexpressed, as is the case during chronic inflammatory conditions, SARS-CoV-2 entry may be facilitated by the glycocalyx. The degree of sulfation and position of the sulfate groups will also affect functions such as immune modulation, the inflammatory response, vascular permeability and tone, coagulation, mediation of sheer stress, and protection against oxidative stress. The rate-limiting factor to sulfation is the availability of inorganic sulfate. Various genetic and epigenetic factors will affect sulfur metabolism and inorganic sulfate availability, such as various dietary factors, and exposure to drugs, environmental toxins, and biotoxins, which will deplete inorganic sulfate. The role that undersulfation plays in the various comorbid conditions that predispose to coronavirus disease 2019 (COVID-19), is also considered. The undersulfated glycocalyx may not only increase susceptibility to SARS-CoV-2 infection, but

Abbreviations: AA, amino acid; ACE2, angiotensin converting enzyme 2; AngP-2, angiopoietin-2; APS, adenosine-5-phosphosulfate; AT, antithrombin; BBB, blood–brain barrier; COVID-19, coronavirus disease 2019; CVD, cardiovascular disease; CyPB, cyclophilin-B; Cys, cysteine; DM, diabetes mellitus; ECM, extracellular matrix; En, endothelial; EnC, endothelial cell; EnGL, endothelial glycocalyx; Ep, epithelial; EpC, epithelial cell; EpGL, epithelial glycocalyx; Ext, exostosin; FasL, Fas ligand; GAG, glycosaminoglycan; GL, glycocalyx; GlcN, D-glucosamine; GlcNAc, *N*-Acetyl-D-glucosamine; GlcNH2, *N*-unsubstituted glucosamine units; GlcNS, *N*-sulfated glucosamine; GSH, glutathione; H_2S, hydrogen sulfide; HAS, human serum albumin; HP, heparin; HPSE, heparanase; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; ICU, intensive care unit; IL, interleukin; IV, intravenous; LMWH, low molecular weight HP; Met, methionine; MMPs, matrix metalloproteinases; NAC, *N*-acetyl-L-cysteine; NaS1, sulfate cotransporter; Ndst, *N*-deacetylase/*N*-sulfotransferase; NO, nitric oxide; NSAIDs, non-steroidal anti-inflammatory drugs; *O*-ST, *O*-sulfotransferase; PAPS, sulfonucleotide 3-phosphoadenosine 5-phosphosulfate; PGs, proteoglycans; RBD, receptor-binding domain; SAA, sulfur amino acid; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; Sat1, sulfate-anion transporter-1; sdc1, syndecan-1; SOD, superoxide dismutase; SP, spike protein; ST or SULT, sulfotransferase; STS, sodium thiosulfate; SULF, sulfatase; T1D, type 1 diabetes; T2D, type 2 diabetes; TEN, toxic epidermal necrolysis; TFPI, tissue factor pathway inhibitor; TMPRSS2, transmembrane serine protease 2; TNFα, tumor necrosis factor alpha; UFH, unfractionated HP; VD, vitamin D.

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would also result in a hyperinflammatory response, vascular permeability, and shedding of the glycocalyx components, giving rise to a procoagulant and antifibrinolytic state and eventual multiple organ failure. These symptoms relate to a diagnosis of systemic septic shock seen in almost all COVID-19 deaths. The focus of prevention and treatment protocols proposed is the preservation of epithelial and endothelial glycocalyx integrity.

KEYWORDS
COVID-19, glycocalyx, heparan sulfate, inorganic sulfate, SARS-CoV-2

1 | INTRODUCTION

From mid-March 2020, the authors determined that sulfation could potentially play a major role in the pathogenesis of coronavirus disease 2019 (COVID-19). Presently, more than a year later, there is still an urgent need to find effective therapeutic solutions to prevent and treat the established disease, which highlights the importance of understanding the pathophysiology underlying COVID-19 and the predisposing factors leading to infection.

In addition to the well-accepted hypothesis[1] of angiotensin-converting enzyme 2 (ACE2) being the main epithelial receptor that is necessary for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection,[2–4] we hypothesize that the degree of sulfation of heparan sulfate (HS), a glycosaminoglycan (GAG) lining the glycocalyx (GL), is also an important variable that will influence susceptibility to infection.[5–8] Undersulfation (less than the normal degree of sulfation) or aberrant sulfation of HS may not only increase susceptibility to viral infection, but may also adversely affect the individual's physiological response to the infection.

The GL is a dense layer of proteins and carbohydrate chains that surrounds all eukaryotic cells. The epithelial GL (EpGL) is the interface between our internal cells with the external environment.[9–12] If our EpGL is compromised, we are vulnerable to the onslaught of viruses, various pathogens, and environmental toxins.[10,13] The GL is especially pronounced on all epithelial cells (EpCs) and lines the apical surface of endothelial cells (EnCs), in a mesh-like structure that floats into the lumen of the vessels' vascular system, known as the endothelial GL (EnGL).[14] In this review, GL will refer to both the EpGL and EnGL, unless specified. HS is the most prominent and studied GAG, and is a highly negatively charged linear heterogeneous polysaccharide (PS), which is attached to a membrane or extracellular matrix (ECM) proteoglycan (PG) to form heparan sulfate proteoglycan (HSPG).[15] HSPGs on the cell surface are involved in developmental, regeneratory, infectious, and inflammatory processes, as a consequence of their interactions with multiple proteins or ligands.[16] These interactions are mediated mainly via the HS moieties of the PGs,[16–18] which form active complexes with the extracellular soluble mediators,[16] such as growth factors, cytokines, matrix components, enzymes, enzyme inhibitors, and pathogen virulence factors,[19] thereby regulating tissue distribution, biological availability, and activity of the proteins. These HS–protein interactions, which mainly determine biological function, are largely dependent on the density and position of sulfate groups in the HS structure.[20]

Sulfation occurs in all tissues and is defined as the transfer of a sulfate group to different substrates, such as GAGs, proteins, lipids, hormones, and drugs.[21–27] The control and degree of sulfation of such a wide range of substances indicate that this pathway is involved in many aspects of life. The rate-limiting factor to sulfation is the availability of inorganic sulfate.[23,28,29] It is, therefore, important to explore the factors contributing to and those that deplete inorganic sulfate, and, therefore, also likely affect sulfation of the EpGL. The intact highly sulfated GL inhibits infection,[30,31] plus it prevents protein and solute exchange and, therefore, regulates barrier permeability, not merely by size and sterical hindrance, but also by its negative electrostatic charge.[18,32] It should be noted that different cell types and tissues vary in their structure of HS, and HS structure, degree of sulfation, and the position of sulfate groups can vary between individuals and with age.[33] These differences in HS composition may be a contributing factor to tissue tropism and host susceptibility to infection by viruses and other pathogens.[7,33,34]

Once airborne SARS-CoV-2 reaches the lungs, it gains entry through the alveolar EpCs.[35] The general consensus is that similar to SARS-CoV-1, SARS-CoV-2 gains entry into cells through spike protein (SP) affinity for ACE2 receptors[36–38] and uses host cell transmembrane serine protease 2 (TMPRSS2)[2] and furin[3] to mediate entry.[6,39,40] ACE2 receptors are expressed in respiratory epithelium, alveolar EpCs, cardiac myocytes, the vascular endothelium, kidney, intestinal tissue,[41] and a small subset of

immune cells, such as macrophages.[39,42,43] It is important to note that even though ACE2 receptors are not expressed in the conjunctival and corneal epithelium,[44] SARS-CoV-2 RNA was nevertheless detected in an ocular swab from a patient diagnosed with COVID-19.[45] It is clear that ACE2 receptors are, therefore, not the only prerequisite for SARS-CoV-2 entry into the cell.[5] Furthermore, for any pathogen to gain access to ACE2 receptors, they need to first penetrate through the GL.[6,7,33,46] Indeed, recent research studies indicate interactions between sulfated HS and SARS-CoV-2 SP,[7,8] which participate in viral adherence and infectivity.[8,47] The GL is the first point of contact for all pathogens that infect animal cells, and it is, therefore, not surprising that many viruses exploit GAGs, such as HS, to facilitate the uptake.[6,12,16,48–52] Through sequence analysis of SARS-CoV-2 SP, it was found that this virus has additional potential GAG binding domains compared to SARS-CoV-1,[50] leading to increased infectivity. SARS-CoV-2–GAG interactions are characterized by immense complexity,[33] with the degree and position of sulfate on HS playing the biggest role in determining viral attachment and penetration into the cell, as outlined in Section 3.1 "Glycocalyx facilitation of SARS-CoV-2 cellular entry".

In this review, we will explore the hypothesis that aberrant or undersulfated HS not only predisposes to SARS-CoV-2 infection, but also results in a dysregulated immune response and subsequent "cytokine storm"[45]; resulting in shedding and degradation of the GL[53]; leading to adhesion of immune cells, increased vascular permeability, inflammation, oxidative stress and coagulation, and increasing the risk of life-threatening organ dysfunction. The main aim of the research is to postulate that undersulfated HS is a predisposing factor to SARS-CoV-2 infection and severe COVID-19. The role that chronic inflammation and undersulfated HS plays in the comorbid conditions that increase susceptibility to COVID-19 will be pointed out and the factors leading to decreased levels of inorganic sulfate will be explored. In addition, therapeutic applications to preserve the GL and increase inorganic sulfate availability will be presented.

2 | GLYCOCALYX AND SULFATION

2.1 | Structural properties of the glycocalyx

The glycocalyx surrounding all eukaryotic cells is a complex external layer of PGs, glycoproteins bound with sialic acid, GAGs, glycolipids, and proteins.[14,54] The glycocalyx is especially pronounced on epithelial (Ep) and EnCs. Plasma proteins such as albumin, fibrinogen, and antithrombin (AT) are also bound within the EnGL.[55] The specific components and detailed structure and the GL have been reviewed before.[32,54,56]

HS is the most structurally heterogeneous[57] and predominant GAG,[58] accounting for 50%–90% of the GAG chains.[42,56] HS is either conjugated to amino acids (AAs), creating HSPGs, or present as unconjugated chains.[15] A GAG chain consists of up to 200 repeating disaccharide units of hexuronic acid and hexosamine.[59] The hexuronic acid is either D-glucuronate (GlcUA) or its epimerized form, L-iduronate, and the hexosamine is either *N*-acetyl-D-glucosamine (GlcNAc) or *N*-acetyl-D-galactosamine (GalNAc), depending on the type of GAG.[59] GAGs can be extensively modified by sulfation and/or (de)acetylation to yield different configurations.[56,60] Modifications include N-sulfation, 6-*O*-sulfation and 3-*O*-sulfation of GlcNAc, 2-*O*-sulfation of glucuronic acid, as well as C5-epimerization of glucuronic acid to iduronic acid.[17,61,62] HS is one of the most complex and information-dense mammalian molecules found in nature,[63] exhibiting high heterogeneity among other GAGs, with regard to structure, sulfate content, the position of the sulfates, and repeat chain structure.[64] This structural heterogeneity forms the molecular basis for its broad specificity[63,65,66] to bind over 400 soluble protein mediators and cell-surface receptors.[67] HSPG, therefore, plays an essential role in maintaining and regulating a wide range of functions, including vascular permeability,[68,69] coagulation activity, inflammatory responses, and viral entry into target cells.[68] The chain length, location, and the number of sulfate groups in the various domains of the GAG determine the binding affinity to the specific proteins[17,42,57,69,70] and thus biological function.[16,19,71] Sulfation sequences of HS at various sites on the carbohydrate chain, impart HS with a landscape of negative charges, allowing HS to bind proteins with specificity[67,72] via positively charged AA residues,[63] thereby regulating their function through electrostatic interactions.[45,58,71,73] Although the spatial arrangement of sulfate groups in given HS sequences is important for optimal binding,[62,64] the degree of sulfation predominantly determines the negative charge of the GL,[17,63,74,75] which defines pathogen evasion, receptor binding sites,[18,56,64,70,74,76] and the electrostatic binding of proteins, including albumin, leukocytes, red blood cells, and platelets.[17,46,77,78]

Even though there are organ-specific trends in HS length and composition,[12] there still remains heterogeneity of HS across individual organ substructures, such as between the alveoli and lung airways.[57] Adding to this complexity, there may be temporal shifts in HS structure, since HS length, degree, and the position of sulfation on the GAG may be dynamically modified in response to cellular and environmental cues.[56,57,72] For example,

extracellular HS domains may undergo endoglycosidase cleavage by the enzyme heparanase (HPSE), thereby releasing extracellular HS fragments[79] and reducing chain length.[17]

Most of the literature refers to HS bound to the core protein as HSPG. However, there are usually HS and CS chains attached to a particular PG, such as in the case of syndecan (sdc),[56,78] and it is, therefore, technically not correct to refer to HSPG, but for simplicity, the term HSPG is used in this review when referring to the sulfated GAG–PG interaction. The main focus of this review will remain on HS, the most studied GAG to date.

In the vascular system, EnCs line the luminal side of blood vessels,[80] thereby modulating the microvascular environment. Abluminally, EnCs are attached to the basement membrane. Endoluminally or luminally, the EnGL coats the EnCs and interacts with the blood, thereby regulating microcirculatory flow[80] and barrier function.[73,81] The large outer tier of the EnGL prevents circulating red blood cells and immune cells, including leukocytes and platelets, from binding to endothelial (En) adhesion molecules under normal physiological conditions.[73] Besides shear stress, EnCs also undergo pulsatile stretch and physical pressure, which will influence the expression of the GL.[73,77,82] Subsequently, the GL can suffer from enzymatic or shear-induced shedding. This dynamic balance between GL biosynthesis and shedding makes it hard to define the GL geometrically.[56] The GL, therefore, incorporates molecules in a dynamic equilibrium, since it continuously binds and replaces both endothelium-derived and flowing blood-derived substances.[72]

2.2 | Sulfation

The sulfation process in the human cell begins with uptake of inorganic sulfate from the extracellular milieu. In mammals, sulfate must be activated to sulfonucleotide 3-phosphoadenosine 5-phosphosulfate (PAPS), prior to reaction with the acceptor molecule.[83,84] PAPS is, therefore, the universal sulfate donor for sulfotransferase (ST or SULT) reactions[21,29,83] (Figure 1). STs transfer sulfate from PAPS to a specific position on the GAG structure[21] and, therefore, contribute significantly to the diversity of GAGs: A simple octasaccharide may have many thousands of different sequences, since sulfation can occur at various positions such as the C4, C6, and/or on nonacetylated nitrogen residues.[74]

The biosynthesis of HS occurs in the Golgi apparatus[67] via the combined actions of more than 25 enzymes.[67] *N*-deacetylase/*N*-sulfotransferase (Ndst) removes *N*-acetyl groups from the subsets of GlcNAc residues and adds *N*-sulfate to the free amino groups.[85] N-sulfation plays a key role in determining the succeeding modifications, through dictating the position of the other sulfated domains.[46] The C5-epimerase converts adjacent GIcUA to IdoUA at C5, and then a group of *O*-sulfotransferase (*O*-ST) add sulfate to C6 and C3 of D-glucosamine (GlcN) units and C2 of uronic acids.[16] These modifications occur in contiguous blocks of sugars along the chain in an incomplete manner, which leads to the occurrence of consecutively *N*-sulfated regions, regions that escape modification and remain *N*-acetylated (neutral charge), and regions of alternating *N*-acetylated and *N*-sulfated disaccharide units.[16,57,59,86] When the *N*-deacetylation/*N*-sulfation reaction is partial, it gives rise to unsulfated *N*-unsubstituted glucosamine units (GlcNH2).[16] GlcNH2 carries a positive charge that may be due to an occasional interruption of the Ndsts-mediated, catalytic linkage between *N*-deacetylation and re-*N*-sulfation, thereby trapping the intermediate GlcNH2.[21] The HS thus contains regions of both high and low sulfation, known as NS and NA domains.[63] This may be exacerbated under conditions of limited availability of inorganic sulfate or PAPS,[28,42] which drives the final sulfation process.[21] Alternatively, residual *N*-acetyl groups can be selectively removed by *N*-acetylase, or *N*-sulfate groups can be selectively eliminated by an unidentified endo-sulfamidase (*N*-sulfatase) at a later stage of HS biosynthesis.[42,85]

The rare GlcNH2 residues are implicated in various important cell biological and pathophysiological phenomena,[85,87] which we hypothesize may include COVID-19. The presence of unsulfated domains correlates with the inability of HS to bind L-selectin,[21,85] and other ligands. The location of GlcNH2 residues and the nature of their surrounding sulfate sequences may contribute to their enzyme susceptibility, as well as protein interactions and antibody recognition.[21] It should be noted that HPSE-I cannot cleave GlcNH2 residues, but in contrast, HPSE II and III can do so if the relevant disaccharide is non-sulfated or *O*-sulfated, respectively.[21] Apart from various enzymes, the protein-coding gene exostosin (Ext) 3 is a candidate regulatory factor of Ndst1 and is involved in the generation of GlcNH2 structures.[85]

While electrostatic interaction seems to be the major driving force, in many cases specific sulfation patterns are required for the recognition of HS by its binding partners.[21] Infrequently, *O*-sulfation may also occur at position 2 of GlcUA and at position 3 of *N*-sulfated GlcN (GlcNS) and of GlcNH2 units,[42,88] where the latter modifications are hallmarks for the respective binding HS domains of AT,[89] glycoprotein D (gD) of herpes simplex virus type I (HSV-1)[16,17,59,62,90,91] and cyclophilin B (CyPB).[91] These modifications reflect a controlled enzymatic system for encrypting functional information into the GAG moiety.[19,46,67,92] The structural heterogeneity of HS[80,89] can

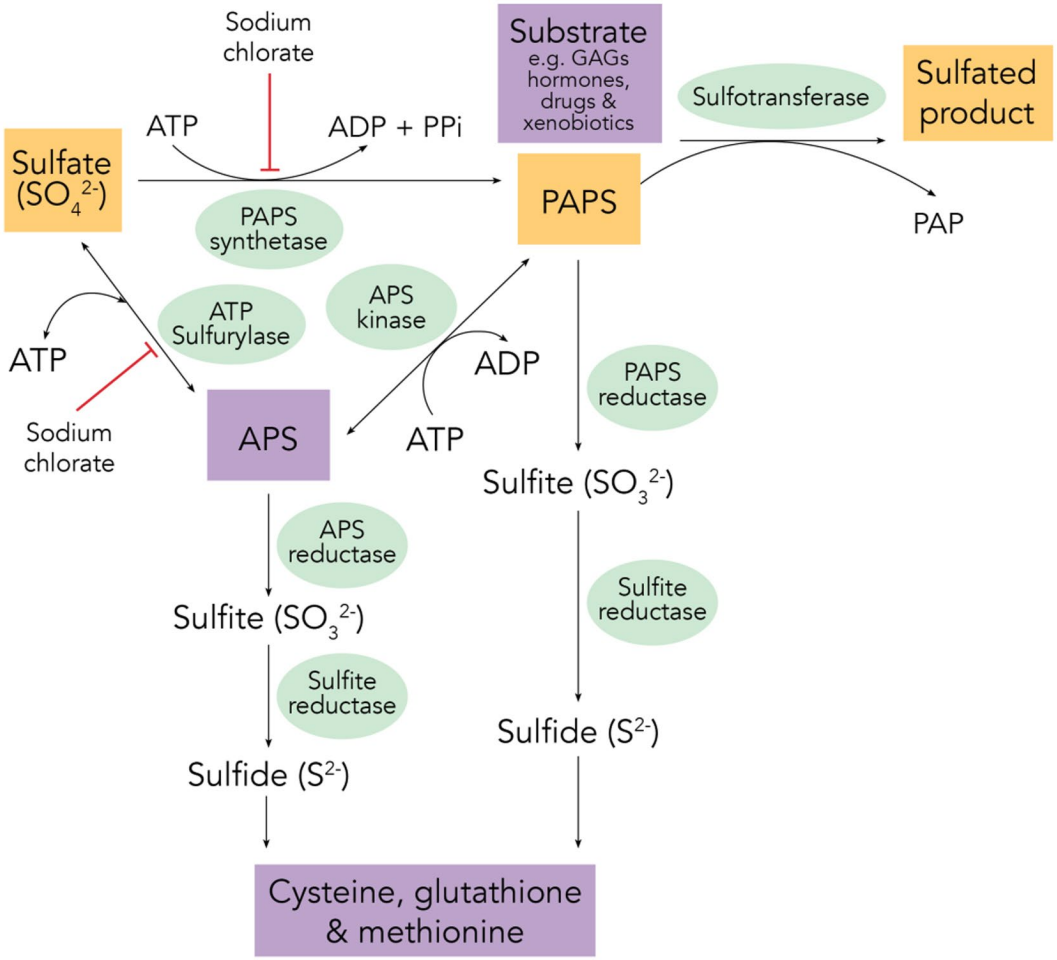

FIGURE 1 Sulfation pathway in mammalians illustrating the conversion of inorganic sulfate (SO_4^{2-}) to the activated form sulfonucleotide 3-phosphoadenosine 5-phosphosulfate (PAPS). The enzyme ATP sulfurylase catalyzes the production of adenosine-5-phosphosulfate (APS) from sulfate and ATP, to provide APS as a substrate to APS kinase, which transfers a phosphate group from ATP to APS to yield PAPS. Sodium chlorate is a selective inhibitor of both ATP sulfurylase and PAPS synthetase, which inhibits PAPS synthesis, resulting in profound undersulfated glycosaminoglycans (GAGs) and impaired detoxification. Sulfotransferase enzymes transfer a sulfate group from PAPS to the substrate molecule to form a sulfated product

explain the remarkable tissue-specific activities of the GL,[16,83,93] influencing cell-, tissue-, and organism-level development, homeostasis,[6,63,74,94] and pathogenesis.[33,89] The importance of sulfation patterning exists at both the HS GAG chain level, as well as at the individual proteoglycan level.[63] In support of this, it was found that in mutant mice lacking 2-*O*-sulfation, HS chains could still support fibroblast growth factor 2, which were possible since they had compensatory increases in *N*- and 6-*O*-sulfation to maintain the charge distribution along the HS chain.[63]

HSPGs are transported from the Golgi apparatus to the cell membrane after completion of polymerization, sulfation, and epimerization, where they contribute to the EnGL and other ECM structures.[46] HS can be further modified at this point, by either HPSE-mediated cleavage or selective removal of 6-*O* sulfates by sulfatase-1 and -2 (SULF).[46] It should be noted that *N*-, 3-*O*-, 2-*O*-sulfation does not have known extracellular SULFs, which indicates dynamic regulation of HS 6-*O*-sulfation as an evolutionary conserved function.[57] When sulfate groups are removed from HS, the release of HSPG-sequestered ligands can degrade the GL, as is evident in a wide range of human tumors where SULFs are overexpressed.[46] Inhibition of PAPS synthesis, the general sulfate donor, with sodium chlorate, a selective inhibitor of both ATP sulfurylase and PAPS synthetase,[21,95] the first enzymes in the sulfate activation pathway, leads to profound undersulfation of GAGs and loss of their function[21,76,95] (Figure 1). The enzyme ATP sulfurylase catalyzes the production of adenosine-5-phosphosulfate (APS) from sulfate and ATP (see Figure 1), to provide APS as a substrate to APS kinase, which transfers a phosphate group from ATP to APS to yield PAPS.[21] Sulfation of GAGs also requires a sulfate transporter, the sulfate transporter gene DTD, to ensure

efficient sulfate incorporation, as well as a PAPS translocase to transport PAPS into the Golgi, and one or more membrane-associated STs to transfer sulfate from PAPS to the GAGs.[21] Although there are many steps and enzymes involved in sulfation, PAPS production is the rate-limiting step for sulfation in most systems, where PAPS synthetase plays a major role,[21] as well as the availability of inorganic sulfate.[23,28,29] It is important to note that sulfation reactions in the human body affect all cells and metabolism of many endogenous and exogenous molecules, such as hormones, drugs, and xenobiotics[21] (Figure 1).

2.3 | Physiological role of the glycocalyx

The biological functions of the GL are comprehensive, since under normal physiological conditions sulfated intact HSPGs interact with and modulate the activity of numerous molecules,[62] such as anticoagulant factors, cytokines,[80] growth factors,[12,60] albumin,[68,73] Hedgehogs, Wingless, and protease inhibitors,[61] as well as protective enzymes such as superoxide dismutase (SOD).[78] These EnGL functions contribute to the regulation of inflammation, vascular permeability and tone, coagulation, the mediation of shear stress,[55,73] lipid metabolism,[60,70] leukocyte adhesion,[77,80,96] and protection against oxidative stress.[55] Therefore, any changes in the GL structure and hence functions, are associated with a wide range of pathophysiological consequences, such as capillary leak syndrome and consequent edema formation,[75] platelet hyperaggregation, hypercoagulation, accelerated inflammation, and loss of vascular responsiveness.[60,73] According to most research studies to date, only few interactions rely on specific oligosaccharide sequences, whereas most of the interactions and functions of the GL rely on the pattern and degree of sulfation or negative charge density.[18,46,97] The affinity and biological activity of the GL increases as the degree of sulfation increases.[83,98] Nevertheless, the GL is a delicate layer, and the removal of one specific component thereof may result in the loss of function of the total.[18,56] The GL and HS do not only regulate physiological processes, but are also implicated in many pathologies, including cancer, infectious, and vascular diseases.[56,79]

2.3.1 | Barrier function and pathogen evasion

The heavily sulfated GAG chains of the GL present a global negative charge that can interact electrostatically with viruses and other pathogens.[59] When HS is undersulfated, viruses exploit these weak interactions to increase their concentration at the cell surface and enhance their chances of binding a more specific entry receptor.[12] Thus, HS lies at a nexus between pathogen invasion and host defense.[52,99,100] The EpCs, together with leukocytes, secrete many defensive compounds into the mucosal fluid, such as mucins, antibodies, protegrins, defensins, collectins, lysozyme, histamines, cathelicidins, and nitric oxide (NO).[73,79] Collectively, these different defensive compounds form a physical barrier with direct antimicrobial activity, and the ability to opsonize pathogens to aid their clearance.[101,102] The HS modulates the expression and release of these defensive compounds.[67,99]

The EnGL is crucial for the maintenance of vascular barrier funtions[56,60,68,80,100] and fluid homeostasis.[4,72,73] An essential function of the EnGL is, therefore, maintaining correct oncotic pressure in the capillary bed, in addition to facilitating the adsorption and reabsorption of molecules across capillary membranes.[4,55]

In the EpGL, HSPGs act as a physical and biochemical barrier, which regulate through passive and active mechanisms, outside-in signaling, protect the cell from external aggression, and maintain tissue integrity.[12,14,74,81] Since GAGs carry a significant number of negatively charged binding sites[79] depending on their degree of sulfation, changes in sulfation will affect protein binding and consequently vascular permeability.[77] Therefore, disruption of the GL can lead to the loss of barrier function with subsequent edema formation.[60,72,77] In various disease conditions, a decrease in *N*- and 6-*O*-sulfate domains in biopsies from patients were associated with albuminuria.[17,72,77] Inflammatory diseases with different etiology may promote EnGL dysfunction by several associated pathways and initiate albuminuria.[17] Intensive care unit (ICU)-treated COVID-19 patients with AKI frequently presented with albuminuria.[103,104]

Enzymatic degradation of HS and HS-associated PGs, such as syndecan-1 (sdc1), leads to barrier dysfunction.[57,71,81] Moreover, HS can modulate sdc1-induced signal activation and subsequent cell function through its ability to bind to cationic mediators.[81] The degree of sulfation will determine these interactions.[60,77] Signals activated via HSPG cross-linking result in GL cytoskeletal reorganization and subsequent barrier dysfunction, where sdc1 and sdc4 HS appear to be the main mediators for these events.[81] This explains some of the mechanisms involved in inflammation and associated changes in vascular permeability and highlights a potential proinflammatory role for specific components of the GL.[81]

2.3.2 | Immune modulation

The degree and specific sulfation patterns of GAGs observed in an intact GL attenuate the binding of chemokines and leukocytes to the cell surface[58,60,105] on the contrary,

but on the other, it also harbors the endothelial cell adhesion molecules, such as E- and L-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1),[56,106] as well as the von Willebrand factor.[14,79] The first critical step in leukocyte migration from the circulation into the tissue is the adhesion of leukocytes to vascular endothelium.[21,60] Galvis-Ramírez et al. demonstrated that both *N*- and 6-*O*-sulfate domains are very important to prevent leukocyte adhesion to the endothelium under normal physiological conditions.[17,33] During infection, the recruitment of leukocytes to specific areas of infection is a very precise multistep process, involving tethering, rolling, adhesion, and transmigration, where all these steps are critical for efficient host defense, regulated by HSPGs.[17,21,80] Under normal conditions, the highly sulfated HS chains and soluble components of the GL seem to act as a shield against adhesion molecules, thereby, preventing interaction with pathogens and subsequent inflammation. Undersulfation or stimuli, such as histones, HPSE and proteases, cytokines, or ischemia and reperfusion,[32,56,60] which degrades the GL, or induce a more open mesh, appear to uncover the adhesion molecules, such as integrins and immunoglobulin super-families, and, therefore, facilitate ligand–receptor interactions.[33] These interactions promote the adhesion of leukocytes to the endothelium,[56,77] the release of sequestered cytokines,[75,78] while released HS and GAG fragments bind to toll-like receptors.[105,107]

In contrast, preservation of the GL mitigates leukocyte adhesion and subsequent cell damage,[58,77,108] highlighting the very important role of the GL, especially sulfated HS, in modulating immune and inflammatory processes.[60,109]

2.3.3 | Coagulation

While coagulation is an essential process under normal physiological conditions, enhanced coagulation will present under several pathological conditions, such as sepsis. During coagulation thrombin, a serine protease, catalyzes the conversion of fibrinogen to fibrin to form blood clots in conjunction with platelets.[110] AT III is a strong inhibitor of procoagulant enzymes such as thrombin and activated factors X and IX (FXa and FIXa),[55,56] where AT's biological activity critically depends on high-affinity binding to 3-*O*-sulfated GAGs.[7,62,64,77,83,100] This demonstrates the importance of specific HS structural domains[17,79] to form electrostatic interactions with AT.[57] Thacke et al. reported that the binding of heparin (HP) to AT induces a conformational change, which consequently increases its anticoagulant catalytic activity by several orders of magnitude.[83] However, it was found that this affinity was decreased by several orders of magnitude (Kd = 30 nM versus 500 μM) in the absence of the 3-*O*-sulfate group, which reduced both the conformational change induced in AT and the inhibition of FXa,[74,83] thereby inducing a proagulatory state. Even though 3-OST-1 is the predominant isoform that produces most of the AT-binding sites, it is likely that other 3-OST isoforms can contribute to HS anticoagulant activity.[111] Intriguingly, even though 3-*O*-sulfates are the rarest of HS modifications, it seems that the degree of sulfation and this arrangement of the sulfation domains, affect anticoagulation activity. Kazatchkine et al. demonstrated that either *N*-desulfation or a combination of *N*- and *O*-desulfation removed the anticoagulant activity of porcine HP.[112]

Tissue factor pathway inhibitor (TFPI) is a potent inhibitor of FVIIa and FXa. TFPI is able to bind to the GL via HS,[79] with other proteins also probably involved. Moreover, uptake and degradation of TFPI–FXa complexes are also dependent on HS in the GL.[79] It is clear that all these anticoagulant molecules present in the intact sulfated GL contribute to the thromboresistant nature of healthy endothelium.[56] Furthermore, adhesion of platelets to endothelium is attenuated by the intact EnGL,[55,72,78] diminishing blood coagulation.[6] An intact GL and degree of sulfation, therefore, clearly play a huge role in thromboregulation that supports physiological blood flow and hemostatic tone.[83,97,109,111] The consequence of an undersulfated GL and shedding is a procoagulant and proinflammatory phenotype of the endothelium, with increased capillary permeability.[73,77,113] Shedding and elevation of plasma sdc1 levels have been associated with not only the severity of sepsis, but also the development of disseminated intravascular coagulation.[77]

2.3.4 | Mechanotransduction

Since the EnGL projects into the vascular lumen, it may deform in the presence of shear stresses that accompany increases in vascular flow.[73] This stress is transduced into the cytoskeleton of the EnCs, triggering induction of En NO synthesis.[79] The subsequent NO-mediated vasorelaxation allows for the accommodation of the increased vascular flow responsible for the inciting shear forces[55] and reduces the adherence of leukocytes and platelets.[73] Enzymatic degradation of HS leads to loss of flow-mediated dilatation in vivo[57,71] through decreased NO production.[14] Voyvodic et al. demonstrated that sdc-1 knock-out mice have a significant effect on the shear stress-induced expression of transcription factors, vasodilatory mediators, inflammatory soluble factors, and receptors,[114] confirming the importance of an intact GL to mediate these responses. It is also important to note that these transcription factors have a genetic

influence that can alter the EnC phenotype and, therefore, affect pathways involved in inflammation, vasodilation, and thrombosis.[114] The intact EnGL, therefore, has a NO-mediated vasoregulation function,[14,56,115] which responds to local conditions and signals, such as endogenous ligands, heat, mechanical (i.e., shear stress), and osmotic stress,[82] for vasodilation or vasoconstriction.[73,82,109,114] The adaptive remodeling of the GL due to shear stress needs a balance between the synthesis of its components such as GAGs and PGs, and their degradation, which is modulated by enzymes such as HPSE[79] and matrix metalloproteinases (MMPs).[73,114]

Moreover, the intact EnGL also exerts a protective function against free radicals.[60,115] The HSPG binding of enzymes, such as extracellular SOD, protects EnCs against oxidative stress from reactive oxygen species (ROS), while maintaining NO bioavailability, thus preventing En dysfunction.[55,56,60] Albumin demonstrates an antithrombotic effect through the reduction of oxidative damage and modulating inflammation, which also seems to be related to the capacity of binding NO. Therefore, the binding of NO to albumin prevents the rapid inactivation of NO and consequently prolongs its effect on preventing aggregation of platelets. It is known that higher shear stress increases albumin uptake and alters GL properties, such as stimulating increased thickness of the GL.[73]

It is evident that an undersulfated GL might not only increase susceptibility to become infected with SARS-CoV-2, but the resulting dysregulated immune response will further disrupt the GL, resulting in a negative feedback destructive loop, increasing viral load, exacerbating the inflammatory response, permeability and coagulation.

3 | PATHOGENESIS OF COVID-19

3.1 | Glycocalyx facilitation of SARS-CoV-2 cellular entry

As with other viruses, the life cycle of SARS-CoV-2 in the host consists of the following steps: attachment, fusion/penetration, biosynthesis, maturation, and release.[116,117] In this review, we will mainly focus on viral attachment. SARS-CoV-2 encodes for four major structural proteins, namely SP, membrane, envelope, and nucleocapsid protein.[118] Attachment and entry of SARS-CoV-2 are mediated by the SP.[50] The SP is composed of a transmembrane trimetric glycoprotein that is protruding from the viral surface and determines the diversity of coronaviruses and influences host tropism.[116] The SP consists of two functional subunits: The S1 subunit is responsible for binding to the host cell receptor and the S2 subunit plays a role in the fusion of the viral and cellular membranes.[6] The C terminal domain of S1 harbors the receptor-binding domain (RBD).[8,119] ACE2 was identified as a functional receptor for SARS-CoV SP.[3,4,116] It is well-established that fusion and internalization mediated by the ACE2 receptor require cleavage of the full-length SP into S1 and S2 units,[36,49] facilitated mainly by the human protease furin[3] and TMPRSS2.[6,39,40]

It is well established that viruses also exploit GAGs, such as HS, as attachment factors.[6,12,16,48,49,51,52,120] These attachment interactions are driven by the overall sulfation of HS at a first electrostatic level,[12,33] thereafter by the specific recognition of structural determinants, therefore, the specific positional arrangement of sulfate groups on HS.[88] It is evident that the physicochemical properties of HS on the EpC surface play a prominent role in modulating the different steps leading to virus uptake by a host cell.[33,49,102] Peerboom et al. extensively reviewed the complexity of these viral–GAG interactions.[33]

More research needs to be done to fully understand the process of initial attachment to the cell surface for many GAG-binding viruses. Little is known about these viral–GAG interactions in vivo, since most of the research performed are in vitro docking studies, focusing only on the viral SP–GAG interactions.[33] Oh et al. hypothesized that these GAG-binding viruses can infect by taking advantage of differences in the GAG structure and charge densities, induced by burst-like appearances of both highly sulfated and undersulfated stretches of HS chains in the GL.[51] Peerboom et al. suggested that the initial attachment of some viruses to HS could be a dynamic process, where they move in a two-dimensional plane in search of secondary receptors before the viruses firmly attach to the membrane and proceed with viral entry. They found that both the degree of sulfation and the arrangement of sulfate groups along the GAG chain, influence viral mobility.[33]

We hypothesize that the degree of HS sulfation, thus, the net negative charge of the GL, is the initial deterrent to SARS-CoV-2 viral attachment. Various researchers found the SARS-CoV-2 SP and net GRAVY score to be highly negative,[121–124] categorizing the SP as a stable, hydrophilic molecule capable of establishing hydrogen bonds.[121] Most viral particles have a net negative charge at neutral pH because they have an isoelectric point below 7.[125,126] An intact, negatively charged, highly sulfated GL will, therefore, repel the virus through electrostatic forces,[127] preventing viral attachment and entry into the cell. Therefore, the higher the degree of GAG sulfation, the more effectively infection is inhibited.[42,59,78] While electrostatic interaction is the major driving force, in many cases, on a secondary level, specific sulfation patterns are required for the recognition of HS by its binding partners.[59,90] Hallak et al. and Tandon have suggested that *N*-sulfation appears

to have a greater effect on viral binding than 2-*O*- and 6-*O*-sulfation.[50,59]

Clausen et al. and Kim et al. found that the ectodomain of SARS-CoV-2 SP interacts with cell surface HS through RBD in the S1 subunit,[6,7] in a length- and sequence-dependent manner.[7,8,47] The binding of HS to SARS-CoV-2 SP shifts the structure to favor the RBD open conformation that binds ACE2 receptors,[128] probably due to the release of furin by the GL after HS binding.[3] SARS-CoV-2 SP binding to cells, therefore, requires the engagement of both cellular HS and ACE2 receptors, suggesting that HS acts as a co-receptor priming the SP for ACE2 receptor interaction (Figure 2). Adjacent to the ACE2-binding site and exposed in the RBD lies a group of positively charged AA residues[129] that represents a potential site that could interact with HS, thus enhancing infectivity.[6,47,130] SARS-CoV-1 SP RBD did not show the same electropositive surface.[40] SARS-CoV-2 has approximately 10–20 times higher affinity for ACE2 than SARS-CoV-1, which could explain the higher infectivity rate of SARS-CoV-2.[40,45] The difference between SARS-CoV-1 and SARS-CoV-2 infectivity is thus dictated by only a few key AA changes in the viral RBD.[100] During docking studies using a tetrasaccharide (dp4) fragment that was derived from HP, it was demonstrated that the preferred interactions were with the SARS-CoV-2 electropositive surface, and based on its dimensions, can accommodate a chain of up to 20 monosaccharides.[7] It should be noted that the putative binding surface for oligosaccharides is adjacent to, but separate from, the ACE2-binding site. This suggests that a single RBD can simultaneously bind both cell surface HS and the ACE2 receptor.[6,7] While the putative HS-binding site is fully exposed in the open state, it is partially obstructed in the "closed" inactive RBD conformation.[6,7]

During in vitro SARS-CoV-2 studies, it was found that cells with the highest expression of cell surface HS had the lowest extent of SP binding, whereas cells with low amounts of cell surface HS had the highest binding of

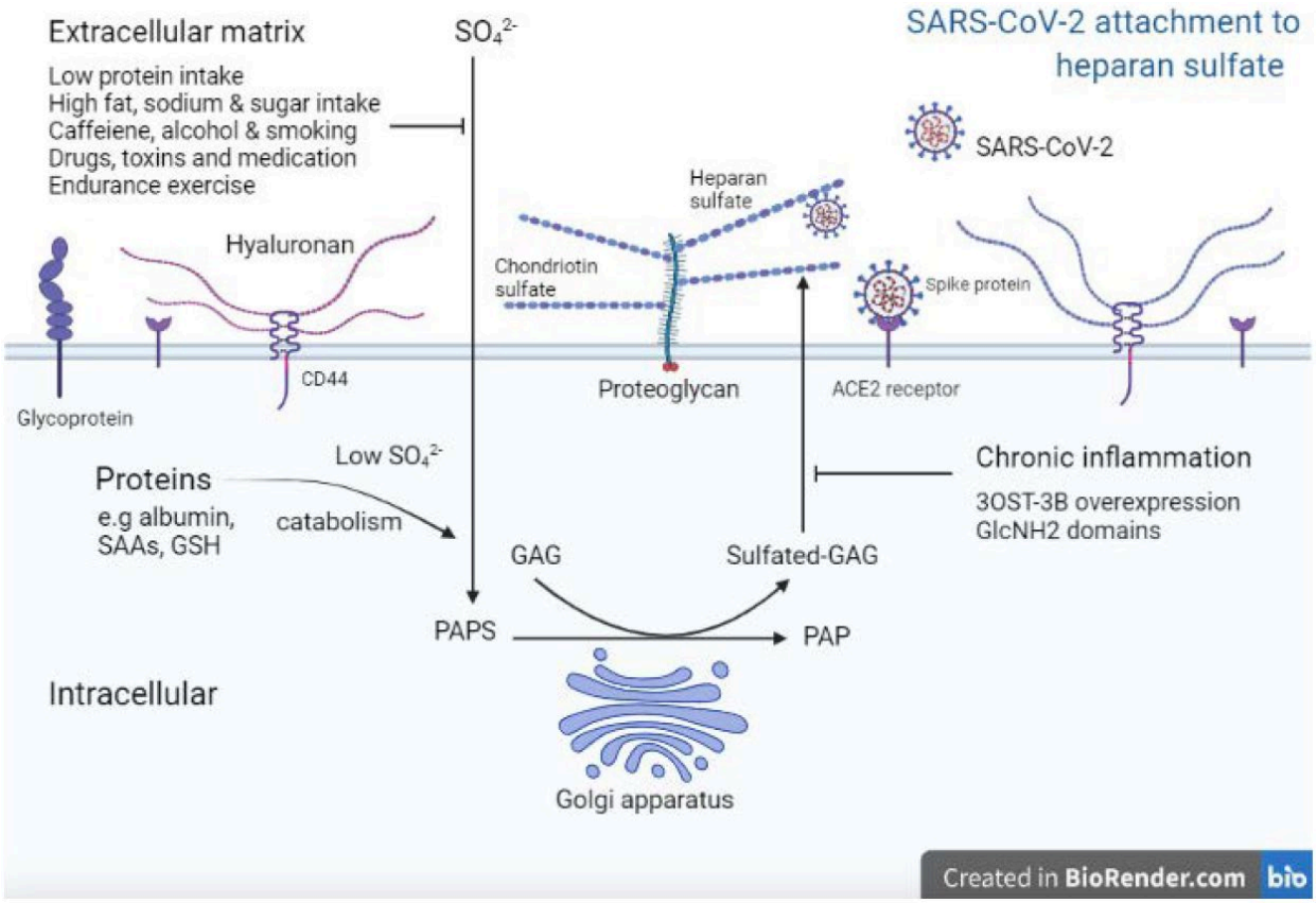

FIGURE 2 During chronic inflammation with overexpression of 3-*O*-sulfotransferase 3B (3OST-3B) and the presence of unsulfated *N*-unsubstituted glucosamine units (GlcNH2), decreased heparan sulfate sulfation will facilitate binding of SARS-CoV-2 to the angiotensin-converting enzyme 2 (ACE2) receptors. Availability of inorganic sulfate (SO_4^{2-}) is the rate-limiting factor to sulfation of the glycosaminoglycan (GAG) heparan sulfate. Various dietary and environmental factors reduce the availability of inorganic sulfate, in which case proteins, such as albumin and glutathione (GSH), would be catabolized to provide sulfur amino acids (SAAs) for inorganic sulfate synthesis

SP.[7] The data from various studies suggest that both the degree and pattern of sulfation of HS affect binding to SP and RBD.[6] Researchers isolated HS from human lung, liver, kidney, and tonsil and it was found that lung HS has a larger proportion of *N*-deacetylated and *N*-sulfated GlcN residues and more 2-*O*-sulfated IdoUA. The different HS preparations also varied in their ability to block the binding of SP RBD to H1299 cells. Remarkably, HS isolated from the lungs was a more potent inhibitor of binding, compared to kidney and liver HS, consistent with the greater degree of sulfation of HS from the lungs.[7] Even though the overall extent of sulfation was less in tonsil HS, both lung and tonsil HS were equally potent in inhibiting binding, supporting the notion that GAG arrangement of the sulfated domains may also affect binding. These findings demonstrate the requirement of cellular HS and the effect of sulfation in mediating SARS-CoV-2 infection of authentic human bronchial EpCs[7] (Figure 2).

Tiwari et al. demonstrated that SARS-CoV-2 RBD of subunit S2 preferentially recognizes 3-*O*-sulfated microdomains generated by HS glucosamine 3-*O*-sulfotransferase 3B (3OST-3B).[49] They found that SP-mediated cell-to-cell fusion arises even in the absence of ACE2, in which case 3OST-3B over-expression enhanced fusion.[49] Over-expression of 3OST-3B results in an increase in 3-*O*-sulfation of cell surface HS[88] that will facilitate SARS-CoV-2 binding to the cell. This finding is of major significance because it has been shown that inflamed cells and tissues of the compromised lung exhibit upregulated 3OST-3B expression,[49] which makes these patients more vulnerable to SARS-CoV-2 infection. Moreover, it was observed that expression of 3OST-3B is up-regulated in many cell types exposed to inflammatory stimuli, including monocytes or macrophages, fibroblasts, and EnCs.[62,88] It can, therefore, be assumed that chronic inflammation can be a predisposing factor to SARS-CoV-2 viral infection; however, the proinflammatory state induced by viral entry into the cell will potentially further upregulate the expression of 3OST-3B, creating a vicious cycle that potentiates infectivity and aggravates the cytokine storm. Furthermore, SARS-CoV-2 may hijack 3-*O*-sulfated HS and eliminate their original functions to facilitate its entry within some target cells,[16] therefore, enhancing functions such as coagulation, which is dependent on 3-OST isoforms for its anti-coagulation effect.[77,83,111] An undersulfated and degraded GL, therefore, seems to enhance viral entry.[131]

3.2 | Lung involvement

The respiratory epithelium is one of the initial sites within the respiratory tract to be exposed to inhaled stimuli and toxins, as well as pathogenic airborne viruses such as SARS-CoV-2.[35] The lung ECM constitutes a three-dimensional scaffold of the alveolar epithelium and the capillary endothelium,[94] with the GL lining both the lung Ep- and EnCs.[4]

The EpGL remains a largely understudied component of the lung, despite its discovery more than 50 years ago.[71] Haeger et al. and Weidenfeld et al. showed that the alveolar EpGL is rich in HS, and mass spectrometry of shed HS indicated a high level of sulfation at both *N* and *O* positions.[13,71] Haeger et al. confirmed the effective barrier function of the intact, highly sulfated negatively charged GL in the lung, intestinal, bladder, and corneal epithelium, where it was suggested that lung endothelium and corneal epithelium-sulfated HS regulate the expression and localization of tight and adherens junction proteins, which are known to be involved in Ep and En barrier function.[71,94] Additionally, Ep HS may act as non-signaling, structural components of the Ep barrier, similar to the "charged meshwork" of the EnGL, which also opposes transvascular protein flux.[71] Oshima et al. indicated that lungs, apart from having predominantly high concentrations of Ep and En HS, feature a uniquely thick EnGL (1.6–1.7 mm), compared to other vascular beds, such as in the cremaster or mesentery (0.6 mm).[57]

HSPGs seem to play a major role in the homeostasis of the lung, due to their huge variability, strategic location on cell surfaces and in basement membranes, and their capacity to bind and modulate a myriad of proteins and effector molecules, such as proteases and protease inhibitors, enzymes involved in neutralizing ROS, cytokines, and growth factors.[89,132] It is well known that the pulmonary EnGL plays a major role in microvascular permeability. Lung EnC HSPGs are key participants in inflammatory cationic peptide-induced signaling that leads to shedding of GAGs with subsequent barrier dysfunction[81] and impaired lung repair due to shed HS fragments that sequester growth factors.[132]

The lung endothelium plays a very important and active role in the recruitment and adhesion of inflammatory leukocyte, erythrocyte, and platelet cells, via increased expression of adhesion molecules and chemoattractant cytokines, once activated by viruses such as SARS-CoV-2 at the blood–gas barrier.[96] Activated immune cells and platelets establish a paracrine communication network between the different immune, Ep-, and EnCs within the injured alveolus that may alter alveolar fluid clearance and permeability, consequently resulting in lung-edema.[94] Therefore, the capillary endothelium of the blood–gas barrier in the lungs and its intact-sulfated GL serves as an imperative gatekeeper, as well as an anti-inflammatory and antithrombotic role in maintaining the oncotic gradient across the En barrier.[96]

The pathological result of SARS-CoV-2 infection is a diffuse alveolar damage[133] with proteinaceous and fibrinoid intra-alveolar exudates with mononuclear inflammation, multinucleated giant cells, and type 2 pneumocyte hyperplasia.[96,133,134] Becker et al. described that post-mortem biopsies of COVID-19 decedents reveal macro- and microvascular thrombosis involving arteries, veins, arterioles, capillaries, and venules in all major organs.[109] Various reports described En cell involvement and endotheliitis across vascular beds. Through both histology and electron microscopy, it was demonstrated that accumulation of viral inclusions and inflammatory cells occurred within the vascular endothelium of the lungs, heart, kidneys, and small bowel. In autopsy and surgical tissues, severe endothelial cell damage with disrupted cell membranes, intracellular virus, diffuse lymphocytic endotheliitis, and apoptotic bodies were found.[109,131] Johnson et al. have shown through COVID-19 autopsies that the lung alveoli were filled with fluid, eukaryotic material, and dead tissue. Concurrent with this pathology, hepatic, renal, and cardiovascular organ failure were observed that were precipitated by sepsis.[4]

In almost all COVID-19 deaths, the prevalence of clotting, multiple organ failure, and all the other symptoms of sepsis signifies systemic septic shock.[4] Alteration in the composition of the GL after exposure to an inflammatory insult is one of the earliest features during sepsis.[77,80] Following infection, acute injury, and inflammatory mediators, glucuronidases (including HPSEs),[12,17,46] ROS,[71,80] NO,[4] and other proteases (such as MMPs),[14,71,94,115] cause shedding of HS and PGs.[60,68] Consequently, adhesion molecules such as E-selectin and intercellular adhesion molecules are exposed on the denuded endothelium,[72,73,75,115] and result in vascular permeability,[17,97] edema, accelerated inflammation,[108] platelet aggregation, hypercoagulation, and a loss of vascular responsiveness[4,13,53,71,73,77,80,82,94,135] (Figure 3). Subsequently, impaired oxygen delivery and altered blood flow may result in organ failure. Even though global oxygen delivery is usually increased during sepsis, many tissue capillary beds do not receive an adequate oxygen supply due to microvascular En injury.[77,80]

In response to inflammatory mediators, shedding of the GL was observed in arterioles, capillaries, and

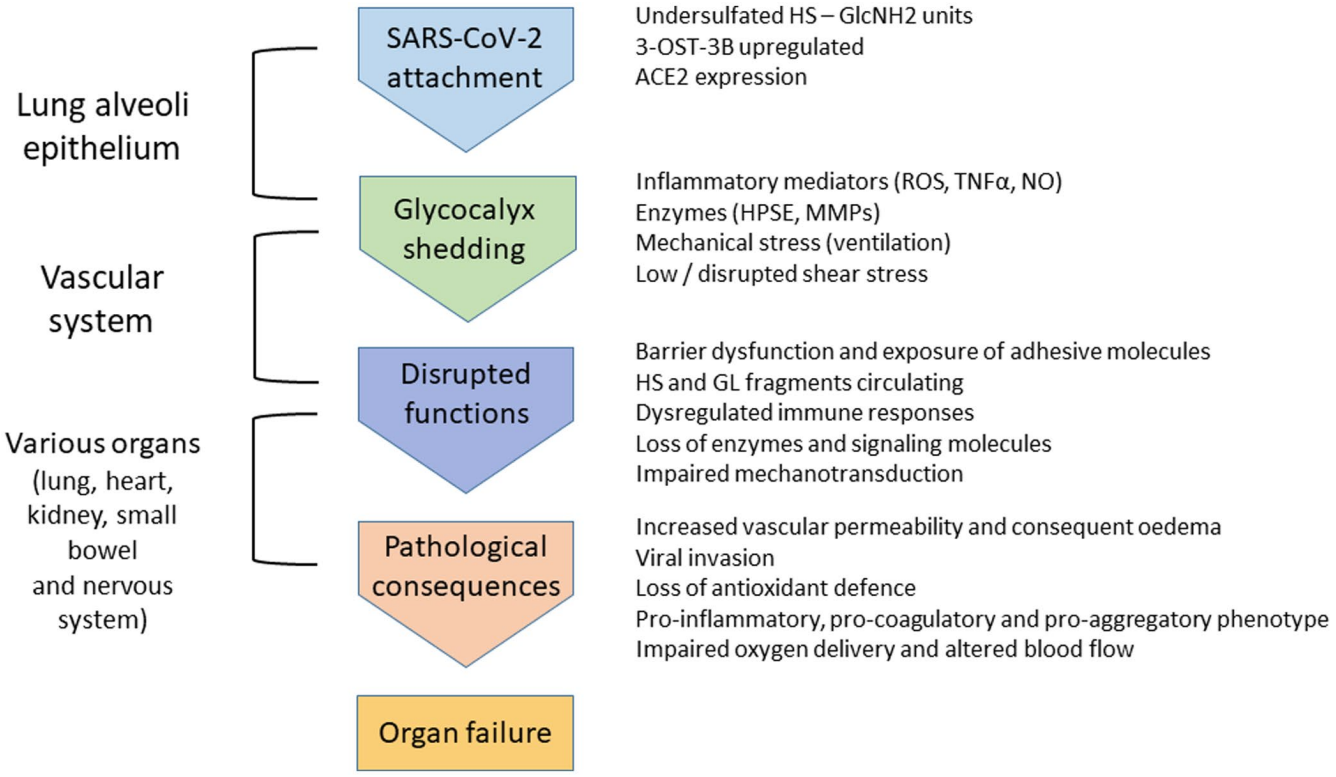

FIGURE 3 Flow diagram summarizing the pathogenesis of COVID-19. Undersulfated heparan sulfate (HS), with upregulated 3-*O*-sulfotransferase 3B (3OST-3B) expression and unsulfated *N*-unsubstituted glucosamine units (GlcNH2), facilitate viral attachment to the angiotensin-converting enzyme 2 (ACE2) receptors. Viral attachment stimulates the release of inflammatory mediators such as reactive oxygen species (ROS), tumor necrosis factor alpha (TNFα), and nitric oxide (NO), and the expression of enzymes such as heparanase (HPSE) and matrix metalloproteinases (MMPs), which in turn cause shedding of the glycocalyx (GL) components. High-tidal volume ventilation and disrupted shear stress will also result in shedding of the GL. The exposure of adhesive molecules in the GL, increased vascular permeability, dysregulated immune responses, and circulating GL fragments will lead to various pathological consequences and eventual organ failure

venules under various experimental models of inflammation.[60] Moreover, circulating HS and PG fragments act as proinflammatory molecules,[17,80] with significant chemotactical properties,[60] through sulfation-based electrostatic interactions, thus capable of influencing growth factor and a variety of homeostatic and/or pathological signaling pathways distant to the site of GL injury, which explains the systemic consequences of GL degradation,[57,109,135,136] and support the relationship between renal damage and the systemic proinflammatory state observed in sepsis.[17,137] These systemic effects may be long-lasting, seeing that circulating HS fragments may persist for more than 5 days in patients with respiratory failure.[57] Several research studies found increased circulating levels of GL fragments or its kinases in patients with severe COVID-19.[138] Released ROS may also cause changes to the GL[82] via depolarizing GAGs, thereby inducing membrane permeability.[17] This creates a vicious feedback inflammatory cascade seen in sepsis and therefore, besides ARDS, severely ill COVID-19 patients may progress to multi-organ dysfunction.[58] We propose that an intact highly sulfated lung GL would have prevented SARS-CoV-2 attachment in the first place, and adequate sulfated HS would have been better equipped to modulate the immune and coagulatory response and prevented vascular and Ep barrier permeability.

Apoptosis of lung EpCs will result in loss of alveolar EpCs and development of ARDS.[94] It is known that in ARDS, the activation of the proapoptotic Fas/Fas ligand (FasL) pathway will cause alveolar Ep injury, through the binding of membrane-bound or soluble FasL to Fas-bearing cells. Therefore, the inhibition of apoptosis by blocking the caspase activity or Fas/FasL pathway may attenuate lung injury and consequent protein-rich edema formation, as well as prevent sepsis and ventilator-induced lung injury, as seen in animal studies.[94] Hirano et al. proposed that the activation of Fas signaling is mediated by 3-*O*-sulfated HS. They suggested that HS chains on sdc2 interact with Fas in the Golgi lumen and mediate the transport of Fas from the Golgi to the cell surface.[139] Therefore, the death receptor-mediated apoptosis pathway, which is mediated through the cell surface receptors for FasL and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), is regulated by HSPGs.[139] In addition to the regulation of apoptosis, a recent study indicated that adequate sulfation of HS is also essential for preventing senescence.[15,140] It is, therefore, important to consider that apoptosis may not necessarily require host cell viral entry, but that viral binding to 3-*O*-sulfated HS on the cell surface may be sufficient to result in proinflammatory and apoptotic pathway signaling.[109]

3.3 | Vascular system and coagulation

In addition to respiratory symptoms, thrombosis and pulmonary embolism have been observed in severe COVID-19 patients.[43,141] These patients exhibited a hypercoagulable phenotype, with prolonged prothrombin time, as well as elevated levels of D-dimer and fibrinogen, with near-normal-activated partial thromboplastin time.[43,94,142] Some COVID-19 patients did eventually progress to overt disseminated intravascular coagulation.[42] A high proportion of acro-ischemia was also observed in deteriorating patients with COVID-19, indicating a hypercoagulable status before the final onset of overt disseminated intravascular coagulation.[43] In patients prone to severe COVID-19, EnGL damage has been identified as a common feature.[138] Seen that the EnGL plays a significant role in thrombotic regulation and vasodilation,[73] hypercoagulable profiles seen in severe COVID-19 illness likely indicate significant En injury and the connection may exist between SARS-CoV-2's ability to bind to 3-*O*-sulfated HS.[8,78,97,142] Coagulopathy has been observed in both adult and pediatric patients,[42] although most patients were older than 60 years, and many had known risk factors for cerebrovascular disease, especially hypertension, diabetes mellitus (DM), hyperlipidemia, and vascular disease.[142] Extensive cross-talk exists between inflammation and coagulation, whereby inflammation does not only lead to activation of coagulation, but coagulation also has a considerable effect on inflammatory responses.[42,83,113,143,144] These two processes may act in a feed-forward manner toward an uncontrolled endpoint. Blood clots may lead to myocardial infarctions, pulmonary embolisms, or renal failure.[143] Furthermore, prolonged states of hypoxia elevate the expression of tissue factors in the monocyte and macrophage lineage, as well as pulmonary vascular EnCs, which may lead to enhanced fibrin accumulation and consequent pulmonary thrombosis.[111]

Various reports suggest a role of En dysfunction and loss of EnGL barrier function in COVID-19 patients.[40,105,107] Microvascular permeability as a result of the En injury can not only facilitate viral invasion,[116,145] but also the spread of HS and PG fragments, cytokines, and other inflammatory mediators[137] (Figure 3). Direct SARS-CoV-2 infection of the En cell and diffuse En inflammation in multiple organ systems were described in a recent report.[146] It was found that HS plasma levels were significantly elevated in COVID-19 patients compared to healthy controls.[105] EnGL barrier function is also crucial in the regulation of fluid and protein extravasation,[14] particularly in the lungs and kidney.[55,58,68,105] Removal of HS by HPSE leads to increased permeability for both ferritin and albumin, and injection of antibodies to HS leads to an

acute selective proteinuria in rats.[93] As pulmonary edema occurs when fluid leaks into alveoli, dysfunction of the endothelium likely contributes to pulmonary edema in COVID-19 patients.[105,109]

No doubt, the binding of SARS-CoV-2 to the ACE2 receptors will influence various processes, such as vasoconstriction, kidney injury, cardiovascular disease (CVD), apoptosis, and oxidative processes,[143] but the effect of the degraded GL and circulating HS is overlooked in most of the COVID-19 research to date. Research studies indicated that 59% of COVID-19 patients had proteinuria upon hospital admission, where 22% of the non-ventilated patients and 90% of the ventilated patients developed AKI.[58] It is known that proteinuria occurs when the En barrier function in the glomerulus of the kidney is compromised.[105]

A known factor in sepsis and ARDS is hypoalbuminemia, which occurs when fluids build-up in the alveoli and it is associated with systemic sepsis, seen that it is related to the fluid and mechanisms of circulation.[4,73] Hypoalbuminemia, vascular disease, and coagulopathy have all been associated in COVID-19 cases and have been linked to the outcome, independent of age, and morbidity.[4] The healthy EnGL is essential in maintaining normal fluid homeostasis. It is physiologically significant in capillary microcirculation and in fluid distribution to the tissues.[73] Human serum albumin (HSA) maintains GL thickness and oncotic pressure, and it transports hormones and free fatty acids,[54,73] but SARS-CoV-2 virions can bind competitively to HSA, thus diminishing its normal transport function[4] and decrease GL thickness, as observed in sepsis.[54] Furthermore, hypoalbuminemia is often seen in patients with conditions such as hypertension, DM, and chronic heart failure, therefore, those most susceptible to SARS-CoV-2 infection.[4] Hypoalbuminemia could, therefore, be a predisposing factor to COVID-19, and/or a consequence of the disease.

3.4 | Immunity

It is clear from the recent research that the innate immune system directly or indirectly impacts COVID-19 disease progression.[32,52,67,99,116] HS has a modulatory effect on both innate[147] and adaptive immune responses.[141,148]

It is proposed that once a virus manages to attach to HS in the alveoli, macrophages migrate to and internalize the viral particles.[52] Sulfated HS, therefore, presents viruses to incoming macrophages for phagocytosis and degradation.[149] Negatively charged molecules were found to penetrate slower into the intact sulfated GL than neutrally or positively charged molecules.[78] This charge repulsion would prevent the neutrophil–Ep interaction.[78] The assumption can, therefore, also be made that once the GL is compromised by undersulfation, therefore less negative charge density, SARS-CoV-2 moves too fast through the EpGL for the immune system to modulate the response. It has been demonstrated that several potential neutrophil bactericidal effectors, including proteases and defensins, also interact with HSPGs, which will affect their intracellular localization and retention within granules.[150] HS shows inhibition activity against HPSE, neutralization of cytokines, inhibition of leukocyte trafficking, and neutralization of extracellular cytotoxic histones in the vascular system.[67,105] HS is also responsible for either activation or inhibition of the complement system and the degree of HS sulfation determines C3b cleavage, which is enhanced when HS is undersulfated.[131] HS, therefore, not only plays a major role in initial infection, but also modulates the immune responses to SARS-CoV-2.[67]

Most individuals recover easily from COVID-19 infection, seemingly producing antibodies to the disease. Nonetheless, it should be noted that antibodies were also produced in most patients who do not recover, and weakened immune function is, therefore, not a cause of their deaths.[4] It is clear that it is the first line of defense that is compromised in COVID-19, being a disrupted GL, and thus a defective innate immune response, more so than adaptive immunity. Sulfated GAGs patrol all the borders of the epithelium and endothelium. GAG undersulfation is an example of the compromised function of a critical barrier that allows pathogens and cells to move into regions from which they are normally excluded.

3.5 | Systemic inflammatory response

The systemic inflammatory response triggered by SARS-CoV-2 is similar to sepsis and can be summarized as Ep- and EnGL degradation, one of the earliest consequences of injury during inflammation. A class of recently identified inflammatory mediators is secreted CyPB, which triggers migration and integrin-mediated adhesion of T lymphocytes[85] and monocytes or macrophages, via interactions with two types of binding sites, CD147 and cell surface HS.[16,20] It was found that the interaction of CyPB with HS is strictly dependent on the presence of GlcNH2 residues and 3-O-sulfation by 3OST-3B isoform, for efficient binding of CyPB to responsive T cells.[16,20] Moreover, HS 3-O-sulfation by 3OST-3B also provides binding sites for SARS-CoV-2,[16,17,49] thus suggesting that HS motifs with binding properties for the virus and CyPB are similar. For efficient infection, SARS-CoV-2 may hijack the binding sites of CyPB that is supposed to protect host cells against apoptosis.[20] Furthermore, it was observed that M2-activated resolving macrophages contained almost twice as much HS than proinflammatory M1 macrophages, with a relatively higher sulfation rate. While

3OST-2 was highly expressed in M2 macrophages, it was replaced by 3OST-3B in M1 macrophages.[20] One can, therefore, speculate that 3OST-3B is expressed with lower HS sulfation, probably as a host T-cell defense mechanism for more effective CyPB binding in the absence of sufficient highly sulfated HS, which also explains the higher expression of 3OST-3B in chronic inflammatory conditions.[16] This seems to confirm our hypothesis that an undersulfated GL with a higher expression of 3OST-3B is a predisposing factor for SARS-CoV-2 infection and severe COVID-19 symptoms.

4 | PATHOPHYSIOLOGY OF COVID-19

From the evidence reviewed thus far, it is not unexpected that changes in the degree of HS sulfation or varied expression of genes coding for HS biosynthetic enzymes[62] and synthesis of inorganic sulfate[151] have been associated with the development of many inflammatory diseases,[152] such as atherosclerosis, stroke, sepsis, DM and related renal diseases, CVD, hypertension, obesity, pulmonary disease, and cancer.[14,46,55,56,74,85,86,96,153,154] These are the very same comorbidities that increase susceptibility to SARS-CoV-2 infection and severe COVID-19. Oxidative stress[155] and chronic inflammation,[30,32,136] as evident in these COVID-19 related comorbid conditions, result in attenuation of the GL.[55,73]

Metabolic syndrome is characterized by symptoms such as glucose intolerance, visceral obesity, dyslipidemia, and hypertension, which are all related to each other and caused by GL dysfunction.[100,153,156] Obesity-induced hepatic inflammation has been implicated in the development of non-alcoholic fatty liver disease, with up-regulation of proinflammatory cytokine expression, such as interleukin (IL)-6 and tumor necrosis factor alpha (TNFα). Non-alcoholic fatty liver disease is also often found in type 2 DM (T2D).[157] Inflammatory diseases whose etiology are different may promote EnGL dysfunction by several associated pathways and initiate albuminuria. Galvis-Ramírez et al. described a study that compared biopsies of mice with lupus nephritis and patients with the same condition. Looking at the glomerular En expression of different HS domains, a decrease in *N*- and 6-*O*-sulfate were observed, which was associated with albuminuria.[17] Although obesity and atherosclerosis are very different, accumulation of M1-like macrophages and low-grade chronic inflammation are characteristics of both conditions. In a mouse-model Ndst1, the inactivation caused a 15% reduction in the overall sulfation of HSPG in macrophages. Even with this modest change in HS sulfation, the Ndst1 deficient mice displayed excessive body weight gain, became profoundly type-2 diabetic, and developed exacerbated atherosclerosis, compared to control mice on a high-fat diet.[154,158] Seen that upregulated expression of 3OST-3B and the presence of GlcNH2 domains have been observed in many cell types exposed to inflammatory stimuli,[16,62,88,152,159] it is evident that HS and its degree of sulfation likely plays a major role in the etiology of the comorbid chronic-inflammatory conditions that predispose to severe COVID-19 illness.

4.1 | Obesity and the role of the glycocalyx

Obesity is a complex and chronic metabolic disorder with a multifactorial etiology, including insulin resistance, hepatic steatosis, inflammation,[157] and an impaired hematopoietic system,[55] all of which are related to a dysfunctional EnGL layer.[82] It is known that cytokines and inflammatory mediators,[154,160] plus other substances involved in insulin resistance, are increased in obese individuals. Adipose tissue is the largest endocrine organ and adipocytes secrete a variety of adipocytokines, such as TNFα, IL-6, and resistin. HSPGs have been implicated in the regulation of the lipid dynamics of adipose tissue,[100] with HS domains playing significant roles.[82,156] In a mouse model with adipose tissue-specific inactivation of Ndst1 and Ext1, both groups gained more weight on a high-fat diet compared to wild-type littermates, and they developed significant glucose intolerance and insulin resistance. It was confirmed through a pyruvate tolerance test that disturbed adipocyte-HS have a major effect on liver glyconeogenesis, where the Ndst1 mice presented with elevated hepatic inflammation, underlining the importance of HS composition in adipose tissue for signaling processes necessary for glucose homeostasis.[161] In addition to inflammation, patients with metabolic disorders and sedentary lifestyles are more susceptible to oxidative stress[82] and endoplasmic reticulum stress,[160] which negatively impact HSPG levels.[162]

HSA also plays an important role in fat metabolism by binding fatty acids and keeping them in a soluble form in the plasma. Hyperlipemia, therefore, occurs in clinical situations of hypoalbuminemia,[163] which has been associated with COVID-19[4] and would indicate low levels of cysteine (Cys), the precursor to both albumin and inorganic sulfate.[23,164]

4.2 | Diabetes and the role of the glycocalyx and sulfation

DM is a clinically well-defined metabolic disease connected with insulin absence or resistance in which obesity,

hyperglycemia, dyslipidemia, and chronic low-grade pancreatic islets inflammation contribute directly or indirectly to beta-cell failure.[162] Patients with DM display a tendency to develop vascular complications, such as microalbuminuria, retino-, and nephropathy, elevated the risk of atherothrombotic cardiovascular and cerebrovascular events.[14,56] Fundamental pathogenic mechanisms in DM-associated vascular disease include accentuated vascular inflammation, increased oxidative stress, and GL degradation.[32,96,109,138] Long-term or induced hyperglycemia is associated with profound thinning and degradation of the GL[14,165] (Figure 4) and increased vascular permeability[56] due to a reduction in HS[17,32,55,60] and decreased GAG sulfation.[22,32,93,166]

Conversely, it seems that HS and dysfunction of the GL play a role in the etiology of DM in the first place. Recent reviews described that human beta cells have a remarkably high intracellular level of highly sulfated HS,[66,86,162] protecting cells in the pancreatic islet from ROS and cell death.[167] This protective anti-apoptotic effect is neutralized when nearby autoreactive T cells secrete HPSE that subsequently degrade HS, leading to an onset of Type 1 DM (T1D).[55,66,85,86] Apart from its antioxidant properties, beta cell HS also regulates insulin secretion. Simeonovic et al. demonstrated that beta cell HS is lost before insulin in human T1D and is a sensitive marker of disease progression. More specifically, 3-O-sulfation of beta cell HS and sdc4 have been shown to play important roles in the secretion of insulin by beta cell lines.[86,162] Moreover, it has been reported that the loss of Ext3 and consequent dysregulation of HS biosynthesis may be a potential factor in the pathogenesis of DM.[85]

Oxidative stress and endoplasmic reticulum stress underlie beta cell failure in T2D, with elevated ROS. In T2D, the antioxidant capacity of beta cell HS is, therefore, impaired. It has been demonstrated in beta cell line studies that undersulfated HS are not protected from hydrogen peroxide-induced death.[162] Dhounchak et al. reported that both beta cell sulfated HS and HSPG core proteins are significantly depleted in prediabetic 3–5 week db/db mice.[162] It was also found that T2D patients present with increased expression of sulfatase (SULF)2, resulting in the removal of 6-O sulfate groups from HS. In a db/db mouse model, reduced HS sulfation on hepatocytes, accompanied by reduced Sdc1-mediated toll-like receptor clearance, were also associated with increased liver SULF2 expression.[168] In addition, the insulin-sensitizing effect of fibroblast growth factor 1 depends on the presence of HS on the plasma membrane and is diminished in Ndst1 and Ext1 mice.[161]

Kidney dysfunction was frequently observed in DM patients with COVID-19. These patients presented with a picture of collapsing glomerulopathy, considered as secondary to viral infection.[169] En HS plays a central role in the development of renal inflammation and fibrosis in diabetic nephropathy. Research indicates that a decrease in En HS sulfation resulted in an increased macrophage infiltration in the glomerulus, glomerulosclerosis, as well as mannose-binding lectin complement deposition.[17] Some DM studies suggest that changes in the level and sulfation

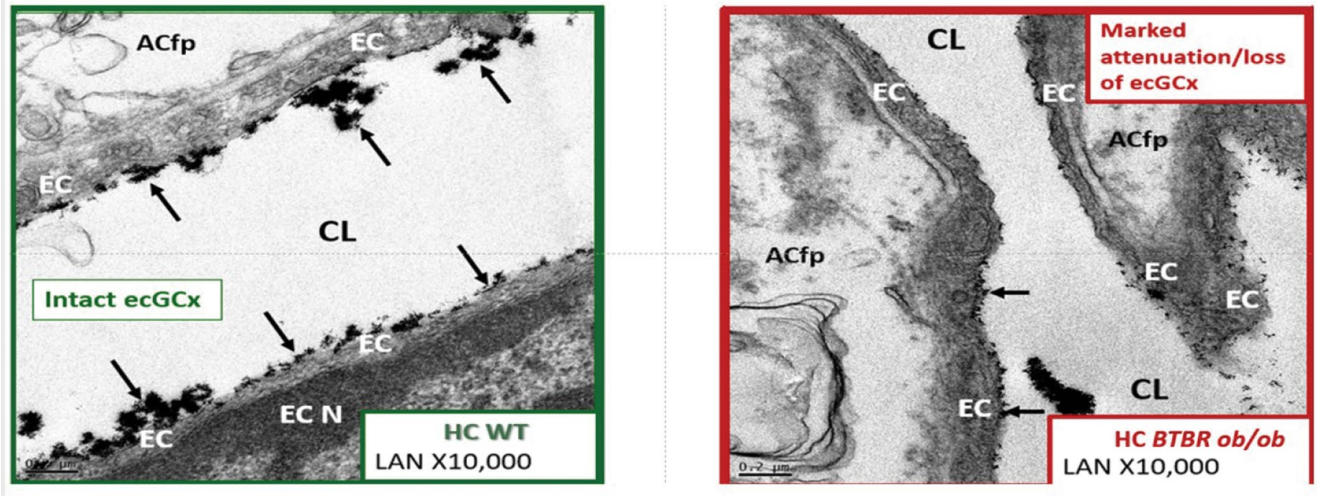

FIGURE 4 Lanthanum nitrate (LAN) staining of the endothelial glycocalyx (EnGL or ecGCx) showing attenuation and loss in the obese diabetic BTBR *ob*/*ob* model: hippocampus CA-1 regions. Note the highly decorated endothelium by LAN staining in the control wild-type hippocampus model with intact EnGL (left-hand figure). In the obese diabetic BTBR *ob*/*ob* model, the EnGL (arrows) when present is markedly attenuated and very thinned and/or lost (right-hand figure). Magnification ×10 000; bar = 0.2 μm. ACfp, astrocyte foot process; CL, capillary lumen; EC, endothelial cell; HC, hippocampus; WT, wild-type control. Adapted with permission by CC by 4.0[165]

patterns of kidney HSPGs can be due to increased kidney levels of HPSE or changes in the regulation of SULFs. Increased levels of HPSE were found in the blood of patients with DM and correlated to the development of kidney complications,[14,166,169] which would be exacerbated during COVID-19. A study focusing on kidney HS showed that the larger glomeruli in kidneys from diabetic rats had a lower degree of N-sulfation when compared to controls.[166] MacArthur reported that livers from insulin-deficient rodents showed reduced HS sulfation and levels, along with decreased expression of Ndst1.[22]

Patients with DM are more vulnerable to infections due to hyperglycemia-induced virulence of various microorganisms,[160] and EnGL degradation.[109] Apart from the fact that people with DM were more susceptible to SARS-CoV-2 infection, recent research indicated that SARS-CoV-2-infected patients with DM were at a higher risk of developing severe pneumonia. These patients presented with uncontrolled inflammatory responses and a hypercoagulable state, associated with dysregulation of glucose metabolism, compared with COVID-19 patients without DM. Furthermore, they displayed remarkably increased serum levels of inflammatory mediators, including IL-6 and C-reactive protein, compared to those of nondiabetic patients, suggesting that diabetic patients are more vulnerable to the cytokine storm leading to rapid deterioration.[160] These findings would be in line with the reduced HS sulfation since highly sulfated HS modulates the immune response and regulate glucose metabolism. It was established that supplementation with sulfur amino acids (SAAs) and sulfur-containing supplements improved glucose homeostasis in diabetic animals and patients.[157] An infusion of N-acetyl-L-cysteine (NAC) attenuated the hyperglycemia-induced reduction of the EnGL volume, which was associated with impairment of flow-mediated vascular dilation and activation of the coagulation system.[115] Even though these benefits of the various sulfur donors on glucose metabolism were ascribed to different mechanisms, it was confirmed that the contribution of thiol compounds to GAG sulfation becomes significant in vivo, by increasing inorganic sulfate plasma concentration.[28,170]

Immunological reaction to exogenous insulin can lead to several clinical manifestations. High levels of anti-insulin IgG antibodies can bind to exogenous insulin and cause immune insulin resistance, which could lead to frequent ketoacidosis.[171] In a case report, it was described that a woman with T1D and on a conventional dose of insulin, presented with brittle DM (both hypoglycemia and hyperglycemia), which was resolved by substituting the conventional insulin with sulfated beef insulin.[157] Sulfation of insulin masks the site recognized by antibodies, but does not eliminate the biological activity of insulin. It also appears that the sulfate would have an insulin-sensitizing effect in insulin-resistance.[157,171] It would be valuable to investigate the effect of unsulfated conventional insulin use in DM on immune modulation in general and the inflammatory response, and how this affects susceptibility to severe COVID-19.

4.3 | Gastrointestinal presentations in COVID-19

SARS-CoV-2 RNA was detected in stool samples of up to 67% of SARS-CoV-2 positive patients, with viral shedding still taking place in the stool for several days after negative conversion in pharyngeal swabs. Gastrointestinal tissue samples also tested positive for SARS-CoV-2 RNA.[172] This, along with the presence of diarrhea in some of these patients, points toward a distinct possibility of gut-lung axis involvement.[41] It has been well-established that gut microbiota influence lung diseases and, conversely, respiratory viral infection causes changes in gut microbiota, therefore, results in dysbiosis. Yeoh et al. found that a gut dysbiosis was more prevalent in COVID-19 patients, compared to non-COVID-19 individuals.[173] A research question, therefore, is whether a gut dysbiosis is a predisposing factor to COVID-19 or a consequence, or both?

Various factors, such as diet, environment, medications, and genetics play an important role in shaping gut microbiota, which in turn influence immunity.[41] Paneth cells in small intestine crypts secrete microbicidal defensins as components of enteric innate immunity[174–176] to protect the host by preventing the intestinal barrier disruption.[147,177–179] The highly cationic defensins interact with HSPGs,[178] which affect their intracellular localization and retention within granules.[150] Since HS modulates the expression of defensins and cytokines, the degree and pattern of HS sulfation will affect immune modulation and SARS-CoV-2 attachment in the gut.[178,180,181]

Elevated HPSE expression in gut EpCs results in colitis or diarrhea, and contributes to local inflammation due to loss of HS.[19,86,182] Gastric epithelium in humans displayed a reduced degree of HS sulfation and lower levels of sulfated GAGs[19] following stress, high alcohol consumption, after aspirin and NSAID administration, inflammation, dysbiosis, and due to low protein/sulfate intake.[23,41,170,182–186]

4.4 | Eye health and its role in viral transmission

Besides the upper respiratory tract, eye exposure to infectious fluids may be associated with increased risk for SARS-CoV-2 transmission. Eye contamination with

coronaviruses was confirmed in previous studies using PCR analysis of tear samples. Recently, clinical observation revealed that SARS-CoV-2 RNA was detected in an ocular swab from a patient diagnosed with COVID-19, while it was undetectable in the nasal swab.[45] Though ACE2 receptors are not expressed in the conjunctival and corneal epithelium,[44] sulfated GAGs will facilitate viral entry.[34,49,74,187] Indeed, because of the synthesis of 3-*O*-sulfated HS by primary human corneal fibroblasts, the eyes will be susceptible to SARS-CoV-2 entry.[83]

4.5 | Neurobiology and COVID-19

SARS-CoV-2 seems to have the capacity to, directly and indirectly, injure the central and peripheral nervous systems.[142,143] The virus can contribute to a number of neurological issues and COVID-19 patients who recovered after severe illness were at higher risk for long-term residual neuropsychiatric and neurocognitive conditions, including obsessive-compulsive disorder, depression, psychosis, dementia, Parkinson's, and Alzheimer's disease. Strokes, as well as other neurological complications, were more common in patients with COVID-19 who also suffered from DM.[143]

The underlying mechanisms for these neurodegenerative conditions may be multifactorial, resulting from the combined or independent effects of sepsis, hypoxia, and immune hyperstimulation.[146] Hippensteel et al. demonstrated that after lipopolysaccharide (LPS) treatment, the accumulation of hippocampal HS fragments persisted for 7 days, a time point normally characterized by impaired cognition, and which eventually normalized after 14 days (Figure 4). These shed HS fragments and cytokines can penetrate the blood–brain barrier (BBB).[90,137,143] The brain vasculature has a continuous layer of EnCs, which reinforces the BBB as a "tripartite" layered structure.[78,107,165] Striking reductions in brain-sulfated HSPGs were observed in kwashiorkor patients, with major pathological implications since BBB integrity is HSPG dependent.[170] Cytokines activated by SARS-CoV-2, such as IL-1, IL-16, and TNFα, are known to cause injury to the BBB.[143,187] There is increasing appreciation for the importance of the EnGL in sepsis pathophysiology,[137] which can lead to microvascular and macrovascular complications in the brain.[142,188] Direct central nervous system infection with SARS-CoV-2 seems unlikely[146,189] when highly sulfated GAGs are present.[150] Even though direct entry into the brain has been described for other coronaviruses[143] and Varga et al. found evidence of direct SARS-CoV-2 infection of En cells and diffuse En inflammation.[136,138] Since nanoparticle delivery to the brain occurs through direct intranasal delivery, drugs introduced into the nasal cavity can reach the brain either via the olfactory bulbs and the trigeminal nerve or via the lymphatic system.[190,191] This could, therefore, most likely also be a route for SARS-CoV-2 infection, seen that viral RNA was detected in olfactory neuronal cells in both animal models and humans. Autopsy results from patients with severe COVID-19 revealed a low level of SARS-CoV-2 invasion across the cribriform plate into the olfactory bulb within the cranium.[192]

Both SARS-CoV-2 and tau's entry into a cell are enhanced by HS 3-*O*-sulfate functional groups,[46] raising the possibility of mechanistic cross-talk between the spread of tau pathology and viral infection in the brain.[90] The degree and pattern of GAG sulfation also affect the binding of Aβ and prion in the cerebral cortex, where a lower degree of *N*-sulfation appears to favor this process.[70,74] The presence of GlcNH2 domains has been associated with prion and Alzheimer's disease.[87] Varki et al. reported that mice deficient in HS6OST1 exhibited defective HS biosynthesis in the brain that resulted in autism-like socio-communicative deficits.[100] HS is also required for the normal functioning of glutamatergic synapses.[193] Recent studies extensively documented the role of HS and HPSE in neuroinflammation. Multiple pathogens, including viruses, exploit variability in HS domains to their benefit to facilitate pathogenesis.[187]

Loss of smell (anosmia) and taste (ageusia) seems to be prevalent in many COVID-19 patients,[142] where taste dysfunction appears to be more common than olfactory dysfunction.[143] Impairment in olfactory and gustatory functions in patients with COVID-19 are likely due to SARS-CoV-2 infection of the EpCs of nasal and oral mucosa.[143,192] Experimental studies on transgenic mice demonstrated that SARS-CoV could reach the brain via the olfactory nerves and then spread to specific areas, including the thalamus and brainstem. Because a recent study reported that SARS-CoV is phylogenetically related to SARS-CoV-2, it is reasonable to speculate that SARS-CoV-2 may infect the olfactory pathways in a similar manner,[44] as demonstrated by Everett et al.[192] There seems to be an interesting relationship between hypogonadotropic hypogonadism 15 (HH15) and sulfation. Mutations of the HS6OST1 gene were recently connected with this disorder. Patients with HH15 also have an impaired sense of smell. It is not yet clear how absent or reduced 6-*O*-sulfation of GlcNS influence the course of the disorder,[74] but it seems probable that a lower degree of GAG sulfation in the nasal and oral EpCs could potentially play a role in anosmia and ageusia.

4.6 | Heart disease and vasculature complications in COVID-19

In addition to respiratory disease, cardiovascular complications are a very prominent threat in COVID-19

patients.[136] The importance of an intact EnGL in vascular integrity and cardiovascular homeostasis has progressively been recognized.[14,107] HSPG mediates uptake and clearance of triglyceride-rich lipoproteins[154] and a reduction of the GL thickness was observed following a cholesterol-rich diet.[56,60] These results suggest that the GL plays a pivotal role in atherosclerosis,[19,56,60,68,114] through inhibition of the coagulation cascade, NO production, lipid clearance, activation of leukocytes,[97,115,134,136,194] and anti-oxidant protection.[60] Degradation of the EnGL plays a role in the development of several CVDs,[34,115] with hypoalbuminemia being a powerful prognostic marker.[73] It was found that sdc1 levels, possibly derived from a degraded EnGL, positively correlated with myocardial infarct severity.[78,115] Decreased En HS sulfation and content were associated with increased atherosclerosis in different species,[22,170,194] while a recent study revealed a positive correlation between HPSE expression in human vulnerable plaques and expression of inflammatory markers, such as CCL5, CCL2, and TNFα.[154] Inactivation of Ndst1 in monocytes and macrophages exacerbated atherosclerosis in mice deficient in LDL receptors. These macrophages were in an activated state, resulting in secretion of proinflammatory cytokines and enhanced macrophage infiltration, foam cell conversion, and development of atherosclerotic lesions. Diet-induced obesity was a consequence of the proinflammatory shift.[154] Martin et al. showed that a strong proinflammatory response in cardiomyocytes was induced by circulating HS in the serum of septic shock patients, leading to cardiac mitochondrial dysfunction.[80] It is speculated that HS may exist in the cardiac conduction system (CCS), where it plays a role in CCS maintenance and its plasticity. However, a build-up of HS in the CCS may trigger atrioventricular block.[195]

4.7 | Skin presentations in COVID-19

The sudden onset of a skin rash is a clinical symptom related to COVID-19, especially in patients who also present with fever and cough. While several skin manifestations of COVID-19 can occur,[109,196] urticarial vasculitis is a form of leukocytoclastic vasculitis with deposition of immunocomplexes.[109] Leukocytoclastic vasculitis is a result of inflammation in the small blood vessels of the skin. It is challenging to confirm whether the cause of skin eruptions is true COVID-19 manifestations, or whether it is drug-induced. It is also known that a viral infection can have a synergistic immunologic effect on an adverse drug reaction.[197] Several of the drugs used in COVID-19 treatment protocols are known to cause cutaneous eruptions.[196] Most of these drugs, such as corticosteroids, NSAIDs, acetaminophen, and aspirin, inhibits or are metabolized through sulfation, and may negatively impact GAG sulfation[23,26,29,198] (Figures 1 and 2). The consequence would be increased vascular inflammation and impaired regulation of keratinocyte proliferation, with increased epidermal differentiation.[199,200] The presentation of dermatitis and vascular degeneration of the basal epidermal layer was confirmed in COVID-19 patients.[109] The skin is abundant in GAG polymerization[115] and sdc1 expression has been reported to be high in the skin.[89] Barbosa et al. observed a decrease in the amount of sulfated GAGs with increased skin ulceration[200] and in noninflammatory peeling skin syndrome, type A.[74] Ban et al. reported six patient cases who presented with toxic epidermal necrolysis (TEN) during a viral infection, induced by the use of acetaminophen.[197] The fact that intravenous (IV) NAC has been successfully used to address TEN in several case studies,[201–203] indicates that decreased GAG sulfation plays a probable role in the pathophysiology of TEN, since NAC is a precursor to both inorganic sulfate and glutathione (GSH) (Figure 5).

In a clinical case, a 9-year-old girl presented with primary cutaneous anaplastic large cell lymphoma (ALCL), where the ALCL lesions appeared all over her body after swimming gala events in chlorinated swimming pools. She has a homozygous mutation for the sulfite oxidase (SUOX) gene, which catalyzes the oxidation of sulfite to sulfate (Figure 5). The combination of chlorine, an inhibitor of PAPS synthesis (Figure 1), together with the SUOX gene mutation and consequently reduced inorganic sulfate levels, had a profound effect on GAG sulfation in the skin. The ALCL lesions cleared up with the administration of a methylsulfonylmethane-based supplement and by avoiding swimming in chlorinated water. The onset of the ALCL lesions was triggered by anesthesia received for a small surgical procedure (HN du Preez, unpublished data).

5 | PREDISPOSING FACTORS INCREASING SUSCEPTIBILITY TO COVID-19

Since we postulate that a decrease in inorganic sulfate and consequent undersulfation of the GL[23,170] will result in increased susceptibility to SARS-CoV-2 infection and affect COVID-19 severity, it is important to take into account the various factors contributing to sulfate synthesis, sulfation, and those depleting inorganic sulfate levels.

Cellular events that will potentially influence sulfation of GAGs include translocation of sulfate ions across the plasma membrane by exchange or conductive pathways;

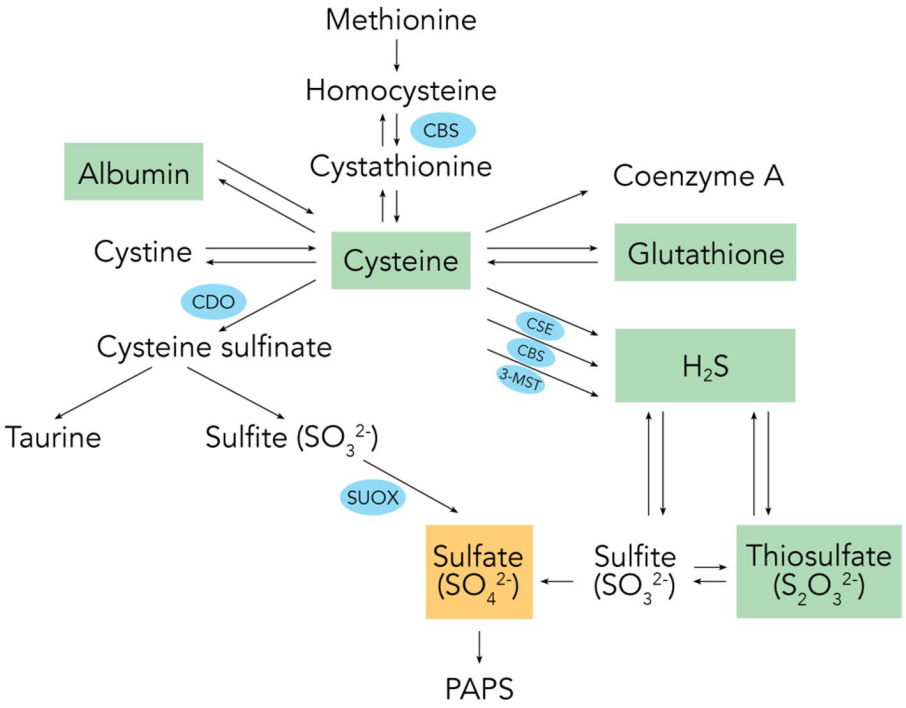

FIGURE 5 Sulfur metabolism diagram. The essential sulfur amino acid methionine converts to cysteine, which is a precursor to albumin, cystine, coenzyme A, glutathione, hydrogen sulfite (H_2S), taurine, and inorganic sulfate (SO_4^{2-}). H_2S can convert to thiosulfate ($S_2O_3^{2-}$) and sulfite (SO_3^{2-}), which is oxidized to sulfate. The enzyme cystathionine-β-synthase (CBS) converts homocysteine to cystathionine, while cysteine dioxygenase (CDO) is responsible for the conversion of cysteine to cysteine sulfinate. Sulfite oxidase (SUOX) oxidizes sulfite to sulfate. H_2S is generated from cysteine with three different pathways through either CBS, cystathionine-γ-lyase (CSE) or 3-mercaptopyruvate sulfotransferase (3-MST)

intracellular generation of sulfate from SAAs (Figure 5); SAA availability; synthesis of PAPS from inorganic sulfate (Figure 1); and transport of PAPS across the Golgi membrane to donate sulfate for the formation of sulfated macromolecules in the Golgi apparatus[21]; as well as the regulation and role of the various SULTs and SULFs in remodeling the GL. The scope of this extensive review mainly focuses on the availability and metabolism of inorganic sulfate and its role in COVID-19.

According to the current evidence, it seems that sulfate metabolism, exclusive of transport, is similar across all species and tissue lines. If, as is most likely, the pool size or turn-over rate of inorganic sulfate is rate limiting for GAG sulfation,[23,28,29,204] it will explain why various drugs, environmental toxins, xenobiotics,[21,24,205] smoking,[27,206,207] and various dietary factors[23,170] that require sulfate for detoxification, can have such a dramatic effect on the integrity of the GL and all the physiological processes involved (Figure 2).

5.1 | Diet and inorganic sulfate

It is clear that multiple and complex interactions can alter GAG sulfation. The generalized nature of these interactions in sulfate metabolism is demonstrated by the finding of undersulfated HS in the brain of patients with kwashiorkor, which was recovered with nutritional rehabilitation.[170] It was also found that fasting or a protein-deficient diet will cause a decrease in albumin synthesis (Figure 5), for as long as the deficiency state is maintained.[163]

The sulfur metabolism pathway summarized in Figure 5 clearly illustrates the conversion of the essential AA methionine (Met) to Cys and the synthesis of inorganic sulfate. Cys serves as the rate-limiting precursor to GSH, hydrogen sulfite (H_2S), albumin, taurine, and inorganic sulfate synthesis. The metabolism and synthesis of inorganic sulfate were extensively covered in the review article titled *NAC and other sulfur-donors as adjunct therapy in COVID-19* (In preparation for publication). Both Pecora et al. and Amadi demonstrated that AA catabolism in vivo contributes to the intracellular sulfate pool when extracellular sulfate availability is low. A mutation in the diastrophic dysplasia sulfate transporter resulted in undersulfation of GAGs as a consequence of reduced extracellular sulfate uptake, which was reversed when hypodermic NAC was administered.[28,170] Therefore, the contribution of thiol compounds and supplements, such as NAC, HSA, and

methylsulfonylmethane, to GAG sulfation becomes significant by increasing SAA plasma concentration.[23,170] Reduction of SAA or sulfur intake may be the cause of undersulfated GAGs observed in some tumor and transformed cell lines.[184] Fuller and Garlicky demonstrated in a detailed review that SAA requirements appear underestimated for both men and women.[208] It seems probable that GAG sulfation may be compromised during marginal intakes of SAAs, and that a preference will then likely be given to the synthesis of proteins and essential metabolic intermediates such as S-adenosyl-L-methionine, coenzyme A, and GSH in the brain and other fundamental organs. It is well-established that the signaling of key enzymes in the sulfur metabolic pathway responds to variations in SAA intake.[23] Low intracellular levels of Met will favor demethylation over trans-sulfuration, thereby conserving Met. While during increased Met intake, trans-sulfuration will dominate to provide Cys as a precursor to GSH and inorganic sulfate. Therefore, to maintain homeostatic balance, protein synthesis will be maintained under conditions of low SAA intake, while synthesis of inorganic sulfate might be inhibited.[23] More research studies are required to establish this important association.

Since the SAAs Cys and Met are not stored in the body, any dietary excess is stored in the form of GSH in the liver. Only once this goal is met, the excess SAAs will be oxidized to inorganic sulfate, excreted in the urine, or reabsorbed depending on dietary levels.[23] Low levels of GSH will, therefore, reflect low levels of inorganic sulfate.[29] A very low intake of SAAs would result in extensive catabolism of tissue proteins to ensure adequate sulfur-supply to maintain physiological function (Figure 2).[29] Any changes in the availability of GSH, and hence inorganic sulfate, are likely to negatively impact the function of the immune system, coagulation, and antioxidant defense mechanisms. This will be mediated via transcription factor activation with consequent up-regulation of proinflammatory cytokines, induced in turn by agents such as mitogens, bacteria, viruses, hydrogen peroxide, and UV or ionizing radiation.[23] It has been demonstrated that patients with moderate to severe COVID-19 illness, the elderly and men, had lower plasma levels of reduced GSH,[23,206,207] higher ROS levels, and thus greater redox status (ROS/GSH ratio),[209] compared to those patients with mild disease.[206] Some of the dietary factors that may contribute to endogenous GSH deficiency would in particular be an insufficient consumption of SAAs[23,170] and fresh vegetables, especially cruciferous, as natural sources of thiol groups.[113,209,210] These dietary influences seem to be an important, though not yet established, risk factor responsible for GSH deficiency in patients with severe COVID-19 illness.[206] The elderly are generally very deficient in SAAs. Ideal protein intake should be around 75–85 g/day and preferably animal protein, which will supply approximately 3.5 to 4.0 g of SAAs per day.[23] Unfortunately, these levels of protein intake are infrequently met by older people and a large segment of the world population, due to cost and availability, changes in taste sensation with preference to fat and carbohydrates, alterations in dentition, social isolation, depression, as well as the fear of consuming too much cholesterol from animal protein. Furthermore, digestion and stomach acidity will play a significant role in the availability of SAAs and inorganic sulfate. Paradoxically, sulfated gastrin stimulates acid secretion from gastric fistulas and pepsin output and sulfation of cholecystokinin stimulate pancreatic responses.[211] A low intake of SAA will, therefore, affect digestion and the converse is also true.

Various dietary factors play a huge role in GL integrity (Figure 2). A diet high in fat, cholesterol,[22,60] and sodium,[115] apart from low protein intake,[23,170] will cause disruption of the GL, while antioxidants will restore or improve measures of En function.[167,209] The addition of caffeine to analgesic drugs was found to exacerbate kidney injury,[212] which could be ascribed to the fact that caffeine requires acetylation for its metabolism[213]; therefore, depleting Cys levels[214] with consequent adverse effects on inorganic sulfate levels and glomerular GL function.[215] Chronic alcohol intake, excessive glucose or carbohydrate consumption, and many food processing chemicals will affect GL function and a reduction in sulfated GAGs.[19,22] A large segment of the population would appear to be sulfur deficient.[23] Various co-factor vitamins and minerals play an important role in the sulfur metabolism pathway that would be obtainable from an organic, balanced wholefood diet.[209] Moreover, diet plays an important role in shaping the composition of beneficial gut microbiota, with evidence that Cys also modulates their abundance and function.[41,216,217]

5.2 | Age- and sex-related considerations

Age is a well-recognized risk factor for SARS-CoV-2 infection that results in severe illness, complications, and death.[206,207] Accumulating research relates dysfunction of the GL to aging.[218] Individuals 60 years and older demonstrate decreased redox potential with lower GSH levels.[39,206,219] Lowered redox status causes alterations in the TNFα receptor activity toward a proinflammatory state.[39] Endogenous H_2S also seems to be playing an important role in COVID-19[220] by supporting basal, physiological cellular bioenergetic functions, where the activity of this metabolic support declines with physiological aging.[221] Multiple innate immunity anomalies have been reported in the elderly,[40] which can be linked to low redox

status and reduced inorganic sulfate levels. The severity of ARDS has been correlated with increased proinflammatory status in older patients.[116] Even though ACE2 expression decreases with age, no difference in ACE, ACE2, and ACE2:ACE activity was found among neonates, children, adults, and elderly patients with ARDS.[40] The differences in infectivity and severity of COVID-19 can be better described through the health state of the GL and degree of GAG sulfation. Kim et al. found that HS decreased in the human aorta with increased age and cholesterol content in the aorta.[115] Another study described the thinning of the GL of mesenteric afferent lymphatic collecting venules in aged rats.[75] The GL layer is very fragile and prone to pathophysiological damage and aging.[4] Furthermore, the expression of several HS-synthesizing enzymes is altered during inflammation, and cartilage expression of HS SULF-1 and SULF-2 is increased with aging.[222]

Apart from a lower intake of SAAs in the elderly,[23] they generally present with low stomach acid levels,[223] which will further impede digestion and availability of inorganic sulfate.[224] Even though the liver will attempt to maintain HSA concentration, a 10%—15% reduction of HSA levels has been observed after the age of 50. Apart from hypoalbuminemia, the unique age-related profile is one of the key factors in COVID-19 infection, with an almost doubling in mortality every 10 years after the age of 50.[4]

Gut microbiota diversity is decreased in old age[41] and the likelihood of gut dysbiosis and other systemic infections can, therefore, be expected in older people. Released microbial biotoxins are high in phenolic compounds, which conjugate with inorganic sulfate for its metabolism.[26,27] Furthermore, during acute viral infection, the compromised immune system allows for the re-emergence of pre-existing, latent pathogens, which will further burden immune responses.[147] Seen that most of the children with COVID-19 showed normal lymphocyte count, their immune system response seems to be sufficient.[40] Pediatric COVID-19 patients presented with relatively milder symptoms in general compared to older patients[42,116] and a much lower infectivity rate. In children diagnosed with COVID-19, a U-shaped curve of severity was observed, where babies younger than one year are at a higher risk of developing severe COVID-19, albeit these infections are infrequent.[42] Even though young children are less likely to have risk factors such as comorbidities, smoking, and obesity,[40] according to USA COVID-19 data, pediatric deaths were associated with underlying chronic comorbidities,[40,42] while genetic variation will also play a pronounced role.[42]

Sulfation predominates over glucuronidation in children, resulting in decreased formation of toxic intermediates and an enhanced ability to inactivate toxic metabolites of drugs, such as acetaminophen. Infants also have a greater capacity to synthesize glutathione. The switch to the adult pattern where glucuronidation dominates occurs at approximately 12 years of age.[205]

In line with the fact that men are significantly more likely to suffer severe effects of COVID-19 infection and experience a higher mortality rate than women,[206] is the fact that the men generally have lower plasma levels of reduced GSH,[207] making them more susceptible to oxidative stress and inflammation.[206] Smoking is also considered a risk factor for severe complications and death from COVID-19,[45,207] and men are more likely to smoke and drink alcohol.[116,136,206] Furthermore, men are more inclined to do high-intensity or endurance exercise, with increased EnGL shedding because of ROS production,[167,219,225] mainly by the skeletal muscles, heart, and liver, but also the lungs, white blood cells, and vessel walls. It should be emphasized that the GL is one of the most oxidation-sensitive components of the internal layer of the vasculature system. ROS has been shown to increase the content of HPSE, therefore, degrading HS.[82] Longitudinal observational studies of COVID-19 patients with acute or chronic heart failure also suggest a correlation to serum-soluble sdc1. Interestingly, the association between sdc1 and mortality was significant in women and marginal in men.[115] Gender may also affect ACE2 expression. ACE2 gene is located on the X-chromosome and circulating ACE2 levels were found to be higher in men than in women.[116] Brzica et al. discussed gender differences in the expression of PAPS and various STs in mice, even though, it seems that environmental and genetic factors in humans may have a much greater impact on these enzymes.[226] It has been reported that GlcNH2 structures are expressed as the predominant disaccharides in male mouse liver in a gender-specific manner.[85]

5.3 | Smoking

Smokers are generally more vulnerable to infectious diseases. An analysis of the literature suggests that smoking is one of the predisposing factors associated with the negative progression and adverse outcomes of COVID-19.[45,206,207] Aldehydes in cigarette smoke deplete the total available intracellular GSH pool by reacting with GSH to form non-reducible GSH-aldehyde derivatives. Cigarette smoke-induced GSH depletion results in increased ROS levels,[206] with consequent inflammation and airspace enlargement, while the GSH adaptive response declines with age.[207] It is well-established that cigarette smoke induces neutrophilic inflammation and injury in the airways of smokers.[227] It has been shown that NAC is able to inhibit, and to some extent reverse, cigarette smoke-induced lung alterations in a rat model.[228] Seen that NAC is not only a

precursor to GSH, but also inorganic sulfate, one can assume that sulfation would be compromised in the lungs of smokers.[89,227] Indeed, smoking influences the expression of many sulfur metabolism enzymes, and a diminished HSPG layer has been observed in the lung tissue of emphysema patients,[89,229] while upregulation of ACE2 receptors has also been detected in the airways of smokers.[45,138] Many of these cigarette smoke toxicants have been shown to contribute to increased Ep permeability,[135] En and vascular dysfunction, inflammation, carcinogenesis, cytotoxicity, and disruption of endocrine homeostasis.[27,30]

5.4 | Drugs and toxins

Sulfation mediated through SULTs are involved in the homeostasis of key endogenous compounds, as well as the detoxification of a wide range of exogenous toxins and xenobiotics, including drugs.[24,205] Polarity and water solubility of the compounds are increased through SULT-mediated transfer of a sulfuryl group from PAPS to an acceptor molecule[27] (Figure 1), to facilitate excretion of the sulfated product via urine and/or bile.[230,231] Acetaminophen is one of the most familiar drugs to be hepatically detoxified through glucuronidation and sulfation.[23,203] It requires large amounts of sulfate for its excretion, where 35% is excreted conjugated with sulfate[84] and 3% with Cys, while the rest is conjugated with glucuronic acid, which also happens to be a major component of GAGs.[23] Endogenous compounds requiring sulfation to be metabolized include steroids, such as estrogen, vitamin D (VD), and thyroid hormones, catecholamines, cholesterol, bile salts, cresols, and phenols.[25–27] Cohen et al. identified a significant effect of the use of acetaminophen, age, sex, and various genetic variants on multiple sulfated metabolites of pregnenolone, androstenediol, and DHEA. Each of these factors made compelling, independent contributions to the sulfated metabolite levels. They indicated that though pregnen-diol disulfate levels decreased with age, the effect of acetaminophen intake on this metabolite was comparable to the effect of around 35 years of aging. They have found that individuals who regularly consumed acetaminophen had very low levels of neurosteroids, such as sulfated-DHEA and pregnenolone sulfate.[26] Similarly, Yasuda et al. indicated that the mechanism underlying the inhibition of 17β-estradiol sulfation by toxicants from cigarette smoke is of a mixed competitive and noncompetitive nature. Being high in phenolic compounds, cigarette smoke toxicants serve as substrates for SULTs, and may, therefore, interfere with the metabolism of 17β-estradiol and other endogenous compounds that require sulfation,[27] such as VD. Similarly, many drugs, biotoxins, xenobiotic compounds,[27] and environmental toxins (e.g., glyphosate,[232–235] PCBs,[230] cyanide,[236] and ethylene glycol[237–239]) that either inhibit sulfation or are metabolized through sulfation, will result in impaired sulfation of the GL and other endogenous metabolites[29] (Figure 2). The effect will be more pronounced with cumulative use and if a combination of drugs is taken.[212,240,241] Interestingly, the Anti-Doping Agency establish differences in the sulfation and sialylation of the *N*-glycans expressed on endogenous against exogenous erythropoietin, to detect illicit drug administration.[100]

During the SARS epidemic in 2003, it was noted that plasma viral titer was enhanced through early treatment with corticosteroids, leading to exacerbated disease.[45,142] Both steroid drugs[23] and NSAIDs require sulfation for their metabolism.[205,242] Though many research studies have been performed to look at the influence of acetaminophen use on sulfation, more research is needed to evaluate the influence of corticosteroid use on specifically GAG sulfation and its effect on infectious disease. Greaves reported reduced content of sulfomucins in the gastric mucosa of laboratory animals after administration of aspirin and other anti-inflammatory agents, such as NSAIDs and corticosteroids.[183] The reduction in GAG sulfation could most likely be the main cause of gastrointestinal bleeding and side effects seen with the administration of NSAIDs, acetaminophen, steroids, and aspirin.[240] Several researchers found that salicylic acid (aspirin) suppressed GAG sulfation, inhibited phenol STs of the human colon mucosa and platelets, and caused salicylate-induced diuresis with a twofold increased excretion of inorganic sulfate, shortly after drug treatment.[198,243,244]

Apart from low SAA intake,[23] elderly COVID-19 patients with comorbid conditions are more likely to be on a variety of chronic medication that requires sulfation to be metabolized, increasing the likelihood that an undersulfated GL may play a role in their etiology.[29,240] Moreover, due to widespread antibiotic use, disruption of gut microbiota has been correlated with an increased risk of lung cancer in humans.[41] It appears that there might be a correlation between an increase in biotoxin phenolic compounds that require sulfate for its metabolism, and lung GAG undersulfation, which requires further investigation.

It was found that the expression of ACE2 receptors was markedly increased in patients with a history of taking angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II type 1 receptor blockers to treat hypertension and DM.[39,203] Furthermore, thiazolidinediones used by diabetics and ibuprofen can also increase ACE2 receptor expression.[203] Furthermore, ACEIs cause an accumulation of bradykinin in the lung,[245] and upon inflammation due to viral infection, bradykinins are further rapidly generated and induce IL-1ß gene expression,[246] a proinflammatory cytokine. ACEIs and other

drugs can, therefore, potentially contribute to viral infection and exacerbate the cytokine storm seen in severe COVID-19. Of note, endothelial HSPGs regulate activation of bradykinin pathways, while degradation of HS by microbial HPSEs promotes proteolytic bradykinin generation. Consequently, increased plasma HPSE activity in COVID-19 patients could aggravate vascular leakage and local inflammation through activation of the bradykinin pathway.[58]

Fontana et al. described two COVID-19 case studies where the male patients, 50 and 71 years old, received high dose IV vitamin C (above 100 g over 96 h). While patient one developed multi-organ failure with hepatic dysfunction and AKI with anuria, the second patient developed progressive kidney dysfunction and needed kidney replacement treatment from day 8. Even after the resolution of COVID-19 lung symptoms, they still failed to recover kidney function after several weeks. A kidney biopsy indicated in both cases that oxalate nephropathy was the probable cause. It is known that vitamin C causes hyperoxaluria through endogenous conversion of ascorbic acid to oxalate.[169] Both vitamin C[247] and oxalates[239,248] require sulfate for its metabolism. These two patients also received IV steroids and broad-spectrum antibiotic therapy, while being placed on enteral nutrition[169] low in Cys.[249,250] All these factors would have had an effect on HS sulfation, which will not only aggravate AKI, but exacerbate severe COVID-19 illness. Conversely, AKI will negatively affect inorganic sulfate homeostasis, which is largely regulated in the kidneys.[226,251] Furthermore, both the sodium-sulfate cotransporter (NaS1) and sulfate-anion transporter-1 (Sat1) are enzymes involved in the transcellular transport of sulfate,[252] while Sat1 also plays an important role in oxalate homeostasis.[253] Gender differences in Sat1 expression were observed, where male rats exhibited higher renal expression of Sat1, compared to female rats.[238] In rats treated with ethylene glycol, a precursor to oxalates, an elevation of Sat1 expression was also observed.[237,238] In the kidney, an upregulated Sat1 or downregulated NaS1 expression, enzymes that are both involved in the reabsorption of inorganic sulfate,[226,253] may result in oxaluria,[237] urolithiasis or renal failure, disrupted sulfate homeostasis, and impaired drug detoxification.[84,239,254] Interestingly, it was found that corticosteroids, thyroid hormone, and VD can modulate serum sulfate levels, renal sulfate handling, and NaS1 expression.[252] The expression of NaS1 is downregulated by corticosteroids and NSAIDs, VD deficiency, hypothyroidism, chronic hypokalaemia, and metabolic acidosis, which will result in lower inorganic sulfate serum levels.[251,253] To compensate for sulfur losses, the adjunct use of sulfur-containing compounds with medications such as acetaminophen, NSAIDs, aspirin, and catabolic agents such as corticosteroids, as well as high-dose vitamin C, should be considered.

It has been hypothesized that mercury toxicity causes susceptibility to COVID-19 since both conditions lead to hypercoagulability, high levels of proinflammatory cytokines, and lymphopenia.[255] Moreover, it should be noted that mercury also has a high binding affinity for sulfur,[256–258] and it is very likely that high mercury levels will result in reduced levels of inorganic sulfate for GAG sulfation. The major source of mercury toxicity in humans is dental amalgam fillings.[259]

5.5 | Demographic and ethnic factors

Surprisingly, and contrary to expectations, the COVID-19 death rate per 1 million population[260] is the lowest in poorer underdeveloped countries. The use of chronic medication and drugs that require inorganic sulfate for its metabolism, are generally much higher in developed countries[261,262] and could be one of the reasons explaining the demographic occurrence of COVID-19. The use of agents with analgesic action, including opioids, acetaminophen, and NSAIDs, are among the most commonly used drugs for self-medication in developed countries, apart from drugs for hypertension.[212,263,264] The differences in COVID-19 treatment protocols adopted by the various countries could also account for demographic variations in mortality rates. Furthermore, cyanide, present mainly as hydrogen cyanide in air pollution, will impede sulfide oxidation in lung EpCs.[221,265] Wu et al. demonstrated that higher exposure to air pollution is positively associated with higher COVID-19 mortality rates.[266]

It seems that COVID-19 is more prevalent in African Americans, Hispanics, and Latinos[42,109,267] and the potential reported reasons were chronic diseases, occupations, housing, and life-time risk exposures,[268] apart from dietary factors and socio-economic reasons. Moreover, Zhan et al. found that the association between NSAID use and adverse kidney disease events was most prominent in black people,[212] indicating that an ethnic genetic component could likely play a role in sulfation[212] and drug metabolism. A reduced GL thickness was observed among whites of European descent and blacks of African descent, compared to Asians and Arabs.[138]

5.6 | Vitamin D (VD) deficiency

VD seems to be involved in many biological events, with an accumulating body of evidence indicating that VD deficiency is associated with DM,[269] cardiovascular,[267] and

kidney diseases.[242] VD deficiency has also been linked with severe illness and death in COVID-19 patients.[206,270] Even though Butler-Laporte et al. did not observe an association between VD levels and COVID-19 susceptibility, severity, or hospitalization through a Mendelian randomization study,[271] several studies reported that GSH levels positively correlate with active VD.[206] It has also been found that lower levels of Cys and GSH correlated with lower VD binding protein and VD levels in diabetic patients. Interestingly, it was shown that GSH deficiency and the consequent increase in oxidative stress epigenetically alter VD regulatory genes resulting in suppressed gene expression and decreased VD biosynthesis, ultimately giving rise to secondary VD deficiency. This can be reversed by the replenishment of GSH through Cys supplementation.[206,269] Cys is a rate-limiting precursor to both GSH and inorganic sulfate. Though it seems probable that sulfation plays an important role in the homeostasis of VD, the mechanism underlying the formation of sulfated VD-related compounds is not well-established. Kurogi et al. demonstrated the sulfating activities toward VD-related compounds, including 7-dehydrocholesterol, VD, 25-OH-VD, and calcitriol.[242] Since circulating concentrations of 25-OH-VD-3-*O*-sulfate is not rapidly secreted by the kidney, this sulfate metabolite may serve as a reservoir of 25-OH-VD in vivo, contributing indirectly to the biological effects of VD.[25,242] In human plasma samples, the 3-*O*-sulfated form of 25-OH-VD has been detected at similar or greater levels (ranging between 10 and 50 nM) than unconjugated 25-OH-VD.[242] It is, therefore, plausible to relate low VD status in COVID-19 patients to low inorganic sulfate levels, which should be established through further research. The fact that SARS-CoV-2 positivity is strongly and inversely associated with circulating 25-OH-VD levels, a relationship that persists across latitudes, races and ethnicities, sexes, and age ranges,[268] means that low inorganic sulfate levels could be the common denominator increasing susceptibility to SARS-CoV-2 infection and affect the severity of COVID-19. Seen that VD deficiency has been implicated in the development of insulin resistance and T2D, which was restored with the administration of NAC, it could suggest that low levels of inorganic sulfate are underlying both VD deficiency and insulin resistance due to undersulfated HS.[269] Interestingly, steroid SULFs are regulated by hypoxic conditions and inflammatory mediators, such as TNFα. Once intracellular, steroid conjugates can therefore be desulfated under hypoxic conditions,[25] which could possibly influence the levels of circulating 25-OH-VD-3-*O*-sulfate in severely ill COVID-19 patients. Boron deficiency has also been correlated with low serum levels of VD.[272]

Moreover, it has been suggested that VD modulates sulfate homeostasis by regulating NaS1 expression, where low VD levels will downregulate NaS1 expression, whereas VD supplementation will result in an upregulated expression of NaS1 mRNA.[253] Besides, Bolt et al. showed that NaS1 expression in the kidney was reduced by 72% in mice lacking the VD receptor, while intestinal NaS1 levels remained unchanged. Correspondingly, in VD receptor knockout mice urinary sulfate excretion was increased by 42%, while serum sulfate concentration was reduced by 50%.[239] Therefore, hepatic GSH levels and skeletal sulfated PGs were also reduced by 18% and 45%, respectively, in the mutant mice.[252] It is evident that there exists an important interrelationship between VD homeostasis and inorganic sulfate.

5.7 | Genetic variability

Evidence of the importance of sulfation in human development and health is mostly based on genetic disorders with mutations in genes involved in sulfur metabolism.[28,46,89] Many of these mutations cause developmental abnormalities and result in various diseases. The genetic basis for undersulfation of the GL is very complex and would involve multigene interactions. Approximately 2% of the human genome encodes enzymes involved in glycan biosynthesis.[74] Glycosylation, sulfation, PG synthesis, SULTs; availability of inorganic sulfate, regulated by methylation, trans-sulfuration, and sulfation pathways will all influence GL integrity. More than 25 enzymes are responsible for HS biosynthesis alone,[18] and their expression is highly regulated.[22,74] Many dozens of different enzymes have been found to be responsible for the synthesis and editing of GAG chains, including deacetylases, *N*- and *O*-STs, epimerases, SULFs, kinases, or hydrolases, besides enzymes involved in the synthesis and transport of precursors.[140] Enzymes that will specifically affect sulfation are PAPS synthase, the bifunctional enzyme with both ATP sulfurylase and APS kinase activity, deacetylases, and endosulfatases.[25,63,89,273] HS biosynthesis enzymes Ext1 and Ext2 affect Ndst1 expression and thus HS sulfation.[22,49,274] The methylation status of genes encoding the SULTs will also have an effect on sulfation.[24] Aberrant expression of 3-*O*-STs as a result of DNA hypermethylation is emerging as a common theme in cancer biology,[83,88] while methylation of the HPSE promoter is a potential regulator of HPSE expression.[79] It is evident that genetic variability and epigenetic factors influencing GAG synthesis and sulfation would underlie the diverse symptoms seen in COVID 19.[27,45]

5.8 | Ventilators

High-tidal volume VT can negatively impact GL thickness and composition, which could subsequently

affect pathogen adhesion and invasion in the context of ventilator-associated pneumonia.[13] During high-tidal volume VT, lung over-distension on the opposite side of the air–blood barrier, enhances the permeability of the Ep barrier. With increased ventilatory pressure, there is a linear increase in the passage of protein from the lung space into the blood,[94,135] with more severe edema formation as a consequence.[81] It has long been known that ventilation with high-tidal volume can cause lung injury.[275] In addition to increased Ep barrier permeability, the mechanical stretch of alveolar EpCs results in the release of inflammatory cytokines and production of reactive oxygen/nitrogen species, superoxide, and NO, which can induce alveolar Ep cell death. Furthermore, during high-tidal volume ventilation, MMP activation is induced, resulting in the shedding of HSPGs.[276] It is, therefore, important to reduce the intensity of mechanical stretch on the lung epithelium by decreasing tidal volume, as a vital protective strategy of VT for patients with acute lung injury, ARDS and COVID-19.[81,94] Of note, low VT may not be the best approach for all patients with ARDS, since hypercapnia was common in patients with COVID-19–associated ARDS while using low tidal volume VT, which resulted in increased pulmonary dead space. An intermediate VT was used to correct hypercapnia effectively in these patients, while not excessively increasing driving pressure.[275,277,278]

6 | THERAPEUTIC APPROACHES FOR PROTECTING THE GLYCOCALYX

In mouse models, full restitution of the microvascular GL after damage by acute enzymatic or cytokine-mediated degradation, requires 5 to 7 days.[60,68] Since the dynamics for the restoration of a degraded EnGL in humans are not clear, it would seem apparent that prevention of damage in the first place should be preferred over cure.[68] Restoration of the GL should limit inflammatory responses in the vascular system. Any approach aimed at improving the GL structure and function would have good therapeutic potential to prevent the pathological processes connected with vascular inflammation,[60] coagulation, and sepsis. There is obviously significant reason for the repurposing of existing and approved medications for the treatment of COVID-19.[96,279]

6.1 | Conventional considerations

6.1.1 | Fluid resuscitation and ventilation

Fluid management has an important influence on the integrity of the GL, with hypervolemia being injurious to the GL, by accelerating EnGL shedding.[77,80,81] Administration of albumin is effective for volume repletion.[73] Hypernatremia also damages the GL, removing the barrier function that facilitates sodium and viral entry into EnCs.[77] Avoid high-tidal volume VT.

6.1.2 | Corticosteroids

The protective effect of corticosteroids in sepsis, ARDS, and COVID-19 remains controversial.[77,80,141] It is believed to provide protection against injury and inflammation in general and prevent shedding of the GL.[68,80] Hydrocortisone has both a direct effect on EnCs, and an inhibitory effect on immune effector cells.[68] It plays an important role in the stabilization of mast cells, which should prevent degranulation and, therefore, proteolytic damage to the GL and the consequent potentiation of inflammation.[60,68] However, these positive findings seem to be limited to a predefined risk group of patients.[80] Controversy still remains regarding the use of high-dose corticosteroids in viremia[80,142,146,280] and it has been reported that the use of steroids had adverse effects on GL integrity and En permeability.[131]

Nevertheless, some reports indicated that better outcomes with COVID-19 were achieved when dexamethasone was used compared to corticosteroids.[42,105,142] Dexamethasone is 25 to 30 times more potent than an equal weight of hydrocortisone. Dexamethasone is also metabolized through sulfation and glucuronidation, but since less drug is used compared to other corticoids, it should have less of an effect on depleting inorganic sulfate compared to corticosteroids. Still, we propose (for appropriate clinical tests) the use of steroid anti-inflammatory drugs in combination with sulfur-donors, such as IV NAC or sodium thiosulfate (STS) (Figure 5), which should result in more favorable outcomes in the treatment of severe COVID-19 illness.

6.1.3 | Plasma protein

Some plasma proteins may protect the GL, such as albumin[68,73,77,78,115] and fresh frozen plasma.[54,55,107] Albumin supplementation significantly attenuated pronounced shedding of the GL,[14,131] and consequently interstitial edema.[55,170] Moreover, albumin has immunomodulatory and anti-inflammatory,[55,60] antioxidant, anticoagulant, and antiplatelet-aggregational properties.[73] Albumin also serves in the binding and transport of a wide range of endogenous and exogenous ligands, such as fatty acids, metal ions, steroids, AAs, bilirubin, hormones, vitamins, and several drugs.[73,163]

6.1.4 | Heparin

Unfractionated HP (UFH), low molecular weight HP (LMWH), and heparinoids are commonly used in many research studies, and proposed as a treatment for COVID-19[6,43,58,105] because of their anticoagulant, antithrombotic,[128] anti-inflammatory,[42,58,273] and anti-viral properties.[42,50] In research studies, HP is often used as a proxy for HS because of its structural similarity, however, HP is considerably more highly charged than HS.[59] The degree of *N*-sulfation is 50% in HS while it is over 70% in HP.[273] The pleiotropic nature of HP can be ascribed to its overall high level of sulfation,[7,80,89] which may mask selectivity. It was observed that in most cases little specificity exists between HP– or HS–protein interactions experimentally, where overall charge density is the predominant factor in determining binding.[63] HS is fundamentally different from HP in many ways.[18,74] The experimental use of HP may, therefore, represent a naïve approach. Formation of the GL is a highly orchestrated and complex process, which involves the chronological expression of numerous growth factors and complex signaling networks.[63] Even though HP may enhance the activity and retention of certain immune-modulatory molecules and growth factors under particular conditions, its binding "promiscuity" may lead to the inhibition of other factors, which may play an important role in tissue maintenance and repair, for example.[63] Furthermore, it is known that HPs interact with HS and cause shedding of HS from the GL competitively.[60,77] Many adverse clinical effects were reported from long-term application of HP as an anticoagulant, such as thrombocytopenia, bleeding,[46] vascular reactions, and osteoporosis.[63] The clinical use of LMWH and ultra-low molecular weight HP may also result in the risk of bleeding due to overdosing.[85] Caution in the widespread use of UFH and its derivatives, both in the clinic and research settings, should be taken.[18,50,60,63,77,80,113,281] Recently, Liu et al. pointed out that thrombocytopenia induced by the use of HP represents a high mortality risk in critically ill COVID-19 patients.[8,119]

LMWHs serve as an inhibitor of HPSE.[46,80] The prophylactic use of LMWH in non-ICU patients was associated with reduced HPSE activity.[58] Since the activation of HPSE can increase the level of MMP expression, LMWH may, therefore, attenuate the increase of MMP expression levels through inhibition of HPSE activity.[54] It was reported by various in vitro studies that SARS-CoV-2 host cell entry was inhibited by the use of soluble UFH and its derivatives.[6,58,128] HP may act as a decoy receptor, diverting SARS-CoV-2 to bind to HP, rather than to cell surface HS on host cells.[42] HP has 3-*O*-sulfation, responsible for its anticoagulant activity,[6] and possibly affinity for SARS-CoV-2. LMWH has also been recommended for the management of coagulopathy in COVID-19.[7,42] LMWH has a decreased risk of bleeding, good predictability, dose-dependent plasma levels, and longer half-life, and is, therefore, preferred over UFH.[105] However, restoring sulfation of HS through the introduction of sulfur-donors, should be a better approach to treat COVID-19, than introducing pharmaceutical HP and its derivatives.

6.1.5 | Glycocalyx components

The potential efficacy of the administration of exogenous GAGs in restoring the GL has been reported.[14,68,74,77,78,89,115] It is not clear if these benefits resulted from the incorporation of the various compounds into the EnGL, or an indirect improvement of its consistency.[68] It has been suggested that some of the physiological effects may be due to exogenous HS limiting the ability of various ligands to bind to endogenous HSPGs, therefore, preventing or regulating their binding to receptors.[63] Oral sulodexide treatment, which consists of mammalian derived HS (80%) and dermatan sulfate (20%), resulted in beneficial changes in EnGL thickness and metabolism of GL constituents,[32,60,77] and was shown to improve vascular barrier function by reducing microalbuminuria.[97,115] Low molecular weight carrageenan, plus its acetylated and sulfated derivatives, reduced human immunodeficiency virus and influenza viral activity by depolymerization and sulfation processes in an in vivo mouse-model.[117]

6.1.6 | Protease inhibitors

An opportunity exists for the therapeutic use of protease inhibitors, since proteases, such as thrombin, have been reported to support the cleavage of sdc1 ectodomains.[68] Doxycycline is one of the tested protease inhibitors[13,60,68] with a strong preclinical rationale and an established safety profile, making it an attractive candidate for a repurposed drug in the treatment of COVID-19.[279] Researchers have demonstrated in various studies that doxycycline inhibited MMP activity and subsequently significantly reduced shedding of the GL, as well as reduced leukocyte adhesion to EnCs, in response to inflammatory and ischemic stimuli.[14,18,60,132] A cross-talk between HPSE and MMP expression also exist.[282] Another candidate as a protease inhibitor is AT III. Apart from its anti-inflammatory properties, it inhibits numerous serine proteases naturally present in the GL,[14,60,68] thereby preventing GL degradation.[131] In silico, molecular docking studies demonstrated that famotidine bind papain-like protease and 3 chymotrypsin-like proteases of SARS-CoV-2.[148] However, famotidine can inhibit the synthesis of both total and sulfated GAGs, along

with histochemical evidence of a reduction in PAS staining of the GL,[183] since famotidine also requires sulfation to be metabolized and might, therefore, negatively affect inorganic sulfate levels.

6.1.7 | TNFα signaling inhibition

TNFα plays a huge role in the development of acute and chronic inflammation. Etanercept, an analog of the TNFα receptor, which is clinically used as an inhibitor of TNFα signaling, significantly reduced the shedding of GL components, coagulation activation, and functional vessel function disturbances during experimental human studies.[60] Although Etanercept has been proposed for use in COVID-19 to reduce mortality of TEN,[283] Atef et al. proposed the use of 600mg IV NAC every 8 h in surgical patients and ICU patients with TEN.[203]

6.2 | Sulfur compounds/thiol donors

Sulfur, the biogenic element, as well as sulfur-containing compounds and SAAs, are widely available, both as synthetic biologically active substances and in natural products.[134] Nikitina et al. describe successful treatment and prevention of thrombophilia and stabilization of blood platelets with sulfur-containing terpene compounds (sulfoxide).[134] The sulfur-rich drug Suramin[284] was found to inhibit SARS-CoV-2 entry into the cell and interfere with the earlier steps of the viral replication process.[285,286]

Cys and NAC, cystine, Met, GSH, H_2S, albumin, and STS can all serve as precursors to inorganic sulfate, to ensure optimal GAG sulfation[23,28,29] (Figure 5). We should rethink standard practices of care, such as the use of acetaminophen, aspirin, and steroids, which either deplete or require sulfate for its metabolism,[26,27,100,212] and enteral feeding low in Cys,[206,249,250] and if used, to consider IV NAC, STS, or other sulfur donors as an adjunct therapy. It was found that after infection the GL is able to reglycosylate to restore the Ep barrier, but sulfated GAGs are not as easily restored, leading to more chronic complications and making them critical in many aspects of physiology.[73,170] Their disruption plays a role in the pathophysiology of many disease processes,[170] as is seen in long-COVID.

Sulfated GAGs and sulfur donors or thiol compounds regulate innate[207] and adaptive immunity[148] at various levels and are well documented in the literature. Moreover, they exhibited beneficial effects on multiple metabolic dysfunctions, including hyperglycemia, hyperinsulinemia, insulin resistance, CVD, obesity, hepatic steatosis, and inflammation.[39,157,287] The fact that the various sulfur donor supplements show many health benefits in these disease conditions, is an indication that inorganic sulfate deficiency is very likely an underlying cause. Even acute lung and liver injuries are attenuated by sulfur donors[287] and they have shown activity in a variety of potential therapeutic target pathways involved in the pathophysiology of SARS-CoV-2 infection.[39] What all the sulfur donors have in common, are the ability to supply sulfur for inorganic sulfate synthesis and, therefore, ensuring optimal GAG sulfation. The degree and specific sulfation patterns of GAGs observed in a healthy EnGL attenuate the binding of pathogens, chemokines, and leukocytes to the EnC surface.[105] Initial inflammation in response to infection should, therefore, be regulated timely to maintain immune homeostasis.[179] It is imperative to stabilize the GL while tackling viral replication. We, therefore, propose that for moderate to severe COVID-19, treatment of patients with IV NAC upon hospital admission might have favorable outcomes. However, in the critically ill, IV STS should be considered, since released cytokines may ameliorate the oxidation of NAC to inorganic sulfate.[151,288] These recommendations should be evaluated through clinical studies. The therapeutic application of NAC and other sulfur donors were extensively reviewed in the article titled *NAC and other sulfur-donors as an adjunct therapy in COVID-19* (In preparation for publication). By understanding the pathophysiological role of sulfation, therapies can be better targeted at the restoration of the GL and sulfation, to ensure prevention and improved outcomes in COVID-19 patients.

7 | POTENTIAL OF GLYCOCALYX CONSTITUENT LEVELS AS DIAGNOSTIC TOOLS

Shed GL constituent levels in plasma are strongly correlated with disease severity and mortality in the critically ill.[60,68,82,145] Given the pathophysiological implications of GL degradation, these shed GL constituents can potentially serve as clinically relevant biomarkers. Promising diagnostic markers in plasma and urine from EnGL degradation are increased syndecans, HS, chondroitin sulfate, dermatan sulfate, hyaluronan, HPSE, hyaluronidase, endocan, soluble endoglin, thrombomodulin, and E-selectin, angiopoietin-2 (AngP-2) and nitrite levels[14,32,54,60,77,80,82,109,115,145]; therefore, for better preservation of the GL layer, one would need to see a decrease in these free-flowing GL constituents. Markers such as HS, soluble endoglin, AngP-2, and E-selectin have been associated with hemostasis, thrombo-inflammatory events, and sepsis.[289] AngP-2 can be rapidly released by a degraded GL and participates in the responsiveness of EnCs to inflammatory, hyperpermeability, and

vasoreactive stimuli, but also induces inflammation and vascular hyperpermeability.[80] Circulating AngP-2 plasma levels were increased in COVID-19 patients, as well as sepsis and ARDS, and predictive of mortality.[80,289] They were correlated with higher fluid overload, coagulation and hepatic dysfunction, acute kidney injury, plasma cytokines, and mortality, probably due to increased vascular leakage[55] as a consequence of EnGL damage.[80] It was shown in general that the plasma concentration of GAGs correlates with the increased concentration of various inflammatory markers.[60,80]

Low urinary excretion of sulfate and sulfated GAGs[170] potentially reflect sulfur metabolism abnormalities, while increased urine GL fragment levels, especially HS, are highly prognostic for mortality.[54] A poly-L-lysine probe seems the best to use in studying the distribution of sulfated GAGs, which is specific for sulfate residues in HS GAG chains.[170] Numerous preclinical and clinical studies showed a decrease in thickness of the GL during sepsis[54,77] (Figure 4). Visualization techniques are also used to determine the integrity of the GL, but there are differences between the GL in vitro and ex vivo, compared to in vivo.[60,78,115] The direct assessment of the EnGL was made possible with in vivo video microscopy technology, by measuring the extent of penetration of RBCs into the EnGL.[77] Measuring the thickness of the GL via imaging techniques has been made possible through recent advances in computer technology and improved computational software.[77] The use of flow-mediated vascular dilation makes it possible to identify En dysfunction and vascular permeability, as well as the noninvasive measurement of microvascular leakage with strain gauge plethysmography.[115] In recent advances, the spatio-chemical organization of the EnGL was identified through the application of in vitro super-resolution fluorescence microscopy (STORM), while detailed and comprehensive characterization of the GL in cells and tissues were made possible by liquid chromatography coupled to mass spectrometry.[107]

Furthermore, albumin levels and heat and moisture exchanger/edema fluid may also serve as a biomarker to quantify the extent of Ep and En injury, respectively, in patients with ARDS and COVID-19.[71,73] Myeloperoxidase is a marker widely linked with neutrophil infiltration during inflammation.[287] Hermans & Bernard reported that the increase in levels of surfactant proteins (SP-A and SP-B), as well as the mucin-associated antigen 17-Q2, correlates negatively with blood oxygenation in ARDS.[135] Moreover, changes in the permeability of the air–blood barrier to macromolecules are best reflected by the levels of lung secretory proteins in serum.[135] These measurements can in principle predict both susceptibilities to COVID-19 and be indicative of disease severity, as well as confirming GL degradation.

However, it may be more worth focusing on the underlying etiology of how the degradation of the GL, represented by elevated shed-soluble GL fragments, leads to adverse events or *vice versa*, rather than solely extrapolating levels of shed-soluble fragments with outcome predictability. Various inflammatory mediators promote shedding of the GL components, including ROS, TNFα, HPSE, and MMPs, during sepsis.[13,77,78,80,97] Furthermore, enzymes such as serine proteases (thrombin, elastase, proteinase 3, plasminogen, and cathepsin B) and hyaluronidase, are also involved in the derangement of the GL.[77] The complexity of GAG signaling and the effect of shed GL fragments require further study. It should be considered that it is not the shed GL fragments *per se* that will affect functionality, but rather the degree of sulfation of these GAG fragments. Since the GL is multi-potential and very complex,[33,74] it is clear that there is no one-size-fits-all biomarker.[77]

8 | VARIATIONS IN GLYCOBIOLOGY

Studies of the GL as a cellular structural element have long been neglected. This can in part be due to the fact that visualizing and studying the cell GL is difficult because of artefactual degradation of the GL during tissue fixation, especially in whole-tissue preparations.[71] Many contradictory results are reflected in glycobiology. Kolálová et al. pointed out that the variation reported by various researchers in the thickness and heterogeneity of structures of the GL could be due to differences in the applied techniques and in sample preparation procedures; the use of in vivo, ex vivo, or in vitro systems or models; the heterogeneity of organs and species; and differences in cultivation conditions.[60] Several studies have shown differences between EnGL in vivo compared to in vitro. Cultured cells are known to have limited formation of the GL in comparison with the extended structure found in vivo in vessels[60,78,114] and EpCs.[52,77] Of note, observations regarding GL-mediated signal transduction and other GL functions, such as viral evasion, have come mostly from cultured EnCs and not from ex vivo or in vivo whole organ studies. The latter would validate a physiological role for the GL in a more complex, as well as clinically relevant model.[77,81] The obvious possibilities to explain these differences are the lack of shear stress, pulsative stretch, and physical pressure that influences the expression of the GL[60] and the effect of the surrounding cells.[77] While cell-culturing medium often contains serum, "standard" commercial tissue culture media have unphysiologically high amounts of glucose,[100] resulting in fundamental differences in media composition. The degree and sequence of sulfation of

stressed cells dividing in a hyperglycemic environment will differ from the in vivo reality.

In vivo evaluations were made possible through intravital microscopy, but it does not allow direct visualization of the GL.[77] In addition, electron microscopy results are strongly dependent on the fixation technique.[60,78] For example, a thicker layer of EnGL ex vivo was revealed using rapid freezing of EnCs and a novel fixation solution, such as lanthanum (III)-nitrate/ glutaraldehyde, compared to preparation with a conventional fixation method for microscopic analysis.[115] Even though it is technically difficult to obtain standardized reagents to work with, given the heterogeneous nature of GAGs,[19,60] Hayden and Ando et al. found that utilizing lanthanum (III)-nitrate to be a reliable and reproducible method in detecting the EnGL in most recent experiments[96,165,290] (Figure 4).

In most studies, HP is used as an analog for HS. Although HP and HS are structurally related, there are some differences, as pointed out before. HS has a lower degree of modification, both epimerization and sulfation, which means higher charge density per HP residue.[70] HS polymer chains are more complex and longer than those of HP macromolecules and functional HS always remains connected to its core protein.[74] The particular protein binding capabilities that are required for specific biological functions under different physiological conditions are made possible by the modifications in HS structure. Studies of the effects of changes in GAG composition and variation in the complex chemical environment in which the GAGs are present, such as ionic strength, pH, and types of associated cations, are only rarely performed.[18] Physicochemical techniques, such as research into the polyelectrolyte nature of the GAGs and the involvement of cation binding to HP and HS chains, only rarely investigate biological activity.[18] Vanpouille et al. confirmed that the binding of particular HP-binding proteins can be dramatically affected through immobilization of HP *via* free intrachain amino groups, likely by blocking interactions with GlcNH2 residues and/or surrounding environment.[16] Limited access to pure HS oligosaccharides with defined sugar chain size, as well as the degree and position of sulfate groups, also hampers studying the variety of biological effects of HP and HS.[42] Moreover, most research evaluating sites of 3-*O*-sulfation have been based on a "bottom-up" approach using degradative techniques. Furthermore, a lack of defined standards limited structural studies and characterization of the STs. Little research has been performed on 3-*O*-sulfation, most likely due to its rarity and the various synthetic and analytical barriers described above.[83]

Most of the SARS-CoV-2 viral binding studies performed to date are in vitro docking studies of the SP only.[50] Binding to SARS-CoV-2 SP is looked at in isolation, and the total charge or GRAVY score of the virus is not considered, thus the net effect of electrostatic forces. Since some expert researchers find "glycan-independent" mechanisms of pathogen interaction with target cells, they sometimes mistakenly assume that the glycan-dependent process is, therefore, unimportant. Moreover, these studies are often done in static in vitro conditions with long contact times, making the initial glycan "handshake" less critical. The situation is obviously very different in vivo, where opportunities for contact and infection may be transient and difficult to establish.[100] Viruses that utilize HSPG for binding can be expected to have broader tropism since HSPG are highly expressed in almost all human tissues. While various reports support an association between HSPG binding and virulence,[7,8,119] others, to the contrary,[48,105] show an inverse correlation between usage of this receptor and virulence or dissemination abilities.[12] These contradictory results could be due to the fact that animal models are often genetically modified to support viral infections, or inoculated viruses are animal-adapted, and the inoculation route and infectious doses used are far from natural in vivo conditions. It is, therefore, essential to do further research under more relevant conditions to aid our understanding of the real impact of HSPG binding on viral pathogenesis in humans.[12]

With most research, there is a bias toward an outcome, and researchers mainly look at glycobiology from an angle of drug development, but not so much in the context of preventative medicine. The reductionistic way we look at science needs to be questioned and experimental data accumulated cannot be summarized by a simple scheme.[46] Defining the GL geometrically is difficult because of the dynamic balance between biosynthesis and shedding of GL components. It is clearly not a static picture, confirmed by the heterogeneity of the membrane-bound mesh of PGs, glycoproteins, and GAGs, as well as the composition of associated plasma proteins and soluble GAGs. Clearly, the GL layer as a whole is very dynamic, with membrane-bound molecules being constantly replaced and with no definite boundary between locally synthesized and associated elements.[80] Furthermore, apart from the variations in sulfation, the high degree of GAG sequence and conformational flexibility, likely support the diversity and complexity of GAG-ligand interactions.[42,63] The myriad of factors that affect inorganic sulfate availability for sulfation, also need to be considered (Figure 2).

The clarification of GAG functional relationships and structure has lagged behind that of proteins and nucleic acids, mostly because of limitations in the research methods available. Furthermore, since GAGs cannot be amplified against a template, they cannot be sequenced in the same manner as RNA or DNA.[63] To overcome many of the research limitations, Liu et al. developed

a chemoenzymatic method to synthesize HS oligosaccharides in vitro using a series of enzymes to mimic the biosynthesis of HS within cells.[42] Although the majority of scientific studies attempt to consider the influence of the GAG structure as a whole, very few consider the effect of the degree of GAG sulfation. Most studies look at HPSE and other cleavage GAG shedding enzymes, but not many use chlorate and other desulfating strategies to determine the influence of the degree of sulfation. As highlighted by this review, a major difference exists in behavior between sulfated PG and the unsulfated or undersulfated PG skeleton. Nevertheless, it should be taken into account that chlorate treatment can indiscriminately affect the degree of sulfation of other glycan classes, as well as proteins.[76]

Previously, experimental design limitations in evaluating the GL, as described in this review, may have resulted in a vicious cycle of poor understanding and consequent poor attention into the field of glycobiology. Progression in glycobiology research was mostly hampered by the fact that during the initial phase of the molecular biology revolution of the 1980s, the study of GAGs got "left out" of the curriculum, not only because of their complexity and difficulty to study, but also because they did not form part of the original "central dogma".[100] Consequently, this led to a peculiar and tragic distortion of the bioscience community, in which an entire generation of biologists, beginning in the 1980s, has been trained without much knowledge nor appreciation about the structure, biosynthesis and roles of GAGs, sulfation, and the GL as a whole. The earlier lack of understanding of the essential biological functions of GAGs could thus be partly blamed for the previous relative lack of interest in GAGs. It was not only the technical difficulties in detection that made it difficult to explore GAG biological function, but also in vivo analysis and manipulation. Besides, current research methods partially or completely destroy or miss labile important modifications, such as acetylation and sulfation.[100] Also, the low number of scientists involved, and the lack of funding and scientific popularity of glycosciences[100] and sulfur metabolism, resulted in a vacuum of knowledge and appreciation of this very important field of science. Sadly, most of the current glycobiology research focuses on the GL from a drug development perspective, and not preventative health. Our clear understanding of the GL has consequently advanced slowly during the past 50 years, from being a seemingly insignificant remnant of the basolateral membrane to being a very prominent and crucial contributor to Ep and En function. It is now realized that GL degradation does not only cause local Ep and En dysfunction, but also releases GL constituents into the systemic circulation, thereby shaping various systemic responses to critical illness.[115]

9 | CONCLUSION

From the compelling evidence in the literature and this review, it is clear that undersulfation of the Ep- and EnGL is a probable predisposing factor making people more susceptible to SARS-CoV-2 infection, and might increase the severity of COVID-19 symptoms experienced. The beneficial role of an intact GL with adequately sulfated GAGs is well established in the control of many Ep and En functions, by way of pathogen evasion, shear stress transduction, control of Ep, and En permeability and coagulation, as well as protecting the GL from ROS, cytokines, and enzymes, with untoward effects. Though knowledge to date on SARS-CoV-2 pathogenesis is still relatively scarce, histological findings confirmed endotheliitis and recent in vivo measurements suggest endothelial dysfunction playing an integral part in severe COVID-19.[131]

The effect of HS on COVID-19 may seem like a paradox. Undersulfated HS will facilitate viral entry into the cell and consequent shed HS fragments released into the circulatory system can aggravate inflammation and coagulation, resulting in multi-organ dysfunction. However, adequately sulfated HS in an intact GL will prevent initial viral entry, modulate inflammation and immunity, and inhibit vascular permeability and coagulation. The overall sulfation of HS drives these interactions at a first electrostatic level, subsequently by the specific recognition of structural determinants, especially the specific arrangement of sulfate groups in a given sequence, such as 3OST-3B expression that facilitates SARS-CoV-2 viral entry. Seen that 3OST-3B is expressed in chronic inflammatory conditions, and when GAGs are undersulfated with the presence of GlcNH2 groups,[16,62] it seems probable that GAG undersulfation is related to chronic inflammatory and metabolic diseases, the very conditions that pave the way for SARS-CoV-2 infection and severe COVID-19 symptoms. It would appear that *N*-sulfation have a major impact on infectivity and is associated with increased sulfation of other domains, resulting in higher affinity for specific ligands with specific physiological consequences.

Both the synthesis and degradation of HS are complex pathways involving various enzymes, temporarily and spatially tuned, which result in heterogeneous GAG chains imparting different degrees of sulfation, with tissue-specific expression of ligand-binding sequences.[46,62] The expression of these enzymes is highly regulated and changes in expression or activity could alter HS fine structure and affect their functional properties.[22] In the functional form, HS always remains connected to its core protein. Due to variation in various factors such as host genetics, nutrition, and the environment, disease progression is difficult to predict. The GL properties will

be dramatically affected by enzymatic removal of any of its constituents, which highlights the importance of considering the synergistic interaction of all GL constituents as a whole,[56] as well as the effect of genetic and epigenetic influences.[36] The pathogenesis of COVID-19 should not be considered from only an undersulfated GL perspective. An undersulfated GL has a more open-mesh configuration, making it more vulnerable to the onslaught of pathogens, various toxins, leukocytes, and cytokines, with degradation of the GL as a consequence. Undersulfation, therefore, increases the vulnerability to shedding, and only once the GL fragments are shed, it has major pathological consequences. It would, therefore, be difficult to distinguish in vivo between the two concepts of GL undersulfation and degradation.

Though infection begins with microbial colonization of host tissues, colonization by most microbes does not necessarily lead to infection. In fact, most microbes are supportive of host well-being and survival. Infection only occurs when several pathogenic microbes breach the protective barriers of the host, entering the cells, multiply, damage cells, and disrupt normal tissue functions.[102] Even though SARS-CoV-2 appears to be more virulent than other viruses, a great percentage of people around the globe who came in contact with the virus experienced no adverse health effects. The fact remains that up to 80% of people exposed to SARS-CoV-2 had no or very mild symptoms.[133,291,292] Generally, only those with symptoms get tested for the virus, therefore, a much greater percentage of the population could be completely asymptomatic. They would appear to have an intact innate defense system, thus uncompromised highly sulfated GL protecting the epithelium from viral entry. For those that are symptomatic, treatment can be directed at restoring and optimizing sulfation of the GL. The main rate-limiting factor to sulfation appears to be the availability of inorganic sulfate.[23,28,29,204]

Administration of NAC, as both preventative and adjuvant treatment in patients with mild to severe COVID-19 symptoms, is worth considering as a cost-effective clinical strategy. Various sulfur donor compounds would prove to be beneficial.[50,117,206,207,293–298] Even though different mechanisms have been ascribed to their functional roles, their likely effect on GAG sulfation becomes apparent too. While this type of physiological intervention is not commercially attractive, it is nonetheless extremely valid, with an extensive history of solid biochemical and physiological research evidence to support it. The beneficial effects of NAC are well-touted in scientific literature. However, NAC is mostly hailed as an important antioxidant, being a precursor to GSH. The fact that NAC is oxidized to inorganic sulfate, and the very important role that sulfation plays in glycobiology, is mostly overlooked. We would like to urge medical professionals and researchers to validate this observation through clinical and research studies, and to focus on GL preservation models to ensure better outcomes for COVID-19 patients.

Although it is important to find effective treatments during a pandemic, more emphasis should be placed on preventative healthcare. The various factors resulting in the undersulfation of the GL, thus compromised innate immunity, should be considered and addressed. May this pandemic be a warning to us all to improve our health, lifestyle, and environment. A quick fix drug and vaccine approach will not address the underlying etiological factors that made us more susceptible to COVID-19. GL degradation is gaining recognition as an important aspect of sepsis and COVID-19 pathophysiology and armed with this knowledge, we should shift our focus more toward preventative healthcare. By becoming a host that is resilient to SARS-CoV-2, COVID-19 patients can improve their odds of a faster and more favorable recovery.[143] Furthermore, we should make sure that the pandemic is not being exploited for profit, and consider the use of effective repurposed drugs. We must make sure we understand the pathophysiology underlying COVID-19, to implement preventative dietary and lifestyle measures. The health benefits of sulfur have been realized since ancient of times, with the beneficial healing effects of sulfur-rich spas and warm baths, but somehow the knowledge became lost with the advent of modern medicine.

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DISCLOSURES

The authors declare that no competing interests exist.

AUTHOR CONTRIBUTIONS

Heidi N. du Preez conceived and researched the hypothesis proposed in this review and wrote the manuscript. Heidi N. du Preez also conceptualized and produced all the figures presented, apart from Figure 4. The other authors extensively reviewed the manuscript.

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