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Abstract

In this literature review, we assess the pathophysiology of severe adverse events observed after vaccination with DNA and mRNA vaccines against COVID-19, despite reports of their overall effectiveness. The focus is on the perspective of an undersulfated and degraded glycocalyx, considering its impact on immunomodulation, inflammatory responses, coagulation, and oxidative stress. The paper explores various factors that lead to glutathione and inorganic sulfate depletion and their subsequent effect on glycocalyx sulfation and other metabolites, including hormones. Components of COVID-19 vaccines, such as DNA and mRNA material, spike protein antigen, and lipid nanoparticles, are involved in possible cytotoxic effects. The common thread connecting these adverse events is endotheliopathy or glycocalyx degradation caused by depleted glutathione and inorganic sulfate levels, shear stress from circulating nanoparticles, aggregation, and formation of protein coronas, leading to imbalanced immune responses and chronic release of pro-inflammatory cytokines, ultimately resulting in oxidative stress and systemic inflammatory response syndrome. By understanding the underlying pathophysiology of severe adverse events, better treatment options can be explored.

COVID-19 vaccine adverse events: Evaluating the pathophysiology from an undersulfated and degraded glycocalyx perspective

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Abstract:

In this literature review, we assess the pathophysiology of severe adverse events observed after vaccination with DNA and mRNA vaccines against COVID-19, despite reports of their overall effectiveness. The focus is on the perspective of an undersulfated and degraded glycocalyx, considering its impact on immunomodulation, inflammatory responses, coagulation, and oxidative stress. The paper explores various factors that lead to glutathione and inorganic sulfate depletion and their subsequent effect on glycocalyx sulfation and other metabolites, including hormones. Components of COVID-19 vaccines, such as DNA and mRNA material, spike protein antigen, and lipid nanoparticles, are involved in possible cytotoxic effects. The common thread connecting these adverse events is endotheliopathy or glycocalyx degradation caused by depleted glutathione and inorganic sulfate levels, shear stress from circulating nanoparticles, aggregation, and formation of protein coronas, leading to imbalanced immune responses and chronic release of pro-inflammatory cytokines, ultimately resulting in oxidative stress and systemic inflammatory response syndrome. By understanding the underlying pathophysiology of severe adverse events, better treatment options can be explored.

Keywords: COVID-19 vaccine; glycocalyx; heparan sulfate; lipid nanoparticles; DNA; messenger RNA; glutathione; albumin; taurine; oxidative stress

Abbreviations: adverse events (AEs); amino acids (AAs); angiotensin-converting enzyme 2 (ACE2); antibody (Ab); antibody-dependent enhancement (ADE); antigen-presenting cell (APC); antithrombin (AT); blood-brain barrier (BBB); B lymphocytes (B cells); cerebral venous sinus thrombosis (CVST); chondroitin sulfate (CS); 5'-cytosinephospho-guanine-3' (CpG); dendritic cells (DCs); double-stranded (ds); endothelial (En); endothelial cells (EnCs); endothelial glycocalyx (EnGL); ephrin (Eph); epithelial (Ep); epithelial cells (EpCs); epithelial glycocalyx (EpGL); extracellular matrix (ECM); extracellular vesicles (EVs); genetic-vaccine-generated (GVG); glutathione (GSH); glycocalyx (GL); glycosaminoglycans (GAGs); Guillain–Barre syndrome (GBS); heparanase (HPSE); heparan sulfate (HS); heparan sulfate 2-O-sulfotransferase (HS2ST); heparan sulfate proteoglycans (HSPGs); heparin (HP); heparin-induced thrombocytopenia (HIT); human serum albumin (HSA); hyaluronan (HA); hypochlorous acid (HOCl); immune-inflammatory and oxidative and nitrosative stress (IO&NS); lactose dehydrogenase (LDH); lipid-nanoparticles (LNPs); matrix metalloproteinases (MMPs); myeloperoxidase (MPO); nanoparticles (NPs); neutralizing antibodies (nAbs); neutrophil extracellular traps (NETs); nitric oxide (NO); nonsteroidal anti-inflammatory drugs (NSAIDs); 3-O-sulfotransferase 3B (3OST-3B); platelet factor 4 (PF4); polyethylene glycol (PEG); proteoglycans (PGs); reactive oxygen species (ROS); receptor-binding domain (RBD); Secreted Protein Acidic and Rich in Cysteine (SPARC); spike protein (Sp); sulfatase (SULF); sulfonucleotide 3-phosphoadenosine 5-phosphosulfate (PAPS); sulfotransferase (SULT); sulfur amino acids (SAAs); syndecan (sdc); T lymphocytes (T cells); vaccine-induced immune thrombotic thrombocytopenia (VITT); vascular endothelial growth factor (VEGF); von Willebrand factor (vWf)

1. Introduction

During the past three years, the deployment of DNA and mRNA vaccines has occurred at an unparalleled pace and scope to fortify immunological defenses against SARS-CoV-2. Despite the documented efficacy of this vaccination strategy [1-3], there have been reports of adverse events (AEs) and unexpected deaths after vaccination [4-9]. Serious AEs reported include acute myocardial infarction, Bell's palsy, cerebral venous sinus thrombosis, Guillain Barré syndrome, myocarditis/pericarditis, mainly in the younger population, pulmonary embolism, hemorrhages, stroke, thrombosis, and immune thrombocytopenia, disseminated intravascular coagulation, lymphadenopathy, appendicitis, herpes zoster reactivation, neurological complications, seizures, autoimmunity, for example, autoimmune hepatitis and autoimmune peripheral neuropathies [4,10-17]. Several studies communicated the existence of symptoms related to rare or never-described before syndromes, which developed after COVID-19 vaccination [18,19]. There were too many serious AEs that warrant attention. A systematic review by Hulscher et al. found a high probability of a causal link between COVID-19 vaccines and death [20]. The critical question therefore arises: What is the underlying pathophysiology of severe AEs and deaths experienced by many COVID-19 vaccine recipients?

Here, we approach this question from an undersulfated or degraded epithelial glycocalyx (EpGL) and endothelial glycocalyx (EnGL) perspective. In a previous review article, we examined how an undersulfated and degraded EpGL and EnGL predispose to COVID-19 [21]. This review focuses on the effect of an undersulfated and degraded glycocalyx (GL) on susceptibility to AEs postvaccination and the impact of the various ingredients of the COVID-19 vaccine on causing epithelial (Ep) or endothelial (En) degradation.

The GL is a complex mesh of proteins and carbohydrates surrounding every eukaryotic cell. Under healthy conditions, adequately sulfated Ep- and EnGL possesses immunomodulating, anti-inflammatory, anticoagulant, and vasodilatory mechanisms. Endotheliopathy [22], evidence of SARS-CoV-2 infection of pulmonary and extrapulmonary endothelial cells (EnCs) [23,24], reports of viremia [25,26], and multiorgan injury [23,27], all confirm the hypothesis that COVID-19 and COVID-19 vaccine injury or AEs are, in part, vascular diseases of the endothelium. Clinical studies have shown that En dysfunction is a major determinant of severe COVID-19 [21,28-30] and vaccine-induced AEs [5,22]. At the vascular surface, the EnGL is essential in regulating barrier function, immunomodulation, nitric oxide (NO) production and vasorelaxation, mechanotransduction, and resistance to oxidative stress, coagulation, and inflammation. The GL is an intricate network composed of glycosaminoglycans (GAGs), such as heparan sulfate (HS), hyaluronan (HA), and chondroitin sulfate (CS), as well as other glycoconjugates, at the interface between the cell surface and the extracellular environment. Proteoglycans (PGs) are macromolecules composed of a core protein with covalently linked GAG side chains that form the major constituents of the GL. Figure 1 represents the various elements of the GL.

In chronic inflammation, high levels of 3-O-sulfotransferase 3B (3OST-3B) and unsulfated N-unsubstituted glucosamine units (GlcNH2) lead to reduced heparan sulfate (HS) sulfation. This facilitates the binding of SARS-CoV-2 to receptors of angiotensin-converting enzyme 2 (ACE2). The availability of inorganic sulfate (SO_4^{2-}) is crucial for GAG sulfation. Various dietary and environmental factors decrease the availability of inorganic sulfate, causing protein catabolism, such as human serum albumin (HSA), glutathione (GSH), and Secreted Protein Acidic and Rich in Cysteine (SPARC), to provide sulfur amino acids (SAAs) for inorganic sulfate synthesis (Figure 1).

The degree of sulfation and the position of the sulfate groups on the GAG chains determine the biological function of the GL [21]. The GL undergoes constant constitutive remodeling, and a balance of synthesis and degradation of the GL components maintains homeostasis. However, various factors, such as infection, inflammation, toxins and heavy metals, malnutrition, ischemia/reperfusion, and hyperglycemia, can lead to degradation of the GL and release, or shed, bioactive GL fragments, exacerbating disease [21]. PGs and GAGs are degraded through the activity of various enzymes that regulate the availability of HS or HA chains for receptor binding. This, together with the degree and conformation of sulfation, controls several important ligand-binding interactions with known roles in leukocyte recruitment, neutrophil function, En behavior, as well as cytokine release, NO, and growth factor release. Furthermore, GL degradation fragments can act as damage-associated molecular patterns, amplifying pro-inflammatory responses and En injury, resulting in barrier dysfunction. Therefore, the GL is an extremely delicate layer, where changing or removing one specific component can result in the loss of function of the total [21].

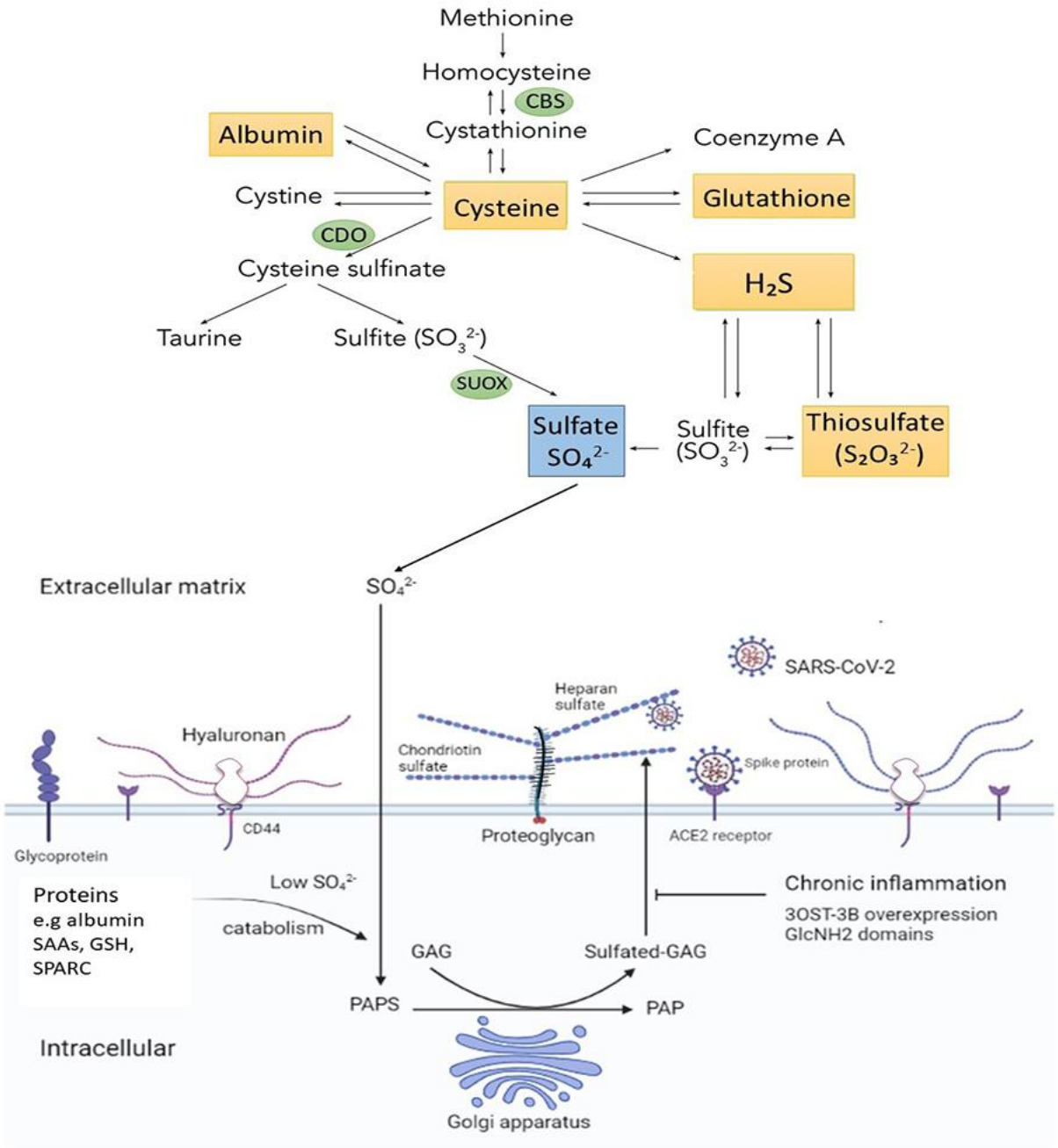

Figure 1: Sulfur metabolism and sulfation diagram. The essential sulfur amino acid (SAA) methionine converts to cysteine, which is a precursor to human serum albumin (HSA), cystine, coenzyme A, glutathione (GSH), hydrogen sulfite (H_2S), taurine and inorganic sulfate (SO_4^{2-}). H_2S can be converted 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 [21]. The availability of inorganic sulfate (SO_4^{2-}) is the rate-limiting factor for sulfation of the glycosaminoglycan (GAG) heparan sulfate (HS). During chronic inflammation with overexpression of 3-*O*-sulfotransferase 3B (3OST-3B) and the presence of unsulfated *N*-unsubstituted glucosamine units (GlcNH2), decreased HS sulfation will facilitate the binding of SARS-CoV-2 to receptors of the angiotensin converting enzyme 2 (ACE2). With limited availability of inorganic sulfate, proteins such as HSA, GSH and Secreted Protein Acidic and Rich in Cysteine (SPARC), would be catabolized to provide sulfur amino acids for inorganic sulfate synthesis. Adapted by CC by 4.0 [21].

The most popular vaccines commercially used against SARS-CoV-2 are the Moderna non-replicating mRNA vaccines from Moderna (mRNA 1273) and Pfizer-BioNTech - BNT162b2 (Comirnaty™), the prophylactic DNA vaccine, INO-4800, as well as the replicating DNA viral vector vaccine, ChAdOx1 nCoV-19 (Oxford/AstraZeneca or Vaxzevria®), for the expression of spike protein (Sp) by target cells *in vivo*. Among others, the non-replicating JNJ-78436735 / Ad26.COV2.S (Johnson & Johnson) vector vaccine, the NVX-CoV2373 (Novavax) subunit vaccine, the BBIBP-CorV (Sinopharm) inactivated whole virus vaccine, the BBV152 / Chevexin and Russian Sputnik V vaccine are also all candidate vaccines based on inactivated viruses or recombinant protein-based vaccines that present the Sp antigen directly. In these viral vector-based vaccines, the viral vector is used to deliver the DNA sequence of the Sp to human cells [31-33]. This review emphasizes the Pfizer-BioNTech (referred to as 'Pfizer' in the rest of this review) and Moderna vaccines, as they are the most widely used and comprehensive published data are available. However, most of the concerns raised also apply to the other COVID-19 vaccines and DNA and mRNA vaccines in general.

Additional complications of GL can be expected because of the introduction of nanoparticles (NPs) into nanomedicine and vaccines. NPs form an integral part of the delivery system of mRNA vaccines. Various liposomal NPs have been approved for inclusion in nanodrugs [34-37], while lipid nanoparticles (LNPs) received FDA approval [38] for inclusion in COVID-19 mRNA vaccines [31,39]. The patents on the Pfizer [40] and Moderna [41] mRNA vaccines can verify this.

We hypothesize that after COVID-19 vaccination, the combination of the genetic-vaccine-generated (GVG) Sp antigen, the genetic material and LNPs, will ultimately contribute to EnC injury and consequent GL degradation, mainly through the generation of chronic, skewed or excessive inflammatory responses, and oxidative stress. Therefore, AEs experienced postvaccination results from compromised barrier functions, circulating pro-inflammatory cytokines, reactive oxygen species (ROS), GL fragments, harmful NPs, and soluble GVG Sp and its fragments, all of which cause various cytotoxic effects. Many studies underscore the importance of adequately sulfated GAGs and other metabolites as endogenous regulators of cancer, thrombosis, myocarditis, neurodegeneration, and other disease conditions. Therefore, GL injury is a pathological manifestation capable of exacerbating the disease, and circulating GL fragments cause GL injury in multiple organs [21,28].

This literature review article aims to highlight the potential effect of COVID-19 DNA and mRNA vaccines on sulfation, the GL and innate immunity; and how these factors could explain the various AEs experienced by many COVID-19 vaccine recipients. Therefore, the main focus of this review article is on understanding the etiological factors and pathogenesis of the AEs experienced by many recipients of the various COVID-19 vaccines.

2. DNA and mRNA vaccines

All current COVID-19 vaccines authorized for general use mainly rely on SARS-CoV-2 Sp as an immunogen, with added adjuvants. In the case of inactivated DNA virus vaccines, other viral proteins/particles are also present in the vaccine. The COVID-19 vaccines provide cells with a blueprint, either DNA or mRNA, to construct the Sp. The Sp is the main focus of COVID-19 vaccine development and antiviral therapeutics, given its central role in viral infection and eliciting humoral and cell-mediated adaptive immune responses in hosts during infection. The exposed subunits of the Sp are the primary target of neutralizing antibodies (nAbs) to inactivate SARS-CoV-2 because of the Sp's essential functions during viral entry, namely receptor binding and membrane fusion [42]. The S1 subunit of the Sp is responsible for receptor binding through the receptor-binding domain (RBD), while subunit 2 (S2) is responsible for viral fusion and entry [21]. The current licensed COVID-19 vaccines present the Sp differently to the immune system. The main categories are mRNA and adenovirus-vector (herein referred to as 'DNA') vaccines, which do not contain the Sp, but provide genetic information for its biosynthesis in cells of the vaccine recipient [43]. The second category encompasses protein-based approaches, such as

classical inactivated whole virus and innovative subunit vaccines containing Sp in different forms and combinations with adjuvants.

mRNA technology is not new. Before the SARS-CoV-2 pandemic, Moderna had already conducted a clinical trial with an LNP-formulated mRNA vaccine against the influenza virus [44,45]. The Pfizer and Moderna COVID-19 mRNA vaccines are synthetic nucleoside-modified mRNA vaccines formulated in LNPs, which encode either the trimerized RBD of the Sp in S1 (BNT162b1) or the prefusion stabilized full-length Sp of SARS-CoV-2 (Moderna and BNT162b2). LNPs ensure stability and facilitate passage of nonreplicating and non-self-amplified RNA through the cell membrane to direct transient expression of the SARS-CoV-2 Sp antigen [10,43,46,47]. With increased improvements and stability of mRNA vaccines, protein expression can be achieved for days after direct *in vivo* administration [48,49]. There exist subtle differences between the Moderna and Pfizer vaccines, in relation to the RNA and LNP carriers, and the Moderna vaccine used a higher amount of RNA per dose (100 µg) compared to the Pfizer vaccine (30 µg) [43].

To improve serum stability, prevent aggregation and extend the circulation time of genetic material, polyethylene glycol (PEG) polymer chains are attached to LNPs (PEGylation) [50]. However, PEG's immunogenicity and shortened biocirculation have been demonstrated by repeated doses [51]. Administration of repeated doses of immune-stimulatory nucleic acids encapsulated with PEG-LNP can generate a robust and long-lived antibody (Ab) response against PEG [36]. Therefore, there is an increased risk of acute hypersensitivity or anaphylaxis upon subsequent administration of mRNA vaccines [43,52].

Nonpathogenic viral vector DNA vaccines use a modified version of a different virus, such as adenovirus, to deliver SARS-CoV-2 Sp DNA to host cells. Genetically modified recombinant viral vectors are designed to enter the cell and then trigger the expression of cloned sequences that encode the desired RNA [53]. The two factors that genetic-based COVID-19 vaccines have in common are the presence of genetic material and the generation of the GVG Sp antigen.

3. Safety concerns

Although preclinical and clinical trials were conducted, several concerns have been raised in the scientific community about the safety and long-term side effects of COVID-19 vaccines [4,10,54-56]. The two main aspects covered in this review about the COVID-19 vaccines that are likely linked to toxic effects are the delivery vehicle of the genes and the GVG Sp antigen - see more regarding the impact of the Sp in Section 4.1. The main ingredients used in the delivery hydrogel that are possibly linked to cytotoxic effects are LNPs [57-59] and PEG [43,52,60].

RNA and DNA viruses are known for their cytotoxicity and potent immunogenicity, leading to the popularity of nonviral vectors such as cationic lipids and polymers as gene carriers. However, until recently, cytotoxicity remained a significant challenge for non-viral vector use in gene therapy [37]. The cytotoxicity of lipid-based nanomaterials will depend on the dose, lipid properties, and cell types studied [37,61]. The *in vivo* application of LNPs has been reported to induce liver and lung injuries in animals [62,63], attributed to lipid-material cytotoxicity and induction of oxidative stress and pro-inflammatory cytokines [64,65]. However, it is crucial to note that *in vitro* toxicity does not always correlate well with *in vivo* cytotoxicity [64], and additional human clinical data are necessary to form a comprehensive understanding of the safety profile of LNPs.

Recent advances in LNP technology have led to significant improvements in its stability, along with efforts to mitigate potential adverse effects. To reduce the cytotoxicity of cationic lipid-based NPs and improve targeted delivery, ionizable and PEGylated LNPs became popular. Ionizable cationic LNPs are pH-sensitive; the lipid-rich ionizable core structure is triggered by a pH change that induces endosomal DNA or RNA release due to the lower pH of the endosome (Figure 2). Therefore, ionizable cationic lipids are deprotonated under neutral conditions and positively charged under low pH conditions, thus below the acid dissociation constant (pKa) of

the lipid [66-69]. The LNPs used in the Pfizer and Moderna COVID-19 vaccines contain an ionizable lipid, a structural phospholipid, cholesterol, and a PEG-lipid in molar ratio [70]. The ionizable lipid used in the Pfizer vaccine is ALC-0315, while SM-102 was used in the Moderna vaccine [71]. They differed in stability due to steric factors: ALC-0315 has four very short tails compared to SM-102, which is more stable with only three tails, while one is long enough to stabilize the lipid in the lipid leaflet [66]. Small changes in LNP chemistry or formulation, the use of modified nucleosides, the 5'-cytosinephospho-guanine-3' (CpG) content, and the length differences between conventional mRNA, plus the molar ratios and excipients used, will affect targetability, transfection, and cytotoxicity [67,72]. The cytotoxicity, structural, and biological properties of LNPs are not attributed to a single lipid component alone, but to the combination of lipids [68].

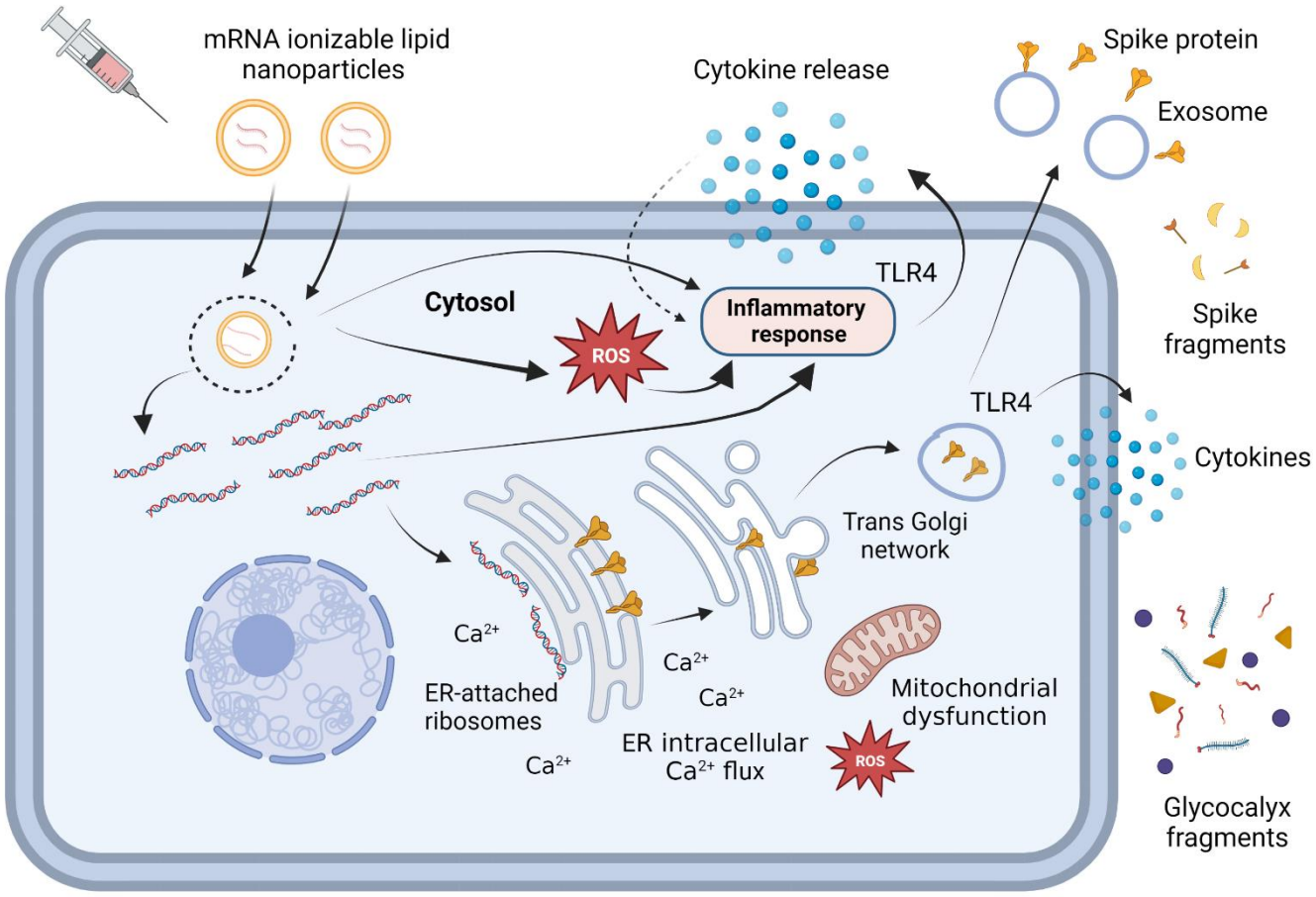

Figure 2: Schematic illustration of the endosomal release of mRNA from ionizable lipid nanoparticles (LNPs); the consequent inflammatory responses and oxidative stress; with cellular release of spike protein (Sp) fragments, cytokines, and glycocalyx fragments into the circulation.

3.1 Adsorption and distribution

The absorption, distribution, and excretion of NPs will be affected by various factors, such as the administration routes, physicochemical properties, particle agglomeration, and surface coatings. After entering the body, NPs can be systemically distributed through the lymph system, blood circulation, and cross biological barriers, resulting in varying degrees of retention in different organs. Due to their very small nanosize, LBNPs can pass through normal physiological barriers, such as the blood-air barrier, blood-testis barrier, blood-brain barrier (BBB) and the blood-placental barrier [10,72-75], thus reaching various organs where they can induce acute or chronic injuries in tissues [76].

LNP's main route of entry into cells is endocytosis or phagocytosis, depending on its size and lipid properties. Once the LNPs are internalized, they are transported through endosomes and lysosomes, where the LNP cargo is digested or exocytosed. Therefore, endosomal escape is a crucial step for effective gene delivery through LNPs [71].

3.2 Membrane and cellular damage

Cationic lipids are known to be excellent surfactants with the potential to cause solubilization, poration, and lysis of the lipid bilayer cell membrane [64,71]. Ionizable LNPs also have endosome lytic properties to ensure the endosomal escape of the cargo [69].

One of the hallmarks of ionizable LNPs is their active approach to endosomal escape. The ionizable lipid component becomes charged upon endosomal acidification, promoting electrostatic interactions between the LNP's ionizable cationic lipids and the anionic lipids in the endosomal membrane, thus destabilizing the endosomal membrane to promote endosomal escape of nucleic acids [68,69,71]. However, interactions of LNPs with cell membranes, in general, can cause systemic cytotoxicity with related toxic side effects *in vivo* if the amphiphilic properties of the lipids and the surface property of the LNP are not accurately controlled in the physiological pH (7.4) and endosomal pH range [71]. Cytotoxicity will also depend on the charge ratio between the cationic lipid species and the nucleic acids, where higher charge ratios are generally more toxic to various cell types [37].

Endosomal signaling is tightly regulated by mechanisms that are not yet fully understood and differ from those originating from receptors on the plasma membrane [77]. Signals from the diverse families of endosomal receptors control essential growth, differentiation, survival, inflammation, and immunity processes. Since defects in these mechanisms can cause disease and drugs that target these mechanisms have potent effects, the potential adverse effects of ionizable LNPs on endosomal membrane destabilization must be considered. However, the mechanism of endocytosis will specify the outcome of endosomal signaling. Endosomes are important sites where receptor signaling can be initiated, sustained, and terminated, such as TLR signaling, which are major mediators of innate immunity [77].

3.3 Inflammation and cell death

LNPs can cause cell death through autophagy, necrosis triggered by inflammatory responses and ROS, or apoptosis through damage to the plasma membrane [78]. It has been shown that the so-called "mix and match" strategies of combining two or more existing vaccine platforms, such as the adenoviral and mRNA-LNP vaccines, resulted in exceptionally potent immune responses [79], with a consequent higher tendency for AEs [80]. When inflammation is activated through ROS or primed by antigens, macrophages trigger a natural innate immune response (Figure 2). However, immunotoxicity can establish after vaccination if the immune response is excessive, chronic, or results in a skewed T cell response, favoring Th1. The innate immune response triggered by an antigen is primarily transient, and an adequately sulfated Ep- and EnGL can successfully modulate the response. However, in individuals with compromised GL barriers and preexisting chronic inflammation, AEs can be expected after vaccination due to a 'systemic inflammatory response syndrome'.

In addition to stabilizing mRNA and facilitating intracellular delivery, LNPs can exert an adjuvant effect on mRNA vaccines [68]. Although LNPs are more effective and demonstrate less immunogenicity and cytotoxicity than liposomes [81], they trigger pro-inflammatory responses. Evidence shows that ionizable cationic lipids within LNPs can induce pro-inflammatory cytokines by activating TLRs within endosomes [82-84]. The autophagy pathway is related to phagocytosis by TLR signaling in macrophages [85]. Endosomes are a site for coordinated activation of signaling pathways stimulated by TLR4 [77]. LNPs were shown to cause TLR4 activation that induces pro-inflammatory cytokines, such as IL-6 [68], and type I IFN [62,77] (Figure 2). Li et al. pointed out that LNP-induced cytotoxicity is caused by the indirect effect of pro-inflammatory cytokines, such as TNFα and IFNγ, which can result in apoptosis of EnCs in a dose-dependent manner *in vitro* and *in vivo* [65].

Kedmi et al. observed *in vitro* that cationic LNPs interact directly with immune cells and lead to immune activation, favoring a Th1 (IL-2, IFNγ, TNFα) and Th17 (IL-17 and IL-6) cytokine response. IL-17 has been shown to play a crucial role in the induction of autoimmune diseases. They confirmed that cationic LNP-siRNA complexes induce immune responses by up-regulating Th1 cytokines and IFN-responsive genes [62]. It was shown that mRNA-LNP immunogenicity would depend on the structure and pKa of the ionizable lipid [68]. Dokka et al. found that highly charged multivalent cationic liposomes caused a marked inflammatory response, determined by neutrophil influx and oxidative burst of lung cells, where the effect was charge-related [64].

In a mouse nucleoside-modified mRNA vaccine study, it was confirmed that LNPs are highly inflammatory, independent of the delivery route, evidenced by excessive neutrophil infiltration, activation of various inflammatory pathways, and production of various inflammatory cytokines and chemokines [57]. It should be noted that inflammatory responses can be exacerbated on a background of preexisting inflammatory conditions, where this effect was proven to be specific to the LNP platform, acting independently of the mRNA cargo [57-59]. Sedic et al. confirmed in animal studies that the observed pro-inflammatory response and mild liver toxicity were primarily driven by the LNP vehicle, with repeated administration of hEPO-mRNA in LNPs. They also found that repeated dosing increases complement activation (C3a, C5b-9). They concluded that given the similarities observed in LNP-related toxicities between rats and monkeys, it is likely that similar effects will translate to the clinic [63].

In vitro, it has been shown that cationic LNPs, whether or not they are complexed with nucleic acids, are highly toxic to macrophages [61]. Macrophages can phagocytose a large amount of LNPs, and this phagocytic activity of macrophages is responsible for the high degree of toxicity. Non-cationic LNPs are also toxic to phagocytic cells but to a lesser extent than cationic LNPs. Although the addition of PEG 2000 might seem to abolish toxicity to some degree, the presence of pH-sensitive lipids in ionizable LNPs can enhance cationic LNP toxicity by destabilizing the endosomal membrane, releasing cationic lipids into the cytoplasm [61]. Yavuz et al. demonstrated that, independently of the type of ionizable lipid used to formulate LNPs, intramuscularly immunized mice induced Th1-biased polarization [70]. This aligns with the Pfizer vaccine that caused a Th1-cellular-biased innate immune response, with the secretion of IL-6, TNFα and IFNγ [86,87]. Therefore, LNPs can induce macrophage M1 polarization through the foreign body response. It is known that continuous M1 polarization can release excessive pro-inflammatory cytokines, such as IL-1, NO and TNFα, as well as ROS, to induce a severe or chronic inflammatory response [88]. In M1-activated macrophages, 3OST-3B is up-regulated, with less HS and a lower degree of sulfation, compared to reparative M2 macrophages [21]. Therefore, M1 macrophages with less HS and lower sulfation, exacerbated by LNPs and ROS, will be more vulnerable to viral and SARS-CoV-2 infection and internalization of the GVG Sp.

3.4 Oxidative stress

Generated ROS leads to oxidative stress capable of activating innate immune responses. However, as noted above, the release of pro-inflammatory cytokines can be induced by LNPs independently of ROS production (Figure 2). LNPs also stimulate ROS generation [62], which induces cytotoxicity and affects intracellular signaling pathways [64,78]. ROS acts as a second messenger in many intracellular signaling cascades. It can lead to cellular macromolecular damage, such as DNA fragmentation, membrane lipid breakdown, protein denaturation, and mitochondrial dysfunction, significantly affecting cell metabolism and signaling [89-91], resulting in deleterious effects on cell viability, proliferation, and cell death [92-94]. The general belief is that the excessive ROS levels produced by NPs are the main reason for their cytotoxicity [95,96].

Oxidative stress and the associated inflammation resulting from increased ROS production and / or decreased antioxidant defense contribute to cytotoxicity. GSH, the most abundant antioxidant that plays a crucial role in antioxidant defense against oxidative damage of ROS cells, regulates various metabolic pathways essential for whole-body homeostasis. GSH is responsible for maintaining mitochondrial function, antiviral defense, regulation of cellular proliferation, apoptosis, DNA synthesis, microtubular-related processes, and immune responses. Variations in GSH levels are a hallmark of many pathological disorders, including cancer, metabolic

abnormalities, and cardiovascular disease [97,98]. The SAA, cysteine (Cys), is a precursor to GSH and inorganic sulfate, hydrogen sulfide (H_2S), HSA, and taurine (Figure 1)[98]. It is important to understand the homeostatic interaction between these sulfur compounds and the effect of inorganic sulfate on cellular health and innate immune defenses against infectious disease. Inorganic sulfate levels are directly correlated with GSH levels and are the rate-limiting factor for sulfation. This topic has extensively been reviewed elsewhere [21,98].

3.5 Functionalization of nanoparticles

Although some of the cytotoxic effects of LNPs are reduced through functionalization with polymers, such as PEG [68,73,99,100], PEG-lipids can dissociate in biological environments, thus increasing the toxicity of the LNPs [68,71,101,102].

Even if LNPs have been functionalized with PEG to decrease toxicity and increase circulation time [68], PEG can cause severe allergy-like symptoms [68,103]. Furthermore, these functionalized surface modifications and cloaking techniques can allow NPs to avoid recognition and clearance through detoxification pathways [52,104], extending circulation time [72,105], or they can accumulate in the system [106]. Although this might be favorable from a drug development perspective, the systemic consequences *in vivo* from the hampered detoxification of NPs are not considered.

Seen that both mRNA and PEG will be degraded over time, the long-term cytotoxicity of COVID-19 vaccines and AE experienced months after vaccination [80] could probably be best ascribed to the presence of NPs and the GVG Sp antigen, as well as the generated ROS [96], protein coronas, pro-inflammatory immune responses, and complement activation [68]. The fact that the failure of the COVID-19 vaccines to determine durable immunity longer than 3-4 months, if at all, for vectorial vaccines and 6 months for mRNA vaccines, required a third boosting dose, without any consideration of the possible build-up of the vaccine toxicological substances in the system. However, it has been shown that, after repeated administration, PEGylated LNPs can undergo accelerated blood clearance and complement activation-related pseudoallergy triggered by the immune system in reaction to PEG. Therefore, accelerated blood clearance is mediated by Abs raised against PEGylated LNPs after the first injection [68].

3.6 Protein coronas

NPs have a high capacity to adsorb small molecules from physiological fluids that are partially hydrophobic, and with low solubility, by interacting with the NP surface through electrostatic, hydrophobic and van der Waals forces [99,100]. It is well established that NPs, due to their high free surface charge, can aggregate and interact with proteins in biological fluids, forming protein coronas, which will have a considerable impact on many physiological processes [99,102,107,108], as well as biodistribution and clearance of the NPs [68,100]. The formed protein coronas will result in protein aggregation, clustering, and fibrillation, affecting innate immune responses, macrophage recognition, circulation, biodistribution, cellular uptake, clearance, and therefore systemic toxicity of the NPs [99,100,102,109-111].

In addition to protein interactions with NPs, interactions exist between neighboring proteins on the protein corona, creating a dynamic system as proteins continuously adsorb and desorb from a protein corona. Furthermore, NP-induced conformational changes in proteins can cause proteins to expose hidden binding sites that may trigger immune responses [100].

Aliakbarinodehi et al. demonstrated that the adsorption of serum proteins to the surface of LNPs is pH dependent [111]. According to the Henderson–Hasselbalch equation, if ionizable LNPs are used with an apparent pKa of 6.4, about 10% of the ionizable LNPs are positively charged in blood (at pH 7.4) [68]. The fact that LNPs form strong bonds with platelets, hemoglobin, antithrombin (AT) III, HSA, and other plasma proteins essential for major physiological functions [68,99,102], means that these bound proteins cannot perform their functions, which will affect many physiological pathways and the availability of these and other molecules (Figure 3). HSA

is a precursor to Cys and consequently affects the availability of GSH and inorganic sulfate [98]. Thus, if HSA is consumed by protein corona formation, it will negatively impact the integrity of the GL.

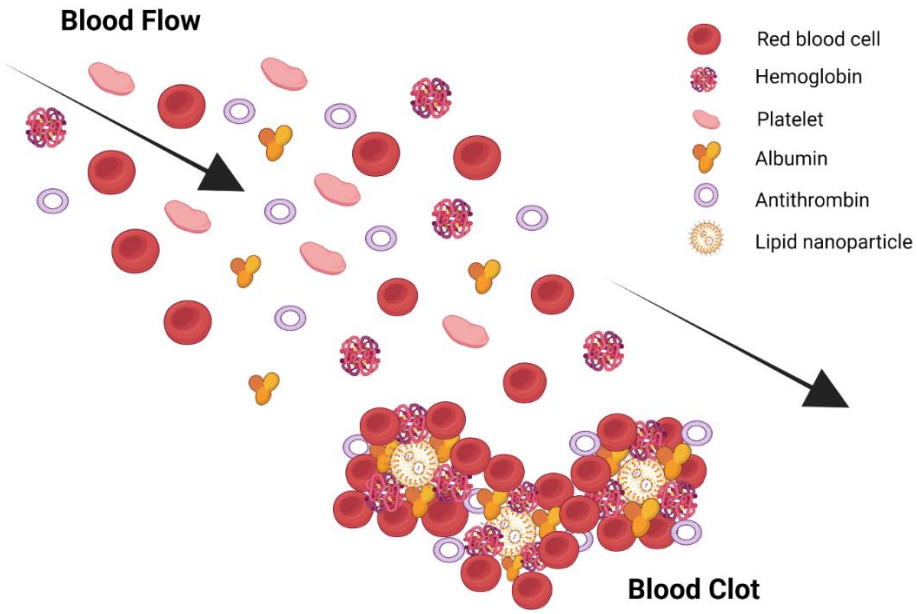

Figure 3: Schematic illustration of protein corona formation and the blood clot inducing potential of lipid nanoparticles (LNPs).

In addition, it has been shown that the secondary structure of HSA changes after adsorption onto NPs, and the presence of HSA and apolipoproteins in protein coronas provides LNPs with a stealth effect, promoting a prolonged circulation time [100]. In addition to HSA and platelets, various proteins and other cellular materials, such as immunoglobulins, fibrinogen, plasma fibronectin, and vitronectin, can form protein coronas on NPs [68,99,100,102,111]. These molecules present in the corona bestow NPs with new properties, transforming their interactions at the bio–nano interface and biodistribution while interfering with both the designed properties of NP and innate biomolecular functions [100,102,109]. For example, NPs that bind to apolipoprotein E are often trafficked to the liver, producing liver toxicity [100]. Bashiri et al. critically reviewed the complexity and effects of protein coronas [100]; however, more *in vivo* studies are needed to understand the complex and dynamic nature of the LNP–protein complex and its interactions with biomolecules.

Many different properties, such as the type of NP, chemical concentration, surface functionalization and molecular composition, shape, curvature, size, and surface charge of the NPs, will all play a role in the composition and evolution of biomaterials that adsorb on their surface, explaining in part the variation in biocompatibility and effects seemingly exerted by NPs [68,99,100,102]. Environmental conditions, such as pH, ionic strength, shear flow and temperature, protein concentration, size, and glycosylation, will impact protein corona formation [100]. Although PEGylation may show antifouling capacity, it cannot fully prevent protein binding and immunogenicity [68,102]. Apolipoproteins have been shown to be enriched in liposome corona–NP complexes, regardless of PEGylation. There has also been evidence of PEG accumulation in protein corona complexes and uncontrolled oxidative degradation of PEG into toxic products [100].

As in the case of Onpattro [68], the FDA-approved liver-targeting LNP, NPs can be exploited for targeted delivery and protein binding. Onpattro was designed to exploit the binding of apolipoprotein E to LNPs in circulation to deliver siRNA to hepatocytes via the LDL R receptor. Before administering Onpattro, a predosing

immunosuppressive cocktail is necessary, consisting of acetaminophen, a glucocorticoid and an H1 / H2 blocker, to offset potential infusion-related reactions [68], which confirms the immunogenicity of LNPs.

The fact that NPs can induce conformational changes in adsorbed proteins, remove them from the circulation, and affect their functionality can cause unwanted effects, such as generating a pro-inflammatory immune response, altering enzyme activity, and causing aggregation of blood components (Figure 3). The design and synthesis of LNPs with enhanced potency and reduced cytotoxicity is a major focus of current LNP research. However, the considerations of intellectual property surrounding them present an additional barrier to clinical translation.

3.7 DNA and synthetic mRNA

Although DNA has a chance of potential genome integration [67,112,113], it is well established that DNA and mRNA can be immunotoxic [62,65,79] (Figure 2). Even though LNPs have been actively recruited to overcome such limitations, they also have drawbacks, such as a short half-life in the body and low loading efficiency [113,114]. To overcome these disadvantages, graphene oxide and graphene quantum dots are often used to transfect genes [113]. Furthermore, Pfizer and Moderna use N1-methyl-pseudouridine-modified mRNA to minimize inherent mRNA immunogenicity; however, internalization of foreign mRNA into the cytosol is detected by intracellular RNA sensors, such as endosomal TLR and cytoplasmic nucleic acid sensors. Binding of mRNA to these host defense receptors will activate innate immune pathways [62], leading to the expression of hundreds of genes [67]. In fact, Abramczyk et al. observed increased cell signaling when cells were incubated with the Pfizer mRNA vaccine [115]. In addition, innate immune responses have been shown to inhibit *in vivo* gene transfer and expression [65].

Antisense RNA may interact directly or indirectly with DNA methyltransferase, interfering with DNA methylation and gene transcription. Synthetic mRNA can, therefore, eventually lead to epigenetic and/or genomic modifications in dividing and nondividing cells. It can lead to modifications of the chromatin structure, chromosomal integration of retrotranscribed synthetic mRNA, genotoxicity, and oncogenesis following mRNA vaccine uptake [55]. Although a peer-reviewed study showed that synthetic genetic vaccine mRNA could activate the expression of endogenous transposable elements, undergo reverse transcription, and enter the cell nucleus [116], more research is needed.

Free plasmid DNA also induces the production of pro-inflammatory cytokines, where the immune response is significantly enhanced when lipid-DNA complexes are used. Cytokine production was observed to be mainly due to unmethylated CpG sequences in plasmid DNA [65]. Therefore, plasmid DNA can serve as a potent immunogen when delivered to immune cells in a 'intact' form, while cationic lipid-DNA complexes largely induce toxicity. McKernan et al. found high levels of DNA contamination that exceed the European Medicines Agency (EMA) 330ng/mg requirement and the FDAs 10ng/dose requirements when they examined the nucleic acid composition of expired vials of the Moderna and Pfizer mRNA vaccines [116]. The exact ratio of linear fragmented DNA versus intact circular plasmid DNA is unknown. However, there is a risk of genome integration, since double-stranded (ds)DNA contamination of the sequence encoding the GVG Sp will not require LINE-1 for reverse transcription. Furthermore, an SV40 nuclear localization signal in Pfizer's vaccine vector [117] will also increase the risk of integration, while SV40 is a cancer promotor. Furthermore, plasmid DNA contamination from E.coli preps is often co-contaminated with lipopolysaccharide, leading to anaphylaxis after injection [116].

The safety profile of nucleoside-modified synthetic mRNA is far from completely understood, and there are no studies available on what happens when mRNA-LNP formulations are stored for long periods [67]. Before the rollout of the COVID-19 vaccines, only limited *in vivo* research studies or trials were conducted to evaluate the biodistribution, cell uptake, translation rates, endosomal escape, functional half-life, and inactivation kinetics of synthetic mRNA and DNA vaccines [118]. Neither were the rates and duration of vaccine-induced antigen expression evaluated in different cell types nor potential interactions with the host genome [55].

In the next section, the possible effects of the genetic material and LNPs, as well as the Sp antigen, are correlated with AEs experienced by the COVID-19 vaccine recipients.

4. Adverse events (AEs)

Since billions of people have been vaccinated with one of the COVID-19 vaccines in a short time frame, it is easier to identify AEs linked to COVID-19 vaccination. Rare events of anaphylactic shock have been reported, above the average normal incidence in the population, after vaccination with the COVID-19 vaccines [4,43,119], in addition to various serious AEs. Although correlation does not necessarily mean causation, active monitoring and awareness of reported post-vaccination AEs are essential. It is important to note that when AEs were analyzed on the EudraVigilance database [120] for the Pfizer, Moderna, AstraZeneca, and J&J vaccines, the percentage frequencies of specific AEs and fatal outcomes for all four COVID-19 vaccines were close to each other. Therefore, the AE profiles of these four vaccines are very similar, if not identical. Although cardiovascular AEs are among the most dangerous, nervous system and musculoskeletal disorders were the most common organ system-related AEs, followed by gastrointestinal, infectious, and skin disorders [120].

Fraiman et al. evaluated the risk of severe AEs in the mRNA COVID-19 vaccine group, relative to placebo, in both the Pfizer and Moderna adult phase III trials and found 10.1 (Pfizer) and 15.1 (Moderna) additional AEs for every 10,000 individuals vaccinated [4]. Furthermore, they identified a 36 % higher risk of severe AEs in vaccinated participants in the Pfizer trial. The excess risk of serious AEs exceeded the reduction in COVID-19 hospitalizations in both the Pfizer and Moderna trials. It is clear from these results that COVID-19 vaccines are associated with more harm than initially estimated at the time of emergency authorization [4].

To draw a parallel between serious AEs [121] and deaths [7,8,122] with COVID-19 vaccines, the etiological factors possibly underlying the various AEs must be explored and better understood. In addition to the effects of the GVG Sp, genetic material, immune responses, and general health status of the vaccinee, it is necessary to consider the possible consequences of the LNPs and ROS generation [96], as well as the impact of the vaccines on the integrity of the GL, when evaluating the pathophysiology of COVID-19 vaccine-induced severe AEs.

4.1 The role of the spike protein in adverse events

Some aspects explicitly of the GVG Sp, such as stability, conformation, charge potential, its immunostimulating properties, and possible systemic toxicity, are highlighted below.

All DNA and mRNA vaccines have to deal with the inherent problem of conformational instability of the GVG Sp, whether the Sp is synthesized in the vaccinee after vaccination or first in cell culture systems. During isolated GVG Sp formation, the absence of interactions with other viral constituents for particle assembly can influence the stability of the S-trimers and glycosylation patterns [43]. To increase immunogenicity, the authentic viral signal sequence was modified through mutations, which can cause inhomogeneity of the N-terminus of the RBD and impair the native folding of S-trimers. The fact that the S-trimer can adopt different conformations will pose a problem for its use in vaccines [43]. The conformation instability of the labile Sp, due to mutations and manufacturing, might affect the specific immune response induced. The fact that older people (> 65) are generally more susceptible to COVID-19 [21,123], while a younger generation (aged 18 to 64) [124] seems to be more at risk for COVID-19 vaccine AEs [10,125], indicates that other factors are also at play, other than the nAb response to the Sp.

Although the ACE2 receptor is the primary entry receptor, various other receptors, ligands, proteases, and cofactors interact with SARS-CoV-2 Sp to facilitate entry into the cell, such as heparan sulfate proteoglycans (HSPGs), integrins, TLRs, neuropilin 1 (NRP1), CD147, CD26, aminopeptidase N, glutamyl aminopeptidase, C-type lectins, DC-SIGN and L-SIGN [26,29,126-129]. It is important to note that the precise mechanisms of viral entry and the role of various receptors are still under investigation, and the available knowledge is evolving. The

SARS-CoV-2 Sp ectodomain was shown to interact with cell surface HS through RBD in the S1 subunit, favoring the RBD open conformation to facilitate receptor binding. Therefore, HS acts as a co-receptor priming the Sp for receptor interaction [21]. CD147, in particular, is widely expressed in human tissues, with higher levels seen primarily in the cardiovascular system. It participates in many physiological and pathological processes due to its numerous interacting partners [127]. CD147, a versatile transmembrane glycoprotein, promotes the activation of matrix metalloproteinase (MMP), myofibroblast differentiation, fibrosis, and oxidative stress [130]. CD147 is also highly expressed in immune cells, EnCs in the brain, tissues of the gastrointestinal tract, platelets, conjunctival tissues, kidney glomerular cells and podocytes, and cardiac pericytes. Its expression level is upregulated during pathological conditions, including several disorders of the central nervous system [131]. During inflammation and oxidative stress, secreted cyclophilins A (CypA) and B (CypB) interact with CD147 and can facilitate viral infection or cellular internalization of GVG Sp. For HIV-1 and SARS-CoV to infect host cells, the viral nucleocapsid protein was found to first binds to CypA, which then in turn recognizes the CD147 receptor expressed on the surface of host cells [127].

In addition, CD147 was shown to be involved in SARS-CoV-2 infection of immune cells [126,132]. In the lungs, small intestine, kidney, and heart, ACE2 is expressed, whereas it is not found in innate and adaptive immune cells. However, CD147 can modulate the abundance of ACE2 [127]. Various researchers demonstrated that CD147 is critical to promoting SARS-CoV-2 infection through interaction with the Sp RBD [127,133,134]. CD147 exerts its influence on tissues through various mechanisms, including: 1) Metabolism modulation - achieved by binding to monocarboxylate transporters and the amino acid (AA) transporter CD98; 2) Permeability regulation - where it controls the levels and activity of MMPs; 3) Vascularization induction - leading to the synthesis and release of vascular endothelial growth factor (VEGF), as well as the expression of the VEGF receptor; and 4) Inflammation mediation - involving leukocyte recruitment and infiltration of leukocytes by interacting with chemokines and adhesion molecules, such as integrins, selectins and CD44 [127].

Therefore, it is possible that the GVG Sp binds to CD147 receptors in cardiovascular tissue, in addition to other inflammatory ligands, resulting in chronic inflammation of the cardiomyocytes and degradation of the EnGL through activation of MMPs. In response to inflammatory stimuli, up-regulation of CD147 mediates leukocyte infiltration by binding to E-selectin. Furthermore, the GVG Sp can potentially act as a signaling molecule, affecting glycosylation, resulting in overexpression of CD147 and consequent cardiac maladaptive hypertrophy and remodeling, as well as increased oxidative stress and ferroptosis [126]. Several studies demonstrated that when SARS-CoV-2 Sp, administered to rodents as a soluble molecule or presented with a carrier, resulted in microvascular damage and induced inflammation [134] and hemagglutination [135]. CD147 has been shown to be involved in various cardiovascular diseases, including atherosclerosis and myocardial infarction, as well as kidney disease during both acute ischemic and chronic fibrotic injuries; and plays a role in pulmonary hypertension, neurological disorders, digestive tract vascular damage, and conjunctivitis [26,127,131].

Furthermore, CD147 plays pleiotropic molecular roles in various physiological conditions, such as spermatogenesis, fertilization, neural networks, and retinal development. It can result in pathological conditions, such as tumor progression, inflammatory response, plasmodium invasion, and rheumatoid arthritis, in addition to facilitating viral infection [126,130]. Endocytosis is a vital entry mode for viral infection, and Zhou et al. demonstrated that SARS-CoV-2 enters host cells through CD147-mediated endocytosis [126]. The assumption can be made that COVID-19 vaccines might cause an upregulation of CD147 due to stimulation of immune responses with consequent inflammation. Although circulating GVG Sp can cause pathological conditions by further upregulating CD147 expression and binding to the receptor, possibly entering host cells through endocytosis. Human platelets express CD147, and it has been observed that SARS-CoV-2 Sp causes platelet activation, aggregation, granule release, and expression of soluble P-selectin, as well as platelet extracellular vesicles (EVs) [135,136].

The involvement of GVG Sp with the various host factors and binding receptors can mediate many signaling pathways that contribute to pathology. It has been shown that SARS-CoV-2 Sp engagement with soluble or

cell membrane-attached ACE2 resulted in depletion of ACE2 from the cell surface, leading to an imbalance in the renin-angiotensin system with consequent inflammatory responses, increased oxidative stress, vasoconstriction, barrier dysfunction, lung injury, hypertrophy of cardiomyocytes and smooth muscle cells and/or thrombosis due to unopposed ACE2 and angiotensin-2-mediated effects [10,29,120,129]. Furthermore, Versteeg et al. demonstrated that expression of SARS-CoV Sp can induce endoplasmic reticulum stress, consequently triggering innate immune responses [137]. It has been documented that SARS-CoV-2 Sp is associated with increased degradation of IκB, resulting in NF-κB signaling pathway activation [138]. The GVG Sp is therefore potentially a potent stimulator of pro-inflammatory processes. More research studies are needed to establish how isolated circulating GVG Sp could affect the various receptors and consequent immune and inflammatory responses and signaling. It is important to note that the soluble GVG Sp can remain engaged with cellular receptors and other cofactors for much longer than the whole coronavirus, with consequent prolonged stimulation of intracellular signaling [134].

Chuang et al. [139] proposed that the Sp-ACE2 interaction can induce vaccine AEs by stimulating MAP4K3/GLK or other signaling molecules. MAP4K3/GLK may be involved in the pathogenesis of hypertension, diabetes, and cardiovascular disease [139]. It has been suggested that the shed Sp particles and the GVG Sp can promote pathology via interactions with the Ep- and EnGL. Biering et al. demonstrated that isolated full-length Sp and RBD from SARS-CoV-2 could mediate barrier dysfunction and vascular leak *in vivo* in an ACE2-independent manner [29]. Shed viral particles, or Sp, can act as 'viral toxins', mediating barrier dysfunction and promoting Sp dissemination and AEs. In a mouse model, it has been observed that administration of Sp into their lungs resulted in a systemic leak in the spleen and small intestine [29]. It seems probable that the binding of Sp to GAGs will cause GL degradation and that both shed GAG fragments and soluble Sp could lead to severe manifestations of disease (Figure 2). Even though Biering et al. and Robles et al. believe that the levels of GVG Sp circulating in patients following COVID-19 vaccination are too low (pg/mL) to trigger vascular leak and AEs [29,129], more research in this area is required. With booster vaccinations recommended every 60 to 90 days, higher circulating levels of GVG Sp can be expected. GVG Sp was no longer detected in the circulation after the second vaccination dose, presumably because Abs generated by the first vaccination quickly and effectively removed the small amounts of Sp reaching circulation [129]. Nonetheless, it would be a combination of circulating GL fragments, pro-inflammatory cytokines, ROS, and soluble GVG Sp that all contribute to various AEs (Figure 2), apart from the vaccine adjuvants and LNPs.

Trougakos et al. suggested that after vaccination, a cell can present the GVG Sp and its subunits, or peptide fragments, to the immune system to stimulate responses or be destroyed via cytotoxic T lymphocytes (T cells) [10]. They believe that the subsequent debris produced and the direct secretion of the transfected cells, including shedding of the Sp antigen, can release large amounts of the Sp and/or its subunits and peptide fragments, into circulation. When the LNPs of the COVID-19 mRNA vaccine are injected into the deltoid muscle, it can affect muscle tissue itself, the lymphatic system [11], and the spleen. However, Sp and its subunits and fragments can also collect in the liver and other tissues, from which it can enter the circulation and distribute throughout the body. The GVG Sp was observed in the plasma of Pfizer and Moderna vaccine recipients, on day 1 after the first vaccine injection, confirming the distribution of the Sp antigen throughout the body [10].

Therefore, an extensive range of interactions could be expected between the soluble free-floating GVG Sp and its subunits and peptide fragments, the pro-inflammatory cytokines, and the various binding receptors and other cofactors in the circulatory system and multiple organs. The fact that numerous AEs occur far from the injection site shows that the GVG Sp, or its production sites are systemically distributed. It is highly probable that some LNPs only release their genetic payload once it enters the systemic circulation. It was proposed that if the vaccinee produces a high amount of Sp at a speed that exceeds the capacity to produce nAbs, the GVG Sp can spread to various tissues throughout the body, including the brain, with the potential of causing inflammation, mitochondrial damage, and coagulopathies [140]. It was demonstrated that in adenovirus-vectored vaccines, the GVG Sp has the native-like mimicry of SARS-CoV-2 Sp's receptor binding functionality and prefusion structure. It is plausible that molecular mimicry may occur through additional interactions with

other proteins in circulation or even the presentation to the immune system of Sp antigenic epitopes mimicking human proteins [10]. Sp antigen is believed to trigger an autoimmune response to the cell itself. At the same time, it was demonstrated *in vitro* that some Abs against SARS-CoV Sp have the potential to mediate FcγRII-dependent entry into B lymphocytes (B cells), thus causing antibody-dependent enhancement (ADE) [141]. Then, there is the concern of possible development of anti-idiotype Ab against vaccination-induced Abs as a means of downregulation; anti-idiotype Abs - in addition to binding to the protective SARS-CoV-2 nAbs – can also mirror the Sp itself and attach to the various binding receptors, possibly triggering a wide array of AEs [10].

The electrostatic forces of the Ep- and EnGL will also play a major role in the binding capacity of soluble GVG Sp to the various receptors. Du Preez et al. indicated previously that an undersulfated GL, with overexpression of 3OST-3B, will facilitate SARS-CoV-2 Sp binding to the various receptors and negatively impact innate immune responses [21]. The Wuhan-strain SARS-CoV-2 Sp is hydrophilic with a grand average hydropathicity (GRAVY) score of -0.079 [142]. One could expect that the whole virus would have a higher negative net GRAVY score [143,144], which will be easier repelled by the negative charge of an intact, highly sulfated GL. However, soluble GVG Sp and its fragments would have a lower charge potential. Therefore, they should be able to penetrate the GL easier and have a higher binding affinity to the various receptors and other cofactors, stimulating immune responses and signaling pathways, with consequent degradation of the barrier functions of the GL [29]. It should be noted that both positive values exist in the Sp domain, while other Sp domains showed negative GRAVY scores [142]. Also, with more Sp mutations, higher negative GRAVY score values could be expected [145], with consequent increase in receptor binding and/or Ab resistance [128] when the GL is compromised. The GVG Sp-induced degradation of the Ep- and EnGL will result in the release of various enzymes, GL fragments, chemoattractants, pro-inflammatory cytokines, ROS and exposure of adhesion molecules, leading to vascular leakage, dissemination of the GVG Sp, clot formation, inflammation, and leukocyte infiltration [10,28,129,135].

These pathological conditions of systemic inflammation will trigger the release of histones, which can exert further cytotoxic activity on the EnGL. There is a reasonable probability that GVG Sp and LNPs induce neutrophil extracellular traps (NETs) after vaccination via a ROS-dependent mechanism [96,146], apart from GVG Sp binding to innate immune receptors [26,147]. SARS-CoV-2, as well as sera from patients with COVID-19, was found to directly triggers NET formation [146]. Neutrophils from these patients seemed activated and primed, making them more sensitive to NET stimuli. This pro-NETotic phenotype can probably continue for a while after vaccination. NETosis has been associated with the formation of thrombosis in patients with vaccine-induced immune thrombotic thrombocytopenia (VITT) [5]. Given that HS plays an important role in modulating the cytotoxic effects of histones within NETs and coagulation [34], it can assume that undersulfation of GAGs would contribute to the hyperinflammatory response and abnormal coagulopathy seen in vaccinees with serious AEs, which needs to be further investigated.

The GVG Sp appears to be highly toxic on its own. Shed GVG Sp has been detected in multiple organs. There is evidence that the GVG Sp can penetrate ovaries, testes, brain, spinal cord, nervous system, heart, lungs, intestines, kidneys, and cross the placenta in pregnant women [118]. The GVG Sp has been found in exosomes four months after the second dose of COVID-19 vaccination [148]. This long persistence of the GVG Sp raises the possibility of sustained inflammation and organ damage. There is a high probability that the circulating hydrophilic GVG Sp affects gene expression and can act as an endocrine disruptor. Fernandes et al. demonstrated through *in vivo* toxicological studies in zebrafish that isolated recombinant SARS-CoV-2 Sp caused adverse effects on the liver, kidney, nervous and reproduction system [149]. Therefore, the isolated Sp and its fragments can interact with HS and various receptors and ligands, mediating and inhibiting various cell signaling and inflammatory pathways [21,149], with pathological consequences.

In summary, the GVG Sp can cause AEs in multiple ways, including 1) signaling and inflammatory cascades; 2) by triggering vascular leak and barrier dysfunction; 3) molecular mimicry with human proteins or as a ligand for various receptors and co-receptors; and 4) by producing NETosis.

4.2 Breakthrough infections and lung diseases

The capacity of the COVID-19 vaccines to protect against SARS-CoV-2 infection and spread is still a matter of debate [150-152]. While various studies demonstrated the efficacy of COVID-19 vaccines against SARS-CoV-2 infection, with less severe symptoms, a reduction in COVID-19-related hospitalization and deaths [153-156], the effect of the vaccines on all-cause mortality rates was not effectively determined. In the initial phase III randomized control adult trials of the Pfizer and Moderna vaccines, 37 people died in the vaccine group, compared to 33 in the placebo arm. This indicates that neither of the vaccines decreased or increased the absolute risk of death by more than 0.08% [157]. Although the study by Ioannou et al. confirmed the very high effectiveness of mRNA vaccines against COVID-19–related death, it did not look at all-cause mortality rates. Furthermore, they found that the effectiveness of the COVID-19 vaccine against SARS-CoV-2 infection was substantially lower than previously reported [153], while several studies indicated that the immunity conferred by the COVID-19 vaccines wanes over time [158,159].

Most published research determining the effectiveness of the COVID-19 vaccines was short-term observational studies with known limitations. Fung & Doshi [160] and Høeg et al. [161] pointed out the bias in the initial observational studies, which overstated the effectiveness of the COVID-19 vaccines. To accurately determine the effectiveness of COVID-19 vaccines, all-cause mortality and the risk of severe AEs, as the most objective and important outcomes, should be determined in long-term quality randomized controlled trials. There is no point in being saved from COVID-19 through COVID-19 vaccination, but dying of a heart attack or crippled lifelong with a neurological disease due to an AE from the vaccines. This review paper focuses on understanding the pathophysiology of documented severe AEs experienced by many vaccine recipients.

Notwithstanding, despite high vaccine coverage, various cases were reported of vaccinated people who became infected with SARS-CoV-2, with breakthrough infections appearing within the first two weeks to several months after injection [150,162]. In a retrospective large Israeli cohort study, conducted in individuals who received two doses of the Pfizer mRNA vaccine, protection appeared to decrease over time and the risk of breakthrough SARS-CoV-2 infection progressively increased. The SARS-CoV-2 infection rate was 4.7% for unvaccinated individuals, compared to a 9.6% infection rate among vaccinated individuals without evidence of past infection [163]. Azzi et al. found that mRNA vaccines induce systemic humoral immunity against the virus, but are not very efficient in promoting specific immunity at the level of the mucosae, the leading viral route of entry [150]. However, seeing that mRNA vaccines appear to demonstrate effectiveness in reducing symptomatic and severe COVID-19, it indirectly indicates their impact on mucosal transmission.

Irrgang et al. found that anti-spike IgG4 Abs and IgG4-switched memory B cells increased after several months post-vaccination with the second Pfizer dose, where the response was boosted after a third vaccination [162]. It has been described that IL-4, together with IL-10, can switch to IgG4. When these Th2-associated cytokines are seen to switch to IgG4, it may negatively impact the anti-inflammatory M2 resolving macrophage phenotype, thereby skewing the immune response. Furthermore, IgG4 has a lower potential to mediate FcγR-dependent secondary effector functions to clear viral infections, indicating a less effective Ab response. Current COVID-19 vaccination regimens do not confer sterilizing protection, as evidenced by the many breakthrough infections caused by the Omicron variant [162]. An IgG4 response can be pathogenic or protective, where IgG4 Abs can lead to cancer and serious illness in several autoimmune disorders [164]. More research is needed to determine the significance of IgG4 Abs in vaccine-induced immunity and viral clearance.

In a previous review, du Preez et al. provided a comprehensive overview of the effect that an undersulfated and degraded lung EpGL would have on susceptibility to SARS-CoV-2 infection [21]. In addition to the GVG Sp, the LNPs, genetic material, pro-inflammatory cytokines, and ROS may cause Ep and En degradation [29], as well as result in depletion of GSH and, consequently, inorganic sulfate, thereby decreasing sulfation ability [98]. Upon inflammation and with an undersulfated EpGL, 3OST-3B would be overexpressed, facilitating the binding of SARS-CoV-2 Sp to the ACE2 receptors (Figure 1). Apart from compromising the EpGL, the first-line of defense, undersulfation also negatively affects innate immune responses. The bactericidal activity of neutrophils and

their recruitment, are influenced by the sulfation pattern of HS. Inactivation of heparan sulfate 2-O-sulfotransferase (HS2ST) in neutrophils substantially reduced their bactericidal activity, increasing susceptibility to systemic infection [34]. Furthermore, GSH depletion has been associated with impaired immune function, especially affecting T cells and macrophages [138]. This is probably associated with the high incidence of secondary infections and viral reactivation in vaccinated individuals. Several cases of herpes zoster [165] and persistent varicella-zoster viral infection [166] have been reported following COVID-19 mRNA vaccination.

Lung macrophages, which reside in the interstitium and alveoli, are recruited by inflammatory stimuli. Various stimuli, such as TNFα and IL-1, were found to affect HS configuration and sulfation in M1 pro-inflammatory macrophages. This resulted in up-regulation of 3OST-3B, with a consequent increase in 3-O-sulfation and hypoxia, which in turn significantly reduces the expression of biosynthetic enzymes and the total HS content [167], thus increasing susceptibility to infection and inflammation. Multiple vaccine platforms and viral infection were shown to induce SARS-CoV–specific immune responses that enhanced lung inflammation, following homologous challenge in mice and African green monkeys [168-170]. Liu et al. previously identified epitopes in the Sp that elicited both nAbs and Abs that enhanced SARS-CoV infection [171].

Chronic inflammation induced by the vaccine platform and excessive ROS generation, may favor the activation of M1 macrophages, producing uncontrolled pro-inflammatory mediators and persistent injury [172]. Indeed, Liu et al. showed that despite markedly reduced SARS-CoV titers, anti–S-IgG caused lung injury during the early stages of infection by abrogating an M2 wound-healing macrophage response and TGF-β production, while promoting pro-inflammatory cytokine IL-8 and accumulation of MCP1 production and inflammatory M1 macrophages [171]. Li et al. also demonstrated that Sp-activated platelets skewed monocytes toward the M1 macrophage phenotype, with increased TNFα levels, bacterial phagocytic activity, and reduced healing capacity [173]. Furthermore, chronic inflammation alone can alter EnGL responsiveness through changes in HS composition and degree of sulfation [174]. Overexpression of 3OST-3B over-expression in inflamed cells of the compromised lung was indicated to enhance Sp fusion with cells, even without binding receptors, thus increasing the chances of infection [21].

Furthermore, it was observed that the expression of 3OST-3B is up-regulated in many cell types exposed to inflammatory stimuli, such as monocytes and macrophages, fibroblasts, and EnCs. Chronic inflammation is therefore a predisposing factor to viral infection, and more so, the pro-inflammatory state induced by the entry of viral or GVG Sp into the cell, will potentially further upregulate the expression of 3OST-3B, creating a vicious cycle that increases infectivity and aggravates the cytokine storm [21]. In vivo, SARS-CoV-2 Sp has been shown to activate macrophages, plus contributed to the induction of acute lung inflammation in mouse studies. While in transgenic mice overexpressing human ACE2, intratracheal instillation of the S1 subunit induced severe COVID-19-like acute lung injury and inflammation [10].

Reactivation of other viruses after COVID-19 vaccination, such as varicella-zoster virus [166], can further be explained by the fact that the GVG Sp promotes a pro-inflammatory activation profile on the most potent antigen-presenting cells (APC), such as the dendritic cells (DCs). Consequent overexpression of 3OST-3B and binding receptors, such as ACE2, CD147, and CD4, will facilitate infection of T helper lymphocytes [10]. Therefore, infected immune cells, such as CD4+ T cells, can also be a source of transmission of SARS-CoV-2 throughout the body and result in lymphopenia. A degraded GL allows immune cells to spread through the circulatory system, and undersulfated immune cells will facilitate better binding and spread of the virus and GVG Sp [175]. The failure of innate immune mechanisms, such as natural killer cells that typically control viral infections, is indicated by unregulated viral replication. Furthermore, viruses, bacteria, microbial fragments, and pro-inflammatory cytokines, such as IL-1β and TNFα, induce the expression of defensins in various cells [176]. The immunomodulatory action of defensins can negatively impact the function of the Ep barrier, thus contributing to viral dissemination and infectivity through increased uptake of viruses via stromal fibroblasts and recruitment of susceptible target cells [175,177].

Furthermore, the GVG Sp can activate the Raf/MEK/ERK signal transduction pathway in cells of the vaccinee. The ERK1/2 signaling pathway plays a crucial role in viral replication and triggers the induction of cyclooxygenase-2, an inflammatory prostaglandin synthetase. Increased inflammation will up-regulate 3OST-3B and expression of the CD147 receptor, resulting in increased susceptibility to SARS-CoV-2 infection [21,134]. Chuang et al. demonstrated through *in vitro* and *in vivo* studies that the SARS-CoV-2 Sp induces serine/threonine kinase MAP4K3 (also known as GLK) overexpression in epithelial cells (EpCs), which can facilitate infection of otherwise less susceptible cells. GLK overexpression in EpCs is positively correlated with COVID-19 severity [139].

4.3 Circulatory problems: Thrombosis, stroke & vasculitis

Damage to the GL substantially increases intravascular adhesion of leukocytes and platelets [167,178], while rolling leukocytes, lymphocytes, and platelets along the vessel wall are supported by an adequate sulfated GL. However, the binding of chemokines and cytokines to the EnGL may represent sequestration, inactivation, or local concentration for presentation to rolling cells [179]. The transcytosis process depends on HS expression. However, the nature of these interactions has been challenging to unravel, as HS is present in both EnCs and immune cells, such as leukocytes and macrophages [167,180]. HS's role in regulating the signaling of various inflammatory mediators through their cell surface receptors is dual functional: they either promote the ligand interaction with its receptor or inhibit receptor–ligand interactions. For example, the EnGL keep neutrophils away from En adhesion molecules.

However, the barrier function of the EnGL can be overcome through mechanical compression by neutrophils and LNPs; and with a protein corona traversing capillaries that are smaller than their own diameter [181]; or shedding of the EnGL through the action of ROS and released enzymes [21]. Modifying HS sulfation patterns and EnGL shedding is important in the regulation of leukocyte / neutrophil rolling in the arterial system. Furthermore, experiments have shown that EnGL shedding and reduced HS sulfation can diminish neutrophil arrest [174]. The systemic knockout of Ndst-1 is lethal in mice, but En inactivation resulted in an impaired response in various inflammatory models. Ndst-1-deficient EnCs showed reduced HS sulfation, reduced IL-8 chemokine presentation on the EnGL, and impaired neutrophil arrest [174]. Another study showed that altering the 2-O-sulfation of uronic acids in HS can enhance inflammation and neutrophil recruitment. The HS2ST enzyme was inactivated in EnCs, resulting in decreased 2-O-sulfation, with increased N-sulfation and 6-O-sulfation of HS. In the mutant, the rearrangement of sulfated sequences resulted in a gain-of-function phenotype, characterized by increased inflammation in the animals. Furthermore, IL-8 exhibited improved binding to the EnGL, leading to the observation of increased neutrophil arrest [174]. Therefore, the response to inflammatory stimuli, shear stress, or tissue damage can alter the configuration and degree of HS sulfation, thus regulating immune cell infiltration. The relevance of the variation of the HS structure in different tissues extends to chemokine binding. For example, aortic and venous EnCs exhibit different degrees of sulfation in their HS, resulting in the formation of chemokine binding sites exclusively in postcapillary venules and small veins where leukocyte migration occurs, while capillaries and arteries lack such sites [167].

During COVID-19, it was observed that an HSA-nutrient deficit could result in degradation of the EnGL, which can improve infection across barriers, such as the gut and BBB [123]. Since Cys is a rate-limiting precursor to HSA and inorganic sulfate [98], hypoalbuminemia may correlate with an undersulfated GL (Figure 1). An undersulfated EnGL will result in a hyperinflammatory response, vascular permeability, and the shedding of GL components, leading to a procoagulant and antifibrinolytic state, with eventual multiple organ failure [21]. Given that GVG Sp and LNPs can induce inflammation and ROS [96], one could expect a global depletion of GSH in vaccinated individuals [182].

Furthermore, HSA is the main component in the regulation of interstitial pressure and is the primary transporter of endogenous and exogenous ligands. It is a large transport protein that binds to nutrient ligands in the intestine and liver and then transports ligands to the small capillaries and interstitial spaces. HSA has a high binding affinity for the GVG Sp and therefore LNPs [99,102,183], and hypoalbuminemia could be expected in

individuals vaccinated with the COVID-19 vaccines [184,185]. A 20% drop in normal HSA levels can cause physiological damage in healthy individuals [123]. In addition to HSA depletion due to increased oxidative stress, oxidative damage can also impair the binding properties of HSA.

Moreover, the potential binding of the GVG Sp, cytokines, Abs and LNPs to HSA, will decrease available nutrient-HSA binding. HSA carries important ions such as Ca^{2+} and heme, so hypoalbuminemia would exhibit a procoagulatory phenotype and can result in venous thromboembolism [186]. Furthermore, reabsorption of HSA in the kidney is usually through clathrin-mediated endocytosis, which thus requires binding of HSA to clathrin. Therefore, any ligand that competes with clathrin for the binding of HSA will change this equilibrium and allow HSA to pass into the urine, further exacerbating hypoalbuminemia [123]. Moreover, the biomolecular corona formed in LNPs can activate coagulation cascades [102] (Figure 3). As is expected in case of long-COVID, the immune-inflammatory processes and decreased antioxidant defenses in vaccinated individuals are accompanied by elevated neutrophils, with reduced HSA, GSH, and inorganic sulfate [187]. This aspect needs further research.

Nonetheless, these oxidative stress pathways will negatively impact the vascular endothelium and result in long-term nonresolving inflammatory processes. Biering et al. found that the Sp alone can mediate En dysfunction and vascular leak [29]. Likewise, it was observed that *in vitro* treatment of vascular EnCs with plasma of COVID-19 patients induced endotheliopathy [188,189], and it is plausible that nonstructural proteins of SARS-CoV-2 might also mediate these effects, apart from activated platelets, enzymes and cytokines [190]. Therefore, increased serum levels of syndecan (sdc)1, HS, and HA in the acute phase of vaccine-induced AEs [5,25], due to dysregulation of the immune response and consequent EnGL degradation can be expected [28]. Seeing that vaccine recipients with only brief and mild symptoms showed elevated levels of sdc1, compared to a 2-fold increase in sdc1 level in VITT patients, it is clear that some initial endotheliopathy has developed, which then increased significantly in the VITT group [5]. As in the case of severe COVID-19 disease, therefore, increased levels of soluble En activation markers, such as sdc1, endocan, selectin and angiopoietin-2, can be expected in vaccine recipients with severe AEs [30,190]. The effect of DNA and mRNA vaccines on the endothelium warrants further research. Angiopoietin-2 can induce the EnGL to secrete heparanase (HPSE), contributing to EnGL shedding and leading to increased vascular leakage and leukocyte diapedesis [191,192]. Angiopoietin-2 levels will be positively associated with increased CRP and D-dimers, the latter possibly reflecting the link between vaccine-induced coagulopathy and En dysfunction. There may also be a positive correlation in kidney microthrombosis between elevated serum creatinine levels and increased D-dimer levels [193].

An increased risk of thrombotic events, including cerebral venous sinus thrombosis, was reported after vaccination with specifically AstraZeneca and J&J adenovirus-vector vaccines [5,43,54,136,194]. At the same time, various cases of thrombocytopenia have been observed in COVID-19 vaccine recipients [14,195]. Collectively, this kind of vaccine-induced AE is designated VITT. A suggested mechanism for VITT was that vaccine constituents, such as viral DNA and/or cellular proteins, can favor the formation of Abs against platelet factor 4 (PF4), thus promoting VITT [43,194]. In response to infections, activated platelets release the small chemokine protein PF4. Although its primary physiological function is to promote blood coagulation, this chemokine is also involved in innate and adaptive immunity when a stimulus activates platelets. Although it seems that VITT resembles heparin-induced thrombocytopenia (HIT), the antigenic target on PF4 differs between HIT and VITT [196].

It was initially reported that VITT and HIT are relatively rare and develop only in a small subset of patients who produce pathogenic anti-PF4 Abs. In vaccine recipients with a genetic variation of class II HLA, one could expect the production of IgG-specific anti-PF4/P and the accelerated generation of thrombin caused by antigen-Ab complexes consisting of PF4, polyanion, and IgG anti-PF4/P-reactive Abs [136]. Many cases of thrombocytopenia have been reported to appear within two weeks of receiving the DNA and mRNA COVID-19 vaccines [190,197-199]. It is noteworthy that adenoviruses themselves can naturally induce thrombocytopenia at a low frequency and it would be essential to analyze all vaccine ingredients that could be associated with VITT [190,195]. Endotheliopathy will probably also play an important role in VITT, while the Sp binds to the same binding

domain as AT in the GL [5,21,195,200]. AT may also be bound to protein coronas [100], further inhibiting its functionality as an anticoagulant. Several reports indicated that S1 could directly induce coagulation by competitive binding to soluble and cellular HS [10]. The interaction between the Sp and HSPGs on the cell membrane can also disrupt factor H protein, which serves as a negative regulator of the complement alternative pathway, thereby triggering an inflammatory response mediated by the downstream C3c convertase protein [195]. Therefore, the full-length GVG Sp could trigger the complement pathway leading to thrombotic events, while its interaction with multiple membrane components might induce thrombocytopenia. Platelets bound in LNP-protein corona (Figure 3) will also be a factor in the development of thrombocytopenia. In the classic model of HIT, PF4 interacts with EnC HSPGs, thus displacing AT. When PF4 binds to HSPGs, it induces a conformational change in PF4, exposing a new antigen. This, in turn, leads to the generation of IgG autoAbs against this complex. The complex formed, comprising heparin (HP), PF4, and auto-Abs, subsequently cross-links numerous FcγRIIa receptors in platelets and monocytes, triggering intracellular signaling, thromboxane biosynthesis, as well as platelet activation and aggregation [190]. The activated platelets degranulate, releasing more PF4 molecules and procoagulant microparticles in the plasma, leading to increased thrombin generation. Moreover, the anti-PF4 Ab activation of monocytes and EnCs leads to accelerated thrombin generation through expression of tissue factor. This can result in hypercoagulation and potentially life-threatening thrombosis [195,196]. It was also demonstrated that the Sp binds the blood coagulation factor, fibrinogen, while the Sp can enhance fibrin-mediated microglia activation and induce fibrinogen-dependent lung pathology in mice [10]. More studies are needed to evaluate the effect of the GVG Sp on coagulation.

Leung et al. demonstrated that VITT was induced by anti-PF4 Abs and mediated by platelet and neutrophil activation and NETosis [194]. NETs are highly prothrombotic *in vivo*, aggregating with platelets and the activated endothelium to form microthrombi, occluding the vasculature, and further perpetuating inflammation. Therefore, there are important interrelations between platelet aggregation, increased neutrophil recruitment, NET formation, and thrombin activation within the microvasculature. While activated neutrophils and platelets have been shown to induce NETosis, NET components can further regulate platelet and neutrophil function. NETosis promotes venous and arterial thrombosis by showing an important procoagulant and prothrombotic activity. They can activate platelets and other immune cells, damage EnCs, and activate blood coagulation pathways [194]. NETs further enhance coagulation by providing a scaffold for platelets, red blood cells, von Willebrand factor (vWf), and tissue factor. This creates a positive feedback loop, resulting in a hypercoagulable state and thrombosis [196]. NETosis is therefore present in patients with active VITT, where NET levels positively correlate with the severity of AEs [5]. It has been demonstrated that VITT IgG triggered a significant increase in DNA release to form NETs to control IgG. Confocal microscopy imaging of thrombi from healthy donors' whole blood, following treatment with VITT IgG, confirmed that platelets, fulminant neutrophil activation, and extracellular DNA formed the thrombi. Data from this study suggested that VITT IgG is primarily responsible for thrombosis and NETosis *in vivo* [194]. Platelet-neutrophil interactions have been proposed to be at the center of the VITT pathology, with platelet-neutrophil aggregation, NETosis, plus platelet EV activation and generation. EV tissue factor and increased D-Dimer levels would correlate with severity, thrombosis, and mortality in VITT [136,194]. Thrombosis and thrombocytopenia in VITT are two distinct processes, where VITT Abs induce thrombocytopenia by binding to platelet FcγRIIa [194]. Hetland et al. found a robust negative correlation between the severity of AEs and platelet counts in COVID-19 vaccinees. In addition, there are negative correlations between inflammatory marker levels and platelet counts [5].

Neutrophils and NETs are present in thrombi in various conditions, such as stroke, acute myocardial infarction, and deep vein thrombosis. It was speculated that the adenovirus in DNA vaccines and/or the GVG Sp could have triggered pronounced inflammatory processes in VITT patients, including NETosis [194,201]. It was also demonstrated that purified recombinant SARS-CoV-2 Sp S1 subunits can elicit unconventional CD147-dependent platelet activation, increasing the risk of thrombosis in various organs [131,134,135,201]. The GVG Sp can play a role in VITT in the same way [195], while binding of S1 to platelet ACE2 receptors triggers its aggregation [10]. The GVG Sp has the potential to activate platelets by binding to TLRs, favoring the occurrence of thrombosis-related cardiovascular events. Activated platelets interact with circulating monocytes, stimulating the release

of pro-inflammatory cytokines. Li et al. demonstrated that such crosstalk indeed occurred, where they found that the Sp, both soluble or as part of a virus envelope, induced platelet activation through engagement with CD42b, in addition to the other receptors, such as PF4, CD147 and CD26 [202]. It was further demonstrated that the Sp competitively antagonizes vWf binding to CD42b and interferes with platelet adherence to vWF. Platelets actively participate in both hemostasis and immune regulation. Sp-activated platelets induced monocyte differentiation to a pro-inflammatory phenotype [173].

Activated platelets degranulate and express various membrane receptors, enabling them to bind through P selectin to circulating leukocytes. This induces monocyte differentiation toward a pro-inflammatory phenotype, featuring a higher expression of CD86, HLA-DR, and IL-1β [173]. VITT, resulting from platelet activation and aggregation, was reported after injections of both DNA and mRNA COVID-19 vaccines [136,196]. It was found that both the AstraZeneca and Pfizer vaccines could elicit anti-PF4 Ab production, even in recipients without clinical manifestation of thrombosis [10]. To abolish VITT IgG-induced thrombosis, one would need to inhibit platelet and neutrophil activation by blocking FcγRIIa, or NETosis and aggregates. It seems probable that an undersulfated GL in VITT will impede *in vivo* modulation of these pathological events [21]. Moreover, extracellular RNA has been established to promote the activation of coagulation proteases. At the same time, different forms of eukaryotic and prokaryotic RNA serve as promoters of pathological blood coagulation and thrombus formation [203]. More research is needed to establish the possible role of injected mRNA in promoting thrombosis.

Cell-derived EVs might also play a role in VITT. Changes in local cytoskeletal rearrangement can result in the budding of the plasma membrane and EV production. EV shedding will be facilitated through various pathological conditions, such as inflammation, coagulation, or activation of the complement cascade, and increased shear stress. It is important to note that EVs have a bilayered phospholipid structure, which exposes coagulant-active phosphatidylserine. It also expresses various membrane bioactive receptors that interact with the coagulatory, complement and immune systems [136].

Cerebral venous sinus thrombosis (CVST), a rare form of cerebrovascular disease, has been reported as an AE and appears to occur with a 4-fold higher frequency in AstraZeneca vaccinees, compared to those who received the J&J vaccine. There have also been reports of CVST cases who received the mRNA vaccines, but the risk of arterial thrombosis in recipients of adenovirus-based vaccines is higher [196,201]. The most common thrombotic event associated with VITT appears to be CVST. However, vaccinees have presented other thrombotic forms, such as venous thromboembolism, abdominal vein clots, and arterial clots [196]. One hypothesis explaining the higher incidence of thrombosis observed in DNA vaccine recipients is that mRNA transcription is more prone to aberrations, resulting in abnormally shorter GVG Sp that is soluble and can easily migrate to the bloodstream [201].

4.4 Myocarditis

Myocarditis, defined as inflammation of heart muscle cells, identified as the infiltration of mononuclear cells into the myocardium, can be caused by infection of various pathogens, or noninfectious triggers, such as toxins and hypersensitivity reactions. Among these triggers, viral infection is the most common cause of myocarditis, particularly in children and young adults. Viral myocarditis can progress to dilated cardiomyopathy, leading to heart failure and cardiac death [204-206]. Myocarditis may affect focal or diffuse areas of the myocardium and can be acute, subacute, or chronic. The immune response is complex, and it would be important to differentiate between a profibrotic response and a healing inflammatory response. Diagnosis is challenging, since the clinical manifestations of myocarditis are heterogeneous, ranging from virtually asymptomatic states or vague signs and symptoms to severe myocardial destruction resulting in cardiogenic shock and arrhythmias. A consistent diagnostic approach is needed to characterize myocarditis [205]. Therefore, the number of patient cases of COVID-19 vaccine-induced myocarditis can be significantly underestimated. Nevertheless, myocarditis in adolescents [13,121,125,207,208] and elderly [209], as well as pericarditis or myopericarditis [13,210,211], has been diagnosed clinically in many recipients of the various COVID-19 vaccines. Yu et al. observed impaired LV

and RV myocardial deformation and persistent late gadolinium enhancement (LGE) in a subset of patients vaccinated against COVID-19 up to 1-year follow-up. The growing evidence indicates a worse prognosis with altered myocardial deformation and LGE in patients with myocarditis [212].

CD147 has been shown to be involved in the development and progression of various cardiovascular diseases, such as atherosclerosis, ischemic cardiomyopathy, and heart failure [130,213]. Since CD147 is expressed in both cardiomyocytes and EnCs, the binding of GVG Sp to these receptors [134,202] may correlate with hemodynamic instability and cardiovascular abnormalities observed in COVID-19 vaccinees. Zhong et al. demonstrated that in response to pressure overload, overexpression of cardiac CD147 promoted cardiac maladaptive hypertrophy and remodeling, along with increased oxidative stress and ferroptosis [130]. Apart from overexpressed CD147 resulting in oxidative stress, it is known that LNPs can also generate excessive ROS [62]. It has been well established that ROS overproduction, resulting in oxidative stress, is a crucial trigger during the pathogenesis of cardiac hypertrophy and the transition to heart failure. In cardiovascular disease, excessive ROS has been shown to cause protein denaturation, lipid peroxidation, DNA damage, and eventual cell death [214]. ROS can directly impair the heart's contractile function by oxidizing proteins central to excitation-contraction coupling. Lipid peroxidation will result in membrane destabilization and ferroptotic cell death, a pathological process associated with ROS-induced heart tissue injury [130,215].

Sp involvement is irrefutable because acute pericarditis and myocarditis, or myopericarditis, were also observed in predominantly men and young people with COVID-19 [215]. In fact, adolescents who developed myocarditis after mRNA vaccination had markedly higher levels of full-length free Sp in their plasma, compared to asymptomatic vaccinated control subjects without detectable free Sp [17]. Avolio et al. found that the shed Sp was more abundant than the whole SARS-CoV-2 particles in COVID-19 patients' serum [134]. It was observed that shed Sp disrupted human cardiac pericyte function and triggered increased production of pro-apoptotic factors in pericytes, resulting in the death of EnCs. The GVG Sp can therefore act as a ligand to induce noninfective cellular stress. To support of this, administration of the Sp promoted dysfunction of human EnCs through increased expression of vWf and CD147 [10,134]. Expressed CD147 promotes MMP activation and will consequently result in considerable EnGL degradation, further exacerbating inflammatory responses, ROS generation and vascular NO abnormalities, resulting in a procoagulant and pro-inflammatory phenotype of the endothelium [17,21,216]. Dursun et al. reported that COVID-19 vaccine-induced acute pericarditis exhibited pleuritic chest pain, pericardial effusion, increased white blood cell count, and increased CRP levels, while serum troponin levels were around normal [215]. However, unlike classical stages of acute pericarditis ECG, vaccinated patients showed no ECG changes. Elevated D-dimer and serum troponin levels were also observed in vaccine-induced acute myocarditis and myopericarditis. Their ECG findings were correlated with those who have stage 1 acute pericarditis [215]. These conditions were typically observed within three days after mainly the second dose of an mRNA vaccine, and mainly in younger and male adults. The prevalence of young adults has been defined in various studies [215,217-219]. Oxidative stress was the underlying cause of these conditions, with low NO levels indicating the inflammatory and procoagulant state in mRNA vaccine-induced heart inflammation [215].

It is well established that the pathogenesis of viral myocarditis is caused by direct virus-mediated injury and/or a toxin and indirect damage through secondary immune and autoimmune responses, as well as influenced by the oxidative state of the host [205,220]. It is very plausible that the GVG Sp and LNPs would stimulate the release of pro-inflammatory cytokines, but that an undersulfated and degraded GL will not be able to modulate the inflammatory response [134], which, together with oxidative stress, will contribute to the pathogenesis of cardiomyopathy [206]. Salah and Mehta speculated that high expression of ACE2 in the heart will facilitate the interaction between HS and the Sp, resulting in HS consumption that will reduce AT activation and anti-inflammatory activity. This may result in endothelialitis, subsequent En injury, and intracardiac thrombus formation [200,221]. However, the fact that the GVG Sp would likely bind to the same binding domain as AT will probably have a greater effect on inflammation and coagulation, in addition to the role of the other Sp binding receptors and the increase in oxidative stress. Avolio et al. demonstrated that the CD147 receptor, not ACE2,

directs Sp signaling in cardiac pericytes [134]. The expression of CD147 was shown to be increased in a variety of cardiovascular diseases, which could serve as compensation for any age or disease-related reductions in ACE2 during viral infections [213].

Nonetheless, Lehmann et al. pointed out that binding of the GVG Sp to ACE2 receptors can induce RAAS activation, particularly an increase in angiotensin-2, resulting in angiotensin-2/NA-dependent acute vasoconstriction, and the progression of inflammatory, fibrotic, and thrombotic processes [120]. A histopathological examination of two teenage boys who died suddenly after their second Pfizer vaccination revealed stress cardiomyopathy caused by catecholamine-induced myocardial injury, which differed from typical findings of myocarditis [120,125]. It is plausible that catecholamines are not deactivated by sulfation. Innate immune responses may also result in excessive caspase-1 activation, resulting in various pathological conditions, such as cardiovascular disease and increased viral infection [204].

The coxsackievirus-adenovirus receptor, an adhesion molecule located predominantly in the heart, is required for viral entry into different cell types. The young adult heart has relatively high levels of coxsackievirus-adenovirus receptors, which may partially explain the increased susceptibility of young adults to myocarditis [205]. However, seeing that COVID-19 vaccine-induced myocarditis predominantly affects young males [13,17,205], other factors would probably play a role. Animal studies indicated that sex differences in TLR signaling play an essential role in differential susceptibility to viral-induced myocarditis [205]. Sex differences in innate immune responses have been observed, where an M1 activating immune response was favored in male mice, compared to an M2 resolving response in female mice [172].

Various studies found an increase in oxidative stress in patients with dilated cardiomyopathic heart failure, with a decrease in circulating GSH concentrations [97]. The fact that myocarditis accounts for 5% to 12% of sudden deaths of young athletes [172], indicates that oxidative stress is probably the main etiological factor underlying myocarditis (see Section 4.5). Research has revealed that males are more susceptible to oxidative stress and possess a lower antioxidant capacity compared to females. Some researchers attribute these sex differences to estrogen, which is known to facilitate the activation of antioxidant systems and regulate the expression and activity of various antioxidant enzymes [97]. However, it is important to note that high testosterone levels, alcohol, smoking, recreational drugs, and certain medications will deplete GSH, altering the redox status [21,222]. It is known that the GSH status of an individual is essential to protect against toxicity. During puberty, there is an approximate twenty-fold increase in endogenous testosterone in males, while there is only a modest increase in females. Testosterone has been shown to decrease cystathione β-synthase (CBS) activity (Figure 1), decrease GSH concentrations, and increase susceptibility to oxidative stress [223]. In steroidogenic cells, one can expect ROS production to be particularly high, since steroid hydroxylations by cytochrome P450 enzymes produce ROS [224], in addition to the mitochondrial electron transport chain.

Furthermore, after biosynthesis, hydrophobic steroids undergo sulfation to facilitate their circulatory transit. Mueller et al. gave a good account of steroid sulfation and desulfation [224]. Most dihydroepiandrosterone (DHEA) is stored as DHEA sulfate, the inactive form, but with decreased levels of inorganic sulfate, DHEA is preferentially converted to testosterone instead of DHEA sulfate [223]. Moreover, higher testosterone levels will not only deplete GSH. It may also reduce available inorganic sulfate levels, exacerbating inorganic sulfate deficiency and altering EnGL sulfation (Figure 1) and steroid hormone sulfation. Therefore, high testosterone levels can stimulate pro-inflammatory cytokines in the vasculature and generate ROS in vascular smooth muscle cells, consequently decreasing NO bioavailability, resulting in increased blood pressure and renal dysfunction, activation of vasoconstrictor signaling pathways, and increased vasoconstriction [222].

Men have been shown to have lower plasma levels of reduced GSH than women, making them more susceptible to oxidative stress and inflammation [225]. Both epidemiological and immunological evidence indicate that steroids can influence the pathogenesis of various chronic inflammatory diseases. However, research on the cardiovascular actions of testosterone is still controversial, showing its effects as protective

to deleterious. Although numerous studies have shown an increased cardiovascular risk and mortality with testosterone deficiency, testosterone therapy has been verified to attenuate cardiovascular risk factors and cardiovascular outcomes [222]. However, high oxidative stress has been associated with adverse testosterone effects, while low oxidative stress is associated with cardioprotective testosterone effects [97].

It also seems that testosterone's positive or negative effects on the heart may depend on the testosterone levels and whether or not testosterone is acting through a nuclear receptor. However, the role of testosterone in the regulation of oxidative stress in cardiomyocytes is far from clear, where testosterone can act as an antioxidant or pro-oxidant [97]. Indeed, a positive correlation has been observed between GSH and testosterone levels. Depletion of the intracellular GSH pool, both in young and old cells, has been found to significantly decrease testosterone production [226]. It is important to consider the regulation of sulfatases (SULFs) by inflammatory mediators, since sex steroids play a role in immune function and inflammatory processes, where SULF activity is often dysregulated and associated with inflammation. For example, in the vascular smooth muscle cells from patients with atherosclerosis, SULF expression was found to be higher in females with mild atherosclerotic changes, compared to severe disease and in male aortas. However, the counterpart of SULF, estrogen sulfotransferase (SULT-1E1), was lower in women with severe disease, indicating the importance of the SULT/SULF ratio in the local regulation of steroid formation in states of inflammatory diseases [224].

Rienks et al. revealed that the ECM protein, SPARC, regulates inflammation, vascular permeability, and consequently mortality in a murine coxsackievirus B3-induced myocarditis model by maintaining the EnGL's integrity (Figure 1) [206]. They found that a lack of SPARC resulted in a loss of GL integrity and consequent barrier function. These alterations in GL integrity resulted in increased cardiac inflammation and mortality during viral myocarditis. They also noted a relationship between SPARC and HS, seeing that both could restore EnGL integrity [206]. SPARC probably serves as a Cys donor for oxidation to both GSH and inorganic sulfate (Figure 1). The degree of GAG sulfation in the GL regulates inflammation, vascular permeability, coagulation, and mechanotransduction. Depleted levels of GSH, through increased inflammation and oxidative stress, would also mean impaired availability of inorganic sulfate, resulting in an undersulfated and degraded GL. In animal studies, taurine deficiency, with Cys as a precursor to taurine (Figure 1), was also shown to result in cardiomyopathy [227].

GVG Sp and LNPs can activate platelets and generate ROS, in addition to the pro-inflammatory response, resulting in degradation of the EnGL and coronary artery lesions [214]. In this model, the overexpression of pro-inflammatory cytokines, such as TNFα and IFNγ, may induce cellular hypertrophy and myocardial damage and possibly causes ventricular remodeling. Since TNFα is a potent inducer of NO and ROS, this inflammatory process can promote cardiac injury, myocardial fibrosis, and electrical remodeling, through a hyperoxidative state [228,229]. As the EnGL degrades, the protective barrier of the EnCs is compromised, leading to increased interstitial edema, capillary leakage, and a higher risk of multiple organ failure. Furthermore, loss of ability to sense shear stress in the endothelium results in NO release, leading to systemic vasodilation [221].

In addition to the function of MMPs in extracellular matrix (ECM) remodeling, a critical process involved in the progression of myocarditis to dilated cardiomyopathy, they are also important modulators of the antiviral immune response. A novel role for MMP-12 in innate immunity is mediating the secretion of IFNα, by transcriptional regulation of NF-κB inhibition [205]. Pro-inflammatory cytokines can also lead to aberrant mitochondrial metabolism of cardiomyocytes, further causing heart dysfunction [204]. EnGL degradation and prolonged and increased activity of MMPs and HPSE would be predominant in systemic vascular leakage [230]. Circulating HS can act as DAMP ligands, binding to TLR4 and increasing the release of pro-inflammatory cytokines. Furthermore, serum HS fragments can induce mitochondrial dysfunction in cardiomyocytes in a TLR4-dependent manner [231]. Degradation of the GL will also result in shedding of endogenous protective enzymes, such as extracellular SOD, which will increase oxidative stress in the endothelium [232,233].

It is also probable that overexpression of IFN in the heart can lead to autoimmune cardiomyopathy. TREX1-deficient mice were shown to develop lethal lymphocytic inflammatory myocarditis, with progressive dilated cardiomyopathy and circulatory failure, in addition to pathological changes in lymphoid organs, consistent with autoimmune cardiomyopathy [234]. Fung et al. gave a good overview of the immunopathogenesis of viral myocarditis [205]. A persistent and excessive immune response after vaccination can have harmful consequences, contributing to the progression of myocarditis and dilated cardiomyopathy [205]. Yonker et al. observed significantly elevated levels of IL-8, IL-6, TNFα, IL-10, IFNγ, and IL-1, with lower IL-4 levels, in adolescents and young adults presenting with myocarditis after COVID-19 mRNA vaccination, compared to healthy vaccinated control subjects [17]. The same Th1-polarized immune response was observed with DNA vaccine (AstraZeneca) immune assays [118], and in a Pfizer COVID-19 vaccination trial [87]. This cytokine and chemokine environment represents a pro-inflammatory M1 macrophage phenotype, with Th1 IFNγ potentiating the macrophage microbicidal activity, thus promoting antigen presentation. When the Th2-associated cytokine IL-4 levels are low, the anti-inflammatory M2 macrophage phenotype is not activated to stimulate myocardial healing or to blunt the inflammatory response [172]. IFNγ–producing CD4+ T cells producing IFN were shown to predominate in autoimmune myocarditis in animal and human subjects, where IFNγ–overexpressing mice developed spontaneous cardiac inflammation [235].

Furthermore, Yonker et al. discovered that total leukocytes, especially neutrophils, were significantly elevated in the post-vaccine myocarditis cohort. On the contrary, platelet counts were found to decrease compared with vaccinated control subjects [17]. These profiles probably suggest innate inflammatory activation in individuals who developed post-vaccine myocarditis. At the same time, adaptive immunity and T cell responses in the postvaccine myocarditis cohort were found to be indistinguishable from those of asymptomatic age-matched vaccinated control subjects. However, the circulating free GVG Sp antigen evaded Ab recognition in postvaccine myocarditis [17].

In addition, dysregulation of specific miRNAs has been demonstrated to contribute to developing viral myocarditis, without altering viral replication dynamics [205]. It seems probable that mRNA vaccines can upregulate miRNAs, which can induce myocarditis [172]. Furthermore, increasing evidence shows that aberrant accumulation of protein aggregates is important in the development of human heart diseases [205]. LNPs are known to aggregate to form protein coronas [236], cross the BBB, and potentially induce strokes and/or cerebral hemorrhages and damage the endothelium of the heart muscle [237]. However, Nahab et al. found that the AstraZeneca DNA vaccine was associated with a higher risk of early post-vaccination ischemic stroke, compared to the Pfizer mRNA vaccine [238]. It is clear that other factors are also at play, apart from the presence of LNPs.

It is well established that GSH depletion, due to oxidative stress, is common in apoptotic cell death. In the pathogenesis of myocarditis, the balance of the oxidative / reductive state of the myocardium plays an essential role. The increased generation of ROS, reactive NO, and peroxynitrite species, and the depletion of enzyme and nonenzymatic antioxidant systems will increase oxidative stress. Oxidative stress can lead to adverse cellular effects in the myocardium, such as impaired regulatory pathways involved in Ca^{2+} homeostasis, conformational changes in myofibrillar proteins and cell membrane properties, alterations in DNA repair mechanisms, mitochondrial dysfunction, and ultimately cell death [97].

Vaccine-induced cardiac injury can, therefore, be related to direct cardiomyocyte damage, coronary plaque destabilization, cytokine inflammatory responses, oxidative stress, EnGL dysfunction and degradation, and intracoronary microthrombi formation [200]. In fact, in addition to inflammation, increased ROS production has been directly associated with various critical characteristics of the pathophysiology of cardiovascular disease. These include vascular remodeling, endothelial dysfunction, altered vasoconstrictor responses, increased inflammation, and modifications of the ECM. All these factors play a significant role in the development and progression of cardiovascular diseases [222]. It is important to note that heart failure is the final mutual outcome of various primary cardiovascular diseases, regardless of the underlying nature of cardiomyopathy. The long-term prognosis of myocarditis in vaccinated individuals remains unclear. Longitudinal studies of COVID-19-

vaccinated patients with myocarditis will be necessary to better evaluate long-term risks. Given the low turnover rate of cardiomyocytes (only 1% annually, even at the age of 25) and the fact that fewer than 50% of cardiomyocytes are exchanged during a normal human lifespan, it becomes evident that loss of cardiomyocytes cannot be adequately replenished in a timely manner. As a result, such loss would be detrimental to maintaining cardiac function. The limited regenerative capacity of cardiomyocytes poses challenges for the heart's ability to recover from significant damage or injury [239]. More research is needed to fully understand the complex interplay between oxidative stress and vaccine-induced myocarditis.

4.5 Adverse events in athletes

Myocarditis is ranked as the third leading cause of sudden cardiac death in competitive athletes by the American College of Cardiology [205,240]. Further investigation is required due to the reported increased incidence of sudden death among athletes after COVID-19 vaccination [208,241].

Because athletes often use testosterone and other anabolic steroids to improve performance, lower levels of GSH and reduced redox status could be expected. Non-medical testosterone has been shown to increase arterial blood pressure and induce left ventricular hypertrophy and myocardial infarction, due to coronary vasospasm or thrombosis [222]. Low levels of GSH have also been established to cause increased inflammation, microglial activation, neuroinflammation, and expression of NO [223], indicating an increase in ROS. Interestingly, steroid SULFs are regulated by hypoxic conditions and inflammatory mediators, such as TNFα. Once intracellular, steroid conjugates can be desulfated during intense exercise [21], increasing levels of active steroid metabolites [224].

At rest and during exercise, the electron transport system linked to the mitochondrial respiratory chain plays a significant role in generating ROS. The mitochondrial respiratory chain could be a potential source of ROS generation in the heart, which during exercise undergoes partial ischemia due to reduced blood supply. However, sufficient oxygen can interact with the increasingly reduced respiratory chain and enhance ROS generation. Even so, blood flow to hypoxic tissues at the cessation of exercise. However, Di Meo et al. found that the number of damaged mitochondria increased with long-term exercise [242]. Mitochondrial dysfunction contributes to inflammation and apoptosis, in addition to playing an essential role in vascular disease. Tostes et al. reported that testosterone also induces mitochondria-associated ROS generation and apoptosis, in vascular smooth muscle cells, by activating androgen receptors [222]. Bille et al. indicated a female/male ratio of 1 / 9 for sudden cardiac death among athletes [240]. Sen et al. [243] studied the association between exercise intensity and related oxidative stress in healthy young men. A 50% decrease in blood GSH levels was observed in moderately trained men during the first 15 minutes of exercise [243]. Numerous studies have indicated that an imbalance of pro-oxidants and antioxidants, and thus a shift in the redox state within a cell, can lead to DNA damage and various functional abnormalities [226]. It was shown that a sulfur donor, such as methylsulfonylmethane (MSM), can dampen IL-1ß and IL-6 in response to intense bout of exercise, while increasing IL-10 levels [244].

Notably, Cys will favor GSH synthesis during exercise-induced oxidative stress. A higher demand for GSH will have an inhibitory effect on inorganic sulfate synthesis. Furthermore, stress and hypoxic states favor H_2S as an 'emergency' substrate for ATP production, while Cys also serves as a precursor for mitochondrial production of H_2S [98]. Under hypoxic conditions, it can be assumed that there will be an increased demand for H_2S as an electron donor. This increased demand may result in the inhibition of inorganic sulfate and GSH synthesis, with Cys acting as the rate-limiting factor (Figure 1). This will affect the degree of HS sulfation and negatively impact GSH levels. Moreover, mediated by hypoxic conditions, increases in intracellular H_2S and ROS levels can synergistically induce membrane depolarization. This may result in increased levels of cytosolic Ca^{2+} ions, leading to activation of the endoplasmic reticulum stress response involved in initiating apoptosis [98] (Figure 2). Furthermore, prolonged hypoxia states increase the expression of tissue factors in monocytes and macrophages, as well as pulmonary vascular EnCs, which can lead to increased fibrin accumulation and consequent pulmonary thrombosis [21].

As discussed in this review, various factors will lead to a lower redox status in recipients of DNA and mRNA vaccines. Coupled with the increased oxidative stress generated during exercise, it is very likely that these events are the perfect storm to result in a heart attack. Increased inflammation and oxidative stress would increase blood viscosity and decrease myocardial perfusion and supply. This would be further exacerbated through dehydration during exercise. In addition, electrolyte imbalances would also affect the renin-angiotensin-aldosterone system, possibly contributing to congestion and worsening of heart failure [120].

4.6 Immunocompromised patients and autoimmune diseases

Many researchers have looked at the possible link between SARS-CoV-2 and autoimmunity, such as vasculitis. Various autoimmune diseases have been reported after COVID-19 vaccination, such as alopecia areata, Guillain–Barre syndrome (GBS), autoimmune-induced hepatitis, acute autoimmune transverse myelitis, idiopathic thrombocytopenic purpura, arthritis, and Ab-associated antineutrophil cytoplasmic vasculitis [6,245,246]. This raises the question of whether COVID-19 vaccines might affect the immune system in the same way that SARS-CoV-2 infection does.

Immunodeficiency can be expected due to abnormal or decreased viability of T cells. On the contrary, the overexpression of the serine / threonine kinase MAP4K3 in T cells induces IL-17A production and T cell hyperactivation, possibly leading to autoimmune diseases [139]. On the other hand, anti-Sp Abs, with cross-reactivity for host proteins, have been hypothesized to contribute to autoimmune pathologies, such as Kawasaki-like disease and GBS [54,245].

Serum from severely ill patients with COVID-19 revealed high autoAb titers, such as antinuclear antibody (ANA), lupus anticoagulant and antineutrophil cytoplasm antibodies (ANCA) [247,248]. In plasma isolated from patients with autoimmune conditions, myeloperoxidase (MPO) and NOX2 were associated with the formation of NET. Enhanced NETosis was associated with the onset of acute and chronic inflammation and autoimmune disorders. At the same time, the presence of auto-Abs inhibited NET degradation, increasing the risk of immune-mediated thrombosis [147]. Although patients affected by immune suppression and immune-mediated inflammatory disorders are encouraged to be vaccinated, there exists a paradox, since it is known that COVID-19 vaccines can trigger disease relapse in patients with an established immune-mediated inflammatory disorder. In patients predisposed to develop an autoimmune disease, vaccination can potentially shift the balance towards self-reaction, leading to the initiation or exacerbation of autoimmune responses [249,250]. Ironically, the initial Pfizer and Moderna vaccine trials excluded immunocompromised patients, including those on immunosuppressive medications, and patients with autoimmune conditions. It has been established that autoimmune reactivity in response to viral antigens, after infection or vaccination, can be easily derived in various tissues from cross-reaction with human tissue antigens that share sequence homology with the virus. Therefore, it seems probable that the GVG Sp is a potential epitopic target for biomimicry-induced autoimmunological processes [251]. Therefore, chronic autoimmune disease can result from excessive Ab production in response to the vaccine. Vaccines have been associated with chronic immune-mediated disorders that can develop only years after vaccination [211,252]. Moreover, IL-17 cytokines, induced by cationic LNPs, will further exacerbate the autoimmune milieu [62].

It is also important to note that the CDO-catalyzed step, which is responsible for producing the majority of inorganic sulfate *in vivo* from Cys, is rate-limiting (Figure 1). Pro-inflammatory cytokines, such as IL-1β, TNFα, and TGFβ, downregulate CDO at the mRNA level. Therefore, higher plasma levels of Cys could be expected, compared to inorganic sulfate concentrations, after vaccination. A lower degree of HS sulfation is evident in various autoimmune and inflammatory health conditions [98].

4.7 Immune-mediated hepatitis

After vaccination, various factors and pathways would cause immune-mediated hepatitis, fibrosis, and liver necrosis, such as increased pro-inflammatory cytokines, oxidative stress [253], circulating LNPs, and soluble GAGs

in circulation due to EnGL damage. Soluble plasma GAGs are cleared from circulation through clearance receptors present in sinusoidal liver EnCs, including stabilin-2. However, in instances of liver cell injury or infection, such as in the case of hepatitis, there may be an associated increase in circulating GAGs due to impaired clearance mechanisms [28]. Decreased sulfation and degradation of the EnGL would be a substantial contributing factor in mediating hepatitis. SAAs play a crucial role in liver health, where increased ROS or lower levels of GSH and the other SAAs will also contribute to hepatitis. The loss of the BPNT1 gene in mice was shown to lead to impaired protein synthesis, which subsequently resulted in impaired liver function and reduced levels of HSA [224].

LNPs can be cleared in a healthy liver through efficient immune responses; for example, by the convectional APCs in the liver, such as DCs, B cells and Kupfer cells. Additionally, sinusoidal EnCs from the liver, liver stellate cells, and hepatocytes can act as APCs. However, sustained expression of GVG Sp-related antigens after vaccination, as well as increased levels of GAG fragments and LNPs, can skew the immune response to autoimmune tissue damage, such as through IL-17 [62], as observed in cases of autoimmune hepatitis after COVID-19 vaccination [10,254]. It has been shown that PEG-lipids with short lipid tails can desorb from LNPs, allowing the NPs to adsorb apolipoprotein E, which will cause LNPs to endogenously target liver hepatocytes [68]. nAb responses elicited after SARS-CoV vaccination have been shown to induce harmful immune responses that result in liver damage [42]. Fernandes et al. [149] injected SARS-CoV-2 rSp into zebrafish and observed mild lobular infiltration of lymphocytes in the liver, centrilobular sinusoidal dilation, patchy necrosis, moderate microvesicular steatosis, and mild inflammatory infiltrates in the hepatic lobule and the portal tract. These changes were similar to those observed in COVID-19 patients [149].

4.8 Neurological symptoms

Various factors, such as genetics, oxidative stress, neuroinflammation, mitochondrial damage, and abnormal protein folding, underlie neurodegenerative disease. Multiple researchers have linked vaccine adjuvants, such as aluminum salts, to neurological symptoms [108]. Even though neurotoxicity due to LNPs crossing the BBB is possible [76], one could more likely expect decreased levels of inorganic sulfate as an underlying etiological factor. Sulfate plays an important role in the brain as a vital component of the GL and ECM [56], and as a deactivator of neurotransmitters, such as serotonin, dopamine, DHEA, and pregnenolone. HSPGs play a crucial role in neurite outgrowth and connectivity between neurons and their target cells. They have been shown to be vital in processes such as neurogenesis, axonal guidance, and synapse development [223]. The depletion of available inorganic sulfate due to vaccine-induced systemic inflammation and oxidative stress will lead to many neurological problems. Reduced serotonin sulfation would impair its inactivation, resulting in increased serotonin levels or serotonin syndrome. Increased serotonin levels would not only affect daily brain functioning, it can also cause focal seizures and motor changes, and a loss of oxytocin-containing cells in the hypothalamus, leading to deficits in oxytocin processing with behavioral consequences.

In addition, sulfated steroids, such as DHEA sulfate, have been shown to inhibit GABAa receptors in the nervous system. Decreased sulfate levels might result in anxiety, depression, apathy, insomnia, and mood disorders. Impaired N-methyl-D-aspartate (NMDA)-dependent long-term potentiation (LTP) was associated with sodium and potassium current perturbations. Sulfate deficiency might alter memory, since the glycoprotein, N-sdc, enables LTP by transmitting signals from ligands binding to attached HSPGs [223]. Sulfated galactocerebroside, known as sulfatide, is a critical myelin lipid component [56] and is involved in various biological processes, such as cell growth, inflammation and immune modulation, protein trafficking, signal transduction, cell-cell recognition, neuronal plasticity, and cell morphogenesis [255]. Mice lacking sulfatides display disorganized paranodes and a deficiency in septate-like junctions, which results in a notable decrease in nerve conduction velocity as a functional outcome. The absence of galactolipid sulfation in the Golgi apparatus appears to disrupt the selective association of NDK/Nm23 proteins with myelin-destined vesicles, leading to random and unregulated associations of NDK/Nm23 with any myelin-destined vesicle. This dysregulation of protein association may contribute to the observed impairment in nerve conduction [256].

Substantial depletion (92 mol%) of sulfatide mass was found in the temporal grey matter of subjects with a clinical dementia rating of 0.5, relative to controls [255].

Although excessive production and release of pro-inflammatory chemokines and cytokines after vaccination, particularly TNF-α, IL-1, and IL-6, can trigger neuropsychiatric symptoms in itself, they also negatively affect thiamine metabolism [257]. A deficiency in sulfur-containing thiamine has been linked to many neuropsychiatric adverse effects.
As in long-COVID, activated immune-inflammatory and oxidative and nitrosative stress pathways (IO&NS) can underlie the physiological symptoms of COVID-19 vaccine-induced chronic fatigue syndrome, major depression, and generalized anxiety disorder [140,258,259], which are characterized by activated IO&NS pathways. Therefore, the combination of toxicity from neuro-oxidative stress and diminished antioxidant defenses can result in increased neurotoxicity, ultimately leading to physiological symptoms [187]. Although Selvakumar et al. did not find a correlation of long COVID conditions between infected and noninfected individuals, the vaccination status was not taken into account as a possible contributing factor to symptoms [260].
An overactive immune system is known to contribute to the emergence or aggravation of neurodegenerative diseases, such as Alzheimer's disease, hemorrhagic and ischemic strokes, multiple sclerosis (MS), and GBS, as well as neuropsychiatric symptoms, as cytokines can pass through the BBB and cause acute necrotizing encephalopathy [10,115,216].

In murine studies, S1 has been shown to act as a pathogen-associated molecular pattern that induces viral infection-independent neuroinflammation through the participation of pattern recognition receptor engagement [10,140]. Furthermore, Sp, S1 of SARS-CoV-2 and mRNA-LNPs can cross the BBB [10,89,149]. Fernandes et al. [149] showed that rSp generated an inflammatory process in the brain, with a severe influx of mononuclear cells. This profile was correlated with acute necrotizing and transverse myelitis related to SARS-CoV-2 infection, confirming the neurotropism of the Sp [149].

Furthermore, circulating shed HS fragments can bind to Aβ fibrils and α-Synuclein, thereby competitively inhibiting FGF2-mediated neuroprotection and promote protein aggregation [261]. Moreover, binding of the GVG Sp and LNPs to HSA can affect HSA transport across the BBB, resulting in increased plaque formation and impaired amyloid processing, thus affecting cognition [123,261]. Low HSA, LNPs, and increased expression of MPO could also exacerbate capillary leak in the brain [262], which can lead to thrombosis, severe encephalitis, toxic encephalopathy, and rupture. The brain contains the largest number of plasmalogens [255], which are susceptible to MPO-induced oxidative stress [263]. Plasmalogen deficiency in cerebral grey matter can be directly related to neurodegeneration and loss of synapses, as a decrease in plasmalogen levels can induce membrane instability [255]. Therefore, by enriching neuronal cell membranes with plasmalogens, neuronal function can be improved through modulation of nonlamellar membrane transformations and synaptic plasticity [264]. A weak BBB, through HSA depletion and MPO-induced oxidative stress, may promote secondary infection, leading to meningitis [89,123,149]. Xu et al. [34] illustrated that loss of brain EnC integrity and decreased host defense mediated by NETs would synergistically render HS2ST-deficient mice susceptible to infection. Furthermore, inactivation of HS2ST in neutrophils affects the binding capacity of histones, which are vital antimicrobial molecules and structural components of NETs, revealing a novel function of HS in neutrophil NET biology [34]. There exists a direct relationship between HSA deficiency and decreased sulfation [98].

Neuronal cells are highly susceptible to exposure to hypochlorous acid (HOCl) induced by MPO, which can release pro-inflammatory cytokines, exacerbating neuroinflammation. HOCl also chlorinates the amine and catechol groups of dopamine, which may selectively kill dopaminergic neurons by inhibiting mitochondrial respiration [262]. It is important to note that endosomes provide a specialized NGF/TrkA platform for sustained signaling, which is required for neuronal survival [77]. The influence of ionized LNPs on these signaling pathways through membrane and endosomal destabilization needs further investigation.

Furthermore, various Sp-binding receptors are expressed in the brain, such as ACE2, ephrin (Eph) receptors and ligands, NRP-1, TMPRSS2 and CD147. The GVG Sp, LNPs and activated IO&NS pathways can result in neuroinflammatory processes characterized by neuron demyelination, hyperactivation of microglia, and stimulation of astrocytes. Specific pathological changes and symptoms vary depending on the type of cell and the brain region affected. Symptoms related to pituitary gland involvement may include headaches and vision changes, while damage to the cerebellum and cortex can cause neurological symptoms such as ataxia, dizziness, and impaired consciousness [216,265]. Numerous cases of acute, temporary, unilateral peripheral facial paralysis, or Bell's palsy, have been reported as AE [49,259,266].

Various reports of Creutzfeldt–Jakob disease have been reported after COVID-19 vaccination [267], with symptoms of rapidly progressive dementia, ataxia, pyramidal symptoms, and akinetic mutism. It has been hypothesized that neuroinflammatory transcriptional signatures and loss of homeostatic identities in astrocytes could be triggered by systemic inflammatory mediators, which can contribute to neurodegeneration and prion disease pathogenesis [216].

Abramczyk et al. observed alterations in the reduction-oxidation pathways associated with Cytochrome c in glial cells of astrocytes, astrocytoma and glioblastoma, incubated with the Pfizer mRNA vaccine, similar to that of brain cancer [115]. Glioblastoma multiforme is a common primary human brain cancer characterized by resistance to apoptosis by chemotherapeutic treatment and radiation. The pathogenesis of glioblastoma multiforme is associated with pro-inflammatory cytokines, chemokines, and Eph receptors. Elevated levels of pro-inflammatory cytokines, such as IL-1β and TNFα, were found in patients with glioblastoma multiforme [217]. It would be important not to misdiagnose glioblastoma multiforme for acute disseminated encephalomyelitis, which could also be associated with COVID-19 vaccination [268].

Reports of acute ischemic stroke cases have emerged after COVID-19 vaccination [6,211,238,269]. There is a long-standing association between systemic infections, inflammation, and acute ischemic stroke. TLRs can also indirectly damage neurons, resulting in ischemic stroke. EphA2 receptors have also been shown to play an important role in ischemic stroke pathology [216].

GBS is a commonly reported vaccine-induced AE [6,259,270-272]. GBS is linked to the release of cytokines and chemokines induced by vaccination, resulting in damage to the central and peripheral nervous system [216]. It is the most common acquired inflammatory neuropathy, characterized by demyelination or damage to the myelin sheath and/or axonopathy. It has been speculated that the GVG Sp can lead to an autoimmune response due to its structural similarities with the ganglioside components of peripheral nerves, thereby damaging them. This hypothesis was confirmed by detecting auto-Abs against gangliosides in the case of tetraparesis [120].

The fact that these neurodegenerative diseases were also observed in COVID-19 patients [216,245,265], indicates the involvement of the Sp with various receptors, with consequent neuroinflammation. Post-vaccination, the integrity of the BBB is probably weakened by LNPs, thereby exacerbating neuroinflammation. Increased immunogenicity can also be expected due to mistranslation of mRNA and protein misfolding, which has been linked to neurodegeneration [273] and heart disease [274].

The acute neurological presentation of Churg–Strauss syndrome following COVID-19 vaccination has been reported [245,249,275]. In a case report discussed by Chan-Chung et al. [275], eosinophilia and a systemic inflammatory process were revealed with baseline investigations and autoimmune tests. On the contrary, renal and liver function tests, as well as lumbar puncture findings, were found to be unremarkable. Although magnetic resonance imaging (MRI) of the brain and spine did not reveal any significant abnormalities, MRI of the orbits showed intense enhancement in and around both optic nerve sheaths, along with extensive nasoethmoidal inflammatory mucosal disease. The patient showed features of small vessel leukocytoclastic vasculitis and eosinophilic myocarditis, without granulomas or giant cells. Although the cause-and-effect association with

COVID-19 vaccines still needs to be proven with certainty, clinicians should suspect autoimmune-related polyneuritis in patients with asthma and rhinosinusitis, who present with sensorimotor symptoms and poor balance after COVID-19 vaccination [245,275].

There exists an important relationship between HS and Churg–Strauss syndrome. It is known that the EnGL is damaged during inflammatory conditions, with very high levels of extracellular histones and pro-inflammatory cytokines, resulting in the degradation and shedding of HS and other GL fragments. However, En-expressed HS plays a role in allergic airway inflammation, such as asthma and sinusitis, by mediating the interaction of leukocytes with the vascular endothelium, thus regulating the recruitment of inflammatory cells to the airways, as well as sequestering and modulating the activity of pro-inflammatory cytokines [276]. In Churg-Strauss syndrome, the membrane basic protein (MBP) and eosinophil cationic protein (ECP) block HS, thereby inhibiting HS binding to AT, and consequently facilitate coagulation through unhindered factor X activation and thrombin generation. Furthermore, MBP/ECP stimulates platelets to release PF4, which also blocks HS with subsequent coagulation [277], and an attenuated ability to regulate inflammation. It is clear that after COVID-19 vaccination, these overstimulated immune-mediated pathways would intensify, with less available HS to modulate the response.

4.9 Endocrine disorders, reproductive health, and pregnancy

During pregnancy, the blood-placental barrier mediates the exchange of nutrients and metabolic waste products, exerts vital metabolic functions, and secretes hormones, and is therefore crucial in maintaining pregnancy. There is a relationship between SAAs, where an HSA deficiency will affect Cys and taurine levels (Figure 1) [98]. Interestingly, lower birth weight and length of infants have been linked to maternal taurine deficiency. Since it has been found that increased size of newborns at birth may be protective against the development of coronary heart disease, taurine or HSA deficiency in utero, and in infants, can lead to the progression of coronary heart disease in adulthood. This link between taurine deficiency and cardiac pathologies was demonstrated in taurine transporter (TauT) gene knockout mice [227]. The hearts of TauT knockout mice had impaired mitochondrial complex I, resulting in elevated superoxide production and thus oxidative stress within mitochondria. Oxidative stress ultimately causes endoplasmic reticulum stress and apoptosis, which were thought to be the cause of cardiac failure [227]. However, a taurine deficiency would also mean lower levels of inorganic sulfate, where undersulfation is also associated with cardiovascular diseases [21]. Therefore, diets that lack adequate SAAs and taurine levels, plus increased oxidative stress after vaccination, can adversely affect the fetus's health status during pregnancy, leading to an increased risk of pathologies in adulthood. Infants also depend on taurine for their neurodevelopment. This was confirmed when a low score on the mental development index at 18 months was associated with low neonatal plasma taurine concentrations [227].

Due to chronic inflammation and oxidative stress, a reduced supply of inorganic sulfate will also impair serotonin sulfation, affecting its inactivation. Increased levels of serotonin are one of the most consistently replicated biochemical findings in autism. Increased serotonin levels would affect day-to-day brain functioning, as well as the development and outgrowth of serotonergic neurons, resulting in a loss of serotonin terminals. Autistic symptoms in offspring, such as reduced social bonding, sensory hyperresponsiveness, seizures, and motor changes, as well as depletion of oxytocin-containing cells in the hypothalamus, can be attributed to elevated serotonin levels during prenatal brain development [223]. This may result in developmental and behavioral changes in children born from COVID-19-vaccinated mothers.

In addition, spontaneous abortions and stillbirths could be expected when the mother has been vaccinated [258,278], either recently before or during pregnancy [279]. The movement of nutrient-rich HSA across the placental barrier is controlled by clathrin-enabled endocytosis. The binding of LNPs and GVG Sp to HSA will reduce the amount of HSA and clathrin binding sites available. This blocks HSA from entering the cell and passing the placental barrier. Therefore, less HSA and nutrients would be available to support healthy growth and development of the fetus and negatively impact the mother [123]. Furthermore, various studies demonstrated

the achievability of designing LNPs as a platform for the delivery of mRNA to the placenta [72,280]. The potential effect of LNPs and mRNA on the fetus post-COVID-19 vaccination needs more research.

Although normal Ep and fetal tissues have low expression of CD147, it is significantly upregulated during inflammatory conditions [131]. Therefore, there is a possibility that the GVG Sp might directly impact the fetus by binding to CD147. While maternal infection is a well-established cause of congenital abnormalities, vertical transmission of SARS-CoV-2 has also been documented. Severe adverse pregnancy outcomes, including miscarriage, premature delivery, intrauterine growth retardation, and maternal death, have been reported in association with other coronaviruses, such as MERS-CoV and SARS-CoV. A systematic review of evidence during the early stages of the COVID-19 pandemic indicated an increased risk of preterm birth, stillbirth, and admission to neonatal intensive care [281].

More research is needed to test the immunogenicity, reactogenicity, and safety of COVID-19 vaccines during pregnancy [282], especially since pregnant and lactating women were excluded from the initial COVID-19 vaccine trials [283]. In recipients of the Pfizer vaccine, a wide-ranging immune response was observed, including stimulation of neutralizing nAb responses, stimulation of CD4 + cells, and expansion of effector memory CD8+ T cells in men and non-pregnant women. However, the extent of a comparable immunological response in pregnant women remains uncertain. This uncertainty is concerning, since favorable perinatal outcomes are highly dependent on enhanced helper T cell type 2 and regulatory T cell activity, combined with reduced Th1 responses. Alterations in CD4+ T cell responses during pregnancy have been associated with unfavorable pregnancy outcomes, such as preterm birth and fetal loss. Furthermore, there is some evidence to suggest that infants born to mothers with variant CD4+ T cell responses may experience long-term adverse consequences [49].

Various research studies have clarified that HSPGs mediate the retention of many growth factors and morphogens. Carboxyl and sulfate groups of the GL contribute to the negative charge essential for interactions between HS and basic AA residues in proteins, such as VEGF-A [284]. In a murine study, it has been demonstrated that dramatically reduced HS sulfation resulted in brain malformations and skeletal defects. This implicates the importance of highly sulfated NS domains for growth factor-binding [284]. Macrophage subsets express differential expression of HS, where reparative M2 macrophages will bind more 2-O-sulfation-dependent FGF-2, augmenting the FGF-2-dependent proliferation of a target cell [167]. This suggests that aberrant regulation of leukocyte HS sulfation during chronic inflammation and oxidative stress, possibly induced by GVG Sp and LNPs, can result in excessive growth factor activity and consequently affect fetal development. Activated macrophages mediate both CD4+ and CD8+ T cell responses. Therefore, HS and its sulfation configuration are essential for cell growth, tissue homeostasis, immune response, and embryonic development [261].

Morris et al. demonstrated through *in vitro* frozen embryo transfer (FET) fertilization that seropositivity to SARS-CoV-2 Sp, whether from vaccination or infection, did not prevent embryo implantation or development during early pregnancy [285]. However, the female zebrafish injected with rSp showed severe damage seven days after inoculation, while the ovarian damage was reversed after 14 days when the zebrafish received a second injection of rSp. For reproductive tissue in humans, there is evidence that ACE2 is expressed during all stages of follicle maturation in the ovary and endometrium [149]. This increases the likelihood that GVG Sp affects female and male fertility, with evidence that the Sp can distribute to the ovaries and testes [286,287]. The dramatic increase in oxidative stress and pro-inflammatory cytokines after COVID-19 vaccination, such as IL-6, can damage spermatozoa. Increased levels of ROS and MPO can damage the sperm membrane [262,263] and induce apoptosis, thus affecting sperm quality, sperm function, and motility. If spermatogenesis is affected, it could eventually result in non-functional sperm, thus negatively impacting male reproductive health [288]. Furthermore, Sertoli and Leydig cells are involved in spermatogenesis and express ACE2, TMPRSS2, and CD147 [130]. The effect that overexpressed binding of CD147 and GVG Sp might have on spermatogenesis and fertilization would require further research.

There have been observations that COVID-19 vaccines caused a decrease in fertility in women [289]. This should be verified with a statistical analysis of data on world birth rate associated with COVID-19 vaccination. *In vitro* fertilization studies would not truly reflect the *in vivo* environment after vaccination. Although the proposed mechanism for infertility is the presumed similarity between GVG Sp and syncytin-1 [285], the upregulation of Sp binding receptors, and the effect of steroid sulfation and desulfation, would likely better explain the variation in fertility issues experienced by COVID-19 vaccinated women. In the fertility study of Morris et al., they used micronized estradiol tablets, followed by a combination of vaginal progesterone and intramuscular progesterone for the preparation of the uterine [285]. However, the study did not reflect the *in vivo* reality of chronic inflammation and oxidative stress after vaccination, its effect on steroid sulfation and HSA availability, and its pathological consequences.

Furthermore, the reduced availability of inorganic sulfate for steroid sulfation could explain the menstrual irregularities and breakthrough bleeding observed in female COVID-19 vaccine recipients [21,290-292]. During the female menstrual cycle, SULF activity peaks in endometrial tissue at the early secretory stage and then declines afterward. The pro-inflammatory cytokine IL-1β increases during the secretory phase of menstruation, which is known to suppress SULF mRNA and activity in human endometrial stromal cells [224]. This implies that IL-1β may control the endometrium steroid microenvironment by reducing estrogen's biological action. However, both hypoxic conditions and inflammatory mediators, such as TNFα and IL-6, also influence steroid SULFs. SULF suppression results in decreased maternal-fetal estrogen during pregnancy due to lack of SULF activity in the placenta. This deficiency of estrogen would result in delayed progression of parturition. Furthermore, it has been found that SULF activity is increased by up to 12 times in endometrial cancer tissue [224]. Sulfation and desulfation play an essential role in balancing the availability of free steroid hormones near target sites and would affect menstruation irregularity. The action of steroid hormones is strongly based on the intricate interplay of the sulfation and desulfation processes and the transport of sulfated steroids across membranes. More research is required to assess the effect of inflammation and oxidative stress on the expression of SULTs and SULFs, and the consequent pathologies if these enzymes are out of balance. The direct biological effects of various steroid sulfates versus their non-sulfated counterparts should also be further explored. The rate-limiting step for all sulfation reactions is the availability of active sulfate in the form of sulfonucleotide 3-phosphoadenosine 5-phosphosulfate (PAPS), where the responsible PAPS synthases are known to be fragile enzymes [21,224]. Cellular efflux of conjugated steroids occurs through the multidrug-resistant protein (MRP) of ABC transporters. Estrone sulfate and DHEA sulfate transport depend on GSH availability, but whether or not the sulfated steroid requires GSH for MRP-mediated efflux remains unresolved [224].

CVST typically affects young women of childbearing age, with a prevalence of prothrombotic disorders being exceptionally high in adolescents. CVST is the most common among female patients younger than 40 years of age, those with thrombophilia, or women who are pregnant, postpartum, or taking hormonal contraception [196]. There is a possible hormonal link to estrogen. Estrogens are known to increase the levels of coagulation factors while decreasing the levels of anticoagulant proteins, such as AT [293]. Since estrogen is inactivated by sulfation, aberrant sulfation after vaccination would be a leading factor predisposing to CVST. At the same time, thromboembolic complications associated with the GVG Sp can lead to fetal vascular malperfusion or fetal vascular thrombosis [281]. In vascular development, peptide growth factors of the VEGF and platelet-derived growth factor (PDGF) families have been found to regulate the migration and proliferation of EnCs and support mural cells, such as pericytes (PC) and vascular smooth muscle cells. HS and the overall degree of sulfation play a vital role in PDGF binding and are responsible for PC recruitment and attachment in vascular development [284].

The fact that insulin could easily interact with LNPs through electrostatic interactions [294,295] may partly explain the high incidence of hyperglycemic crisis and type 2 diabetes after COVID-19 vaccination, apart from the destructive effect that oxidative stress, endoplasmic reticulum stress and low inorganic sulfate levels would have on beta cells in pancreatic islet [21,137,261,296-298]. It is also necessary to note that HSA transports both insulin and glucose. With more HSA being consumed during inflammation and oxidative stress and through binding

to LNPs, a reduced concentration of insulin delivered to the liver could be expected, with the subsequent elevation of glucose. Moreover, this excess glucose can result in HSA glycosylation, which further reduces the binding sites. A reduction in HSA is associated with type 2 diabetes [261]. Glycated HSA also suppresses glucose-induced insulin secretion by altering glucose metabolism and result in pancreatic beta cell dysfunction through autophagy. Glycated HSA has a very high binding affinity; therefore, with more GVG Sp and LNPs binding to glycated HSA, glucose and insulin metabolism would be further impaired [52,123].

Type 1 diabetes have also been reported as an AE after vaccination [298,299]. Seen that high levels of sulfated HS protect beta cells in the pancreatic islet from ROS and cell death, this protective antiapoptotic effect would be neutralized during excessive oxidative stress and inflammation, when nearby autoreactive T cells secrete HPSE that subsequently degrade HS, leading to the onset of Type 1 diabetes [21,222].

Some cases of thyroid disorders have been documented after COVID-19 vaccination, such as subacute thyroiditis and Graves disease [300,301]. Evidence of the presence of ACE-2 receptors and TMPRSS2 in thyroid cells [265] increases the probability that the GVG Sp would activate these receptors in the thyroid. Furthermore, thyroid hormones are also sulfated during metabolism, and with reduced inorganic sulfate levels, active thyroid hormones can negatively impact thyroid health [302].

4.10 Cutaneous adverse effects

Various skin reactions were reported in recipients of the COVID-19 vaccines, such as petechiae, bruising or hemorrhages, hemangiomas, rashes, increased spider veins, redness, blue discoloration, and / or peeling of the skin [120,303]. These symptoms mainly occurred associated with other complex symptoms and were usually clustered. The onset of symptoms occurred from 12 hours to 9 weeks after vaccination, where Moderna elicited the most frequent reactions, followed by AstraZeneca and Pfizer [120].

A case of Darier's disease flare-up was reported after the first dose of the AstraZeneca vaccine. Darier's disease is a rare autosomal dominantly inherited dermatosis, which is due to a mutation in the ATP2A2 gene on chromosome 12q23-24 that encodes SERCA2, a Ca^{2+} ATPase in the sarco/endoplasmic reticulum [304]. Other mechanisms would also be involved, since an antigen-immune-mediated flare was observed after vaccination. Syndecan HSPGs (sdc) regulate cytosolic Ca^{2+} to control cell adhesion, actin cytoskeleton, and junction formation. This is achieved by controlling transient receptor potential canonical (TRPC) channels. An sdc–TRPC4 complex in epidermal keratinocytes controls adhesion, adherent junction composition, and early differentiation. HSPGs and the degree of HS sulfation will determine the binding of growth factors, morphogens, cytokines and chemokines, which synergistically combine with high-affinity receptors to affect intracellular signaling [305]. It is yet again clear that sulfation would affect practically every cellular process. A shift in sulfur metabolism, due to inflammation and oxidative stress, would exacerbate underlying genetic vulnerabilities, such as Darier's disease.

CD147 has been observed to increase with age in the skin [213]. Chronic inflammation after COVID-19 vaccination would increase the expression of CD147. When CD147 induce extracellular MMPs, an excessive breakdown of connective tissue components in the skin could be expected. Even though, most of the skin eruptions following vaccination could be drug-induced, where vaccination might have a synergistic immunologic effect on an adverse drug reaction. Most drugs used to treat inflammatory conditions, such as corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), acetaminophen, and aspirin, inhibit or are metabolized, through sulfation and can therefore negatively impact GAG sulfation in the epidermis. The consequent vascular inflammation would alter the regulation of keratinocyte proliferation, with increased epidermal differentiation. Toxic epidermal necrolysis (TEN) is often observed when using acetaminophen, which is associated with inorganic sulfate depletion [21].

Conventional DCs constitute the major resident DC population in the normal human dermis. DC maturation is crucial to the effectiveness of mRNA-based vaccines, where TLR locations in different DC subsets, and the DC

locations in various organs can affect the immune response, resulting in possible cutaneous AEs. Intradermal or subcutaneous delivery of the mRNA vaccine would result in antigen translation in skin monocytes and DCs [306].

4.11 Ocular adverse events

The COVID-19 vaccines had associated ocular AEs [16,49,307,308]. There have been reports of the GVG Sp triggering a pro-inflammatory response in human corneal EpCs, with increased oxidative stress and DNA double-strand breaks in human peripheral blood mononuclear cells [10]. MPO-induced HOCl will damage plasmalogens in the lens and ocular neurons [262,309].

Li et al. established that after the first and second doses of mRNA vaccines, these vaccinated individuals had a significantly increased risk of retinal vascular occlusion up to two years after vaccination [310]. Sp-induced disruption of retinal capillary barrier function is similar to En damage following intravitreal administration of vascular En growth factor, a prominent vascular hyperpermeability factor in diabetic retinopathy, an inflammatory eye disease [129]. The visual disturbances observed after vaccination [310,311] could be caused by microvascular supply disturbances of the retina and / or optical center, after exposure to GVG Sp and cytokines. Acute visual disturbances with retinal hemorrhages have been reported in association with AstraZeneca vaccination and autoimmune thrombocytopenia [120]. Given that a diet deficient in taurine is associated with the development of retinopathy [227], there is a clear link between the availability of SAAs, inorganic sulfate, inflammation, vascular and ocular health.

4.12 Cancer

Carcinogenesis is a complex multifactorial and multistage process that consists of initiation, promotion, and progression. Very specific interactions between tumor cells and the microenvironment are required during cancer development, growth, metastasis, and invasion. The GL is the critical effector of the tumor cell surface and microenvironment [261]. Therefore, the GL is involved in tumor growth and metastasis, by interacting with growth factors, growth factor receptors, and cytokines. Chronic inflammation is underlying many cancers and is a driving force for metastasis of cancer cells. HSPGs play a central role in regulating cell behavior and cancer progression, where the pattern and degree of GAG sulfation are strongly related to the type of cancer, the tumor regulatory effect, and the level of differentiation [261].

Significant alteration of steroid metabolism in many endocrine-related cancers is a well-established fact. Evidence suggests that sulfation pathways are down-regulated [312], while SULF activity is up-regulated in numerous tumors [313]. This leads to a preference for desulfation and subsequent conversion of steroids into more active metabolites [224]. Many factors can contribute to dysregulation of sulfation pathways [21]. However, with COVID-19 vaccination, the chronic pro-inflammatory response and oxidative stress generated by GVG Sp and LNPs, would probably be the main factors influencing sulfation. Furthermore, various factors, in addition to pro-inflammatory cytokines, would affect the signaling of SULTs and SULFs [224]. There has been an increase in breast and lung cancer cases reported to VAERS, while pancreatic, ovarian, and bladder cancers were directly linked to the COVID-19 vaccines [56]. One could also expect more cancers related to sarcoma, leukemia, and lymphoma after COVID-19 vaccination [314-322]. It is still unclear whether the increase in lymphadenopathy diagnosed post-COVID-19 vaccination is due to an immune response to the vaccine or an underlying malignant process. Although most lymphadenopathies diagnosed after vaccination in a study appeared benign, there was a 2.4% increase in new breast cancer malignancies diagnosed after vaccination [323]. Since cancer generally takes months or years to progress, from an initial cellular malignant transformation to the development of a clinically identifiable tumor, more randomized controlled trials are needed in this area (by screening before and after vaccination).

The fact that the ECM protein SPARC regulates tumor development, progression, and angiogenesis [208], confirms the vital role of SAAs in cancer. SPARC would serve as a Cys donor to maintain homeostatic balance

in the sulfation pathways (Figure 1). SPARC, which is overexpressed during cancer, exhibits anticancer properties and modulates inflammatory processes [324].

It was shown that the Sp increases human cell syncytium formation, triggering pyroptosis of syncytia formed, by fusion of Sp and ACE2-expressing cells, possibly the founding tumorigenic event. Furthermore, it was demonstrated that the Sp inhibits DNA damage repair processes, induce changes in Snail-mediated epithelial-mesenchymal transition markers, and promote lung metastasis in a breast cancer mouse model [10]. Furthermore, the Sp S2 subunit interacts with BRCA-1/2 and 53BP1. In particular, BRCA-1 is commonly found to be mutated in breast cancer in women and prostate cancer in men, while 53BP1 is a well-established tumor suppressor protein [325].

CD147, a stimulator of MMP1 production in fibroblasts, plays well-characterized roles in tumor metastasis, angiogenesis, and chemoresistance. Glycosylation plays a key role in regulating the pro-hypertrophic effects of CD147, such as the involvement of glycosylated CD147 in tumor metastasis [130]. When Sp can initiate gene expression changes, it might also affect glycosylation. Liu et al. showed [133] that CyPA and CD147 have higher levels of expression in pancreatic cancer tissues. Seeing that this proliferation of pancreatic cancer cells can be effectively blocked by a CD147 Ab [133], confirms that the GVG Sp might be a driving factor in carcinogenesis by upregulating the expression of CD147. It has also been shown that the SARS-CoV-2 Sp increases GLK levels in EpCs. GLK overexpression in lung EpCs is correlated with human lung cancer recurrence and poor prognosis, while upregulated expression of GLK has been observed in human liver cancer. Chuang et al. demonstrated that GLK directly phosphorylates the cytoskeleton regulator IQGAP1, leading to increased cell migration and promoting cancer metastasis [139].

The formation of NETs has been observed in some cancers [201]. DNA enriched with 8-hydroxy deoxyguanosine in NET was shown to bind to a transmembrane protein like Cdc25 in tumor cells, thereby facilitating its potential for metastasis. NETs induce the production of pro-inflammatory cytokines, which in turn stimulate more NETosis, leading to a cascading "feedback" effect. Elevated levels of NETs were found in metastatic lesions. NETosis and increased NET formation were shown to precede cancer metastasis, with the NET-DNA complex acting as a chemotactic factor, attracting metastatic cells to new sites. Enhanced NET formation compromises the adaptive immune system. Additionally, NETs coat tumor cells, providing protection against cytotoxic lymphocytes and/or natural killer cells that typically target tumor cells. Mounting evidence suggests that neutrophils promote tumor growth and metastatic progression through the formation of NETs. Therefore, NET-dependent mechanisms play a crucial role in promoting the invasive and proliferative properties of tumors [147]. Therefore, the formation of new tumors, and the aggravation of existing malignancies, can be expected post-vaccination.

Cationic LNPs cause TLR4 activation, which was shown to induce proliferation, activation of the NF-kB pathway and chemo-resistance in ovarian cancer, and increased migration and invasion in both colon and pancreatic cancer cells [62]. Activation of TLR4 by cationic LNP-RNAs can lead to cancer promotion and progression, and increase angiogenesis. Although PEG is relatively stable at physiological pH, it is collapsible under acidic conditions because of the protonation of imidazole groups. Therefore, PEG-coated ionizable LNPs will be destabilized in the acidic tumoral environment [326], thus amplifying the cytotoxic effects of these NPs.

It has also been postulated that COVID-19 vaccines may interfere with the target cell genome, activating oncogenes and/or deactivating anticarcinogenic gene sequences, thus increasing cancer risks [327,328]. In fact, numerous studies documented how dysregulation of miRNA is associated with cancer development and metastasis. Cancer pathogenesis is related to several biomolecular processes, for example, genomic alterations, transcription of oncogenic factors, and inhibition of repressor transcription, such as P53 and hypoxia [329]. Miyashita et al. suggested that miR-92a-2-5p and miR-148a play a role in immune responses to components of the Pfizer vaccine. Of particular interest, miR-92a-2-5p has been identified as a biomarker for small cell lung cancer [330]. However, the direct induction of miRNA dysregulation resulting from mRNA vaccines

can potentially have significant consequences for millions of people, including children, by triggering the pathogenesis of tumors or cancer relapses. This warrants careful monitoring and further investigation to ensure vaccine safety and efficacy in the long term.

4.13 Variation in adverse events

Many factors would contribute to the variation in AEs experienced by COVID-19 vaccine recipients. Although most recipients of the COVID-19 vaccines seem to have experienced no adverse effects, more and more reports of severe AEs, and death, due to COVID-19 vaccination are being confirmed. Factors contributing to the variation in AEs experienced by COVID-19 vaccine recipients include underreporting, limitations of adverse event reporting systems [122,331], and the difficulty in determining the true effects of vaccines. To further complicate matters, Haas et al. reported that although significantly more AEs were reported in the vaccine recipient groups compared to the placebo groups, the rates of reported AEs in the placebo arms were still considerable [332]. Although there has been an increase in chronic disease, cancer, and excess death in the past three years, [7,121,322], these events were mainly not related to the COVID-19 vaccines.

Underlying genetic mutations will predispose to certain AEs. Salter et al. described cases of rhabdomyolysis experienced after vaccination [333]. The first case was a 30-year-old woman with rhabdomyolysis after her second dose of the Moderna mRNA vaccine. She had a history of episodes of rhabdomyolysis, usually triggered by viral infections. She has a mutation in the ryanodine receptor 1 (RYR1) gene, increasing her risk of developing malignant hyperthermia and rhabdomyolysis. Furthermore, a 34-year-old man diagnosed with carnitine palmitoyltransferase II deficiency experienced rhabdomyolysis, with creatine kinase (CK) levels reaching 250,000 U/L, within 24 hours after receiving the AstraZeneca vaccine. The individual also possessed a pathogenic mutation of the RYR1 gene. This gene is responsible for encoding the ryanodine receptor located in the sarcoplasmic reticulum membrane of skeletal muscle cells. In fact, dysfunction in the RYR1 gene lowers the threshold for rhabdomyolysis. While preexisting diseases and genetic variations can predispose individuals to rhabdomyolysis, the administration of the vaccine may trigger the release of myotoxic cytokines like TNFα. This release of myotoxic cytokines can lead to the breakdown of skeletal muscle, exacerbating the risk of rhabdomyolysis after vaccination [333].

AEs will also be aggravated by specific medications taken by the vaccinee. Drugs used to treat AEs, such as fever, inflammation, and pain, can exacerbate the cytotoxicity of vaccines and immune responses, leading to more severe AEs. In an AstraZeneca study, higher rates of solicited reactogenicity were observed in vaccinated participants who received acetaminophen prophylaxis, compared to the no acetaminophen control [118]. Medications that require sulfation for their metabolism, such as acetaminophen and NSAIDs, can deplete the liver PAPS pool in 2 hours [334]. This will negatively impact sulfation of the GL, and other molecules, due to the decreased levels of available inorganic sulfate or PAPS (Figure 1) [21,224], exacerbating AEs.

The sulfation pathways' important role in disease prevention has been highlighted in this review and elsewhere [21]. In this review, the effect of COVID-19 vaccines on inorganic sulfate depletion has been discussed, where the pathological consequences would be aggravated by existing underlying degradation of the GL, sulfate deficiency or dysregulated sulfation pathways. The various epigenetic and genetic factors that influence sulfation should be considered, such as dietary factors / nutritional deficiencies, aging, male sex, comorbidity, genetic variation, inflammatory insults and oxidative stress, and various environmental factors [21]. Where adolescents are more inclined to drink, smoke and use recreational drugs that will deplete GSH and sulfate, athletes' overall health and redox status will determine their response to vaccination. Impaired redox homeostasis and associated oxidative stress seem critical to explaining increased susceptibility to AE post-vaccination.

Tostes et al. demonstrated the variability in individual responses and testosterone actions as contributing factors to cardiovascular disease [222]. They found that testosterone increased ROS levels in cells from hypertensive, compared to normotensive animals. They suggested that a genetic vascular predisposition can

further lead to the development or aggravation, of cellular dysfunction in response to testosterone [222]. High testosterone levels correlate with increased oxidative stress, while low testosterone levels contribute to redox imbalances [97]. The variation in responses to COVID-19 vaccines can also be attributed to the initial metabolic-energetic-redox status of the cell that can exacerbate cardiovascular risk; the global or local increases in testosterone and other hormones may produce differential effects based on specific cell types that are stimulated; the availability of inorganic sulfate to activate and inactivate steroid hormones, as well as regulate the sulfation of GAGs; and the 'sex' of the individual or cell/tissue may determine differential effects. The complexity of steroids' effects on cardiovascular health is evident [222].

Variations in AEs could also be expected due to variations in different tissue expression, cellular distribution of enzymes, and the effect of COVID-19 vaccines on gene expression. Different host factors will coordinate the responses to synthetic DNA and mRNA in COVID-19 vaccines differently, via intrinsic RNA and DNA sensory pathways, and interindividual genetic variations, such as SNPs or splice variants of transcripts of key signaling molecules. This may hamper the accurate elimination of foreign and self-cytosolic nucleic acids. This can result in sustained up-regulated pro-inflammatory responses, and increased risk of autoinflammatory and autoimmune conditions, genomic instability, and cancer [55]. The presence of dsRNA, which must be removed during transcription and mRNA purification *in vivo*, can lead to excessive innate immune responses and associated reactogenicity. Adenovirus vector vaccines are more complex than mRNA vaccines, involving additional layers of complexity, such as production in mammalian cell cultures, leading to diverse immune reactions and AEs. Variations encompass the type of adenovirus used as a vector, genetic modifications, cell lines for production, purification procedures, and specific gene designs expressing the GVG Sp. Significant variation also exists within vaccine candidates from a specific company, such as Pfizer, which has four vaccine candidates. Although BNT162b1 and BNT162b2 are nucleoside-modified mRNA vaccines, BNT162b1 encodes a dimerized RBD of Sp, while BNT162b2 encodes a full-length Sp. On the contrary, BNT162a1 is an uridine-based mRNA vaccine, and BNT162c2 is a self-amplifying mRNA-based vaccine. Variations in efficiency and reactogenicity have been observed between the different candidates [335]. Even though it appears that BNT162b2 has been mostly rolled out in mass vaccination, there is no distinct evidence or clarification on which candidate was used in most of the research and published case studies.

Independent investigations have revealed that some lots or batches of COVID-19 vaccines are associated with severe AEs, and death. On the contrary, other lots have resulted in very few or no AEs, related to their use [336,337]. It seems that there is variation among the different lot numbers of a particular vaccine and between the different COVID-19 vaccines [43]. Krutzke et al. found that AstraZeneca vaccines contained significantly higher than expected levels of host cell proteins (HCPs) and free viral proteins [338]. The HCP content exceeded the 400 ng specification limit per vaccine dose by at least 25 times, while the manufacturer's batch release data in some of the lots, indicated several hundred times. On the contrary, many of the J&J vaccine contained only very low amounts of HCP [338]. After evaluating monovalent Pfizer vaccines from the same lot, it became apparent that they contained excessive DNA, exceeding the specified limit by an 18-70-fold magnitude [116]. More studies are needed to compare the DNA content of different mRNA vaccine lots.

Furthermore, during the AstraZeneca clinical trials, a subset of participants received a lower vaccine dose than intended, due to unexpected interference of the excipient, polysorbate 80, with spectrophotometry assays [118]. Early commercial batches of the Pfizer vaccine were suggested to have lower levels of intact mRNA [337]. Furthermore, for large-scale manufacturing and distribution of COVID-19 vaccines, smaller biotechnological companies and institutions collaborated with large biopharmaceutical companies to mass-produce the vaccines. This will allow for a greater risk of contamination and manufacturing inconsistencies, since the choice of the DNA or mRNA purification technique used differs, depending on whether they are to be employed on a small or an industrial scale [339]. The variation in manufacturing and possible contamination, could also explain the differences in severity of AEs experienced due to batch-dependent variation of the COVID-19 vaccines [337].

It is important to note that the response in vaccine recipients after exposure to the same antigen differs, ranging from an asymptomatic course to 'cytokine storm' and death. The most severe AEs experienced are mainly due to the immune system's hyperactive response to the antigen. Apart from being a predisposing condition to AEs, preexisting chronic disease would be exacerbated, and the inflammatory response is usually concentrated in areas of previous trauma in the body. Due to variations in host factors, each vaccinee would respond differently to the COVID-19 vaccines. In contrast, some people can produce higher levels of GVG Sp, compared to others, and/or at a faster rate. A more robust immune response to vaccines could be expected in a younger generation, which correlates to the higher degree of AE experienced in this age group (age 18 to 64) [10,124,125].

Furthermore, various host factors will influence the biodistribution of NPs [84]. It was shown that after DNA and mRNA vaccination, the vaccine ingredients disperse rapidly from the injection site and can be found in most parts of the body [118]. After intramuscular mRNA vaccination, it was observed that mRNA spread to the brain [10]. The effect of COVID-19 vaccines will also depend on the formulation, dose, and route of administration [84]. Incorrect or variation in vaccination technique can lead to intravascular injection, increasing the risk of AEs. Although COVID-19 vaccines are administered intramuscularly [10], Friedensohn et al. found a higher seroconversion rate with accidental subcutaneous administration [340]. This is in line with the study by Yavuz et al., who observed ionizable LNPs induced Ab Th2-biased immunity when subcutaneously administered [70]. Direct intramuscular and intradermal injections of LNP-mRNA were shown to offer the best levels of antigen expression and duration of effect, with the production of antigen protein peak at 4 hours, and maintained locally 8–10 days after injection, depending on the dose.

In addition, the efficacy of mRNA administration with a needleless device was found to be much better than with direct needle injection [306]. De Beuckelaer et al. demonstrated that intranodal injection of mRNA lipoplexes provoked the most potent cytolytic T-cell responses [48]. Murine studies have revealed that intravenous injection of an mRNA vaccine produced myopericarditis [202]. Intravenous injection of mRNA can activate granulocytes, potentially leading to their entrapment in capillaries and causing tissue damage. Studies have shown that NETs from granulocytes can block microcirculation more effectively than thrombi [5]. The location of TLRs in different DC subsets and the distribution of DCs in various organs play a crucial role in the effectiveness of mRNA-based vaccines. Understanding this relationship can shed light on the immune response, efficacy and impact of factors such as route of administration, dose, and formulation of mRNA vaccines [306].

Various factors will affect NP biomolecular corona formation and, therefore, the *in vivo* behavior of NPs. Apart from the NP composition, the biofluid protein concentration, temperature, pH, ionic strength and disease state of the host, are all critical parameters that can influence the corona and, therefore, the subsequent nano-bio interactions [102,111]. This will potentially have important implications for LNP clearance, tissue accumulation, efficacy and effect on AEs. Since proprietary ingredients and manufacturing processes are being used in the COVID-19 vaccines, it is difficult to match the outcome with the physicochemical properties of NPs, and other possible ingredients in DNA and mRNA vaccines. As intellectual property or proprietary novel functional excipients, graphene-based NPs can be functionalized with the complex multilayered polymeric lipid delivery structures [101,341,342] used in COVID-19 vaccines [38,343-346].

5. Diagnostic tests

Increased D-dimer levels would give a good indication of disseminated intravascular coagulation. Elevated serum D-dimer has been reported among COVID-19 patients and many COVID-19-vaccinated individuals [347]. Through anecdotal evidence, a high tendency of *Helicobacter pylori* infection has been noted among COVID-19 vaccinated individuals. Physicians should, therefore, consider screening for this pathogen, especially if gut-related symptoms accompany AEs. Since sulfated gastrin stimulate acid secretion from gastric fistulas and

pepsin production [348], undersulfation and low stomach acid levels could predispose to *Helicobacter pylori* infection.

Markers of NETosis and serum NET levels, will be elevated levels of cell-free DNA, MPO or MPO-DNA, citrullinated histone H3 (CitH3) and neutrophil elastase [146,147,194]. MPO forms HOCl, by catalyzing the reaction of H_2O_2 with chloride ions (Cl^-), to facilitate the destruction of pathogens. HOCl rapidly depletes GSH. Glutathione sulfonamide is a sensitive marker for MPO-mediated reactions, while 3-chlorotyrosine and elevated levels of chlorinated aldehydes would indicate HOCl-mediated damage [262]. Cell-free DNA levels should show a strong correlation with other markers of inflammation, such as C-reactive protein (CRP) and D-dimer [147]. Furthermore, higher levels of dsDNA and calprotectin could be expected, together with DNA histone, granular enzymes, and sdc1, as found in VITT [5].

High serum levels of CRP would indicate Macrophage Activation Syndrome, while high serum levels of cytokines, especially IL-6, would be another biomarker of inflammation and robust immune response. Increased activity of pro-oxidative enzymes, such as MPO, points to oxidative stress, while oxidative damage is linked to higher production of malondialdehyde (MDA), protein carbonyls, and advanced protein oxidation products. Elevated levels of NO metabolites and IgM directed to nitroso neoepitope adducts indicate increased nitrosative stress. The lower total antioxidant capacity (TAC) of plasma is reflected in reduced levels of glutathione peroxidase (GPx), zinc, GSH and H_2S [187]. Increased serum transaminase levels are correlated with low systemic toxicity. AST and ALT levels were elevated in mice treated with PEG-LNP [36]. To identify EnGL damage, elevated plasma levels of sdc1, endocan, CS, HA, and HS would be useful markers [221], as in the case of COVID-19 [30].

Furthermore, increased levels of fibrinogen, fibrin degradation products, D-dimer, vWf, and soluble thrombomodulin indicate En injury. At the same time, impaired En function would be related to apoptotic ECs, decreased NO availability, and vascular leakage [28]. When sdc4 is expressed by EnCs, smooth muscle cells, and cardiac myocytes, there is a positive correlation between increased sdc4 serum levels of sdc4 and heart failure, making it a possible useful biomarker for predicting cardiovascular events. Sdc4 expression and shedding would increase due to mechanical stress on the walls of the vessel [349], such as the physical forces that LNPs and protein coronas would apply. Elevated troponin I level or abnormal wall motion on echocardiography may indicate myocardial involvement [350]. Cardiac troponin levels can therefore support a diagnosis of myocarditis [172].

6. Treatment considerations

Various antioxidants will be beneficial in the treatment of COVID-19 vaccine-induced AEs, by scavenging or chain-breaking ROS and peroxynitrite species, thereby converting them to less reactive products. Enzymatic antioxidants, such as superoxide dismutase, glutathione peroxidase, catalase, glutathione reductase, and glutathione transferase, as well as nonenzymatic endogenous molecules, such as N-acetylcysteine (NAC), taurine, and GSH, could be used. Thiols and low molecular weight antioxidants, such as methylsulfonylmethane, tocopherols, ascorbate, retinol, urate, and reduced GSH, would act as 'the second line of defense' against ROS [222].

The application of taurine will be an important treatment consideration for cardiovascular-related AEs. The beneficial effects of taurine have been demonstrated in many cardiovascular conditions, such as decreased serum low-density lipoprotein, decreased progression of atherosclerosis, anti-inflammatory effects, regulation of blood pressure and protection against ischemia–reperfusion injury of the myocardium. Therefore, taurine protects against coronary heart disease [227,351]. An increase in metabolic stress has been reported to induce a decrease in myocardial taurine. Cardiac metabolic stress can lead to necrosis, hypertrophy, and eventual heart failure. It has also been demonstrated *in vitro* that taurine reduces angiotensin-2's effect on rat cardiomyocytes' hypertrophy. Most of the beneficial effects of taurine on

particularly cardiac hypertrophy are due to its ROS scavenging properties, as well as its impact on intracellular Na^+ and Ca^{2+} overloads. Taurine deficiency has been shown to lead to retinopathy, cardiomyopathy, and cardiac abnormalities [227,351]. Taurine also significantly reduced pro-inflammatory cytokines in traumatic brain injuries, alleviating the severity of injury [227].

Furthermore, studies have demonstrated that taurine supplementation can reduce macrophage infiltration, elastin fragmentation, and MMP activation, which are linked to MPO overexpression. Taurine acts as a competitive target for HOCl [262]. The antioxidant and scavenging effect of taurine makes it an important treatment option to combat vaccine-induced oxidative stress, restore mitochondrial dysfunction, and improve cardiac energy metabolism.

Anaphylaxis events after Pfizer vaccination have responded to epinephrine treatment, although many cases required more than one dose of epinephrine [49]. Imai et al. described a case of vaccine-induced cytopenia in the presence of clozapine, which resolved after discontinuing the drug [352]. Immunological mechanisms of drug-related leukopenia have previously been reported after vaccination [352]. This phenomenon could be expected with drugs such as steroids, non-steroidal anti-inflammatory drugs (NSAIDs), and aspirin, which deplete or require sulfation for their metabolism [21]. Furthermore, suppose a vaccine recipient presents with any signs of serotonin syndrome, such as focal seizures or tremors, fasciculation, and muscle weakness; they should not be given a selective serotonin reuptake inhibitor (SSRI). Inorganic sulfate is needed to inactivate serotonin through sulfation.

A case of vaccine-induced eosinophilic granulomatosis with Polyangiitis (EGPA) was effectively treated with pulsed intravenous corticosteroids, starting with 250 mg of methylprednisolone for three days and transitioning to oral prednisone at a dose of 1 mg/kg/day. The patient experienced gradual recovery from myositis and blood eosinophil counts decreased significantly. Prednisone was reduced and eventually discontinued after six months [249]. This case indicates that COVID-19 vaccines might potentially overstimulate immune-inflammatory pathways involved in the pathophysiology of the disease. Adding a sulfur donor, such as NAC, would ensure a better outcome during steroid treatment [21,98]. Patients taking moderate or high doses of corticosteroids, have an increased risk of COVID-19 and vaccine-induced complications [49,353]. In acute viral myocarditis, the use of NSAIDs remains controversial and has also shown increased myocardial inflammation and mortality in murine models and human case studies [205,350]. NSAIDs deplete inorganic sulfate, thus exacerbating the underlying condition of inflammation, En damage, and thrombosis [21]. Drugs, such as acetaminophen, with limited anti-inflammatory effects, may have inadequate analgesic function, increase blood pressure, deplete a vast amount of inorganic sulfate, impair renal function and affect left ventricular performance [350,354].

Although IFNs are commonly used to treat viral myocarditis [205], they are not recommended for vaccine-induced myocarditis, in which case the antigens are responsible for robust inflammatory responses and oxidative stress. Since adequately sulfated HS is important in regulating the threshold for IFN stimulation of macrophages [21], one could expect that undersulfated macrophage HS, and a degraded GL, will drive the immune response towards a pro-inflammatory state when IFNs are used as treatment.

Inhibition of MMP13 in CVB3 infected mice has been shown to increase myocarditis [229]. Since HS plays a role in modulating MMPs, the application of NAC should be considered. NAC is the acetylated form of Cys. Cys would serve as a decoy, blocking the binding of GVG Sp to the cell surface and soluble HS, and as a potent anti-inflammatory, antioxidant and anticoagulant, plus precursor to inorganic sulfate [21,98]. Furthermore, due to its excellent antioxidant properties, NAC's application significantly reduced ROS [355]. It is important to address mitochondrial-dependent damage that could be triggered via oxidative stress, ischemia, and DNA damage. At the same time, mitochondrial dysfunction could induce mitoROS burst, further aggravating mitochondrial disorders, in turn [355]. Since thiol groups are established targets for MPO-derived oxidants, thiol-based therapeutics have broad applications in biomedicine [262].

NAC is bioavailable and can cross the BBB, where it acts via multiple pathways in the brain. Several animal studies have shown evidence of increased brain GSH after oral administration of NAC. Oral and transdermal GSH supplementation, administered to children on the autism spectrum, has been found to lead to significant increases in plasma reduced GSH, inorganic sulfate, Cys, and taurine levels [223]. NAC has been shown to be effective in treating many psychiatric and neurological disorders, and can be beneficial in preventing cognitive decline associated with acute physiological insults and dementia-related conditions. NAC has been shown to modulate several neurological pathways, including glutamate dysregulation, oxidative stress, and inflammation [356]. NAC scavenges ROS and several reactive nitrogen species, which play a role in the oxidation of lipids, proteins, and DNA. NAC also has anti-inflammatory activity, independently of its antioxidant activity [98,357]. NAC has antithrombotic effects and potentiates En NO's vasodilator and antiaggregatory effects. Intravenous NAC has been shown to promote lysis of arterial thrombi that is resistant to conventional methods. It has been suggested that the main molecular target of the antithrombotic activity of NAC is vWF, which cross-links platelets in arterial thrombi [357]. However, since NAC also serves as a precursor to inorganic sulfate, the various antithrombotic properties of an adequately sulfated GL must also be taken into account [21]. In hospitalized patients, with moderate to severe COVID-19 pneumonia, NAC 600 mg bid orally for 14 days, improved the PO2/FiO2 ratio over time, and decreased the white blood cell, CRP, D-dimer and lactose dehydrogenase levels [357].

To effectively decrease elevated D-dimer levels, it is necessary to first address the underlying inflammation as well as NETosis. Low-dose naltrexone offers promise in the treatment of severe inflammatory conditions in patients injected with a COVID-19 vaccine, as it can dampen innate immune responses and TLR signaling, and reduce IL-1, TNFα and IFN levels [358]. The application of nattokinase also shows promise in the treatment of vaccine-induced AEs [353], with anticoagulatory and neuroprotective properties [359-361]. Moreover, an *in vitro* study showed the potential to degrade the Sp [362]. More research needs to be performed to evaluate its *in vivo* effect on the GVG Sp. However, if administered with NAC, nattokinase shows great promise in the treatment of AEs induced by the COVID-19 vaccine.

Research has shown that patients with coronary heart disease, stroke, and myocardial infarction exhibit significantly reduced total GSH levels, making their erythrocytes more susceptible to hemolysis. This suggests that the administration of NAC or GSH could serve as a potential therapeutic strategy to prevent adverse cardiovascular events. Additionally, GSH levels are found to be decreased in asymptomatic cardiac patients with structural abnormalities, even before the onset of full-blown heart failure. Clinical data have demonstrated that intravenous administration of GSH during the acute phase of myocardial infarction, prior to coronary recanalization, can ameliorate reperfusion damage. This indicates that timely exogenous administration of GSH or NAC can potentially improve outcomes after myocardial infarction and slow the progression of cardiac abnormalities leading to heart failure [97]. Niwano et al. demonstrated that NAC suppressed myocarditis and electrical remodeling in a dose-dependent manner in immunized rats [228], which confirms that hyperoxidative stress plays an important role in promoting electrical and structural remodeling after vaccination.

NAC has also effectively treated acute liver failure, spermatogenesis disorders, and dermatological diseases [138]. It has been established that NAC alleviated viral-induced liver injury and has been suggested as a safe and effective cardioprotective therapeutic option for viral-induced myocarditis [204]. Furthermore, NAC is a rate-limiting precursor to hydrogen sulfide (H_2S), which plays an important physiological role in the nervous, circulatory and cardiovascular systems, along with renal physiology. H_2S has antiviral properties by down-regulating TMPRSS2 expression and inhibiting ACE2 activity. It exerts excellent anti-inflammatory actions by down-regulating IL6, inhibiting NF-κB and NLRP3 inflammasome, consequently reducing pro-inflammatory cytokines. A negative correlation has been observed between serum H_2S and levels of IL 6 and CRP [138]. The various antiviral, anti-inflammatory, antioxidant and anticoagulatory properties of NAC, and its role and application in COVID-19, have been discussed elsewhere [21,98].

Hypercatabolic states, such as cytokine storm and sepsis, are known to result in thiamine deficiency [363,364]. In addition to having direct antioxidant properties, thiamine is also essential for glutathione production [365]. Oliveira et al. observed immediate neurological improvement of encephalopathy in COVID-19 patients with high-dose intravenous thiamine administration. They hypothesized that the high catabolic states associated with the cytokine storm in critically ill patients with COVID-19 would result in thiamine depletion [363]. Sulaiman et al. found that the use of thiamine as an adjunct therapy in critically ill patients with COVID-19 was associated with a lower incidence of thrombosis [364]. Chen et al. successfully treated a patient with right facial weakness, after the second dose of the Moderna vaccine, with thiamine, riboflavin, and prednisolone [366]. Thus, postvaccination inflammatory mechanisms can thus also trigger neuropsychiatric symptoms via thiamine deficiency. High-dose intravenous thiamine, or benfotiamine, is recommended upon the presentation of neurological symptoms after vaccination.

Although dexamethasone has previously been shown to reduce neutrophil recruitment and NETosis, both in *in vitro* and *in vivo* murine models [147], Dowey et al. found that dexamethasone did not affect NETosis in neutrophils isolated from hospitalized patients with COVID-19 [146]. Since NETs drive inflammation, thrombosis, and disease severity, finding effective solutions to address NETosis is important. Ruboxistaurin, a protein kinase C (PKC) inhibitor, could reduce NET formation, thus diminishing airway inflammation and other events, including microvascular thrombosis [146]. Rapid initiation of treatment similar to severe HIT is recommended for patients with suspected or confirmed VITT. However, it is recommended to avoid HP and HP-containing products in VITT management due to case reports describing thrombosis progression after HP use, similar to HIT and autoimmune HIT complications. Non-HP anticoagulants are currently considered better therapeutic options. Aspirin as prophylaxis for VITT in vaccine recipients is not recommended, as a population-based study in Scotland demonstrated an increased frequency of hemorrhagic events up to nearly one month after vaccination by approximately 50%. Aspirin increases the risk of bleeding and has no clear benefit [196], plus it improves urinary excretion of inorganic sulfate [21].

To remove NETs, treatment with deoxyribonuclease-1, or pharmacological inhibitors, has also been suggested, which was shown to inhibit tumor-induced inflammation and metastasis [147]. Emerging evidence indicates that neutrophils play a role in promoting tumor growth and metastatic progression by forming NETs. Interestingly, the proposed mechanism for tumor enhancement involves NETs that stimulate mitochondrial biogenesis and bioenergetics in tumor cells through the induction and activation of TLRs and peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α), a key regulator of cellular energy metabolism. It has been suggested that this mechanism is how metformin inhibits the adhesion of cancer cells to NETs and consequently prevents metastasis [147].

Bioidentical progesterone will decrease the activity of SULFs, which have been implicated as the main drivers of hormonal cancers and metastasis. Sulfur donors, such as NAC, GSH, and methylsulfonylmethane, will increase the availability of inorganic sulfate and upregulate SULTs, resulting in the inactivation of estrogen through sulfation [21,224,367].

Elevated oxidative stress, as a result of increased expression of MPO, LNP activity, and inflammatory mediators, can result in phospholipid oxidation. LNP and HOCl-induced hemolysis, and cell lysis in general, can be addressed by the application of plasmalogens. HOCl-induced cytotoxicity has also been reported in immune and EnCs, lung and bronchial EpCs, chondrocytes, fibroblasts, and vascular smooth muscle cells [262]. Cellular plasmalogen lipids are a target for HOCl, which cleaves the plasmalogen vinyl ether linkage, resulting in elevated levels of chlorinated aldehydes [262,368]. Plasmalogens are especially abundant in neuronal, cardiac, and immune cells [264]. A deficiency, or destruction, of the antioxidant of the cellular membrane, plasmalogen, has been linked to various diseases, such as respiratory disorders, neurodegenerative, cardiovascular, cancer, and various inflammatory diseases, and metabolic syndrome. Both oxidative stress and chronic inflammatory conditions will result in plasmalogen deficiency [263,369-372], resulting in membrane defects [255]. Plasmalogen

replacement therapy will be an important treatment option to consider, by administering purified plasmalogens, and/or plasmalogen precursors, to increase plasmalogen levels. Chimyl alcohol and alkylglycerol are important precursors that have been shown to restore plasmalogen levels, while DHA-enriched lipids effectively increased plasmalogen levels in the brain [263,264,369,373]. Precursors are better absorbed than purified plasmalogens [368,373]. Since low-density lipoprotein (LDL) is a major transport protein of plasmalogens, the use of statin drugs might not be advisable to treat vaccine-induced AEs [372,374]. Furthermore, plasmalogen replacement therapy has been shown to be approximately twice as effective as statins in lowering cholesterol levels, where plasmalogen precursors reduced membrane cholesterol levels through increased membrane cholesterol esterification and transport [372,375]. More research is needed on the influence of statins on the physicochemical properties of cell membrane plasmalogens.

Low-dose methylene blue is a multimodal drug that has been used successfully to treat severe COVID-19 [376-378]. It has antiviral, anti-inflammatory and antioxidant properties, making it a promising treatment option to address mitochondrial dysfunction, chronic and neuroinflammation, oxidative stress, endotheliitis, and metabolic disturbances in COVID-19 vaccine AEs. Methylene blue improves mitochondrial respiration; it has inhibitory actions on activation of the NLRP3 inflammasome, NF-kB signaling, NO synthesis, and ROS production; it has applications in neurology as a neuroprotective agent; and it improves circulation and oxygen consumption of cells [376-380]. Since methylene blue promotes HOCl production [376,378], it would be recommended to use it with plasmalogen replacement therapy. Methylene blue should not be used in patients with glucose-6-phosphate dehydrogenase deficiency due to an increased risk of hemolytic anemia. Concomitant use of antidepressants, such as MAO inhibitors and serotonin reuptake inhibitors, is also contraindicated [376-378].

The protease inhibitor, doxycycline, significantly reduces GL PG shedding and should be investigated as a treatment option in AE associated with the vascular system. HPSE-2 should also be up-regulated, as it will inactivate HPSE-1 and thus reduce EnGL degradation [221]. Yuan et al. successfully treated EnGL degradation with HS mimetics [189]. Since a degraded EnGL will favor the migration of cytokines, vaccine antigens, and ingredients, focusing treatment options on restoring the GL will be necessary. To effectively address the various AEs caused by COVID-19 vaccination, it is clear that a comprehensive and personalized treatment protocol is needed, rather than a single-drug approach.

7. Conclusions

With substantial evidence indicating that the GVG Sp antigen, genetic material, and LNPs used in COVID-19 vaccines can lead to endotheliopathy [5], a re-evaluation of DNA and mRNA vaccination is warranted. Components in the vaccines, including the GVG Sp antigen, LNPs, and DNA/RNA, trigger pro-inflammatory cytokines and excessive oxidative stress, negatively affecting the integrity of the GL through reduced sulfation and degradation. This impairment of GL function may result in skewed inflammatory responses, compromised immunity, a procoagulatory state, and disease processes. In particular, COVID-19 vaccines did not prevent infection [381,382] or transmission [151,383] and were associated with a significant risk of chronic disease, serious AEs or death [7,20,121,384].

Nanotechnology, which has been hailed as a major advancement in medical research and nanovaccinology, has been rapidly deployed in the fight against COVID-19 [81,385]. However, lipid-based mRNA delivery systems, despite demonstrating high transfection efficiency *in vitro*, faced toxicity issues and poor pharmacokinetic profiles *in vivo*, resulting in unwanted inflammatory and immune responses. Inadequate research on the biocompatibility of NPs prior to clinical application led to severe adverse reactions and fatalities related to COVID-19 vaccination [7]. It is most likely that the LNP platforms resulted in acquired cellular immunopathology and severe oxidative stress. Although there is scope for using NPs in precision medicine with intelligent NP design, much more research is needed to gain valuable insights regarding its possible adverse effects *in vivo* at the molecular level. It is also essential to fully understand the molecular–cellular basis of the rare and severe AEs experienced after vaccination with the first generation COVID-19 vaccines, with more research urgently

needed in this area. However, it will only be possible to conclude by fully disclosing the precise content of the various COVID-19 vaccines and manufacturing procedures.

The maintenance and restoration of integrity of the GL is proposed as a primary therapeutic approach against COVID-19. GAGs and PGs, essential components of the GL, are highly complex biomolecules with significant structural and functional heterogeneity. Taking into account the complexity of the innate immune system, GL, and inflammatory responses, the current focus on adaptive immune responses in vaccinology should be revisited.

Synthetically induced chronic inflammation, as seen in some diseases, can have detrimental effects on tissues, organs, and normal cells, leading to increased morbidity and mortality. While a natural inflammatory response is beneficial, the pathological consequences of chronic inflammation highlight the importance of addressing the underlying causes of susceptibility to infectious diseases, such as promoting balanced wholefood nutrition, effective supplementation, moderate exercise, hygiene, rest, and autonomic balance.

Concerns arise from the continued development of various COVID-19 vaccines and plans for DNA and mRNA vaccines for other diseases. The risks associated with gene therapy and vaccination may outweigh the potential benefits, necessitating careful consideration and critical evaluation of their impact on the innate immune system and the general body system. The focus should be on addressing the root causes of diseases rather than pursuing profit-driven approaches. Additionally, the compatibility of synthetically engineered particles with the complexity of the human body's physiology must be thoroughly examined to ensure patient safety and well-being.

Data availability: Data sharing is not applicable to this review article as no datasets were generated or analyzed during the current study. All articles referred to in this review of the literature are listed in the list of references and are in the public domain.

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