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Dysregulation of Protein S in COVID-19

Martha M.S. Sim [a], Jeremy P. Wood [a,b,c,*]

[a] Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY, USA
[b] Gill Heart and Vascular Institute, Division of Cardiovascular Medicine, Department of Internal Medicine, University of Kentucky, Lexington, KY, USA
[c] Saha Cardiovascular Research Center, University of Kentucky, Lexington, KY, USA

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ABSTRACT

Coronavirus Disease 2019 (COVID-19) has been widely associated with increased thrombotic risk, with many different proposed mechanisms. One such mechanism is acquired deficiency of protein S (PS), a plasma protein that regulates coagulation and inflammatory processes, including complement activation and efferocytosis. Acquired PS deficiency is common in patients with severe viral infections and has been reported in multiple studies of COVID-19. This deficiency may be caused by consumption, degradation, or clearance of the protein, by decreased synthesis, or by binding of PS to other plasma proteins, which block its anticoagulant activity. Here, we review the functions of PS, the evidence of acquired PS deficiency in COVID-19 patients, the potential mechanisms of PS deficiency, and the evidence that those mechanisms may be occurring in COVID-19.

In early 2020, it was recognized that Coronavirus Disease 2019 (COVID-19), caused by infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is associated with an increased risk of thrombotic events [1]. Coagulation (Fig. 1) is the process by which soluble fibrinogen becomes converted to an insoluble fibrin clot. Under haemostatic conditions, this process is initiated by the exposure of extravascular tissue factor (TF) to flowing blood [2]. TF binds coagulation factor VIIa (FVIIa) and promotes the activation of factors IX and X to factors IXa (FIXa) and Xa (FXa), respectively [3]. FIXa, in complex with factor VIIIa (FVIIIa), activates additional FXa. FXa forms the prothrombinase complex with factor Va (FVa), which generates thrombin from its precursor prothrombin [4]. Thrombin is the serine protease that converts fibrinogen to fibrin, among other functions. Thrombosis, pathological blood clot formation, likely occurs through a similar pathway to haemostasis, though the initiator of the process is less clear. TF may become exposed on the surface of stimulated immune or endothelial cells, under inflammatory conditions [5]. Alternatively, thrombin may be produced through the intrinsic or contact pathway, in which exposure of negatively charged molecules, such as DNA, can lead to TF-independent activation of FIXa and FXa [6]. As excessive or insufficient thrombin generation leads to thrombosis or bleeding, respectively, the coagulation system is tightly regulated by anticoagulants, including TF pathway inhibitor (TFPI), activated Protein C (APC), and Protein S (PS) [2]. This review will focus on PS and the PS deficiency that occurs in patients with viral infections, including SARS-CoV-2.

* Corresponding author. University of Kentucky, 741 S Limestone, BBSRB B359, Lexington, KY, 40536, USA.
E-mail address: Jeremy.Wood@uky.edu (J.P. Wood).

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1. PS and the anticoagulant system

PS is a Vitamin K-dependent glycoprotein that circulates in plasma as a free soluble form (~40%, normal range ~5–15 mg/L) or as part of a high-affinity complex with complement component C4-binding protein/C4BP (~60%, normal range~10–20 mg/L) – bringing the total plasma PS to 15–35 mg/L – and is also packaged in platelet alpha-granules (~2.5%) and released upon platelet activation [7–9]. Plasma total and "free" PS (the pool not bound to C4BP) are commonly used as diagnostic tests. PS (Fig. 2) contains an N-terminal Gla domain, a thrombin sensitive region (TSR), four Epidermal Growth Factor (EGF)-like domains, and a sex hormone binding globulin (SHBG)-like region, which consists of two laminin G-type (LG) domains [10]. These domains provide PS with functions in the coagulation and inflammatory responses. The Gla domain mediates interaction with membrane phosphatidylserine and is critical for all known functions of PS. The EGF1-2 domains bind APC, and the LG domains bind C4BP, TFPIα, and FIXa [10]. C4BP binding blocks most of PS's anticoagulant function, and thus free PS is considered to be the anticoagulant pool [11].

PS (Fig. 1) is best known as a cofactor for APC, the serine protease that degrades the cofactors FVa and FVIIIa [12]. PS enhances APC-mediated FVa inactivation by promoting its binding to negatively charged phospholipids and subsequently increasing the rate of FVa cleavage [13]. PS also moderately enhances the rate of APC-mediated FVIIIa degradation [14]. More recently, PS was shown to promote the anticoagulant activity of the alpha isoform of TFPI (TFPIα), which inhibits the active sites of the serine proteases FXa and FVIIa [15,16]. PS enhances TFPIα-mediated inhibition of FXa by ~10 fold and inhibition of TF-FVIIa by ~9 fold [15]. The enhancement of FXa inhibition is dependent on the ability of PS to bind membranes and is abolished by the tethering of TFPIα to membranes, suggesting that PS promotes the binding of TFPIα to membrane surfaces, where FXa is localized [16]. The inhibition of FXa is further enhanced by interaction of PS and TFPIα with an alternatively spliced form of factor V called FV-short [17]. Finally, PS also has direct anticoagulant activity. It binds FIXa and blocks assembly of the intrinsic tenase complex, thereby preventing FXa activation [18,19]. In short, PS can be thought of as a master anticoagulant, regulating all three coagulation complexes.

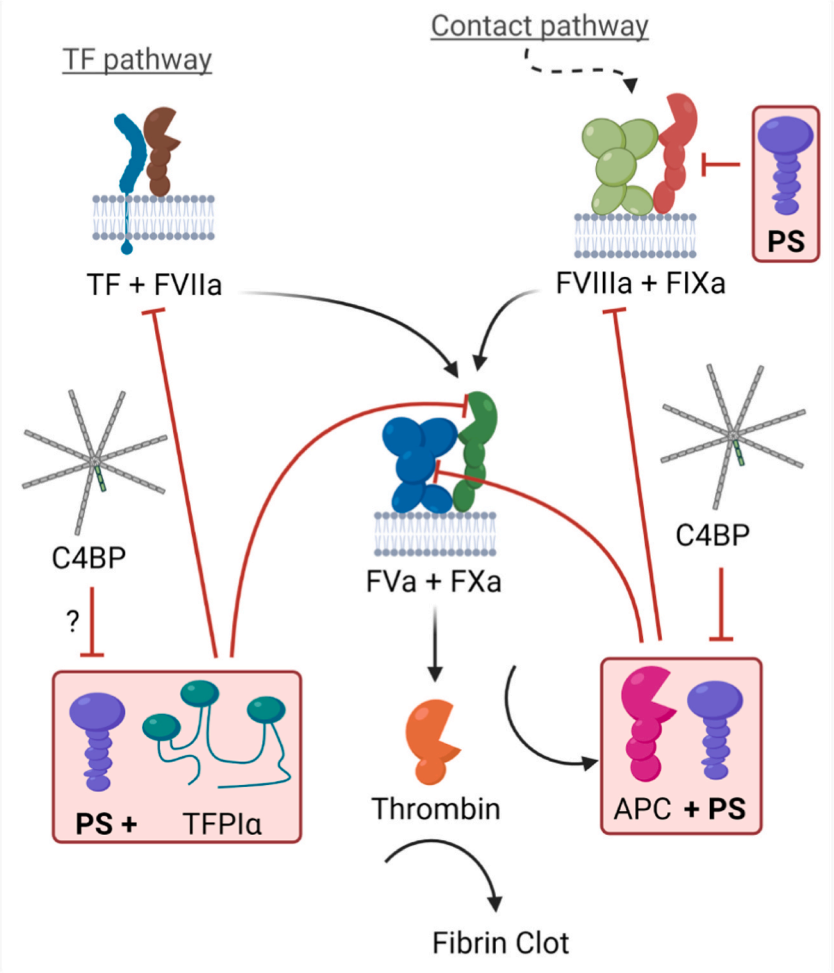

Fig. 1. Protein S anticoagulant functions. Protein S is a master anticoagulant regulating all three coagulation complexes, via its cofactor function with Tissue Factor Pathway Inhibitor-α (TFPIα) and activated Protein C (APC), and its direct anticoagulant acitivity. (TF = Tissue Factor; FVIIa = factor VIIa; FVIIIa = factor VIIIa; FIXa = factor IXa; FVa = factor Va; FXa = factor Xa; C4BP = complement component C4b-binding protein).

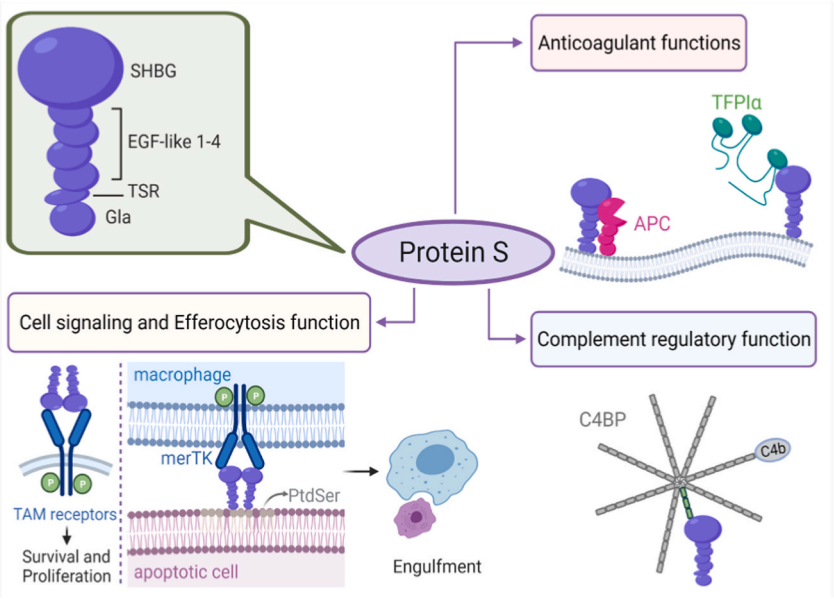

Fig. 2. Diverse functions of Protein S. Protein S consists of an N-terminal Gla domain, a thrombin sensitive region (TSR), four Epidermal Growth Factor (EGF)-like domains, and a sex hormone binding globulin (SHBG)-like region. These domains provides Protein S with functions in regulating coagulation, complement activation, cell signalling, and efferocytosis.

2. PS as a regulator of complement

PS also possesses anti-inflammatory functions. ~60% of plasma PS circulates in a noncovalent high-affinity complex with C4BP [20]. Only the free fraction of plasma PS has full anticoagulant properties, as C4BP blocks APC cofactor activity of PS [11]. Due to the high affinity interaction (K_d of ~0.1 nM), the concentration of free PS is largely dictated by the plasma concentration of C4BP β-chain, the PS-binding component [7,21,22]. C4BP is a 570 kDa protein composed of seven α-chains that are the binding sites for C4b and one β-chain that binds PS, held together by disulfide bonds. C4BP is synthesized primarily in the liver and secreted into the blood (average plasma concentration = 200 mg/L; 300 nM). There are three isoforms of C4BP: (a) 7 α-chains 1 β-chain (the most abundant; ~160 mg/L), (b) 6 α-chains 1 β-chain, and (c) 7 α-chains without β-chain [21,23]. *In vitro,* successful secretion of C4BP β-chain depends on its intracellular complex formation with PS in the endoplasmic reticulum [21]. C4BP inhibits the C3 convertase, the C4b2b complex of the complement system, by promoting degradation of C4b. C4BP is an acute phase protein and its level may increase up to 4-fold during an inflammatory response [7]. This rise in C4BP largely pertains to an increase in α-chain synthesis, while synthesis of the β-chain is less affected, due to differential regulation of expression by cytokines [23]. As such, an increase in C4BP during inflammation will not necessarily result in a decrease of free PS.

While the exact function of the PS-C4BP complex is largely unknown, PS is proposed to regulate the complement system by localizing C4BP to negatively charged phospholipids present on the surface of apoptotic cells [24]. PS has been shown to enhance C4BP binding to neutrophils and to control complement activation at sites where coagulation has initiated [25]. Cell surface-associated PS-C4BP can bind to C4b and inhibit phagocytosis [26]. C4BP also binds to necrotic cells via PS-PtdSer binding [27]. Furthermore, C4BP binds DNA via a patch of positively charged amino acids on the second component control domain of its α-chains, limiting DNA release from necrotic cells and inhibiting DNA-mediated complement activation *in vitro* [27]. It is unclear whether PS binding affects the C4BP/DNA interaction. Overall, however, the PS/C4BP complex reduces the pro-inflammatory state.

3. PS and TAM receptors

In addition to its functions with C4BP, PS has C4BP-independent anti-inflammatory activity, which is mediated through its SHBG domain. PS and its homolog, growth arrest-specific factor 6 (Gas6), are ligands of TAM (Tyro3, Axl, and MerTK) receptor tyrosine kinases, key modulators of the innate immune system and part of an important anti-inflammatory signaling axis. PS activates Tyro3 and MerTK, with limited evidence of effect on Axl, while Gas6 binds to all three TAM receptors [28]. Both proteins interact with the TAM receptors via their SHBG-like region. TAM receptor activation leads to intracellular signaling pathways regulating cell differentiation, metastasis, angiogenesis, and other processes that culminate in cell survival, proliferation, and anti-inflammatory effects [29–32].

PS and Gas6 are also bridging molecules that facilitate binding between exposed phosphatidylserine on apoptotic cells and TAM receptors on macrophages, triggering receptor activation and subsequent cell signaling to promote efferocytosis; the phagocytosis of dead or apoptotic cells, which is essential for normal homeostatic cell-turnover and for clearance of infected cells [33]. Activation of

Tyro3 and Mer by PS attenuates the response of macrophages and dendritic cells to pathogens and reduces the secretion of proinflammatory cytokines such as IL-6, TNFα, and type 1 interferons [34]. Gas6/PS-stimulated efferocytosis contains cell death and dampens the innate immune response within the affected tissue, preventing inflammation and autoimmunity and protecting the surrounding healthy tissues [35,36]. The immune system also uses efferocytosis as a way to protect the host from pathogens; by mediating phagocytic clearance of infected cells. For example, PS and Gas6 mediate macrophage efferocytosis of HIV-1-infected T-cells by bridging phosphatidylserine exposed on the infected cells to macrophage-expressed Mer tyrosine kinase [37]. In that *in vitro* study, efferocytosis was shown to remove not only dead infected T-cells, but also live virus-producing cells.

4. PS deficiency

Based on its status as a master anticoagulant, one might expect PS deficiency to be associated with thrombosis. Indeed, homozygous PS deficiency is associated with lethal thrombosis shortly after birth and heterozygous deficiency with a 5–11.5-fold increased risk of thrombosis [38]. While genetic PS deficiency in the general population is relatively rare (~0.03–0.13% allelic frequency) [39], it is more prevalent in East Asian populations, and in populations with familial history of venous thrombosis [40]. Several biological factors also influence plasma PS concentration: men have higher free PS and total PS than women, neonates have lower total PS but higher free PS than adults, and total PS increases further with age [41].

Acquired PS deficiency is more common than hereditary deficiency and is associated with conditions such as pregnancy, nephrotic syndrome, liver disease, disseminated intravascular coagulation, autoimmune diseases, multiple myeloma, the use of oral contraceptives, chemotherapy, or vitamin K-antagonists, and several viral infections [39,42].

Acquired PS deficiency is a relatively common complication of infection with human immunodeficiency virus 1 (HIV-1) [43–46], varicella [47], dengue [48], and SARS-CoV-2 [49,50], all of which are associated with an increased risk of thrombosis. The best studied of these is HIV-1. Acquired PS deficiency is the most common coagulation abnormality in HIV-1 patients, with frequency as high as ~76% [46]. It correlates with decreased T-cell count and progression to acquired immunodeficiency syndrome [44]. We recently showed that PS deficiency in people living with HIV-1 correlates with increased thrombin generation potential [45]. As Brummel-Ziedins et al. showed that increased thrombin generation correlates with all-cause mortality in people living with HIV-1 [51], this suggests that PS deficiency is a contributing risk factor [46,52]. The cause of the acquired PS deficiency is unknown, but may involve decreased biosynthesis, increased coagulation consumption, production of inhibitory PS autoantibodies, increased C4BP, or a combination of these mechanisms [52–54], some of which may also be occurring following SARS-CoV-2 infection.

5. PS deficiency in COVID-19

Soon after the identification of thrombotic risk in COVID-19 patients, acquired PS deficiency was hypothesized to be a contributing factor. Since then, multiple studies have described PS deficiency (Fig. 3) in this population, lending credence to this hypothesis. Here, we review the evidence for dysregulation of PS antigen or function in COVID-19, and discuss the possible mechanisms for this

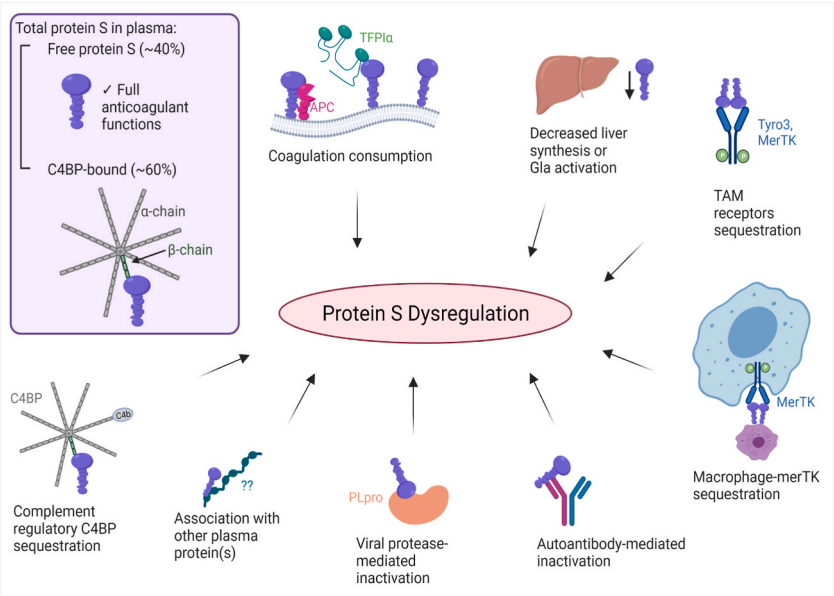

Fig. 3. Possible causes of Protein S dysregulation in COVID-19. Plasma Protein S can be dysregulated through coagulation consumption, decreased liver synthesis or Gla activation, sequestration by the TAM (Tyro3, Axl, Mer) receptors or by complement component C4b-binding protein (C4BP), inactivation by autoantibody or by viral papain-like protease (PLpro), or by increased association with other plasma protein(s).

dysregulation.

Stoichitoiu et al. reported a decrease in PS activity in 65% of a 91-inpatient cohort at admission [49], which was confirmed to be secondary due to COVID-19 by a follow up measurement at ~2.5 months post infection [50]. This deficiency correlated with the extent of pulmonary damage, disease severity and mortality. Ferrari et al. found a 20% prevalence of PS deficiency in a cohort of 89 hospitalized COVID-19 patients [55]. A case report by Ali et al. discussed a patient with PS deficiency and COVID-19 infection presenting with a recurrent stroke [56]. Lemke et al. suggested that procoagulant activity and immune hyper-reaction in COVID-19 might be exacerbated by PS deficiency [57].

Martín-Rojas et al. [58] reported a free PS reduction (median 56.6%) in 206 hospitalized COVID-19 patients, with 33% of patients showing levels below 50%. The lowest levels of free PS were observed in patients receiving low-flow oxygen therapy ($p < 0.001$) [58]. Panigada et al. measured free PS antigen in 11 ICU patients with mechanical ventilation and saw a marginal decrease (69 IU/dL; reference range 60–140) as well. On the other hand, Voicu et al. [59] measured PS activity and free PS antigen concentrations in their cohort of 82 mechanically ventilated COVID-19 patients and found a slightly reduced activity (58 IU/dL; reference range 50–130) with preserved antigen level (79 IU/dL; reference range 50–130). Here, they compared the PS activity or antigen level to the absence or presence of venous thromboembolic event and found no statistical difference [59].

In a cohort of thirty ICU patients, Corrêa et al. [60] reported low free PS at baseline that occurred to a similar extent in patients with SOFA (Sequential Organ Failure Assessment) scores ≤ 10 or > 10. The low free PS was observed to recover over time up to 14 days post-ICU admission [60]. Sehgal et al. [61] showed that free PS is reduced in a cohort of eighty hospitalized patients, but is not different between the non-survivors and survivors, between those with SOFA scores of 0 or ≥ 1, or between those with or without Acute Respiratory Distress Syndrome. Free PS concentration was in the 60–70% range (reference range 60–140%) and remained so through day 7 and at discharge [61]. Hardy et al. [62], on the other hand, followed ICU stay of 21 severe patients and observed that free PS antigen level were still within normal range, albeit lower at day 1–10 (median 80%), compared to either day 11–20 (median 114%) or day 21–30 (median 110%) of care [62]. White et al. [63] also looked at free PS antigen and compared it between 34 noncritical and 75 critical COVID-19 patients and observed similar extent of reduction (mean 52.1 vs 54.5 IU/dL; reference range 65–135). Fan et al. [64] also looked at PS activity between 10 mild and 10 severe patients and found mild reduction, but no difference between mild and severe patients (median 74% vs 71%; reference range 55–130%).

Bauer et al. [65] analysed 58 hospitalized adult patients with clinically suspected COVID-19 and reported no statistical difference in PS activity in 41 non-COVID-19 (mean 93%) and 17 COVID-19 (mean 74%) patients ($p = 0.06$), with 22% of non-COVID-19 and 35% of COVID-19 patients being below reference range [65]. Overall, these studies demonstrate a mild to moderate decrease in either free PS antigen or PS activity in COVID-19 patients. This decrease is dependent on infection, but appears in many studies to be independent of disease severity. This suggests that, while PS deficiency may make a patient more prone to thrombosis, a second procoagulant hit is required for a thrombotic event to occur.

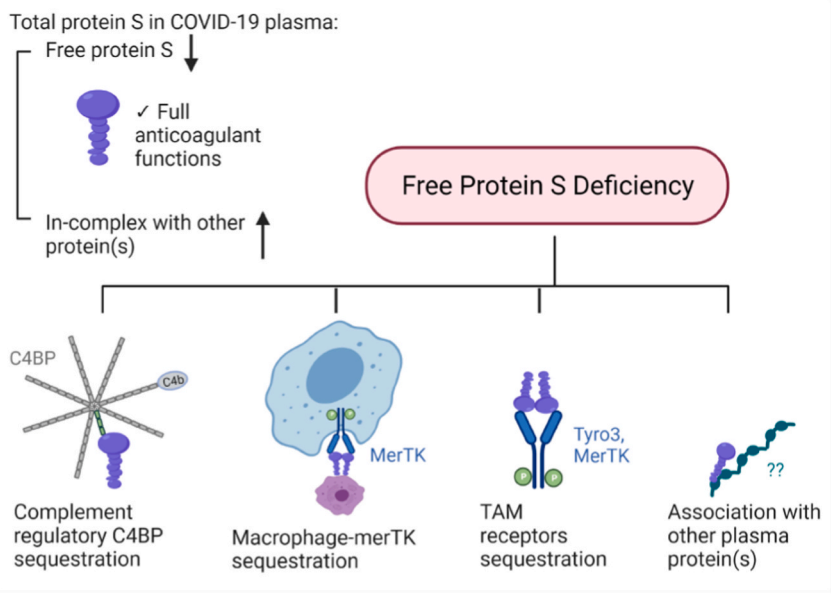

Fig. 4. Possible causes of free Protein S deficiency in COVID-19. Free Protein S deficiency can occur through increased sequestration of plasma Protein S by either complement component C4b-binding protein (C4BP), TAM (Tyro3, Axl, Mer) receptors, or by increased association with other plasma protein(s).

6. Possible mechanisms of acquired PS deficiency

There are many potential causes of acquired PS deficiency, which vary depending on whether Total PS or only free PS is reduced. Total PS deficiency (Fig. 3) may result from increased consumption, clearance, or degradation, or from decreased synthesis. By contrast, free PS deficiency (Fig. 4) is caused by binding of PS to another protein, generally presumed to be C4BP.

6.1. Loss due to consumption

PS may be consumed through any of its described functions, all of which are relevant to COVID-19 pathology. COVID-19 has been associated with macro- and microthrombosis, along with disseminated intravascular coagulation-like pathophysiology [66,67]. Dysregulation of coagulation factors has been widely reported, as well as consumption of fibrinogen in severely ill patients [68], indicating that PS may be consumed through its actions as an anticoagulant.

COVID-19 is also associated with a local and systemic hyperinflammatory response, which includes dysregulation of the PS/Gas6-TAM receptor tyrosine kinase axis and activation of the complement system. Morales et al. found that higher plasma Gas6 and Axl were predictive of COVID-19 severity and mortality [69]. In a more recent study, Tonello et al. showed that baseline plasma Gas6 concentration was higher in patients with adverse outcome while soluble MerTK was lower in patients who had quicker resolution of the disease [70]. While both of these studies did not look at PS, contribution of PS as ligand for TAM receptors in COVID-19 has been acknowledged [57,71]. In COVID-19, hyperactivation of the complement system is also evident [72,73]. Patients with severe COVID-19 have high circulating levels of complement activation markers C5a and C5b-9, which correlate with disease severity [74, 75]. Complement component C5 and C5a receptor activation on endothelial cells, neutrophils, monocytes and macrophages and the resulting release of TF on neutrophil extracellular traps are recognized to be important drivers of COVID-19 thromboinflammation [76, 77]. Data from UK Biobank indicate single-nucleotide variants in the gene encoding the C4BP α chain as risk factors for morbidity and death from SARS-CoV-2 infection [78]. More studies are needed to understand the extent of PS depletion that might result from activation of TAM receptors and the efferocytic process, along with complement dysregulation.

6.2. Loss due to degradation by viral protease

Studies have also suggested that PS may be degraded directly by the SARS-CoV-2 virion. Group IV and VI viral cysteine proteases specifically recognize conserved cleavage site sequences of ~6–8 amino acids and have been shown to target approximately 40 host proteins [79]. SARS coronaviruses, including CoV-2, encodes two cysteine proteases; 3C-like protease and papain-like protease [80]. The papain-like protease of SARS-CoV-2 has been shown to target PS *in vitro*. Ruzicka et al. identified a prospective cleavage site in the first laminin G domain, which would result in removal of ~80% of the SHBG domain from PS, likely eliminating most of its function [81]. Reynolds et al. showed cleavage of PS following prolonged treatment with the papain-like protease (65–72 h at room temperature) [79]. By contrast, treatment of pooled human serum with the protease did not result in readily detected cleavage products [79]. Thus, it is not clear whether PS may be proteolytically degraded by the papain-like protease *in vivo*.

6.3. Loss through antibody-mediated clearance

Acquired PS deficiency with autoantibodies against PS and severe thrombosis are occasionally observed following varicella infection in children [47], and has been suggested to contribute to PS deficiency in HIV-1 patients [53].While autoantibodies against PS have not been demonstrated with COVID-19, there is evidence of other autoantibody formation. Persistent presence of lupus anticoagulant, anti-cardiolipin or β2-glycoprotein antibodies are risk factors for thrombosis in patients with antiphospholipid syndrome and have been associated with coagulation abnormalities and hyperinflammation in COVID-19 [82]. Antiphospholipid antibodies (aPL) target phospholipids and phospholipid-binding proteins, including proteins involved in coagulation, such as prothrombin, thrombomodulin, antithrombin III, protein C, and PS [82,83]. Oosting et al. investigated IgG fractions from patients with aPL who suffered thrombotic complications, and found subpopulations of IgG directed to prothrombin, APC, and PS, and confirmed the inhibitory effects of these antibodies [84]. Ferrari et al. found a 20% prevalence of PS deficiency and 72% prevalence of antiphospholipid antibodies, mainly lupus anticoagulant, in a cohort of 89 hospitalized COVID-19 patients. However, these findings did not correlate with abnormal coagulation activation markers [55].

6.4. Loss due to decreased synthesis

Apart from increased consumption or clearance, decreased total PS could be caused by reduced synthesis, resulting from the inflammatory and respiratory nature of COVID-19. Chatterjee et al. showed that hypoxia and interleukin-6 downregulate PS hepatic expression via stabilization of transcription factor hypoxia inducible factor 1 [85,86]. Mast et al. [87] showed evidence of downregulation of PS gene expression (~54-fold transcript reduction) in broncho alveolar lavage fluid samples of COVID-19 patients. Dofferhoff et al. found evidence of extrahepatic vitamin K insufficiency in a cohort of hospitalized COVID-19 patients, which was related to poor outcome [88]. Vitamin K is necessary for synthesis of PS. As vitamin K is preferentially transported to the liver for the activation of vitamin K-dependent procoagulant factors, including PS, this mechanism might result in a loss of PS activity [89,90]. Finally, it is possible that PS is co-translationally processed by the papain-like protease, resulting in either intracellular degradation or secretion of an inactive protein [79].

6.5. Free PS deficiency

The majority of studies that have reported PS deficiency in COVID-19 patients have either reported free PS or PS activity, a clot time-based measurement of the APC cofactor activity of free PS [49,50,55–65]. It is possible for total PS antigen to remain normal but free PS, and PS anticoagulant activity, to decrease. Indeed, we recently reported specific free PS deficiency in a cohort of 82 COVID-19 patients, including mild and severe patients [91].

Due to its diverse functions, PS interacts with many proteins discussed above, including C4BP, APC, TFPIα, FIX, Tyro3, and MerTK. C4BP is known to block PS anticoagulant activity and is commonly increased during infection. This increase can lead to a shift between the free and bound PS pools and result in reduced PS anticoagulant function, as was shown in an HIV-1-infected patient cohort [54]. In that study, Bello et al. showed that free PS deficiency correlated with elevated C4BP. The patients also showed evidence of thrombophilia, as indicated by a prolonged euglobulin clot lysis time compared to controls [54]. Conversely, C4BP deficiency can result in increased PS activity, as reported by Mulder et al., in a patient with ischemic retinopathy, who had increased free PS and decreased C4BP and PS-C4BP complex [92].

C4BP is well-described to decrease free PS and block anticoagulant activity, but other as-yet-unidentified proteins could have similar effects. We recently showed, using native gel electrophoresis, that PS complexes with many other protein(s) in its native plasma environment [91]. These proteins include the ones described above, but also others that we have yet to identify. It will be important to understand how these binding partners alter PS functions and the results of clinical PS assays.

7. Summary

PS is a key regulator of the coagulation and inflammatory responses. Thus, PS deficiency is a common result of inflammatory conditions, including viral infections such as SARS-CoV-2, but also including brain injury, autoimmune diseases, and cancers. Many studies have shown that acquired PS deficiency occurs in SARS-CoV-2+ patients, though the mechanism(s) leading to this deficiency are unclear. They may involve consumption, degradation, antibody-mediated clearance, decreased synthesis, or binding to another plasma protein, such as C4BP, that blocks PS anticoagulant function. More studies are necessary to understand which among these processes are the most relevant and tributary to the cause, in order to establish the appropriate clinical strategy for treatment.

8. Practice points

- Acquired Protein S deficiency commonly occurs in COVID-19, with variable contribution to thromboembolic occurrence.
- The cause of Protein S deficiency varies depending on whether total, or only free, Protein S is reduced.

9. Research agenda

- Protein S anti-inflammatory and complement regulatory functions, and their contributions to deficiency, are poorly understood.
- As more studies rely on free Protein S antigen and activity measurements, it is essential to understand what plasma proteins modulate these tests.

Declaration of competing interest

J.P.W. has an investigator-initiated grant through Pfizer Inc. that is unrelated to this work. M.M.S.S. has no conflict of interest to disclose.

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