Peer-reviewed version available at *Int. J. Mol. Sci.* **2020**, *21*, 5168; doi:10.3390/ijms21145168

Type of the Paper: Review

COVID-19: the rollercoaster of fibrin(ogen), D-dimer, von Willebrand Factor, P-selectin and their interactions with endothelial cells, platelets and erythrocytes

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Abstract: Severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2), coronavirus disease 2019 (COVID-19)-induced infection is strongly associated with various coagulopathies that may result in either bleeding and thrombocytopenia or hypercoagulation and thrombosis. Thrombotic and bleeding or thrombotic pathologies are significant accompaniments to acute respiratory syndrome and lung complications in COVID-19. Thrombotic events and bleeding, often occurs in subjects with weak multiple risk factors and co-morbidities. Of particular interest are the various circulating inflammatory coagulation biomarkers involved directly in clotting, with specific focus on fibrin(ogen),

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D-dimer, P-selectin and von Willebrand Factor (vWF). Central to activity of these biomarkers are their receptors and signaling pathways on endothelial cells, platelets and erythrocytes. In this review, we discuss vascular implications of COVID-19, and relate this to circulating biomarker, endothelial, erythrocyte and platelet dysfunction. During the progression of the disease, these markers may either be within healthy levels, upregulated or eventually depleted. Most significant is that patients need to be treated early in the disease progression, when high levels of vWF, P-selectin and fibrinogen are present with still low levels of D-dimer. Progression to vWF and fibrinogen depletion with high D-dimer levels and even higher P-selectin levels, followed by the cytokine storm, will be indicative of a poor prognosis. We conclude by looking at point-of-care devises and methodologies in COVID-19 management and suggest that a personalized medicine approach should be considered in the treatment of patients.

Keywords: keyword 1 COVID-19; keyword 2 Fibrin(ogen); keyword 3 Thrombosis; keyword 4 Bleeding (List three to ten pertinent keywords specific to the article; yet reasonably common within the subject discipline.)

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2), coronavirus disease 2019 (COVID-19)-induced infection is strongly associated with various coagulopathies [1-5]. This is entirely consistent with infection-induced inflammatory changes as observed in patients with disseminated intravascular coagulopathy [6]. Because of limited clinical patient data and the current lack of clinical trial data, it is important to investigate all possible adjuvant therapies that may contribute to a better patient outcome, specifically with regards to the coagulation profile. Of particular interest are the various circulating inflammatory coagulation biomarkers involved directly in clotting, with specific focus on fibrin(ogen), D-dimer, P-selectin and von Willebrand Factor (vWF). Changes in their levels can lead to an imbalance between pro-coagulant and anticoagulant factors, e.g., fibrinogen contributes to thrombus formation, while loss of high molecular vWF causes a bleeding tendency [7]. During COVID-19 pathology, depending on the severity of the condition, dysregulation has been noted in all of the mentioned biomarkers, where increased levels of P-selectin [8], fibrinogen and D-dimer accompany COVID-19 progression [4, 5, 9-15] and vWF [16, 17] have been found. D-dimers are normally not present in blood unless coagulation has occurred, and the D-dimer therefore serves as

a biomarker for thrombosis [18, 19]. The normal range of D-dimer is < 0.50 µg/mL. Interestingly, during early onset of the condition, D-dimer is normal (clinical observation by co-author Laubscher).

Although both low and high levels of fibrinogen (normal levels are between 2 – 4 mg/mL) have been reported in COVID-19 [4], central to the presence of high levels of fibrinogen is the presence of increased blood viscosity. COVID-19-associated hyperviscosity has been reported and its presence ascribed to a potentially severe consequence of infection [20]. An important marker of COVID-19 disease severity might thus also be erythrocyte sedimentation rate (ESR). It can be expected that where fibrinogen levels are increased, there will be greater tendance for rouleaux formation and thus a raised ESR, as the relatively dense rouleaux will sink to the bottom faster [21]. Rouleaux formation may also happen due to the presence of inflammation and an increase in acute phase proteins in circulation [21]. Al-Samkari and co-workers reported that ESR levels of >40 mm/h [adjusted OR, 2.64 (1.07-6.51)] [4], as well as increased levels CRP, fibrinogen, ferritin, and procalcitonin, could be found in patients with thrombotic complications, but the opposite could also accur in a bimodal phenomenon, where pathologically lower levels may lead to bleeding. The authors also mentioned that clinically-relevant thrombocytopenia, and reduced fibrinogen were rare but when present, were associated with significant bleeding manifestations [4]. An important clinical dilemma in this pandemic, is that the patient cohort is extremely diverse, with no two patients with the same clinical profiles. A one-treatment-for-all regime is therefore not a useful clinical approach (and may be dangerous). Rather, a personalized patient-orientated clinical approach should be followed, where various biomarker analysis, including clotting profile point-of-care analysis, would be the most successful approach. However, there is a fine balance between time, number of patients and viable options during this pandemic. Here we focus on some of the important biomarkers in coagulation pathology.

Fibrinogen, D-Dimer, vWF and P-selectin are central in the development of coagulopathies, and coagulopathies with diverse aetiologies have been described in COVID-19 patients. An example is the augmented risk of venous thromboembolism [7]. In a cohort study involving 201 patients with confirmed COVID-19 pneumonia, risk factors associated with the development of acute respiratory distress syndrome (ARDS) and progression from ARDS to death included among others coagulation dysfunction [22]. For patients with ARDS who died, coagulation function indices including D-dimer ($p = 0.001$) were significantly elevated compared with patients with ARDS who survived; elevated D-dimers were prognostic of worse outcome in other reports as well [22]. As mentioned previously, during early onset of the condition, D-dimer is normal, and as the patient disease severity progresses, D-dimer levels are significantly increased (clinical observation by co-author). It was also suggested that the early evaluation and continued monitoring of D-dimer levels after hospitalization may identify patients with cardiac injury and predict further COVID-19 complications [23]. Furthermore, it was

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noted that D-dimer on admission greater than 2.0 µg/mL (fourfold increase; the normal range of D-dimer is < 0.50 µg/mL) could effectively predict in-hospital mortality in patients with Covid-19 [24]. One dilemma in using D-dimer or fibrinogen levels as biomarkers, was pointed out by Favaloro and Thackil in 2020 [14]. The authors argue that care should be taken with regards to the units and to use standardized D-dimer and fibrinogen assays, when looking at COVID-19 patient data [14]. Recent recommendations also suggest that all hospitalized COVID-19 patients should receive thromboprophylaxis, or full therapeutic-intensity anticoagulation if such an indication is present [25]. However, we argue here that detailed measurement of levels of fibrin(ogen), D-dimer and other markers of hypercoagulation, especially P-selectin and vWF are of importance during thromboprophylaxis.

Platelet levels are typically in the range 150,000 to 450,000 platelets per µL. A platelet count of less than 150,000 platelets per µL is lower than normal and if it is below normal, the patient has thrombocytopenia. Importantly, in the context of COVID-19, the risk for serious bleeding occurs when the levels are as low as 10,000 or 20,000 platelets per µL. Together with fibrin(ogen) and D-dimer analysis, thrombocytopenia in COVID-19 is also well recognized [26-28]. Low platelet count is associated with increased risk of severe disease and mortality in patients with COVID-19, and thus should serve as a clinical indicator of worsening illness during hospitalization [26]. Thrombocytopenia is a well-known pathology during viral (and bacterial) infections [29]. One cause of depleted platelet numbers might be because of an increase in circulating biomarkers (including fibrin(ogen), D-dimer, P-selectin and vWF) that may directly bind to platelet receptors, followed by platelet hyperactivation and aggregation. During such hyperactivation, platelet count is lower, as hyperactivated and aggregated platelets are not counted during platelet count analysis. Thrombocytopenia was also noted in COVID-19 pathogenesis [30]. During COVID-19 pathogenesis, endothelial activation and interaction with the various inflammatory biomarkers, as well as the virus material, may also be crucial.

In this paper, we discuss the nexus between COVID-19 and circulating inflammatory biomarkers, with a particular focus on fibrin(ogen), its breakdown products (especially D-dimer), P-selectin, and vWF. We review literature that shows how circulating biomarkers could be used in the early detection of risk of increased disease severity, and argue that they should therefore be helpful markers to improve the management of COVID-19 patients. We first discuss how fibrin(ogen) D-dimer, vWF and P-selectin interact with platelets, endothelial cells and erythrocytes. We then propose a mechanism regarding how these biomarkers, and particularly fibrin(ogen), may be involved in COVID-19 hypercoagulation and thrombocytopenia. See Figure 1 for a general layout of this review. Figure 2A shows the typical pathology in bleeding and clotting, while Figure 2B shows the fine balance between these biomarkers

and the development of hyperclotting and thrombosis followed by thrombocytopenia and bleeding and the cytokine storm during COVID-19.

Figure 1: 1) Vascular implications of acute respiratory syndrome coronavirus 2 (COVID-19). 2) may result in clotting protein and circulating biomarker, endothelial and erythrocyte and platelet dysfunction. 3) We review the various biochemical processes associated with vascular dysfunction, focussing on fibrin(ogen), D-Dimer, P-selectin and von Willebrand Factor. 4) We conclude by looking at point-of-care devises and methodologies in COVID-19 treatment and suggest that each patient should be treated using a 5) personalized medicine approach. This image was created with BioRender (https://biorender.com/).

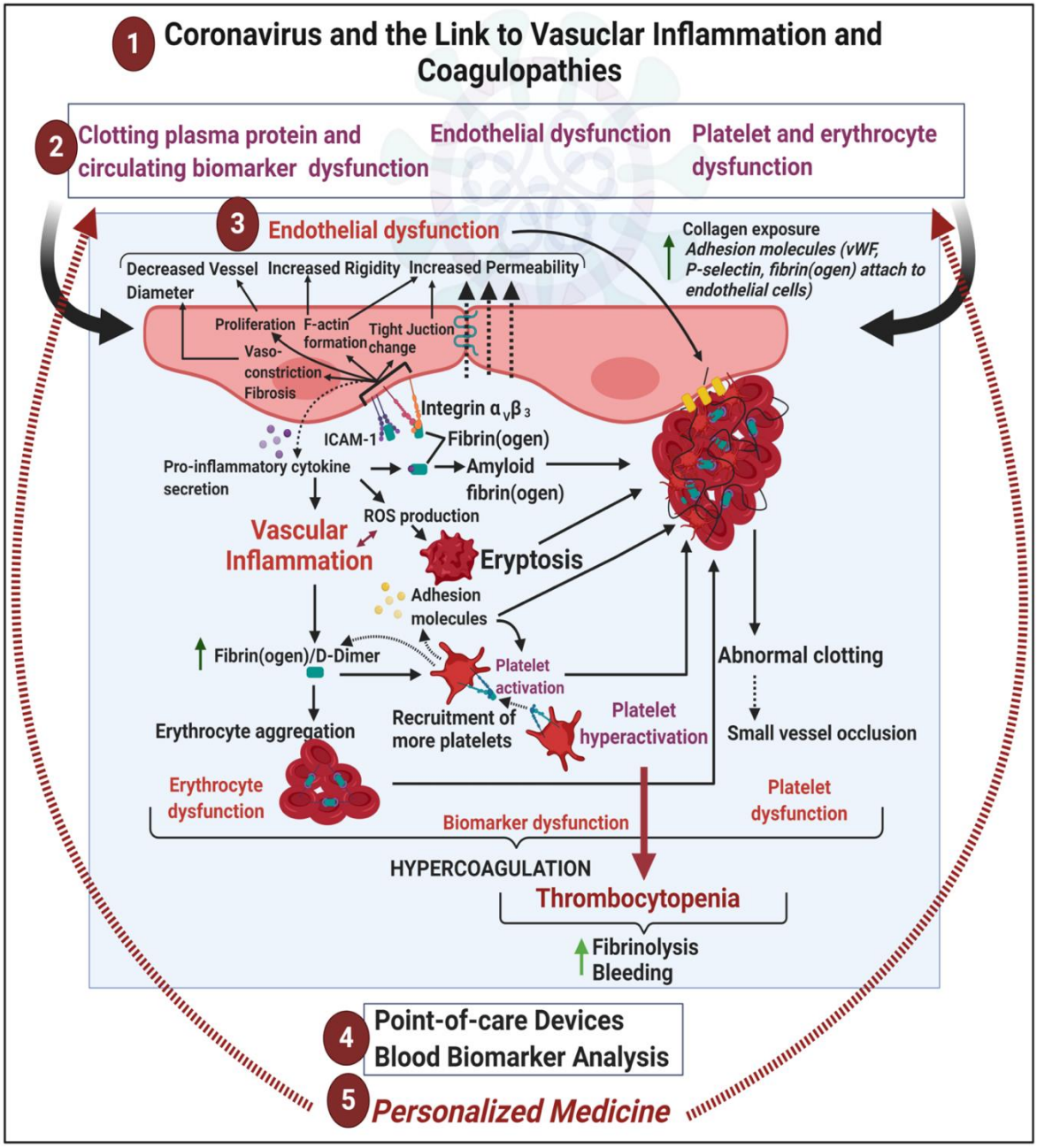


Figure 2A: Typical pathology in bleeding and clotting, the seesaw balancing act between bleeding and thrombocytopenia and hypercoagulation. This image was created with BioRender (https://biorender.com/).

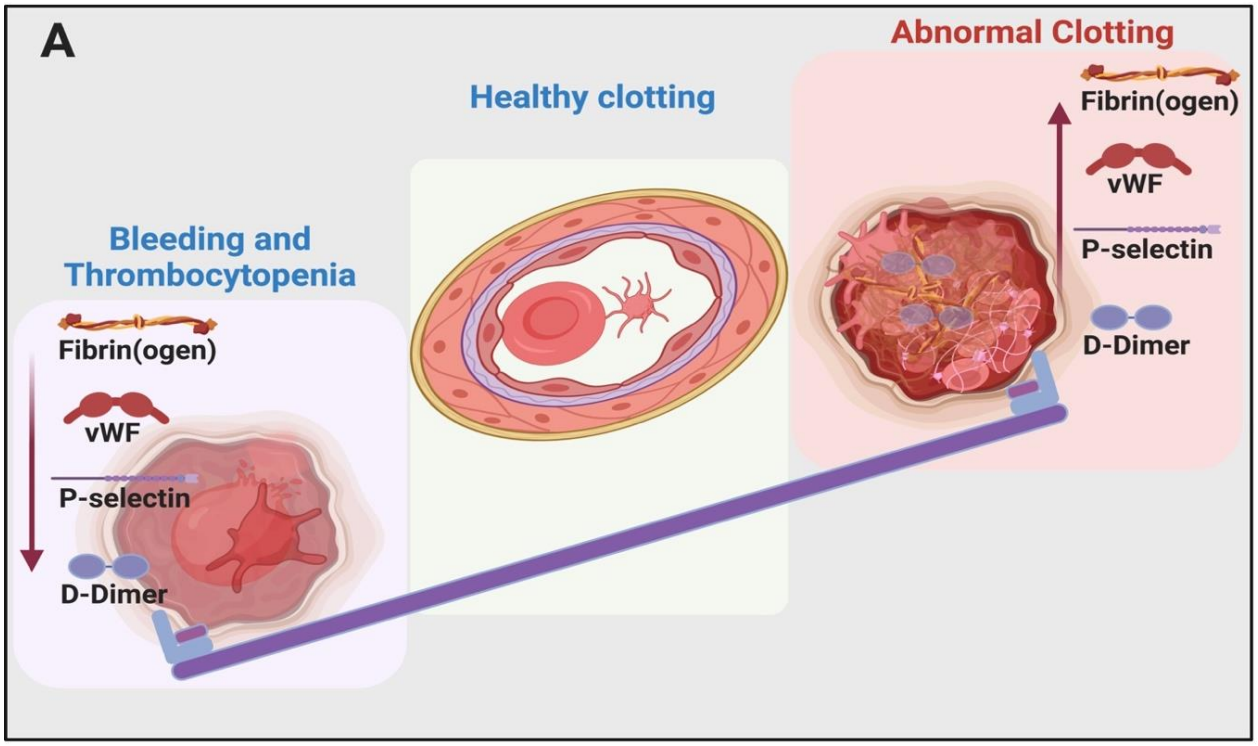
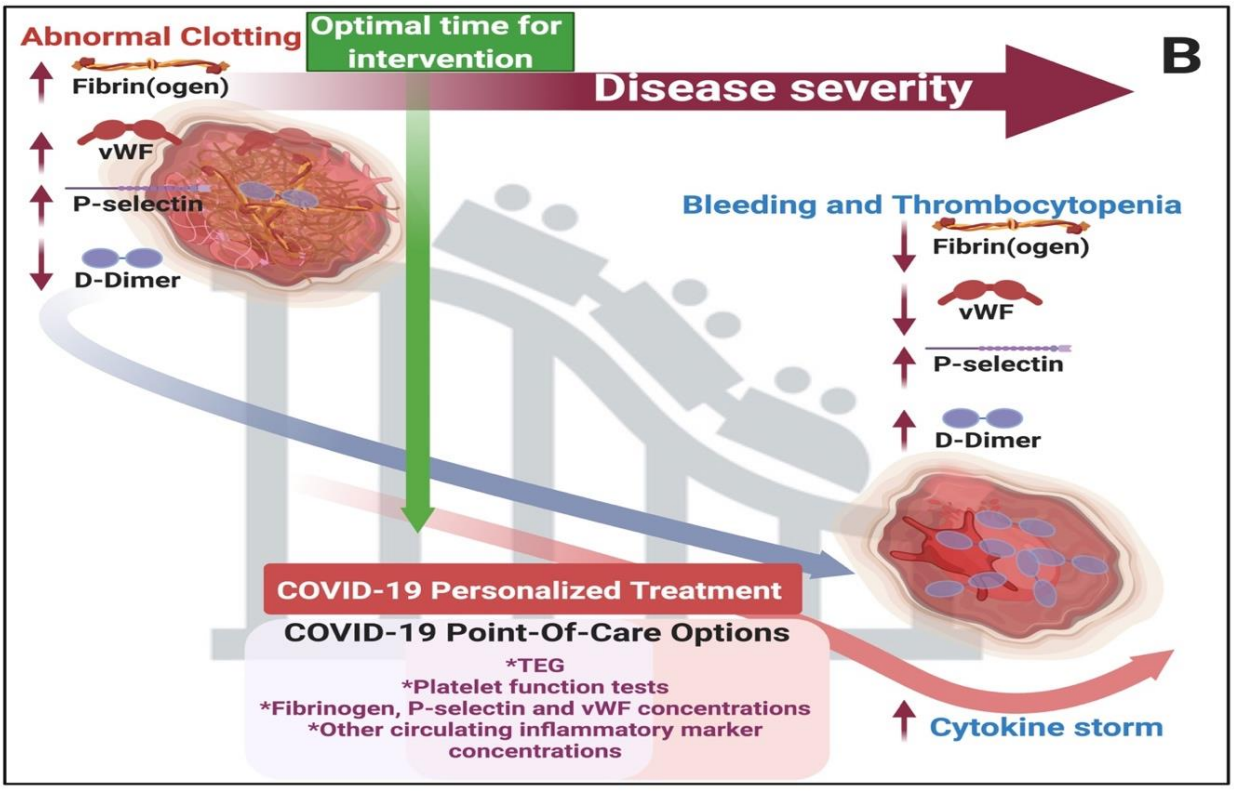


Figure 2B: Clinical manifestation of hypercoagulation, thrombocytopenia and bleeding during COVID-19, as well as clinical care options, and optimal time for intervention. This image was created with BioRender (https://biorender.com/).



2. Discussion

2.1 The importance of fibrin(ogen) and is breakdown product, d-dimer, as circulating biomarkers

Figure 3 shows the structure of soluble fibrinogen and how it polymerises into insoluble fibrin fibre (blood) clots, under the action of thrombin. Fibrinogen is a large, extracellular protein, synthesised by the liver and mainly found in the blood [31-33].) Fibrinogen (normal levels between 2 – 4 mg/mL) is also an acute-phase protein that is upregulated during inflammation [31, 34, 35]. Upregulation of fibrin(ogen) is associated with hypercoagulability and endothelial dysfunction. Increased fibrinogen levels are also associated with many inflammatory conditions including hypertension, diabetes, and thrombotic strokes [31, 32, 36-38]. Blood clots are dissolved by plasmin, a protein which degrades fibrin networks, producing fibrin degradation products [31], which include D-dimer [39]. D-dimer is also an important circulating inflammatory biomarker [40-44]. The D-dimer protein contains two cross-linked D fragments from the fibrinogen protein formed upon degradation of the fibrin gel, the core component of blood clots [18]. Fragment D consists of all three (α-, β-, and γ-) chains that are components of intact fibrinogen [45].

Enhanced fibrin synthesis activates plasminogen and the resulting plasmin cleaves the fibrin network into soluble fragments [39]. Plasmin cleavage between the D and E domains yields (DD)E, the noncovalent complex of D-dimer (DD) and fragment E. Further proteolysis liberates fragment E from DD [39]. Lysis of crosslinked fibrin by plasmin therefore produces D-dimer containing γ-γ crosslinks that hold 2 D-regions together [46]. In short, the morphology of D-dimer is characterised by the cross-linked, cleaved, identical monomers which each serve as D-dimer domains (see Figure 3). We also recognise the widespread prevalence in infection-related disease of an abnormal pathway of blood clotting to create an amyloid form of fibrin that is highly resistant to degradation [47-62]. Assessing this in COVID-19 patients would seem to be an important direction.

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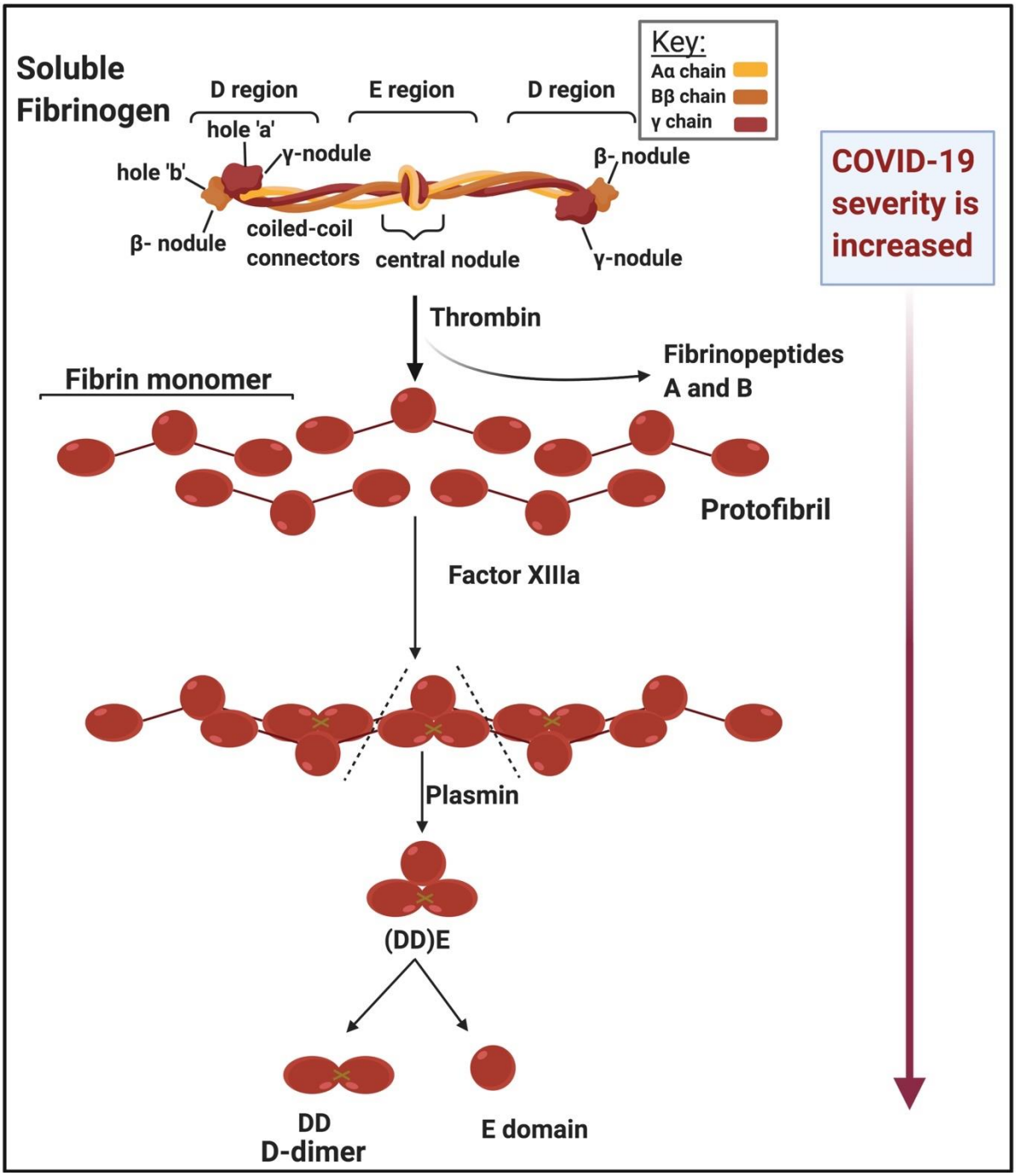
Figure 3: Structure of soluble versus insoluble fibrin(ogen), and the action of thrombin amd D-Dimer formation Adapted from [39]. D-dimer levels are increased in very ill patients (clinical observation. This image was created with BioRender (https://biorender.com/).



2.1.1 Interaction of fibrin(ogen) and d-dimer with cellular receptors

Although coagulation is the primary function of fibrinogen, it also interacts with other plasma components such as platelets, endothelial cells (ECs), erythrocytes and extracellular proteins [32]. Fibrin(ogen) receptors are of particular importance, as binding of their ligands, causes the activation of various inflammatory signalling pathways. These pathways are important in healthy physiological processes, but play crucial roles in pathophysiology, including the cytokine storm in COVID-19. Poor outcomes in COVID-19 correlate with clinical and laboratory features of cytokine storm syndrome [63] and increased D-dimer levels. However, when the cytokine storm is present in a patient, bleeding are prevalent and a low survival rate is then noted (clinical observation). See Table 1 for fibrin(ogen) platelet receptors.

Table 1: Receptors known to bind fibrinogen and D-dimer and the effects they elicit within different cell. types.

Fibrin(ogen)			
Cell Type	**Receptor**	**Effect**	**References**
Endothelial cells (EC)	Integrin $\alpha_V\beta_3$, $\alpha_5\beta_1$	Endothelial cell proliferation, Endothelial cell activation, Angiogenesis, Increased EC permeability, Vasoconstriction	[64-66] [32, 67]
	Integrin $\alpha_M\beta_2$	Facilitates interaction fibrinogen with ICAM-1 during leukocyte transmigration	[68]
	ICAM-1	Platelet adhesion, Leucocyte adhesion and transmigration to site of infection, Mitogenesis, Angiogenesis, Cell survival, Release of pro-inflammatory cytokines, ICAM-1 receptor recruitment to EC membrane, Vasoconstriction,	[68-73]
Platelets	Glycoprotein IIb/IIIa	Platelet activation and spreading, Integrin activation, Granule secretion,	[69]
	Glycoprotein VI (GPVI)	Platelet activation and spreading, Integrin activation, Granule secretion	[38, 46, 74-76]
	Integrins $\alpha_{IIb}\beta_3$ $\alpha_V\beta_3$	Outside-in signalling in platelets – platelet spreading and granule secretion	[32, 38, 65, 77-79]

Erythrocytes	αIIbβ3-related type integrin? CD47	Erythrocyte aggregation and adhesion	[80] [45, 81, 82]
Endothelial cells	ICAM-1	(i)D-dimer and /or Fragment D Arterial constriction	[45]
Platelets	Integrin αIIbβ3	Platelet spreading and aggregation	[76]
	GPVI (Monomeric/dimeric?)	Platelet spreading	[46, 83]

2.2.2 Fibrin(ogen) and D-dimer receptors and pathways in platelets

Platelets contain three types of granules - α-granules, dense granules, and lysosomes [84]. Ligand binding, including fibrin(ogen) and D-dimer to platelet receptors, followed by the activation of signaling pathways, leads to the secretion of molecules stored in these granules. Granule secretion, results in platelet activation, aggregation, and thrombus growth.

Soluble fibrinogen mainly binds to integrins on platelets, activating the platelets and promoting the formation of a platelet clot [38, 84, 85]. Integrins are cell-surface transmembrane receptors responsible for platelet aggregation and adhesion of cells to vessel walls [77, 84, 85]. Integrin αIIbβ3 signaling is one of the important platelet processes where fibrinogen binding is involved [78] and is involved in platelet spreading [86] (see Figure 4 for the Integrin αIIbβ3 structure). Other integrins to which fibrinogen binds have also been identified, not only on platelets, but also on endothelial cells, see Table 1 for such receptors.

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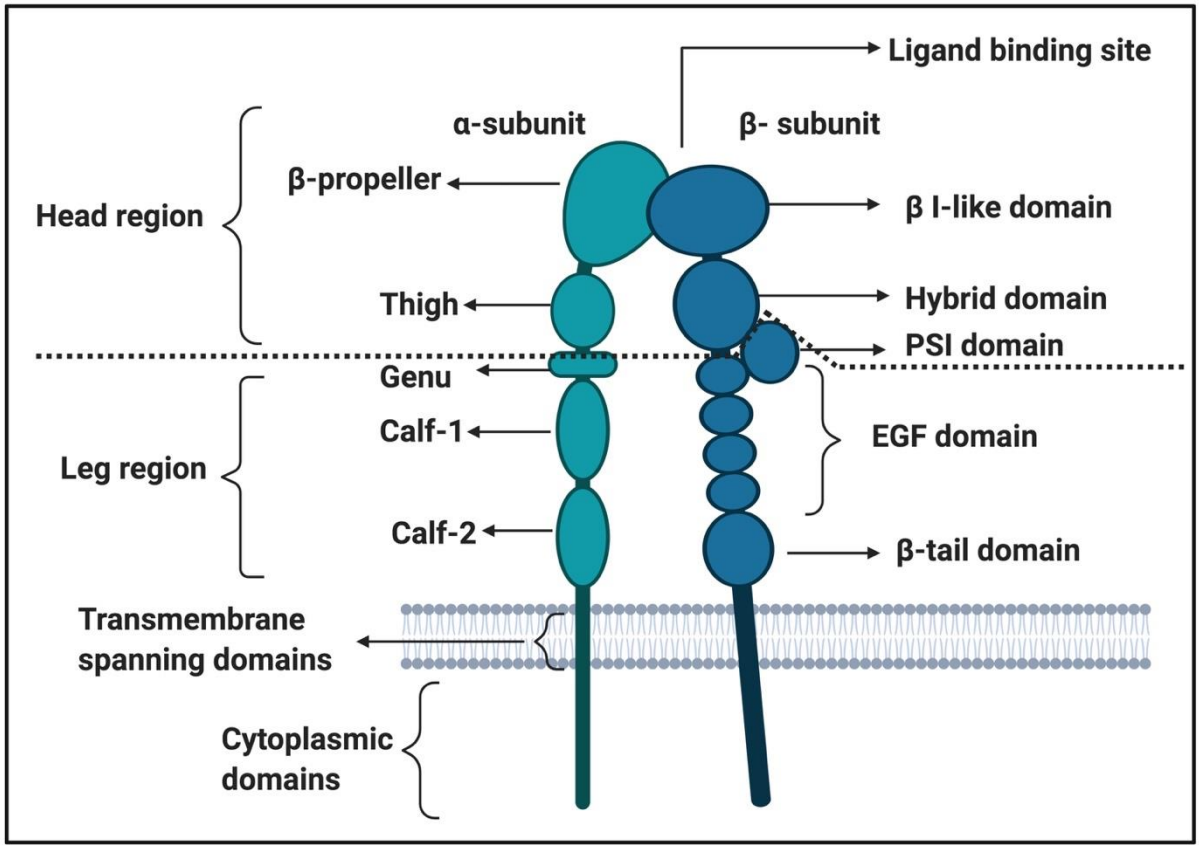
Figure 4: Integrin αIIbβ3 structure. Adapted from [77, 87-89]. This figure was created using BioRender (https://biorender.com/).



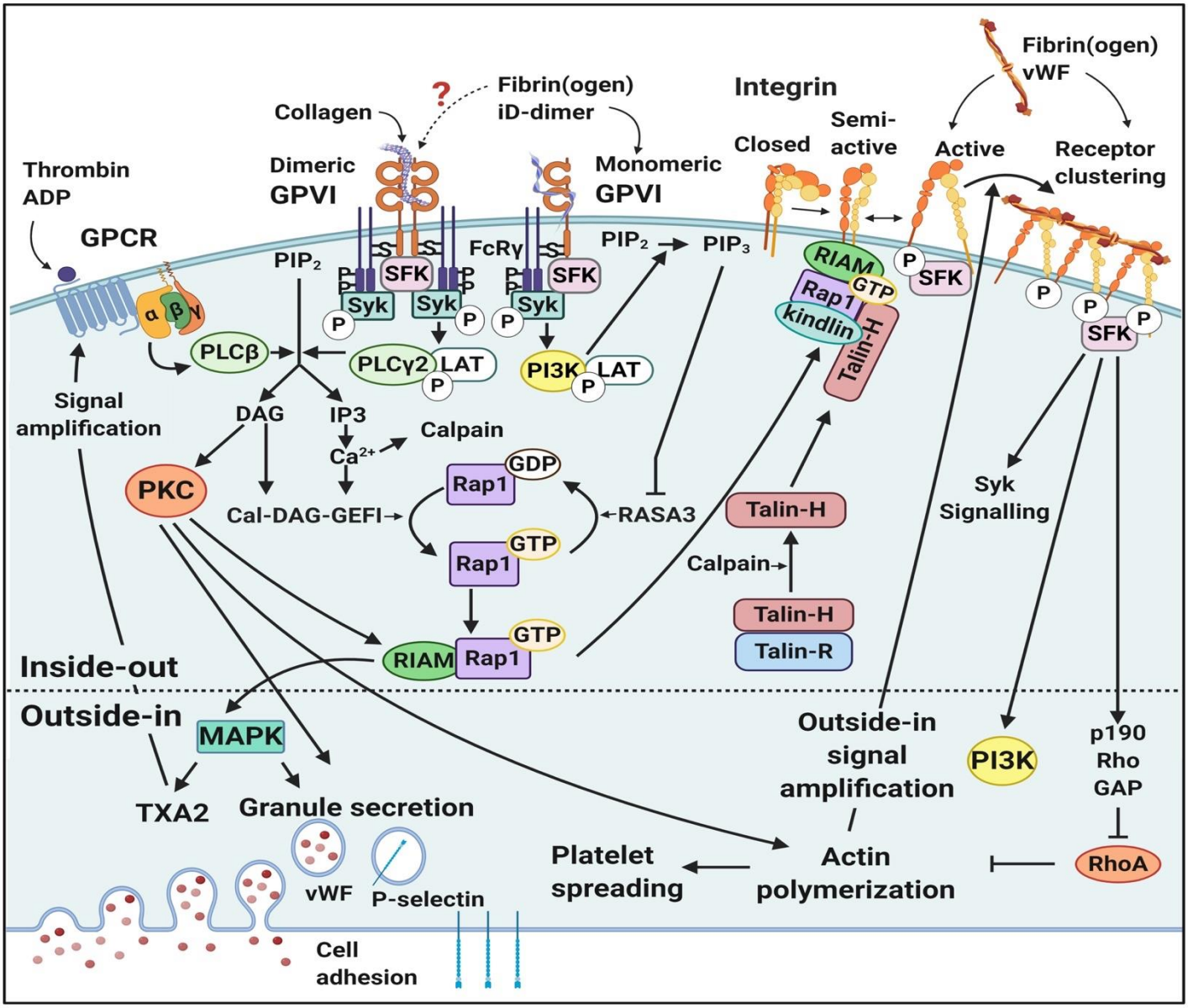
Integrin αIIbβ3 receptors in the membranes of platelets are usually inactive, resulting in a low affinity for ligands [77]. Activation of a platelet by other ligand-receptor binding events, can convert integrins to a higher affinity state resulting in conformational changes to the receptors - and allows for additional signaling events [38]. Such a signaling event is called "*inside-out*" signalling/activation [77]. When platelets are activated due to binding of biomarkers to a membrane receptor, *inside-out* signaling pathways increase the affinity of αIIbβ3 for fibrinogen (and other ligands). The binding of biomarkers to active integrin αIIbβ3 receptors then results in *outside-in* signaling. An example of this process is where αIIbβ3 receptor binding is then dependent on FcγRIIa ITAM (immunoreceptor tyrosine based activation motif)/Syk/PLCγ2, and PI3K/Akt to amplify the platelet activation [38, 90, 91]. This integrin receptor activation is in many cases caused by receptor clustering and also where the integrins form complexes (or heteroclusters) with other receptors (such as GPIb, GPVI, or FcγRIIa) Kaneva, Martyanov, Morozova, Panteleev and Sveshnikova [77].

Both fibrin and fibrinogen may interact with αIIbβ3 each acting through distinct epitopes [38]. Fibrinogen binds to αIIbβ3 via the carboxy-terminal peptide sequence of the γC-peptide (GAKQAGDV), while fibrin binds to the integrin through a unique sequence in the γC -peptide, ATWKTRWYSMKK, which binds to the αIIb β-propeller [75]. It was also noted that platelet activation through αIIbβ3 is required for expression of both immobilized D-dimer (iD-dimer) and fibrinogen binding [76].

Platelets also express glycoprotein VI (GPVI). GPVI is the main platelet receptor for collagen, which is exposed during endothelial damage or dysfunction. GPVI may be found in two states, monomeric and dimeric GPVI [46, 76, 92]. Collagen, and other substrates, binds monomeric GPVI, which induces its dimerization with an adjacent GPVI [93]. Dimerization of GPVI is required for collagen binding and initiation of signalling through the associated FcR-γ chain [83]. It was found by [76] that only dimeric GPVI can interact with fibrinogen D-domain, at a site proximate to its collagen binding site, to support platelet adhesion/activation/aggregate formation on immobilized fibrinogen and polymerized fibrin. However, contrasting observations have been reported on whether fibrin(ogen) binds to monomeric or dimeric GPVI, or to neither form [83]. Both fibrinogen [74, 76] and fibrin [46, 94] can interact with GPVI on platelets [95].

Figure 5 shows some of the relevant signaling pathways where fibrin(ogen) and d-dimer are involved in platelet activation [74, 77, 84, 85]. These signaling events initially cause platelet activation and aggregation, and also conformational shape change, clot formation, and eventually clot retraction [38, 77, 84, 85, 88, 96].

Figure 5: Activation of a platelet, inside-out and outside-in signalling upon ligation of major platelet membrane receptors. *Abbreviations:* GPCR, G-protein coupled receptor; GPVI, Glycoprotein VI; vWF, von Willebrand's factor; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; SFK, Scr family kinases; Syk, spleen tyrosine kinase; PLC, phospholipase C; LAT, linker for activation of T-cells; DAG, diacylglycerol; IP3, inositol triphosphate; PKC, protein kinase C; Ca^{2+}, calcium ions; Cal-DAG-GEFI, diacylglycerol regulated guanine nucleotide exchange factor I; GDP, guanine diphosphate; GTP; guanine triphosphate; RASA3, Ras GTPase-activating protein 3; RIAM, Rap1-GTP interacting adapter molecule; MAPK, mitogen activated protein kinase; TXA2, thromboxane A2, GAP, GTPase activating protein; PI3K, phosphatidylinositide 3-kinase. This image was made using BioRender (https://biorender.com/).

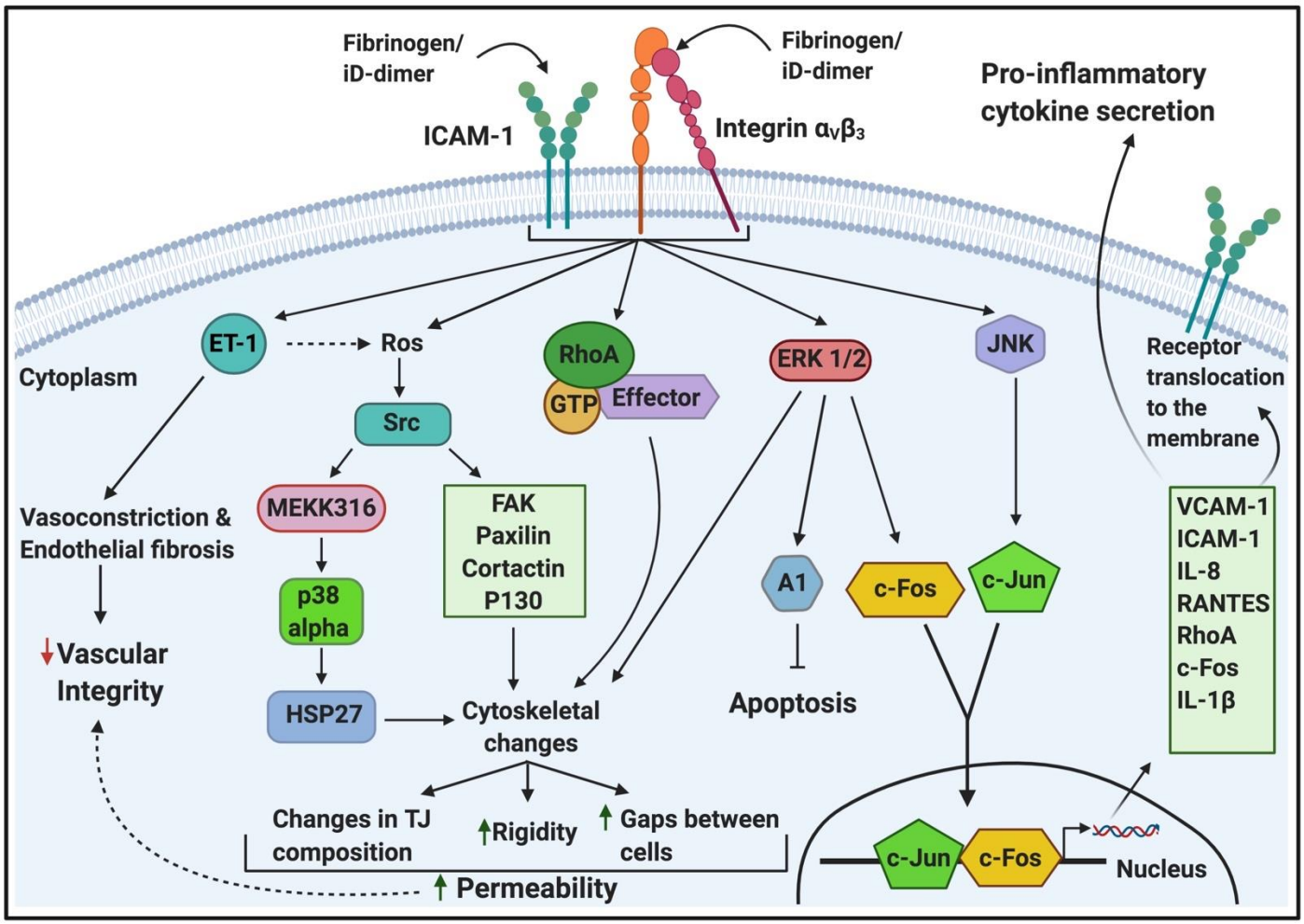


2.2.3 Fibrin(ogen) binding to endothelial cells

Fibrinogen binds to endothelial cells (ECs) via the interactions with 'intracellular' adhesion molecule 1 (ICAM-1), integrin $\alpha_V\beta_1$, and integrin $\alpha_V\beta_3$ [97]. ICAM-1 is an immunoglobulin (Ig)-like adhesion molecule expressed on the membrane of leukocytes and endothelial cells. Extracellularly, ICAM-1 consists of five Ig-like domains, which are mostly hydrophobic, and form β-sheets when folded. ICAM-1 has short transmembrane and cytoplasmic regions (24 and 28 amino acids, respectively) [73]. Activation of ICAM-1 leads to EC structure changes, creating gaps between ECs [68, 70, 73, 97]. These gaps are thought to facilitate the final steps of leukocyte transmigration [73]. Leucocyte transmigation is an important occurance in the inflammatory response, and involves the recruitment of blood leukocytes to a site of injury or infection, resulting in leukocyte adhesion to the endothelial lining, diapedesis (the passage of blood cells through the intact walls of the capillaries) and known as or transmigration across the endothelial monolayer, followed by directed migration to a site of infection or injury that often involves transmigration across epithelia [98].

During inflammation, fibrin(ogen) contributes to the transmigration of leukocytes from blood vessels to inflamed tissues. This is achieved by simultaneously binding to ICAM-1 and integrin $\alpha_M\beta_2$ on ECs and leukocytes [68]. However, during hyperfibrinogenaemia, fibrinogen contributes to endothelial dysfunction, proliferation and angiogenesis [64, 68-73, 97]. Endothelial dysfunction also increases the risk of thrombus formation [68-70, 73, 97]. Vasoconstriction was also shown to be mediated by fibrinogen and fragment D (early degradation product of fibrin(ogen)), when they bind to vascular ICAM-1 [45]. This might suggest that iD-dimer, too, may bind to ICAM-1 and that it is in part responsible for increased vascular tone and resistance which compromises blood circulation. Figure 6 depicts some of the main intracellular pathways elicited upon fibrinogen (and possibly iD-dimer) binding to their receptors on endothelial cells.

Figure 6: The intracellular signalling of endothelial cells upon ligation of fibrinogen and iD-dimer. Adapted from (Kowalczyk et al., 2015, Lawson and Wolf, Patibandla et al., 2009, Pluskota and D'Souza, 2000, Tyagi et al., 2008, Altieri et al., 1995, Haidari et al., 2012, Koenig, 2003, Rahman and Fazal, 2009). *Abbreviations:* ICAM-1, Intercellular adhesion molecule-1; RhoA, activated Rho factor protein; iD-dimer, immobilised D-dimer; ROS, Reactive Oxygen Species; Src, sarcoma; MEKK316, Endothelial MAP kinases 3 and 6 (MKK-3 and -6); FAK, Focal Adhesion Kinase; p130, Retinoblastoma like protein-2; p38, Mitogen-activated protein kinase; HSP27, Heat shock protein 27; ERK, extracellular signal-regulated kinase/ extracellular receptor kinase; c-Fos, proto-oncogene; JNK, stress-activated protein kinase; c-Jun, protein encoded by the JUN gene; VCAM-1, Vascular cell adhesion protein-1; IL-8, Interleukin-8; RANTES, Regulated upon Activation Normal T Cell Expressed and Presumably Secreted; IL-1 beta, Interleukin-1 beta. This image was made with BioRender (https://biorender.com/).



2.2.4 Fibrin(ogen) and D-dimer binding to erythrocytes

Fibrinogen is of course a major component of the coagulation cascade as well as a significant determinant contributing to plasma viscosity and ESR levels [81]. Fibrin(ogen) is also considered the main plasma protein responsible for increased erythrocyte shear-dependant reversible aggregation, contributing largely to vessel occlusion [37]. Fibrin(ogen) is thought to serve as a

bridging molecule between erythrocytes during aggregation [37]. The transient bridging of two erythrocytes, promoting erythrocyte aggregation, can represent an important cardiovascular risk factor [99]. Although a search on Pubmed did not show any publications yet on the presence of Rouleau formation in Covid-19 infection, it would be an interesting phenomenon to investigate.

Fibrinogen may also play an important role in erythrocyte deformability [100]. Fibrinogen might interact with erythrocytes via integrin-like receptors, however, there are no consensus on the presence of such receptor [81]. [80] suggested that a $\alpha_{IIb}\beta_3$-like might be present on erythrocytes; [45] also argued that there might be such a integrin-like receptor. However, [81] could not find any evidence of such integrin-like receptor, and rather suggested that CD47 is a fibrinogen ligand on erythrocytes, and CD47 expression was found to be decreased on the surface of erythrocytes in obese individuals [82]. The authors suggested that changes in CD47 expression on the erythrocyte surface may be an adaptive response to hyperfibrinogenemia associated with obesity [82]. iD-dimer might also possibly bind to such a integrin-like receptor or CD47 on erythrocytes.

In addition to directly binding to erythrocytes, fibrin(ogen) influences erythrocyte functionality by increasing circulating inflammatory biomarkers by binding to ECs. These biomarker are associated with ROS production, which cause erythrocyte eryptosis and pathological deformability [73, 101]. The increased viscosity of blood due to hyperfibrinogenaemia may also increase shear flow rates [69]. This, along with inflammation leads to a phosphatidyl serine (PS) flip on the erythrocyte membrane. The exposure of PS on erythrocytes are known to be present during pathological coagulation, and can inturn be involved in the production of thrombin [37]. Under pathological conditions, such as chronic inflammation, the PS flip contributes to increased erythrocyte aggregation [37, 101]. PS also mediates the adhesion of erythrocytes to vessel walls, promoting occlusion of small vessels [37]. During COVID-19 infection, pathological levels of thrombin, fibrin(ogen), D-dimer and increased circulating inflammatory molecules, may interact with erythrocytes, resulting in fragile erythrocyte membranes, with pathological elasticity. These erythrocytes may inturn be trapped in embolisms and

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clots formed in COVID-19 patients. Normal levels of D-dimer are noted early in the progression of the disease; however, as the disease progresses D-Dimer levels increase (clinical observation).

2.3 The importance of von Willebrand Factor (vWF), as a circulating biomarker

Von Willebrand factor (vWF) is a multimeric glycoprotein present in plasma and the subendothelial matrix [102]. It is stored in the form of ultra-large (UL) vWF multimers in Weibel-Palade bodies and platelet α-granules for secretion upon stimulation. In response to high shear stress and other inflammatory mediators [103] resting ECs are activated and release large amounts of long vWF multimers into circulation. These vWF multimers are cleaved, and can be activated by the metalloprotease ADAMTS-13 [104]. Therefore, after ADAMTS-13 activation, vWF will now have an exposed binding site for GPIbα (which is part of the GPIb-IX-V receptor complex) [104]. ADAMTS-13 is produced in the liver, and its main function is to cleave vWF anchored on the endothelial surface and in circulation [105]. Subsequently, platelets bind to these activated UL-vWF string via GpIbα interaction with the exposed A1 domain (in the GPIb-IX-V complex), initiating the thrombogenic process which is summarized in Figure 7 and details of the pathway and receptors are discussed in the next section.

The production of vWF is exclusive to endothelial cells and megakaryocytes [106]. vWF is involved in platelet aggregation and thrombus formation and due to its important role in inflammation, vWF is identified as an acute phase reactant [107]. In addition, vWF is identified as a crucial player in the propagation of atherosclerosis by promoting plaque formation and inflammation [108]. This is important because atherosclerotic lesions cause obstruction, which further promotes thrombus formation (and embolization), resulting in reduced cerebral blood flow (cerebral ischemia) [108]. In addition, vWF tethers circulating platelets to the endothelium as part of the processes of coagulation, inflammation, and also tumor progression [109]. vWF also acts as a carrier – and stabilizer – of the procoagulant factor VIII (FVIII) in circulation [110] which is achieved by the formation of a non-covalently bound vWF-FVIII complex that protects FVIII from being degraded by activated protein C [111]. A most important consideration for COVID-19 pathology, is that, under normal conditions,

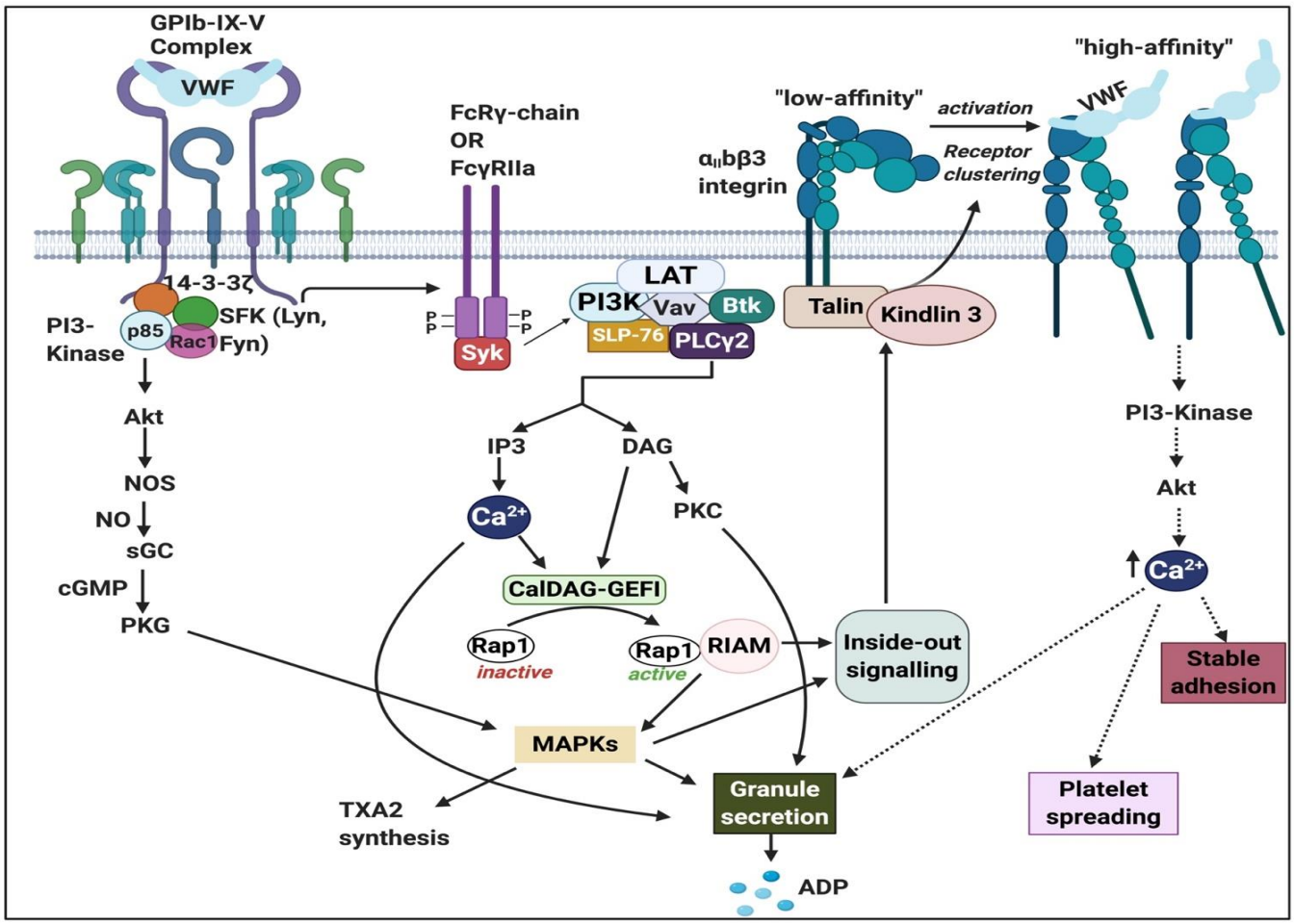
vWF is both a bleeding and thrombotic marker [103]. However, the the stage of the disease is of significant importance for treatment (see also our discussion in the Conclusion section).

2.3.1 Von Willebrand Factor (vWF) receptors and pathways on platelets

vWF binds to two distinct platelet receptors which are localized on the platelet membrane, they are GPIbα in the GPIb-IX-V complex [106, 112] and integrin αIIbβ3 (GPIIb-IIIa complex) [106]. In previous sections we discussed $\alpha_{II}b\beta3$ in detail. The GPIb-IX-V complex comprises of 2 chains of GPIbα (135kDa), 2 GPIbβ (26 kDa), 2 GPIX (20 kDa) and 1 GPV (82kDa), ratio of 2:2:2:1. All 4 proteins belong to the leucine-rich repeat (LRR) superfamily [113]. Binding of vWF to GPIbα causes activation of tyrosine protein kinases LYN and FYN, which are members of the Src family kinase (known as non-receptor tyrosine kinases). Activation of these kinases leads to tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) on the FcRγ or FcγRIIa receptors with which GPIb physically associates [113].

Engagement of GPIb-IX-V activates intracellular signaling events that lead to full platelet activation and aggregation through the $\alpha_{II}b\beta3$ integrin [113]. The binding of vWF to GPIb-IX-V leads to the upregulation of $\alpha_{II}b\beta3$ integrin affinity [114]. vWF can then bind to $\alpha_{II}b\beta3$, thus enhancing platelet adhesion, platelet aggregation, and contributing to thrombus formation by binding to fibrinogen [114] which is mediated via different pathways. Intracellular signaling that induces changes in the extracellular ligand-binding domain of integrins from a low-affinity state to the activated or high-affinity resulting inside-out signaling [115]. Figure 7 shows the some of the signaling pathways of vWF.

Figure 7: The signaling pathway of von Willebrand factor (vWF) in platelets via the GPIb-IX-V complex (with GPIbα) leading to integrin $\alpha_{II}b\beta3$ activation and the important role of FcRγ-chain and FcγRIIa ITAM pathway. Adapted from [106, 112]. *Abbreviations:* ADP, Adenosine triphosphate; Btk, Bruton tyrosine kinase; CalDAG-GEFI, Ca^{2+}-dependent guanine nucleotide exchange factor; DAG, Diacylglycerol; IP3, Inositol triphosphate; LAT, Linker of activated T-cells; MAPK, mitogen-activated protein kinase; NO, Nitric oxide; NOS, Nitric oxide synthase; PI3K, Phosphoinositide 3; PLC-γ_2, phospholipase C-γ_2, PKC, Protein kinase C; PKG, Protein kinase G; Rac 1, Ras-related C3 botulinum toxin substrate 1; Rap1, Ras-related protein 1; RIAM, Rap1-GTP-interacting adaptor molecule; SFK, Src family kinases; SLP-76, SH2 domain-containing leukocyte phosphorylation of 76 kDa; SYK, spleen tyrosine kinase; sGC, soluble guanine cyclase; TXA2, Thromboxane A2; vWF, von Willebrand factor. Figure created using Biorender (https://biorender.com/).



2.3.2 Von Willebrand Factor receptors and pathways on endothelial cells

Although the pivotal physiological role of vWF is to activate platelets, binding to ECs has been demonstrated. αvβ3 is the major integrin expressed on ECs [116]. Although it binds multiple ligands such as vitronectin, fibrinogen, and fibronectin, it is the best-characterized EC receptor for vWF [116]. The most extensively studied function of αvβ3 in vascular biology relates to endothelial cell (and

smooth muscle cell) adhesion, migration proliferation, differentiation, and survival [110]. The complex responses which rely on these functions of αvβ3 include angiogenesis, vasculogenesis, and vascular cell survival [116, 117]. It has been suggested that this αvβ3 integrin may be central in the inflammatory endothelial responses [117]. However, very little is known about the signaling events that follow VWF binding to αvβ3 on EC [118].

2.3.3 Von Willebrand Factor signaling in erythrocytes

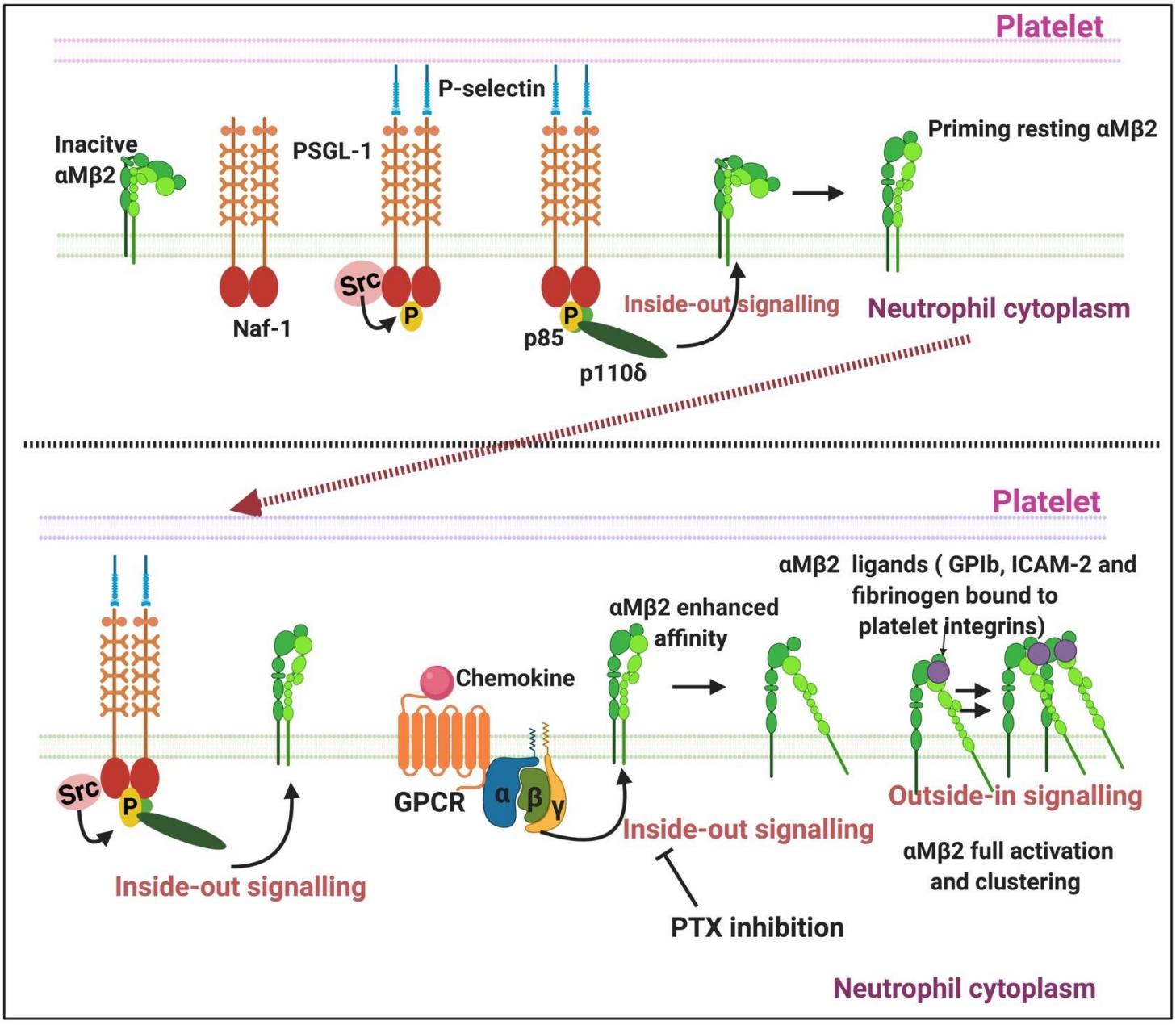
VWF can bind to erythrocytes under conditions such as reduced shear rates [119]. The erythrocyte surface receptor(s) subdomains in vWF that mediate this adhesion are unknown [120]. Upon an inflammatory insult and increased production of reactive oxygen species (ROS), EC are prompted to release vWF from the Weibel-Palade bodies, resulting in elevated vWF levels observed in an inflammatory state [121]. Following secretion from Weibel-Palade bodies, a portion of vWF enters into the circulation while another portion remains bound to the endothelial surface [122]. During inflammation (and oxidative stress), Erythrocytes can also undergo eryptosis [101]. Eryptosis is characterized by three distinct physiological processes which are cell shrinkage, membrane blebbing, and cell membrane scrambling [101, 123]. Subsequent to membrane scrambling, phosphatidylserine (PS) translocates from the inner leaflet of the cell membrane and is exposed on the erythrocyte surface [124]. Nicolay et al. (2018) [125] showed that the exposure of PS and Annexin V, which avidly binds to PS, mediates binding of eryptotic RBCs to vWF. The A1 domain of vWF is mainly responsible for mediating this vWF-erythrocyte adhesion [125]. Thus, vWF promotes erythrocyte-erythrocyte linking [125]. Moreover, intraluminal vWF mediates platelet-independent erythrocyte adhesion to ECs thus mediating microvascular occlusion and impaired dynamic blood flow [119].

2.4 The importance of p-selectin as a circulating biomarker

P-selectin, also known as CD62P, play an important role in modulating interactions between blood cells and endothelial cells [126]. P-selectin is constitutively present in α-granules of platelets and Weibel-Palade bodies in endothelial cells [126-128]. P-selectin also found in human plasma – here it is alternatively spliced and lacks the transmembrane domain [128]. This is referred to as soluble P-

selectin (sP-selectin) [126]. As membrane receptor, P-selectin acts as an adhesion receptor to support leukocyte rolling and emigration at sites of inflammation [128]. Figure 8 shows a simplified overview of P-selectin interactions with platelets and neutrophils.

Figure 8: The signalling pathways involved in neutrophil and platelet activation. Adapted from [129]. *Abbreviations:* GPCR: G protein-coupled receptor; Mac-1, macrophage antigen-1; P-sel, P-selectin; PSGL-1, P-selectin glycoprotein ligand-1; PTX, pertussis toxin. Figure created using BioRender (https://biorender.com/).



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Since P-selectin is stored in and expressed endothelial cells and the platelets, there has been substantial debate whether raised plasma levels of P-selectin indicate endothelial dysfunction, platelet activation, or both [126]. Elevated levels of sP-selectin may also reflect platelet activation, since P-selectin is proteolytically shed from the plasma membrane in vivo shortly after activation [38]. Plasma levels of sP-selectin have also been considered a useful biomarker in in cardiovascular diseases since it is constantly elevated in such patients [130]. Increased sP-selectin can therefore also reflect endothelial cell activation and damage [131]. Pathological levels of sP-selectin consistently promote leukocytes to adhere to endothelial via the activation of the leukocyte integrin Mac-1 [132]. Circulating sP-selectin is also thought to trigger signalling in leukocytes that has a direct contribution to inflammation and thrombosis. However, sP-selectin likely circulates as a monomer, and in vitro studies propose that sP-selectin must dimerize to induce signalling in leukocytes [130]. When sP-selectin is dimerized, it can trigger activation the leukocytes in vitro, manifest as leukocyte adhesion to ICAM-1 and to fibrinogen, and the release of citrullinated histones and neutrophil extracellular traps (NETs) [133].

2.4.1 P-selectin signaling in platelets

P-selectin glycoprotein ligand-1 (PSGL-1) is the primary receptor for P-selectin binds PSGL-1 [132] and is a 120kDA transmembrane protein that is mostly expressed as a homodimer rich in O- as well as N-glycans [126, 134]. P-selectin from endothelial cells also binds to the GPIbα [135], which is part of the platelet receptor complex GPIb-V-IX that promotes platelet aggregation. P-selectin plays an important role in neutrophil-platelet, platelet-platelet, and monocyte-platelet interactions [135]. P-selectin on activated platelets in suspension can also bind to PSGL-1 on neutrophils or monocytes, contributing to the formation of mixed cell aggregates [136]. When platelets are exposed to agents such as adenosine or epinephrine, the platelet can become activated and there is an increase of P-selectin expression on the platelet surface [126]. Thus P-selectin is a most important signal molecule during pathological coagulation as well as infection.

2.4.2 P-selectin signaling in endothelial cells

Early inflammatory mediators like histamine, thrombin, hypoxia or phorbol esters can stimulate EC *in vitro*, causing endothelial damage or endotheliopathy. These agonists mobilize P-selectin to the apical membranes of EC where it initiates the rolling adhesion of flowing neutrophils [137]. Although not the focus of this review, the interactions of the rolling of neutrophils, P-selectin and the endothelial cells are shown in a simplified diagram, Figure 9. P-selectin in Weibel-Palade bodies are mobilized in a process of degranulation [126]. P-selectin has at least two waves of aggregation at the cell surface: one 10 min and the other 12 h after endotoxic or oxidative stress [138]. In addition, neutrophils rolling on P-selectin secrete the cytokine oncostatin M . The released oncostatin M can also triggered signals through glycoprotein 130 (gp130)-containing receptors on ECs that, resulting in a further clustered P-selectin and markedly enhanced its adhesive function [137]. P-selectin also interacts with platelet sulfatides, thereby stabilizing initial platelet aggregates formed by GPIIb/IIIa-fibrinogen bridges [139]. Oxidatively-modified fibrinogen can also cause platelet aggregation and potentiates ADP-induced platelet aggregation and production of active oxygen forms in zymosan-stimulated leukocytes [140]. The oxidized form of fibrinogen impairs microrheological properties of the blood, significantly reduces erythrocyte deformability, increases blood viscosity, and reduces suspension stability of the blood [140].

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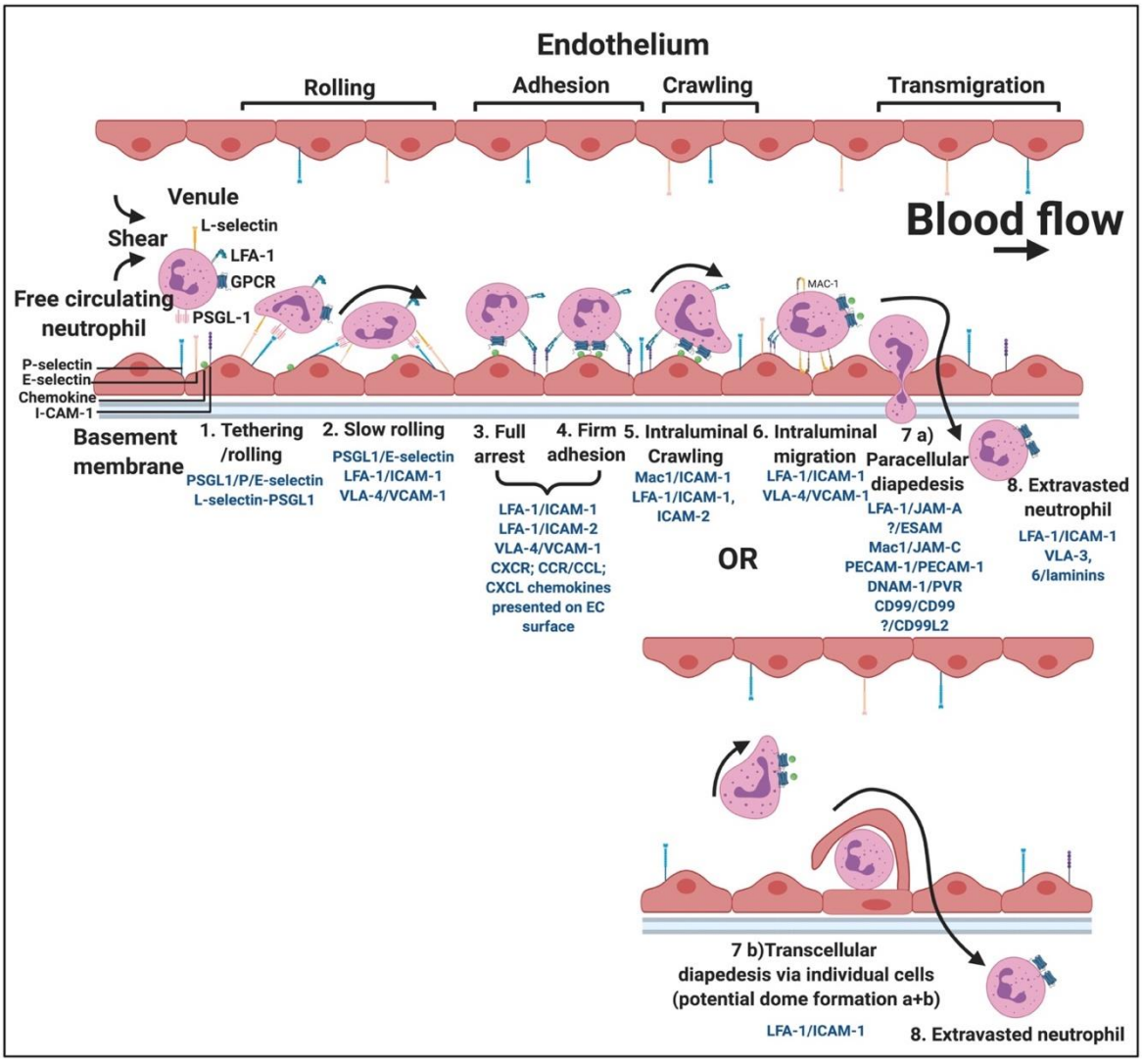
Figure 9: Simplified Leukocyte extravasation cascade and rolling of neutrophils over the endothelium. Adjusted from [141] and [142-145]. For more detail, under each step, the known adhesion receptor interactions are given with the leukocyte receptor being named first. Unknown ligands are indicated with question marks. Adhesion Molecule *Abbreviations:* GPCR- G-protein coupled receptor, LFA-1, lymphocyte function-associated antigen 1; PSGL-1, P-selectin glycoprotein ligand-1; I-CAM-1, Intercellular adhesion molecule 1. Figure created using BioRender (https://biorender.com/)



2.4.3 P-selectin interaction with erythrocytes

Erythrocytes are not considered to participate in the receptor-mediated processes seen in endothelial cells and platelets, since normal red blood cells (RBCs) are not known to bear selectin ligands or to bind to P-selectin [146]. However, studies focused on sickle cell disease have indicated that the adhesion of sickle erythrocytes to the vascular endothelium may be potentiated by the upregulation

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of adhesion molecules on activated endothelial cells [146]. Normal, and to a greater extent, sickle erythrocytes adhere to endothelial P-selectin. The adherence of sickle erythrocytes is problematic since it may contribute to vaso-occlusion [146]. The binding of sickle cells is much higher, than normal cells, since in sickle cell disease there are multiple adhesion systems involved [146]. Abnormal adhesion of erythrocytes to the endothelial layer is linked to the pathophysiology of various vascular disorders [147]. It leads to several biochemical changes, like exposure of PS on erythrocytes outer membranes and plasma protein levels [147]. Adhesion between EC, erythrocytes and fibrin(ogen) plays and important role in the hyperactivation of the coagulation system during inflammation.

When P-selectin is upregulated in circulation, it therefore suggests that endotheliopathy, as well as platelet hyperactivation is present. In addition, P-selectin plays a fundamental role in adhesion of erythrocytes to damaged endothelia, as well as to adjacent erythrocytes and to hyperactivated platelets. Recently it was reported that endotheliopathy is present in patients with COVID-19, and that it is likely to be associated with critical illness and death [148]. The authors came to this conclusion after studying endothelial cell damage, levels of platelet activation, vWF antigen, soluble thrombomodulin, soluble P-selectin, and soluble CD40 ligand, various coagulation factors, endogenous anticoagulants, and fibrinolytic enzymes [148].

3. Conclusion

It is very well-known that pathological levels (both decreased and increased levels) of fibrin(ogen), D-Dimer, vWF and P-selectin play crucial roles in abnormal coagulation and endothelial dysfunction. These molecules may also be significantly dysregulated in patients with COVID-19, as reviewed in the introduction. Specifically, dysregulation during COVID-19, has been noted in P-selectin [8], fibrinogen and D-dimer [4, 5, 9-15], and vWF [16, 17]. Depending on the direction, dysregulation of fibrin(ogen) D-Dimer, VWF and P-selectin may result in either hypercoagulation or excessive bleeding and thrombocytopenia. During typical bleeding and abnormal clotting diseases, low fibrinogen levels are indicative of a higher propensity for bleeding while high levels are known to be associated with hypercoagulation [149]. We summarized these phenomena in Figure 2A. Bleeding, as well as thrombotic events, often occurs in subjects with multiple weak risk factors which

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interact to produce the symptoms [103]. Both bleeding, thrombocytopenia and thrombotic pathologies have been reported in COVID-19 patients, and are significant accompaniments to acute respiratory distress syndrome and lung complications [3-6, 12, 150, 151] Therefore, fibrin(ogen) levels, D-Dimer, vWF and P-selectin could all be valuable biomarkers that might provide clinicians with the correct clinical diagnosis and assist in deciding the method of treatment.

Our understanding of this process is explained in Figure 2B, and clinical observation suggests the following: during ealy stage COVID-19, patients present with normal D-dimer levels, increased levels of fibrinogen, VWF and P-selectin, and slightly activated platelets. if untreated, the clinical picture changes to an increase in D-dimer, still higher levels of fibrinogen, vWF and P-selectin, and hyperactivation of platelets. This is in line with hyperclotting or thrombosis. In the critically ill patient, D-dimer and P-selectin levels are high, while fibrinogen and vWF levels are decreased as these molecules are depleted from either the circulation or the damaged endothelial cells and hyperactivated platelets, that now show thrombocytopenia. It is during these late stages of the progression of the disease that the cytokine storm is also prevalent.

Although D-dimer assays to determine the levels of D-dimer in circulation are also very helpful, as is an indirect marker of fibrinolysis and fibrin turnover [152]. However, in COVID-19 patients, D-dimer levels are normal during the early stages of the disease (clinical observation). Increased levels of circulating P-selectin is associated with a higher risk of a thrombotic event [153]. However, P-selectin expression on platelets may also be used to diagnose mild bleeding disorders and increased bleeding might be associated with very suppressed P-selectin expression [154]. A dilemma is that P-selectin expression varies considerably between individuals.

If the vWF level is increased, it predicts a thrombotic phenotype, and when these levels are low in plasma, the phenotype is indicative of bleeding [103]. Thrombotic risk may be more prevalent when vWF is activated and increased and able to bind to the various receptor complexes (Figure 7). Heparin inhibits VWF-GP1b binding, and it might be because heparin overlaps the binding site within

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the vWF A1 domain [103]. Heparin has also been found in some circulstances to be a helpful treatment for COVID-19 [155, 156]. Heparin interveres with vWF platelet activation, and possibly assists in the prevention of thrombotic events. When the bleeding phenotype is more prevalent, it may be due to abnormalities in subendothelial collagen, which may alter its interaction with platelets and vWF [157]. However, if vWF is depleted, it results in bleeding. During the late stages of COVID-19, vWF levels are indicatinve of depletion, and this is due to large scale endothelial damage. Endotheliopathy is also prevalent in patients with COVID-19 [148] and is significantly linked to coagulopathies and clinical observations.

Personalised medicine has never been so important as during this epidemic. This is mainly because the patient cohort is so extremely diverse, and many may have pre-existing thrombotic disease and cardiovascular co-morbidities [150]. Point-of-care devices and diagnostics like the thromboelastograph (TEG) or point-of-care ultrasound (POCUS) [158] allows for frequent testing of the coagulation/bleeding profiles as well as blood clot fibrinolysis of patients at the bedside [158]. Recently, [151] reported that fibrinolysis shutdown, as evidenced by elevated D-dimer and a complete failure of clot lysis at 30 minutes on TEG, predicts thromboembolic events and a need for hemodialysis in critically ill patients with COVID-19. Most importantly, we need clinicians to have access to adequate point-of-care devices and encourage them to use parameters of haemostasis [10], to allow them to determine if their patients are in need of either therapeutic antithrombotic prophylaxis/treatment or fibrinolytic therapy to prevent which ever coagulopathy is present, be it hypercoagulation, fibrinolysis shutdown or bleeding. Most significant is that patients need to be treated early in the disease progression, when high levels of vWF, P-selectin and fibrinogen are present with still low levels of D-dimer. Progression to vWF and fibrinogen depletion with high D-dimer levels and even higher P-selectin levels, followed by the cytokine storm, will be indicative of a poor prognosis.

Author Contributions: Conceptualization, EP and Y.Y.; X.X.; validation, G.J.L. and A.S. writing—original draft preparation, C.G, J.C.B, L.M.G., S.C.M, EP ; writing—review and editing, E.P, D.B.K; supervision, E.P.; funding acquisition, E.P.. All authors have read and agreed to the published version of the manuscript.

Funding: Please add: This research was funded by Medical Research Council of South Africa (MRC), grant number: A0X331 Self-initiated research and The Novo Nordisk Foundation, Grant number: NNF10CC1016517 .

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

vWF	Von Willebrand Factor
EC	Endothelial cells
COVID-19	Coronavirus disease 2019
ICAM-1	'intracellular' adhesion molecule 1
sP-selectin	Soluble P-selectin
GPCR	G-protein coupled receptor
GPVI	Glycoprotein VI
PIP2	phosphatidylinositol 4,5-bisphosphate
SFK	Scr family kinases
Syk	spleen tyrosine kinase
PLC	phospholipase C
LAT	linker for activation of T-cells
DAG	Diacylglycerol
IP3	inositol triphosphate
PKC	protein kinase C
Ca2+	calcium ions
Cal-DAG-GEFI	diacylglycerol regulated guanine nucleotide exchange factor I
GDP	guanine diphosphate
GTP	guanine triphosphate
RASA3	Ras GTPase-activating protein 3
RIAM	Rap1-GTP interacting adapter molecule
MAPK	mitogen activated protein kinase
TXA2	thromboxane A2
GAP	GTPase activating protein
PI3K	phosphatidylinositide 3-kinase

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