



**Review Article**

**The Prothrombotic State Associated with SARS-CoV-2 Infection: Pathophysiological Aspects**

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**Competing interests:** The authors declare no conflict of Interest.

**Abstract.** Severe coronavirus disease-2019 (COVID-19) is frequently associated with microvascular thrombosis, especially in the lung, or macrovascular thrombosis, mainly venous thromboembolism, which significantly contributes to the disease mortality burden. COVID-19 patients also exhibit distinctive laboratory abnormalities that are compatible with a prothrombotic state. The key event underlying COVID-19-associated thrombotic complications is an excessive host inflammatory response to severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) infection generating multiple inflammatory mediators, mainly cytokines and complement activation products. The latter, along with the virus itself, the increased levels of angiotensin II and hypoxia, drive the major cellular changes promoting thrombosis, which include: (1) aberrant expression of tissue factor by activated alveolar epithelial cells, monocytes-macrophages and neutrophils, and production of other prothrombotic factors by activated endothelial cells (ECs) and platelets; (2) reduced expression of physiological anticoagulants by dysfunctional ECs, and (3) suppression of fibrinolysis by the endothelial overproduction of plasminogen activator inhibitor-1 and, likely, by heightened thrombin-mediated activation of thrombin-activatable fibrinolysis inhibitor. Moreover, upon activation or death, neutrophils and other cells release nuclear materials that are endowed with potent prothrombotic properties. The ensuing thrombosis significantly contributes to lung injury and, in most severe COVID-19 patients, to multiple organ dysfunction. Insights into the pathogenesis of COVID-19-associated thrombosis may have implications for the development of new diagnostic and therapeutic tools.

**Keywords:** SARS-COV-2; Thrombosis; COVID; Infection; Prothrombotic state.

**Citation:** Semeraro N., Colucci M. The prothrombotic state associated with SARS-CoV-2 infection: pathophysiological aspects. *Mediterr J Hematol Infect Dis* 2021, 13(1): e2021045, DOI: <http://dx.doi.org/10.4084/MJHID.2021.045>

**Published:** July 1, 2021

**Received:** May 18, 2021

**Accepted:** June 8, 2021

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**Introduction.** Coronavirus disease-2019 (COVID-19) is a viral illness caused by severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2). Since its emergence in late 2019, the disease has rapidly achieved pandemic proportions causing remarkably high mortality worldwide. Although most people infected with SARS-CoV-2 are totally asymptomatic or have a mild illness, some patients (about 5%) usually present with

progressive respiratory failure (acute respiratory distress syndrome, ARDS), and even multiple organ dysfunction.<sup>1,2</sup> Accumulating clinical and pathological evidence indicates that severe SARS-CoV-2 infection is frequently associated with a prothrombotic state which can manifest as microvascular or macrovascular thrombosis, and that these complications significantly contribute to the mortality burden of COVID-19 patients.

Microvascular thrombosis occurs mainly in the lung, as documented by several autopsy reports.<sup>3-6</sup> Indeed, in addition to diffuse alveolar damage, platelet-fibrin thrombi are frequently seen in the small pulmonary vasculature in almost all the examined lungs. Importantly, alveolar-capillary microthrombi were 9 times as prevalent in patients with Covid-19 as in patients who died from ARDS secondary to influenza A (H1N1) infection.<sup>7</sup> Pulmonary microvascular thrombosis also appears more pronounced in severe SARS-CoV-2 infection than in other human coronavirus infections targeting the lower respiratory tract, namely SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV).<sup>8</sup> In COVID-19 patients with more severe disease, thrombosis of the microcirculation may also be seen in other organs (heart, kidney, brain, and liver).<sup>4-6</sup>

Among macrovascular thrombotic events reported in COVID-19, venous thromboembolism (VTE), which includes deep vein thrombosis (DVT) and pulmonary embolism (PE) is the most frequent, with a cumulative incidence of 16,7 to 49% in critically ill patients admitted to the intensive care unit (ICU), and with PE being the most common complication.<sup>9-13</sup> Notably, VTE may occur despite standard thromboprophylaxis. Moreover, COVID-19 ARDS patients develop more thrombotic complications, mainly PE, than non-COVID-19 ARDS patients, and patients suffering from a thrombotic complication had more than a 5-fold increase in all-cause mortality.<sup>10,12</sup> Because the frequency of PE far exceeds that of DVT in most reports on COVID-19 patients, it has been proposed that the occlusion of pulmonary vessels in these patients results from pulmonary thrombosis rather than embolism.<sup>13,14</sup> In hospitalized, non-severely ill patients receiving standard thromboprophylaxis, the incidence of VTE is obviously much lower, ranging from 0 to about 6%.<sup>9,14-16</sup> Arterial thrombosis has also been reported in patients with COVID-19, including myocardial infarction,<sup>11,17</sup> ischemic stroke<sup>11,18</sup> and peripheral thrombosis,<sup>19,20</sup> with rates < 3%.<sup>10,11,15</sup> Patients with COVID-19 may also experience bleeding complications. A multicentre study of 400 hospitalized patients with COVID-19 reported an overall bleeding rate of 4.8% and a severe bleeding rate of 2.3%.<sup>15</sup>

Based on the extensive clinical evidence summarized above, thrombotic events emerge as critical issues in severe COVID-19 and can be listed among life-threatening complications of the disease. This implies that patients suffering from severe COVID-19 have haemostatic abnormalities that predispose to thrombosis, commonly referred to as hypercoagulability or prothrombotic state.

In this review, we will 1) shortly summarize the distinctive laboratory haemostatic abnormalities in patients with COVID-19, 2) discuss the possible pathogenetic mechanisms of COVID-19-associated

thrombosis, and 3) describe the new diagnostic and therapeutic tools that are being developed.

### Laboratory Haemostatic Abnormalities

*Routine assays.* The most frequent finding in patients with COVID-19-associated coagulopathy is an increased plasma D-dimer concentration, which is found in almost 50% of patients and has attracted particular attention because of its prognostic significance. Markedly higher D-dimer levels (usually more than three-fold the upper limit of normal) were consistently observed in severely affected patients (requiring critical care support) and in nonsurvivors. Significantly, exceedingly high D-dimer levels on hospital admission or a progressive elevation during the hospitalization are associated with an increased need for mechanical ventilation and an increased risk of death.<sup>21-24</sup> Therefore, COVID-19 patients who have markedly raised D-dimer on admission should be carefully checked even in the absence of other laboratory abnormalities or severe symptoms because the presence of high D-dimer is strongly suggestive of clotting activation and increased thrombin generation.

Thrombocytopenia is uncommon in COVID-19 patients, and, when present, it is usually mild. Even in patients with the most severe illness, the platelet count is generally greater than  $100 \times 10^9/L$ ,<sup>12,21,23,25-27</sup> and less than 5% of patients displays a lower count.<sup>23,25</sup> In very advanced cases, a progressive decrease in platelet count may occur, indicating platelet consumption.<sup>21,22,26</sup> However, despite the relatively small changes in platelet count in patients with COVID-19, two studies suggest that thrombocytopenia may have prognostic significance. Lippi et al., in a meta-analysis of nine studies, showed that the platelet count was significantly lower in patients with more severe COVID-19 and correlated with mortality.<sup>28</sup> Moreover, in the most extensive so-far analysis of thrombocytopenia in patients with COVID-19 (1476 patients, 238 of whom were nonsurvivors), Yang et al. found a significant association between thrombocytopenia and in-hospital mortality.<sup>29</sup> The latter study is also important because it confirms the relatively low frequency of thrombocytopenia which, with  $125 \times 10^9/L$  as the lower limit of the normal range, occurred in about 20% of the patients.

Prothrombin time (PT) is normal in non-critically ill COVID-19 patients, whereas it is only mildly prolonged in severe cases.<sup>22,23</sup> When performed on admission, PT is more prolonged, albeit modestly, in patients who need critical care support and in nonsurvivors.<sup>23</sup>

Fibrinogen is markedly increased in Covid-19 patients,<sup>12,22,30,31</sup> which likely reflects the inflammatory acute phase response. However, a sudden drop in fibrinogen level below 1.0 g/L has been reported in terminal COVID-19 patients shortly before they died.<sup>22</sup>

The coagulation abnormalities of patients with

Covid-19 in relation to the disease severity are often heterogeneous and somewhat difficult to interpret, particularly with respect to platelet count and PT. A meta-analysis of 34 studies (with a pooled population of 6,492 COVID-19 patients) showed that elevated D-dimer levels, low platelet count, and prolonged PT occur more often in severe COVID-19 patients and in nonsurvivors.<sup>32</sup> Therefore, monitoring these biomarkers in COVID-19 patients might aid in the early detection of severe disease. As a matter of fact, the guidance for the management of coagulopathy in COVID-19 released by the International Society on Thrombosis and Haemostasis (ISTH) recommends testing and monitoring routine haemostatic markers, including, in decreasing order of importance, D-dimer, prothrombin time, platelet count and fibrinogen.<sup>33</sup> The alterations of routine haemostatic tests, when combined, are reminiscent of the pattern seen in DIC. However, there are significant differences from classical DIC associated with sepsis. First, thrombocytopenia is the most common haemostatic abnormality in the latter condition and is usually much more pronounced ( $\leq 50 \times 10^9/L$ );<sup>34,35</sup> of note, severe thrombocytopenia is strongly associated with mortality either when present at admission or when appearing during the hospital stay.<sup>35</sup> In addition, patients with sepsis-associated DIC have much lower levels of several coagulation factors, including those involved in the PT assay, namely prothrombin, factor (F)V, FVII, FX, and fibrinogen, because of consumption, and a marked reduction in plasma levels of physiological anticoagulant proteins antithrombin (AT) and protein C (PC).<sup>36,37</sup> These changes are almost absent in COVID-19 patients (see below). It is worth mentioning that one previous study<sup>22</sup> reported that 71% of non-survivors COVID-19 patients fulfilled the ISTH criteria for DIC, compared with only 0.6% among survivors, suggesting that DIC is common in severe SARS-CoV-2 infection. However, several other investigators did not find evidence of DIC in their patients,<sup>12,15,30,31,38,39</sup> leading to the conclusion that DIC is rare in COVID-19.

*Other assays.* Global coagulation assays on whole blood, including thromboelastography (TEG) and thromboelastometry (ROTEM), have consistently demonstrated a hypercoagulable profile in patients with COVID-19,<sup>30,31,38,40</sup> except for one study reporting ROTEM parameters in the normal range.<sup>41</sup> Thrombin generation assay (TGA), a global test performed in plasma with or without thrombomodulin, has been used by some investigators to evaluate the COVID-19-associated coagulopathy with results that are difficult to interpret because the assay is sensitive to anticoagulants and the majority of COVID-19 hospitalized patients receive either standard thromboprophylaxis with low molecular weight heparin (LMWH) or, in more severe cases, higher doses of LMWH or standard heparin. In

general, TGA parameters were reported to be normal despite the use of anticoagulants,<sup>41,42</sup> a finding interpreted as evidence of hypercoagulability.<sup>42</sup> Using plasma samples from COVID-19 patients treated to neutralize anticoagulants prior to the assay, White et al.<sup>43</sup> found normal thrombin generation in both critical and noncritical patients, whereas Hardy et al.<sup>44</sup> observed increased thrombin potential over the first week after ICU admission.

Thrombin-antithrombin complex (TAT) and prothrombin fragment 1+2 (F1+2) have also been measured in COVID-19 patients as in vivo thrombin formation markers. TAT plasma concentration was elevated in some studies,<sup>41,45,46</sup> with significantly higher values in ICU compared with non-ICU patients,<sup>45</sup> but normal<sup>43</sup> or moderately increased<sup>47</sup> in others. F1+2 was normal<sup>41,43</sup> or marginally increased.<sup>48</sup> In the latter study, in a cohort of 114 critically ill COVID-19 patients, F1+2 levels were significantly higher in those with thrombosis and were more discriminant than D-dimer in identifying thrombotic manifestations. FVIII and von Willebrand Factor (vWF) are consistently and considerably elevated in COVID-19 patients, particularly in severe cases,<sup>12,31,41,45</sup> likely reflecting endothelial cell activation (see next section). Taken together, these results indicate that most hospitalized COVID-19 patients, especially those with severe disease, have haemostatic abnormalities that are compatible with a prothrombotic state.

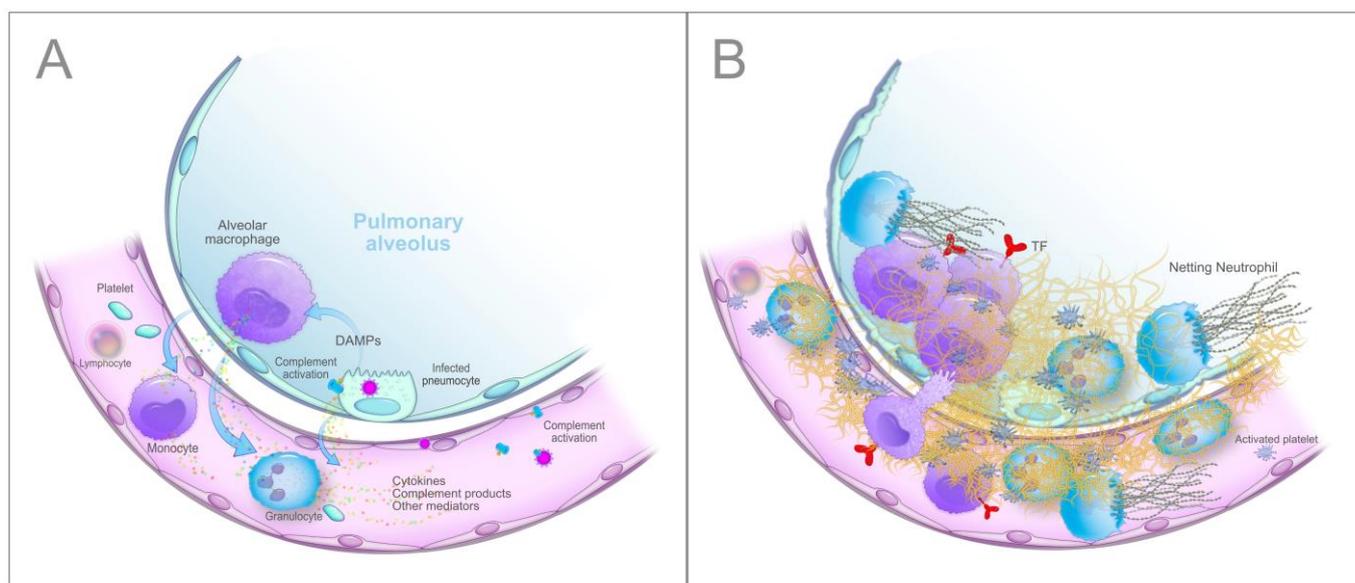
**Pathophysiology.** The urgent need to understand how SARS-CoV-2 induces a prothrombotic state and to develop effective therapeutic approaches for COVID-19-associated thrombosis has prompted an unprecedented number of studies. Despite this enormous effort, the mechanisms underpinning haemostatic abnormalities and thrombosis in COVID-19 patients still remain to be fully elucidated. Being COVID-19 a severe infection, it can be reasonably postulated that they may overlap, at least in part, with those of sepsis-induced DIC. Like classical bacterial sepsis, severe SARS-CoV-2 infection is characterized by an excessive inflammatory response of the host which is thought to drive, together with a virus-induced lowering of antiviral defences (low interferon response, lymphocytopenia), the progressive worsening of lung function and, in most severe cases with systemic inflammatory response, multiorgan failure ultimately leading to the death of COVID-19 patients. Several recent reviews have been published on this topic.<sup>49-51</sup> Briefly, SARS-CoV-2, through its surface spike (S) protein, primarily infects alveolar epithelial cells, especially type 2 cells, which express the highest levels of angiotensin-converting enzyme 2 (ACE2), the best characterized entry receptor for the virus.<sup>52</sup> This leads to cell activation and/or death by apoptosis and pyroptosis and to the release of damage-associated

molecular patterns (DAMPs).

Given the close proximity to pneumocytes, alveolar macrophages are the first immune cells that recognize DAMPs and probably also the virus and/or its unique constituents (PAMPs, pathogen-associated molecular patterns) through specific receptors (PRRs, pattern recognition receptors, primarily the TLRs, Toll-like receptors), and respond with the synthesis and release of large amounts of proinflammatory mediators, mainly cytokines and chemokines. Cytokines secreted by alveolar macrophages, i.e., interleukin (IL)-6, IL-10 and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), have been detected in the lung by immunohistochemistry<sup>53,54</sup> and increased circulating levels of numerous cytokines, including IL-6, IL-8, IL-1 $\beta$ , and TNF- $\alpha$ , that correlate with disease severity have been reported in COVID-19 patients.<sup>23,25,27,49</sup> Moreover, SARS-Cov-2 activates the complement (C) alternative and lectin pathways through at least 2 surface proteins, i.e., the S protein, which, being heavily glycosylated with L-fucose and mannose, binds mannose-binding lectin (MBL), and the nucleocapsid protein (N protein), which binds MBL-associated serine protease 2 (MASP-2),<sup>55,56</sup> thus generating other potent proinflammatory mediators including the anaphylatoxins C3a and C5a, C3b and C5b-9 complex (membrane attack complex, MAC). The involvement of the complement system in COVID-19 is strongly supported by the extensive deposition of C5b-9, C4d, and MASP-2 within the lung microvasculature.<sup>57</sup> Strikingly, the SARS-CoV-2 S glycoprotein colocalized with C5b-9 and C4d in a subset of cases. In addition, patients with COVID-19 have elevated serum C5a and sC5b-9 levels.<sup>58,59</sup> All the generated inflammatory mediators then cause the recruitment into the alveolar and interstitial spaces of monocytes-macrophages and neutrophils, which are activated and, in their turn, release additional cytokines

(the so-called "cytokine storm"), proteases and other mediators, such as reactive oxygen species (ROS), which work in concert to trigger a self-amplifying inflammatory cascade eventually resulting in a further injury of alveolar epithelial cells and hypoxic respiratory failure. In this setting, the vascular endothelium also plays a pivotal role. ACE2 is widely expressed on endothelial cells, and the presence of SARS-CoV-2 elements within these cells has been well documented.<sup>60</sup> Direct viral infection, combined with the milieu of proinflammatory mediators, will cause endothelial dysfunction/injury that strongly contributes to inflammation and tissue damage. Finally, platelets are activated by cytokines and complement products, as well as by the interaction with injured vessels and activated leukocytes, and release the content of their granules, including numerous proinflammatory mediators that further amplify the inflammatory reaction.<sup>61,62</sup> As expected, COVID-19 patients exhibit a marked elevation of acute-phase reactants (e.g., C-reactive protein, fibrinogen and ferritin). A schematic summary of the host inflammatory response to SARS-CoV-2 infection is reported in **Figure 1**.

In the light of these findings, a plausible hypothesis is that the plethora of inflammatory mediators generated during the persistent inflammatory status, along with the virus-derived components, drives thrombus formation in severe COVID-19 patients mainly through 1) the upregulation of the cellular procoagulant pathways, 2) the downregulation of physiological anticoagulant pathways and 3) the impairment of fibrinolysis. Virtually all cells participating in the inflammatory process, including lung epithelial cells, resident macrophages, monocytes, neutrophils, platelets, and endothelial cells, may variably contribute to these mechanisms.



**Figure 1. A.** Schematic overview of the main steps of the hyperinflammatory response to SARS-CoV-2 infection in the lung. **B.** Tissue damage and fibrin deposition in the lung induced by inflammatory cells recruited by cytokines and complement activation products. Details in the text.

**Upregulation of Cellular Procoagulant Pathways: the Role of Tissue Factor.** Currently, the aberrant *in vivo* expression of tissue factor (TF) is thought to play a pivotal role in almost all forms of thrombosis, including the sepsis-associated widespread microthrombosis.<sup>36,37,63</sup> TF appears to be the major trigger of the coagulation cascade also in several viral infections as inferred from *in vitro* findings and animal studies.<sup>64,65</sup>

*Lung epithelial cells.* Lung epithelial cells are the first target of SARS-CoV-2. *In vitro* studies suggest that the basal expression of TF in these cells may be upregulated by infection with some viruses<sup>64</sup> or exposure to a mixture of cytokines.<sup>66</sup> Moreover, mice infected with influenza A virus H1N1 show both an increased TF expression in lung epithelial cells and signs of clotting activation.<sup>64</sup> Concerning COVID-19, a preprint study suggests that lung epithelial cells infected *in vitro* with SARS-CoV-2 undergo transcriptional changes in several coagulation genes that make them prothrombotic.<sup>67</sup> Specifically, TF is upregulated while two major coagulation inhibitors, TFPI (tissue factor pathway inhibitor) and protein S, are either unmodified or downregulated. Similar transcriptional changes were found *ex vivo* in cells isolated from bronchoalveolar lavage fluid (BALF) of COVID-19 patients, although the cell type(s) undergoing these changes were not identified. If confirmed, these findings could contribute to explain the initial fibrin deposition in the alveoli.

*Monocytes and macrophages.* Monocytes and macrophages have long been recognized as major players in the activation of blood coagulation, through the synthesis of TF in response to several inflammatory mediators. These cells, like many others, upon activation or during apoptosis, release small-membrane vesicles bearing TF, called microparticles (TF<sup>+</sup> MPs) or microvesicles, that can be transferred to the surface of other cells via specific receptors (for instance, P-selectin glycoprotein ligand-1, PSGL-1 on leukocyte-derived MPs and P-selectin on activated platelets or endothelial cells), making the recipient cell capable of triggering and propagating coagulation.<sup>68</sup> The best example illustrating the role of monocytes-macrophages in clotting activation is sepsis-associated DIC, in which the aberrant expression of TF by these cells has been amply documented both in experimental and human studies.<sup>36,37</sup> TF can also be induced in these cells after viral infection.<sup>64</sup> As mentioned above, monocytes and macrophages play a key role in the overwhelming inflammatory response observed in COVID-19 patients,<sup>53,69</sup> being responsible, once activated, for the unrestrained release of cytokines, ROS and proteases, which sustain the inflammatory process and aggravate lung injury. Therefore, although direct proof is still lacking, it can be argued that, in patients with severe

COVID-19, alveolar macrophages and recruited monocytes-macrophages synthesize and express active TF on their surface, in response to several inflammatory mediators, mainly cytokines and complement activation products (**Figure 1B**). Among cytokines, IL-6 appears of particular importance because a) it stimulates TF synthesis by human monocytes *in vitro*,<sup>70</sup> and seems to be critical for *in vivo* activation of blood coagulation in animal models;<sup>71</sup> b) it is present in the lung of patients with COVID-19, as shown by immunohistochemistry;<sup>53</sup> c) its serum level is significantly elevated in nonsurvivors and progressively increases with illness deterioration.<sup>21,72</sup> In addition, IL-6 is one of the key mediators of the cytokine storm and can suppress normal T cell activation, which may partly explain the occurrence of lymphopenia.<sup>49,72</sup> Concerning the complement-derived mediators, C5a is known to induce TF expression in human monocytes,<sup>73,74</sup> and its plasma level is significantly increased in patients with COVID-19.<sup>58,59</sup> The possible involvement of monocyte TF in SARS-CoV-2 infection is supported by a recent study of Hottz et al.<sup>75</sup> These investigators demonstrated that patients with severe COVID-19, but not those with mild disease or asymptomatic, exhibit platelet activation, as assessed by increased P-selectin and CD63 surface expression, increased plasma levels of thromboxane B<sub>2</sub> (TXB<sub>2</sub>, a metabolite from platelet TXA<sub>2</sub> synthesis), and a greater number of platelet-monocyte aggregates. Interestingly, exposure to plasma from severe COVID-19 patients increased the activation of control platelets *in vitro*. More relevant in the context of this discussion, platelet-monocyte interaction resulted in strong TF expression by the monocytes. Both platelet activation and monocyte TF expression were associated with fibrinogen and D-dimers levels, and were increased in patients requiring invasive mechanical ventilation or patients who died in hospital. Notably, platelets from severe COVID-19 patients were able to induce TF expression in monocytes from healthy volunteers, a phenomenon that was inhibited by platelet P-selectin neutralization or integrin  $\alpha$ IIb/ $\beta$ 3 blockade (with the aggregation inhibitor abciximab). The plasma factor(s) responsible for platelet activation and platelet-dependent monocyte TF expression were not identified, but they could well be cytokines and/or complement activation products. The concept that activated platelets form heterotypic aggregates with monocytes and neutrophils in the setting of infection and inflammation is rather well established<sup>76</sup> and has been confirmed in COVID-19 patients also by other investigators.<sup>62</sup> These aggregates might play a role in mechanical occlusion of the microvasculature in addition to altering the functional cell responses, eventually leading to TF expression. A definitive understanding of the *in vivo* expression of monocyte-macrophage TF and the role thereof in blood clotting activation and thrombosis associated with

COVID-19 remains to be settled. In ARDS secondary to conditions other than COVID-19, studies performed with BALF and pulmonary tissues have consistently reported that activation of coagulation with fibrin formation is mainly due to TF upregulation in alveolar and recruited macrophages as well as in lung epithelial cells, likely as a response to inflammatory mediators.<sup>66,77,78</sup> Therefore, studies at the tissue level, particularly in the lung, which is the main target organ of SARS-CoV-2, are warranted.

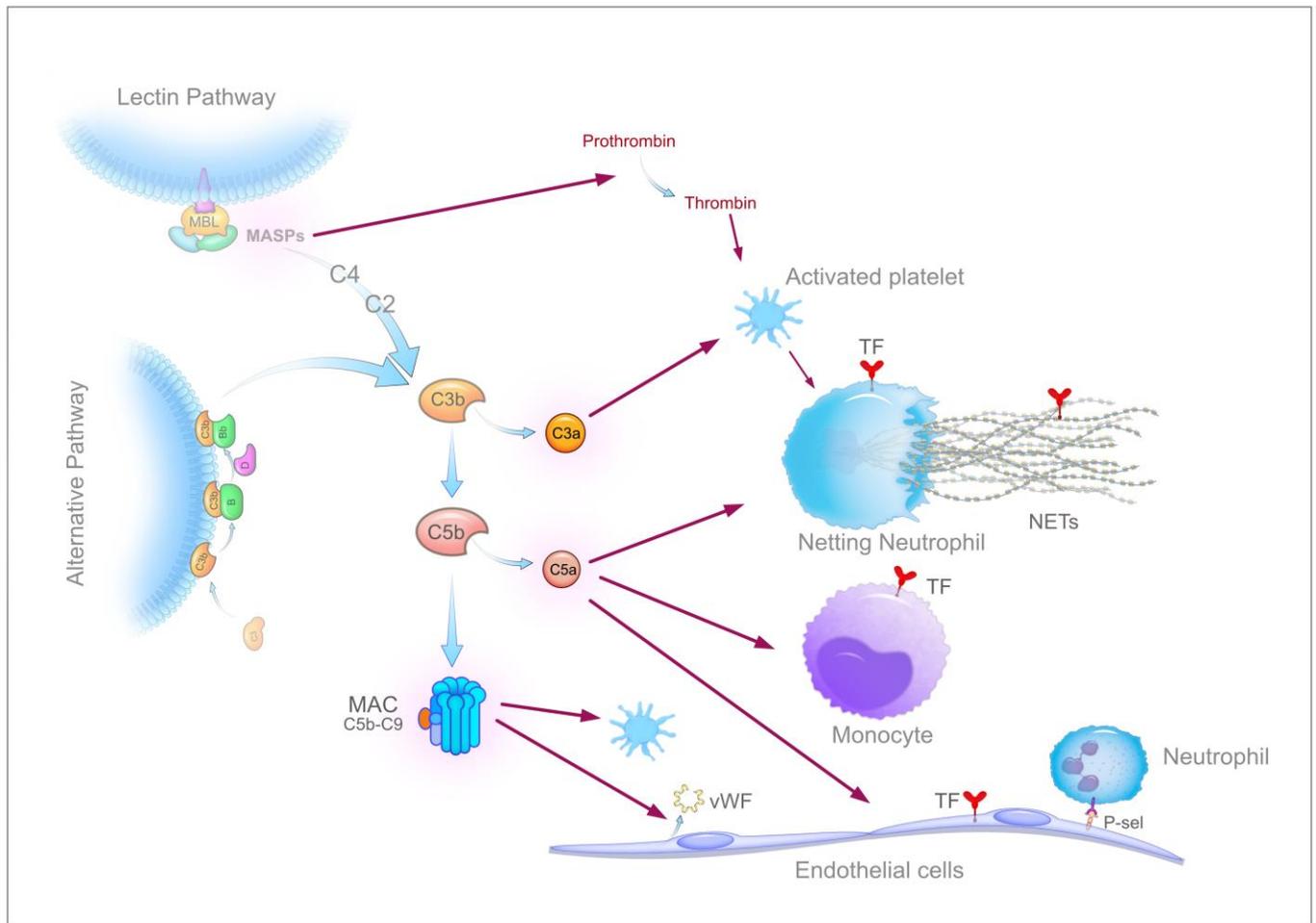
**Neutrophils.** Increased neutrophil counts have been associated with disease severity and poor prognosis in COVID-19<sup>27,79</sup> and the neutrophil-to-lymphocyte ratio is an independent risk factor for severe disease.<sup>80</sup> In addition, neutrophil infiltration of pulmonary capillaries has been described in autopsy specimens.<sup>4,57</sup> Neutrophils reportedly express TF in response to inflammatory stimuli.<sup>81,82</sup> Another remarkable feature of activated neutrophils is the capacity to release neutrophil extracellular traps (NETs), a process named NETosis.<sup>83</sup> NETs are networks of chromatin filaments made up of histones and DNA strands, decorated with proteins and lysosomal enzymes (myeloperoxidase, elastase and cathepsin G, among others) and are released by neutrophils upon exposure to a variety of stimuli such as microorganisms and their products, inflammatory mediators, including cytokines and complement activation products, and ROS. Noteworthy, activated platelets are potent inducers of NET formation as a consequence of their interaction with neutrophils. Extracellular traps can be actively extruded also by other activated innate immune cells, such as mast cells, eosinophils, and mononuclear phagocytes, and individual components (histones and DNA, mainly as nucleosomes) can be passively released by dying cells. NET formation involves the unwinding of nuclear DNA fibres and the nuclear membrane breakdown before the final active discharge in the extracellular milieu. This process is mediated by NF- $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells) signalling, peptidyl arginine deiminase 4 (PAD4), and neutrophil elastase (NE), which cooperate to modify histones and enable DNA decondensation, and by ROS via NADPH oxidase. Originally characterized as a major innate immunity mechanism to trap and eventually neutralize invading microorganisms,<sup>83</sup> NETs, when not properly regulated, have the potential to propagate inflammation, to cause cell and tissue injury, and to trigger thrombus formation, and thus they play a major role in numerous diseases,<sup>84</sup> including viral infections.<sup>85</sup> NETs are now emerging as important players in COVID-19 as well, as clearly suggested by several lines of evidence. First, numerous investigators demonstrated that patients with COVID-19 exhibit elevated serum or plasma levels of known markers of NETs, namely myeloperoxidase (MPO)-DNA complex, citrullinated histone H3 (cit-H3),

and cell-free DNA.<sup>46,86-89</sup> Cell-free DNA and/or MPO-DNA levels were found to be consistently correlated with disease severity, being higher in hospitalized patients undergoing mechanical ventilation than in those breathing room air,<sup>86,87,89</sup> and in nonsurvivors as compared to survivors.<sup>87</sup> Plasma NETs also correlate with respiratory failure severity and the Sequential Organ Failure Assessment (SOFA) score.<sup>87</sup> In patients who recover from COVID-19, plasma NETs decrease to levels similar to those of healthy subjects, suggesting the normalization of NET formation.<sup>87</sup> In a case-control study of hospitalized COVID-19 patients, the serum levels of NET markers and calprotectin, a marker of neutrophil activation, were associated with a higher risk of thrombotic events despite prophylactic anticoagulation.<sup>90</sup> Interestingly, plasma or serum from patients with COVID-19 was able to stimulate NET release when added to neutrophils isolated from healthy donors,<sup>86,87</sup> suggesting the presence of factors capable of triggering NETosis, such as cytokines and complement activation products. Second, neutrophils isolated from COVID-19 patients with severe disease presented signs of in vivo activation, for example, a significant decrease in granularity<sup>87</sup> or a significant increase of "low-density granulocytes," a specific form of neutrophils characterized by a high propensity to spontaneously form NETs.<sup>89</sup> As a matter of fact, when cultured in vitro in the absence of any stimulus, these cells released markedly higher levels of NETs compared to normal neutrophils.<sup>86-88</sup> Third, examination of autopsy lung specimens from COVID-19 patients by immunofluorescence revealed the presence of microvascular thrombi containing, in addition to platelets and fibrin, numerous neutrophils releasing NETs.<sup>87,88,91,92</sup> Colocalization of cit-H3-positive neutrophils with platelets suggests that the interaction between these cells contributes to the release of NETs. Such interaction is further supported by the significantly higher levels of circulating platelet-neutrophil aggregates in COVID-19 patients compared with healthy adults.<sup>62,87</sup> In a detailed immunohistochemical study of post-mortem lung specimens from 4 COVID-19 patients,<sup>92</sup> NETs were consistently located in the airway compartment, often associated with fibrin that occluded some alveoli or bronchioles, in the interstitial compartment, especially in areas infiltrated by neutrophils and macrophages, and in the vascular compartment, mainly in arterioles containing neutrophil-rich microthrombi. Notably, NETs were not detected in lung specimens of 4 patients who died from a COVID-19-unrelated cardiac cause. In COVID-19 patients, neutrophil-enriched thrombi were restricted to the lung in one study<sup>92</sup> but present also in renal and cardiac microvessels in another.<sup>91</sup> The concentration of NETs was about 10 times higher in tracheal aspirate fluid than in plasma samples from COVID-19 patients and airway fluid from healthy

controls.<sup>87,88</sup> Finally, infection of neutrophils from healthy individuals with SARS-CoV-2 induced the release of NETs, which were cytotoxic for lung epithelial cells *in vitro*.<sup>88</sup> Altogether, these studies strongly suggest a key role of NETs in the pathophysiology of lung injury and microthrombosis (**Figure 1B**); but how do NET-releasing granulocytes promote thrombus formation? NETs are endowed with tremendous thrombogenic potential. They *per se* can promote thrombosis by providing a three-dimensional scaffold for recruitment of platelets and other cells and by adsorbing several prothrombotic proteins such as vWF, fibronectin, fibrinogen, and even cell-derived TF.<sup>93</sup> NETs colocalize with fibrin and interact closely with fibrin strands in the thrombus, thus potentially influencing thrombus organization and stability. Mechanistically, NET's constituents are primarily responsible for thrombus formation because they display a variety of prothrombotic activities.<sup>36</sup> Histones, the most abundant proteins in NETs, induce platelet activation and expression of procoagulant properties, phosphatidylserine exposure on erythrocyte membrane, TF expression on monocytes and endothelial cells, prothrombin autoactivation, impairment of the protein C pathway and interference with the anticoagulant activity of heparin-like substances.<sup>36,94</sup> Most of these effects are attributable to histone H4 and, to a lesser extent, H3. In addition, histones were shown to promote prothrombin activation by FXa in the absence of FVa and surface phospholipids, both *in vitro* and in mice, thus providing a new mechanism to disseminate intravascular coagulation.<sup>95</sup> Double-stranded DNA serves as a suitable negatively charged surface that initiates the intrinsic pathway of coagulation by favouring the auto-activation of FXII and potentiating FXI activation by thrombin. Among proteins and enzymes hosted in NETs, elastase cleaves the major physiological anticoagulants TFPI, AT, and TM, myeloperoxidase oxidizes and inactivates TM, and cathepsin G augments platelet activation on the NET surface. Finally, NETs can harbour neutrophil or blood-derived TF. The relative role of each of these mechanisms in triggering COVID-19-associated thrombosis is difficult to establish. Skendros *et al.*<sup>46</sup> unravelled the combined importance of TF and complement. They found a significant increase of plasma MPO/DNA complexes in COVID-19 patients, which positively correlated with TAT level, suggesting a possible role in the activation of blood coagulation. More importantly, using confocal immunofluorescence microscopy, they observed spontaneous formation of NETs bearing high amounts of TF in neutrophils collected from patients with severe COVID-19. Patients' neutrophils also exhibited a 5-fold increase in TF mRNA levels as compared to normal cells. In addition, when neutrophils isolated from healthy individuals were treated with COVID-19 platelet-rich plasma (PRP), they

expressed increased levels of TF mRNA and generated TF-bearing NETs, two phenomena that could be prevented by a specific C5a receptor antagonist. Furthermore, targeting C3 with the specific inhibitor compstatin also decreased neutrophil TF at both the mRNA and protein levels. These findings, coupled with the high plasma levels of sC5b-9 and C5a in COVID-19 patients,<sup>58,59</sup> clearly suggest a pivotal role of complement and NETs in COVID-19-associated thrombosis. Furthermore, the complement system may exert additional prothrombotic effects<sup>56,96</sup> (**Figure 2**). MASP-1 and MASP-2, two serine proteases that initiate the lectin complement pathway, can activate prothrombin to thrombin, and MASP-1 can also directly convert fibrinogen to fibrin and activate FXII. C3a and MAC induce platelet activation and increase platelet prothrombinase activity, thus contributing to clotting activation. C5a and MAC also activate ECs leading to increased P-selectin expression, release of vWF, and expression of cell surface prothrombinase activity. C4b-binding protein (C4BP) binds to and inhibits protein S.

Like NETs and/or their constituents, another nuclear product, namely high-mobility group box 1 (HMGB1), can be actively secreted by a variety of stimulated immune cells or passively released by necrotic cells. HMGB1 displays strong proinflammatory properties in the extracellular milieu and is thought to play an essential role in numerous acute and chronic systemic inflammatory diseases, including sepsis.<sup>36,97</sup> In systemic inflammation, HMGB-1 levels rise into the circulation and in other biological fluids and exhibit a direct correlation with disease severity. In this context, two observations are of particular interest and suggest a possible involvement of this protein in microvascular thrombosis of different aetiologies: 1) the direct correlation between serum HMGB1 and severity of pneumonia caused by various agents, including viruses; 2) the capacity of HMGB1 to stimulate TF expression in monocytes and ECs and to reduce the activity of thrombin-TM complex with a consequent reduction in protein C activation.<sup>36,97</sup> In COVID-19 patients, the levels of serum HMGB1 are elevated and correlate with the severity of the disease and with a poor clinical outcome.<sup>98,99</sup> Moreover, *in vitro*, exogenous HMGB1 induces the expression of SARS-CoV-2 entry receptor ACE2 in alveolar epithelial cells in a RAGE (receptor for advanced glycosylation end products)-dependent manner. Significantly, genetic or pharmacological inhibition of the HMGB1-RAGE pathway blocks ACE2 expression.<sup>99</sup> This mechanism adds to the known ability of HMGB1 to stimulate the production of inflammatory cytokines by immune cells (e.g., monocytes-macrophages) via TLRs.<sup>97</sup> Therefore, HMGB1 might play an important role in COVID-19, although more studies are required.



**Figure 2.** Overview of the thrombogenic potential derived from the activation of the complement system. Details in the text.

**Platelets.** Activated platelets closely cooperate with monocytes-macrophages and neutrophils to promote thrombosis during SARS-CoV-2 infection. Platelets, however, may participate in the activation of coagulation and thrombus formation by additional mechanisms.<sup>36,63</sup> First, in quiescent platelets, variable levels of TF mRNA and protein have been detected, increasing upon stimulation with different agonists. Moreover, activated platelets provide a suitable phospholipid surface (anionic phospholipids, mainly phosphatidylserine) that accelerates the coagulation reactions many folds and renders clotting enzymes less susceptible to fluid phase protease inhibitors. Finally, stimulated platelets release from dense granules soluble polyphosphates that are composed of 60-100 linear-linked phosphate subunits and represent a suitable surface for the activation of the contact system, thereby inducing thrombin generation independently of TF and bradykinin formation. The role of the contact system in COVID-19-associated thrombosis has not been addressed so far. However, the potential benefits of targeting the contact system activation are discussed in recent reviews.<sup>100,101</sup>

**Endothelial cells.** As alluded to above, there is compelling evidence that endothelial cells play a pivotal role in COVID-19, as they are the target of both the virus

and the multiple inflammatory mediators generated by the hyperinflammatory response and, as a consequence, undergo activation/injury. In vitro infection of endothelial cells with some viruses has been shown to induce TF expression.<sup>64,65</sup> Likewise, cultured endothelial cells have long been known to respond to cytokines, including TNF $\alpha$ , IL-1 $\beta$ , and IL-6, as well as to complement activation products with the expression of TF.<sup>36,37</sup> To our knowledge, data on the direct and/or indirect effects of SARS-CoV-2 on endothelial TF are unavailable. DiNicolantonio and McCarty<sup>102</sup> hypothesized that SARS-CoV-2, after incorporation into endosomes within endothelial cells, can activate endosomal NADPH oxidase and that the resulting local production of superoxide/hydrogen peroxide leads to activation of NF- $\kappa$ B and increased TF expression. Though unproven, this hypothesis is interesting based on the finding that Covid-19 patients display overactivation of NADPH oxidase 2 (Nox2), which is more intense in severe than non-severe patients.<sup>103</sup> Moreover, patients with thrombotic complications have higher Nox2 activation than those without, suggesting the possible involvement of Nox2 in thrombus formation. Despite the lack of evidence for the in vivo TF expression during SARS-CoV-2 infection, activated endothelial cells may contribute to thrombus formation by other important

mechanisms. They adopt a proinflammatory phenotype that supports the recruitment of monocytes-macrophages and neutrophils (both expressing a great procoagulant potential) through the exposure of adhesion molecules (P-selectin and intercellular adhesion molecule 1, ICAM-1, among others), thus favoring local thrombus formation. In this context, P-selectin is of particular importance because it is per se a potent stimulus for induction of TF in monocytes<sup>104</sup> and, as mentioned above, it binds TF<sup>+</sup> MPs of leukocyte origin. MPs are then internalized, and the TF moiety is recycled to the cell surface, thus inducing a substantial increase in the cell procoagulant potential.<sup>68,105</sup> Endothelial ICAM-1, on the other hand, is overexpressed in COVID-19 post-mortem lung samples compared to H1N1 and control lung samples.<sup>54</sup> In addition, activated endothelial cells are known to secrete von Willebrand factor (vWF) in its highly platelet-agglutinating form (i.e., ultra-large vWF multimers) from Weibel-Palade bodies, eventually resulting in platelet activation and platelet-mediated clotting stimulation (**Figure 2**). As a matter of fact, increased plasma levels of vWF along with a decrease in vWF-cleaving protease ADAMTS-13 (a disintegrin and metalloprotease with thrombospondin type 1 motif, member 13, that reduces the thrombogenicity of released vWF by cleaving ultralarge multimers) have been reported by several investigators.<sup>12,31,41,106-108</sup> The extent to which changes in ADAMTS-13/vWF ratio contribute to thrombosis and illness severity in COVID-19 patients remains to be fully elucidated. Bazzan et al.,<sup>107</sup> in a cohort of 88 COVID-19 patients, found lower ADAMTS-13 (and higher vWF) levels in nonsurvivors compared with survivors, and ADAMTS-13 levels < 30% were significantly associated with higher mortality. Cugno et al.,<sup>59</sup> in a cohort of 148 patients with COVID-19 of different severity, reported that plasma levels of sC5b-9 and vWF were strongly correlated with each other and disease severity. Interestingly, sC5b-9 and vWF declined simultaneously during remission, suggesting that, in addition to proinflammatory cytokines and direct SARS-CoV-2 infection, complement activation plays a key role in mediating COVID-19-associated endotheliopathy. Several studies have reported the increase of other markers of endothelial activation/damage, including soluble TM (sTM),<sup>45,59,109</sup> soluble E-selectin,<sup>59</sup> soluble P-selectin (sP-selectin, a marker of endothelial and platelet activation),<sup>45,110</sup> and TFPI.<sup>43</sup> In one of these studies,<sup>45</sup> the levels of sP-selectin, like those of vWF, were significantly higher in ICU than in non-ICU COVID-19 patients, suggesting a more severe endothelial activation-damage, although a contribution of activated platelets cannot be excluded. sTM, instead, was not different in ICU versus non-ICU patients. However, its plasma concentration, like vWF antigen, correlated with clinical outcome and was a predictive marker of mortality in

COVID-19. In another study,<sup>110</sup> ICU COVID-19 patients had significantly and persistently elevated plasma levels of hyaluronic acid and syndecan-1, besides sP-selectin, suggesting degradation of the endothelial glycocalyx with profound consequences on vascular function, such as, for example, a decrease in nitric oxide production that will promote platelet recruitment to vascular endothelium. Moreover, the plasma levels of heparan sulfate and heparanase were significantly higher in COVID-19 patients than in healthy controls, and heparanase activity was associated with disease severity.<sup>111</sup> Of note, LMWH prophylaxis in these patients reduced heparanase activity, which translates into a beneficial effect because heparanase can activate macrophages and induce the secretion of cytokines. Finally, circulating endothelial cells, which are considered reliable markers of endothelial injury, were significantly higher in COVID-19 patients than in healthy subjects at earlier phase of the disease, further supporting the role of endothelial damage in COVID-19.<sup>112</sup>

During SARS-CoV-2 infection, at least two additional mechanisms may contribute to the prothrombotic state. First, COVID-19 patients reportedly have increased plasma levels of angiotensin II (ATII) due to the downregulation of cell surface ACE2 expression that follows the interaction with SARS-CoV-2 S-protein.<sup>52,113</sup> ATII contributes to the clinical and pathological manifestations of COVID-19 and, more importantly, it is endowed with proinflammatory and prothrombotic properties, including the capacity to induce TF expression in both monocytes and endothelial cells.<sup>113,114</sup>

Second, a rather common finding in patients with COVID-19 is severe hypoxia. Although not formally tested in the context of SARS-CoV-2 infection, hypoxia, through the release of HIFs (hypoxia-inducible transcription factors), is likely to induce a prothrombotic state by stimulating TF synthesis in monocytes-macrophages and endothelial cells, and by downregulating natural anticoagulants protein S and TFPI.<sup>115, 116</sup>

### **Changes in Physiological Anticoagulant Mechanisms.**

Downregulation of physiological anticoagulant pathways, characterized by a marked reduction in plasma levels of major anticoagulant proteins (antithrombin, protein C and protein S) and by a decreased expression of endothelial anticoagulant properties (heparan sulphate, TM and endothelial protein C receptor), is a common feature of sepsis-associated haemostatic alterations and contributes to widespread thrombosis of the microcirculation seen in this condition.<sup>36,37</sup> In striking contrast, plasma concentration of antithrombin, proteins C and protein S are not significantly altered in patients with COVID-19, even in those with severe

disease.<sup>12,42,43,45</sup> Some investigators reported a slight decrease of these parameters, particularly in non-surviving patients, but the plasma levels rarely dropped below 80% of normal.<sup>22,31,41</sup> The anticoagulant activities of endothelial cells have not been formally investigated in SARS-CoV-2 infection, neither in cell cultures nor at the tissue level. Considering that endothelial cells are major targets of the virus and of the numerous proinflammatory mediators present in severe COVID-19 patients, impairment of endothelial anticoagulant properties can be safely hypothesized in the context of endothelial dysfunction-injury. The reported increase of plasma levels of sTM, heparan sulphate and TFPI in COVID-19 patients (see above) would support this view, although more data are needed to reach definitive conclusions.

**Changes in Fibrinolysis.** Suppression of fibrinolysis is widely recognized as a significant player in the pathophysiology of sepsis-associated DIC, as it contributes to widespread microvascular thrombosis by preventing fibrin removal. It has been attributed mainly to upregulation of plasminogen activator inhibitor-1 (PAI-1) and, more recently, to activation of thrombin activatable fibrinolysis inhibitor (TAFI) by enhanced thrombin formation, a hallmark of sepsis.<sup>35-37</sup> Likewise, impaired fibrinolysis, mainly due to increased PAI-1 levels, seems to play a critical role in viral diseases.<sup>64</sup> Unfortunately, studies evaluating fibrinolysis in COVID-19 are scarce and little information is available so far, both at tissue/cellular and systemic level.

In the lung, the main target of SARS-CoV-2, where microvascular thrombosis and alveolar fibrin deposition are most prominent and substantially contribute to ARDS, the role of fibrinolysis has not been investigated. In ARDS, secondary to other causes, fibrin formation is not adequately counterbalanced by fibrin removal because fibrinolysis is impaired. Measurements in BALF from patients with ARDS or severe pneumonia requiring mechanical ventilation have shown a marked increase in PAI-1, the most powerful fibrinolysis inhibitor, which was associated with mortality in ARDS patients.<sup>77,78</sup> Whether a similar mechanism might operate in COVID-19 patients remains a matter of speculation.<sup>117,118</sup> It should be considered that NETs and their components (histones, DNA, and particularly histone/DNA complexes), abundantly released during COVID-19, modify the fibrin structure by increasing the fiber thickness and stabilize the clot by binding to large fibrin degradation products, thereby exerting an inhibitory effect on fibrinolysis.<sup>119</sup> Clearly, specific investigations with BALF and pulmonary tissue from COVID-19 patients are required to shed light on this important issue.

At systemic level, a decreased fibrinolytic capacity, commonly referred to as fibrinolysis shutdown, has been proposed by several investigators based on global assays

performed with whole blood, such as TEG and ROTEM.<sup>42,120-123</sup> However, the interpretation of these findings is difficult, considering that the ability of standard viscoelastic tests to detect hypofibrinolysis is rather limited. Using a variant of the ROTEM assay, in which blood is challenged with exogenous t-PA, two studies reported a marked resistance to fibrinolysis in severe COVID-19 patients,<sup>42,120</sup> which was more pronounced in ICU COVID-19 patients with thrombosis than in ICU patients without thrombosis.<sup>42</sup> A similar fibrinolytic resistance was shown by Blasi et al.<sup>41</sup> in a small group of COVID-19 patients, using a global assay which measures the t-PA induced lysis time of plasma clots (plasma clot lysis time, CLT), but no difference was seen between patients admitted to ICU and ward. Because all patients received LMWH, which shortens CLT at clinically relevant concentrations, the fibrinolytic defect might have been underestimated in these patients.<sup>41</sup> In a longitudinal study of 21 COVID-19 patients with daily measurements during the ICU stay, reduced fibrinolysis, assessed by another global plasma assay named global fibrinolytic capacity test, was frequently observed.<sup>44</sup>

Data on the main proteins of the fibrinolytic system and on markers of fibrinolysis activation, which might help in better understanding the fibrinolytic state of COVID-19 patients, are rather limited. In general, the studies on plasma fibrinolytic proteins reported increased levels of PAI-1 and, when measured, also of t-PA<sup>41,42,45,59,124,125</sup> with some exceptions.<sup>39,126</sup> Some investigators found that t-PA and/or PAI-1 were significantly higher in ICU than in non-ICU COVID-19 patients,<sup>42,124,125</sup> whereas others found no difference.<sup>41,45,59</sup> White et al.<sup>43</sup> reported significantly increased levels of t-PA, but not of PAI-1, in critical COVID-19 patients. The plasma levels of t-PA/PAI-1 complexes were increased in a series of 147 COVID-19 patients as compared to controls, and patients with thrombotic disease had higher values than those without; moreover, t-PA/PAI-1 complexes were correlated with disease severity and, like D-dimer, were an independent risk factor for death.<sup>127</sup> In the study of Zuo et al.,<sup>124</sup> involving 118 COVID-19 patients, both t-PA and PAI-1 were associated with poor clinical outcomes. Interestingly, plasma samples from COVID-19 patients with high t-PA, when tested with a spontaneous clot lysis assay (i.e., in the absence of exogenous t-PA), exhibited significantly enhanced fibrinolysis compared to samples with low t-PA and samples from healthy controls. According to the authors, there is a subset of COVID-19 patients with exceedingly high levels of t-PA, which is prone to fibrinolysis, suggesting a possible mechanism that explains the enhanced bleeding risk, at least in part, observed in some patients with COVID-19. The role of TAFI activation as a mechanism that impairs fibrinolysis and facilitates fibrin deposition has not been sufficiently

addressed so far, despite the knowledge that COVID-19 is associated with a hypercoagulability state and a consequent increase in thrombin generation. In this respect, only Nougier et al.,<sup>42</sup> in their study on 78 COVID-19 patients, reported not only raised t-PA and PAI-1 but also increased plasma levels of TAFIa/i (a combination of activated TAFI and its inactivated form). The latter, like t-PA and PAI-1, was significantly higher in ICU than in non-ICU patients. Plasma levels of  $\alpha_2$ -antiplasmin were reported to be in the normal range both in ICU and non-ICU patients,<sup>42,45</sup> whereas plasminogen level was normal at admission and slightly, albeit significantly, reduced in ICU patients, suggesting a mild consumption with the progression of the disease.<sup>128</sup>

Concerning the evaluation of fibrinolysis activation, plasma levels of plasmin- $\alpha_2$ -antiplasmin complex (PAP), also called plasmin- $\alpha_2$ -plasmin inhibitor complex (PIC), which reflect ongoing in vivo plasmin generation, were found to be significantly increased in COVID-19 patients.<sup>41,109,127</sup> One study reported that PAP level did not differ between ICU and general ward patients,<sup>41</sup> while another<sup>127</sup> showed that it was higher in patients with thrombosis and correlated with disease severity. Ranucci et al.,<sup>126</sup> in a study of 20 COVID-19 patients, observed increased levels of PAI-2, normally absent from plasma (except in pregnant women), especially in nonsurvivors, but normal t-PA (PAI-1 was not measured); at follow-up, PAI-2 increased further in nonsurvivors reaching a level 6-fold higher than that of survivors, with a significant difference between the two groups. PAP levels were not significantly modified; however, the baseline PAI-2/PAP ratio was significantly higher in nonsurvivors than in survivors. Finally, in another study of 20 critical COVID-19 patients, Tang et al.<sup>129</sup> evaluated the trajectory of fibrinolytic changes and found that PAP levels decreased over time and became significantly lower in nonsurvivors during the late stage in ICU, which coincided with a significant increase of t-PA-PAI-1 complex. The findings were interpreted as a PAI-1-mediated hypofibrinolysis status that might suggest the use of thrombolytic therapy with t-PA.

The mechanisms behind the changes in t-PA and PAI-1 have not been specifically investigated in COVID-19 patients. However, considering the pivotal role of the endothelium in the pathophysiology of SARS-CoV-2 infection, increased t-PA and PAI-1 plasma levels most probably reflect endothelial activation-injury induced by the virus itself and by the numerous inflammatory mediators released in response to the infection. Among the latter, IL-6 seems, once again, one of the most important as suggested by Kang et al.,<sup>125</sup> who showed, albeit in few patients, that treatment with tocilizumab (a monoclonal antibody targeting the IL-6 receptor) decreased PAI-1 and improved the clinical features. Moreover, angiotensin II might also contribute to fibrinolytic changes because it is increased in COVID-

19 patients<sup>130</sup> and is known to induce the expression of PAI-1 in endothelial cells.<sup>131</sup> In ARDS secondary to conditions other than COVID-19, lung macrophages have been reported to be the main cellular source of PAI-1 and, in some studies, also of PAI-2,<sup>77,132</sup> although the contribution of damaged alveolar epithelial cells and activated platelets cannot be overlooked.<sup>77,133</sup> In a preprint study,<sup>67</sup> lung epithelial cells infected in vitro with SARS-CoV-2 showed an upregulation of several fibrinolytic genes, including t-PA, u-PA, the u-PA receptor, and PAI-2. Therefore, while it can be safely concluded that SARS-CoV-2 infection is associated with multifaceted changes of the fibrinolytic system, we need to understand how these changes impact the fibrinolytic process in the lung parenchyma of COVID-19 patient.

**Conclusion and Perspectives.** Over the last months, considerable progress has been made in the understanding of the complex events involved in the pathological derangement of the haemostatic process that leads to macrovascular and/or microvascular thrombosis associated with COVID-19. The extent to which the current knowledge will prove useful for developing new diagnostic and therapeutic tools remains to be established. Although many laboratory tests are frequently altered in COVID-19 patients (e.g., global assays, markers of coagulation activation, and markers of endothelial activation), the diagnosis and monitoring of haemostatic abnormalities are still based on routine assays, such as D-dimer level, prothrombin time, fibrinogen level, and platelet count. Interestingly, measuring D-dimer in combination with IL-6 greatly improves the specificity and sensitivity for early prediction of the severity of COVID-19.<sup>134</sup> A recent study in septic patients reported that D-dimer corrected for thrombin and plasmin generation provides a better picture of the in vivo coagulation-fibrinolysis balance and displays a much higher prognostic value than standard D-dimer.<sup>135</sup> Whether the "corrected" D-dimer may represent a useful prognostic marker in patients with COVID-19 deserves investigation. Some new emerging parameters that could be of clinical utility await further investigation. For instance, elevated plasma levels of complement activation products (C5a and sC5b-9) and markers of NETs (cell-free DNA, MPO-DNA complex, and cit-H3) paralleling the disease severity have been consistently reported in COVID-19 patients.<sup>58,59,86,87,89</sup> Moreover, circulating HMGB-1 is augmented in COVID-19 patients and may have prognostic value.<sup>98,99</sup> These proteins, therefore, might be new sensitive biomarkers of disease progression and useful predictors of outcome in SARS-CoV-2 infection.

Regarding the supportive treatment for haemostatic abnormalities associated with SARS-CoV-2 infection, different strategies can be envisaged in light of insights into the pathogenetic mechanisms underlying macro and

microthrombosis in COVID-19 patients. Considering the high rate of thromboembolic complications among hospitalised patients, prophylactic administration of heparin, particularly LMWH, is recommended for all hospitalised COVID-19 patients in the absence of contraindications. The use of more intense intermediate to therapeutic LMWH or heparin dose, especially in ICU patients, is still a matter of debate.<sup>136</sup> The central role of cytokines, particularly IL-6, in the pathophysiology of SARS-CoV-2 infection and the associated thrombotic complications provides a rationale for using tocilizumab and similar drugs in COVID-19 patients.<sup>137</sup> Several studies have indeed reported beneficial effects of the drug in these patients.<sup>138</sup> Clinical trials on the efficacy and safety of tocilizumab in COVID-19 are underway. Besides cytokines, also the complement system and the inflammatory mediators derived from its activation appear to play a major pathophysiological role in COVID-19 hyperinflammatory response and ensuing thrombotic complications. Therefore, targeting complement at different levels, especially at C5 or C3 level, may represent a therapeutic option.<sup>139,140</sup> Consistent with this notion, blocking C5 with eculizumab (a monoclonal antibody that binds to C5 with high affinity and inhibits its cleavage into C5a and C5b, thus preventing the generation of the terminal complement complex C5b-9) or C3 with AMY101 (a small peptide that binds to C3 and impairs the cleavage of the protein by C3 convertases, thus preventing

propagation and amplification of complement activation) has shown promising results in terms of reduction of inflammatory responses and improvement of clinical conditions, eventually leading to successful disease outcomes. As expected, AMY101 exhibited a broader anti-inflammatory effect.<sup>141</sup> These and other complement inhibiting drugs are being studied in clinical trials.<sup>139</sup> Finally, considering the emerging role of NETs and/or their constituents in dysregulation of haemostasis and consequent micro or macrothrombosis associated with SARS-CoV-2 infection, targeting these compounds at different levels could be a novel therapeutic strategy.<sup>142</sup> Inhibitors of the enzymes PAD4, NADPH-oxidase, and elastase, all of which are involved in NETs formation, are possible candidates for active regulation of NETs. Dismantling of NETs by deoxyribonuclease (DNase) 1 could be another potential treatment of COVID-19. In small series of patients with severe disease, administration of nebulized Dornase alfa, a recombinant human DNase 1, resulted in a significant improvement of pulmonary function and a reduction of inflammatory markers.<sup>143,144</sup> Therefore, appropriately designed clinical trials are required. Since histones, the most toxic NET components, along with HMGB-1, seem to be critical mediators of organ dysfunction and death in COVID-19 patients, an attractive therapeutic approach could be the development of effective histone and HMGB-1 antagonists.

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