Current and Novel Biomarkers of Thrombotic Risk in COVID-19

-A Consensus Document from The International COVID-19 Thrombosis Biomarkers Colloquium

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ABSTRACT

COVID-19 predisposes to thrombotic and thromboembolic events, due to excessive inflammation, endothelial cell activation and injury, platelet activation and hypercoagulability. Patients with COVID-19 display a prothrombotic or thrombophilic state, with elevations in several thrombotic biomarkers, which are related to disease severity and prognosis. While biomarkers of COVID-19-associated coagulopathy (CAC), including high levels of fibrinogen and D-dimer, were recognized early during the pandemic, many new biomarkers of COVID-19 thrombotic risk have emerged. In this review, we aim to delineate the thrombotic signature and present the most up-to-date biomarkers and platforms to assess thrombotic risk, including markers of platelet activation, platelet aggregation, endothelial cell activation or injury, coagulation and fibrinolysis as well as biomarkers of the newly recognized post-vaccine Thrombosis with Thrombocytopenia Syndrome (TTS). Consensus recommendations are then made regarding clinical use of these biomarkers to inform prognosis, assess disease acuity and predict thrombosis and in-hospital mortality. A thorough understanding of these biomarkers may aid risk stratification and prognostication, guide interventions and serve as a platform for future research.

Abbreviations

ACE = angiotensin-converting enzyme

ACT = activated clotting time

ADP = adenosine diphosphate

aPTT = activated partial thromboplastin time

CAC = COVID-19-associated coagulopathy

COVID = coronavirus disease

CRP = C-reactive protein

EV = extracellular vesicles

ICU = intensive care unit

IL = interleukin

LMWH = low-molecular-weight heparin

NETs = neutrophil extracellular traps

PAI = plasminogen activator inhibitor

PF = platelet factor

PT = prothrombin time

TF = tissue factor

t-PA = tissue plasminogen activator

TEG = thromboelastography

TTS = thrombosis with thrombocytopenia syndrome

Tx = thromboxane

UFH = unfractionated heparin

VITT = vaccine-induced thrombotic thrombocytopenia

VTE = venous thromboembolism

vWF = von Willebrand factor

INTRODUCTION

Whilst the conventional coagulation abnormalities in COVID-19 patients mimic other systemic coagulopathies associated with severe infections, such as disseminated intravascular coagulation, COVID-19 has some very distinct features.[1,2]

Deep venous thrombosis and pulmonary embolism are frequently reported, with incidence depending on whether routine (ultrasound) screening is instituted and the severity of the disease, being more prevalent in intensive care unit (ICU) patients.[3–5] In a recent meta-analysis of 49 studies, comprising 18,093 patients, the reported pooled incidence of venous thromboembolism (VTE) was 17.0%, with studies employing systematic screening (28% of studies) reporting a 33% incidence and those relying on clinical diagnosis reporting 9.8% incidence.[6] More recent large randomized controlled trials in hospitalized COVID-19 patients, which did not screen for VTE, have reported lower incidence of VTE at 6-10% when prophylactic and 4-8% when treatment dose anticoagulation was used.[7–9] In ICU patients, systematic reviews suggest a VTE incidence of 28%, although more recent reviews suggest incidences of 19-24% when clinical diagnosis is used and 36-46% when routine screening is employed.[10,11]

Additionally, superficial vein thrombosis and catheter-related thrombosis are frequently reported.[12] Akin to venous thrombosis, arterial thrombosis can affect all organs, with ischaemic stroke, systemic arterial embolism, acute coronary syndrome, limb and mesenteric ischemia reported to occur in 1-5% of patients.[13–17] Furthermore, there is evidence of *in situ* microvascular thrombus formation, particularly in the pulmonary circulation.[18,19]

In addition to disease severity, the occurrence of thrombosis and elevation of coagulation markers are important determinants of prognosis in patients with COVID-19.[20,21] Early in the pandemic, it was recognized that hospitalized patients with COVID-19 frequently exhibit derangements in coagulant biomarkers including fibrinogen, D-dimer, and activated partial thromboplastin time (aPTT), and it was recommended that these be routinely performed in hospitalized patients.[22–24] However, a number of other markers of coagulation have emerged that have helped refine our understanding of the thrombotic signature of COVID-19 (Figure 1). In addition to helping understand the pathophysiological processes underlying the disease and its complications, assessment of these markers, including with more pathway-specific platforms (Figure 2), can identify individuals at higher risk and potentially guide thromboprophylaxis and treatment approaches.

In this Consensus Statement, we describe the thrombotic signature of COVID-19 and present the most up-to-date biomarkers and platforms to assess thrombotic risk, based on the review of the available clinical evidence. We include markers of platelet activation, platelet aggregation, endothelial cell activation or injury, coagulation and fibrinolysis as well as biomarkers of the newly recognized post-vaccine thrombosis with thrombocytopenia syndrome. We review the evidence supporting the use of the available biomarkers in guiding prognosis, including disease acuity and in predicting thrombotic risk and in-hospital mortality. We make evidence-based Consensus Statements for the clinical use of these markers in patients with COVID-19 according to the latest available clinical data and expert review (Tables 1 and 2).

Methods for consensus recommendations

The authors conducted a search for literature on biomarkers associated with thrombosis in patients with COVID-19. The evidence-based literature was synthesized using the PubMed, Embase and Cochrane library databases, with no restriction on language. In addition, the reference lists of selected articles were checked for further relevant articles. Case reports, animal studies, comments and author replies were excluded. Since a systematic review of all the literature on potential biomarkers was not logistically feasible, we aimed to synthesize studies that identified serologic or cellular markers that corelated with clinical outcomes up to October 2021. Since the literature search yielded a very large number of studies (>100,000), the authors prioritised reporting of larger studies over smaller ones. Systematic reviews and meta-analyses were reported where available and, again, larger cohorts prioritised over smaller preliminary reviews.

All authors reviewed all sections of the manuscript and participated in the Delphi process (conducted virtually) and judged the available tests of thrombosis, leading to consensus recommendations. The Consensus Statements were formulated after review of available evidence synthesized by the experts. Each author participated in the final evaluation of each method and reconsideration of his or her own judgements were incorporated for the Delphi consensus process. There were no disagreements between authors.

This Consensus Statement summarizes the final conclusions and recommendations agreed by the expert panel, based on best available evidence and expert opinion, and are graded according to the Oxford Centre for Evidence-Based Medicine (OCEBM) level of evidence.[25] In particular, the evidence base was evaluated against the diagnostic criteria in the OCEBM

"is this diagnostic or monitoring test accurate?" and the screening criteria "is this (early detection) test worthwhile?" with grading levels 1-5.

Soluble markers of inflammation, coagulation and immune system activation

In a minority of cases, infection with SARS-CoV2 leads to a severe multisystem disease characterized largely inflammatory and thrombotic processes that damage host tissues. The cumulative evidence now suggests that the extent to which specific pathways are triggered determines, or at least predicts, a more severe COVID-19 trajectory. In this regard, several biomarkers associated with specific inflammatory and coagulation pathways have been identified that correlate with, or that predict progression to, severe COVID-19. Here, we review the markers that are relevant to thrombotic risk in COVID-19.

IL-6 and CRP

The cytokine interleukin (IL-)6 is is a pleotropic mediator of inflammation and is a central stimulus in the acute-phase response. Data support a key role for IL-6 in the pathological inflammatory response that drives severe COIVD-19 as high levels are prognostic of more severe outcomes in hospitalized COVID-19 patients[26] and IL-6 receptor blockade improves survival in this group.[27] Thus, IL-6 is a biomarker of severe disease that is also important in pathogenesis. While IL-6 is prognostic of more severe outcomes in COVID-19,[26] it cannot be used to discriminate between COVID-19 and other causes of severe illness (similar to other markers discussed here) as a meta-analysis of 19 studies (1,245 patients) showed that although IL-6 levels were elevated in patients with severe COVID-19, levels were significantly lower than in non-COVID-19-related sepsis and acute respiratory distress syndrome.[28] Overall, the data support IL-6 levels as a predictive biomarker of more severe outcomes in hospitalized COVID-19 but a specific link with the development of thrombosis has not been established.

C-reactive protein (CRP) is a known marker of acute inflammation across many disease states. It is produced in the liver in response to inflammatory cytokines, especially IL-6. Both CRP and IL-6 are increased in COVID-19 patients and levels of both molecules are positively correlated with disease severity and mortality.[28–30] [28]A meta-analysis of 20 studies, including 4,843 COVID-19 patients, noted a 4-fold higher risk of poor outcomes in COVID-19 patients with elevated CRP (pooled-OR: 3.97; 95% CI: 2.89–5.45; $p<0.00001$).[31] In a large analysis of

2,782 patients from a health system in New York City, more than 97% of patients were found to have elevated CRP at presentation, and baseline CRP was associated with progression to critical illness, acute kidney injury, VTE and all-cause mortality.[32] IL-6 measured at hospital presentation was associated with progression to critical illness and with all-cause mortality in patients with CRP above and below the median suggesting additional prognostic information, although the relation to the occurrence of thrombotic events was not specifically examined.[32] Overall, studies show a positive correlation between CRP and COVID-19 severity and, in contrast to IL-6, CRP has been shown to predict risk of thrombosis.[32]

D-dimer

D-dimer is the principal breakdown fragment of fibrin and is used as a biomarker of coagulation and fibrinolysis.[33] In the setting of COVID-19, D-dimer has been widely investigated and reported to be elevated in most patients hospitalized with COVID-19[34] with a peak approximately 5 days into hospitalization, and is higher in subjects with critical illness or those who subsequently died.[29,35,36] An in-depth investigation of D-dimer in 2,377 hospitalized COVID-19 patients found a stepwise association between baseline and peak D-dimer with critical illness, thrombosis, acute kidney injury, and all-cause mortality.[34] D-dimer level could be used to screen for thrombosis, with higher levels of D-dimer found in COVID-19 patients with VTE than in those without, in multiple individual studies and in a systematic review of 47 studies.[37] Another review of 71 studies suggested that D-dimer could be used to identify COVID-19 patients needing CT pulmonary angiogram to diagnose pulmonary embolism, using D-dimer cut-off levels of at least 1000 μg/L.

In a retrospective analysis of outcomes in 195 patients admitted to ICU with COVID-19, in which two of five ICUs managed patients with a protocol-based escalation of anticoagulation based on D-dimer levels, D-dimer-driven anticoagulation was associated with reduced mortality and renal failure.[38] In a prospective study of 803 patients hospitalized with COVID-19, all receiving anticoagulant thromboprophylaxis, a protocolized escalation of anticoagulation dose based upon a combination of illness severity, body weight, and D-dimer was associated with reduced mortality (6.3% vs. 11.8%, $p = 0.02$) and fewer thrombotic events (4.4% vs. 10.7%, $p = 0.002$) compared to patients treated off-protocol.[39] While several trials investigating anticoagulation have used D-dimer in the inclusion criteria,[40,41] the multiplatform trial tested the effect of therapeutic anticoagulation with heparin in 2,219 hospitalized patients with COVID-19 with either high (≥2 times the upper limit of the normal range [ULN]) or low D-dimer (<2 times the ULN) levels and found a benefit in both groups.

However, the probability of superiority of therapeutic-dose heparin over usual-care thromboprophylaxis was 97.3% in the high D-dimer cohort compared to 92.9% in the low D-dimer cohort.[9] In another large open-label randomized controlled trial of 615 hospitalized patients with COVID-19 with D-dimer above the normal range at presentation, therapeutic anticoagulation did not improve clinical outcomes, regardless of the actual level of D-dimer at enrolment.[7]
Overall, a majority of studies support use of D-dimer as a marker of thrombosis.

Coagulation activation markers
More specific information about the coagulation pathways may be obtained with biomarkers reflecting specific protease-inhibitor concentrations or activation peptides. In COVID-19 coagulopathy is thought to be driven by cellular tissue factor expression, which, as a plasma marker, is detectable on extracellular vesicles (see below). The tissue factor-factor VII(a) complex engages the extrinsic coagulation route, resulting directly, or indirectly via factor IX, in factor X activation and subsequent prothrombin conversion to thrombin. The activation of prothrombin yields an F1+2 fragment that can be quantified in blood. The generation of thrombin can be indirectly probed by measuring thrombin-antithrombin complexes (TAT). Both F1+2 and TAT were elevated in patients with acute respiratory distress syndrome (with or without COVID-19) and showed further increases during extracorporeal membrane oxygenation.[42] Elevated F1+2 levels were associated with increased risk of clinical deterioration[43,44] and increased TAT levels persisted despite detectable anti-Xa activity during thromboprophylaxis.[45] Utilizing markers for contact and intrinsic system activation, Busch and colleagues provided evidence that COVID-19 severity-dependent increases in the fraction of elevated kallikrein are linked to increments in markers for downstream activation of factors XI, IX and prothrombin. These changes in contact and intrinsic system coagulation were closely associated with activation of neutrophils and complement.[46] These data support the postulate that contact activation due to persistent NETosis and complement activation may be an important accelerator of thrombo-inflammation in COVID-19[47] and points to potential targets for therapy.[48]

Tests of coagulation

COVID-19-associated coagulopathy (CAC) is characterized by near-normal platelet count

and prothrombin time (PT) in the majority of patients, and uniformly high D-dimer and fibrinogen levels.[49,50] In contrast, disseminated intravascular coagulation, a consumptive coagulopathy, which is only occasionally observed in patients with severe COVID-19, is characterized by decreased platelet count, elevated D-dimer, prolonged prothrombin time and decreased fibrinogen levels,[50] and was mainly only reported at the start of the pandemic. Like in other severe diseases, the occurrence of DIC laboratory features is associated with a poor prognosis in COVID-19.[51]

Prothrombin time

PT, which assesses the extrinsic coagulation pathway, measures the time taken for plasma to clot following the addition of an excess of thromboplastin plus an optimal concentration of calcium. While earlier in the pandemic, it was recommended that PT should be checked routinely to assess for CAC,[23] it is normal or near normal in most patients, with occasional prolonged values seen in those with severe disease.[50,52]

Partial thromboplastin time

The aPTT is usually normal in patients with COVID-19 and is not associated with disease severity.[53] Prolongation of the aPTT may indicate a clotting-factor deficiency, the presence of specific coagulation inhibitors (e.g. antibody to factor VIII), or a laboratory artefact due to an antibody that interferes with phospholipid (e.g. lupus anticoagulant). In patients with COVID-19, artefactual aPTT prolongation has been documented secondary to lupus-like inhibitors or the increased prevalence of heparin resistance due to high fibrinogen and factor VIII levels.[54]

Anti-Xa assay

The anti-Xa assay is used to monitor the effect of treatment with low-molecular-weight heparin (LMWH), unfractionated heparin (UFH), and factor Xa (FXa) inhibitors. The anti-Xa assay better correlates with UFH concentration compared to the activated clotting time (ACT) or aPTT. In this functional assay, citrated platelet-poor plasma is mixed with a known amount of FXa and, depending on the level of Xa inhibition, a clotting-based FX assay or an assay with a specific chromogenic substrate is used to measure the residual FXa levels. The residual FXa level is directly related to the UFH/LMWH concentration. Therefore, whilst not recommended for routine monitoring, assessment of LMWH effect with anti-Xa levels in

patients with severe renal impairment is mentioned in the guidelines,[54] with a recommended target anti-Xa level of 0.3–0.7 IU/mL. A retrospective study in 56 patients showed that personalization of LMWH dose based on the anti-Xa assay was independently associated with a lower risk of COVID-19-related death (OR 0.040 [95%CI 0.002–0.90], p=0.043).[55]

Thrombin generation

Thrombin generation can be assessed using commercial *ex vivo* assays (e.g. Calibrated Automated Thrombogram®, Diagnostica Stago, Parsippany, NJ, USA) and *in vivo* by measuring prothrombin fragment 1 + 2 levels. In the *ex vivo* assays, thrombin generation is probed upon stimulation of plasma with tissue factor; this way the potential to form thrombin is assessed. Since most hospitalized patients with COVID-19 will be on anticoagulant thromboprophylaxis, thrombin generation can be assessed in the presence of polybrene or heparinase as a neutralizing agent.[56] Thrombin generation analysis was further modified for the COVID-19 coagulopathy to enable measurements in plasma containing very high levels of fibrinogen as well as heparins.[57]

Whilst some studies have shown similar levels of thrombin generation between patients with COVID-19 and healthy controls, other have shown elevated thrombin generation in patients with severe COVID-19 despite anticoagulation.[44,58,59] Thrombin peak levels were associated with poor outcome in a retrospective study of 99 patients.[43] In another study of 127 patients with COVID-19, longer lag time and lower endogenous thrombin potential (ETP) were more often associated with DIC;[58] in this study, a D-dimer/ETP ratio indicated a higher risk of major adverse events. Daily follow-up of thrombin generation analysis (in conjunction with viscoelastic tests, see further) may be helpful to tailor antithrombotic medication.[60] One study showed dose-dependent heparin effects on thrombin generation parameters, including ETP and thrombin peak.[61] One study showed persistently enhanced thrombin generation in convalescent patients at a median follow up of 68 days after SARS-CoV2 infection.[62]

Viscoelastic assays (TEG and ROTEM)

In contrast to conventional plasma-based assays that reflect a specific pathway of coagulation, viscoelastic assays such as thromboelastography (TEG, Haemonetics, Boston, MA, USA) and rotational thromboelastometry (ROTEM, Instrumentation Laboratories, Bedford, MA, USA) provide a global assessment of dynamic changes in clot characteristics, from the initiation of clot formation to platelet-fibrin clot generation, stability, and lysis in whole blood. In ROTEM, the tension that is generated following platelet-fibrin clot formation

in a cup is recorded using a pin suspended in the cup. This tension is recorded as "clot strength/amplitude" and displayed on the time vs. amplitude graph (Figure 3). The new point-of-care, cartridge-based TEG6s assay uses microfluidic resonance frequency technology to assess real-time clot characteristics and provides similar clot tensile strength information as ROTEM[63] (Supplementary Table 1).[52] These assays are more sensitive to UFH, LMWH, and direct thrombin inhibitors and less sensitive to warfarin and direct Xa inhibitors. In patients with COVID-19, viscoelastic assays can be used to assess: 1) hypercoagulability indicated by high platelet-fibrin clot strength and/or short reaction time (an indicator of enzymatic coagulation), 2) response to anticoagulants indicated by prolongation of reaction time, 3) high functional fibrinogen levels, 4) high fibrin clot strength, and 5) impaired fibrinolysis. Multiple studies have performed viscoelastic tests in patients with COVID-19, in both prospective and retrospective studies, mainly on the ICU, generally enrolling 20-50 patients. Whilst there is variability in definition and cut-offs, a consistent theme is the detection of hypercoagulability marked by reduced clot formation time and increased fibrin clot strength (demonstrated by increased maximal amplitude or maximal clot firmness), despite anticoagulant prophylaxis.[52,64,65] The latter markers were reported to better discriminate patients with COVID-19 from COVID-19-negative patients with pneumonia than standard COVID-19 biomarkers.[66] In patients monitored with ROTEM during ICU admission, >80% of EXTEM and FIBTEM MCF values remained above the upper reference value up to 6 weeks of follow-up.[67] Upon discharge from ICU, complete normalization of viscoelastic testing at 3 and 6 months, respectively, was shown in limited case series.[68,69] A systematic review that included data on a total of 1,063 hospitalized COVID-19 patients showed that studies using viscoelastic assays, mainly ROTEM, could not demonstrate a retrospective relationship between clot formation characteristics and occurrence of VTE.[64] On the other hand, a systematic review of 15 studies, mainly small observational cohort studies in ICU patients, showed that utilization of TEG can predict thrombotic complications.[70] In a study of 119 hospitalized COVID-19 patients, fibrin clot strength >40 mm measured with TEG was independently associated with a greater than 3-fold increase in the risk of the composite of death and thrombotic events.[66] Based on the measurement of reaction time in the TEG6s assay, an inadequate anticoagulant response was noted in patients treated with enoxaparin compared to heparin prophylaxis.[53,71] These data provide preliminary evidence supporting the use of ROTEM and TEG for diagnostic and prognostic utility, as well as to personalize anticoagulant therapy in patients with COVID-19, although prospective validation is needed (Table 1).

Tests of fibrinolysis

Impaired fibrinolysis is central to the coagulopathy observed with SARS-CoV-2 infections.[72] The balance between coagulation and fibrinolysis is lost in patients with COVID-19. Profound hypercoagulability, which is exacerbated by a significant state of fibrinolysis shutdown, appears to be mediated by overexpression of plasminogen activator inhibitor (PAI)-1 and thrombin-activatable fibrinolysis inhibitor. Despite the hypofibrinolytic state, the overall processes of clot formation and lysis are augmented in SARS-CoV-2, explaining elevated levels of D-dimers, as discussed earlier.[72]

Viscoelastic assays (TEG and ROTEM)

Hypofibrinolysis and fibrinolysis shutdown, based on the estimation of lysis at a fixed time in viscoelastic assays, have been reported in patients with COVID-19 and correlated with the severity of COVID-19 and COVID-19-related adverse outcomes.[73–75] The degree of clot lysis detected at 30 minutes after maximal clot strength is achieved (LY30) is a measure of the efficiency of fibrinolysis. In one study, complete fibrinolysis shutdown, defined as an Ly30 of 0% in the TEG and a D-dimer of > 2.6 ug/mL, was seen in 57% of patients with COVID-19 on the ICU, and was associated with the development of renal failure, VTE, and other thrombotic events, which were seldom observed in patients without fibrinolysis shutdown.[73] In fact, LY30 significantly predicted the occurrence of VTE in COVID-19 patients, with an area under the receiver operating characteristic (ROC) curve (AUC) of 0.742 ($p = 0.022$). Amongst 52 patients with COVID-19 in the ICU, all receiving anticoagulant thromboprophylaxis, a hypercoagulable state with fibrinolysis shutdown was demonstrated in 31% and was related to the occurrence of VTE.[76] In another prospective study of 40 patients with COVID-19 admitted to the ICU, substantially impaired fibrinolysis was detected in all patients, but this was more pronounced in patients with subsequent thromboembolic complications.[77] Furthermore the authors showed that assessment of maximum lysis in the EXTEM test resulted in an AUC of 0.8 [95% CI 0.7–0.9] for the prediction of thromboembolic events ($p = 0.001$), while assessing maximum lysis in INTEM resulted in an AUC of 0.79 [95% CI 0.6–0.9] ($p = 0.002$), with the combination of D-dimer and EXTEM maximum lysis resulting in an AUC of 0.92 [95% CI 0.8–1]. Another prospective study showed hypofibrinolysis in all 24 patients who were tested on the ICU.[52] In a retrospective study in 40 ICU patients, testing with ROTEM showed that maximum lysis at 60 minutes

was significantly decreased.[78] Similarly, fibrinolysis shutdown, defined as having EXTEM maximum lysis of <3.5% in ROTEM, was seen in 44% of 25 ICU patients and was associated with a 73% incidence of thrombotic complications.[74] In patients monitored with ROTEM during ICU admission, t-PA-ROTEM, an assay that also probes fibrinolysis showed a persistent hypofibrinolytic pattern.[67] These observations suggest that viscoelastic assays are useful as prognostic methods to detect fibrinolysis shutdown and predict adverse outcome in the patient with COVID-19.

Soluble markers of fibrinolysis

A key mechanism for hypofibrinolysis is the elevation of plasma PAI-1 levels, secondary to release by endothelial cells and activated platelets.[79] Additional mechanisms include elevated plasma levels of thrombin-activatable fibrinolysis inhibitor and protein C inhibitor.[80] This hypofibrinolytic environment is evident despite raised tissue plasminogen activator (t-PA) levels, emphasizing the strength of the anti-fibrinolytic response in SARS-CoV-2.[79]

Two crucial questions arise: do changes in the fibrinolytic system have a prognostic value and can they alter patient management?

An early case-control study demonstrated that PAI-1, t-PA and thrombin-activatable fibrinolysis inhibitor levels are elevated in SARS-CoV-2 infections and has also shown significantly higher plasma levels in those requiring admission to an ICU (n=48) compared with non-ICU patients (n=30).[81] In a larger study of 118 patients, Zuo and colleagues confirmed that elevated plasma PAI-1 levels are associated with more severe disease[82] but the wide range observed made PAI-1 an inaccurate prognostic marker. However, the same group found a possible prognostic value for t-PA levels, although large-scale studies are warranted before concrete conclusions can be made. A systematic review and meta-analysis of 17 small mainly retrospective studies, including 3 which assessed t-PA, 4 which measured PAI-1 and 3 which assessed soluble thrombomodulin, found that elevation of each of these markers was associated with adverse prognosis in COVID-19.[83] A small study (n=50) suggested that soluble thrombomodulin, a complex endothelial-derived anticoagulant and anti-fibrinolytic factor, may predict clinical outcome in COVID-19, with levels above 3.26 ng/ml associated with increased in-hospital mortality [HR 5.9 (95% CI 1.9-18.4)] or longer inpatient stay,[84] and increased levels have also been shown in convalescent patients a median of 68 days following infection.[62] Elevated thrombomodulin levels may be a marker of endothelial dysfunction, a key abnormality in COVID-19 infection and this is further discussed below.

Platelet count and function

Numerous studies have assessed platelet count and markers of platelet reactivity and activation in COVID-19. The potential utility of these is summarized in Table 2.

Platelet count

Many studies have compared platelet count between patients with COVID-19 and healthy controls. Generally, the most severe manifestations of COVID-19 (e.g. ventilated/fatal) were associated with lower platelet counts than seen in milder forms of the disease,[32,85,86] this association being supported by a meta-analysis.[87] A platelet count <100 x10^9/L was much more frequent in those with critical COVID-19 than in those with severe or moderate disease,[88] with serial measurements indicating that the difference in platelet count between non-survivors and survivors increases during hospitalization.[89] The immature platelet fraction has also shown some value in predicting outcomes such as length of hospital stay and ICU admission, including in relatively large cohort studies.[90–92] Mean platelet volume, a biomarker of platelet hyperactivity, was also found to be associated with disease severity and the occurrence of thrombosis in patients with COVID-19 in small studies and in a meta-analysis of 15 studies, with the presence of larger, more immature platelets associated with increased thrombotic risk.[93,94]

Platelet activation

Post-mortem studies have noted a high incidence of platelet-fibrin microthrombi in organs such as the lung, heart, liver and brain.[95–97] Platelet aggregates and granules have been observed in blood films and may indicate severity; in one study, films from all patients with COVID-19 requiring ICU showed aggregates and granules, whereas this was not seen outside the ICU setting.[98] Electron microscopy has demonstrated platelet clumping, membrane fragmentation and degranulation.[99,100]

Upon activation, platelets express P-selectin, which binds/activates leukocytes and is cleaved to a soluble form.[101] In small case control studies of less than 50 patients each, platelet P-selectin expression was consistently significantly higher in COVID-19 patients than in healthy controls,[85,102–105] and in severe COVID-19 compared to mild or asymptomatic disease.[106] Circulating levels of soluble P-selectin were significantly higher in ICU-requiring COVID-19 patients compared to healthy controls, but not higher than that of non-ICU

COVID-19 patients in one study,[84] although higher in ICU than non-ICU patients in another.[107] Another study found soluble P-selectin a reasonable predictor of intubation and death on ROC analysis.[108] In a post-hoc analysis of samples from more than 300 patients with COVID-19, plasma P-selectin level on hospital admission was strongly associated with the subsequent diagnosis of VTE and was independent of disease severity.[109]

Platelet activation also leads to surface expression of CD40L, which is subsequently cleaved to a soluble form, that can stimulate leukocytes and stabilize platelet aggregates. In case control studies of 50-100 patients, circulating soluble CD40L was significantly increased in COVID-19 patients compared to healthy controls.[84,107,110] Analysis of 100 hospitalized patients with COVID-19 found soluble CD40L to be independently associated with the composite of thrombosis or death, but not with thrombosis alone,[94] whilst another cross-sectional study of 68 patients showed no difference in CD40L in relation to disease severity.[84] Higher levels of platelet cytosolic calcium and phosphatidylserine externalization have also been associated with ICU vs. non-ICU COVID-19 patients or healthy controls.[111] A very small study showed greater platelet expression of glycoprotein Ib and IX in eight COVID-19 patients compared to healthy controls.[112] A small study of 36 patients indicated that markers of platelet activation return to normal 2-3 months after COVID-19.[102]

Levels of urinary 11-dehydoxy-thromboxane (Tx)B_2, a marker of TxA_2 generation that occurs upon platelet activation, are higher in patients with COVID-19 than in patients with non-COVID-19 pneumonia, and in COVID-19 complicated by adverse events compared to those with less severe disease, in studies of more than 100 patients.[71,113] Similarly, serum TxB_2 was significantly elevated in patients with severe disease compared to those with mild or asymptomatic COVID-19 or compared to healthy volunteers in one study,[106] though not another.[110] A retrospective assessment showed that raised serum TxB_2 levels were independently associated with the occurrence of thrombosis in 100 patients hospitalized with COVID-19.[94]

The virus and antibody to SARS-CoV-2 may also have a direct role in platelet activation. A recent study has shown that immune complexes containing recombinant SARS-CoV-2 spike protein and anti-spike IgG, were able to enhance platelet-mediated thrombosis on vWF, especially in response to high shear, when the glycosylation of the Fc domain was modified to correspond with the aberrant glycosylation previously identified in patients with severe COVID-19.[114]

It has been generally accepted that SARS-CoV-2 requires host cell expression of angiotensin-converting enzyme (ACE) 2 to facilitate entry and transmembrane protease, serine 2 (TMPRSS2) to prime the spike protein.[115] A number of studies have failed to demonstrate evidence of ACE2 and TMPRSS2 expression in platelets.[103,110,116] Conversely, another group observed 'robust' expression of both proteins,[117] also demonstrating that binding of SARS-CoV-2 to platelets enhanced a range of processes relating to platelet activation/aggregation. Further, SARS-CoV-2-associated mRNA was detected in the platelets of 2 of 25 COVID-19 patients, suggesting that platelets may allow virus entry independent of ACE2.[103] A recent study analysing platelet RNA showed presence of fragmented SARS-CoV-2 viral genome in the platelets of all tested COVID-19 patients.[118] Furthermore, platelets were shown to rapidly internalize SARS-CoV-2 virions, regardless of the presence of ACE2, leading to programmed cell death and a release of extracellular vesicles.[118] Most recently, active virus uptake has been demonstrated in megakaryocytes.[119]

Platelet aggregation

There is significant heterogeneity in studies reporting on platelet aggregation in patients with COVID-19. In a study of 54 patients, of whom ~10% of patients received aspirin and none received a $P2Y_{12}$ inhibitor, adenosine diphosphate (ADP)-induced platelet aggregation, as assessed by light transmittance aggregometry, was enhanced in ICU versus non-ICU patients, whereas no difference was noted between non-ICU patients and healthy controls.[107] In another study, in which 41 patients received no antiplatelet therapy, platelet aggregation in response to a range of agonists was significantly increased compared with healthy volunteers, and most marked in those patients on ICU.[103] A further study has shown greater responses in those hospitalized with COVID-19 than healthy controls.[119] It is unclear whether any patients in these studies were receiving unfractionated heparin, which can potentially confound findings by increasing platelet aggregation.[120] In contrast, another small study utilizing multiplate electrode aggregometry (MEA) with ADP, arachidonic acid (AA) and thrombin-receptor-activating peptide (TRAP) as agonists, showed no difference in aggregation between 27 ventilated COVID-19 patients and 12 healthy controls; although ADP-induced aggregation was lower in COVID-19 patients, this difference was no longer significant after adjusting for gender.[121] Antiplatelet therapy was not fully described in this study, making interpretation of the results problematic. Another study showed significantly lower platelet reactivity in patients with COVID-19 compared to controls when assessed by MEA using AA

and TRAP, but not ADP, as agonists.[122] A study of TEG platelet mapping in 24 hospitalized COVID-19 patients showed ADP-induced responses in the high-normal range, which was not related to disease severity.[123] Another study in which 100 hospitalized COVID-19 patients had TEG platelet mapping on admission showed that platelet hyper-reactivity was associated thrombotic/ischaemic complications and antiplatelet treatment guided by TEG platelet mapping was associated with a significant reduction in mortality,[124] although this was a non-randomised protocol. Assessment of 20 hospitalized COVID-19 patients not treated with antiplatelet therapy with the Platelet Function Analyser 100, showed mean platelet aggregation values in the normal range, similar to that of healthy controls.[104] A small study using the Total Thrombus formation Analysis System (T-TAS) in hospitalized patients demonstrated impaired platelet thrombus formation in the early phase of COVID-19 compared to later stages and to healthy controls, independent of illness severity.[125]

Platelet-omics (transcriptomics, proteomics and metabolomics)

A study of the platelet transcriptome of hospitalized COVID-19 patients found significant differences in mRNA expression of 3,325 genes, including many relevant to protein ubiquitination, antigen presentation and mitochondrial dysfunction, compared to healthy controls.[103] Notably, there was significant upregulation of interferon-induced transmembrane protein 3, which has an important role in limiting viral infectivity. Another study of serum proteomics and metabolomics in patients with severe COVID-19 showed broad differences compared to controls, including a striking downregulation of 15 out of 17 proteins involved in platelet degranulation, particularly chemokines pro-platelet basic protein and platelet factor 4.[126] A retrospective study of 3,915 patients with COVID-19 demonstrated a distinct transcriptomic profile characteristic of prothrombotic large and immature platelets, and further showed that the interaction of SARS-CoV-2 with megakaryocytes alters the platelet transcriptome, and its effects are distinct from the coronavirus responsible for the common cold.[119]

Platelet-leukocyte interactions

Platelet-monocyte and platelet-neutrophil aggregates are elevated in blood from patients with COVID-19,[104] especially in those that require hospitalization[127] with the highest levels observed in patients on the ICU,[106] indicating that platelet-leukocyte aggregates may serve as a surrogate for severity of illness in COVID-19. Plasma P-selectin levels in patients predicted subsequent venous thromboembolic events independently of COVID disease severity.[128]

Moreover, platelet engulfment by white blood cells has been detected in blood from patients with COVID-19.[104] In individuals with COVID-19, platelet adhesion to monocytes is associated with monocyte tissue factor (TF) expression, and platelets from individuals with COVID-19 trigger TF expression by normal monocytes. Monocyte-TF expression correlates with D-dimer levels in patients with COVID-19. Together these observations support a role for platelet-induced TF expression in COVID-19 thrombosis.

Endothelial cell activation and injury

Endothelial dysfunction, in particular the consequent effects on coagulation and angiogenesis, appears to be a critical mediator of pathogenesis in COVID-19.[129] In patients with severe COVID-19, there is a relationship between the extent of endotheliopathy and the magnitude of the immune inflammatory response, as well as the extent of organ dysfunction, even after adjustment for other risk factors such as age, sex, and body mass index.[130] The addition of plasma from critically-ill patients with COVID-19 to cultured pulmonary microvascular endothelial cells *in vitro* was shown to trigger endothelial damage[131] suggestive of immune-mediated endotheliopathy. Incubation of platelet releasates isolated from patients with COVID-19 with microvascular endothelial cells induced an inflammatory hypercoagulable endotheliopathy demonstrating an underappreciated role of platelets in the pathogenesis of COVID-19-induced endothelial activation, that was not seen in control platelet releasates.[132] ACE2 is highly expressed on endothelial cells,[133] and more so in COVID-19 patients compared to influenza and control groups. Although ACE2 has been considered essential for entry of SARS-CoV-2 into host cells, further research will evidence whether direct endothelial infection is the most common trigger of activation or whether paracrine pathways are the dominant driver of endothelial cell inflammation.[134]
In our earlier section on "Soluble markers of fibrinolysis", we have already discussed the roles of t-PA, PAI-1 and thrombomodulin, which are also markers of endothelial dysfunction, and discuss von Willebrand factor below.

Von Willebrand Factor
Von Willebrand Factor (vWF) is a large glycoprotein ranging in size from 600,000 to 20 million Daltons, synthesized by megakaryocytes and vascular endothelial cells, that participates actively in platelet adhesion to injured, disrupted, or activated vascular endothelial cells, high-shear stress associated platelet aggregation, inflammation and immune

activation.[135] Plasma concentrations of vWF are primarily reflective of its synthesis and release from vascular endothelial cells following activation or injury.[136]
Other stimuli for vWF release include hypoxemia, angiogenesis, inflammation and tissue injury. The greater the stimulus for virus-induced cytotoxicity, proinflammatory cytokine production, cell-free nucleic acid-associated cellular injury and impaired vWF clearance mechanisms, the higher the level measured in peripheral blood.[137] Fluid shear stress dynamically regulates vWF by promoting aggregation of multiple vWF units, while at the same time reducing multimer size through force-dependent cleavage by ADAMTS13.[138]
VWF is a common factor within three pathophysiological themes for the acute phase of COVID-19: immuno-inflammation, vasculopathy and coagulopathy. Several groups have reported increased vWF antigen levels, heightened vWF activity, including increased collagen binding, and increased vWF antigen to ADAMTS13 ratio among patients with COVID-19.[84,131] In a single centre cross-sectional study of 68 COVID-19 patients, of whom 48 were on the ICU, vWF antigens levels were markedly elevated, and higher in those on ICU, and were found to correlate with mortality.[84] A relative reduction in ADAMTS13 activity for the level of vWF antigen suggests a processing abnormality that could contribute to altered vWF multimer profiles and a heightened state of thrombosis potential.[139–141] In small cross-sectional studies of hospitalized patients with COVID-19, an elevated VWF antigen to ADAMTS13 activity ratio was strongly associated with disease severity and complications.[142,143] Whilst many investigators in cross sectional cohort studies have observed a direct correlation between vWF and disease acuity, morbidity and in-hospital mortality,[86,140,144–146] there are sparse data to support the usefulness of vWF markers as diagnostic or prognostic markers of thrombotic events.[146] While routine measurement at the time of hospitalization in patients with moderate-to-severe COVID-19 is not recommended, future clinical trials with vWF inhibitors should be considered and, with them, measurements will be needed.[147,148]

Other markers of endothelial injury and pro-angiogenesis

In addition to the effects mediated by vWF, endothelial cell dysfunction induces hypercoagulation through PAI-1, soluble thrombomodulin (an integral membrane-bound glycoprotein expressed at the surface of endothelial cells), and TF pathway inhibitor. Furthermore, elevated levels of tissue-type plasminogen activator (t-PA), the chief pro-fibrinolytic serine protease, found on endothelial cells, its principal inhibitor PAI-1, and

soluble thrombomodulin have been associated with adverse outcomes in COVID-19 patients, including respiratory failure, multiorgan failure and death.[83,84,130]

Furthermore, increased expression of pro-angiogenic factors including vascular endothelial growth factor, hypoxia-inducible factor 1 α, IL-6, TNF receptor super family 1A and 12, have been found in both serum and the lung biopsies from COVID-19 patients.[129,130,149] Moreover, hypercytokinaemia has been documented in patients with COVID-19, manifesting in raised levels of IL-1β,-7,-9,-10, and TNF-α.[149] In particular, elevated levels of IL-6 and TNF-α are a marker of adverse prognosis,[150] and likely contribute to endothelial dysfunction in COVID-19.[151]

Extracellular Vesicles

Released by blood, vascular and other cells, extracellular vesicles (EV) circulate in blood where they may serve many functions. Inside the lipid bilayer, the particles can contain proteins, metabolites and nucleic acids that are being eliminated, recycled or serve as signalling molecules. Additionally, the lipid bilayer can contain membrane-associated proteins, including adhesion receptors like integrins and selectins, and EVs may dock to and deliver content to tissues. Pro-coagulant EVs, largely produced by endothelial cells and monocytes, express phosphatidyl serine, TF, and other coagulation factors. Classic activation of the complement system, which may be stimulated by SARS-CoV-2 antibodies or other factors, can trigger the release of EVs.[152 153] Once formed, EVs theoretically could induce a number of cellular events.[154] In 100 hospitalized COVID-19 patients, EV-TF activity correlated with disease severity and thrombosis, and D-dimer levels[154] as well with PT, fibrinogen levels, plasmin-antiplasmin complexes, vWF, ADAMTS13, circulating leukocytes and inflammatory markers.[155] An observational study of 111 hospitalized patients showed much higher EV-TF activity in severe compared to moderate COVID-19 and EV-TF levels strongly correlated with the occurrence of clinical thromboembolic events, with AUC of 0.851 ($p< 0.0001$).[155] Another observational study of 84 COVID-19 patients showed EV-TF activity to be markedly higher in the 5 patients with arterial or venous thrombosis than those without.[156]

Both mean particle size and volume of EVs are higher in patients with more severe COVID-19,[157] and the EVs are enriched in pro-thrombotic factors (TF, t-PA, vWF), TNF superfamily and IL-6 family members. Platelet-derived EVs are also higher in patients requiring

hospitalization.[110,158] Importantly, there is no evidence from retrospective analysis that EV levels are altered in patients receiving anticoagulant or corticosteroid therapy.[154]
While studies of EV may eventually shed lights on the pathogenesis of COVID, methods for EV are not automated yet making routine measurements in patients challenging at this time.

Novel soluble biomarkers

Neutrophil extracellular traps
COVID-19 is characterized by a high prevalence of immunothrombotic complications.[11,18,159] Neutrophil extracellular traps (NETs) were first recognized as a pro-thrombotic scaffold in 2010, consisting of neutrophil-derived chromatin associated with pro-coagulant proteins and anti-microbial proteins such as myeloperoxidase (MPO) or neutrophil elastase (NE).[160,161] Ten years later, NET components were found abundantly in plasma, serum and post-mortem specimens from patients with COVID-19.[162,163] The detection of NETosis in severe COVID-19 not only opened diagnostic and prognostic possibilities (with NET biomarkers), but also helped to define new therapeutic targets.
Autopsy specimens from patients with lethal COVID-19 have revealed NET-containing microthrombi in many cases.[164–168] Histopathology of lungs and other organs consistently showed microvascular obstruction by aggregated NETs, associated with endothelial cell disruption. A prospective autopsy cohort study revealed neutrophilic plugs in 10 out of 21 patients.[95] Clinically, thromboembolic events had been diagnosed in 48% of these patients. NETs were often co-localized with platelets and detected in lungs, heart, kidneys, liver, spleen, and brain (median disease course 22 days). NETs were subsequently found in approximately half of autopsy cases, suggesting that NETosis contributes to severe COVID-19.
Increased plasma or serum levels of cell-free DNA, MPO/DNA complexes, NE/DNA complexes and citrullinated histone 3 (H3Cit) have been found in a number of inflammatory disorders and, although their diagnostic value may therefore be limited, these NET markers may reflect disease severity in COVID-19. Interestingly, compared to MPO/DNA or NE/DNA, both cell-free DNA and H3Cit correlated more strongly with disease severity.[167] NET markers should preferably be measured in plasma rather than in serum, because neutrophils release NETs during clotting of the blood sample *in vitro*. Accordingly, cell-free DNA levels were consistently higher in serum compared to plasma and showed greater variability.[167]

However, owing to better accessibility, NET markers were also investigated in serum. Cell-free DNA, MPO/DNA and H3Cit were increased two-fold in patients with COVID-19 compared to healthy controls, with concentration relating to disease severity.[162] It is mechanistically interesting that serum from COVID-19 patients induced NET formation in control neutrophils *in vitro*. Cell-free DNA, MPO/DNA and H3Cit were also associated with a higher risk of developing thrombotic events.[162]

In plasma, MPO/DNA and H3Cit strongly correlated with disease severity and were also associated with thrombotic events in most studies.[102,166,168–172] These observational studies enrolled 19-135 patients with COVID-19 at all disease stages. *In vitro*, plasma-derived antibodies from COVID-19 patients induced NET formation in neutrophils isolated from healthy donors. Moreover, NET release could also be induced by direct interaction of SARS-CoV-2 with neutrophils.[168] Cell-free DNA, H3Cit and NE concentrations in plasma were associated with respiratory support requirement and mortality,[170] indicating their prognostic value. In critically ill patients, cell-free DNA showed a rather weak negative correlation with oxygenation parameters, but its decrease over time predicted the number of ventilator-free days.[173] Together, these data show that NETosis is not disease-specific and therefore not useful to assess the presence of SARS-CoV-2 infection, but NET parameters reflect disease severity. Their prognostic value should now be investigated in prognostic studies.

Complement factors (C3, C5, C5a, sC5b-9)

Several complement factors were found to be elevated in the plasma of patients with COVID-19 (C3,[171] C5,[171] C5a,[174–177] sC5b-9[175–178]) and levels showed an association with disease severity. Subsequently, an open-label, non-randomized clinical trial compared the addition of the C5 inhibitor eculizumab to standard of care against standard of care alone in 80 patients with severe COVID-19 on the ICU, and showed significantly improved 15-day survival and oxygenation in those receiving eculizumab.[179] In line with the consistently strong association between plasma complement factors and disease severity, a prospective observation of 134 patients showed increased complement activation in critically ill patients (soluble C5b-9 and C5a were increased in critically ill patients), suggesting valid prognostication.[180] A prospective cohort study of 219 patients found C5a elevation in patients who did not survive COVID-19.[181]

MicroRNAs

An early study found 35 microRNAs to be upregulated and 38 miRNAs to be downregulated in whole blood from COVID-19 patients compared to healthy controls.[182] Hsa-miR-16-2-3P was most promising with a 1.6-fold upregulation. In a larger study of 84 patients with COVID-19, miRNA levels were correlated with disease severity and specific circulating miRNA profiles (e.g. miR-148a-3p, miR-451a and miR-486-5p) appeared to have prognostic predictive value.[183]

Angiotensin-converting enzyme 2

ACE2 gene expression was upregulated in bronchoalveolar lavage fluid some cases of COVID-19,[184] and a case report found increased serum ACE2 levels in a patient with COVID-19 ARDS.[185] In contrast, a study of 85 patients showed no difference in serum ACE2 levels between SARS-CoV-2-positive patients and matched SARS-CoV-2-negative control patients,[186] indicating that serum ACE2 may not be useful as a diagnostic or prognostic tool in COVID-19.

High-mobility group box protein 1

The chromatin protein and transcription regulator high-mobility group box protein (HMGB1) was elevated in serum and plasma of patients with severe COVID-19, and related to adverse prognosis.[187,188] HMGB1 induces the expression of ACE2 in alveolar epithelial cells and inhibition of HMGB1 was shown to block ACE2 expression, indicating that HMGB1 inhibition may be a potential treatment strategy in COVID-19.

Progranulin

Progranulin (PGRN) is predominantly expressed in epithelial cells, neurons and macrophages and promotes inflammation and cell proliferation. PGRN was recently identified in a serum protein expression screen of 85 emergency room all-comers with COVID-19 symptoms and positive versus negative SARS-CoV-2 status.[189] PGRN was upregulated in COVID-19 patients and associated with adverse outcomes, suggesting prognostic value.

Calprotectin

Calprotectin (S100A8/S100A9), a neutrophil cytosolic component, was found to be elevated in serum and plasma from COVID-19 patients.[162,190–193] In these observational studies of up to 172 patients, calprotectin concentration was associated with disease severity[190–193] and thrombotic risk,[162] suggesting that it may be of prognostic value, pending prospective

validation. A retrospective study found that circulating calprotectin levels were significantly higher in 291 hospitalised COVID-19 patients than in SARS-CoV-2 negative diseased controls and among COVID-19 patients, levels were significantly higher in patients who went on to develop thrombosis or critical illness.[132] Following adjustment for age, sex, race/ethnicity, body mass index, diabetes, chronic obstructive pulmonary disease /asthma, history of coronary artery disease or cancer, antiplatelet and anticoagulant therapy, calprotectin levels were independently associated with thrombosis, with those in the highest quartile exhibiting a greater than threefold higher odds of thrombosis.

Novel Urinary Markers

In addition to urinary 11-dehydoxy-TxB_2, patients with COVID-19 who at hospital admission had higher levels of 8-hydroxy-2'-deoxyguanosine, a proposed biomarker of oxidative damage of deoxyribonucleic acid and liver-type fatty acid binding protein, a more accurate indicator of acute kidney injury than serum creatinine, had longer hospitalization, and a higher risk of thrombotic events and death, than those with lower urinary levels of these biomarkers.[113]

Vaccine-Induced Thrombotic Thrombocytopenia and Immune Thrombocytopenic Purpura

Vaccine-induced Thrombotic Thrombocytopenia (VITT), also known as Thrombosis with Thrombocytopenia Syndrome (TTS), has been described as an uncommon complication of COVID vaccination administered 4 to 30 days prior to symptom onset.[194] The diagnosis requires the presence of venous or arterial thrombosis, most often splanchnic or cerebral venous sinus thrombosis, mild-to-severe thrombocytopenia and a positive platelet-factor 4 (PF4) ELISA.

VITT is mostly commonly associated with the ChAdOx1 CoV-19 vaccine (AstraZeneca, University of Oxford) and the Ad26.COV2.S vaccine (Janssen; Johnson & Johnson), both recombinant adenoviral vector vaccines. A recent report suggests that the incidence is 3.6 per million people after the ChAdOx1 CoV-19 vaccine and 0.9 per million people after the Ad26.COV2.S vaccine.[195] A single case of *possible* VITT related to the mRNA-1273 (Moderna) vaccine has been published, although the diagnosis has been contended.[196] VITT

has not been reported with other adenoviral vaccines or the other mRNA-based vaccine, BNT162b2 (Pfizer-BioNTech).

The presentation and pathophysiology is highly similar to heparin-induced thrombocytopenia (HIT) with a similar presence of antibodies binding platelet factor 4 (PF4). These antibodies are immunoglobulin G (IgG) molecules that activate platelets via low-affinity platelet FcγIIa receptors leading to extensive platelet activation and a hypercoagulable state that causes VTE.

We summarize hematological biomarkers associated with the syndrome of VITT including platelet concentration, fibrin split-products including d-dimer and fibrinogen, antibodies to PF4 and whole blood impendence aggregometry.

Patients with VITT commonly have a platelet count in the region of 20 x 10^9/L but a platelet count of <15 x 10^9/L is sufficient to consider a diagnosis of VITT in the presence of clinically-evident arterial or venous thrombosis.[197] Very low platelet counts below 10 x 10^9/L following vaccination suggest an alternative diagnosis of acute immune thrombocytopenic purpura (ITP), another rare post-vaccination complication that presents as bleeding rather than thrombosis.[198] In most patients, D-dimer concentration is often markedly elevated, but fibrinogen concentration may be reduced in some patients, and other coagulation markers such as INR and aPTT tend to be normal. ELISA revealed high levels of antibodies to PF4-polyanion complexes in almost every reported case of VITT, with optical density greater than 2.0 to 3.0. Because the turnaround time of PF4-heparin ELISA assays can be slow, treatment may first be initiated in patients with thrombosis, thrombocytopenia and elevated D-dimer, while awaiting PF4 ELISA results.[198,199] Rapid non-ELISA assays for heparin-induced thrombocytopenia are presently not recommended for diagnosis because of the poor diagnostic accuracy for VITT.[200]

Whole blood impedance aggregometry using patient serum/plasma with donor platelets have also proposed as to diagnose VITT.[201] In contrast to heparin-induced thrombocytopenia, in which platelet aggregation only occurs in the presence of low-concentration heparin and is subsequently attenuated with high-concentration heparin, patients with VITT demonstrate enhanced platelet aggregation in the absence of heparin; the addition of high-concentration heparin also does not attenuate the enhanced platelet aggregation observed in VITT.

DISCUSSION AND FUTURE DIRECTIONS

The pro-thrombotic profile, that is now well recognized as a hallmark of COVID-19, appears to be mediated by excessive inflammation, endothelial activation and injury, platelet activation, impaired or dysfunctional fibrinolysis, immune-related molecular events and yet to be fully characterized systemic hypercoagulability. These processes predispose to thrombotic and thromboembolic events that affect small, medium and large-sized veins and to a lesser degree arteries.

Circulating biomarkers, often referred to as blood biopsies, have been associated with early phase disease acuity and outcomes, including the occurrence of venous thrombosis, the need for ICU or mechanical ventilation, and in-hospital mortality. The role of serial measures and its relation to longer-term outcomes, including post-acute sequelae of SARS-CoV-2 infection, is less clear.

The prognostic potential of D-dimer, platelet count, CRP or IL-6, vWF antigen or vWF antigen:ADAMTS13 ratio measurements is strong and well-aligned with the severity of disease and prognosis. However, the evidence is based predominantly on observational, retrospective studies using tests that were, and still are, readily available in most hospital laboratories.

There are several other biomarkers which require more specialized assays and testing platforms. These include, *inter alia*, markers of platelet activation, hypofibrinolysis, and NETs. These markers have shown potential in a number of small, retrospective studies, and shown to relate to disease severity and prognosis. However, while of potential prognostic usefulness, many of these markers require specialist laboratory expertise and are not easily measurable within a clinically relevant timeframe. Whilst automated point-of-care testing may overcome this limitation, instruments are not in widespread clinical use and data supporting usefulness in guiding prognosis with point-of-care techniques are scarce, although there may be potential to use viscoelastic assays to personalize antithrombotic therapy in the ICU setting.

In addition to the lack of prospective validation, there is lack of clarity with many biomarkers over the optimal cut-off level that defines increased risk, and since these markers have generally been measured at a single timepoint, it is not clear whether dynamic changes could be used to guide prognosis with changing clinical acuity. Furthermore, most of these biomarkers have been measured in isolation, and their additive value in guiding prognosis is unknown. Finally, a very important limitation of grouping together these findings is that data

have been obtained during the first, second and third waves of the disease, during which both the predominant viral strains and the therapeutic approaches, notably anticoagulation and use of anti-inflammatory treatments including dexamethasone and tocilizumab, changed significantly.

A novel avenue is the use of proteomics in the setting of COVID-19, which has enabled identification of large numbers of proteins associated with SARS-CoV-2 pathogenesis and clinical outcomes.[202] Using a machine learning-based pipeline, an early proteomics study from Wuhan, China, found that 11 host proteins and a set of biomarker combinations were able to predict COVID-19 outcomes.[203] These proteins and pathways relate to immune or inflammatory responses, platelet degranulation and coagulation, and metabolism, that likely contribute to pathogenesis. A recent analysis used state-of-the-art proteomics to investigate the infection process between SARS-CoV-2 and its cell targets.[204] Altogether, proteomics is fostering a better understanding of the virus and its pathogenesis and finding additional therapeutic targets.

A major unmet need is understanding the role of biomarkers in determining optimal treatment to include drug(s) selection, dose and duration of administration. Prospective, randomized trials must include blood sampling and targeted assays are needed to answer many lingering questions about routine measurement in clinical practice. We have highlighted those biomarkers that have an established routine role and others where potential is evident. We hope this review will underpin future larger prospective studies to assess the prognostic and diagnostic value of these biomarkers, alone and in combination. Additionally, studies assessing antithrombotic treatments (specifically antiplatelet and anticoagulant strategies) should assess the value of these biomarkers at baseline and over-time to risk-stratify patients who may benefit most from these targeted approaches.

In addition to CAC, the recent recognition of vaccine-induced TTS has highlighted the need to identify individuals at risk of thrombotic events following vaccination. In addition to a heightened index of clinical suspicion, prompt recognition of TTS requires measurement of platelet count, CRP, and PF4 ELISA. Future studies are needed to investigate whether biomarkers predicting thrombotic risk can identify individuals at risk of TTS, not only to enable closer vigilance and possible thromboprophylaxis, but also since booster shots are likely to be needed.

Conclusions

In addition to conventional tests of coagulation, several novel biomarkers and platforms are available to assess the risk of thrombosis and prognosis in patients with COVID-19. Although most have not been sufficiently tested or validated prospectively for adoption into routine clinical practice, there are growing clinical data supporting the use of these tests and markers to aid risk stratification and prognostication. Furthermore, these biomarkers could potentially guide therapy and current data should serve as a platform to underpin future research to enable optimization of treatment to reduce the risk of thrombosis.

Conflicts of interest

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Fig. 1 | **Pathophysiology of SARS-CoV-2-associated coagulopathy**

Following the entry of SARS-CoV-2 into the host cell by binding to the ACE2 receptor, expression, and enzymatic activity of ACE2 are significantly reduced, resulting in increased vascular permeability and TF expression in subendothelial cells, as well as in leukocytes and platelets, triggering coagulation. ACE2 may exert antithrombotic effects, through a number of mechanisms, including the renin-angiotensin pathway, in which Ang I is converted by angiotensin converting enzyme to Ang II, which is then broken down by ACE2 to Ang(1–7). Reduction in ACE2 leads to an increase in Ang II, which stimulates PAI-1 expression in a variety of cells, including smooth muscle cells, endothelial cells, and adipocytes. The increase in PAI-1 results in hypofibrinolysis. Endothelial cell activation/dysfunction results in a generalised inflammatory state, characterized by high levels of inflammatory cytokines, vWF release, and enhanced surface expression of adhesion molecule E-selectin, promoting thrombus formation and leukocyte recruitment. Inflammation is an important driver of thrombosis, through several mechanisms. Inflammatory cytokines and viral specific TLRs induce TF expression, resulting in activation of the coagulation cascade. Platelet activation by TLR results in enhanced platelet reactivity and platelet aggregation. Activation of neutrophils results in release of NETs, resulting in activation of coagulation and serving as a scaffold for the adhesion of platelets, red blood cells and platelet adhesion molecules. Ang: angiotensin; ACE: angiotensin converting enzyme; CAC: covid-associated coagulopathy; CRP: C-reactive protein; DAMPS: Damage-associated molecular patterns; HIF: hypoxia-inducible factor; I-CAM: inter-cellular adhesion molecule; IL: interleukin; MCP: monocyte chemotactic protein; NET: neutrophil extracellular traps; PAI: plasminogen activator inhibitor; RANTES: Regulated on activation, normal T expressed and secreted; TF: tissue factor; TFPI: tissue factor pathway inhibitor; TLR: toll-like receptor; TMPRSS: Transmembrane serine protease

2; TNF: tumour necrosis factor; t-PA: tissue plasminogen activator; V-CAM: vascular cell adhesion molecule; VWF: von Willebrand factor.

Fig. 2 | **Available platforms to assess different pathways contributing to thrombosis risk**

Platforms can be broadly grouped into those that assess coagulation, fibrinolysis, platelet activation and platelet aggregation, with some overlap. aPTT: activated partial thromboplastin time; ELISA: enzyme-linked immunosorbent assay; PAI: plasminogen activator inhibitor; PFA: Platelet Function Analyser; PT: prothrombin time; ROTEM: rotational thromboelastometry; sCD40L: soluble CD40 ligand; TEG: thromboelastography; TFPI: tissue factor pathway inhibitor; TMPRSS: Transmembrane serine protease 2; t-PA: tissue plasminogen activator.

Fig. 3 | **Viscoelastometry tracings**

ROTEM tracing from (A) a healthy individual and (B) a critically-ill patient with COVID-19.

Patient with COVID-19 demonstrates more rapid coagulation as evidenced by shortened clotting time and clot formation time, increased maximal amplitude, greater fibrin clot strength (maximum clot firmness) and complete absence of fibrinolysis ("fibrinolysis shutdown").

X = clotting time, Y = clot formation time, MA = maximum amplitude, Ly30 = lysis achieved in 30 min.

Table 1. Consensus Statements regarding Diagnostic, Prognostic and Management Capabilities of Coagulation Assays and Associated Thrombosis Biomarkers in COVID-19

Abbreviations: DIC: disseminated intravascular coagulation; HC: healthy control; LoE: level of evidence; ICU: intensive care unit; TEG: thromboelastography

Table 2. Consensus Statements regarding Diagnostic, Prognostic and Management Capabilities of Platelet Markers in COVID-19

Abbreviations: DIC: disseminated intravascular coagulation; EV: extracellular vesicles; HC: healthy control; ICU: intensive care unit; LMWH: low molecular weight heparin; LoE: level of evidence; NETs: Neutrophil extracellular traps; PAI-1: plasminogen activator inhibitor; TEG: thromboelastography; t-PA: tissue plasminogen activator; VTE: venous thromboembolism; vWF: von Willebrand factor