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International COVID-19 thrombosis biomarkers colloquium: COVID-19 diagnostic tests

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Hypercoagulability is a hallmark of COVID-19 and is accompanied by microvascular thrombosis, mainly in small pulmonary and renal vessels, and an elevated risk of venous thromboembolism, stroke and myocardial injury [1, 2]. Unlike conventional sepsis, a mortality benefit associated with anticoagulation therapy suggests an important role of hypercoagulability in COVID-19 outcomes [3]. In the absence of robust evidence from randomized clinical trials, physicians are often implementing institutional guidelines for antithrombotic therapy. Various consensus guidelines and recommendations have also been written addressing specific laboratory functional analyses to facilitate clinical decision-making (Table 1) [4–8]. Both prophylactic and therapeutic doses of anticoagulants, depending on the severity of the disease, have been used in an attempt to attenuate the risk of thrombosis [4–8]. Unlike other thrombotic diseases with longer disease progression, COVID-19 has a very rapid progression, reaching peak severity within weeks. Preliminary studies suggest an inadequate effect of prophylactic anticoagulant therapy in a substantial percentage of patients. Therefore, monitoring with coagulation and platelet function tests may optimize antithrombotic therapy management and reduce thrombotic risk during the critical initial course of the disease.

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Coagulation tests

Anti-factor (F)Xa assay

The anti-FXa assay is used to monitor effects of low-molecular-weight heparin (LMWH), unfractionated heparin (UFH), and FXa inhibitor therapy. The anti-FXa assay better correlates with UFH concentration compared to the activated clotting time (ACT) or activated partial thromboplastic time (aPTT). In this functional assay, citrated platelet-poor plasma is mixed with a known amount of FXa, and a clotting-based FX assay with a specific chromogenic substrate is used to measure the residual FXa levels. The residual FXa level is inversely related to the UFH/LMWH concentration.

In patients with COVID-19, LMWH monitoring based on anti-FXa levels is noted in the society guidelines in patients with severe renal impairment but not for routine monitoring (Table 1). This recommendation is based on the artefactual prolongation of aPTT secondary to lupus anticoagulants and the high prevalence of heparin resistance (nearly 80%) in patients with COVID-19 due to elevated levels of fibrinogen and factor VIII [7, 9, 10]. The recommended target for anti-FXa level is 0.3–0.7 IU/mL. The personalization of LMWH doses based on anti-FXa assay has been reported to be independently associated with a lower risk of COVID-19-related deaths (OR = 0.040, 95% CI = 0.002–0.90, p = 0.043) [10].

Prothrombin time and activated partial thromboplastin time

The prothrombin time (PT) assesses the effectiveness of the extrinsic pathway and is indicated by the time required for the plasma to clot after an excess of thromboplastin plus an optimal concentration of ionized calcium has been added. Although PT is recommended in the guidelines to diagnose disseminated intravascular coagulation (DIC), it is normal or near-normal in most patients with COVID-19, with few patients exhibiting prolonged values. aPTT is often normal

Table 1 Major recommendations for coagulation tests

Centers for Disease Control and Prevention (CDC) guidelines [4]	Hospitalized adults with COVID-19 should receive VTE prophylaxis per the standard of care; hematologic and coagulation parameters are commonly measured, although there is insufficient data to recommend for or against using laboratory values to guide management
International Society for Thrombosis and Haemostasis's interim guidance (ISTH-IG) [5]	Monitoring D-dimer, partial thromboplastin time (PTT), platelet count, and fibrinogen levels for all patients who present with COVID-19 as the measurements may be helpful as more aggressive critical care treatment is warranted and experimental therapies should be considered (D-dimer markedly raised three- to fourfold, prothrombin time prolonged, platelet count $< 100 \times 109$, and fibrinogen < 2.0 g/L)
American Society of Hematology (ASH) [6]	Recommends monitoring D-dimer, PTT, platelet count, and fibrinogen Anti-Xa activity assay, not aPTT, is recommended to monitor unfractionated heparin therapy Thromboelastography and rotational thromboelastometry are currently under investigation for COVID associated coagulopathy and should not be used routinely to guide management
American College of Chest Physicians (ACCP) [7]	Insufficient data to guide clinical practice for coagulation tests
American College of Cardiology (ACC) [8]	Regular monitoring of platelet count, prothrombin time, D-dimer, and fibrinogen is important to diagnose worsening coagulopathy

VTE receive venous thromboembolism, *PTT* partial thromboplastin time, *aPTT* activated PTT

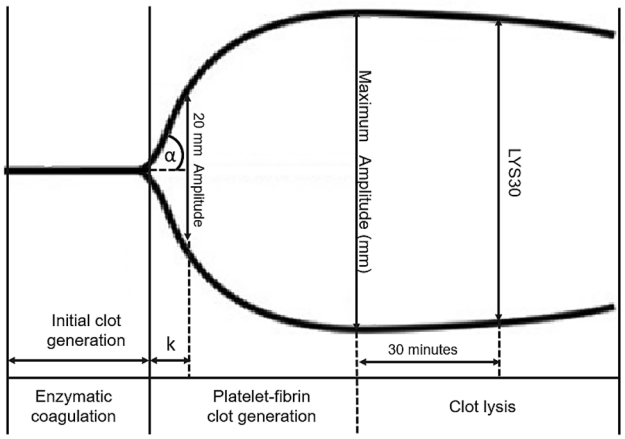

Fig. 1 Thromboelastography/rotational thromboelastometry tracings

in patients with COVID-19 and is not associated with the severity of COVID-19.

Viscoelastic assays

The standard coagulation assays described above assess specific pathways of coagulation. Viscoelastic hemostatic assays, such as thromboelastography (TEG) and rotational thromboelastometry (ROTEM) provide a global assessment of hemostasis. Detailed information on dynamic changes in clot characteristics from the initiation of clot formation to platelet–fibrin clot generation, stability, and lysis is provided (Fig. 1; Table 2). These characteristics can be used to assess the relative contribution of coagulation proteins and platelets to clot formation. They can also be used to estimate hyper- or hypocoagulability and to assess response to antiplatelet or anticoagulant agents [11]. Since kaolin and other intrinsic/contact phase activators are often used to initiate clotting in standard viscoelastic assays, these assays are more sensitive to UFH, LMWH, and direct thrombin inhibitors but less sensitive to warfarin and direct FXa inhibitors [12]. High platelet–fibrin clot strength and short reaction time (an indicator of enzymatic coagulation) despite anticoagulation prophylaxis, high fibrinogen concentrations, and high fibrin clot strength have all been reported in patients with COVID-19 [13–17]. The diagnostic utility of the bedside TEG6s assay has been demonstrated where fibrin clot strength, fibrinogen levels, and platelet–fibrin clot strength were better able to discriminate patients with COVID-19 from patients admitted with pneumonia and high D-dimer levels who were COVID-negative [14]. It was also demonstrated that, compared to patients on enoxaparin prophylaxis (subcutaneous enoxaparin < 80 mg BID) ($n = 50$), COVID-19 patients on heparin prophylaxis (subcutaneous unfractionated heparin ≤ 7500 units TID) ($n = 21$) and therapeutic anticoagulation (intravenous unfractionated heparin or subcutaneous enoxaparin ≥ 80 mg BID) ($n = 17$) exhibited a significantly greater difference in reaction time (0.31 ± 0.8 versus 1.2 ± 1.7 and 1.5 ± 1.4 min, $p < 0.004$ for both comparisons). A higher incidence of poor anticoagulant response (difference in reaction time (ΔR) < 1 min) with enoxaparin prophylaxis compared to heparin prophylaxis and intravenous heparin (84% versus 62% and 53%, $p < 0.05$ for both comparisons) was also observed. Thus, there was an inadequate pharmacodynamic response to anticoagulants in a high percentage of COVID-19 patients, most of whom were African Americans [18].

Table 2 Viscoelastic assay characteristics

Indices		TEG	ROTEM	COVID-19 findings
Clot Initiation: Initial clot generation- Time to develop 2 mm clot amplitude	Enzymatic phase; depends on concentration and function of coagulation factors	R (Reaction time) Normal: 4.6–9.1 min; Citrate/kaolin	CL (clotting time) Normal-INTEM:122–208 s EXTEM:43–82 s	Shorter R-value despite widespread use of anticoagulants
Clot Kinetics: Time to develop 20 mm from 2 mm amplitude	Depends on coagulation factors, fibrinogen, platelets, FVIII activity	K—Normal: 0.8–2.1 min	CFT (clot formation time) Normal-INTEM:45–110 s EXTEM: 48–127 s	–
	Fibrinogen concentration/function	A (angle), Normal: 63–78 degrees	αA (alpha angle) Normal: INTEM:70–81 degrees EXTEM:65–80 degrees	Shorter angle reflecting elevated fibrinogen concentration/function
Clot Strength: Maximum clot strength/firmness	Platelet and fibrinogen function	MA (maximum amplitude) Normal: 52–69 mm	MCF (Maximum clot firmness) Normal-INTEM:51–72 mm EXTEM:52–70 mm	Elevated clot strength
Clot Stability: Lysis (%) at fixed time	Fibrinolytic activity Depends on plasmin/plasminogen and activators	LYS30/LYS60—(lysis at 30/60 min) LYS30 = 0–2.6%	LY30/45/60 (Lysis at 30/45/60 min)	Absence of lysis indicate fibrinolysis shutdown (?)
Fibrinogen Level	Functional fibrinogen	M-FCS): (maximum fibrin clot strength measured in the presence of glycoprotein inhibitor Normal: 15–32 mm FLEV (Functional fibrinogen level) Normal: 278-581 mg/dL	FIBTEM-Normal:7–24 mm	Very high levels of fibrinogen and fibrin-clot strength

In a study of 100 patients with COVID-19, the TEG platelet mapping assay (TEG-PM) results (maximum amplitude-arachidonic acid/adenosine diphosphate > 50 mm) were associated with thrombotic/ischemic complications and TEG-PM assay guided antithrombotic therapy (n = 72) was associated with decreased mortality (p = 0.0002) as compared to patients receiving non-guided therapy (n = 28) [19]. These results provide evidence to support the use of bedside viscoelastic assay in patients with COVID-19, but more evidence is needed to include this assay for routine measurement as a diagnostic and prognostic assay.

Platelet tests

The role of platelets in COVID19 has been less studied than inflammation and coagulation. Platelets are critically involved in vascular homeostasis via specific receptors and granule release that subsequently regulate hemostasis and thrombosis. In addition, platelets also play a role in infection and innate and adaptive immunity [20]. Although bone marrow is the main source of platelets, direct imaging of the lung microcirculation in mice revealed that a large number of megakaryocytes that originated in the bone marrow circulate through the lungs and ultimately are released into circulation. Approximately 50% of total platelet production is attributed to lung contribution [21]. The presence of angiotensin converting enzyme 2 (ACE2) receptors or other pathways of SARS-CoV-2 entry on platelets is controversial with some, for example, finding no mRNA for this receptor in platelets whilst others have reported that binding of SARS-CoV-2 to platelet ACE2 receptors causes platelet activation and associated pro-inflammatory responses [22, 23].

Platelet count

Near normal levels of platelet count are usually observed in COVID-19 that may be due to a balance between increased consumption by microthrombosis and increased production driven by hepatic thrombopoietin release due to cytokine storm. Thrombocytopenia becomes progressively more frequent with increasing severity of disease [23–25]. Liao et al. reported 49% of patients with critical disease having platelet count $< 100 \times 10^9$/L compared with only 9% of patients with moderate disease [24]. COVID-19 patients have been found to have increased mean platelet volume and increased platelet reactivity [22, 23]. In particular, adult non-survivors have been noted to have progressive increase in platelet distribution width coupled with progressive thrombocytopenia whereas these indices may remain normal in survivors [25]. Consequently, determination of platelet count and indices may help with characterising severity of the disease. An

increased incidence of immune thrombocytopenia has also been associated with SARS-CoV-2 infection [26].

Circulating platelet-leukocyte aggregates as well as other markers of in vivo platelet activation point to a contribution of platelets in the inflammatory response in COVID-19 [27, 28]. The potential role of antiplatelet agents such as aspirin and oral $P2Y_{12}$ receptor antagonists is the subject of numerous ongoing clinical studies.

Platelet activation markers

During innate immunity, platelets are activated by pathogens and release inflammatory and bioactive molecules stored in dense and alpha granules that promote inflammation and coagulation. These processes stimulate thrombotic and thromboembolic complications [27–29].

In vivo platelet activation can be determined by studying the activation-dependent platelet surface receptor expression using flow cytometry in whole blood samples or by released platelet activation markers in plasma samples by enzyme-linked immunoassay. Some of the most widely investigated markers in clinical studies are P-selectin and the active conformation of the GPIIb/IIa receptor. In these assays, washed platelets or whole blood samples are incubated with fluorescence-labeled monoclonal antibodies directed against the specific surface receptor and receptor expression is quantified using flow cytometry. In addition, flow cytometry can also be used to quantitate microparticles released by activated platelets and circulating platelet-leukocyte/monocyte aggregates [30]. Flow cytometry is an expensive, time-consuming assay and requires careful collection and handling of blood samples to avoid laboratory artifacts.

Elevated levels of platelet surface expression of P-selectin, soluble P-selectin and circulating microparticles released from platelets has been reported in patients with COVID-19 compared to healthy controls indicate hyperactive platelets [31]. Activated platelets crosstalk with neutrophils and thereby induce the release of neutrophil extracellular traps (NETs) and microparticles that are involved in inflammation and endothelial damage. Moreover, normalization of platelet and neutrophil activation in patients recovered from COVID-9 further indicates their potential role in COVID-19 pathophysiology [31]. It has also been reported that elevated levels of platelet surface expression of P-selectin and CD63 are more evident in patients with severe COVID-19 and strongly associated with D-dimer and lactate dehydrogenase (LDH), but others could not find a similar relation between P-selectin expression and D-dimer [32].

In a study of 54 patients with COVID-19, soluble CD40L but not P-selectin levels were higher in patients with COVID-19, and, by day 14, both CD40L and P-selectin levels decreased but remained higher in patients with severe COVID-19. Both CD40L and P-selectin levels were higher in patients with myocardial injury (37% of patients) and in those who died (30%) [33]. In another study of 100 randomly selected patients with COVID-19, soluble P-selectin, CD40L, serum thromboxane (Tx) B_2 and mean platelet volume were independently associated with the composite of thrombosis or death [34].

The stable urinary metabolite of thromboxane A2 (TxA_2), urinary 11-dehydro thromboxane B_2 (u 11-dh $TxB_{2)}$ has been used as a marker of platelet activation and also response to aspirin therapy. U 11-dh $TxB_{2,}$ in addition to platelet COX-1 activity, also reflects a low-grade inflammatory process of atherosclerosis and activation of inflammatory white cells thus reflecting the whole-body inflammatory state [35]. In a recent study of 120 patients with COVID-19, patients who were on chronic aspirin therapy (mostly 81 mg/day, 29% of patients) had lower u11-dh TxB_2 levels compared to patients not on aspirin therapy ($p = 0.003$). However, an inadequate therapeutic aspirin response (> 1520 pg/mg creatinine) was observed in 91% of COVID-19 patients on 81 mg daily aspirin [18]. The frequency of thromboinflammation (> 4200 pg/mg creatinine) was highest in COVID-19 patients not on aspirin (81%) and lower on 81 mg daily (55%). In addition, compared to COVID-19 patients not on aspirin, patients on 81 mg daily aspirin exhibited a trend for lower whole blood aggregation in response to 5 µg/mL collagen ($p = 0.08$) and 50 µmol/L epinephrine ($5.0 \pm 4.3\%$ versus $6.7 \pm 4.1\%$, $p = 0.1$). With Total thrombus generation assay with platelet chip, COVID-19 patients on 81 mg daily aspirin exhibited lower area under the curve compared to patients not on aspirin ($p = 0.01$). These results suggest that low dose aspirin is insufficient to provide a meaningful clinical effect in the presence of elevated systemic inflammation (cytokine storm) and hypercoagulability [18].

Expression of ACE2, a receptor for SARS-CoV-2, and TMPRSS2, a serine protease for spike protein priming, has been demonstrated on platelets and is associated with platelet activation, platelet aggregation, PAC-1 binding, P-selectin expression, α-granule secretion, dense granule release, platelet spreading, and clot retraction in in vitro experiments using blood samples from patients with COVID-19 [32]. Following internalization, SARS-CoV-2 activates platelets by binding to Toll-like receptor (TLR) present in the platelet endolysosomes [36].

Platelet aggregation

Platelet aggregation in response to various agonists is the gold standard to study platelet reactivity and response to antiplatelet agents in various disease conditions. Although light transmittance aggregometry with platelet-rich plasma is the most widely accepted measure to assess platelet function, due to concerns regarding safety during sample handling, whole blood aggregometry using Chronolog,

Multiplate analyzer, or Aggredyne may be considered. The response to antiplatelet agents can be evaluated with the VerifyNow assay, TEG with platelet Mapping assay, or PFA-100 with collagen/EPI or collagen/ADP cartridges. Moreover, since platelet function assays need to be performed within 2–3 h of blood collection and require experienced laboratory technicians, very few studies have assessed platelet aggregation in patients with COVID-19. Platelet aggregation under arterial flow with α-thrombin as an agonist suggested that platelets might be hyperresponsive in COVID-19 compared to platelets from healthy subjects [33]. It has been reported that platelet aggregation in response to adenosine diphosphate, arachidonic acid, or thrombin-receptor-activating peptide was similar between the patients with COVID-19 and patients with respiratory failure but without COVID-19 and also did not change over 14 days after hospital presentation in COVID-19 patients [33].

Platelet proteomics

Proteomics, the study of various proteins during disease, may provide a "blueprint" of the risk of the disease and underlying mechanistic pathways [37]. Stable isotope-labeled proteomics strategy with TMTpro (16plex) assay, ultra-performance liquid chromatography/tandem mass spectrometry, and immunoassays followed by machine-learning models have been used in proteomics studies. Among 894 proteins and 941 metabolites analyzed in sera from 46 patients with COVID-19 and 53 controls, substantial downregulation of proteins related to platelet degranulation, importantly platelet-expressing chemokines proplatelet basic protein (PPBP; also called macrophage-derived growth factor), and platelet factor 4 (PF4) was found in patients with severe disease [38]. In another study of 22 patients with COVID-19, 11 among 8472 proteins analyzed including acute-phase proteins (APPs), cholesteryl ester transfer protein (CETP), and peptidase inhibitor 16 (PI16) (that are involved in platelet dysfunction/aggregation and activation of coagulation) were associated with COVID-19 [39]. In a cohort of 31 hospitalized patients with COVID-19, 27 potential proteins associated with the complement system, coagulation, and inflammation markers upstream and downstream of interleukin-6 but not PF4 were differentially expressed depending on the severity of COVID-19 [40].

Platelet histopathology studies

Histology and autopsy studies provide further insight into the role of coagulation, complement system, inflammation (cytokine storm), and platelet function in COVID-19. The presence of dense granules and platelet aggregates in blood films suggests heightened platelet reactivity [41]. A review of 40 postmortem examinations found that intravascular fibrin or platelet-rich aggregates in lungs are present in 90% of cases followed by acute lung injury in 73% of cases. These observations suggest the critical role of platelets in COVID-19 [42]. A review of 27 publications involving 226 autopsies and 9 biopsies revealed that the main histopathological findings involve the lung (diffuse alveolar damage and microthrombi/thrombi), heart (lymphocytic myocarditis), kidney (acute tubular injury), central nervous system (microthrombi, ischemic necrosis, acute hemorrhagic infarction, congestion, and vascular edema), and vasculature (deep vein thrombosis) [43]. These results further highlight the potential central role of platelets in COVID-19 (Table 3).

Conclusions

The presence of hypercoagulability has been unequivocally demonstrated in many patients with COVID-19. An inadequate response to guideline-based antithrombotic therapy has also been observed in many patients and the latter phenomenon may be associated with poorer clinical outcomes including, mortality. Preliminary data also suggests a role for platelets in ischemic complications and mortality associated with COVID-19. Assays to assess coagulation and platelet function have been proposed to better discriminate patients with COVID-19 from patients admitted with COVID-19-like symptoms (influenza and high D-dimer levels) at the time of admission, and to assist in early identification of high-risk patients, and most importantly, to guide antithrombotic therapy. In this line, United States, Food and Drug Administration (FDA) issued a guidance to regarding the availability of coagulation systems for measurement of whole blood viscoelastic properties that are used to assess hemostasis in COVID-19 patients [44]. However, more focused studies are needed to establish the precise role of these tests in patients with COVID-19 with respect to diagnostic, prognostic, and management capabilities.

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Table 3 Diagnostic, prognostic and management capabilities of coagulation and platelet assays in COVID-19

Tests	Diagnostic capability	Prognostic Capability	Management Capability
Coagulation Tests			
Anti-FXa assay	No	Personalization of LMWH doses based on anti-FXa levels associated with lower risk of mortality	Preferred assay to monitor LMWH, UFH, FXa inhibitor therapy
Prothrombin Time	Normal or near normal in most patients	No	No
Activated partial prothrombin time (aPTT)	No	No	No
Viscoelastic assays			
Thrombo-elastography/Thromb-elastometry	High platelet–fibrin clot strength, high fibrinogen levels and high fibrinogen clot strength discriminate COVID-19 patients	Insufficient evidence	Preliminary data suggest use of TEG to personalize antiplatelet/antithrombotic therapy to improve outcomes, but more data needed to implement in routine practice
Platelet studies			
Platelet Aggregation	Insufficient evidence	No	No
Platelet proteomics	Insufficient evidence	PF4 found in severe COVID19 patients	No
Platelet histopathology studies	Insufficient evidence	Presence of intravascular fibrin or platelet rich aggregates in lungs in post-mortem studies	No

Declarations

Conflict of interest Dr. Gurbel reports grants and personal fees from Bayer HealthCare LLC, Otitopic Inc, Amgen, Janssen, and US WorldMeds LLC; grants from Instrumentation Laboratory, Haemonetics, Medicure Inc, Idorsia Pharmaceuticals, and Hikari Dx; personal fees from UpToDate; Dr Gurbel is a relator and expert witness in litigation involving clopidogrel; in addition, Dr. Gurbel has two patents, Detection of restenosis risk in patients issued and Assessment of cardiac health and thrombotic risk in a patient. Dr. Tantry reports receiving honoraria from UptoDate. RF Storey reports institutional research grants/support from AstraZeneca, Cytosorbents, GlyCardial Diagnostics and Thromboserin; consultancy fees from Amgen, AstraZeneca, Bayer, Bristol Myers Squibb/Pfizer, Cytosorbents, GlyCardial Diagnostics, Haemonetics, HengRui, Idorsia, PhaseBio, Portola, Sanofi Aventis and Thromboserin; and honoraria from AstraZeneca, Bayer, Bristol Myers Squibb/Pfizer, Intas Pharmaceuticals and Medscape.

Consent to publication All the authors provide consent to publish this article.

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