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RESEARCH ARTICLE

Evaluation of Covid-19-Associated Hypercoagulability with Functional Coagulation Assays and Extracellular Vesicles

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ABSTRACT

Most patients affected by the novel coronavirus SARS-CoV-2 — responsible for the Coronavirus disease, COVID-19 — remain asymptomatic or develop mild symptoms. Only a small percentage of cases develop a severe disease that may lead to a fatal outcome. Since the early reports published in the literature by Wuhan colleagues, we have learned that patients hospitalized for acute COVID-19 infection have different clinical and laboratory pictures of coagulopathy. In particular, a marked increase in blood clotting capacity has been reported in most hospitalized patients – COVID-19-associated coagulopathy, CAC – which in turn increases the risk of developing thrombotic complications. The main pathophysiological mechanisms underlying this hypercoagulable state are inflammation, endothelial damage, hypofibrinolysis and hypoxemia. Traditional coagulation tests fail to fully characterize the nature and severity of COVID-19-associated hypercoagulability (CAH). Hence the need for functional coagulation assays (i.e. tromboelastometry/graphy, thrombin generation, platelet function test) and circulating extracellular vesicles to better understand these peculiar conditions. Moreover, it would be very helpful to use these tests to identify patients at increased risk of developing thrombotic complications or with a worse prognosis as well as to ascertain the effectiveness of the anticoagulant treatment. The aim of our narrative review was to describe the main pathophysiologic mechanisms of CAH and to summarize the current knowledge on functional coagulation assays and extracellular vesicles tests in CAH.

INTRODUCTION

The novel coronavirus SARS-CoV-2 is responsible for the Coronavirus Disease 2019 (COVID-19), which often remains asymptomatic or causes mild symptoms including fever, myalgia, cough, dyspnea and less frequently headache, diarrhea, nausea and vomiting[1]. However, up to 14% of patients require hospitalization in a medical ward (MW) or intensive care unit (ICU) and 5% may develop acute respiratory distress syndrome (ARDS) leading to death[2]. The virus penetrates endothelial cells, lymphocytes and pneumocytes, the main target cells of SARS-CoV-2, by binding to angiotensin converting enzyme (ACE)-2 receptors on their surfaces[3]. COVID-19-associated coagulopathy (CAC) is characterized by an activation of the coagulation resulting in both a hypercoagulable state and hypocoagulability the latter due to markedly increased consumption of platelets and coagulation factors[4-6]. Traditional coagulation assays in patients with CAC show elevated levels of D-dimer (most severe cases), Factor VIII (FVIII) and Von Willebrand Factor (VWF). Prothrombin time (PT) and activated Partial Thromboplastin Time (aPTT) may be slightly prolonged in few cases. Fibrinogen levels may be both normal or increased, due to acute inflammation. Most patients have a normal platelet count, though it may be increased or decreased in others. These peculiar findings allow to differentiate CAC from disseminated intravascular coagulation (DIC)[7]. COVID-19 patients who develop a severe hypercoagulability may experience both venous (e.g. microthrombi, pulmonary embolism) and arterial (e.g. myocardial infarction, stroke) thrombotic complications[8,9]. Recent studies have shown that the risk of venous thromboembolism (VTE) in COVID-19 patients is estimated at up to 30% in MW and up to 70% in ICU vs. 17% and 30%, respectively in non-COVID-19 patients[10-13]. Deep vein thrombosis (DVT) has been reported to be more frequent than pulmonary embolism (PE), which correlates strongly with disease severity and mortality[14]. It was also observed that almost in 60% of patients with DVT the localization of thrombosis was in multiple sites. The VTE risk remains high despite ongoing thromboprophylaxis[15]. The purpose of this review is to summarize the principal pathophysiologic mechanisms of COVID-19-associated hypercoagulability (CAH) and to describe the main findings gleaned from functional coagulation assays (i.e. tromboelastometry/graphy, thrombin generation, platelet function test) and circulating extracellular vesicles.

PATHOPHYSIOLOGIC MECHANISMS OF COVID-19-ASSOCIATED HYPERCOAGULABILITY

Inflammation

SARS-CoV-2 binds to cell surfaces, thus activating inflammatory processes via macrophages, monocytes, neutrophils. The subsequent cytokine storm may cause the most severe disease, by promoting and sustaining the inflammation[16,17]. Several studies have reported increased levels of pro-inflammatory cytokines (e.g. IL-6, TNF-α, Type 1 IFN), as well as reduced IFN-γ that result in the apoptosis of CD4+ lymphocytes[18]. COVID-19 patients with ARDS often show elevated levels of D-dimer, IL-6 and fibrinogen, and have poor prognosis[19]. Pro-inflammatory cytokines, in particular TNF-α, stimulate the expression of tissue factor (TF) on the surface of endothelial and inflammatory cells with up-regulation of the coagulation cascade. The overactivation of the complement system releases C3 and C5 proteins, thus maintaining the inflammation and enhancing a hypercoagulable state. The point of convergence between the complement system and the coagulation cascade may lie in the presence of circulating ultra-large VWF molecules stemming from the reduced activity of the metalloproteinase ADAMTS13 caused by the SARS-CoV-2 infection[20]. It is hypothesized that these ultra-large VWF molecules may provide an additional surface for platelet adhesion and complement activation. In addition, other components of the complement system such as MASP-1 and MASP-2 proteins are involved in the activation of the coagulation cascade by increasing thrombin formation[21]. The inflammation also activates neutrophils which in turn may release neutrophil extracellular traps (NETs) that can directly activate the intrinsic coagulation pathway and promote platelet activation[22].

Endothelial Injury

As mentioned earlier, endothelial cells are an important target of SARS-CoV-2 via ACE-2 receptors. The endothelial cells infected by the virus lose their ability to produce nitric oxide (NO), a potent inhibitor of the activation of inflammatory response, able to prevent platelet and leukocyte adhesion[23]. In addition, the increase in angiotensin II, resulting from ACE-2 receptors depletion, causes vasoconstriction. These events lead to microcirculatory damage in the lungs

and other vascular districts. In particular, the concurrency of several pathological mechanisms – e.g. microcirculatory alterations, alveolar destruction, microthrombi formation, fibrin deposition, etc – is responsible for the severe lung damage observed in COVID-19 patients. Furthermore, hypercoagulability in the pulmonary endothelium may also be caused by a peculiar mechanism involving the decreased expression of anticoagulant proteins such as thrombomodulin and endothelial protein C receptor (EPCR)[24]. Moreover, pro-inflammatory stimuli cause the infected endothelial cells to release important players (e.g. FVIII, VWF, etc) directly involved in the activation of the coagulation cascade and platelet aggregation. Angiopoietin 2 is another molecule that seems to play an important role in the severity of the endothelial injury[25]. Similarly to FVIII and VWF, Angiopoietin 2 is also stored in Weibel-Palade bodies and released upon inflammatory stimuli. Another mechanism that may cause endothelial perturbation leading to hypercoagulability is the degradation of the glycocalyx, a glycoprotein covering cell membranes. It is believed that the glycocalyx acts as a binding surface, via heparan sulfate, for antithrombin which is one of the most important natural coagulation inhibitors[26]. The degradation of the glycocalyx due to SARS-CoV-2 infection leads to an imbalance between pro- and anticoagulant mechanisms resulting in increased endothelial permeability, and excess of circulating thrombin thus culminating with the activation of coagulation and platelets.

Hypofibrinolysis

The physiological role of the fibrinolytic system is to lyse fibrin in blood clots. The main enzyme of the fibrinolytic system is plasminogen which is transformed into plasmin (active enzyme) by two specific activators: the tissue plasminogen activator (tPA) and urokinase (uPA). The plasminogen activator inhibitor (PAI-1) has the task of inhibiting the activity of tPA and uPA and all three work synergistically to ensure a balance between hyper- and hypofibrinolysis. It has been demonstrated that COVID-19 who develop ARDS show significantly increased PAI-1 levels, resulting in hypofibrinolysis[27]. There have also been reports that increased levels of ACE and Angiotensin II may promote the release of PAI-1 from endothelial cells, as observed in some COVID-19 patients who presented hypofibrinolysis. A marked reduction of fibrinolytic activity — defined by some Authors as "fibrinolysis shutdown"[28] — appears to contradict countless previous studies that reported markedly increased D-dimer levels in COVID-19 patients[29]. The increase in D-dimer levels normally denotes an activation of the fibrinolytic system resulting in fibrin degradation, and therefore, we hypothesized that this discrepancy may be attributable to an increase in tissue-level fibrinolytic capacity in the absence of an increase in systemic fibrinolytic capacity[30].

Hypoxemia

Hypoxemia has been extensively reported as one of the main negative prognostic factors in COVID-19 patients, as it has been associated with increased disease severity and higher risk of death. Hypoxemia in COVID-19 patients is mainly caused by lung damage and may be further worsened by the presence of microthrombi in the pulmonary vasculature. Moreover, persistent hypoxemia may lead to hyperviscosity which is a known hypercoagulable and prothrombotic condition in both venous and arterial circulation[31,32]. Finally, low oxygen levels promote the synthesis and release in the blood stream of the hypoxia-inducible factor (HIF), a pro-inflammatory cytokine that may contribute to sustaining hypercoagulability[33].

FUNCTIONAL COAGULATION ASSAYS

Thromboelastometry/graphy

The peculiarity of point-of-care coagulation assays such as rotational thromboelastometry (ROTEM, Werfen) and thromboelastography (TEG System, Haemonetics Corporation) is to globally assess coagulation function and capacity. These apparatuses analyze viscoelastic changes that occurred during whole blood clot formation[34]. Previous studies on ROTEM and TEG have characterized a hypercoagulable state in patients with COVID-19 defined by the following main features (Table 1): *i*) early clot initiation and/or shortened propagation phase; *ii*) increased clot strength (mainly referring to increased fibrinogen); *iii*) severe hypofibrinolysis (or fibrinolysis shutdown)[35-39]. These features were reported, by ours and by several other groups, in patients admitted both in intensive care units and in medical wards[40-43]. Interestingly, it was clear from the very beginning that the coagulation picture described by these tools was not that of consumptive coagulopathy as previously suggested by traditional coagulation tests. Moreover, another salient point that emerged from an article recently published by our

group is that the thromboelastographic profile in patients with COVID-19-related pneumonia is significantly more hypercoagulable as compared to that observed in patients with pneumonia from different etiologies[44]. The hypercoagulable state detected via thromboelastometry/graphy does not appear to predict the risk of developing VTE in patients admitted to MWs or ICUs for acute COVID-19 pneumonia[45-48]. On the contrary, a significant association has been reported between hypofibrinolisis and VTE risk[28,49,50].

Viscoelastic profiles	**Parameters**
Early clot initiation and/or reduced propagation phase	ROTEM: shortened Clotting Time (CT, sec) and/or Clot Formation Time (CFT, sec)
	TEG: shortened Reaction time (R, sec) and/or Clot Kinetic (K, sec)
Increased clot strength (mainly referred to fibrinogen contribution)	ROTEM: increased alfa angle (a) and/or Maximum Clot Firmness (MCF, mm)
	TEG: increased alfa angle (a) and/or Maximum Amplitude (MA, mm)
Severe hypofibrinolysis (or fibrinolysis shutdown)	ROTEM: reduced Maximum Lysis (ML, %), LI30, LI45, LI60
	TEG: reduced LY30 (%)

Table 1. Viscoelastic tests in COVID-19-associated hypercoagulability

Clotting time and Reaction time: the time from the beginning of the coagulation analysis until an increase in amplitude of the thromboelastographic trace of 2 mm; Clot Formation Time and Clot Kinetic: the time elapsed for an increase in amplitude of the thromboelastogram from 2 to 20 mm; Maximum Clot Firmness and Maximum Amplitude: the maximum amplitude in millimeters reached in the thromboelastogram; Maximum Lysis: the maximum percentage of clot lysis; LI30, LI45, LI60 and LY30: the percentage reduction in amplitude 30 min, 45 min, 60 min after maximum amplitude

Thrombin Generation

TG is a global coagulation assay able to measure the overall tendency of plasma to form thrombin[51]. Recent studies have evaluated the possible association between thrombin generation (TG) parameters and the inflammatory/hypercoagulable state in patients with COVID-19 and found a hypercoagulable state before initiating heparin at thromboprophylaxis dose[52]. However, studies by Nougier C et al. and by de la Morena-Barrio ME et al. found similar TG profiles in COVID-19 patients and healthy controls despite thromboprophylaxis[50,53]. The addition of thrombomodulin, a natural anticoagulant released in vivo by damaged endothelial cells, causes a lower reduction of TG in COVID-19 patients than in healthy controls, thus confirming a hypercoagulable state due to SARS-CoV-2 infection.

Platelet function

Several studies have reported an alteration of both platelet count and function in COVID-19 patients[54]. Although platelet count remains normal in most patients, it may nevertheless increase or decrease during the disease. The mechanisms underlying these differing trends in platelet count are for the most part unknown, except that decreased platelet counts may stem from consumptive coagulopathy. As regards platelet function, there is in the literature laboratory data consistent with an increase of platelet activity[55]. Several mechanisms may cause platelet hyperaggregability, among which the most important may be the reduced capability of infected endothelial cells to produce NO and the degradation/derangement of the glycocalyx[56]. Moreover, the endothelial damage caused by SARS-CoV-2 promotes platelet adhesion, activation and aggregation[57]. Platelet hyperaggregability also has a predominant role in the activation of the coagulation cascade via the increased TF expression on activated platelets.

EXTRACELLULAR VESICLES

Extracellular vesicles (EVs) are cell-derived lipid structures formed by shedding of cytoplasmic membranes. EVs bear specific surface markers depending on their origin and have procoagulant properties. Moreover, they may also express TF leading to the activation of the extrinsic coagulation pathway. There is much debate about the role of EVs in the pathophysiology of COVID-19 (Table 2)[58-64]. Our group reported increased levels of EVs derived from activated endothelial cells, platelets, pericytes, leukocytes, and neutrophils expressing ACE-2 receptors and/or TF on their surface, some of which in correlation with disease severity[60]. Phosphatidylserine-exposing EVs, as well as endothelium-derived and leukocyte-derived EVs expressing TF drop significantly over time. In contrast, high levels of platelet-derived EVs (P-Selectin+) and leukocyte-derived EVs (CD-45+) persist long after healing. Moreover, leukocyte-derived EVs expressing TF+ increase during the acute phase of the disease and unlike leukocyte-derived EVs (CD45+), decrease once the healing process is completed, thus indicating that the inflammatory state and cellular activation (including platelet) persists over time, even after the acute phase. Elevated baseline levels of platelet-derived EVs appear to be strongly associated with VTE[62]. Phospholipid-dependent clotting time appears to be correlated with PS+EVs demonstrating the phospholipid-mediated role in clotting activation in this setting.

Study	**Main findings**
Balbi C et al., 2021[59]	• Surface antigen profile of EVs in COVID-19 patients is characterized by a combination of seven surface molecules (CD49e, CD209, CD86, CD133/1, CD69, CD142, and CD20). • Significant association between CD142+ EVs and poor outcome
Campello E et al., 2022[60]	• From baseline to 30-day post-discharge - significant decrease of endothelium-derived EVs - significant increase of platelet- and leukocyte-derived EVs • Significant association between - P-Selectin+ EVs and thrombosis - E-Selectin+ EVs and worsening/death
Cappellano G et al., 2021[61]	• Significantly higher platelet-derived EVs - in SARS-CoV-2 positive vs. negative patients - in SARS-CoV-2 positive patients vs. healthy controls
Guervilly C et al., 2021[62]	• Significant increase of TF+ EVs activity - in patients with severe vs. moderate COVID-19 disease - in patients with vs. without thromboembolic events
Kudryavtsev I et al., 2021[63]	• Significant increase of CD235a+ and CD14+ EVs in patients with moderate COVID-19 infection vs. healthy donors • Significant decrease of CD8+ and CD19+ EVs in COVID-19 patients vs. healthy donors • Significant decrease of CD4+, CD19+, and CD146+ EVs in severe COVID-19 infection vs. healthy donors
Traby L et al., 2021[64]	• Significantly higher platelets, endothelial cells, leukocytes, or neutrophils derived EVs in COVID-19 patients vs. healthy controls • Significantly higher alveolar-macrophages and alveolar-epithelial cells-derived EVs in COVID-19 patients vs. healthy controls

Table 2. Extracellular vesicles in COVID-19-associated hypercoagulability

CONCLUSIONS AND PERSPECTIVES

Studies published in the Literature so far have shown that patients hospitalized for acute COVID-19 present altered traditional coagulation tests that are often non-specific and are not able to thoroughly describe the patient's coagulation profile. Moreover, the use of aPTT and/or Ativated Clotting Time (ACT) for monitoring heparin therapy may be limited due to the acute-phase condition. Hence the need for functional coagulation tests (i.e. thromboelastometry/graphy, thrombin generation, platelet function test) and the measuring of circulating extracellular vesicles which allow to better characterize the marked hypercoagulable state — resulting in increased risk of developing thrombotic complications — in hospitalized COVID-19 patients. Unfortunately, these tests are very expensive and not currently widely available in daily clinical practice as they require devices and expertise (especially thrombin generation and extracellular vesicles) limited to specialized coagulation laboratories. Despite solid evidence that these tests can accurately identify the state of hypercoagulability in hospitalized COVID-19 patients, there is scarce data in the literature as regards their ability to identify patients at increased risk of developing thrombotic complications or with a worse prognosis. Further studies are needed to ascertain the effectiveness of the anticoagulant treatment in relation to the functional tests and the risk of developing thrombotic complications, so as to readjust the dosage if needed. Finally, it would be helpful to create standardized algorithms based on integrated data gathered from traditional coagulation tests as well functional tests and extracellular vesicles towards optimizing the management of COVID-19-related hypercoagulability.

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