Understanding COVID-19-associated coagulopathy

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Abstract | COVID-19-associated coagulopathy (CAC) is a life-threatening complication of SARS-CoV-2 infection. However, the underlying cellular and molecular mechanisms driving this condition are unclear. Evidence supports the concept that CAC involves complex interactions between the innate immune response, the coagulation and fibrinolytic pathways, and the vascular endothelium, resulting in a procoagulant condition. Understanding of the pathogenesis of this condition at the genomic, molecular and cellular levels is needed in order to mitigate thrombosis formation in at-risk patients. In this Perspective, we categorize our current understanding of CAC into three main pathological mechanisms: first, vascular endothelial cell dysfunction; second, a hyper-inflammatory immune response; and last, hypercoagulability. Furthermore, we pose key questions and identify research gaps that need to be addressed to better understand CAC, facilitate improved diagnostics and aid in therapeutic development. Finally, we consider the suitability of different animal models to study CAC.

In the early days of the SARS-CoV-2 pandemic, reports from clinical investigators in Wuhan, China indicated a markedly increased risk of thrombosis among patients with COVID-19 (REF.[1]). This finding was confirmed by subsequent reports and is consistent with the idea that COVID-19-associated coagulopathy (CAC) contributes to the morbidity and mortality of patients infected with SARS-CoV-2 (REFS.[1–11]). CAC can manifest as microthrombi and macrothrombi, resulting in damage to multiple organs, including the lungs, heart, brain and kidneys (FIG. 1). Recognizing the critical risk of CAC to public health, the National Heart, Lung, and Blood Institute (NHLBI) at the National Institutes of Health (NIH) organized a 2-day workshop on CAC in May 2021. The goal was to explore the current understanding of the molecular and cellular mechanisms that trigger CAC, identify the scientific gaps and recommend high-priority research topics.

Compared with diseases caused by other common respiratory viral infections, patients with COVID-19 have a higher frequency and severity of clotting events, which in most cases are associated with elevated plasma levels of D-dimer, C-reactive protein, P-selectin and fibrinogen (see BOX 1 for a glossary of terms)[12–14]. D-dimer is generated by plasmin cleavage of cross-linked fibrin and is therefore a marker of both coagulation events and fibrinolysis. It is notable that elevated D-dimer levels in patients with COVID-19 are accompanied by only occasional prolongation of the prothrombin time and activated partial thromboplastin time[15], together with moderate changes in platelet counts. A recent report from the ACTIV-4B clinical trial suggests that D-dimer levels are elevated in approximately 10% of all patients with COVID-19, indicating a high frequency of coagulopathy[16]. The haematological findings are not consistent with classical disseminated intravascular coagulation[17] but, instead, suggest a different aetiology for CAC that could include perturbations in fibrinolysis[18].

Several lines of evidence indicate that CAC involves complex dysregulated interactions between the inflammatory, immune, coagulation, fibrinolytic, complement and kallikrein–kinin systems[18–22]. This association of the innate immune response with coagulation — particularly in the microvasculature where endothelial cell dysfunction promotes clot formation and inflammation — has been termed 'immunothrombosis'[19,23–25]. It is an evolving concept, suggesting complex relationships between circulating immune cells, vascular endothelium and a host of soluble and membrane-bound factors that regulate coagulation and innate and adaptive immunity. This paradigm is increasingly being incorporated into our understanding of thrombosis in multiple settings, including in cancer, sepsis and acute respiratory distress syndrome[26]. However, in the context of COVID-19, a detailed understanding of the underlying molecular and cellular interactions of each of these systems is still lacking. In this Perspective, we discuss three pathways that are emerging as key pathological mechanisms in CAC: first, vascular endothelial cell dysfunction; second, a hyper-inflammatory immune response; and last, hypercoagulability. Although limited in scope, our aims are to summarize the key ideas raised during the NHLBI workshop, to highlight major gaps in our current knowledge and to provide a road map for future research on CAC.

SARS-CoV-2 and vascular dysfunction

Disruption of the vascular endothelium and antithrombotic function. Under normal conditions, the vascular endothelium provides an antithrombotic surface by expressing molecules that prevent both platelet activation (for example, nitric oxide, prostacyclin and ectonucleotidases) and coagulation (for example, tissue factor pathway inhibitor (TFPI), thrombomodulin and endothelial cell protein C receptor (EPCR)). The vascular endothelium is also lined by a carbohydrate-rich glycocalyx that includes anticoagulant molecules such as heparan sulfate, and serves to protect against pathogen invasion[27]. These finely regulated antithrombotic systems may be disrupted in the setting of bacterial and viral infections, with functional loss of anticoagulant molecules such as TFPI, thrombomodulin and EPCR from the
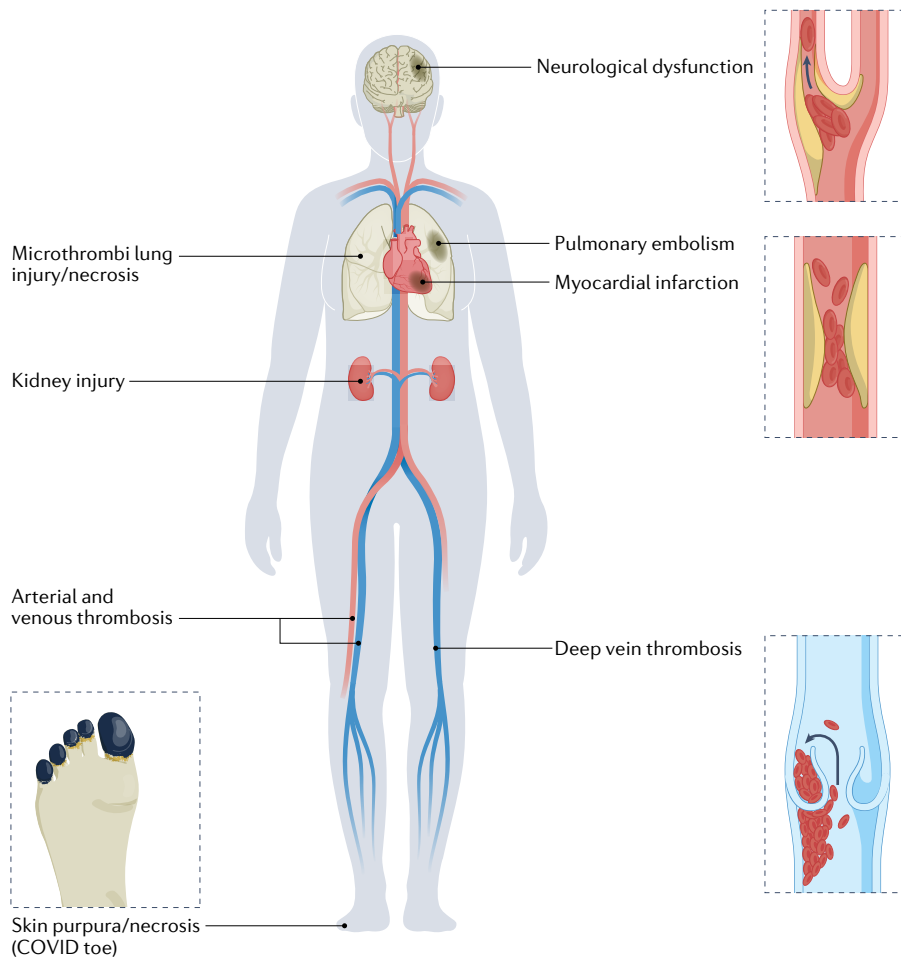
Fig. 1 | **Potential clinical consequences of COVID-19-associated coagulopathy.** COVID-19-associated coagulopathy (CAC) is characterized by coagulation disorders that affect multiple tissue and organ sites, and vary from skin purpura (also known as 'COVID toe') to myocardial infarction and neurological dysfunction. Circulating microthrombi and/or macrothrombi can lead to multi-organ injury or failure.

endothelial surface, heightened expression of procoagulants (most notably tissue factor (TF)) and damage to the glycocalyx. With SARS-CoV-2 infection, dysfunction of the vascular endothelium is thought to be a major contributor to the pathogenesis of COVID-19 vasculopathy[28–30]. In the earliest stages of disease, this occurs primarily in the lungs, where SARS-CoV-2, via angiotensin-converting enzyme 2 (ACE2), infects type II pneumocytes, cells that are in direct apposition to the alveolar vascular network. Resultant damage to the alveoli and the microvascular endothelium features heightened inflammation, infiltration of activated neutrophils, release of neutrophil extracellular traps (NETs), activation of complement and diffuse microvascular thrombosis, all of which contribute to the diminished respiratory function and worsened prognosis[19,31]. It is not yet known whether the endothelial damage is initially caused by direct infection of the endothelium by SARS-CoV-2 (REFS.[20,32]) and/or indirectly as a result of the so-called 'cytokine storm'[33–35], or by other innate, adaptive or autoimmune phenomena. However, as the disease progresses, it is likely that multiple factors contribute to the severity of the endothelial damage, and the extent of thrombosis and organ damage. Delineating the specific triggers and determinants of disease severity are under investigation.

Dysregulation of the kallikrein–kinin system and endothelial cell injury. Although vascular endothelial dysfunction can occur in any severe infection, SARS-CoV-2 has unique interactions with its host cell surface receptor, ACE2, that might induce endothelial cell dysfunction at an early stage of the infection. SARS-CoV-2 binds to ACE2 present on lung epithelial cells[36], triggering endocytosis of the SARS-CoV-2–ACE2 complex by a similar mechanism to that seen with SARS-CoV-1 (REFS.[37,38]). Consequently, ACE2 expression levels on the cell surface fall at the site of the infection, resulting in local ACE2 deficiency.

ACE2 has dual functions, as it regulates both the renin–angiotensin–aldosterone system and the kallikrein–kinin system[39,40]. Kinins are vasoactive peptides that cause relaxation of vascular smooth muscle cells and increased vascular permeability[41,42]. Dysregulated kinin degradation, owing to direct effects of the virus on ACE2, could increase kinin activity leading to angioedema[43]. The kallikrein–kinin system may thus be activated systemically during COVID-19, resulting in a 'kinin storm'[44] leading to increased vascular permeability, inflammation, fluid accumulation and organ damage (FIG. 2).

The markedly elevated D-dimer levels seen in severe COVID-19 (REF.[3]) implicate fibrin deposition and subsequent degradation as playing roles in the pathophysiology of COVID-19. However, the relative contributions of intravascular versus extravascular fibrin formation and lysis to D-dimer are not clear. Plasmin breakdown of this extravascular fibrin and leakage of D-dimer into the bloodstream may contribute to the observed elevated levels of D-dimer in patients with COVID-19. Indeed, during SARS-CoV-2 infection, prothrombotic TF and extracellular vesicles are released from multiple cell types. The predominant source of TF-positive (TF+) extracellular vesicles found in broncho-alveolar lavage fluid (BALF) from patients with COVID-19 is likely activated lung epithelial cells. Although the cellular origin of TF+ extracellular vesicles in the blood is not definitively known, they are probably mainly derived from activated monocytes, with endothelial cells, neutrophils and epithelial cells also contributing[45]. Whatever the source, elevated circulating levels of TF+ extracellular vesicles are associated with increased disease severity, including an increased incidence of venous thromboembolism in patients with COVID-19 (REFS.[46,47]), strongly implicating their involvement in CAC.

SARS-CoV-2 and the blood–brain barrier. In addition to the lungs, clinically important targets of SARS-CoV-2 include the kidneys, liver, heart, skin and central nervous system (CNS), particularly the brain[48–50] (FIG. 1). In serial histological analyses of 100 COVID-19-positive autopsies, 58 brains revealed widespread microthrombi and microinfarcts in the neocortex, and the presence of microthrombi was often associated with

small and patchy infarctions. Moreover, immunohistochemical staining revealed robust expression of the ACE2 receptor in the intraparenchymal blood vessels[51]. Drawing from these observations, it is not surprising that patients infected with SARS-CoV-2 often encompass a wide spectrum of neurological symptoms, including headache, sleep disorders, alterations in sense of smell and taste, ischaemic stroke, major cognitive defects, delirium and diminished levels of consciousness[52–54]. These neurological manifestations of SARS-CoV-2 infections, which are associated with an increased risk of in-hospital mortality[55], are believed to be primarily immunothrombosis-dependent[51], resulting in so-called dysregulated 'neurocoagulation' with excess thrombin generation, alterations in blood–brain barrier endothelial cell function and neuroinflammation[48,56,57] (FIG. 2).

Although somewhat controversial, SARS-CoV-2 may potentially infect brain tissue directly, thereby triggering cellular damage[58]. However, it seems likely that it also affects the brain via indirect mechanisms. In some post-mortem studies of patients with COVID-19, multifocal microvascular endothelial brain injury was detected without any evidence of SARS-CoV-2 in the brain, implicating an indirect effect via host defence mechanisms[50]. By contrast, in another post-mortem series, SARS-CoV-2 RNA was detected in some patients in one or more brain regions, suggesting that the virus might elicit direct neurocytopathic effects[59]. In vitro studies support the latter hypothesis, in that SARS-CoV-2 can directly infect murine and human neurons with accompanying metabolic effects on infected and neighbouring neurons[60]. It remains unclear, however, which brain regions or cell types are most susceptible to either direct or indirect SARS-CoV-2-mediated damage, and how the virus most effectively accesses the brain tissues via the blood–brain barrier.

When expressed by different cells in the CNS, including blood–brain barrier endothelial cells, SARS-CoV-2-derived molecules may trigger neuroinflammatory or behavioural responses via pattern-recognition receptors (PRRs), such as Toll-like receptor 2 (TLR2) and TLR4. For example, the S1 subunit of the SARS-CoV-2 spike protein, when dissociated from the virion, can cross the blood–brain barrier in mice[61]. Even in the absence of viral RNA, the S-protein can be found localized to microvascular endothelial cells, where its S1 subunit can trigger pro-inflammatory cytokine expression and complement

activation that could provoke a thrombotic outcome[62]. In vivo, the S1 subunit can induce abnormal behavioural responses and neuroinflammation, putatively by triggering TLR2-dependent and TLR4-dependent cellular responses[63]. The S-protein is also highly glycosylated, providing a means of modulating the immune response locally and systemically. The main SARS-CoV-2 protease, M[pro], has also been proposed to contribute to CNS pathology, partly by cleaving endothelial cell-expressed NF-κB essential modulator (NEMO), thereby resulting in cellular damage and setting the stage for a hypercoagulable state[64]. Recognizing the heterogeneity of the neurovasculature, and the endothelial cells that form the blood–brain barrier, further investigation is needed to determine which specific regulatory factors are critical in modulating the passage of SARS-CoV-2 and its component proteins into the CNS and their ultimate role in triggering CNS pathology[65].

In summary, there is now substantial evidence that SARS-CoV-2 infection can cause vascular endothelial cell dysfunction and that this underlies the pathology seen in many patients with severe COVID-19. BOX 2 lists some of the major scientific questions that still need to be addressed in this area. In the following section we discuss how hyper-inflammation can contribute to CAC.

Hyper-inflammation and coagulopathy

Neutrophils, NETs and complement.

Neutrophils represent a major cellular component of the innate immune system and are recruited early after viral infection in response to chemotactic factors produced at sites of virus-mediated injury (FIG. 2). In addition to being phagocytic, neutrophils release various proteolytic enzymes,

chemokines, cytokines and reactive oxygen species (ROS). They also contribute significantly during early immune responses to viral infections through the formation of NETs[66]. NETs are formed when neutrophils undergo programmed cell death, referred to as NETosis. Elevated in the blood of patients with COVID-19, and often in conjunction with neutrophilia[67], NETs are web-like structures comprising DNA, histones, microbicidal proteins, oxidant enzymes, coagulants and complement factors[66]. During early activation of innate immunity, NETs can promote clotting, in part via expression of functional TF and by capturing platelets. This has been documented in COVID-19, where increased plasma levels of NETs and TF+ extracellular vesicles correlate with thrombin generation, severity of respiratory status and risk of death[24,67]. Moreover, sera from severely ill patients with COVID-19 can stimulate NET formation in neutrophils from uninfected controls, and can trigger heightened complement activation[25,68]. Indeed, excess complement activation and NETosis are well established in COVID-19 (REF.[69]). Potential relevance to CAC is underscored by reports showing that ex vivo inhibition of complement component C3 disrupts TF expression in neutrophils from patients with COVID-19 (REF.[70]). Also, pilot studies in patients with COVID-19 have reported that anti-C3 and anti-C5 agents dampen neutrophilia, attenuate NET release and reduce inflammation[70].

Post-mortem analyses of tissues from patients with COVID-19 consistently revealed deposition of complement activation fragments in the vasculature of multiple organs together with microvascular and macrovascular endotheliopathy and thrombosis[71]. Clinically, microvascular

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Box 1 | Glossary of terms

- **D-dimer:** the soluble degradation product of cross-linked fibrin; elevated levels indicate ongoing activation of the clotting system
- **Prothrombin time:** a plasma clotting test that reports the integrity of the tissue factor and final common pathways of blood clotting
- **Activated partial thromboplastin time:** a plasma clotting test that reports the integrity of the contact and final common pathways of blood clotting
- **Disseminated intravascular coagulation:** the systemic activation of blood coagulation, commonly resulting in fibrin deposition, microvascular thrombi and consumption of clotting proteins and platelets; can cause multiple organ failure
- **Tissue factor (TF):** the major trigger for coagulation
- **Tissue factor pathway inhibitor (TFPI):** a major negative regulator of coagulation
- **Endothelial protein C receptor (EPCR):** a cofactor for activation of protein C
- **von Willebrand factor (vWF):** a large multimeric, pro-haemostatic factor released by activated endothelial cells and platelets
- **a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13):** a zinc-containing metalloproteinase that cleaves ultra-large vWF multimers
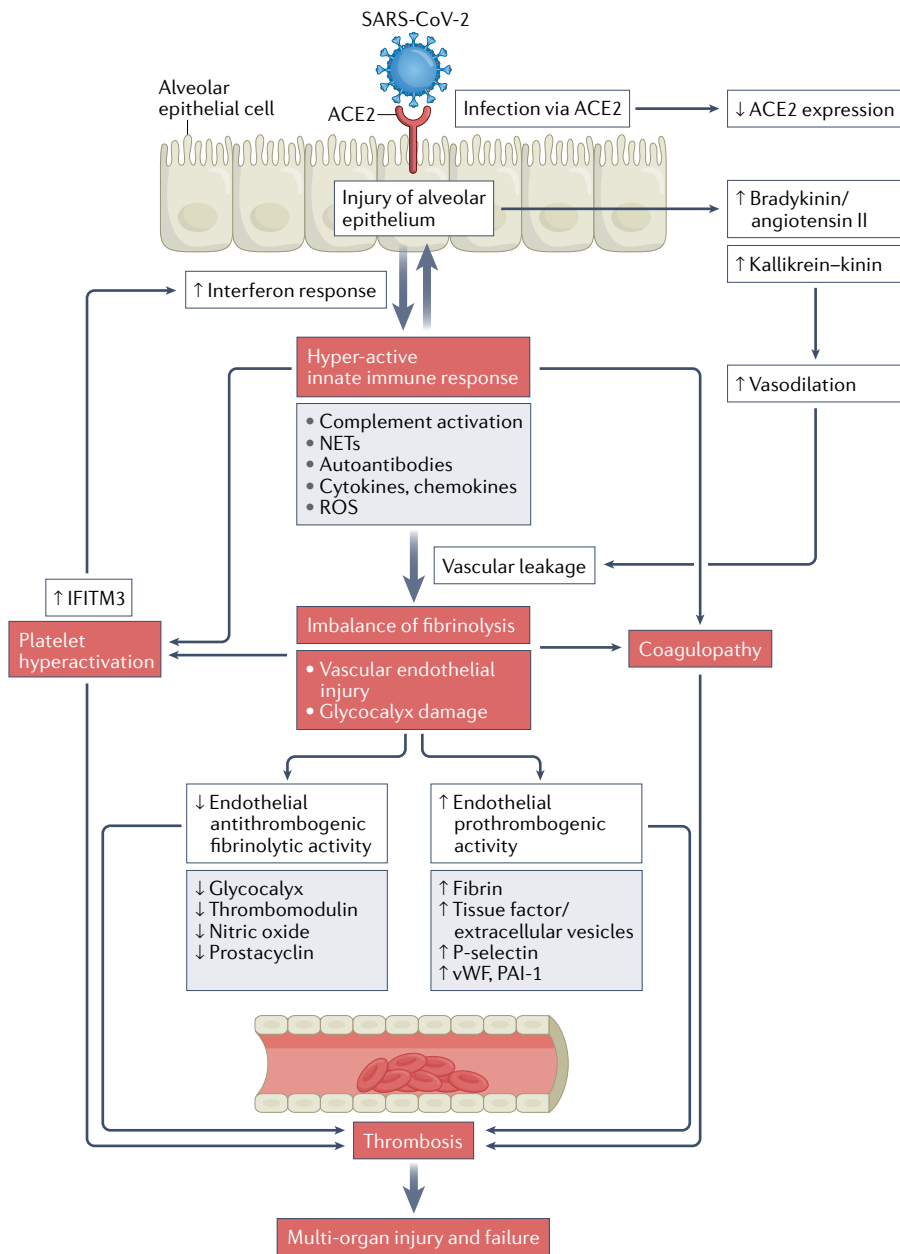

Fig. 2 | **Pathway contributing to COVID-19-associated coagulopathy.** COVID-19-associated coagulopathy (CAC) likely involves the dysregulation of numerous pathways that, in ways that are not currently well understood, culminate in endothelial damage, thrombosis and multi-organ failure. ACE2, angiotensin-converting enzyme 2; autoantibodies, anti-phospholipid-targeting autoantibodies; IFITM3, interferon-induced transmembrane protein 3; NET, neutrophil extracellular trap; PAI-1, plasminogen activator inhibitor 1; ROS, reactive oxygen species; vWF, von Willebrand factor. Adapted with permission from REF. [35], Elsevier.

thrombosis can manifest itself as skin purpura or the condition known as 'COVID toe' (FIG. 1). Serial studies of serum from patients with COVID-19 revealed early and persistent hyperactivation of complement, with increased circulating levels of proteolytic fragments[72–75] that are predictive of poor outcomes. These findings suggest SARS-CoV-2 can trigger complement activation via all three major pathways: first, the classical pathway through formation of complement-fixing IgG and IgM antibody immune complexes[73]; second, the alternative pathway by competing with the negative regulator factor H[76]; and last, the lectin pathway through direct interaction of the viral spike protein with mannose-binding lectin (MBL), ficolin 2 and collectin 11, and by viral nucleocapsid protein-mediated enhancement of MASP2 cleavage of complement component C4 (REFS.[77,78]). SARS-CoV-2 can also induce synthesis and

release of complement factors from infected respiratory epithelial and endothelial cells via Janus kinase 1 (JAK1)-dependent and/or JAK2-dependent pathways, interestingly in parallel to the release of procoagulant factors[79].

In addition to complement-triggered release of TF-carrying NETs, there are multiple other mechanisms by which SARS-CoV-2-induced complement hyperactivation may initiate coagulation[80,81]. For example, cleavage of complement component C5 leads to release of prothrombotic factors from platelets. Also, C5 cleavage can result in TF activation and increased P-selectin expression on endothelial cells, both of which recruit inflammatory leukocytes. COVID-19 complications in seriously ill patients are proposed to involve secondary tissue damage due to a cycle of dysregulated and/or hyperactive coagulation and inflammatory pathways, fuelled by positive feedback loops between complement, neutrophils, NETs and coagulation (FIG. 2). Several clinical trials involving treating patients with COVID-19 with anti-complement drugs have been performed (reviewed in REFS.[19,81]). These have yielded promising results but were confounded by the small numbers of patients recruited and, also, the challenges of treating a dynamically changing disease.

Platelet dysfunction contributing to immunothrombosis. Although thrombocytopenia is common in many viral illnesses, it is not frequently observed in COVID-19 (REFS.[2,82–85]). Nonetheless, heightened platelet activation is often detectable in COVID-19, triggered by multiple factors including soluble coagulation and complement factors (for example, thrombin, C3a and C5a), inflammatory cytokines, anti-SARS-CoV-2 immunoglobulins, changes in shear stress and exposure to activated endothelial cells and neutrophils[86,87]. Through the release of their intracellular granule constituents and externalization of the prothrombotic membranous phosphatidylserine surface, platelets help trigger and sustain an immunothrombotic amplification loop that contributes to CAC[86]. Platelets from patients with COVID-19 exhibit enhanced aggregation, accompanied by increased cell surface P-selectin expression[88–90] (FIG. 2), but with reduced activation of αIIbβ3 integrin[89,91,92]. Patients with COVID-19 also have increased circulating levels of soluble platelet activation markers (for example, CC-chemokine ligand 5 (CCL5; also known as RANTES) and platelet factor 4)[24,88,92,93], as well as increased platelet–neutrophil and platelet–monocyte aggregates[24,89,94]. With

increased vascular endothelial activation, these aggregates may readily adhere to the vessel wall. Adherence of platelet–neutrophil aggregates is likely to result in the release of pro-thrombotic and pro-inflammatory NETs[67,86,95], whereas platelet–monocyte aggregates can promote hypercoagulability via increased monocyte TF expression[94]. Overall, strong evidence points to activated platelets, neutrophils and NETs playing significant and complementary roles in thrombus formation, and as predictors of more severe disease[8,96].

Mechanisms underlying the augmented platelet reactivity in COVID-19 appear to involve increased MAPK signalling and thromboxane A2 production[88,89,97], responses that are likely due to reported SARS-CoV-2-induced changes in the platelet transcriptome[98–100]. RNA-sequencing analyses comparing platelets from patients with COVID-19 with those from healthy controls revealed more than 3,000 differentially expressed genes[89]. By contrast, only a subset of these genes was differentially expressed in the platelets of non-intensive care unit versus intensive care unit-admitted patients with COVID-19 (REF.[89]), indicating that these changes in platelet gene expression are most likely an early response to SARS-CoV-2 infection and not necessarily associated with disease severity.

It is not known whether SARS-CoV-2-induced changes to the platelet transcriptome occur as a result of direct viral infection of platelets, or indirectly secondary to hyper-inflammatory responses to the virus. Initial studies suggested that SARS-CoV-2 mRNA could be detected in platelets from some patients with COVID-19 (REFS.[88,89]). However, it remains unclear whether platelets express ACE2, or whether there are other viral uptake pathways, such as via extracellular vesicles[101,102]. Comparative analyses of platelet transcriptomes from patients with COVID-19 with platelet transcriptomes from patients with other viral infections suggest that there are likely alternative mechanisms for viral entry into host cells. For instance, dengue virus, influenza virus and SARS-CoV-2 infections are all marked by increased levels of interferon-induced transmembrane 3 (IFITM3), an antiviral protein critical for regulating endocytosis, viral entry and replication[89,101,103] (FIG. 2). Furthermore, SARS-CoV-2 alters the megakaryocyte transcriptome through an ACE2-independent but CD147-dependent mechanism, which could explain reports of SARS-CoV-2 uptake in platelets and megakaryocytes[101].

Although platelet hyperactivity is common in COVID-19, platelet inhibitors have not convincingly provided benefit in preventing organ failure or CAC[104,105]. For example, in a randomized trial of more than 500 hospitalized non-critically ill patients with COVID-19, addition of P2Y$_{12}$ inhibitors to therapeutic doses of heparin did not increase the odds of being free of acute respiratory distress syndrome, reduce the need for organ support, improve survival at 21 days or decrease thrombotic events[105]. Also, in a randomized, controlled trial of approximately 15,000 patients with COVID-19, it was similarly concluded that aspirin (which limits platelet function by inhibiting cyclooxygenase 1) had little effect on the incidence of mechanical ventilation or mortality[104]. However, the group receiving aspirin did experience a slight reduction in the number of thrombotic events and a small increase in the rate of being discharged within 28 days. BOX 3 summarizes key questions that remain to be addressed in terms of understanding the contribution of hyper-inflammation and platelet dysfunction to CAC. In the following section we address the role of hypercoagulation in CAC.

Hypercoagulability in CAC

Changes in plasma procoagulant and fibrinolytic activity. Multiple studies have documented elevated procoagulant activity and suppressed fibrinolytic activity in patients with COVID-19, which together could drive CAC[17,106,107]. A study that compared the extent of these abnormalities in hospitalized patients with COVID-19 with patients with sepsis detected elevated plasma levels of soluble thrombomodulin in both groups, consistent with heightened endothelial cell activation[17] (FIG. 2). Both patient groups also exhibited delayed but enhanced fibrin formation and increased fibrin resistance to fibrinolysis. However,

plasma from patients with COVID-19 and from patients with sepsis showed different perturbations in the kinetics of thrombin generation and fibrinolytic activity. Whereas plasma from patients with sepsis had normal thrombin generation potential, plasma from patients with COVID-19 had enhanced rates of thrombin generation[17,108,109]. Plasma from patients with sepsis also showed delayed plasmin generation, whereas those with COVID-19 had normal plasmin-generating potential. Finally, plasma from patients with COVID-19 showed delayed times to thrombin, plasmin and fibrin formation with an increased sequential organ failure assessment (SOFA) score (a marker of disease severity).

Transcriptional profiling of BALF from patients with COVID-19 versus individuals without COVID-19 demonstrated differential regulation of genes encoding coagulation and fibrinolytic proteins[110]. Elevated TF transcripts were detected in BALF cell samples from patients with COVID-19, and TF expression may be higher in severely ill patients versus moderately ill patients or healthy controls[111]. SARS-CoV-2 was also associated with upregulation of genes encoding procoagulant proteins (for example, factor XI, factor VII and von Willebrand factor (vWF)) together with the downregulation of genes encoding anticoagulants (thrombomodulin and protein S) and profibrinolytic proteins (urokinase and urokinase plasminogen activator receptor).

Changes in urokinase pathway gene expression were previously implicated in a mouse model of SARS-CoV, including increased expression of genes encoding plasminogen activator inhibitor 1 (PAI-1), tissue plasminogen activator (tPA) and urokinase plasminogen activator receptor[112]. Notably, changes in gene expression were not completely congruous in these studies, which may reflect differences in the host (human versus mouse), the virus (SARS-CoV-2

Box 2 | **SARS-CoV-2 and vascular dysfunction: major scientific gaps and questions**

- What are the mechanisms underlying SARS-CoV-2-induced endothelial dysfunction, activation and vasculopathy?

- How does SARS-CoV-2 differentially affect the vasculature at different sites in different organs?

- What are the implications of endothelial glycocalyx disruption in different vascular beds? What are the mechanisms underlying glycocalyx disruption, how can it be monitored and what mitigating treatment modalities can be designed?

- What roles do circulating tissue factor and extracellular vesicles play in COVID-19-associated coagulopathy (CAC), what are the mechanisms for their release, can they be targeted and are they useful predictive biomarkers for CAC?

- What are the mechanisms for direct and indirect SARS-CoV-2 infection of the central nervous system and what is the pathogenesis of neuronal damage?

- What features of the blood–brain barrier endothelium regulate the entry of SARS-CoV-2, viral proteins and inflammatory mediators into the brain?

- What role do neutrophil extracellular traps (NETs) play in COVID-19-associated coagulopathy (CAC)? Are circulating NETs a marker of disease severity? Can NETs be safely targeted to improve clinical outcomes?
- What are key complement activation products and pathways that promote CAC? Are these the same in all vascular beds?
- Which complement pathways and components are the most efficacious and safest therapeutic targets?
- Assuming SARS-CoV-2 can infect platelets and megakaryocytes, what are the mechanisms of viral entry into the cells? Are there specific viral receptors, aside from angiotensin-converting enzyme 2 (ACE2)? Could modified glycans participate in viral entry?
- Can platelet transcriptomics be used as a tool to predict risk of thrombosis?
- What 'new' molecular pathways regulating platelet activation might be targeted to reduce thrombosis in COVID-19, either alone or in combination with other interventions?
- When in the course of COVD-19 is it safe and most efficacious to therapeutically target the innate immune response, while still ensuring an adequate host response to the infection?

versus mouse-adapted SARS-CoV), the disease stage (moderate versus severe disease), the sample (BALF versus lung) and/or other determinants of coronavirus infection or severity. Moreover, it is difficult to anticipate the functional implications of these changes, particularly given observations that fibrinolytic pathway genes with opposing function (for example, PAI-1 and tPA) are coordinately regulated in certain settings[113].

Dysregulation of the protein C system contributing to CAC. Activated protein C is a naturally occurring plasma serine protease that has both anticoagulant activity and direct cell signalling activities that require, primarily, EPCR and the protease activated receptors (PARs) PAR1 and PAR3 (REFS.[114,115]). There is growing evidence that the integrity of the protein C system, required for generation of activated protein C, is impaired in CAC. Low levels of protein C in hospitalized patients with COVID-19 were associated with worse disease progression and increased mortality[116]. This mirrors observations in patients with sepsis and disseminated intravascular coagulation where low protein C levels were a useful biomarker to identify patients at risk for developing severe illness[117,118]. Low protein C levels, which confer a heightened risk of ischaemic stroke[119] and dementia[120], may also increase the risk of stroke in patients with COVID-19 (REF.[116]) or 'brain fog', one of the features of long COVID (also known as post-acute sequelae of SARS-CoV-2 infection). Increased levels of soluble thrombomodulin — which correlate directly with lung endothelial cell damage and disease severity — may indicate that the generation of activated protein C is impaired[17,110,121,122]. Also consistent with

reduced generation of activated protein C, as well as endothelial cell damage and activation, are reports of elevated levels of soluble EPCR in patients with COVID-19 (REFS.[110,123]). EPCR shedding from inflamed endothelium may not only reduce the ability to generate activated protein C but also reduce the ability of activated protein C to induce cytoprotective signalling. Thus, shedding of endothelial thrombomodulin and EPCR may induce a protein C pathway dysfunction that, in turn, induces cellular resistance to activated protein C and limits its ability to rebalance dysregulated inflammatory and coagulant pathways.

From the preceding narrative, it is evident that the protein C system represents a potential target for mechanistic and therapeutic studies of CAC. The cytoprotective effects of activated protein C and/or its variants may be uniquely effective in blunting immunothrombosis in COVID-19, particularly as EPCR-dependent PAR1 and PAR3 signalling also elicits anti-inflammatory effects.

Anti-phospholipid antibodies. An unusual feature of CAC, among thrombotic disorders in general, is that it involves thrombosis in arteries, veins and the microcirculation — a spectrum reminiscent of patients with severe anti-phospholipid syndromes[124]. Indeed, there have been multiple case reports of patients with COVID-19 developing severe thrombotic disorders accompanied by anti-phospholipid antibodies and lupus anticoagulant[124–126]. However, transient development of anti-phospholipid antibodies can accompany other viral infections, so it remains to be established whether an anti-phospholipid syndrome is truly induced in patients with COVID-19 and what role this may have in driving thrombosis

and organ damage in these patients[124,126]. Interestingly, some mechanistic studies have reported that isolated IgG from the serum of patients with COVID-19 who have high titres of anti-phospholipid antibodies is strongly prothrombotic when injected into mice[127,128], and is capable of activating cultured human endothelial cells[129]. Clearly, more research is required to determine whether anti-phospholipid antibodies in patients with COVID-19 are driving CAC, and if so, whether such antibodies persist in these patients[124,126]. BOX 4 lists key questions that need to be addressed to better understand the hypercoagulability that occurs in COVID-19. In the final section of this article, we consider the suitability of the available animal models of COVID-19 for studying the mechanisms of CAC.

Animal models of COVID-19
Preclinical animal models of SARS-CoV-2 infection are important tools for improving our understanding of host–virus interactions and COVID-19. They are essential for rapid testing of novel therapies and can allow for controlled and detailed examination of the host response to infection. Two years into the pandemic, various small and large animal models have been developed, all with distinct advantages and disadvantages[130].

Mice are the most commonly used small animal model due to their short gestation period and time to sexual maturity, minimal space requirements, ease of manipulating the mouse genome and relatively low cost. Additionally, mice models are useful tools to investigate mechanisms of underlying comorbidities, such as diabetes, hypertension, obesity and the impact of other factors such as age[130]. An initial limitation to using mice to study COVID-19 was that the original isolates of SARS-CoV-2 did not infect wild-type mice, as the virus did not bind to the murine ACE2 (mACE2) entry receptor[131]. Consequently, several laboratories adapted the SARS-CoV-2 spike protein to bind to mACE2. This approach combined with a serial passage in mice resulted in mouse-adapted SARS-CoV-2 strains that caused moderate to lethal lung disease[132–134]. Thus, mouse-adapted SARS-CoV-2 (MA10) could infect wild-type mice and drive acute lung injury and acute respiratory distress syndrome in a dose-dependent manner[133]. Infection also caused a decrease in lung surfactant production and significant respiratory dysfunction in mice. Similarly to SARS-CoV-2, MA10 also induced a pronounced hyper-immune, pro-inflammatory cytokine and chemokine response, leading to recruitment of inflammatory cells to the

lung. Immunohistochemical analyses of the lungs from MA10-infected mice revealed increased TF expression that is accompanied by extravascular fibrin staining[135]. These initial studies suggest that the MA10 mouse model recapitulates some of the coagulo-pathic features observed in humans infected with SARS-CoV-2, and thus might prove a useful tool in deciphering mechanisms and identifying novel therapies.

To circumvent the challenge of having to manipulate the infecting virus, mice have also been engineered or transduced to express human ACE2. K18-hACE2 trans-genic mice express hACE2 (REF.[136]), albeit restricted primarily to the respiratory epi-thelium and brain. Notably, they are highly susceptible to SARS-CoV-2 infection follow-ing intranasal exposure, and as such might serve as a good model for gaining novel insights into infection via this route. High levels of SARS-CoV-2 were detected in the lungs of SARS-CoV-2-infected K18-hACE2 mice, with lower levels of the virus found in other tissues. Over a period of 2–7 days, in concert with increased D-dimer levels and a prolonged prothrombin time, there was evidence of a progressive inflammatory process, again affecting predominantly the lungs[137]. Indeed, the pathology seen in this mouse model was similar to that observed in patients with severe COVID-19, with histo-logical evidence of diffuse broncho-alveolar damage with oedema, fibrin deposits, leukocyte infiltration and pneumocyte hyperplasia[137]. This model is also proving to be an excellent tool to study SARS-CoV-2 neuroinvasion and the subsequent clinico-pathologic manifestations, featuring menin-goencephalitis, thrombosis, haemorrhage and vasculitis[138].

Hamsters have emerged as a useful model for SARS-CoV-2 infection owing to their susceptibility to disease following infection and the ability to transmit the virus between animals via aerosolization[139,140]. They are a promising model, although some drawbacks are the lack of readily available genetic and biochemical reagents, their relatively high cost and their high space requirements. Underlining the value of this model, and particularly interesting in view of the heightened risk of COVID-19 in individuals with diabetes and obesity, hamsters on a high-fat and high-sugar diet developed worse lung disease following SARS-CoV-2 infection than mice fed a regular diet, with lung histological evidence of vasculitis, haemorrhage, oedema and notable fibrin deposition[141]. In spite of their limitations, hamsters are likely to be an essential tool for SARS-CoV-2 research and may

yield important insights into COVID-19 pathogenesis and CAC. Indeed, these animal models are already holding promise to be instructive, as infection of K18-hACE2 mice and hamsters with the B.1.1.529 Omicron variant causes significantly milder disease, with less lung inflammation, findings that are in line with the apparent reduced pathogenicity of Omicron variants in humans[142,143].

Studies of non-human primate infections of SARS-CoV-2 are limited by space and facility requirements and by the high cost of experiments. However, as the closest related species to humans, non-human primates remain an important consideration for preclinical vaccine and drug development, while also offering important insights into human pathophysiology and the immuno-thrombotic response to SARS-CoV-2 infec-tions. Rhesus and cynomolgus macaques, as well as African green monkeys, experi-ence mild (in the case of the macaque) to moderate (in African green monkeys) lung disease after SARS-CoV-2 infection[144,145]. Aged rhesus macaques develop more severe symptoms, reflecting what is seen in humans with COVID-19. Notably, research-ers found that African green monkeys infected with SARS-CoV-2 also developed a coagulopathy[141]. Although not extensively evaluated, in the days following infection with SARS-CoV-2, circulating levels of fibrinogen and pro-inflammatory cytokines rapidly rose, and post-mortem analyses of the lungs revealed broncho-alveolar epithe-lial damage, with neutrophilic inflammation, alveolar oedema and haemorrhage, fibrin deposition and microthrombi within alveo-lar capillaries[146]. Involvement of other organs and tissues for evidence of small and large vessel thrombosis has also not been fully studied.

Animal models remain a cornerstone for gaining insights into mechanisms underlying the immunothrombosis associated with COVID-19 and for strategic development of novel and effective therapies. Moreover, they afford the opportunity to evaluate how CAC can be modified by known determinants

of disease severity, including age, sex and epigenetic and genetic factors, and by underlying comorbidities, such as diabetes and obesity. A notable shortcoming of all current models is the apparent lack of phenotypes that fully recapitulate the CAC that occurs in humans, particularly in terms of modelling microvascular and macrovascular thrombosis. Recognition of the strengths and weaknesses of any animal model is therefore vital before extrapolating the results to humans.

Conclusion

The NHLBI's workshop on CAC was convened in May 2021, soon after the first SARS-CoV-2 variants of concern began to emerge. Highlighting the urgency of assembling such a conference, it was already widely known that thrombosis in the microvasculature and macrovasculature was a life-threatening complication of COVID-19, particularly for those with severe disease[7–11,147]. Equally disconcerting was the increasing recognition that a high percentage of patients with COVID-19 suffer from long-term sequelae (long COVID)[48,49]. Whether excessive coagulation, impaired fibrinolytic capacity and/or a prominent endotheliopathy might contribute to the pathogenesis of long COVID by extending an acute CAC to a chronic condition remains to be determined[148,149].

Although much has been learned in the past 2 years about the pathophysiology of COVID-19, insights into the triggering events of CAC remain elusive, as do effective preventative and therapeutic strategies (see TABLE 1). This is evident from the uncertainties and controversy surrounding the use of several therapeutic agents in different clinical scenarios[150,151]. This is not surprising, given that CAC is a highly complex and dynamic process, involving the interplay between multiple biochemical, proteolytic and cellular pathways. This makes it difficult to differentiate which processes are causal, consequential or merely coincidental. It is thus a challenge to discern which pathway to therapeutically target, and

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Box 4 | Hypercoagulability in COVID-19: major scientific gaps and questions

- Why is disseminated intravascular coagulation not a major feature of COVID-19?

- Can combination therapies be considered to 'rebalance' alterations in coagulation and fibrinolysis in COVID-19, thereby minimizing the risk of COVID-19-associated coagulopathy (CAC)?

- What is the relative contribution of respiratory epithelial cells in the release of coagulation, fibrinolytic and complement proteins that impact on local and systemic thrombosis and CAC? Can these cells be targeted to reduce thrombotic risk?

- Which components of the protein C system and its downstream effectors are the best candidates as therapeutic targets to prevent and/or treat CAC? Can combination therapies be considered?

- What are the mechanisms by which anti-phospholipid antibodies might contribute to CAC?

Table 1 | **Selected current treatments for non-pregnant adult patients hospitalized for COVID-19 (adapted from REF.[153], Springer Nature Limited)**

Immunothrombosis therapy	Drug/target	Treatments and responses	Limits/ recommendations	Refs.
Corticosteroids	Dexamethasone	Hospitalized patients; increases organ support-free days and reduces 28-day mortality	Recommended	[154–156]
Janus kinase (JAK) inhibitors	Baricitinib (with IL-6 inhibitor and dexamethasone)	Patients with moderate–severe illness; rapid declines of C-reactive protein, ferritin and D-dimer with gradual improvement in haemoglobin, platelet counts and clinical status	At least 2 large RCTs; high likelihood of benefit	[157]
IL-6 inhibitors	Toclizumab	Hospitalized patients; reduces inflammatory markers, D-dimer and fibrinogen; reduced 21 or 28-day mortality and organ support-free days	For critically and severely ill patients; widely used	[158]
Anticoagulants	Low molecular weight heparin (LMWH) — prophylactic intensity	Critically ill patients (hospitalized, intensive care unit) for venous thromboemboli prophylaxis and to increase the number of organ support-free days; no effect on survival to hospital discharge	Certainty of evidence from multiple trials is low; but widely used	[154–156,159]
	LMWH — therapeutic dose	Patients with moderate-severity illness (hospitalized, low-flow oxygen use, elevated D-dimer) for venous/ arterial thromboemboli prophylaxis and to reduce organ support-free survival; uncertain if an effect on survival; consider bleeding risk	Certainty of evidence from multiple trials is low; but widely used	[154–156,159]
	Rivaroxaban (or other direct oral anticoagulants)	Patients with moderate-severity illness; no effect on survival or need for supplemental oxygen	Not recommended	[154,160]
Anti-platelets	Aspirin, P2Y12 inhibitors	Early use of ASA may lower odds of 28-day in-hospital mortality; no benefit from P2Y12 inhibitors	Not recommended	[104,161,162]
Thrombolytics	Alteplase (tPA)	For respiratory failure with heparin; evidence of moderate improved oxygenation	Early phase	[163]
Anti-spike protein monoclonal antibodies	Casirivumab and imdevimab	Hospitalized patients; may reduce 28-day mortality	Uncertain which patient groups will benefit most	[164]
Anti-complement	Inhibitor of C5 activation: ravulizumab	Critically ill patients, phase III study; stopped due to lack of clinical benefit	No clinical benefit	[81]
	Inhibitor of C5 activation: zilucoplan	Patients with moderate-severity illness; improved oxygenation at day 15, reduced cytokine levels and reduced 28-day mortality	Underpowered	[165]
	C5a blockade: (e.g., violbelimab, BDB-001); C5a-receptor blockage (avdoralimab)	Hospitalized patients; evidence of improvements in oxygenation	Early phase or underpowered studies	[81]
	C3 inhibitors (e.g., AMY-101, APL9), C1-esterase inhibitor, MASP2 antibody (narsoplimab)	Hospitalized patients; evidence of improvements in oxygenation	Early phase or underpowered studies	[70,160]
Bradykinin-targeted	Icatibant (bradykinin 2 receptor antagonist), ecallantide (kallikrein inhibitor)	Icatibant ± C1-esterase inhibitor; evidence of improvements in oxygenation	Underpowered	[164]

Detailed, updated therapy guidelines for patients with COVID-19 and associated coagulopathy are available from several organizations. tPA, tissue plasminogen activator.

when to do so in the course of the disease, without disrupting natural protective mechanisms. Indeed, it is likely that one or more CAC mechanisms predominate at distinct stages of the disease and at different organ sites and vascular beds. In that regard, we have a better handle on the early stages of COVID-19 following infection, where the virus damages the alveoli and adjacent microvasculature, with an initial local hypoxic and immunothrombotic response[19,31]. With subsequent dissemination of the disease, identifying key triggers within the complex immunothrombotic web becomes more of a challenge.

It is important to recognize that all of the preceding discussion refers to information gathered from patients infected with the Alpha or Delta variants. With few solid data[143,152], the impression is that the more recent Omicron variants are less immunothrombotic and cause less severe disease. Animal studies support these observations[142,143]. In spite of the apparent reduced pathogenicity of these more recent variants, scientists in the field must nonetheless continue to be vigilant and persist in delineating the underlying mechanisms so that we can be ahead of the curve with effective diagnostics and therapeutics.

Adding to the complexity, we have few insights into the factors — acquired, environmental and/or epigenetic — that render some populations (for example, older people, individuals with obesity, individuals with diabetes) at increased risk of severe disease and CAC. The workshop provided a unique and important forum to gather wide expertise in diverse disciplines, with the common aim of integrating our knowledge to gain new insights into CAC, to identify gaps and opportunities in the science and to explore practical lines of study that might yield clinical benefit. With the continued uncertainty of the emergence of new

SARS-CoV-2 variants, such focused research continues to be crucial for the development of effective anti-immunothrombotic preventative and therapeutic strategies for patients infected with SARS-CoV-2.

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Competing interests
The authors declare no competing interests.

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