

Procoagulant platelets: linking coagulation and thromboinflammation in cardiovascular disease

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Abstract

Platelets are the key cell types in haemostasis after vascular injury and crucially contribute to thrombus formation. In addition to their aggregation and clot contraction functions after stimulation by soluble agonists or extracellular matrix proteins, platelets can adopt a highly activated state known as procoagulant activation. Procoagulant platelets influence the pathophysiology underlying various cardiovascular diseases, including myocardial infarction, stroke and deep vein thrombosis. Findings described in the past decade position procoagulant platelets at the dynamic intersection between thrombosis and inflammation. In this Review, we discuss the expanding research on procoagulant platelets, describing how this platelet activation state contributes to macrovascular and microvascular clot formation in cardiovascular diseases. We summarize the key receptors and signalling pathways that control platelet procoagulant activation and that distinguish the procoagulant phenotype from other platelet activation states. Finally, we highlight the clinical significance of platelet procoagulant activation and discuss how the individual pathways involved in this activation can be targeted with the use of both readily available and novel therapeutic approaches, providing a framework for future research that might lead to new diagnostic and therapeutic applications in cardiovascular disease, septic inflammation and immune complex-mediated diseases.

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Key points

- Platelet procoagulant activation is a unique effector function characterized by membrane ballooning and phosphatidylserine exposure, allowing the recruitment of coagulation factors to the platelet surface.
- Procoagulant activity depends on distinct signalling cascades, including the mitochondrial protein cyclophilin D and membrane scramblase transmembrane protein 16F.
- Triggers of procoagulant activation include high shear stress rates and parallel stimulation of platelets by multiple agonists, leading to sustained rises in intracellular Ca^{2+} levels.
- Procoagulant activity is crucially involved in arterial and venous thrombosis, as well as thromboinflammatory diseases, including COVID-19, vaccine-induced thrombocytopenia and thrombosis and heparin-induced thrombocytopenia.
- Inhibition of procoagulant activity by specific targeting of the involved cellular machinery has shown efficacy in animal models of thrombosis, with minor effects on the risk of bleeding.

Introduction

After their release from the bone marrow, platelets continuously patrol the circulatory system searching for vascular breaches to fulfil their main functions: sense vessel injury, adhere to the site of exposed extracellular matrix, aggregate and build a thrombus that prevents potentially lethal blood loss^{1,2}. To execute these tasks, platelets are equipped with a unique repertoire of surface receptors, a rapidly responsive set of signalling cascades, and a plethora of cytoplasmic granules that enable the release of stimulating agents and the formation of thrombi within seconds¹. The ever-alert platelet is thus perfectly suited to respond to vascular injury, and the high number of platelets present in mammalian blood ensures constant surveillance and rapid platelet aggregation^{3,4}. On the downside, platelets cannot differentiate the upstream cause of the activating stimuli; for example, whether stimuli such as extracellular matrix components exposed on the vessel lumen are a result of traumatic vessel injury (in which clot formation and the prevention of blood loss are beneficial) or are due to atherosclerotic plaque rupture (in which intravascular clot formation can lead to vessel occlusion and ischaemia) is unknown⁵. Atherothrombosis and its sequelae remain the most frequent cause of death in Western countries⁶, and current treatment strategies focus on preventing platelet activation through inhibition of their main signalling hubs: thromboxane generation and ADP receptor agonism⁷. However, these treatments target receptors and pathways that are also important for haemostasis after traumatic injury, which explains the risk of bleeding associated with current antiplatelet regimens and underlines the need to develop new therapeutic approaches⁸. Furthermore, arterial thrombosis is traditionally targeted by inhibiting platelet aggregation, whereas low-flow-induced clot formation (for example, deep vein thrombosis (DVT), pulmonary embolism and cardiac thrombus formation in atrial fibrillation) is treated or prevented by targeting the plasmatic coagulation cascade. However, insights from animal models indicate that both effectors – platelets and coagulation – are involved in clot formation independently of the vascular bed and shear stress rates^{9,10}.

A crucial link between platelets and coagulation is mediated by a specialized subtype of platelets known as procoagulant platelets¹¹. This highly activated platelet state is characterized by the exposure of negatively charged phospholipids, drastic changes in cell shape and enlargement of cell surface area, all of which in turn enable the formation of coagulation factor complexes on the platelet surface and thereby the rapid generation of thrombin, thus uniquely connecting cellular haemostasis and plasmatic coagulation. The signalling cascades underlying procoagulant platelet formation, a process termed procoagulant activation, and their role in various settings of immunity and thrombosis have gained increasing attention in the past decade. Consequently, new triggers, signalling cascades and downstream mediators of procoagulant activation have been defined. These advances, in combination with the central role of this activation state in integrating platelet activation and coagulation, have paved the way for the development of novel therapeutic strategies that target both platelets and coagulation.

In this Review, we summarize the latest advances in our understanding of procoagulant platelet biology. We first define classical versus procoagulant activation and describe receptors and molecular pathways that contribute to and distinguish platelet procoagulant activation from classical aggregatory responses. We describe how procoagulant platelets contribute to venous and arterial thrombosis, contrast these platelet roles with their protective functions in haemostasis and highlight their involvement in immunothrombosis and thromboinflammation, with a focus on septic inflammation, COVID-19 and the sequelae of vaccine-induced thrombocytopenia and thrombosis (VITT) and heparin-induced thrombocytopenia (HIT). Finally, we summarize the potential of diagnostic tools and therapeutic strategies directed at procoagulant activation for the treatment of thrombotic and thromboinflammatory disorders. This Review provides an updated framework for future research into the role of procoagulant platelets in clinically relevant disorders, including cardiovascular disease and septic inflammation, for which new treatment approaches are needed.

Definitions of procoagulant activation and procoagulant platelets

The signalling events that occur in classical platelet activation in haemostasis and thrombosis are well defined, including the binding of the glycoprotein (GP) Ib–V–IX complex to exposed von Willebrand factor (vWF), binding of collagen receptors to its ligands, and the subsequent release of granule content that mediates autocrine and paracrine activation^{1,12,13}. During this activation, the discoid, quiescent platelet undergoes morphological changes mediated by cytoskeletal rearrangements, including the formation of thread-like filopodia, and structural changes in main platelet integrins, including the inside-out signalling-mediated conformational switch of $\alpha\text{IIb}\beta\text{3}$ integrin into an ‘open’ state that can bind to fibrin and fibrinogen¹². These key events foster platelet–platelet interactions, result in the formation of a $\alpha\text{IIb}\beta\text{3}$ integrin–fibrinogen network known as a white thrombus¹⁴ and activate additional signalling cascades in the platelet, including integrin-mediated outside-in signalling¹⁵.

In contrast to the formation of these classically activated platelets, which have an aggregatory phenotype and work as a multicellular syncytium¹³, the formation of procoagulant platelets only occurs when different activation cascades are simultaneously activated^{11,16}. Simultaneous stimulation of platelet receptors, for example, proteinase-activated receptors (PARs) and GP receptors such as GPVI, leads to swift elevations in cytoplasmic Ca^{2+} concentrations

that trigger downstream events, such as mitochondrial depolarization, that do not routinely occur after stimulation by a single agonist. Procoagulant activation results in the formation of a highly activated platelet characterized by morphological changes to a balloon-like shape, an anionic phospholipid inversion of the plasma membrane with exposure of high levels of phosphatidylserine in the outer layer, high expression of P-selectin, and recruitment and accumulation of plasmatic coagulation factors, such as tenase and prothrombinase complexes, on the negatively charged plasma membrane^{11,17}. Thus, flow-cytometry-based detection of procoagulant platelets relies on the detection of phosphatidylserine^{high}P-selectin^{high}CD41⁺ cells (as defined by the International Society for Thrombosis and Hemostasis (ISTH) Scientific and Standardization Committee guidelines¹⁸), whereas imaging-based approaches for fixed or live procoagulant platelets focus on detecting morphological changes (platelet ballooning and microvesicle formation)¹⁹. Adhering to these definitions in the study of procoagulant platelets is important given that both phosphatidylserine and P-selectin are also expressed by other platelet subtypes, such as apoptotic platelets (for phosphatidylserine) or aged, activated and secretory platelets (for P-selectin)^{20–23}.

From a historical point of view, two concepts are noteworthy. First, what we define today as a procoagulant platelet was described previously using a plethora of different names, including collagen and thrombin platelets (COAT)^{24,25}, zombie platelets²⁶, fibrinogen-capped platelets (FIB-CAP)²⁷, necrotic platelets^{28,29}, ballooned platelets³⁰ and sustained Ca²⁺-induced platelets (SCIP)³¹. Second, although procoagulant activation manifests rapidly, it is a step-wise process and, therefore, different stages of procoagulant activation can occur simultaneously. For example, a 2015 study described different stages of procoagulant

activation, including ballooned non-spread platelet and ballooned and procoagulant-spread platelet phenotypes¹⁷, and more recent work revealed rapid temporal differences in cytosolic Ca²⁺ levels during the process of procoagulant activation^{19,32}. Therefore, despite the different names, the term procoagulant platelet comprises platelets that match the aforementioned descriptions, even though whether different paths for procoagulant activation exist (for example, paths that predominantly lead to microvesiculation versus coagulation factor binding) remains debatable¹¹.

In summary, procoagulant platelets are distinguishable from classically activated platelets through various parameters, including changes in morphology, plasma membrane composition and Ca²⁺ currents.

Receptors and pathways in platelet procoagulant activation

Generally, procoagulant activation requires a high activation threshold in vitro and can be induced by simultaneous stimulation by different ligands. One hypothesis for this observation is that these characteristics prevent host damage, given that an unrestricted rise in procoagulant platelet numbers could lead to disseminated activation of coagulation and subsequent thrombotic complications, as observed in severe COVID-19 (ref. 33). A classic example of two strong ligands is the simultaneous engagement of PARs (for example, by the serin protease thrombin) and surface receptors of the immunoreceptor tyrosine-based activation motif (ITAM) receptor family (such as GPIIb/IIIa by collagen or the snake venom convulxin)^{24,25} (Fig. 1). The signalling cascades downstream of these receptors result in the same procoagulant platelet phenotype regardless of the platelets being in suspension

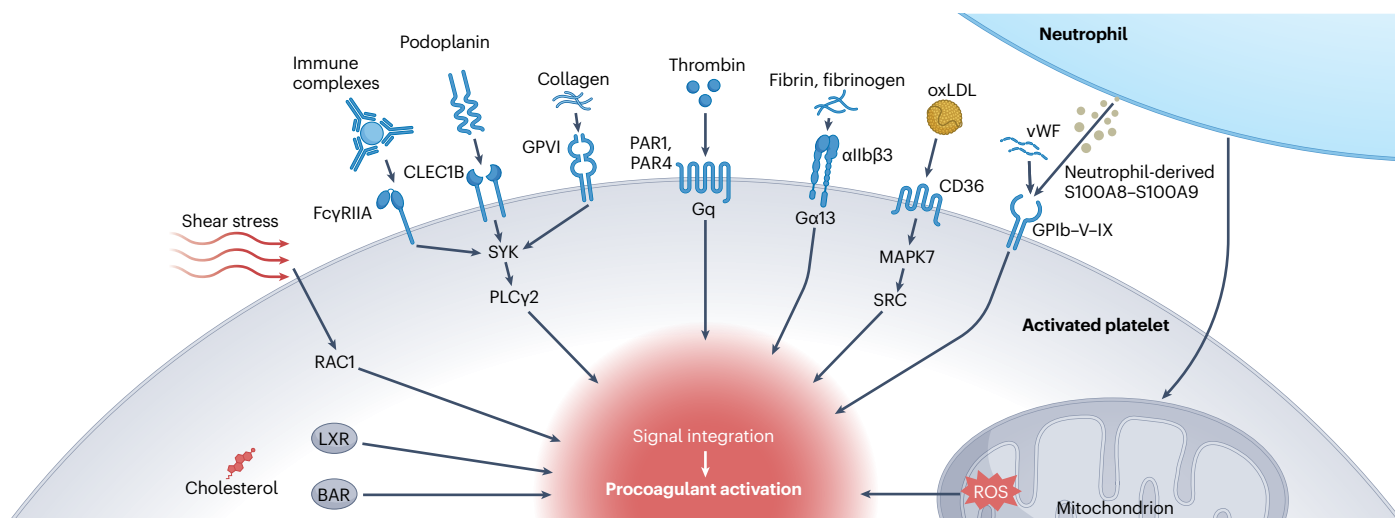


Fig. 1 | Receptors and signalling pathways that promote platelet procoagulant activation. Platelet procoagulant activation can be induced by various agonists and their respective receptors. In contrast to other forms of platelet activation, procoagulant activation usually only occurs upon simultaneous co-stimulation by different ligands, including coagulation factors such as thrombin, fibrin, fibrinogen and von Willebrand factor (vWF); extracellular matrix components such as collagen; and physical stimuli such as shear stress. Crosstalk with the immune system in the setting of septic inflammation or immune complex-mediated diseases can also induce platelet procoagulant activation, for example, through binding of immune complexes to Fcγ receptor IIA (FcγRIIA), direct interaction with podoplanin on leukocytes or

through binding of neutrophil-derived S100A8–S100A9 to platelet glycoprotein Iba (GPIIb). Of note, the relative contributions of receptor–ligand pairs to procoagulant activation are variable and remain poorly understood. BAR, bile acid receptor (also known as farnesoid X receptor); CD36, platelet glycoprotein 4; CLEC1B (also known as CLEC2), receptor C-type lectin domain family 1 member B; ERK/MAPK7, mitogen-activated protein kinase 7; GPIIb, glycoprotein VI; LXR, liver X receptor; oxLDL, oxidized LDL; PAR, protease-activated receptor; PLCγ2, 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase-γ2; RAC1, RAS-related C3 botulinum toxin substrate 1; ROS, reactive oxygen species; SRC, proto-oncogene tyrosine-protein kinase Src; SYK, tyrosine-protein kinase SYK.

or adhered to a substrate, except for the 'procoagulant spreading' phenotype, which occurs only in adherent platelets^{11,17}. However, *in vitro*, suspended platelets require higher concentrations of the respective agonists (such as PAR agonists) to undergo procoagulant activation compared with adherent and spread platelets, in which integrin signalling is already engaged^{28,34,35}.

A high concentration of the agonist is not required when additional stimuli are present. Both mechanical stressors, such as high shear stress^{36,37}, and exposure to inflammation-associated molecules, such as reactive oxygen species (ROS)³⁸, markedly reduce the threshold for procoagulant activation. Likewise, platelets adhered to fibrinogen or migrating on fibrinogen–albumin matrices adopt a procoagulant phenotype when GPVI is activated¹⁹. Given that circulating platelets encounter high shear stress (such as at sites of vessel stenosis), it is reasonable to assume that the agonist threshold for procoagulant activation is likely to be lower *in vivo* than the high amounts of agonists that have been described to induce procoagulant activation *in vitro*. Nevertheless, much of the mechanistic evidence on procoagulant activation stems from *in vitro* models, whereas the exact signalling events (and disease-specific upstream effectors) that occur *in vivo* remain incompletely understood.

Several platelet GPs and other receptors have a role in inducing procoagulant activation (Fig. 1). GPVI, a bona-fide collagen receptor, is a prominent example^{19,26,39–41}, with several studies showing that its absence or inactivation reduces the formation of procoagulant platelets^{19,42}. Activation of GPVI through the binding of collagen, fibrin or the snake venom toxin convulxin leads to phosphorylation of YXXL motifs, which are located in the intracellular domains of its associated Fcγ receptor^{43,44}. The phosphorylation of Fcγ receptors promotes the binding of SRC family kinases, such as LYN and FYN, to GPVI intracellular domains and of tyrosine-protein kinase SYK to the Fcγ receptor, which in turn activates the downstream effector 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase-γ2 (PLCγ2) in a multistep process. PLCγ2 is also activated after binding of podoplanin and immunoglobulins to the (hemi)ITAM receptor C-type lectin domain family 1 member B (also known as CLEC2) and Fcγ receptor IIA (FcγRIIA), respectively, through similar mechanisms. PLCγ2 then unleashes several effector pathways, including the generation of second messengers such as inositol trisphosphate and diacylglycerol (DAG) that foster cytosolic Ca²⁺ increases and further activate molecules such as the small GTPase RAC1, which is also implicated in platelet procoagulant activation³⁷.

PARs also boost platelet procoagulant activation, with PAR1 being the predominant effector in humans^{25,45–47}. Cleavage of the extracellular domain of PARs by coagulation factors, such as thrombin and factor Xa⁴⁸, induces a conformational rearrangement of transmembrane helices that allow the binding of heterotrimeric G proteins to PAR intracellular domains to initiate signalling. Downstream effectors include increases in cytosolic Ca²⁺ levels, platelet degranulation and the release of potent platelet activators such as ADP and ATP. PAR1 and PAR4 signalling are crucial for the generation of procoagulant platelets in humans⁴⁵, whereas PAR3 and PAR4 mediate procoagulant activation in mouse platelets⁴⁹. Procoagulant activation is also mediated by transactivation of GPIIbα by neutrophil-derived calprotectin (also known as S100A8–S100A9 proteins) in humans and mice, partially through the scavenger receptor CD36 (ref. 50), although the downstream mechanisms are unknown. Furthermore, in adhering and migrating platelets, platelet integrin αIIbβ3 mediates procoagulant activation through outside-in signalling induced by guanine nucleotide-binding protein subunit-α13 (Gα13) in both human and mouse platelets^{19,36}.

Consistently, platelet-specific knockout of the genes encoding integrin β3 or Gα13 in mice decreased shear-induced platelet procoagulant activation³⁶.

In addition to these classical platelet receptors, other surface molecules have been implicated in phosphatidylserine exposure following platelet activation. Binding of oxidized LDL to platelet CD36 activates mitogen-activated protein kinase 7 (also known as ERK5) and proto-oncogene tyrosine-protein kinase SRC, which leads to caspase activation and phosphatidylserine exposure⁵¹. Interfering with these pathways, for example, by blockade of CD36 or with antibodies or small-molecule inhibitors targeting SRC, decreases phosphatidylserine exposure and thrombin generation^{50,52}. Of note, these signalling events depended on apoptotic signalling (specifically caspase cleavage) and, therefore, these platelets might be a subset of apoptotic platelets with procoagulant properties rather than platelets with 'true' procoagulant activation⁵¹. Intracellular receptors, such as bile acid receptor (also known as farnesoid X receptors) and liver X receptors, can also promote procoagulant activation upon binding by their ligands (cholesterol and bile acids)³⁸, a mechanism that links hyperlipidaemia and thrombosis.

Activation of the aforementioned receptors culminates in elevations in cytosolic Ca²⁺ concentrations. Importantly, procoagulant activation requires sustained high levels of cytosolic Ca²⁺, which have been described as 'supramaximal' Ca²⁺ levels³² *in vitro*, and depends on simultaneous cytosolic influx of Ca²⁺ from several storage sites. Platelets have multiple Ca²⁺ storage compartments, including the dense tubular system (DTS)–endoplasmic reticulum (ER) as the major intracellular Ca²⁺ pool, and lysosome-like acidic organelles. However, mitochondria are the main Ca²⁺ hub implicated in platelet procoagulant activation. Under steady-state conditions, sarcoplasmic–ER Ca²⁺ ATPase ensures the maintenance of low cytosolic Ca²⁺ levels by pumping Ca²⁺ into the DTS–ER in an ATP-dependent manner⁵³. Activation of G protein-coupled receptors or ITAM receptors leads to PLCγ2-mediated generation of inositol trisphosphate and DAG, which then induce rapid spikes in cytosolic Ca²⁺ levels. DAG increases cytosolic Ca²⁺ concentrations by promoting the entry of extracellular Ca²⁺ through protein kinase C and Ca²⁺ channels such as short transient receptor potential channels (TRPC3 and TRPC6)^{53–55}. Inositol trisphosphate increases cytosolic Ca²⁺ levels by promoting the release of Ca²⁺ from the DTS–ER. Subsequent low luminal Ca²⁺ concentrations in the DTS–ER activate stromal interaction molecule 1 (STIM1)⁵⁶, which multimerizes and binds to calcium-release-activated calcium channel protein 1 (ORAI1)⁵⁷, an integral part of Ca²⁺ release-activated channels that are crucial for store-operated Ca²⁺ entry⁵⁶. This signalling leads to sustained, highly Ca²⁺-selective ion entry from the extracellular space and further increases cytosolic Ca²⁺ levels, which trigger Ca²⁺ uptake into mitochondria via the mitochondrial calcium uniporter (MCU) complex⁵³ (Fig. 2). As mitochondrial Ca²⁺ load changes via MCU during strong or sustained stimulation, the probability of a transient or permanent opening of the mitochondrial permeability transition pore (mPTP) increases. Pharmacological inhibition of either of these molecules (STIM1, ORAI1 or TRP channels) implicated in platelet calcium homeostasis does not abolish platelet procoagulant activation¹⁹. However, the combined inhibition of these proteins, or the selective blockade or genetic depletion of MCU (and thus the reduced probability of mPTP opening), decreases the generation of procoagulant platelets in human and mouse platelets *in vitro*^{26,32,56,58}, with some evidence indicating that platelets from mice with deficiency in STIM1 or ORAI1 have mildly reduced procoagulant potential *in vitro*⁵⁶. Conversely, Ca²⁺ ionophores such as ionomycin, which elevate membrane permeability to Ca²⁺, have been shown to

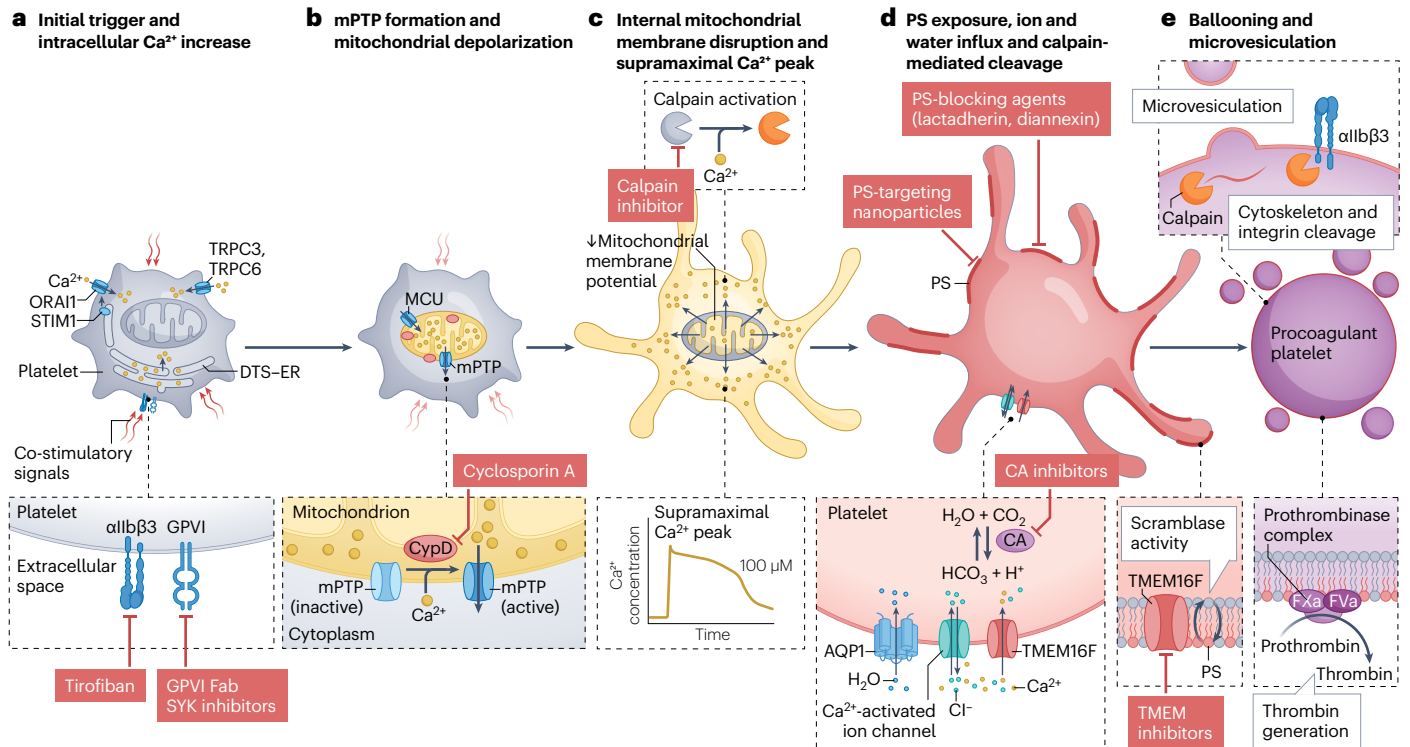


Fig. 2 | Mechanisms of platelet procoagulant activation and its therapeutic targeting. **a**, After co-stimulation by different ligands, platelet activation initially leads to increases in cytosolic Ca^{2+} concentrations through Ca^{2+} entry from the extracellular space, for example, through short transient receptor potential channels (TRPC3 and TRPC6) and release from the dense tubular system (DTS)–endoplasmic reticulum (ER). Stromal interaction molecule 1 (STIM1) senses the drop in intraluminal DTS–ER Ca^{2+} concentration and binds and activates calcium release-activated calcium channel protein 1 (ORAI1), which boosts further increases in cytosolic Ca^{2+} levels. **b**, Elevations in cytoplasmic Ca^{2+} content and increased mitochondrial Ca^{2+} uptake (for example, through the mitochondrial calcium uniporter (MCU)) promote the formation of the mitochondrial permeability transition pore (mPTP) and increase its opening probability, which is, among other partially unknown mediators, induced by the activation of cyclophilin D (CypD; also known as mitochondrial peptidyl-prolyl *cis-trans* isomerase F), leading to mitochondrial depolarization. **c**, Consequently, the mitochondrial membrane potential is reduced, which leads to an efflux of Ca^{2+} stored in the mitochondria into the platelet cytoplasm, resulting in high cytoplasmic Ca^{2+} concentrations ($\sim 100 \mu\text{M}$), a phenomenon known as

supramaximal Ca^{2+} peaks. In parallel, Ca^{2+} -dependent, non-lysosomal cysteine proteases called calpains are activated. **d**, Supramaximal Ca^{2+} peaks activate, among others, the transmembrane protein 16F (TMEM16F; also known as anoctamin 6), a scramblase that flips negatively charged phosphatidylserine (PS) to the outer layer of the plasma membrane. Other Ca^{2+} -activated ion channels and aquaporins (AQPs) are also activated, leading to both ion and water influx. **e**, This influx of water and ions and calpain 2-mediated proteolysis to cleave cytoskeletal proteins and receptors (including the cytoplasmic tail of $\alpha\text{IIb}\beta 3$ integrin) promote the swelling or ‘ballooning’ of the activated, procoagulant platelet, which now assumes a large, balloon-like shape. In addition, coagulation factors bind to these platelets via the Ca^{2+} bound to the negatively charged PS-rich surface of the plasma membrane. Finally, proteolysis of cytoskeletal proteins also facilitates microvesiculation, another hallmark of platelet procoagulant activation. Red boxes indicate potential pharmacological interventions to target platelet procoagulant activation. CA, carbonic anhydrase; Fab, fragment antigen binding; FVa, activated factor V; FXa, activated factor X; GPVI, glycoprotein VI; SYK, tyrosine-protein kinase SYK.

trigger platelet procoagulant activation *in vitro*^{25,35}, suggesting that Ca^{2+} are ‘gatekeepers’ of procoagulant activation¹⁶. Consequently, the activity of the individual Ca^{2+} channels is tightly regulated. For example, in mouse platelets, bridging integrator 2, which couples STIM1 and ORAI1 channel function⁵⁹, is inhibited via protein kinase C-mediated phosphorylation, reducing procoagulant platelet activity and thrombin generation⁶⁰.

Upon reaching a critical threshold, MCU-mediated increases in mitochondrial Ca^{2+} levels lead to disruption of the inner mitochondrial membrane and the formation of the mPTP^{35,61,62}. This process depends on cyclophilin D (CypD; also known as peptidyl-prolyl *cis-trans* isomerase D)⁶³, which is present on the inner side of the mitochondrial membrane. Genetic ablation or pharmacological inhibition

(for example, using the clinically approved calcineurin inhibitor cyclosporin A) of CypD abrogates platelet procoagulant activation *in vivo* and *in vitro*^{28,35,61,64,65}. Of note, MCU and CypD are not the only mitochondrial proteins with a role in procoagulant activation signalling. Mitofusin 2, a protein involved in mitochondrial homeostasis by regulating mitochondrial fusion, is essential for procoagulant platelet activation⁶⁶. In addition, mitochondrial ROS production sensitizes platelets to procoagulant activation^{66,67}. Moreover, although platelets exhibit time-dependent reductions in mitochondrial abundance and function with advanced circulatory age, aged platelets have been shown to shift towards a higher procoagulant potential compared with younger platelets²³.

Once the mPTP is established, the resulting mitochondrial depolarization and sudden release of mitochondrial Ca^{2+} into the cytoplasm

lead to the simultaneous activation of several processes in platelets. High Ca^{2+} levels activate the cytosolic thiol protease calpain, which then cleaves several cytoskeletal and membrane linker proteins, including actin, myosin and vinculin^{68,69}, resulting in structural reorganization of the activated platelet. Of note, calpain 2, but not calpain 1, seems to be essential for procoagulant activation-associated integrin inactivation in mice, given that integrin $\alpha\text{IIb}\beta\text{3}$ inactivation was not altered in mouse *Capn1*^{-/-} platelets⁷⁰. By contrast, phosphatidylserine exposure itself is largely unaffected in platelets treated with calpain inhibitors, confirming that calpain activity is not required for the lipid-scrambling event itself⁷⁰.

In parallel to structural reorganization, α -granules are released and contribute to procoagulant activation, as evidenced by reduced procoagulant potential in *Nbeal2*-knockout platelets, which are characterized by an absence of α -granules^{71,72}. High and sustained Ca^{2+} levels also affect the plasma membrane composition. High Ca^{2+} levels and the ATP depletion that occurs following mitochondrial depolarization inhibit flippases, which ensure membrane asymmetry and translocate negatively charged phospholipids (such as phosphatidylserine) to the inner membrane leaflet under steady-state conditions, but increase the activity of membrane scramblases, which form a hydrophilic groove that enables bidirectional movement of phospholipids across the plasma membrane bilayer, leading to loss of membrane asymmetry^{73,74}. The most prominent scramblase required for platelet procoagulant activation and phospholipid exposure is transmembrane protein 16F (TMEM16F; also known as anoctamin 6 and encoded by *ANO6*)^{73,75–79}. Importantly, TMEM16F has two main functions in platelets undergoing procoagulant activation: its scramblase activity leads to high levels of phosphatidylserine on the outer platelet membrane, and its function as a Ca^{2+} -dependent unselective ion channel allows for rapid entry of chloride anions^{78,80}. Subsequently, membrane-bound aquaporins, including aquaporin 1, allow water influx, which boosts the formation of procoagulant platelet ‘balloons’^{11,81}. This process is facilitated by calpain-mediated cleavage of cytoskeleton and linker proteins, as described earlier. Calpain also contributes to the formation of procoagulant microvesicles that are released from the forming balloons⁸². In line with the aforementioned findings, pharmacological inhibition of TMEM16F activity or its selective genetic deletion from the megakaryocyte–platelet lineage decreases platelet procoagulant activation^{19,64,75,76,83}. Similarly, genetic or pharmacological blockade of aquaporins, specifically aquaporin 1, interferes with platelet ballooning^{11,17,64,81}. Interestingly, physical distortion of the platelet plasma membrane is also sufficient to trigger procoagulant activation. In individuals with severe polytrauma, extracellular histone H4 released after tissue damage can disrupt the plasma membrane, leading to the formation of procoagulant balloons⁸⁴. However, whether this phenomenon is induced by pore formation in the platelet membrane (as shown for smooth muscle cells exposed to free histone H4 (ref. 85)) or by histone-mediated, unselective increases in Ca^{2+} membrane permeability^{86,87} remains unclear.

The resulting procoagulant platelet balloon is characterized by high levels of phosphatidylserine and P-selectin and a receptor expression profile marked by reduced integrin expression (mediated by mPTP-dependent calpain activation and cleavage of integrins and their associated cytoskeletal proteins) and closure of integrin $\alpha\text{IIb}\beta\text{3}$ conformation (caused by TMEM16F-mediated phospholipid scrambling)^{70,88}. This observation is intriguing, because these changes separate procoagulant function from platelet aggregation, meaning that procoagulant platelets can no longer contribute to clot formation or its direct stabilization per se. Despite consistent reports of integrin closure as a

hallmark of procoagulant activation, an infrequent, low-calcium procoagulant platelet subpopulation has been shown to retain its integrin activation state *in vitro*⁸⁹.

After phosphatidylserine exposure in procoagulant platelets, Ca^{2+} binds to the negatively charged membrane surface, which then serves as a binding surface for vitamin K-dependent plasmatic coagulation factors via their γ -carboxyglutamate (Gla) domain and facilitates formation of the tenase and prothrombinase complexes. These complexes then foster factor Xa and thrombin generation at the cell surface^{11,19,24,25,27,30,90–93}, thus positioning procoagulant platelets at the intersection between primary and secondary haemostasis and bridging both haemostatic systems^{1,94} (Fig. 2). Finally, whether procoagulant platelet clearance from the circulation is an active (for example, uptake by phagocytes) or passive (such as disintegration of the structurally unstable platelet balloon) process remains unknown.

Importantly, exogenous factors can affect the propensity of circulating platelets to turn procoagulant. In diabetic mice, hyperglycaemia led to increased mitochondrial ROS levels in platelets and decreased the threshold of procoagulant activation⁹⁵. Platelet-specific deletion of both *Glut1* and *Glut3*, which encode glucose transporters, rendered mouse platelets resistant to dual agonist-induced procoagulant activation⁹⁵. Increases in systemic ROS levels were also found to foster procoagulant activation in patients with diabetes mellitus, through hyperglycaemia-induced aldose reductase activation and subsequent mitochondrial dysfunction⁹⁶. As mentioned earlier, the extent of shear stress also contributes to the propensity of platelets to turn procoagulant and subsequently generate fibrin³⁶ and can also be affected by binding of GPIb to vWF⁹⁷.

Procoagulant platelets in venous and arterial thrombosis

Current models propose a physiological role of procoagulant platelets in bridging cellular and plasmatic coagulation, supporting haemostatic clot formation to avoid blood loss. For example, one study proposed that during arterial clot formation after mechanical injury, clot contraction drives procoagulant platelets to the outside of the growing thrombus, where they are involved in recruiting plasmatic coagulation factors to enhance thrombus stability⁹⁸. Although the underlying mechanism has been questioned in favour of thrombin-driven induction of procoagulant activation in the thrombus periphery⁹⁹, it seems physiologically relevant to recruit coagulation factors at sites of the thrombus that are exposed to blood flow. In this sense, a study using 4D intravital imaging in a mouse model of haemostasis revealed a crucial role for platelet procoagulant activation in fibrin formation in the growing haemostatic plug¹⁰⁰.

The thrombotic potential of procoagulant platelets comes with a price; for example, given the presence of strong triggers of procoagulant activation in the setting of atherothrombosis, including high shear stress and extracellular matrix components such as collagen, procoagulant platelets have been described in many conditions involving arterial thrombosis in experimental models and in clinical samples. A study reported an increased number of procoagulant platelets in the circulation in patients with coronary artery disease compared with healthy individuals¹⁰¹. Moreover, a study in mice indicated that procoagulant platelets provide a procoagulant surface during arterial thrombosis and showed that platelet-specific deletion of *Ppid* (which encodes CypD) reduced ferric-chloride-induced carotid thrombosis *in vivo*²⁸. Our group confirmed these findings *in vivo* in mice¹⁹. However, another study showed increased arterial thrombus formation in mice with global *Ppid* deficiency, a finding that was attributed to reduced

integrin inactivation and concomitant increases in local accumulation of aggregating platelets³⁵. Further strengthening the notion of a prothrombotic role of procoagulant platelets in arterial thrombosis, two studies found reduced arterial thrombus formation in platelet-specific *Ano6*-knockout mice^{19,83}. GPVI-mediated procoagulant activation has also been shown to have a role in driving disease progression of abdominal aortic aneurysm in mice¹⁰².

Although several studies have assessed procoagulant activation in arterial thrombosis, the role of procoagulant platelets in venous thromboembolism is not well defined. Platelets are recruited early during venous thrombus formation and aid in the recruitment and activation of immune cells such as neutrophils and monocytes^{10,103,104}. Systemic levels of procoagulant platelets have been shown to be elevated in mice with flow-reduction-induced venous thrombi compared with control mice and in patients with confirmed DVT or pulmonary embolism compared with individuals without these venous thrombosis conditions but in whom both entities had been suspected owing to clinical evidence and plasma D-dimer elevations⁶⁴. Furthermore, procoagulant platelets were also detected in mouse DVT clots and in retrieved human emboli⁶⁴. Both genetic depletion of either *Ppid* or *Ano6* and pharmacological inhibition of platelet ballooning using the clinically approved carbonic anhydrase inhibitor methazolamide (which blocks aquaporin-1-mediated water influx into platelets undergoing procoagulant activation) reduced the incidence and severity of DVT in mice⁶⁴, offering a potential new therapeutic approach for this disease.

The upstream mechanisms and molecular triggers that induce platelet procoagulant activation in venous thromboembolism remain unclear, given that venous thrombus formation usually occurs in the absence of exposure of extracellular matrix components. However, thrombin might have a role given that mice with a mutation-mediated decrease in PAR4 activity have reduced procoagulant platelet formation and flow-reduction-induced venous thrombosis compared with mice with normal PAR4 activity, and mice with platelet-specific *Par4* deletion were protected from laser-induced venous thrombosis¹⁰⁵. Leukocyte-induced inflammation has an important role in DVT¹⁰⁶, with neutrophil extracellular traps (NETs)^{10,107,108} and neutrophil-derived soluble factors such as high mobility group protein 1 (ref. 109) directly affecting thrombus size and incidence. Future studies will be necessary to identify whether these factors and other elements, such as antibodies and complement components¹¹⁰, affect platelet procoagulant activation in DVT.

Ischaemic stroke is a major health-care problem and remains one of the most frequent causes of cardiovascular death and disability worldwide¹¹¹. Several studies have addressed the role of procoagulant platelets in patients with stroke and in mouse models^{65,112,113}. Furthermore, clinical evidence suggests that high systemic levels of procoagulant platelets are predictive of the risk of ischaemic stroke after a transient ischaemic attack, whereas low levels of circulating procoagulant platelets are associated with intracranial bleeding and haemorrhagic transformation, suggesting a delicate balance between protective and detrimental roles for platelet procoagulant activation^{114–119}. Although the study in platelet-specific *Ano6*-knockout mice described earlier found reduced carotid thrombus formation with platelet-specific TMEM16F deficiency, it found no difference in functional stroke outcomes and brain infarction volumes in these mice⁸³. A study in a mouse model of stroke induced by transient middle cerebral artery occlusion described a detrimental role of platelet procoagulant activation, showing that procoagulant platelets promoted neutrophil recruitment to the infarcted brain regions⁶⁵. In this mouse model,

genetic deletion of *Ppid* in platelets decreased neutrophil recruitment and vascular occlusion, leading to improved cerebral blood flow, smaller stroke volumes and improved neurological outcomes as assessed by functional scoring compared with mice with intact *Ppid* in platelets. Interestingly, the protective effects of preventing procoagulant platelet activation were not observed with depletion of neutrophils^{16,120}, suggesting that neutrophils and NETs are downstream effectors of procoagulant platelet-mediated thromboinflammation in stroke.

Taken together, these findings suggest an important role of platelet procoagulant activation in (immuno)thrombosis across different cardiovascular diseases in both the arterial and venous systems (Fig. 3).

Procoagulant platelets in thromboinflammation

Platelets are important for immunity and aid in host responses through a plethora of functions, including direct antimicrobial effects, serving as landing pads for leukocyte recruitment and participating in immunothrombosis to prevent pathogen dissemination^{121–124}. Consequently, thrombocytopenia has been shown to aggravate disease severity during septic inflammation¹²⁵. The interaction of procoagulant platelets with leukocytes, specifically neutrophils, has been documented by several studies. An *in vitro* study showed increased platelet–neutrophil interactions after platelet procoagulant activation¹²⁶. A seminal study described the detrimental role of the procoagulant-platelet–neutrophil interplay in remote thrombosis after intestinal ischaemia–reperfusion injury in mice¹²⁷. Ischaemia–reperfusion injury induced the formation of procoagulant platelets in the ischaemic gut vasculature in a CypD-dependent manner, which led to abundant interactions of procoagulant platelets with neutrophils and the formation of procoagulant-platelet–neutrophil macroaggregates that were responsible for subsequent occlusion of pulmonary vessels¹²⁷. Additional studies have described that phosphatidylserine exposure on the outer layer of the plasma membrane in platelets facilitates their interaction with neutrophils, such as in the context of thrombus formation^{126,128}.

Research on COVID-19 has led to crucial findings on the role of procoagulant activation in thromboinflammation¹²⁹. Patients with severe COVID-19 were found to have increased levels of procoagulant activation in circulating platelets compared with healthy controls and patients with less severe COVID-19, and the level of procoagulant activation positively correlated with plasma D-dimer levels, thrombotic complications and mortality³³. Mechanistically, circulating immunoglobulins (namely, IgG) induced platelet procoagulant activation via FcγRIIA, phosphoinositid-3-kinase (PI3K) and protein kinase B (AKT). Blockade of either signalling hub (including antibody-mediated blockade of FcγRIIA, antagonizing FcγRIIA downstream signalling through increasing cyclic AMP levels and prevention of PI3K or AKT phosphorylation) reduced platelet procoagulant activation^{33,130,131}, suggesting a new therapeutic approach to COVID-19-associated thrombotic complications. Another proposed mechanism of COVID-19-associated procoagulant activation is that the neutrophil-derived alarmin S100A8–S100A9 induces procoagulant activation in a GPIIbα-dependent manner⁵⁰. However, other studies have provided evidence that isolated platelets from patients with severe COVID-19 show markedly reduced procoagulant responses upon dual agonist stimulation, suggesting an exhausted platelet phenotype^{132,133}. Whether these discrepancies are a result of patient heterogeneity, disease severity or the underlying severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variant remains unclear. No therapeutic intervention

studies in mouse models of COVID-19 have been conducted so far; thus, whether alterations in procoagulant activation are an epiphenomenon of disease severity or are distinct drivers of thromboinflammatory complications is unknown.

VITT linked to vaccines for SARS-CoV-2 is associated with platelet procoagulant activation¹³⁴. High levels of antibodies against platelet factor 4 (PF4) were found in sera from patients with VITT, and these antibodies activated human platelets *in vitro* through binding FcγRIIA and induced procoagulant activation¹³⁵. Platelet procoagulant activation *in vitro* was dependent on the presence of both heparin and PF4 and particularly increased after platelet priming with PARI agonists¹³⁶. Interestingly, platelet procoagulant activation has also been described in anti-PF4-negative thrombosis and thrombocytopenia syndromes, whereby platelet-activating histone–antihistone IgG complexes were found to induce procoagulant platelet activation *in vitro*¹³⁷.

Similar to VITT, HIT is characterized by a systemic procoagulant state with disseminated thrombosis and a hyperactivated platelet state^{138,139}. However, in HIT, immune complexes target a PF4 epitope outside the heparin-binding pocket, whereas in VITT antibodies target the PF4 heparin-binding pocket¹⁴⁰. In HIT, heparin binds to platelets through FcγRIIA and leads to strong platelet activation, particularly the formation of procoagulant platelets in a calpain-dependent manner¹⁴¹. The ensuing increase in platelet phosphatidylserine exposure was shown to contribute to the procoagulant state observed in HIT and, therefore, has been proposed to serve as a diagnostic screening tool for patients with suspected HIT^{142,143}. Antibody-mediated blockade of platelet FcγRIIA or increasing platelet intracellular cyclic AMP levels with a prostacyclin analogue (which inhibits platelet activation) were shown to prevent HIT immune complex-dependent thrombin generation and procoagulant activation-associated thrombus formation *in vitro*¹⁴³. However, the *in vivo* relevance of these findings remains unclear. Considering the important role of neutrophil activation and NET formation in HIT¹⁴⁴, as well as the observed increases in procoagulant–platelet–neutrophil interaction described earlier, future research into how procoagulant activation might shape leukocyte recruitment and activation in the context of HIT is warranted.

Despite the mechanistic evidence available thus far, whether platelet procoagulant activation is truly a driving force in all the disorders described earlier or is merely an epiphenomenon of systemic hyperinflammation is not completely clear. In addition, although platelet procoagulant activation is associated with a prothrombotic state and (remote) collateral damage, this platelet subset can also mediate protective effects during inflammation. In septic inflammation and subsequent neutrophil extravasation, localized formation of procoagulant platelet sentinels was shown to protect from inflammatory bleeding in mice¹⁹. Furthermore, aged platelets, which are skewed towards a procoagulant state, possess superior antimicrobial capability compared with their younger counterparts²³. Given these findings and the notion that low procoagulant platelet levels are associated with an increased risk of bleeding in patients with stroke-associated thromboinflammation¹¹⁵, procoagulant activation should not be considered as purely detrimental and might indeed contribute to preserve vascular integrity and pathogen containment in inflammation¹⁴⁵. We hypothesize that the net effect of procoagulant platelets depends on both location and abundance of procoagulant activation: locally formed procoagulant platelets might exert a protective role, whereas procoagulant platelets activated in the circulation or in great abundance might cause and propagate systemic thromboinflammation (Fig. 3).

Diagnostic and therapeutic potential

Assessing the activation state of circulating platelets or the cargo released by activated platelets can provide diagnostic insights in the setting of thrombotic disorders, as shown for cardiovascular diseases such as stroke¹⁴⁶, venous thrombosis⁶⁴, pulmonary embolism¹⁴⁷ and coronary artery disease^{101,148,149}, and in autoimmune diseases such as HIT¹⁴² and VITT¹³⁶. The observation of increased platelet procoagulant activation in these different disease settings highlights the broad involvement of platelet procoagulant activation in human thromboinflammatory diseases and might prove to be useful in ruling out certain thrombotic disorders in which the pathophysiology relies on procoagulant platelet formation, in estimating disease severity or for monitoring therapeutic efficacy after targeted intervention. In addition to classical molecules such as annexin V and lactadherin, new procoagulant activation-related molecules have emerged as valuable for both diagnostic testing and experimental approaches such as *in vivo* imaging. In particular, tripeptide trivalent arsenical 4-(*N*-(*S*-glutathionylacetyl) amino) phenylarsenoxide (GSAO)^{28,136} and the C1 multimer Apotracker Tetra^{19,64,150–152} can be useful for the detection of phosphatidylserine^{high} procoagulant platelets *in vitro* and *in vivo*.

In terms of therapeutic targeting, the predominant association of platelet procoagulant activation with acute thrombotic disorders such as ischaemic stroke makes the interference with procoagulant platelet generation ('antiprocoagulant antithrombotics')¹⁵³ an attractive therapeutic strategy. Of note, previous work has found little-to-no effect of clinically available antiplatelet drugs on procoagulant activation (including aspirin and P2Y purinoceptor 12 inhibitors^{28,34,150,154}). In contrast to these antiplatelet drugs, interference with signalling cascades leading to procoagulant activation is generally considered to be safer in terms of bleeding complications. In patients with Scott syndrome, a hereditary disorder characterized by TMEM16F deficiency, procoagulant platelets or phosphatidylserine-rich procoagulant membranes in other cell types cannot be formed owing to systemic inability to flip phosphatidylserine to the outer membrane, and these patients have a comparably mild bleeding phenotype unlike that in patients with haemophilia^{155,156}. Compared with wild-type mice, mice with whole-body knockout of *Ano6* have markedly increased haemorrhage following tail vein injury⁷⁵, whereas mice with platelet-specific deletion of either *Ppid* or *Ano6* show no overt bleeding or a mild bleeding phenotype after traumatic tail vein injury^{19,83}.

Several studies have highlighted different treatment strategies for selective targeting of platelet procoagulant activation. Physical blockade of phosphatidylserine exposed on platelets using lactadherin¹⁵⁷ and (di)annexin^{158–160} reduced thrombus formation but, to some degree, also aggravated haemorrhage in mice *in vivo*. An integrin αIIbβ3-binding and phosphatidylserine-binding recombinant fusion protein efficiently blocked phosphatidylserine-mediated thrombosis *in vivo* in mice, but was also associated with prolonged bleeding time after tail injury¹⁶¹. Phosphatidylserine-targeting nanoparticles were shown to reduce procoagulant platelet-mediated thrombosis without affecting bleeding times in mice *in vivo*¹⁶², offering an exciting treatment strategy. In a different approach, maintaining flippase activity and thus membrane asymmetry using a small molecule impaired procoagulant activation and reduced thrombin generation after stimulation of platelets with the Ca²⁺ ionophore A23187 *in vitro*¹⁶³. In mice, pharmacological inhibitors of carbonic anhydrases, which also interfere with aquaporin 1 function¹⁶⁴, reduced arterial and venous thrombosis without affecting haemostasis after tail vein injury by inhibiting platelet water influx and ballooning^{17,64,81}. However, given the dual role of integrin αIIbβ3

and GPVI in promoting procoagulant activation, combined low-dose administration of available drugs that target both receptors might selectively increase the threshold for procoagulant activation without affecting haemostasis^{19,165,166}.

In addition to these classic targets, targeting the non-canonical platelet repertoire, including ITAM receptors and their downstream signalling cascades, might have clinical utility in reducing platelet procoagulant activation, as shown for the antibody-mediated blockade of FcγRIIA in vitro¹³⁵ or inhibition of tyrosine-protein kinase BTK in vitro and in vivo^{167,168}. However, most of the currently available mechanistic data is derived from in vitro models, and for most (cardiovascular) diseases, the upstream mechanisms culminating in procoagulant activation in vivo remain unknown. Unravelling these upstream factors might contribute to the identification of disease-specific therapeutic targets of procoagulant activation.

In the setting of bleeding and coagulopathies, boosting procoagulant activity might be desirable¹⁶⁹. Given the capacity to rapidly recruit and cluster coagulation factors on their surface, increasing local procoagulant platelet accumulation might be a beneficial strategy whenever haemostasis is warranted. In this sense, a study in mice and rats showed that platelet-mimicking procoagulant nanoparticles can augment haemostasis in vivo¹⁷⁰. Similarly, platelet-like nanoparticles ameliorated bleeding in mouse models of von Willebrand disease¹⁷¹ and other animal models of bleeding, including traumatic liver laceration^{172,173}. In patients with mild congenital platelet function defects, administration of desmopressin, which is clinically used to increase the availability of large vWF multimers in von Willebrand disease, selectively enhanced the potential to turn platelets procoagulant through increasing Ca²⁺ levels¹⁷⁴, suggesting that desmopressin might also be of clinical use in platelet disorders associated with reduced procoagulant activation.

Beyond thrombosis and haemostasis, a study in mice showed that GPVI-induced platelet procoagulant activation in the tumour microvasculature led to an upregulation of immune checkpoint molecules on procoagulant platelets, which hampered T cell-mediated immunity and promoted tumour evasion¹⁷⁵. These data highlight the importance of context-specific therapeutic targeting as well as the Janus-faced role of procoagulant platelets during (thrombo)inflammation.

Conclusion

Our understanding of the mechanisms leading to procoagulant activation and the pathophysiological consequences of procoagulant platelet generation across several thrombotic and inflammatory diseases have markedly increased over the past decade. Furthermore, the field has identified both diagnostic and therapeutic tools that might enter clinical investigation for select indications to broaden our therapeutic options for thrombotic disease. Despite the increased bleeding susceptibility observed in patients with Scott syndrome, experimental evidence suggests that selective targeting of procoagulant activation could be a useful alternative to contemporary pharmacological platelet modulation, given that this approach could be used to treat and prevent arterial and venous thrombosis, as well as inflammatory diseases. Importantly, platelet-selective targeting will be required to avoid systemic bleeding (as observed in whole-body *Ano6* knockout animals⁷⁵). This targeted approach would be key for any antithrombotic therapy to surpass the overall clinical efficacy and safety of currently used antithrombotics.

Before advancing to clinical trials, several issues need to be addressed, including in which settings physiological platelet responses, such as procoagulant activation, become detrimental to the host;

whether different subsets of procoagulant activation in terms of procoagulant activity exist (for example, depending on the stimulating agonists); or how agents that either inhibit or boost platelet procoagulant activation can be safely and selectively administered, without causing either haemorrhage or disseminated coagulopathy. Finally, although some studies have shown mechanistic evidence of platelet procoagulant activation being harmful (for example, in ischaemia–reperfusion injury¹²⁷ and ischaemic stroke^{65,95}), the delicate interplay between platelet procoagulant activation and immune cells is still incompletely understood and will require further attention in future studies. In summary, despite many open questions, the exciting field of procoagulant platelet research has advanced and will probably soon lead to translationally relevant therapeutic opportunities.

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R.K. wrote the initial manuscript draft. R.K. and L.N. edited the manuscript, contributed to the discussion of its content and designed the figures before submission.

Competing interests

The authors declare no competing interests.

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