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REVIEW ARTICLE

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Integrating platelet and coagulation activation in fibrin clot formation

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Abstract

Platelets interact with the coagulation system in a multitude of ways, not only during the phases of thrombus formation, but also in specific areas within a formed thrombus. This review discusses current concepts of platelet control of thrombin generation, fibrin formation and structure, and anticoagulation. Indicated are how combined signalling via the platelet receptors for collagen (glycoprotein VI) and thrombin induces the secretion of (anti)coagulation factors, as well as surface exposure of phosphatidylserine, thereby catalysing thrombin generation. This procoagulant platelet response is also facilitated by the adhesive complexes glycoprotein Ib-V-IX and integrin $\alpha_{IIb}\beta_3$. In the buildup of a platelet-fibrin thrombus, the extrinsic, tissue factor–driven coagulation pathway is predominant in early stages, while the intrinsic, factor XII pathway seems to promote at later time points. Already early generation of thrombin enforces platelet responses and stimulates intra-thrombus heterogeneity with patches of loosely aggregated, contracted, and phosphatidylserine-exposing platelets. Fibrin actively formed on the surface of activated platelets supports thrombus growth, but also captures thrombin. The fibrin distribution in a thrombus appears to rely on the local procoagulant trigger and the blood flow rate. Clinical studies support the importance of the platelet-coagulation interplay, by showing beneficial effects of combination therapy in the secondary prevention of cardiovascular disease.

KEYWORDS

coagulation, fibrin, platelets, thrombin, thrombus formation

Essentials

- Activated platelets secrete coagulation factors, expose PS, and support thrombin and fibrin formation.
- Platelet receptors for collagen and thrombin are complementary and enforce each other's activity.
- The extrinsic and intrinsic pathways are inversed balanced in the formation of a platelet-fibrin thrombus.
- Clinical studies support the high degree of interactions between platelets and coagulation.

1 | INTRODUCTION

Over the last decade, it has become clear that the conventional concept of hemostasis and thrombosis, relying on platelets first and coagulation second, needs to be revised. Platelet and coagulation activation are not separate processes, but need to be considered as highly reciprocal and interconnected processes.[1,2] The importance in (patho)physiology of the interplay between platelets and coagulation is evident from in vivo experimental thrombosis models, from clinical samples of dissected thrombi in arteries and veins ex vivo, as well as from complementary effects of antiplatelet and anticoagulant therapies. Mouse studies have shown that: (i) collagen as well as thrombin via tissue factor (TF) initiate arterial thrombus formation,[3] (ii) both platelet and coagulation activation contribute to the thrombotic process in arteries and veins,[4] (iii) the coagulation end product fibrin is present in initial stages of a thrombus,[5] and (iv) essentially all thrombosis models are sensitive to deficiencies in either platelet or coagulation factors.[6]

How platelets interact with the coagulation system can be understood traditionally as a phased process during thrombus formation.[7,8] Or rather, as we begin to understand it now, as a sequence of interactions occurring in specific areas within the developing thrombus. The general consideration is that thrombus formation starts with exposure of collagen and TF in the vessel wall, triggering platelet adhesion along with formation of first traces of thrombin. In amplification phases, procoagulant platelets in various forms promote thrombin accumulation, and aggregated platelets contract. Then there is, what we thought to be the final stage, the phase of fibrin formation, although it is now realised that the "end product" fibrin can have a role in both platelet and coagulation activation.

2 | HOW DO PLATELETS CONTROL THROMBIN GENERATION?

Platelets regulate coagulation reactions leading to thrombin generation in multiple ways; by phosphatidylserine (PS) exposure; by binding coagulation factors via the glycoprotein complexes GPIb-V-IX, integrin $\alpha_{IIb}\beta_3$ and GPVI; and via thrombin-induced activation of the protease-activated receptors (PARs) (Figure 1A). How to conceptually integrate these interaction models, is an active area of research.

2.1 | Establishment and roles of PS exposure

Exposure of the negatively charged phospholipid PS at the membrane surface has shown to be a controlling process in hemostasis, as recently observed in mice carrying platelets with deficient PS exposure.[9] Yet, upon injury or activation, also the endothelium and other vascular cells can provide a PS-exposing surface. In platelets, the PS exposure is triggered by strong agonists (via prolonged elevated cytosolic Ca^{2+}), as well as in apoptosis (Ca^{2+}-independently via caspases) or necrosis.[10] It was established that the ion channel, anoctamin-6 (gene *ANO6* or *TMEM16F*), is a crucial player in Ca^{2+}-dependent PS exposure (also indicated as phospholipid scrambling), as well as swelling and ballooning of activated platelets.[11] Defective anoctamin-6 expression, such in patients with Scott syndrome (*ANO6* mutations) or deficient mice, leads to a mild bleeding phenotype, and platelets with failure to Ca^{2+}-dependent PS exposure and ballooning.[12] A typical agonist combination causing PS exposure is that of collagen plus thrombin, relying on signalling via GPVI. Downstream signalling components required for a prolonged Ca^{2+} elevation are the Fc receptor γ-chain (FcRγ), LAT, Src-family kinases (SFK), Syk, phospholipase Cγ2 (PLCγ2), and phosphatidylinositol 3-kinase (PI3K) isoforms β and γ.[2] Other contributing pathways are entry of extracellular Ca^{2+} via Orai1 channels and the STIM1 sensor, and Ca^{2+} liberation from mitochondria.[13] Activation of the C-type lectin-like receptor 2 (CLEC-2), by the snake venom toxin rhodocytin or the endogenous ligand podoplanin, results in a similar signalling cascade. Interestingly, thrombi formed on a rhodocytin surface showed relatively high PS exposure, pointing to a role of CLEC-2.[8] Structurally, PS-exposing platelets rapidly swell and transform to a balloon shape, thereby increasing their procoagulant surface.[14]

The procoagulant role of PS-exposing platelets have been attributed to the high-affinity binding of Gla-domain containing coagulation factors, i.e. (activated) prothrombin, FVII, FIX, and FX.[15] Cofactor FV can bind to PS membranes via its C2 domain.[7] Microscopy studies indicated a near complete colocalization of the constituents of the tenase (FVIIIa and FIXa, activating FX) and prothrombinase (FVa and FXa, activating prothrombin) complexes with PS-exposing platelets.[16,17] Of note, for FVIII(a) only sparse colocalization was observed, with the majority of FVIII costaining with von Willebrand factor (VWF), likely acting there as a supply pool.[17] Kinetic studies have shown that PS-containing membranes enhance the activities of tenase and prothrombinase complexes by up to 1000-fold.[18] PS-exposing platelets also shed extracellular vesicles (microparticles) via a mechanism that is still partly unclear. Such vesicles can accumulate under pathophysiologic conditions.[19]

The PS-exposing platelets are often confused with coated platelets.[20] The latter form a subpopulation, also arising after strong agonist stimulation, which is characterized by the formation of a covalent coat, containing transglutaminase-anchored platelet-derived proteins.[21] Conceptually, it is now believed that, after initial PS exposure, FXIII activation by thrombin is required to de-encrypt the transglutaminase activity, and to allow cross-linking of multiple proteins including fibrin at the platelet surface.[20] Both transglutaminase and integrin $\alpha_{IIb}\beta_3$ interactions are required for a platelet control of fibrin formation.

Activated platelets carry and secrete multiple coagulation factors (prothrombin, FV, an *F8* transcript, FXIII, fibrinogen) and anticoagulation factors (antithrombin, various serpins). The picture that emerges from the abundance analysis of coagulation factors in both plasma and platelets (Figure 2) is that especially factors implicated in later stages of the coagulation process are stored in platelets. It can be hypothesized that especially the latter are important for formation of a sufficiently "strong" thrombus supporting hemostasis.

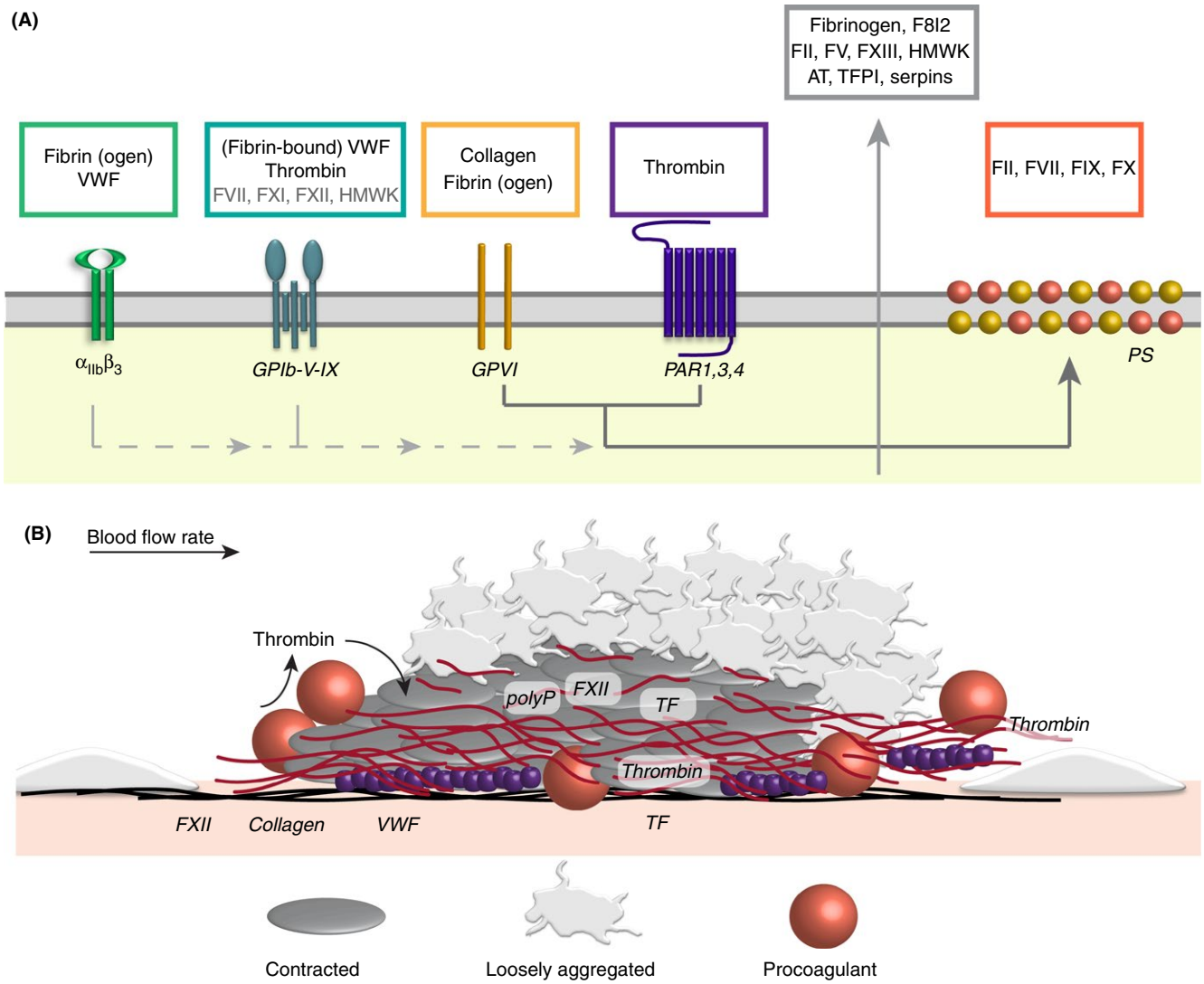

FIGURE 1 Platelet-coagulation interactions on individual platelets and in a platelet-fibrin thrombus. (A) Key platelet receptors and their (procoagulant) ligands as constituents of the platelet procoagulant function. Indicated is the contribution of platelet receptors to PS exposure (straight line, inducing; dashed line, supporting) and the binding of procoagulant factors to the PS-exposing surface, magnifying thrombin generation. Also by secreting a variety of (anti)coagulant factors, activated platelets modulate the coagulant response. (B) Platelet-fibrin thrombus showing the dynamic interaction between platelets and the coagulation system with: local platelet heterogeneity, thrombin feedback and dense vascular-oriented fibrin. Highlighted herein is the extension of the fibrin network recruiting and activating additional platelets. For further description, see text.

Exemplary is a patient with severe congenital FV deficiency, in whom residual platelet-stored FV was considered to be sufficient to prevent major bleedings. In this patient, also thrombin generation was detected in platelet-rich plasma.[22]

Together, this leads to a concept, where activated platelets (coactivated by thrombin) secrete coagulation factors, expose PS, support thrombin generation, and form a fibrin coat at their surface.

2.2 | Interactions of GPIb-V-IX with VWF/FVIII and other factors

Interaction of the GPIb-V-IX complex with immobilized VWF is an initial step in shear-dependent thrombus formation and, by implication, for ensuing platelet procoagulant activity. The GPIb complex is tightly connected with the cytoskeleton, thus defining the platelet structure.[23] On its own, GPIb-V-IX interaction with VWF induces merely weak intracellular signals (involving 14-3-3, SFK, PI3K, and small GTPase isoforms), causing only limited integrin $\alpha_{IIb}\beta_3$ activation and platelet spreading.[24] An initial report suggested that this interaction, in coagulating plasma, enhances platelet (and PS)-dependent thrombin generation.[25] However, more recent data point to a role of the glycoprotein complex in platelet procoagulant activity independent of VWF and GPIbα.[26] Intriguingly, also a role of 14-3-3ζ in mitochondrial-dependent PS exposure appeared to be independent of VWF-GPIb interactions.[27] Hence, the last word on this topic has not yet been said.

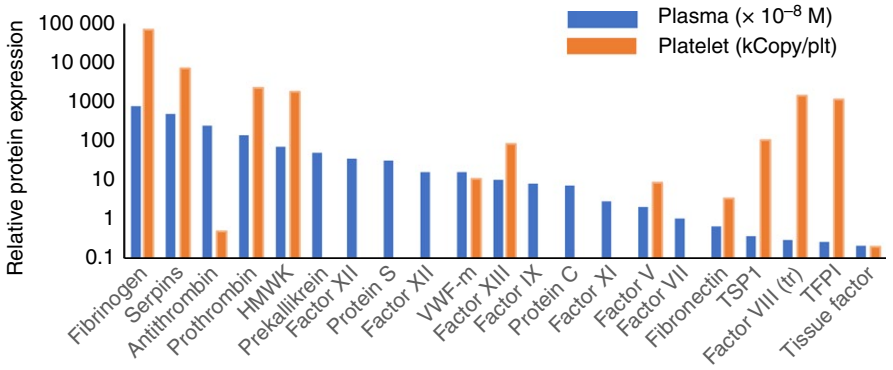

FIGURE 2 Relative abundance of (anti)coagulation proteins in platelets and plasma. Shown are the protein copy numbers (kCopy) of indicated (anti)coagulant factors per platelet (grey) as determined by mass spectrometry.[110] In addition, average factor levels in normal plasma (×10^{-8} mol L^{-1}, black). For FVIII, the platelet copy number refers to the *F8* transcript F8I2. HMWK, high molecular weight kininogen; VWF-m, VWF-monomers; TSP1, thrombospondin 1; TFPI, tissue factor pathway inhibitor

The platelet GPIb-V-IX complex furthermore provides a high-affinity interaction site for thrombin, thus potentiating platelet activation through PAR1 and PAR4 receptors.[28] In addition, the GPIb complex can bind multiple other coagulation factors, such as FVII(a), FXI, FXII, and high molecular weight kininogen. The functional consequences of coagulation factor binding (other than thrombin) to GPIb-V-IX (or alternatively the apolipoprotein E receptor 2) is still largely unclear.[7] A recent paper indicated that thrombin-stimulated FXI activation, in a way depending on GPIb, contributes to vascular inflammation in hypertensive mice,[29] thus pointing to a complex interaction system of GPIb, thrombin and FXI.

2.3 | Interactions of integrin $\alpha_{IIb}\beta_3$

Integrin $\alpha_{IIb}\beta_3$ is expressed abundantly with an estimated 80 000 copies per platelet, also with links to the actin cytoskeleton. The integrin binds fibrinogen, fibronectin, VWF, and other plasma proteins, after an agonist-induced conformational change that results in an open, activated state.[30] Integrin activation is pivotal for platelet aggregation, and hence for thrombus buildup and stability under flow.[31,32] Subsequent outside-in signalling of ligand-occupied $\alpha_{IIb}\beta_3$ can trigger several coagulation-stimulating platelet responses. Recent evidence shows that $\alpha_{IIb}\beta_3$ can mechanically sense soluble fibrinogen versus platelet-bound fibrinogen to avoid "spontaneous" platelet activation.[33]

During thrombus formation, integrin outside-in signalling via Src and Syk tyrosine kinases can enforce the initial thrombin-induced Ca^{2+} rises that mediate PS exposure and ensuing massive thrombin generation.[34] This explains why integrin antagonism causes a decreased thrombin generation in platelet-rich plasma and, thus, prolongs the clotting time.[35] In flow studies, treatment of patients with unstable coronary syndrome with the $\alpha_{IIb}\beta_3$ antagonist, abciximab, did not only suppress platelet accumulation, but also fibrin formation.[36] Overall, integrin signalling appears to enhance procoagulant PS exposure, but is insufficient to cause this by itself.

Integrin outside-in signalling is also required for the retraction of a platelet-fibrin thrombus.[37] Current understanding is that the activation of $\alpha_{IIb}\beta_3$ is a highly dynamic process, requiring persistent platelet stimulation provided by autocrine and paracrine factors, especially ADP (via $P2Y_{12}$ receptors).[38] A negative feedback mechanism is provided by $\alpha_{IIb}\beta_3$ in-activation, which in high-Ca^{2+}, PS-exposing platelets is mediated by calpain cleavage of the β_3 chain and associated proteins, including Src and talin.[39]

Summarizing, the roles of the adhesive complexes GPIb-V-IX and $\alpha_{IIb}\beta_3$ in coagulation appear to be threefold, namely: (i) facilitating (thrombin delivery, enhanced signalling), (ii) structural (cytoskeletal linked, anchoring fibrin), (iii) providing binding sites for multiple coagulation factors.

2.4 | Initiating and feedback interactions of GPVI

Stable platelet adhesion to collagen type I (to which VWF binds) relies on the receptors GPVI and integrin $\alpha_2\beta_1$.[40,41] Thrombin clearly supports the signalling processes, as it potentiates the rises in Ca^{2+}, activation of integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$, and exposure of PS.[18] In mice, blocking of GPVI or deficiency in either FcRγ, LAT, Syk or PLCγ2, resulted in diminished collagen-induced thrombus formation, PS exposure as well as thrombin generation.[42] On the other hand, a gain-of-function mutation in PLCγ2 increased collagen-induced thrombus formation and PS exposure.[43]

First evidence for an additional role of GPVI in later stages of thrombus formation came from the observation that, in a mouse model of $FeCl_3$-induced injury, GPVI deficiency or depletion mostly affected later stages of thrombus formation and vessel occlusion.[44] This agrees with a recently identified role of GPVI as receptor for fibrin, likely to be relevant for continued growth of a platelet-fibrin thrombus.[45] For both human and mouse platelets, fibrin adhesion leads to formation of a GPVI signalosome, independently of $\alpha_{IIb}\beta_3$.[45] Increased exposure of PS is measured in this condition as well. Furthermore, in platelet-rich plasma from GPVI-deficient patients, a

marked impairment was seen of thrombin generation, along with reduced platelet adhesion to fibrin at low (300 s^{-1}) and high (1500 s^{-1}) shear rates.[46]

In addition to these positive feedback loops between platelet and coagulation activation, there is evidence for a negative feedback process, realised by FXa-dependent shedding of GPVI on activated platelets.[47] Interestingly, no role of the protease thrombin was found in this GPVI proteolytic cleavage.

Taken together, this indicates that fibrin can play its own role in the mutually stimulatory effects of collagen and thrombin on platelets.

2.5 | Interactions by thrombin receptors

Thrombin is a key protease implicated in the initiation and propagation of the coagulation cascade, in platelet activation and in fibrin formation.[2] Prothrombin binds to procoagulant platelets via its Gla-domains. Once cleaved, the thrombin strongly interacts with the platelet GPIb and PAR receptors (PAR1/4 in human, PAR3/4 in mouse), thus inducing multiple responses. In the human situation, higher concentration of thrombin are required for the cleavage of PAR4, when compared to PAR1.[48,49] Both isoforms are Gq-coupled receptors and signal via PLCβ, causing quantifiable cytosolic Ca^{2+} spiking,[50] and only limited PS exposure. Thrombin-induced PAR activation enhances the GPVI-induced PS exposure of platelets.

The blood flow rate was found to be a modulating factor determining the contribution of PAR isoforms to platelet activation, i.e., declining at (pathologically) high shear rates.[51] Under flow conditions, thrombin initially binds to PS-exposing platelets, but then relocalizes to the newly formed fibrin fibers.[16] Hence, in a developed thrombus, most of the thrombin appears to be captured by fibrin. In other words, the fibrin fibers extending from a platelet thrombus function as a thrombin sink, confining this protease to the thrombus proximity. Early findings suggested that fibrin-bound thrombin is protected from inactivation by antithrombin,[52] implicating that thrombin's activity near a thrombus is relatively high.

The PAR-type thrombin receptors can additionally be cleaved by several matrix metalloproteinases including ADAM17[53,54] and neutrophil-derived cathepsin G.[55] Cleavage of PARs can also be accomplished by activated protein C (APC, specific for PAR1), and the fibrinolysis protease plasmin (specific for PAR4).[56] However, the relative contribution of PAR cleavage by proteases other than thrombin under flow conditions is still unknown.

The platelet-activating receptors for collagen (GPVI) and thrombin (PAR1/4 human, 3/4 mouse) are complementary in signalling and thus enforce each other's activity (e.g., in PS exposure). Both receptor types can act in both early stages (TF-produced thrombin, collagen exposure) and late stages (fibrin as a thrombin sink, GPVI as fibrin receptor) of thrombus formation and clotting.

3 | HOW DO PLATELETS CONTROL FORMATION AND PROPERTIES OF FIBRIN?

Fibrin is actively formed on the surface of activated platelets, with triggering via both the extrinsic (TF, FVII) and intrinsic (FXII, FXI) coagulation pathways. Platelets furthermore alter the fibrin network structure and coordinate the contraction of a clot (Figure 1B).

How platelets and flow change the formation and structure of fibrin clots is still largely unknown.

3.1 | Fibrin formation at the platelet surface

The traditional view is that the growth of a platelet thrombus is stabilized by thrombin and fibrin, likely formed via both the extrinsic and intrinsic routes.[57,58] Mechanistic studies also showed that, at sufficient thrombin generation, the fibrin network can extend from the platelet and thrombus area.[59] The platelet-dependent fibrin formation is in particular triggered by TF, and contributes to the formation of a densely packed thrombus core.[60] Extensive fibrin formation can also be observed on coated platelets and, here, relies on transglutaminase (FXIII) activity and $\alpha_{IIb}\beta_3$ binding.[20]

3.2 | Roles of TF and FVII

Triggering of the extrinsic pathway occurs through TF, a membrane protein highly expressed on subendothelial cells (smooth muscle cells, fibroblasts, monocytes, macrophages) and at a limited extent on the inflamed endothelium.[2,61] Thrombin generation via TF requires the presence of procoagulant membranes, which can be provided by PS-exposing platelets.[2] Platelets can furthermore deliver protein disulphide isomerases, which help to de-encrypt TF into its active form. There is a long debate whether the limited amount of TF expressed by platelets is capable to trigger thrombin generation.[62] Platelets most likely inherit TF from their precursor cells, since functionally active TF could be identified in proplatelets shed from megakaryocytes.[63] However, given the presence of relatively high amounts of tissue-factor pathway inhibitor (TFPI) in platelets, the actual contribution of platelet-derived TF likely is limited to specific environments.[64] The physiological role of TF-expressing extracellular vesicles (microparticles)[65] is not well understood.

During the in vivo buildup of a platelet-fibrin thrombus, the TF-FVII(a) complex can play a rate-limiting role.[66] Also under flow conditions ex vivo, immobilized TF (and FVII) together with collagen supports high levels of thrombin generation, with a late-stage contribution of FXI and with fibrin serving a potent thrombin-capturing mechanism.[67]

3.3 | Roles of FXI, FXII and polyphosphates

The importance of the intrinsic coagulation pathway, accomplished by FXII and FXI, in platelet-fibrin thrombus formation has been shown in vivo using deficient mice upon injury of healthy[68,69]

or atherosclerotic[66] arteries. Especially at low TF levels, this pathway can be considered pivotal for the formation of platelet-fibrin thrombi.[66] Experimental mouse models support a role of FXII and FXI in (later stages) of thrombus stabilization, since deficiencies in prekallikrein, FXII, or FXI promoted thrombus embolization.[70] Similarly, pharmacological inhibition of the FXII pathway resulted in detachment of platelets from the surface of arterial thrombi.[71]

Intrinsic triggers of the FXII/FXI pathway include binding to collagen,[58] while it also binds to fibrin fibers but not to PS-exposing platelets.[66] The presence of FXII at fibrin fibers in a developing thrombus may ensure fibrin-dependent progression of the intrinsic coagulation pathway. However, this FXII pool can also modify the fibrin clot structure.

Platelets can enhance coagulation via FXII (auto)activation through secreted polyphosphates.[72] However, the FXII-activating role has been questioned, as soluble polyphosphates secreted by platelets do not contain the proper longer chain length to enable this.[73] Kinetic studies indicated that the platelet-derived, short-chain polyphosphates rather enhance FV and FXI activation, and thus promote clot stability. The controversy may be resolved by recent insight that the FXII-activating potential can be provided by nanoparticles of clustered polyphosphates, associated with the platelet membrane.[74]

Overviewing the current findings, there appears to be an "inversed balance" between the contribution of extrinsic (TF, FVII) and intrinsic (FXII, FXI) pathways in the formation of a platelet-fibrin thrombus. The extrinsic route is most strongly active near the vessel wall with perhaps a small role of platelets (extracellular vesicles) expressing TF later on; the intrinsic route is moderately stimulated on collagen surfaces, and possibly enhanced later via polyphosphate clusters.

3.4 | Regulation of fibrin formation and properties

Several observations have shown that platelet-dependent fibrin formation starts at the thrombus base (near the site of TF), then growing upwards within and outside the thrombus, depending on the local procoagulant trigger.[75] Analysis of the elastic-mechanical properties pointed to a relatively low elasticity of the fibrin inside platelet thrombi. Key elements for high elasticity, outside of platelet thrombi, were a relatively low blood flow and a low TF trigger. When reducing the elasticity, e.g., by inhibiting fibrin polymerization, thrombi become vulnerable to shed emboli.[76]

By regulating the local thrombin concentration,[77] platelets can also indirectly influence the structure of the surrounding fibrin network. The prevailing concept is that, at low thrombin concentrations, thick fibrin fibers are formed in a loose network (more susceptible for fibrinolysis), while at high thrombin concentrations thin fibrin fibers pack into a tight network (more resistant to lysis).[78] An explanation for the lytic resistance is the low tissue plasminogen activator (tPA)-mediated plasmin generation at thinner fibrin fibers. Incorporation of red cells may lead to distinct regions of highly packed fibrin fibers next to areas of limited fiber formation.[79] Under specific circumstances, also neutrophils and red blood cells can contribute to thrombin generation. This is not further discussed here.

The complexity of platelet-fibrin interactions becomes evident from studies with patients with complete fibrinogen deficiency. These patients can suffer from both arterial and venous thrombotic events.[80] This is explained by a higher circulating thrombin activity, as generated thrombin is no longer retained by fibrin, with as a result increased thrombin-induced platelet activation.[81] On the other hand, a lack of fibrin in the patients' thrombi reduces the stability and increases embolization.[82] In platelets from Scott syndrome patients or anoctamin-6 deficient mice, with defective PS exposure, fibrin formation is also impaired.[9,75]

3.5 | Clot retraction

After the formation of a platelet-fibrin thrombus, clot retraction is essential for proper hemostasis as it tightens the wound edges. Clot retraction can also be considered as a way of platelet-platelet contact-dependent signalling.[83] Most likely, thrombus contraction refers to the same process of tightening platelet-platelet contacts in a fibrin environment,[37] although thrombus contraction can also occur at limited extent without fibrin.[84]

Clot retraction, in the presence of thrombin, relies on the binding of fibrin(ogen) to activated $\alpha_{IIb}\beta_3$ and subsequent outside-in signalling events, ultimately leading to actin-myosin rearrangements.[30] Platelets from multiple mouse strains with deficiencies in (integrin) signalling proteins, or lacking cytoskeleton-associated proteins show an impairment in clot retraction.[37] Strikingly, the nonaggregated, PS-exposing platelets do not contribute to this process, likely because of calpain degradation of the actin cytoskeleton.[39]

Taking this together, clot retraction may ensure that the vascular-oriented fibrin within a thrombus shows: (i) low elasticity (high stiffness), (ii) tight packing, (iii) and thrombus stabilising effect. The underlying mechanisms still need further elucidation.

4 | THE FEEDBACK LOOPS OF THROMBUS HETEROGENEITY

Multiple factors have been listed explaining the structural and functional heterogeneity of platelets, while in circulation or when assembled into thrombi.[7] In a growing thrombus, individual platelets become exposed to different micro-environments (e.g., binding to collagen or adjacent to TF). However, response variation has also been examined between single adhered platelets, seemingly exposed to the same micro-environment.[42] The intrinsic factors of platelet heterogeneity are largely unknown, but likely include variation between megakaryocytes and the ageing of platelets.[85] In vivo observations also point to an overall heterogeneity in thrombus buildup.[60] Platelets (with secretion and integrin activation) appear to be densely packed platelets in the thrombus core, which is

surrounded by loosely adhered platelets in the outer thrombus shell. This implies a certain degree of cross-cellular communication in the thrombus core, such as indeed has been reported.[86] Neighboring platelets can form gap junctions, which might be a requirement for dense packing and platelet contraction.[87]

Platelets with high Ca^{2+} fluxes, for instance upon collagen and thrombin costimulation, appear as another population in thrombi in vitro and in vivo. These platelets are characterized by PS exposure, ballooning, and microparticle shedding.[88] In mice, the injection of a PS scavenger (annexin A5 or lactadherin) resulted in a reduced arterial thrombus formation, underlining the procoagulant function this population.[4,89] Typically, these platelets are unable to $\alpha_{IIb}\beta_3$ activation,[39] and are separated from the patches of aggregated platelets.[16] This thrombus heterogeneity likely is caused—next to intrinsic differences between individual platelets—by different exposure of the platelets to soluble agonists such as ADP, thromboxane and thrombin, as well as by a varying hemodynamic environment.[2] Other factors for heterogeneous thrombus growth can be the vascular bed and the (patho)physiological state of the injured vessel.

Microfluidics studies have pointed to a positive feedback loop between PS-exposing platelets and thrombin activity under flow conditions. Thus, where coagulation is restricted—in hemophilia blood or dilutional coagulopathy—especially the population of PS-exposing platelets becomes reduced.[75] This points to a thrombin-dependent enhancement of PS exposure that results in additional thrombin generation. Intrathrombus heterogeneity is also observed for the fibrinolysis factor, plasmin, preferably binding to PS-exposing platelets.[90]

Conclusively, thrombus formation relies on a continuous and dynamic interaction between platelets and the coagulation system. Intrathrombus heterogeneity with different patches of (loosely) aggregated, contracted and PS-exposing platelets is enforced by the positive feedback loop of thrombin generation and thrombin responses.

5 | HOW DO PLATELETS CONTROL ANTICOAGULATION?

Coagulant activity is tightly balanced by both pro- and anticoagulant factors. Several anticoagulation factors are present in the blood, including the protein C/protein S complex, TFPI and antithrombin. Limited information is available on a role of platelets in the anticoagulant processes.

5.1 | Protein C, protein S and TFPI

The vitamin K-dependent proteins C and S are known to bind to PS-exposing membranes via their Gla-domains, with protein C as protease and protein S as membrane-binding cofactor. Thrombin-activated protein C (APC) selectively inactivates FVIIIa and FVa, thus suppressing tenase and prothrombinase activities. How the protein C/S anticoagulant pathway contributes to thrombus formation is unknown. Clinically, the importance of APC cleavage is emphasised by the fact that patients with a FV-Leiden mutation (carrying a FVa mutation that cannot be inactivated by APC) have an increased risk of venous thromboembolism. On the other hand, mice with a homozygous FV-Leiden mutation displayed normal mesenteric arterial thrombus formation.[4] A meta-analysis revealed a relative risk increase of myocardial infarction and coronary stenosis for carriers of the FV-Leiden allele of only 1.17, suggesting a no more than moderate association with arterial thrombosis.[91]

The anticoagulant TFPI, a Kunitz-type protease inhibitor, reversibly inhibits the TF/FVIIa, FXa, and protein C pathways, attenuating thrombin generation through proteolytic inhibition of the cofactors FVa and FVIIIa. The isoform TFPIα, carrying all three Kunitz domains, actively inhibits TF/FVIIa and FXa, interestingly in a protein S-dependent manner.[92] While TFPIα circulates in the blood at low concentrations, levels may locally increase due to platelet secretion. TFPI has indeed been detected on the surface of (coated) platelets. In vivo, mouse TFPI was shown to suppress thrombus growth.[64] Ex vivo, the role of (plasma) TFPI appeared to be confined to conditions of low coagulant strength.[93]

5.2 | Antithrombin

Antithrombin is a key serpin (serine protease inhibitor) that targets multiple activated coagulation factors, especially thrombin and to a lesser extent FVIIa and FIXa-XIIa.[2] Antithrombin is an effective inhibitor of thrombin generation in plasma, and its activity is greatly enhanced by heparins. Antithrombin has not yet been identified in developing thrombi.

Interestingly, PS-exposing platelets can serve as assembly sites for both coagulant and anticoagulant factors. How anticoagulants compete with the coagulant factors, however, remains unclear. For the active-site inhibitors, antithrombin and C1 inhibitor, no platelet binding sites are known.

Taken together, no more that little is known how anticoagulant factors interact with platelets, and how anticoagulant mechanisms can restrict thrombus growth and stability.

6 | PARADOXICAL EFFECTS OF BLOOD FLOW RATE

In general, arterial thrombi (formed at high wall shear rates) are rich in white platelets and fibrin fibers, whereas venous thrombi (formed at low shear rates) are usually more red with a fibrin network, platelet clumps, and incorporated red blood cells.[94] Microfluidics studies have provided insight into these processes.

Both platelet and coagulant activity rely on the local blood flow and shear conditions. A relevant parameter here is the wall shear rate (near-wall sliding rate), which is low in the venous part of the circulation, and gradually rises from large arteries to small arterioles of the microcirculation.[95] At arterial flow conditions, platelet deposition and hence thrombus formation increases with the wall-shear

rate (and hence flow rate) through GPIb-V-IX interaction with VWF. Also the contribution of GPVI and $P2Y_{12}$ receptors increases at higher shear rate.[96] At pathological, very high wall shear rates up to 10 000 s^{-1}, at sites of stenosis, VWF is released from the endothelium, thus further enforcing platelet deposition.[97] On the other hand, coagulation processes are enhanced at lower flow rates, as this limits dilution and facilitates thrombin accumulation. Mathematical models show that flow rate determines the transport rates of coagulation factors and thereby the extent of fibrin polymerization.[98]

Accordingly, depending on the flow conditions, either platelet adhesion (high shear) or thrombin/fibrin generation (low flow) can act as driving factors for thrombus formation.[16,75] By extension, local differences in shear and flow rate may result in a differential formation of thrombin and fibrin within a growing thrombus.[99] Typically, shear and flow rates will drastically increase during thrombus growth, a condition fostered by stenotic sites, which promote platelet adhesion, but also embolus shedding. On the other hand, interstitial flow rates can be relatively low near developing thrombi and stenotic sites, which will prevent coagulation factors from dilution.[100] This may explain the heterogeneous fibrin buildup often seen in and around a thrombus. Blood flow also stimulates the fibrinolysis process by enhancing the dissolution of platelet-fibrin thrombi, provided the presence of a fibrinolysis trigger such as tPA.[90]

The prevailing flow and wall shear conditions may, reciprocally, determine not only the global buildup of a platelet-fibrin thrombus, but also part of the heterogeneity in thrombin and fibrin accumulation within the thrombus.

7 | SOME CLINICAL CONSIDERATIONS

Antithrombotic therapy for the secondary prevention of cardiovascular events typically consists of two antiplatelet drugs, given for a certain period of time. In multiple studies, dual antiplatelet therapy (aspirin with $P2Y_{12}$ receptor blocker) has shown to be beneficial, although recurrent thrombotic events are not completely eliminated and the risk of bleeding events is relatively high.[101] As a possible improvement, the PAR1 antagonist vorapaxar has been examined. Vorapaxar only blocks the platelet effects of thrombin, while leaving unaffected thrombin's effects in coagulation.[102] The TRA2P-TIMI 50 study evaluated a combination of vorapaxar with aspirin and clopidogrel ($P2Y_{12}$ antagonist) in patients with stable acute coronary syndrome, and reported a lowering in cardiovascular events, but at the expense of increased major bleeding.[103] Complications here can be platelet-independent effects of vorapaxar, i.e., on endothelial and smooth muscle cell PAR receptors.

Concerning coagulation targeting, the early WARIS[104] and ASPECT[105] trials demonstrated a clinical benefit of vitamin K antagonists for the secondary prevention of coronary artery disease, although bleeding increased. Yet, dual-antiplatelet therapy was found to be more effective. More recently, direct thrombin and FXa inhibitors were evaluated in such patients, mostly in combination with dual antiplatelet therapy. The APPRAISE-2 trial, using apixiban (FXa inhibitor) on top of aspirin plus $P2Y_{12}$ inhibitor, was terminated prematurely, due to negative effects on recurrence and higher major bleeding.[106] The ATLAS ACS-2 trial tested a low dose of rivaroxaban (FXa inhibitor), again for the majority of patients on top of dual antiplatelet therapy (aspirin plus thienopyridine), and had a more positive outcome on the primary endpoint—a composite of myocardial infarction, cardiovascular death or stroke—albeit again at the expense of bleeding events.[107]

More recently, multiple studies have combined low rivaroxaban with either a $P2Y_{12}$ antagonist or aspirin. The recently published COMPASS trial reports a superiority of low-dose rivaroxaban plus aspirin versus aspirin alone regarding the primary endpoint.[108] No difference was found between the groups in intracranial or fatal bleeding, but major bleeding again was by the combined treatment. The ATLAS ACS-2 study indicated that joint inhibition of platelets (aspirin plus $P2Y_{12}$ antagonist) and coagulation (rivaroxaban), when compared to dual antiplatelet therapy alone, reduced the risk of instent thrombosis.[109]

Clinical studies thus support the high degree of interactions between platelet activation (by soluble agonists ADP and, thromboxane) and coagulation (thrombin) in recurrent cardiovascular disease, and also in the control of hemostasis. For combination therapies, fine-tuned targeting will be needed to achieve maximal suppression of thrombus formation with minimal bleeding risk.

RELATIONSHIP DISCLOSURE

P.v.d.M. reports a collaboration with Bayer. F.S., H.S., and J.H. have nothing to disclose.

AUTHOR CONTRIBUTIONS

F.S. reviewed the literature and drafted the manuscript. H.S., J. H., and P.v.d.M. completed and revised the manuscript.

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