

# Platelet-based coagulation: different populations, different functions

## Citation for published version (APA):

Heemskerk, J. W. M., Mattheij, N. J. A., & Cosemans, J. M. E. M. (2013). Platelet-based coagulation: different populations, different functions. *Journal of Thrombosis and Haemostasis*, 11(1), 2-16. <https://doi.org/10.1111/jth.12045>

## Document status and date:

Published: 01/01/2013

## DOI:

[10.1111/jth.12045](https://doi.org/10.1111/jth.12045)

## Document Version:

Publisher's PDF, also known as Version of record

## Document license:

Taverne

## Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

## General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

[www.umlib.nl/taverne-license](http://www.umlib.nl/taverne-license)

## Take down policy

If you believe that this document breaches copyright please contact us at:

[repository@maastrichtuniversity.nl](mailto:repository@maastrichtuniversity.nl)

providing details and we will investigate your claim.

## REVIEW ARTICLE

# Platelet-based coagulation: different populations, different functions

J. W. M. HEEMSKERK, N. J. A. MATTHEIJ and J. M. E. M. COSEMANS

Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, the Netherlands

**To cite this article:** Heemskerk JWM, Mattheij NJA, Cosemans JMEM. Platelet-based coagulation: different populations, different functions. *J Thromb Haemost* 2013; **11**: 2–16.

**Summary.** Platelets in a thrombus interact with (anti)coagulation factors and support blood coagulation. In the concept of cell-based control of coagulation, three different roles of platelets can be distinguished: control of thrombin generation, support of fibrin formation, and regulation of fibrin clot retraction. Here, we postulate that different populations of platelets with distinct surface properties are involved in these coagulant functions. Platelets with elevated  $\text{Ca}^{2+}$  and exposed phosphatidylserine control thrombin and fibrin generation, while platelets with activated  $\alpha_{\text{IIb}}\beta_3$  regulate clot retraction. We review how coagulation factor binding depends on the platelet activation state. Furthermore, we discuss the ligands, platelet receptors and downstream intracellular signaling pathways implicated in these coagulant functions. These insights lead to an adapted model of platelet-based coagulation.

**Keywords:** coagulation factors, clot retraction, fibrin formation, platelet receptors, procoagulant activity, thrombin generation.

## Introduction

Since the discovery of platelets as essential blood constituents of an arterial thrombus, it has rapidly become clear that platelets also contribute to fibrin clot formation. On the other hand, coagulation is often still regarded as a merely plasmatic process, characterized by initiation, propagation and termination phases of thrombin generation [1]. In the conventional scheme, the extrinsic coagulation cascade starts with tissue factor binding to factor VIIa, whether or not in complex with FXa, resulting in the cleavage of traces of prothrombin into thrombin. Thrombin amplifies its own generation by proteolytically activating other coagulation factors. In a next phase, these active factors are inactivated by anticoagulation factors, such as tissue factor pathway inhibitor (TFPI), activated protein C, antithrombin, and C1 inhibitor.

It is widely accepted that phospholipid membranes containing the negatively charged lipid phosphatidylserine (PS) are required to propagate and enhance the coagulation reactions. This membrane dependency implies ultimate cellular control of the coagulation process as a whole. In the blood system, activated platelets with surface-exposed PS cleave high amounts of FX and prothrombin into the proteolytically active forms, FXa and thrombin [2,3]. However, platelets also have other roles in coagulation. They provide a scaffold for the formation of fibrin fibers [3], and, once fibrin clots are formed, they regulate the process of clot retraction [4].

The interwoven nature of platelet activation and the coagulation system has been observed in numerous studies. For instance, in the most common *in vivo* thrombosis models, i.e. ferric chloride-induced damage of arteries or arterioles, the thrombotic process is similarly sensitive to defects in platelet activation and to inhibition of coagulation [5]. This *in vivo* work has collectively shown that: thrombus formation is triggered by collagen, as well as by tissue factor and thrombin [6–9]; both platelets and coagulation contribute to arterial and venous thrombus formation [10,11]; and fibrin formation already occurs at initial stages of thrombus formation [12].

On the basis of literature evidence, we postulate that different platelet populations may have different roles in the coagulation process, depending on their activation state and surface properties. In the following, we will discuss this from the perspectives of: (i) interaction of platelets with (anti)coagulation factors; (ii) heterogeneity of platelet populations with respect to control of thrombin generation, fibrin formation, and clot retraction; and (iii) receptors and signaling processes leading to these different platelet populations.

## Platelet interactions with (anti)coagulation factors

For coagulation control, platelets need to be able to interact with coagulation factors. The reported evidence for such interactions is summarized below.

### Fibrinogen and fibrin

The fibrinogen receptor, integrin  $\alpha_{\text{IIb}}\beta_3$ , is the most abundantly expressed platelet glycoprotein (GP). Platelet–fibrinogen-binding

Correspondence: Johan W. M. Heemskerk, Department of Biochemistry (CARIM), Maastricht University, PO Box 616, 6200 MD Maastricht, the Netherlands.

Tel.: +31 43 3881671; fax: +31 43 3884159.

E-mail: jwm.heemskerk@maastrichtuniversity.nl

requires activation of  $\alpha_{IIb}\beta_3$  via conformational changes, a response that is induced by most platelet agonists [13]. This integrin is also supposed to bind fibrin. However, whether integrin-bound fibrinogen is directly converted into fibrin is unclear. Specific fibrin receptors have not been reported, although a role for platelet GPIb in fibrin binding has been proposed [14].

#### *Prothrombin and other vitamin K-dependent factors*

Phospholipid membranes with negatively charged phospholipids, i.e. PS and, to a lesser extent, phosphatidylethanolamine, greatly facilitate the binding of coagulation factors and the generation of FXa and thrombin [2,3]. Platelet activation by strong agonists, e.g. thrombin and collagen, is required for PS exposure. As shown by flow cytometry, the PS-exposing platelets characteristically display high-affinity binding sites for vitamin K-dependent (anti)coagulation factors, i.e. prothrombin, FVII, FIX, FX, and proteins C, S, and Z [3,15]. Gamma-carboxyglutamate (Gla) domains, which are present in all vitamin K-dependent factors, mediate  $Ca^{2+}$ -dependent factor binding to the PS surface. Hence, PS-exposing platelets serve as assembly sites for components of the tenase complex (FVIIIa, FIXa, and FX) and the prothrombinase complex (FVa, FXa, and prothrombin).

Investigations with fluorescence microscopy have shown that labeled FX and prothrombin only bind to PS-exposing platelets in a thrombus [16,17]. However, once cleaved into thrombin, the prothrombin label appears to redirect to the sites of fibrin fibers [17], which is in agreement with indications that fibrin acts as a site for thrombin. So far, specific protein receptors on platelets for vitamin K-dependent factors such as FIX or FX have not been identified [2,3]. A recent proposal, however, is that these factors may stay bound to the platelet surface after the formation of cysteine bridges, catalyzed by protein disulfide isomerase isoforms [18]. Unlike some other cell types, platelets lack the signaling FXa receptor, protease-activated receptor (PAR)2 [19]. An unexpected finding was that FXa, similarly to ADAM-10/17, influences platelet activation by cleaving the collagen receptor GPVI [20,21].

#### *Thrombin*

Thrombin is the only vitamin K-dependent factor known to bind to specific receptor proteins on platelets. Thrombin interacts with high specificity with PARs, i.e. the PAR1 and PAR4 isoforms in human platelets, and the PAR3 and PAR4 isoforms in mouse platelets. Of these, only PAR1 and PAR4 are cleavable by thrombin. In addition, high-affinity thrombin-binding sites are present on GPIb, whereas GPV serves as a thrombin cleavage substrate [22].

#### *Tissue factor*

Similarly to megakaryocytes, platelets express limited amounts of tissue factor, e.g. upon sepsis [23,24]. It is, however, doubted

whether the levels of tissue factor present in platelets are physiologically relevant [25]. Furthermore, activated platelets release considerable amounts of TFPI, which restricts thrombus growth [26]. Hence, platelet-derived TFPI is expected to rapidly inactivate any tissue factor present at the platelet surface.

#### *FV*

Platelet  $\alpha$ -granules contain  $\sim 20\%$  of the blood content of FV. Once released, platelet FV becomes activated by traces of thrombin. It has been reported by one group that platelet-derived FVa is more resistant to protease inactivation than the FVa in plasma [27], but this finding needs confirmation. FVa binds to PS-exposing (platelet) membranes via its C2 domain. Bound to PS-exposing platelets, it interacts with FXa to form local prothrombinase complexes [17,28]. With flow cytometry, a subpopulation of (thrombin-activated) platelets has been detected with intermediate FVa binding [29]. This is in agreement with the observation that FVa is incorporated into the membranes of coated platelets [30], and this may point to gradual accumulation of FVa at the platelet surface.

#### *FVII*

In platelets stimulated by collagen and thrombin, binding of recombinant FVIIa to GPIb $\alpha$ , is described, subsequently leading to FX activation [31]. The proposed mechanism is that FVIIa interaction with the GPIb–V–IX complex facilitates the Gla domain-dependent binding to PS-exposing membranes. Platelet-bound FVIIa can restore thrombus formation of platelets lacking  $\alpha_{IIb}\beta_3$  by enhancing PS exposure [32]. This may explain why recombinant FVIIa can be an effective prohemostatic drug in patients with Glanzmann's thrombasthenia.

#### *FVIII and FIX*

Although it is not a vitamin K-dependent factor, FVIIIa is able to bind to PS-exposing membranes via its C2 domain, probably in a similar manner as FVa [33]. This binding of FVIIIa enhances FIXa binding, and thus stimulates tenase complex formation and FXa generation on PS-exposing platelets. As FVIII in plasma is carried by von Willebrand factor (VWF), FVIII can also interact with platelets via VWF and GPIb–V–IX. However, in kinetic studies of fibrin formation, a role for VWF-bound FVIII in platelet coagulant activity could not be detected [34]. The binding of FIXa appears to be confined to PS-exposing platelets, as determined by annexin A5-labeling studies, and relies on prior elevation of cytosolic  $Ca^{2+}$  [35,36].

#### *FXI and FXII*

Decades ago, it was proposed that platelets promote fibrin formation in an FXII-dependent manner [37]. Recent observations point to marked roles of FXI and FXII in arterial thrombus formation in mice, suggesting that platelets, in some

way, stimulate the intrinsic pathway of coagulation [38]. One explanation for the thrombus-stimulating effect of FXI and FXII is the binding of FXII to collagen, and its subsequent activation in the presence of prekallikrein and high molecular weight kininogen [39]. There is, however, also some evidence that FXI and FXII can bind to the platelet surface via GPIIb/IIIa [40]. However, an early suggestion that platelet-bound FXIa is active and cleaves FIX could not be confirmed. Recent studies with human and mouse platelets point to binding of FXI to the platelet apolipoprotein E receptor 2 (LRP8) [41]. It is still not quite clear whether FXII can be activated on the surfaces of platelets. Some studies suggest that polyphosphates released from platelets may fulfil such a role [42], but recent data indicate that the size of platelet-derived polyphosphates (60–100 phosphate residues) is too small for this action [43]. On the other hand, platelet-size polyphosphates do accelerate the activation of FXI by thrombin [44].

### FXIII

The coagulation factor FXIIIa acts as a transglutaminase in the cross-linking of fibrin fibers. Experiments with immobilized FXIIIa have shown that platelets bind to this protein via  $\alpha_{IIb}\beta_3$  and  $\alpha_v\beta_3$  [45]. FXIIIa has also been implicated in the chemical cross-linking of secretory proteins from the  $\alpha$ -granules at the surfaces of so-called coated platelets (see below) [46]. However, other tissue-type transglutaminases may also contribute to this process [47].

### Anticoagulation factors

Platelets secrete significant amounts of the anticoagulant proteins TFPI and protease nexin-1 (PN-1, gene *SERPINE2*).

Binding of TFPI has been detected at the surfaces of coated platelets [48]. PN-1 inactivates thrombin, tissue-type plasminogen activator, and plasmin, thereby acting as a platelet-dependent, negative regulator of coagulation and fibrinolysis [49]. The anticoagulant vitamin K-dependent factors protein S and protein C, both of which bind to PS membranes, can suppress thrombin generation at the platelet surface. It has been reported that activated protein C binds to the platelet LRP8 receptor [50], but the importance of this interaction for the regulation of anticoagulation is unknown. The anticoagulant protein Z acts as an FXa inhibitor [51,52]; whether it binds to platelets is unclear. Similarly, binding sites are not known for anticoagulant proteins such as antithrombin and C1 inhibitor, which block the active sites of coagulation factors.

As summarized in Table 1, most coagulation factors seem to interact preferentially with PS-exposing platelets. These highly activated platelets provide the assembly sites for coagulation factor complex formation, causing greatly enhanced generation of FXa and thrombin. How the anticoagulation factors operate at the platelet surface is not clear. Another unresolved issue is whether all (anti)coagulation factors bind to a PS-exposing platelet at the same time, or whether, perhaps, fibrin or anticoagulation binding to platelets displaces other coagulation factors.

### Different platelet populations controlling distinct coagulation steps

Research on the role(s) of platelets in coagulation is complicated by the formation of distinct populations of activated platelets, e.g. with or without PS exposure. Below, we discuss evidence from the literature on how this heterogeneity can determine the different coagulant roles of platelets.

**Table 1** Reported interactions of (anti)coagulation factors with platelets; for explanation and references, see text

(Anti)coagulation factor	Binding site on platelets and result	Platelet population
Fibrin(ogen)	Fibrinogen: binding to $\alpha_{IIb}\beta_3$ . Precursor for fibrin formation. Fibrin receptor unknown	Aggregating and clot-retracting platelets
Factor II	FII (prothrombin): binding to PS (via Gla domain) FIIa (thrombin): binding to PAR1, PAR3, PAR4, and GPIIb-V-IX	FII: PS-exposing platelets FIIa: all platelets
Tissue factor	Weakly expressed. Physiologic role unclear	NA
Factor V	Released by platelets. Binding to PS (via C2 domain); other receptors unknown. Activation of prothrombin	PS-exposing and coated platelets
Factor VII	Binding to PS (via Gla domain) and GPIIb-V-IX Activation of FIX and FX	PS-exposing platelets
Factor VIII	Binding to PS (via C2 domain), indirectly via GPIIb-V-IX. Activation of FX	PS-exposing platelets
Factor IX	Binding to PS (via Gla domain). Activation of FX	PS-exposing platelets
Factor X	Binding to PS (via Gla domain); other receptors unknown. Activation of prothrombin	PS-exposing and coated platelets
Factor XI	Binding to LRP8 and GPIIb-V-IX	Not described
Factor XII	Binding site unclear	Not described
Factor XIII	Binding to $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$	PS-exposing and coated platelets
TFPI	Released by platelets; binding site unclear	Coated platelets
Protease nexin-1	Released by platelets; binding site unclear	NA
Protein C	Binding to PS (via Gla domain) and LRP8	PS-exposing platelets
Protein S	Binding to PS (via Gla domain)	PS-exposing platelets

GP, glycoprotein; NA, not applicable; PAR, protease-activated receptor; PS, phosphatidylserine; TFPI, tissue factor pathway inhibitor.

### Procoagulant platelets (agonist-induced PS exposure)

Within a thrombus, formed in a damaged vessel *in vivo* or on a collagen matrix *in vitro*, platelets with different surface properties are clearly distinguishable [53,54]. Aggregated platelets in the inner thrombus core, close to activating components of the vessel wall, have formed pseudopods, undergone secretion, and bind fibrinogen (see below). Discoid platelets at the outer part of growing thrombi may easily detach and lack these activation markers. A distinct population of highly activated platelets is present close to collagen fibers and as patches around a thrombus. These platelets are characterized by surface-exposed PS (detected with the probes annexin A5 and lactadherin), prolonged cytosolic  $\text{Ca}^{2+}$  rises, a rounded structure, and the ability to bind coagulation factors such as FV and FX [16,17]. They also produce procoagulant microparticles. Particularly under coagulant conditions, large patches of PS-exposing platelets are formed, implying a role of thrombin as a platelet agonist. The population of PS-exposing platelets has also been described as procoagulant platelets and platelets with a sustained calcium-induced platelet morphology [55,56]. Several studies have shown that *in vivo* injection of annexin A5 or lactadherin (chelating exposed PS) significantly impairs thrombus formation in mouse vessels [10,57], indicating the functional importance of this platelet population.

Studies with isolated coagulation factors have shown that PS-exposing platelets enhance the activities of the tenase and prothrombinase complexes by almost 1000-fold [2,58]. Recent *ex vivo* flow studies have indicated that platelets with prolonged high  $\text{Ca}^{2+}$  and PS exposure also catalyze fibrin network formation, although fibrin formation does not appear to be limited to these sites [34]. Jointly, these findings indicate that PS exposure is a prerequisite for platelet-dependent thrombin generation, with fibrin formation not being directly linked to this response.

### Coated platelets

Dale *et al.* [30,59] have identified a population of platelets, formed after stimulation with collagen plus thrombin, that have been termed coated platelets. A characteristic of these platelets is their ability to irreversibly bind several  $\alpha$ -granular proteins, including FV, thrombospondin, fibrinogen, fibronectin, and VWF [60]. It is considered that these proteins cluster together at the platelet surface and form an immobilized coat. The first reports proposed that FXIII activity is required for chemical cross-linking of these granular proteins to serotonin residues on the platelet membrane [46]. However, in studies with mice deficient in FXIII, it was shown that at least fibrinogen and VWF can bind to coated platelets in the absence of FXIIIa, suggesting (additional) involvement of a tissue-type transglutaminase expressed by platelets [47]. Considering that coated platelets also expose PS [30], the question arises of whether these two populations are identical. This is suggested by the findings that coated and PS-exposing platelets are both formed in response to strong agonists (e.g. collagen and thrombin),

through the same cellular processes (e.g. mitochondrial permeability transition pore [MPTP] formation), and that both are accompanied by shedding of microparticles [59,61–64]. Partial overlap of these two populations has indeed been observed in studies using dual labeling of thrombi with annexin A5 and a serotonin probe [16]. On the other hand, stimulation of washed platelets with the  $\text{Ca}^{2+}$  ionophore A23187 resulted in the formation of PS-exposing platelets without a coat of FVa [30] or high fibrin(ogen) [47]. Together, these findings suggest that coat formation occurs secondary to PS exposure, most likely as a consequence of the assembly of platelet secretion products and plasma factors at the outer membrane. How the formation of this protein coat affects the binding, activation and activity of coagulation factors is unknown.

### Aggregating and clot-retracting platelets

Aggregate-forming platelets in a thrombus are characterized by fibrinogen binding and the presence of active  $\alpha_{\text{IIb}}\beta_3$  at their surfaces (determined with PAC-1 antibody for human platelets, and with JON/A for mouse platelets). As indicated above, this population of platelets differs from the PS-exposing platelets in that the latter do not show activated  $\alpha_{\text{IIb}}\beta_3$  [53,55]. Interestingly, under coagulant conditions, the aggregated platelets with integrins in the active conformation play a predominant role in fibrin clot retraction. The latter process, which is dependent on platelet fibrin(ogen) binding and integrin outside-in signaling, translates the contractile forces generated in the platelet actin–myosin cytoskeleton to connecting fibrin fibers around platelets [65]. A diffuse fibrin network is thereby converted into a small and dense platelet–fibrin clot. Under flow conditions, this process is observed as contraction of platelet aggregates, and reflects the initial phases of coagulation (Video S1). It is seen as a mechanism for narrowing the gaps between platelets to allow contact-dependent signaling [66]. As the PS-exposing platelets are non-adhesive, have closed integrins, and have a disturbed actin cytoskeleton [56], it is quite unlikely that they will participate in clot retraction. This points to a major difference between the aggregating and PS-exposing platelet populations with respect to the regulation of coagulation processes. Whereas the former produce FXa and thrombin outside the platelet plug, the aggregated platelets consolidate the plug by clot retraction.

### Apoptotic and necrotic platelets

In a pioneering paper, Schoenwaelder *et al.* [67] reported that the anti-tumor drugs ABT-737 and ABT-263 induce platelet PS exposure in response to relatively minor rises in cytosolic  $\text{Ca}^{2+}$ . These compounds inhibit the antiapoptotic proteins Bcl-2 and Bcl- $\chi_L$ , and cause activation of the Bak–Bax pathway to start a mitochondrial-dependent route of apoptosis and subsequent PS exposure. The mechanism differs from that of agonist-induced PS exposure, as apoptotic PS exposure appears to rely on caspase activation. Other studies have indicated that the ABT compounds disrupt normal platelet activation responses, such as secretion, integrin activation, and platelet aggregation

[68]. Interestingly, the same or a similar apoptotic pathway is triggered in aging or stored platelets, which show a gradual decline in Bcl-x<sub>L</sub> expression and also liberate the proapoptotic Bak/Bax proteins [69,70]. The aging process is slow, requiring a time span of several days to yield PS-positive platelets, as examined with the probes annexin A5 and lactadherin [71–73]. Although in vitro tests have shown that aging, apoptotic platelets have a coagulant potential and support thrombin generation, it is questionable whether these PS-exposing platelets play a role in coagulation under physiologic conditions, as they will be rapidly taken up by scavenging cells in the circulation.

Because of clear distinctions between apoptotic and agonist-stimulated PS-exposing platelets, e.g. with respect to caspase activation, it has been suggested that the latter are activated by a necrotic cell death pathway [74]. Given the common definition of necrosis as premature death of cells caused by external sources (e.g. hypoxia or injury), we do not prefer this terminology. Instead, we find the term ‘procoagulant platelets’ more appropriate, as it refers to their active participation in the support of thrombin and fibrin generation.

#### *Platelet microparticles*

Both procoagulant PS-exposing [75] and aging [76] platelets can release microparticles. As these microparticles, in part, expose PS, they have a thrombin-generating potential. This seems to be particularly relevant under pathophysiologic conditions, when elevated levels of platelet-derived microparticles have been measured [77]. Under normal conditions, their role may be restricted, because of rapid interaction with leukocytes and other scavenging cells [78].

#### *Different platelet populations*

This overview points to the presence of two types of activated platelet with fundamentally different roles in the coagulation process. On the one hand, there are PS-exposing and coated platelets (with partial overlap) that are characterized by a high activation state, binding of multiple coagulation factors, and the ability to stimulate thrombin generation. These platelets also actively form fibrin, with the coated platelets having a fibrin layer at their surface. On the other hand, present are aggregating platelets, which characteristically express activated integrins and have a role in fibrin clot retraction, which implies that they bind fibrin as well. Although apoptotic (aging) platelets also expose PS, it is unclear whether these have a physiologic role in coagulation. As reviewed elsewhere, the mechanism for this heterogeneity in platelet fate is still incompletely understood [53]. It seems that intrinsic platelet factors, such as platelet size and structure, protein composition, genetic factors, and platelet age, account for only part of the response heterogeneity. Platelet environmental factors, such as the local rheology, exposure to agonists, surrounding cells, and plasma, are probably at least as important. This is discussed further below.

### **Ligands and receptors mediating platelet PS exposure and fibrin formation**

Most studies investigating the roles of platelets in coagulation have been carried out to determine the regulation of PS exposure and the ensuing thrombin generation. Less is known about the mechanisms controlling fibrin formation by platelets. An overview of the ligands and receptors involved in these two processes is given below.

#### *VWF and GPIb–V–IX*

A role of the GPIb–V–IX complex to platelet PS exposure, thrombin generation and subsequent fibrin formation has been reported by several groups, claiming that this relies stringently on the interaction of GPIb with VWF [34,79,80]. Particularly under coagulant conditions and with a low shear rate (implying the presence of thrombin), VWF binding to GPIb can lead to prolonged Ca<sup>2+</sup> responses, PS exposure, assembly of coagulation factors, and formation of fibrin at the platelet surface [34]. The signaling mechanism downstream of GPIb may involve the actin cytoskeleton.

Initially, platelet procoagulant activity via GPIb was considered to rely on the presence of fibrin, interacting with GPIb via VWF [14]. However, studies with platelets from Bernard–Soulier patients pointed to a fibrin-independent signaling role of GPIb–V–IX to enhance thrombin-induced Ca<sup>2+</sup> mobilization [81]. In mouse platelets, GPIbβ was found to potentiate the Ca<sup>2+</sup> rises and PS exposure induced by either thrombin or collagen receptor agonists. This procoagulant effect was independent of the N-terminal part of GPIbα, as it was not affected by endopeptidase cleavage of this glycoprotein [82]. Interestingly, deficiency in GPV, which is also part of the GPIb–V–IX complex, increased rather than decreased the responses of mouse platelets to thrombin by a still undisclosed mechanism [83].

#### *Thrombin and receptors*

The thrombin receptors of human platelets, PAR1 and PAR4, belong to the class of G-protein-coupled receptors, and elevate cytosolic Ca<sup>2+</sup> via Gq and phospholipase C (PLC)β stimulation [84]. The consequence is an oscillatory (spiking) Ca<sup>2+</sup> signal, which, by itself, is insufficient to evoke PS exposure [85]. However, thrombin markedly enhances collagen-induced PS exposure, in which case binding to PAR1 appears to be the main activation mechanism [2,86], rather than binding of thrombin to GPIb [87]. Some authors, however, have reported a role for GPIb in PS exposure, e.g. in thrombin-stimulated gel-filtered platelets, where residual VWF may be present [79].

An explanation for the primary role of PAR1 in thrombin-mediated PS exposure comes from the recent observation that inhibition of protein kinase C (PKC) negatively regulates Ca<sup>2+</sup> rises and PS exposure elicited by PAR1, but not by the other thrombin receptor, PAR4 [88]. However, PAR4 activation also has a known supporting role in potentiating Ca<sup>2+</sup> rises and PS exposure [89]. In mouse platelets, where only PAR4 – and not

the other receptor, PAR3 – is cleaved by thrombin [90], thrombin-dependent PS exposure seems to rely on the presence of both isoforms.

The literature contains some evidence that the PAR isoforms on platelets can be cleaved by proteases other than thrombin. For example, PAR1 cleavage by ADAM-17, a related plasma protease [91], or by matrix metalloproteinase-1, has been reported [92]. At present, the physiologic relevance of these alternative mechanisms of thrombin receptor cleavage is unclear. The same holds for the reported cleavage of PAR4 by the fibrinolysis protease plasmin [93] or the neutrophil-derived cathepsin G [94].

#### *Thromboxane and TP receptors*

In spite of the prominent role of thromboxane  $A_2$  in collagen-induced platelet aggregation, this autocrine agonist is only marginally effective in stimulating PS exposure. In fact, blockade of thromboxane production by aspirin affected PS exposure of collagen-adhered platelets only in combination with ADP receptor blockers [95]. Along the same line, aspirin treatment only slightly affected thrombin generation in coagulating platelet-rich plasma [96]. In agreement with these findings, stimulation of the platelet TP receptors is known to cause only limited  $Ca^{2+}$  mobilization.

#### *ADP, fibrinogen, and receptors*

The receptors for ADP (in particular  $P2Y_{12}$ ) and fibrinogen ( $\alpha_{IIb}\beta_3$ ) appear to have supporting and partially redundant roles in PS exposure and platelet-dependent thrombin generation. This was most clearly observed under coagulant conditions, e.g. in tissue factor-activated platelet-rich plasma. In this case, autocrine-produced ADP was found to prolong  $Ca^{2+}$  rises and to increase PS exposure via  $P2Y_{12}$  mediated signaling towards phosphoinositide 3-kinase (PI3K) [97,98]. Blockade of  $P2Y_{12}$  by intake of the prodrug clopidogrel significantly suppressed the procoagulant effect of platelets in coagulating plasma [99]. Similarly, blockade of this receptor by the active metabolite of prasugrel impaired ADP/collagen-induced  $Ca^{2+}$  rises, PS exposure and thrombin generation in whole blood [100,101].

Recent work has indicated that  $\alpha_{IIb}\beta_3$  outside-in signaling via PLC and the tyrosine kinase Syk also support thrombin-induced  $Ca^{2+}$  rises and PS exposure, along with platelet-dependent thrombin generation [102]. The contributory role of  $\alpha_{IIb}\beta_3$  partly overlaps with that of autocrine mechanisms via  $P2Y_{12}$ , which not surprising, given the prominent role of  $P2Y_{12}$  in integrin activation. These findings explain why integrin antagonists cause marked suppression of tissue factor-induced thrombin generation, and prolong the clotting times in platelet-rich plasma [103,104].

#### *Immune receptor Fc $\gamma$ RIIA*

There is limited evidence that stimulation of the immune receptor Fc $\gamma$ RIIA on human platelets induces PS exposure and

microparticle formation, e.g. via antibodies against the heparin–platelet factor 4 complex [105]. Platelet activation via this receptor, which probably signals via the Syk–PLC $\gamma_2$  pathway, is considered to contribute to the pathology of heparin-induced thrombocytopenia.

#### *Collagen and GPVI*

Circulating platelets will encounter vascular collagen only after endothelial damage and luminal exposure of the extracellular matrix. Under in vitro conditions, stable adhesion of platelets to immobilized fibers of collagen type I is an effective trigger of PS exposure via the immunoglobulin-type collagen receptor GPVI [106]. Studies of platelet interaction with collagen fibers under flow have indicated that all key constituents of the GPVI signalosome contribute to GPVI-induced PS exposure. In line with this, platelets from mice lacking the FcR  $\gamma$ -chain, LAT, Syk or PLC $\gamma_2$ , or with blocked GPVI, showed a marked reduction in collagen-dependent PS exposure and thrombin generation [107,108]. On the other hand, mouse platelets with a gain-of-function mutation in PLC $\gamma_2$  were more active in collagen-dependent PS exposure and thrombus formation [109]. The adhesive collagen receptor  $\alpha_2\beta_1$  was found to support procoagulant activity in an indirect way, by enforcing collagen–platelet interactions via GPVI [110].

In line with this, inhibition of the protein tyrosine kinase Syk, which, together with Src family kinases, controls PLC $\gamma_2$  activity, has been found to suppress GPVI-induced PS exposure, whereas inhibition of protein phosphatases increases this response [16,111]. The responsible phosphatase has been unknown for a long time, but recent data point to involvement of the phosphatase TULA2, which associates with Syk and can negatively regulate GPVI-induced signaling events [112]. This phosphatase might also control PS exposure.

Platelet adhesion to immobilized collagen causes appreciable PS exposure. However, costimulation with collagen and other agonists such as thrombin is needed to obtain substantial fractions of PS-exposing platelets [2,113]. This costimulation probably has a dual effect: it potentiates the  $Ca^{2+}$  rises in platelets, and also activates the integrins ( $\alpha_2\beta_1$  and  $\alpha_{IIb}\beta_3$ ) needed for stable platelet adhesion to collagen fibers and for signaling via the low-affinity GPVI receptors. This is in contrast to the process of collagen-induced platelet aggregation, which is less dependent on high  $Ca^{2+}$  rises, and only requires  $\alpha_{IIb}\beta_3$  activation via autocrine agonists such as ADP and thromboxane. Other ligands of GPVI, such as cross-linked collagen-related peptide (low affinity) and, particularly, the snake venom convulxin (high affinity) also provoke PS exposure, but again this response is markedly enhanced by costimulation with thrombin [106].

#### *Other ligands and receptors*

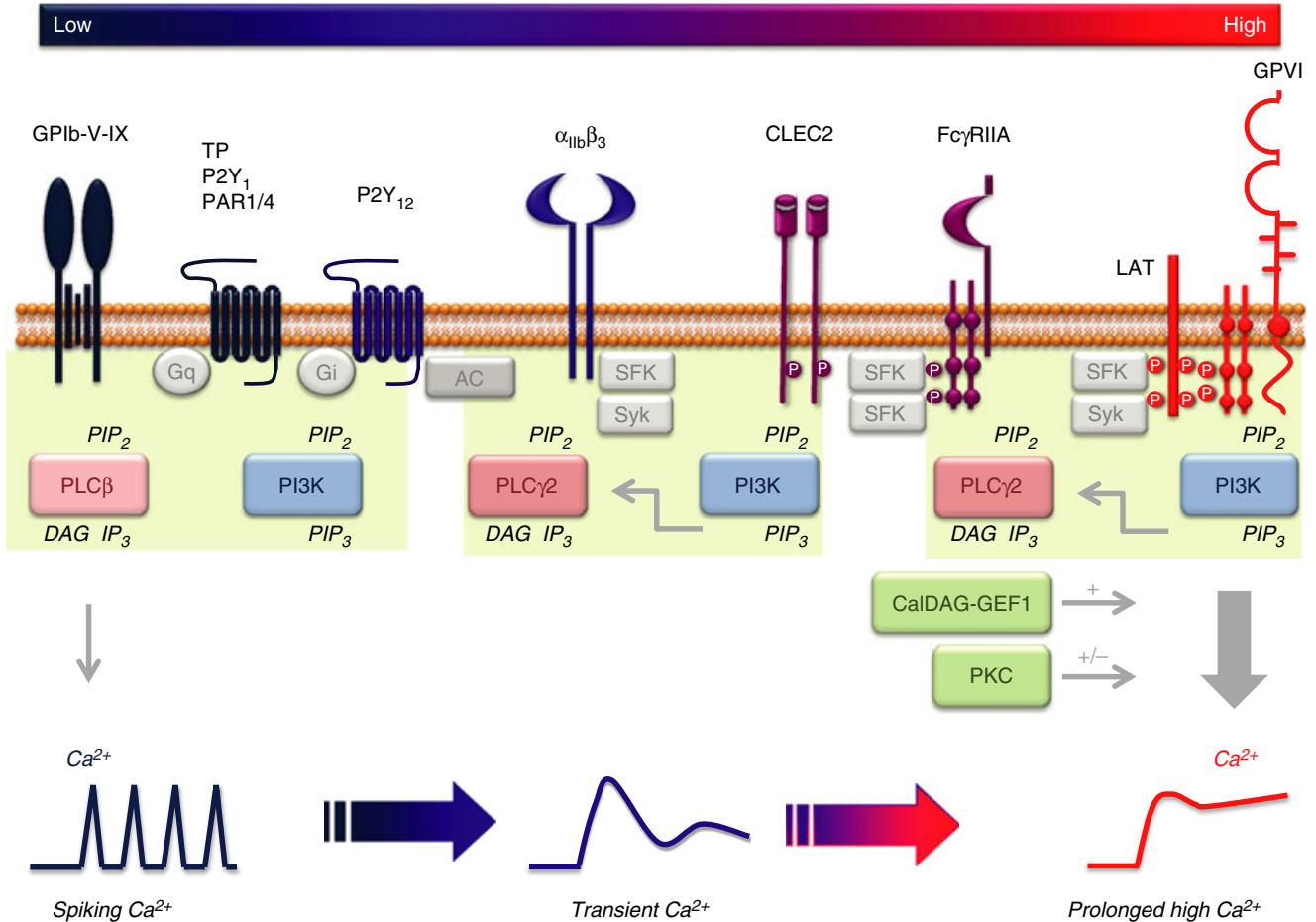
A newly described platelet receptor acting via a similar activation pathway of LAT–Syk–PLC $\gamma_2$  is the protein CLEC2, which can be activated by the snake venom rhodocytin and the

lymphatic protein podoplanin [114]. There are no reports yet on platelet procoagulant activity induced by CLEC2 activation.

In addition to collagen, a number of other adhesive proteins have been found to promote PS exposure, especially after immobilization at a surface. For instance, immobilized thrombospondin-1 and oxidized LDL can stimulate PS exposure of adhered platelets to a limited extent via the receptor CD36 [115]. Furthermore, under coagulant conditions with thrombin present, surface-immobilized fibrinogen [17] and laminin [116] can stimulate PS exposure of adhered platelets via  $\alpha_{IIb}\beta_3$  and FXII, respectively. The findings that platelet adhesion favors procoagulant activity are clearly relevant, not only because stable adhesion permits more continued signaling, but also because this provides a way to locally confine the coagulation process to sites of platelet adhesion and activation.

In summary, only a few physiologic agonists, particularly those stimulating GPVI, are capable of inducing PS exposure

by themselves. On the other hand, a larger number of agonists, including VWF, fibrinogen, thrombin, ADP, antibodies, and thrombospondin, appear to enhance the process of (GPVI-induced) PS exposure by stimulating their respective receptors. In most cases, the co-agonists act by increasing the  $Ca^{2+}$  signal, leading to a prolonged high  $Ca^{2+}$  rise (Fig. 1). Apparently, to generate a sufficiently high  $Ca^{2+}$  signal for PS exposure, costimulation is needed of both receptors linked to PLC isoforms ( $\alpha_{IIb}\beta_3$ , Fc $\gamma$ RIIA, and GPVI) and receptors linked to PLC $\beta$  isoforms (GPIb and PAR1/4). The requirement for 'full' PLC stimulation is supported by the observation that PI3K isoforms support PLC-dependent  $Ca^{2+}$  mobilization by producing phosphatidylinositol trisphosphate, which acts as membrane-binding site and enhancer of PLC $\gamma$  isoforms [117]. Regarding the control of fibrin formation, a contribution of the VWF-GPIb axis has been clarified so far. Given the high-activation conditions required for PS exposure, it is reasonable to assume that not all platelets in a thrombus encounter



**Fig. 1.** Platelet-based coagulation: signaling for prolonged high  $Ca^{2+}$  rises, which are required for phosphatidylserine (PS) exposure. The gradient color bar indicates the range of receptors with low  $Ca^{2+}$ -mobilizing potency, owing to phospholipase C (PLC) $\beta$  activation, causing spiking  $Ca^{2+}$  signals (blue), to receptors with high  $Ca^{2+}$ -mobilizing potency, owing to PLC $\gamma$ 2 activation, causing prolonged  $Ca^{2+}$  signals (red). Isoforms of phosphoinositide 3-kinase (PI3K) produce the phosphoinositide phosphatidylinositol trisphosphate (PIP<sub>3</sub>), which can stimulate PLC $\gamma$ 2 activity. Co-signaling via multiple receptors will result in accumulated  $Ca^{2+}$  signals, reaching the threshold levels of prolonged high  $Ca^{2+}$  required for PS exposure. The diacylglycerol (DAG) and  $Ca^{2+}$ -dependent effector proteins protein kinase C (PKC) and CALDAG-GEFI act as modulators of the  $Ca^{2+}$  signal. See also text. AC, adenyl cyclase; GP, glycoprotein; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; PAR, protease-activated receptor; PIP<sub>2</sub>, phosphatidylinositol bisphosphate.



sufficient levels of agonists, thus explaining part of the response heterogeneity. On the other hand, intrinsic platelet factors may contribute as well, e.g. different numbers of receptors or signaling molecules [53].

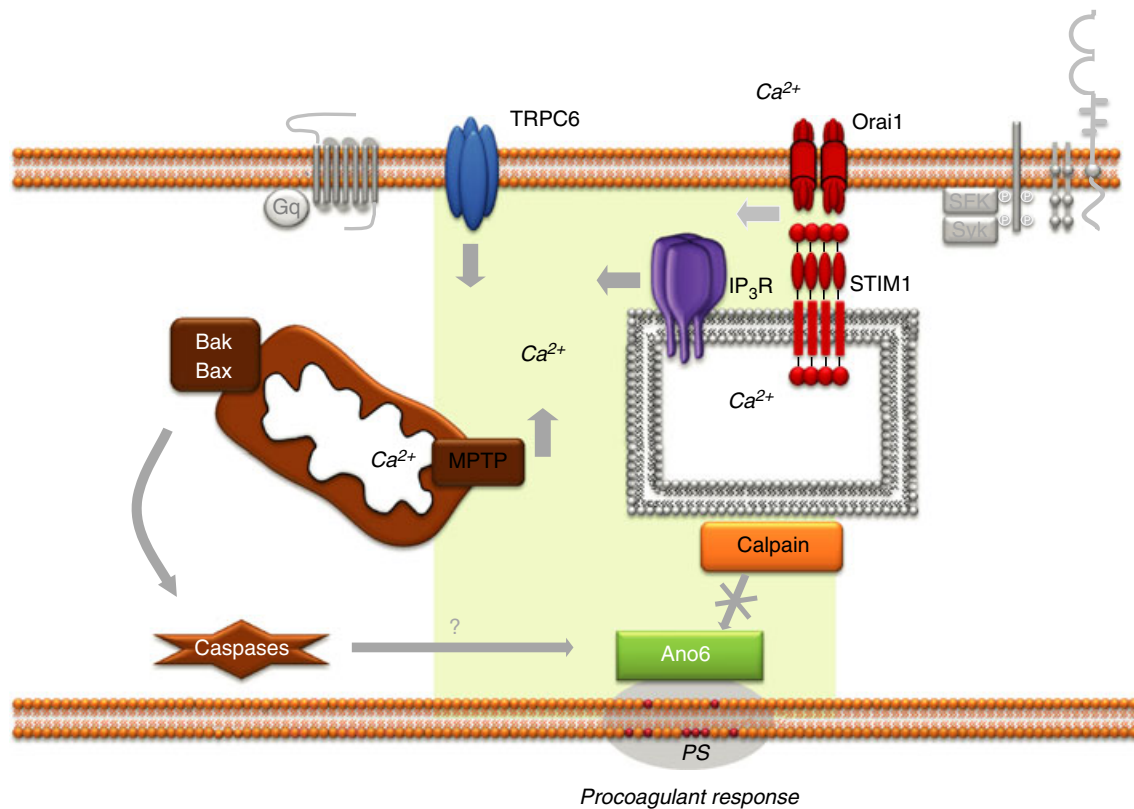
### Platelet signaling to PS exposure and fibrin formation: central role of $\text{Ca}^{2+}$

Since the early finding that  $\text{Ca}^{2+}$  ionophores (A23187 or ionomycin) cause rapid and full PS exposure in essentially all platelets, owing to phospholipid membrane scrambling, it has become clear that a prolonged high  $\text{Ca}^{2+}$  signal is a sufficient trigger for this platelet response [2,58]. This is confirmed by the observation that most physiologic agonists or combinations provoking PS exposure also induce sustained  $\text{Ca}^{2+}$  rises. The recent literature provides novel insights into the signaling pathways involved (Fig. 2).

#### Store-operated $\text{Ca}^{2+}$ entry (SOCE)

An initial finding was that  $\text{Ca}^{2+}$  entry via SOCE not only prolongs the platelet  $\text{Ca}^{2+}$  signal, but also promotes PS exposure [118]. In the last few years, by the use of murine

knockout and pharmacologic approaches, a central role of two membrane proteins in SOCE has been demonstrated [9]. Stromal interaction molecule (STIM)1 was identified as a central  $\text{Ca}^{2+}$  sensor in the platelet reticular membrane, monitoring  $\text{Ca}^{2+}$  store depletion, and Orai1 was identified as a  $\text{Ca}^{2+}$  entry channel that is activated through coupling to STIM1 [119,120]. The chaperone protein cyclophilin A is proposed to act as a positive  $\text{Ca}^{2+}$  modulator of STIM1 [121]. With mouse platelets lacking STIM1 or Orai1, collagen-dependent thrombus formation was greatly impaired, along with GPVI-induced PS exposure [119,120,122,123]. In contrast, no role was found for the paralogs STIM2 and Orai3, which are both present in platelets. Interestingly, Orai1 makes only a small contribution to  $\text{Ca}^{2+}$  entry in platelets activated by thrombin and  $\text{PLC}\beta$ . This may explain why, in the presence of high thrombin concentrations, PS exposure is only marginally dependent on STIM1 and Orai1 [122]. Observations such as these have raised the question of whether another  $\text{Ca}^{2+}$  entry mechanism (receptor-operated  $\text{Ca}^{2+}$  entry) could operate downstream of thrombin receptors to facilitate PS exposure [124]. Recent findings with human and mouse platelets suggest that transient receptor potential cation channel (TRPC)6 could play such a role [125,126]. Earlier reports on a role of the



**Fig. 2.** Platelet-based coagulation: central role of elevated  $\text{Ca}^{2+}$  in phosphatidylserine (PS) exposure. The mechanisms for  $\text{Ca}^{2+}$  elevation contributing to PS exposure include:  $\text{Ca}^{2+}$  mobilization from the endoplasmic reticulum via  $\text{IP}_3$  receptors ( $\text{IP}_3\text{Rs}$ );  $\text{Ca}^{2+}$  entry via store-operated  $\text{Ca}^{2+}$  entry via stromal interaction molecule 1 (STIM1) and Orai1 channels; and release of mitochondrial  $\text{Ca}^{2+}$  via mitochondrial permeability transition pore (MPTP) formation. Elevated  $\text{Ca}^{2+}$  activates the protease calpain, and triggers phospholipid scrambling via anoctamin 6 (Ano6), thereby resulting in PS exposure. The apoptotic pathway of PS exposure via Bak/Bax-induced caspase activation is relatively independent of  $\text{Ca}^{2+}$ . TRPC6, transient receptor potential cation channel 6.

isoform TRPC1 in  $\text{Ca}^{2+}$  entry [127] were ruled out because of the complete lack of a phenotype in mouse *Trpc1*<sup>-/-</sup> platelets [126,128].

Transmembrane fluxes of ions other than  $\text{Ca}^{2+}$  may contribute to the procoagulant activity. Prolonged platelet treatment with ouabain, which inhibits the  $\text{Na}^+/\text{K}^+$ -ATPase, was found to provoke limited PS exposure and thrombin generation via a small but persistent  $\text{Ca}^{2+}$  rise that was dependent on  $\text{Na}^+$  entry [129]. In addition,  $\text{Na}^+$  loading via the  $\text{Na}^+/\text{H}^+$  exchanger [130], or  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  currents (Gardos channels) [131] may increase platelet PS exposure and prothrombinase activity. The precise mechanisms are not understood.

#### Mitochondrial collapse

Mitochondrial collapse caused by MPTP formation can be induced by various forms of cellular stress, e.g. free oxygen radicals. Several groups have examined a role for this mitochondrial process in platelet procoagulant activity. In  $\text{Ca}^{2+}$  ionophore-stimulated platelets, inhibition of MPTP formation with cyclosporin A was found to prevent depolarization of the mitochondrial inner membrane potential, as well as microparticle formation. However, PS exposure was only moderately inhibited, indicating that mitochondrial collapse as such is not a strict requirement for PS exposure [132]. In agreement with this,  $\text{Ca}^{2+}$  ionophore-induced PS exposure was unchanged in platelets from mice deficient in cyclophilin D, which is a component of the MPTP [62]. However, in the cyclophilin D-deficient platelets, PS exposure and thrombin generation induced by the receptor agonists convulxin and thrombin were strongly diminished. Along the same line, cyclosporin A suppresses the formation of coated platelets in response to convulxin/thrombin [63]. This implies a role of mitochondrial collapse in fibrin (coat) formation. In other cells, MPTP formation abolishes the sequestration of  $\text{Ca}^{2+}$  in mitochondria [133]. Hence, also in platelets, mitochondrial collapse may lead to a loss of  $\text{Ca}^{2+}$  flux control, thus provoking agonist (convulxin/thrombin)-induced PS exposure. In contrast, the apoptosis-induced PS exposure, while also involving mitochondria, is essentially  $\text{Ca}^{2+}$ -independent, and specifically relies on Bak/Bax and caspase activation [74].

#### Signaling downstream of $\text{Ca}^{2+}$

The majority of platelet agonists signal via  $\text{PLC}\beta$  or  $\text{PLC}\gamma$  isoforms, which produce diacylglycerol (DAG) and the  $\text{Ca}^{2+}$ -mobilizing second messenger inositol 1,4,5-trisphosphate, resulting in activation of PKC and CalDAG-GEFI (Fig. 1). Detailed studies using knockout mice and specific inhibitors point to a two-sided role for PKC isoforms in platelet activation and PS exposure [134]. The conventional isoforms  $\text{PKC}\alpha$  and  $\text{PKC}\beta$ , which are activated by DAG and  $\text{Ca}^{2+}$ , positively contribute to platelet secretion,  $\text{Ca}^{2+}$  signaling, and PS exposure [135–137]. The novel isoform  $\text{PKC}\theta$ , being activated by DAG alone, acts differently, in that it suppresses

GPVI-induced  $\text{Ca}^{2+}$  mobilization and PS exposure [136]. This negative role of  $\text{PKC}\theta$  could explain why general PKC stimulation with phorbol ester downregulates these platelet responses [134]. However, some authors have reported slight stimulation of platelet function by  $\text{PKC}\theta$  [138]. In summary, the role of PKC isoforms in platelet PS exposure (procoagulant response) can be understood in terms of PKC-dependent fine-tuning of the  $\text{Ca}^{2+}$  signal.

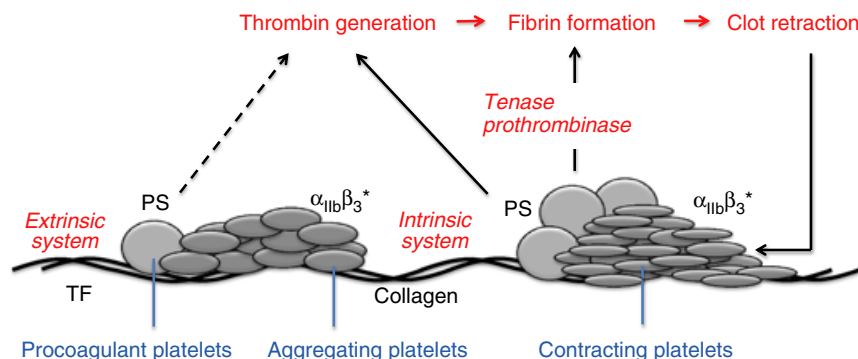
Mouse experiments also point to a separate role in procoagulant activity of the regulatory protein CalDAG-GEFI [139]. This activates the small GTPase Rap1b, thereby supporting integrin activation and other platelet functions. Platelets from CalDAG-GEFI-deficient mice were found to be substantially impaired in PS exposure during thrombus formation on collagen, by a mechanism that is not yet understood [140]. It was concluded that CalDAG-GEFI, like  $\text{STIM1}$ , contributes to the first wave of thrombin generation catalyzed by PS-exposing procoagulant platelets. Furthermore, platelet CalDAG-GEFI appeared to regulate fibrin clot formation.

With the recent discovery of the platelet protein anoctamin 6 (gene *TMEM16F*) as a key regulator of  $\text{Ca}^{2+}$ -dependent phospholipid scrambling and hence PS exposure [141], other speculations on protein PS translocators have been ruled out [77]. In platelets from patients with Scott syndrome, a moderate bleeding disorder, anoctamin 6 is lacking, and ionomycin-induced and agonist-induced PS exposure are greatly impaired [141,142], in spite of normal  $\text{Ca}^{2+}$  rises [143]. Anoctamin 6 is a multiple membrane-spanning protein with a supposed  $\text{Ca}^{2+}$ -binding site, but whether it operates alone is unclear. Recent data obtained with mice containing inactivated anoctamin 6 support a role of this protein in platelet PS exposure and arterial thrombus formation [144]. Another  $\text{Ca}^{2+}$ -dependent protein contributing to microparticle formation is the protease calpain, but inhibitor studies have indicated that its role in PS exposure is moderate at best [145].

Taken together, the available evidence indicates that PS exposure in response to agonist stimulation is accomplished by multiple pathways acting synergistically to cause a rise in  $\text{Ca}^{2+}$  (Fig. 2). In other words, platelet stimulation with multiple agonists that cause massive  $\text{Ca}^{2+}$  store depletion and SOCE, owing to the  $\text{STIM1}$ – $\text{Orai1}$  interaction, may result in a loss of the normal control of  $\text{Ca}^{2+}$  handling by  $\text{Ca}^{2+}$  pumps,  $\text{PKC}\theta$ , and MPTP formation, so that cytosolic  $\text{Ca}^{2+}$  remains high, and  $\text{Ca}^{2+}$ -dependent phospholipid scrambling via anoctamin 6 becomes activated. How this facilitates extracellular fibrin formation is unclear.

#### Platelet signaling for clot retraction: central role of integrins

The regulation of clot retraction by platelets has been less extensively studied than that of PS exposure. Despite the only sparse mechanistic studies, clot retraction is generally considered to be an  $\alpha_{\text{IIb}}\beta_3$ -dependent event and a marker of integrin



**Fig. 3.** Model of platelet-based coagulation. Vascular damage initiates the extrinsic coagulation pathway via tissue factor (TF) and the intrinsic pathway via collagen. Collagen-adhered platelets and, in a later stage, patches of platelets in a thrombus expose phosphatidylserine (PS), and thereby serve as a membrane substrate for the tenase and prothrombinase complexes, resulting in massive thrombin generation. These platelets can also support fibrin formation and form a fibrin coat. Populations of PS-exposing platelets are characteristically high in cytosolic  $\text{Ca}^{2+}$ , whereas the remaining aggregated platelets in a thrombus display activated  $\alpha_{\text{IIb}}\beta_3$ . By interacting with fibrin in the presence of thrombin, the latter platelets contract and cause clot retraction.

outside-in signaling. While we do not aim to repeat other reviews on platelet integrin signaling [13], here we summarize what is known of the signaling processes implicated in platelet-dependent clot retraction.

#### Signaling downstream of $\alpha_{\text{IIb}}\beta_3$

Several investigations, mostly using mouse blood, have indicated that clot retraction is mediated by signaling proteins that also contribute to  $\alpha_{\text{IIb}}\beta_3$  outside-in signaling. Specific platelet proteins implicated in clot retraction include the protein tyrosine kinases Src and Fyn, the adapter protein Lnk, AMP-activated protein kinase, and the effector proteins PLC $\gamma$ 2 and PI3K $\beta$  [146–148]. Recent studies point to two phases in platelet-dependent clot retraction upon stimulation with thrombin: the initial formation of high-adhesion  $\alpha_{\text{IIb}}\beta_3$  contacts with fibrin, and a subsequent phase in which the platelet contractile forces are transmitted to the fibrin clot [65,149]. The force generation occurs via the actin cytoskeleton, and appears to depend on activation of myosin IIA by Rho kinase and myosin light-chain kinase [150]. An extracellular process required for clot retraction is the cross-linking of platelet-associated fibrin fibers by the transglutaminase FXIIIa [151]. The  $\text{Ca}^{2+}$ -dependent protease calpain suppresses clot retraction, probably by cleaving cytoskeletal proteins [152]. In this context, it is relevant to note that calpain causes considerable degradation of cytoskeletal proteins in PS-exposing platelets [153], thus providing another explanation for why the PS-exposing platelet population is not involved in clot retraction. Next to the already established role of thrombin in platelet PS exposure [154], these data also point to a physiologic role of thrombin in integrin-dependent clot retraction.

Hence, the signaling processes contributing to platelet-regulated clot retraction, as far as they have been unraveled, rely on  $\alpha_{\text{IIb}}\beta_3$ -dependent signaling processes controlling actin–myosin interactions, which are strikingly different from those leading to PS exposure.

#### Conclusion

Taken together, all of these findings lead to an adapted model of platelet-based coagulation, as schematized in Fig. 3. Collagen-adhered platelets and, in a later stage, patches of platelets in a thrombus, activated by thrombin and other agonists, display with prolonged high  $\text{Ca}^{2+}$ , and expose PS. PS-exposing platelets serve as membrane substrate for multiple coagulation factors, with massive propagation of thrombin generation as a result. These platelets can also support fibrin formation and form a fibrin coat. The remaining aggregated platelets with active  $\alpha_{\text{IIb}}\beta_3$  contract and cause clot retraction by interacting with fibrin in the presence of thrombin. This scheme may help to resolve many unanswered questions regarding platelet and coagulation activation in arterial and venous thrombosis.

#### Disclosure of conflict of interests

This work was supported by the Cardiovascular Centre (HVC) Maastricht, the Center for Translational Molecular Medicine (CTMM) INCOAG, and the Netherlands Heart Foundation (2011T006).

#### Supporting information

Additional Supporting Information may be found in the online version of this article:

**Video S1.** Whole-blood thrombus formation on collagen under coagulating conditions.

#### References

- 1 Mann KG, Brummel K, Butenas S. What is all that thrombin for? *J Thromb Haemost* 2003; **1**: 1504–14.
- 2 Heemskerk JW, Bevers EM, Lindhout T. Platelet activation and blood coagulation. *Thromb Haemost* 2002; **88**: 186–93.
- 3 Monroe DM, Hoffman M, Roberts HR. Platelets and thrombin generation. *Arterioscler Thromb Vasc Biol* 2002; **22**: 1381–9.

- 4 Jurk K, Kehrel BE. Platelets: physiology and biochemistry. *Semin Thromb Hemost* 2005; **31**: 381–92.
- 5 Jackson SP. Arterial thrombosis: insidious, unpredictable and deadly. *Nat Med* 2011; **17**: 1423–36.
- 6 Chou J, Mackman N, Merrill-Skoloff G, Pedersen B, Furie BC, Furie B. Hematopoietic cell-derived microparticle tissue factor contributes to fibrin formation during thrombus propagation. *Blood* 2004; **104**: 3190–7.
- 7 Dubois C, Panicot-Dubois L, Merrill-Skoloff G, Furie B, Furie BC. Glycoprotein VI-dependent and -independent pathways of thrombus formation in vivo. *Blood* 2006; **107**: 3902–6.
- 8 Kalia N, Auger JM, Atkinson B, Watson SP. Critical role of FcR  $\gamma$ -chain, LAT, PLC $\gamma$ 2 and thrombin in arteriolar thrombus formation upon mild, laser-induced endothelial injury in vivo. *Microcirculation* 2008; **15**: 325–35.
- 9 Stegner D, Nieswandt B. Platelet receptor signaling in thrombus formation. *J Mol Med* 2011; **89**: 109–21.
- 10 Kuijpers MJ, Munnix IC, Cosemans JM, van Vlijmen BJ, Reutelingsperger CP, oude Egbrink MG, Heemskerk JW. Key role of platelet procoagulant activity in tissue factor- and collagen-dependent thrombus formation in arterioles and venules in vivo. Differential sensitivity to thrombin inhibition. *Microcirculation* 2008; **15**: 269–82.
- 11 Brill A, Fuchs TA, Chauhan AK, Yang JJ, de Meyer SF, Köllnberger M, Wakefield TW, Lämmle B, Massberg S, Wagner DD. von Willebrand factor-mediated platelet adhesion is critical for deep vein thrombosis in mouse models. *Blood* 2011; **117**: 1400–7.
- 12 Furie B, Furie BC. Thrombus formation in vivo. *J Clin Invest* 2005; **115**: 3355–62.
- 13 Shattil SJ, Kim C, Ginsberg MH. The final steps of integrin activation: the end game. *Nat Rev Mol Cell Biol* 2010; **11**: 288–300.
- 14 Béguin S, Kumar R, Keularts I, Seligsohn U, Coller BS, Hemker HC. Fibrin-dependent platelet procoagulant activity requires GPIb receptors and von Willebrand factor. *Blood* 1999; **93**: 564–70.
- 15 Zwaal RF, Schroit AJ. Pathophysiological implications of membrane phospholipid asymmetry in blood cells. *Blood* 1997; **89**: 1121–32.
- 16 Munnix IC, Kuijpers MJ, Auger JM, Thomassen CM, Panizzi P, van Zandvoort MA, Rosing J, Bock PE, Watson SP, Heemskerk JW. Segregation of platelet aggregatory and procoagulant microdomains in thrombus formation. Regulation by transient integrin activation. *Arterioscler Thromb Vasc Biol* 2007; **27**: 2484–90.
- 17 Berny MA, Munnix IC, Auger JM, Schols SE, Cosemans JM, Panizzi P, Bock PE, Watson SP, McCarty OJ, Heemskerk JW. Spatial distribution of factor Xa, thrombin, and fibrin(ogen) on thrombi at venous shear. *PLoS ONE* 2010; **5**: e10415.
- 18 Jurk K, Lahav J, van Aken H, Brodde MF, Nofer JR, Kehrel BE. Extracellular protein disulfide isomerase regulates feedback activation of platelet thrombin generation via modulation of coagulation factor binding. *J Thromb Haemost* 2011; **9**: 2278–90.
- 19 Daubie V, Cauwenberghs S, Senden NHM, Pochet R, Lindhout T, Buurman WA, Heemskerk JW. Factor Xa and thrombin evoke additive calcium and proinflammatory responses in endothelial cells subjected to coagulation. *Biochim Biophys Acta* 2006; **1763**: 860–9.
- 20 Gardiner EE, Karunakaran D, Shen Y, Arthur JF, Andrews RK, Bernd MC. Controlled shedding of platelet glycoprotein (GP)VI and GPIb/IX/V by ADAM family metalloproteinases. *J Thromb Haemost* 2007; **7**: 1530–7.
- 21 Al-Tamimi M, Grigoriadis G, Tran H, Paul E, Servadei P, Berndt MC, Gardiner EE, Andrews RK. Coagulation-induced shedding of platelet glycoprotein VI mediated by factor Xa. *Blood* 2011; **117**: 3912–20.
- 22 Ravanat C, Freund M, Mangin P, Azorsa DO, Schwartz C, Moog S, Schuhler S, Dambach J, Cazenave JP, Lanza F. GPV is a marker of in vivo platelet activation: study in a rat thrombosis model. *Thromb Haemost* 2000; **83**: 327–33.
- 23 Panes O, Matus V, Saez CG, Quiroga T, Pereira J, Mezzano D. Human platelets synthesize and express tissue factor. *Blood* 2007; **109**: 5242–50.
- 24 Rondina MT, Schwertz H, Harris ES, Kraemer BF, Campbell RA, Mackman N, Grissom CK, Weyrich AS, Zimmerman GA. The septic milieu triggers expression of spliced tissue factor mRNA in human platelets. *J Thromb Haemost* 2011; **9**: 748–58.
- 25 Kretz CA, Vaezzadeh N, Gross PL. Tissue factor and thrombosis models. *Arterioscler Thromb Vasc Biol* 2010; **30**: 900–8.
- 26 Maroney SA, Cooley BC, Ferrel JP, Bonesho CE, Mast AE. Murine hematopoietic cell tissue factor pathway inhibitor limits thrombus growth. *Arterioscler Thromb Vasc Biol* 2011; **31**: 821–6.
- 27 Gould WR, Silveira JR, Tracy PB. Unique *in vivo* modifications of coagulation factor V produce a physically and functionally distinct platelet-derived cofactor. *J Biol Chem* 2004; **279**: 2383–93.
- 28 Majumder R, Quinn-Allen MA, Kane WH, Lentz BR. A phosphatidylserine binding site in factor Va C1 domain regulates both assembly and activity of the prothrombinase complex. *Blood* 2008; **112**: 2795–802.
- 29 Fager AM, Wood JP, Bouchard BA, Feng P, Tracy PB. Properties of procoagulant platelets: defining and characterization of the subpopulation binding a functional prothrombinase. *Arterioscler Thromb Vasc Biol* 2010; **30**: 2400–7.
- 30 Alberio L, Safa O, Clemetson KJ, Esmon CT, Dale GL. Surface expression and functional characterization of  $\alpha$ -granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin. *Blood* 2000; **95**: 1694–702.
- 31 Weeterings C, de Groot PG, Adelmeijer J, Lisman T. The glycoprotein Ib–V–IX complex contributes to tissue factor-independent thrombin generation by recombinant factor VIIa on the activated platelet surface. *Blood* 2008; **112**: 3227–33.
- 32 Lisman T, Adelmeijer J, Cauwenberghs S, van Pampus EC, Heemskerk JW, de Groot PG. Recombinant factor VIIa enhances platelet adhesion and activation under flow conditions at normal and reduced platelet count. *J Thromb Haemost* 2005; **3**: 742–51.
- 33 Gilbert GE, Novakovic VA, Kaufman RJ, Miao H, Pipe SW. Conservative mutations in the C2 domains of factor VIII and factor V alter phospholipid binding and cofactor activity. *Blood* 2012; **120**: 1923–32.
- 34 Cosemans JM, Schols SE, Stefanini L, de Witt S, Feijge MA, Hamulyak K, Deckmyn H, Bergmeier W, Heemskerk JW. Key role of glycoprotein Ib/V/IX and von Willebrand factor in platelet activation-dependent fibrin formation at low shear flow. *Blood* 2011; **117**: 651–60.
- 35 London FS, Marcinkiewicz M, Walsh PN. A subpopulation of platelets responds to thrombin- or SFLLRN-stimulation with binding sites for factor IXa. *J Biol Chem* 2004; **279**: 19854–9.
- 36 London FS, Marcinkiewicz M, Walsh PN. PAR1-stimulated factor IXa binding to a small platelet subpopulation requires a pronounced and sustained increase of cytoplasmic calcium. *Biochemistry* 2006; **45**: 7289–98.
- 37 Walsh PN. Platelet coagulant activities in thrombasthenia. *Br J Haematol* 1972; **23**: 553–69.
- 38 Renné T, Pozgajova M, Grüner S, Schuh K, Pauer HU, Burfeind P, Gailani D, Nieswandt B. Defective thrombus formation in mice lacking coagulation factor XII. *J Exp Med* 2005; **202**: 271–81.
- 39 Van der Meijden PE, Munnix IC, Auger JM, Govers-Riemsag JW, Cosemans JM, Kuijpers MJ, Spronk HM, Watson SP, Renné T, Heemskerk JW. Dual role of collagen in factor XII-dependent thrombus and clot formation. *Blood* 2009; **114**: 881–90.
- 40 Baglia FA, Shrimpton CN, Emsley J, Kitagawa K, Ruggeri ZM, Lopez JA, Walsh PN. Factor XI interacts with the leucine-rich repeats of glycoprotein Ib $\alpha$  on the activated platelet. *J Biol Chem* 2004; **279**: 49323–9.
- 41 White-Adams TC, Berny MA, Tucker EI, Gertz JM, Gailani D, Urbanus RT, de Groot PG, Gruber A, McCarty OJ. Identification of coagulation factor XI as a ligand for platelet apolipoprotein E receptor 2 (ApoER2). *Arterioscler Thromb Vasc Biol* 2009; **29**: 1602–7.
- 42 Müller F, Mutch NJ, Schenk WA, Smith SA, Esterl L, Spronk HM, Schmidbauer S, Gahl WA, Morrissey JH, Renné T. Platelet

- polyphosphates are proinflammatory and procoagulant mediators *in vivo*. *Cell* 2009; **139**: 1143–56.
- 43 Smith SA, Choi SH, Davis-Harrison R, Huyck J, Boettcher J, Reinstra CM, Morrissey JH. Polyphosphate exerts differential effects on blood clotting, depending on the polymer size. *Blood* 2010; **116**: 4353–9.
- 44 Choi SH, Smith SA, Morrissey JH. Polyphosphate is a cofactor for the activation of factor XI by thrombin. *Blood* 2011; **118**: 6963–70.
- 45 Magwenzi SG, Ajjan RA, Standeven KF, Parapia LA, Naseem KM. Factor XIII supports platelet activation and enhances thrombus formation by matrix proteins under flow conditions. *J Thromb Haemost* 2011; **9**: 820–33.
- 46 Dale GL, Friese P, Batar P, Hamilton SF, Reed GL, Jackson KW, Clemetson KJ, Alberio L. Stimulated platelets use serotonin to enhance their retention of procoagulant proteins on the cell surface. *Nature* 2002; **415**: 175–9.
- 47 Jobe SM, Leo L, Eastvold JS, Dickneite G, Ratliff TL, Lentz SR, di Paola J. Role of FcRg and factor XIIIa in coated platelet formation. *Blood* 2005; **106**: 4146–51.
- 48 Maroney SA, Haberichter SL, Friese P, Collins ML, Ferrel JP, Dale GL, Mast AE. Active tissue factor pathway inhibitor is expressed on the surface of coated platelets. *Blood* 2007; **109**: 1931–7.
- 49 Boulaftali Y, Ho-Tin-Noe B, Pena A, Loyau S, Venisse L, Francois D, Richard B, Arocas V, Collet JP, Jandrot-Perrus M, Bouton MC. Platelet protease nexin-1, a serpin that strongly influences fibrinolysis and thrombolysis. *Circulation* 2011; **12**: 1326–34.
- 50 White TC, Berny MA, Tucker EI, Urbanus RT, de Groot PG, Fernandez JA, Griffin JH, Gruber A, McCarty OJ. Protein C supports platelet binding and activation under flow: role of glycoprotein Ib and apolipoprotein E receptor 2. *J Thromb Haemost* 2008; **6**: 995–1002.
- 51 Van't Veer C, Butenas S, Golden NJ, Mann KG. Regulation of prothrombinase activity by protein S. *Thromb Haemost* 1999; **82**: 80–7.
- 52 Dayer MR, Ghayour O, Dayer MS. Mechanism of protein Z-mediated inhibition of coagulation factor Xa by Z-protein-dependent inhibitor: a molecular dynamic approach. *ISRN Hematol* 2012; **2012**: in press.
- 53 Munnix IC, Cosemans JM, Auger JM, Heemskerk JW. Platelet response heterogeneity in thrombus formation. *Thromb Haemost* 2009; **102**: 1149–56.
- 54 Nesbitt WS, Westein E, Tovar-Lopez FJ, Tolouei E, Mitchell A, Fu J, Carberry J, Fouras A, Jackson SP. A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. *Nat Med* 2009; **15**: 665–73.
- 55 Kulkarni S, Jackson SP. Platelet factor XIII and calpain negatively regulate integrin  $\alpha$ Ib $\beta$ 3 adhesive function and thrombus growth. *J Biol Chem* 2004; **279**: 30697–706.
- 56 Heemskerk JW, Kuijpers MJ, Munnix IC, Siljander PR. Platelet collagen receptors and coagulation. A characteristic platelet response as possible target for antithrombotic treatment. *Trends Cardiovasc Med* 2005; **15**: 86–92.
- 57 Shi J, Pipe JT, Heegaard CW, Gilbert GE. Lactadherin blocks thrombosis and hemostasis *in vivo*: correlation with platelet phosphatidylserine exposure. *J Thromb Haemost* 2008; **6**: 1167–74.
- 58 Bevers EM, Comfurius P, Zwaal RF. Platelet procoagulant activity, physiological significance and mechanisms of exposure. *Blood Rev* 1991; **5**: 146–54.
- 59 Dale GL. Coated-platelets: an emergent component of the procoagulant response. *J Thromb Haemost* 2005; **3**: 2185–92.
- 60 Szasz R, Dale GL. Thrombospondin and fibrinogen bind serotonin-derivatized proteins on COAT platelets. *Blood* 2002; **100**: 2827–31.
- 61 Dale GL, Remenyi G, Friese P. Quantitation of microparticles released from coated-platelets. *J Thromb Haemost* 2005; **3**: 2081–8.
- 62 Jobe SM, Wilson KM, Leo L, Raimondi A, Molkenin JD, Lentz SR, diPaola J. Critical role for the mitochondrial permeability transition pore and cyclophilin D in platelet activation and thrombosis. *Blood* 2008; **111**: 1257–65.
- 63 Remenyi G, Szasz R, Friese P, Dale GL. Role of mitochondrial permeability transition pore in coated-platelet formation. *Arterioscler Thromb Vasc Biol* 2005; **25**: 467–71.
- 64 Yakimenko AO, Verholomova FY, Kotova YN, Ataulkhanov FI, Pantelev MA. Identification of different proaggregatory abilities of activated platelet subpopulations. *Biophys J* 2012; **102**: 2261–9.
- 65 Schoenwaelder SM, Ono A, Nesbitt WS, Lim J, Jarman K, Jackson SP. Phosphoinositide 3-kinase p110 $\beta$  regulates integrin  $\alpha$ Ib $\beta$ 3 avidity and the cellular transmission of contractile forces. *J Biol Chem* 2010; **285**: 2886–96.
- 66 Brass LF, Zhu L, Stalker TJ. Minding the gaps to promote thrombus growth and stability. *J Clin Invest* 2005; **115**: 3385–92.
- 67 Schoenwaelder SM, Yuan Y, Josefsson EC, White MJ, Tao Y, Mason KD, O'Reilly LA, Henley KJ, Ono A, Hsiao S, Willcox A, Roberts AW, Huang DCS, Salem HH, Kile BT, Jackson SP. Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function. *Blood* 2009; **114**: 663–6.
- 68 Vogler M, Hamali HA, Sun XM, Bampton ET, Dinsdale D, Snowden RT, Dyer MJS, Goodall AH, Cohen GM. BCL2/BCL-XL inhibition induces apoptosis, disrupts cellular calcium homeostasis and prevents platelet activation. *Blood* 2011; **117**: 7145–54.
- 69 Kodama T, Takehara T, Hikita H, Shimizu S, Shigekawa M, Li W, Miyagi T, Hosui A, Tatsumi T, Ishida H, Kanto T, Hiramatsu N, Yin XM, Hayashi N. BH3-only activator proteins Bid and Bim are dispensable for Bak/Bax-dependent thrombocyte apoptosis induced by Bcl-xl deficiency. Molecular requisites for the mitochondrial pathway to apoptosis in platelets. *J Biol Chem* 2011; **286**: 13905–13.
- 70 Mason KD, Carpinelli MR, Fletcher JI, Collinga JE, Hilton AA, Ellis S, Kelly PN, Ekert PG, Metcalf D, Roberts AW, Huang DCS, Kile BT. Programmed nuclear cell death delimits platelet life span. *Cell* 2007; **128**: 1173–86.
- 71 Curvers J, van Pampus EC, Feijge MA, Rombout-Sestriekova E, Giesen PL, Heemskerk JW. Decreased responsiveness and development of activation markers of platelets stored in plasma. *Transfusion* 2004; **44**: 49–58.
- 72 Rand ML, Wang HH, Bang KW, Poon KS, Packham MA, Freedman J. Procoagulant surface exposure and apoptosis in rabbit platelets: association with shortened survival and steady-state senescence. *J Thromb Haemost* 2004; **2**: 651–9.
- 73 Albanyan AM, Murphy FM, Rasmussen JT, Heegaard CW, Harrison P. Measurement of phosphatidylserine exposure during storage of platelet concentrates using the novel probe lactadherin: a comparison study with annexin V. *Transfusion* 2009; **49**: 99–107.
- 74 Jackson SP, Schoenwaelder SM. Procoagulant platelets: are they necrotic? *Blood* 2010; **116**: 2011–18.
- 75 Sims J, Wiedmer T, Esmon CT, Weiss HJ, Shattil SJ. Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome, an isolated defect in platelet procoagulant activity. *J Biol Chem* 1989; **264**: 17049–57.
- 76 Cauwenberghs S, Feijge MA, Harper AG, Sage SO, Curvers J, Heemskerk JW. Shedding of procoagulant microparticles from unstimulated platelets by integrin-mediated destabilization of actin cytoskeleton. *FEBS Lett* 2006; **580**: 5313–20.
- 77 Morel O, Jesel L, Freyssinet JM, Toti F. Cellular mechanisms underlying the formation of circulating microparticles. *Arterioscler Thromb Vasc Biol* 2010; **31**: 15–26.
- 78 Vasina E, Heemskerk JW, Weber C, Koenen RR. Platelets and platelet-derived microparticles in vascular inflammatory disease. *Inflamm Allergy Drug Targets* 2010; **9**: 346–54.
- 79 Dörmann D, Clemetson KJ, Kehrel B. The GPIb thrombin-binding site is essential for thrombin-induced platelet procoagulant activity. *Blood* 2000; **86**: 2469–78.
- 80 Béguin S, Keularts I, al Dieri R, Belluci S, Caen J, Hemker HC. Fibrin polymerization is crucial for thrombin generation in platelet-rich plasma in a vWF-GPIb-dependent process, defective in Bernard-Soulier syndrome. *J Thromb Haemost* 2004; **2**: 170–6.

- 81 McNicol A, Sutherland M, Zou R, Drouin J. Defective thrombin-induced calcium changes and aggregation of Bernard-Soulier platelets are not associated with deficient moderate-affinity receptors. *Arterioscler Thromb Vasc Biol* 1996; **16**: 628–32.
- 82 Ravanat C, Strassel C, Hechler B, Schuhler S, Chicanne G, Payrastré B, Gachet C, Lanza F. A central role of GPIb-IX in the procoagulant function of platelets that is independent of the 45-kDa GPIb $\alpha$  N-terminal extracellular domain. *Blood* 2010; **116**: 1157–64.
- 83 Ramakrishnan V, Reeves PS, DeGuzman F, Deshpande U, Ministri Madrid K, DuBridge RB, Phillips DR. Increased thrombin responsiveness in platelets from mice lacking glycoprotein V. *Proc Natl Acad Sci USA* 1999; **96**: 13336–41.
- 84 Offermanns S. Activation of platelet function through G protein-coupled receptors. *Circ Res* 2006; **99**: 1293–304.
- 85 Heemskerk JW, Feijge MA, Henneman L, Rosing J, Hemker HC. The Ca<sup>2+</sup>-mobilizing potency of  $\alpha$ -thrombin and thrombin-receptor-activating peptide on human platelets. Concentration and time effects of thrombin-induced Ca<sup>2+</sup> signaling. *Eur J Biochem* 1997; **249**: 547–55.
- 86 Andersen H, Greenberg DL, Fujikawa K, Xu WF, Chung DW, Davie EW. Protease-activated receptor 1 is the primary mediator of thrombin-stimulated platelet procoagulant activity. *Proc Natl Acad Sci USA* 1999; **96**: 11189–93.
- 87 Keuren JF, Wielders SJ, Ulrichs T, Hackeng T, Deckmyn H, Heemskerk JW, Bevers E, Lindhout T. Synergistic effect of thrombin on collagen-induced platelet procoagulant activity is mediated through protease-activated receptor-1. *Arterioscler Thromb Vasc Biol* 2005; **25**: 1499–505.
- 88 Harper MT, Poole AW. PKC inhibition markedly enhances Ca<sup>2+</sup> signaling and phosphatidylserine exposure downstream of protease-activated receptor-1 but not protease-activated receptor-4 in human platelets. *J Thromb Haemost* 2011; **9**: 1599–607.
- 89 Dorsam RT, Tuluc M, Kunapuli SP. Role of protease-activated and ADP receptor subtypes in thrombin generation on human platelets. *J Thromb Haemost* 2004; **2**: 804–12.
- 90 Coughlin SR. Thrombin signalling and protease-activated receptors. *Nature* 2000; **407**: 258–64.
- 91 Andrews RK, Karunakaran D, Gardiner EE, Berndt MC. Platelet receptor proteolysis. A mechanism for downregulating platelet reactivity. *Arterioscler Thromb Vasc Biol* 2007; **27**: 1511–20.
- 92 Trivedi V, Boire A, Tchernychev B, Kaneider NC, Leger AJ, O'Callaghan K, Covic L, Kuliopulos A. Platelet matrix metalloprotease-1 mediates thrombogenesis by activating PAR1 at a cryptic site. *Cell* 2009; **137**: 332–43.
- 93 Quinton TM, Kim S, Derian CK, Jin J, Kunapuli SP. Plasmin-mediated activation of platelets occurs by cleavage of protease-activated receptor 4. *J Biol Chem* 2004; **279**: 18434–9.
- 94 Sambrano GR, Huang W, Faruqi T, Mahrus S, Craik C, Coughlin SR. Cathepsin G activates protease-activated receptor-4 in human platelets. *J Biol Chem* 2000; **275**: 6819–23.
- 95 Lecut C, Schoolmeester A, Kuijpers MJ, Broers JLV, van Zandvoort MA, Vanhoorelbeke K, Deckmyn H, Jandrot-Perrus M, Heemskerk JW. Principal role of glycoprotein VI in  $\alpha 2\beta 1$  and  $\alpha I Ib\beta 3$  activation during collagen-induced thrombus formation. *Arterioscler Thromb Vasc Biol* 2004; **24**: 1727–33.
- 96 Vanschoonbeek K, Feijge MA, van Kampen RJW, Kenis H, Hemker HC, Giesen PL, Heemskerk JW. Initiating and potentiating roles of platelets in tissue factor-induced thrombin generation in the presence of plasma: subject-dependent variation in thrombogram characteristics. *J Thromb Haemost* 2004; **2**: 476–84.
- 97 Léon C, Alex M, Klocke A, Morgenstern E, Moosbauer C, Eckly A, Spannagl M, Gachet C, Engelmann B. Platelet ADP receptors contribute to the initiation of intravascular coagulation. *Blood* 2004; **103**: 594–600.
- 98 van der Meijden PE, Schoenwaelder SM, Cosemans JM, Wetzker R, Heller R, Jackson SP, Heemskerk JW. Dual P2Y<sub>12</sub> receptor signaling in thrombin-stimulated platelets: involvement of phosphoinositide 3-kinase  $\beta$  but not  $\gamma$  isoforms in Ca<sup>2+</sup> mobilization and procoagulant activity. *FEBS J* 2008; **275**: 371–85.
- 99 van der Meijden PE, Feijge MA, Giesen PL, Huijberts M, van Raak EP, Heemskerk JW. Platelet P2Y<sub>12</sub> receptors enhance signalling towards procoagulant activity and thrombin generation: a study with healthy subjects and patients at thrombotic risk. *Thromb Haemost* 2005; **93**: 1128–37.
- 100 Frelinger AL, Jakubowski JA, Li Y, Barnard MR, Linden MD, Rarnow I, Fox ML, Sugidachi A, Winters KJ, Furman MI, Michelson AD. The active metabolite of prasugrel inhibits adenosine diphosphate- and collagen-stimulated platelet procoagulant activities. *J Thromb Haemost* 2008; **6**: 359–65.
- 101 Judge HM, Buckland RJ, Sugidachi A, Jakubowski JA, Storey RF. The active metabolite of prasugrel effectively blocks the platelet P2Y<sub>12</sub> receptor and inhibits procoagulant and pro-inflammatory reactions. *Platelets* 2008; **19**: 125–33.
- 102 van der Meijden PE, Swieringa S, Feijge MA, Gilio K, Hamulyák K, Heemskerk JW. Integrin  $\alpha I Ib\beta 3$  outside-in signaling causes platelet procoagulant activity and thrombin generation. *Cell Mol Life Sci* 2012; **69**: 3481–92.
- 103 Iiveskero S, Lassila R. Abciximab inhibits procoagulant activity but not the release reaction upon collagen- or clot-adherent platelets. *J Thromb Haemost* 2003; **1**: 805–13.
- 104 Goto S, Tamura N, Li M, Handa M, Ikeda Y, Handa S, Ruggeri ZM. Differential effects of various anti-GPIIb/IIIa agents on shear-induced platelet activation and expression of procoagulant activity. *J Thromb Haemost* 2003; **1**: 2022–30.
- 105 Lhermusier T, van Rottem J, Garcia C, Xuereb JM, Ragab A, Martin V, Gratacap MP, Sie P, Payrastré B. The Syk-kinase inhibitor R406 impairs platelet activation and monocyte tissue factor expression triggered by heparin-PF4 complex directed antibodies. *J Thromb Haemost* 2011; **8**: 2067–76.
- 106 Siljander P, Farndale RW, Feijge MA, Comfurius P, Kos S, Bevers EM, Heemskerk JW. Platelet adhesion enhances the glycoprotein VI-dependent procoagulant response: involvement of p38 MAP kinase and calpain. *Arterioscler Thromb Vasc Biol* 2001; **21**: 618–27.
- 107 Munnix IC, Strehl A, Kuijpers MJ, Auger JM, van der Meijden PE, van Zandvoort MA, oude Egbrink M, Nieswandt B, Heemskerk JW. The glycoprotein VI-phospholipase C $\gamma 2$  signaling pathway controls thrombus formation induced by collagen and tissue factor in vitro and in vivo. *Arterioscler Thromb Vasc Biol* 2005; **25**: 2673–8.
- 108 Auger JM, Kuijpers MJ, Senis YA, Watson SP, Heemskerk JW. Adhesion of human and mouse platelets to collagen under shear: a unifying model. *FASEB J* 2005; **19**: 825–7.
- 109 Elvers M, Pozgai R, Varga-Szabo D, May F, Pleines I, Kuijpers MJ, Heemskerk JW, Nieswandt B. Platelet hyperactivity and a prothrombotic phenotype in mice with a gain-of-function mutation in phospholipase C $\gamma 2$ . *J Thromb Haemost* 2010; **8**: 1353–63.
- 110 Siljander PR, Munnix IC, Smethurst PA, Deckmyn H, Lindhout T, Ouwehand WH, Farndale RW, Heemskerk JW. Platelet receptor interplay regulates collagen-induced thrombus formation in flowing human blood. *Blood* 2004; **103**: 1333–41.
- 111 Heemskerk JW, Vuist WM, Feijge MA, Reutelingsperger CP, Lindhout T. Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine and procoagulant activity of adherent platelets. Evidence for regulation by protein tyrosine kinase-dependent Ca<sup>2+</sup> responses. *Blood* 1997; **90**: 2615–25.
- 112 Thomas DH, Getz TM, Newman TN, Dangelmaier CA, Carpino N, Kunapuli SP, Tsygankov AY, Daniel JL. A novel histidine tyrosine phosphatase, TULA-2, associates with Syk and negatively regulates GPVI signaling in platelets. *Blood* 2010; **116**: 2570–8.
- 113 Bevers EM, Comfurius P, van Rijn JL, Hemker HC, Zwaal RF. Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. *Eur J Biochem* 1982; **122**: 429–36.
- 114 Séverin S, Pollitt AY, Navarro-Nunez L, Nash CA, Mourao-Sa D, Eble JA, Senis YA, Watson SP. Syk-dependent phosphorylation of

- CLEC-2. A novel mechanism of hem-immunoreceptor tyrosine-based activation motif signaling. *J Biol Chem* 2011; **286**: 4107–16.
- 115 Nergiz-Unal R, Lamers MM, van Kruchten R, Luiken JJ, Cosemans JM, Glatz JF, Kuijpers MJ, Heemskerk JW. Signaling role of CD36 in platelet activation and thrombus formation on immobilized thrombospondin or oxidized low density lipoprotein. *J Thromb Haemost* 2011; **9**: 1835–46.
- 116 White-Adams TC, Berny MA, Patel IA, Tucker EI, Gailani D, Gruber A, McCarty OJ. Laminin promotes coagulation and thrombus formation in a factor XII-dependent manner. *J Thromb Haemost* 2010; **8**: 1295–301.
- 117 Gilio K, Munnix IC, Mangin P, Cosemans JM, Feijge MA, van der Meijden PE, Olieslagers S, Chrzanoska-Wodnicka MB, Lillian R, Schoenwaelder S, Koyasu S, Sage SO, Jackson SP, Heemskerk JW. Non-redundant roles of phosphoinositide 3-kinase isoforms  $\alpha$  and  $\beta$  in glycoprotein VI-induced platelet signaling and thrombus formation. *J Biol Chem* 2009; **285**: 33750–62.
- 118 Smeets EF, Heemskerk JW, Comfurius P, Bevers EM, Zwaal RF. Thapsigargin amplifies the platelet procoagulant response caused by thrombin. *Thromb Haemost* 1993; **70**: 1024–9.
- 119 Varga-Szabo D, Braun A, Kleinschnitz C, Bender M, Pleines I, Pham M, Renné T, Stoll G, Nieswandt B. The calcium sensor STIM1 is an essential mediator of arterial thrombosis and ischemic brain infarction. *J Exp Med* 2008; **205**: 1583–91.
- 120 Braun A, Varga-Szabo D, Kleinschnitz C, Pleines I, Bender M, Austinat M, Bösi M, Stoll G, Nieswandt B. Orail (CRACM1) is the platelet SOC channel and essential for pathological thrombus formation. *Blood* 2009; **113**: 2056–63.
- 121 Elvers M, Herrmann A, Seizer P, Münzer P, Beck S, Schönberger T, Borst O, Martin-Romero FJ, Lang F, May AE, Gawaz M. Intracellular cyclophilin A is an important  $\text{Ca}^{2+}$  regulator in platelets and critically involved in arterial thrombus formation. *Blood* 2012; **120**: 1317–26.
- 122 Gilio K, van Kruchten R, Braun A, Berna-Erro A, Feijge MA, Stegner D, van der Meijden PE, Kuijpers MJ, Varga-Szabo D, Heemskerk JW, Nieswandt B. Roles of STIM1 and Orail in glycoprotein VI- and thrombin-dependent procoagulant activity and thrombus formation. *J Biol Chem* 2010; **285**: 23629–38.
- 123 Bergmeier W, Oh-hora M, McCarl CA, Roden RC, Bray PF, Feske S. R93W mutation in Orail causes impaired calcium influx in platelets. *Blood* 2009; **109**: 6875–8.
- 124 Harper MT, Poole AW. Store-operated calcium entry and non-capacitative calcium entry have distinct roles in thrombin-induced calcium signaling in human platelets. *Cell Calcium* 2011; **50**: 351–8.
- 125 Ramanathan G, Gupta S, Thielmann I, Pleines I, Varga-Szabo D, May F, Mannhalter C, Dietrich A, Nieswandt B, Braun A. Defective diacylglycerol-induced  $\text{Ca}^{2+}$  entry but normal agonist-induced activation responses in TRPC6-deficient mouse platelets. *J Thromb Haemost* 2012; **10**: 419–29.
- 126 Hassock SR, Zhu MX, Trost C, Flockerzi V, Authi KS. Expression and role of Trpc proteins in human platelets: evidence that Trpc6 forms the store-independent calcium entry channel. *Blood* 2002; **100**: 2801–11.
- 127 Lopez JJ, Salido GM, Pariente JA, Rosado JA. Interaction of Stim1 with endogenously expressed human canonical Trp1 upon depletion of intracellular  $\text{Ca}^{2+}$  stores. *J Biol Chem* 2006; **281**: 28254–64.
- 128 Varga-Szabo D, Authi KS, Braun A, Bender M, Ambily A, Hassock SR, Gudermann T, Dietrich A, Nieswandt B. Store-operated  $\text{Ca}^{2+}$  entry in platelets occurs independently of transient receptor potential (TRP) C1. *Pflügers Arch* 2008; **457**: 377–87.
- 129 Tomasiak M, Stelmach H, Rusak T, Ciborowski M, Radziwon P. The involvement of  $\text{Na}^+/\text{K}^+$ -ATPase in the development of platelet procoagulant response. *Acta Biochim Pol* 2007; **54**: 625–39.
- 130 Bucki R, Pastore JL, Giraud F, Janmey PA, Sulpice JC. Involvement of the  $\text{Na}^+/\text{H}^+$  exchanger in membrane phosphatidylserine exposure during human platelet activation. *Biochim Biophys Acta* 2006; **1761**: 195–204.
- 131 Wolfs JL, Wielders SJ, Comfurius P, Lindhout T, Giddings JC, Zwaal RF, Bevers EM. Reversible inhibition of the platelet procoagulant response through manipulation of the Gardos channel. *Blood* 2006; **108**: 2223–8.
- 132 Leytin V, Allen DJ, Mutlu A, Gyulkhandanyan AV, Mykhaylov S, Freedman J. Mitochondrial control of platelet apoptosis: effect of cyclosporin A, an inhibitor of the mitochondrial permeability transition pore. *Lab Invest* 2009; **89**: 374–84.
- 133 Starkov AA. The molecular identity of the mitochondrial  $\text{Ca}^{2+}$  sequestration system. *FEBS J* 2010; **277**: 3652–63.
- 134 Strehl A, Munnix IC, Kuijpers MJ, van der Meijden PE, Cosemans JM, Feijge MA, Nieswandt B, Heemskerk JW. Dual role of platelet protein kinase C in thrombus formation: stimulation of pro-aggregatory and suppression of procoagulant activity in platelets. *J Biol Chem* 2007; **282**: 7046–55.
- 135 Konopatskaya O, Gilio K, Harper MT, Zhao Y, Cosemans JM, Karim AZ, Whiteheart SW, Molkenin JD, Verkade P, Watson SP, Heemskerk JW, Poole AW. PKC $\alpha$  regulates platelet granule secretion and thrombus formation in mice. *J Clin Invest* 2009; **119**: 399–407.
- 136 Gilio K, Harper MT, Cosemans JM, Konopatskaya O, Munnix IC, Prinzen L, Leitges M, Liu Q, Molkenin JD, Heemskerk JW, Poole AW. Functional divergence of platelet protein kinase C (PKC) isoforms in thrombus formation on collagen. *J Biol Chem* 2010; **285**: 23410–19.
- 137 Harper MT, Poole AW. Diverse functions of protein kinase C isoforms in platelet activation and thrombus formation. *J Thromb Haemost* 2010; **8**: 454–62.
- 138 Nagy B, Bhavaraju K, Getz T, Bynagari YS, Kim S, Kunapuli SP. Impaired activation of platelets lacking protein kinase C- $\theta$  isoform. *Blood* 2009; **113**: 2557–67.
- 139 Stefanini L, Roden RC, Bergmeier W. CalDAG-GEFI is at the nexus of calcium-dependent platelet activation. *Blood* 2009; **114**: 2506–14.
- 140 Ahmad F, Boulaftali Y, Greene TK, Ouelette TD, Poncz M, Feske S, Bergmeier W. Relative contributions of stromal interaction molecule 1 and CalDAG-GEFI to calcium-dependent platelet activation and thrombosis. *J Thromb Haemost* 2011; **9**: 2077–86.
- 141 Suzuki J, Umeda M, Sims PJ, Nagata S. Calcium-dependent phospholipid scrambling by TMEM16F. *Nature* 2010; **468**: 834–8.
- 142 Castoldi E, Collins PW, Williamson PL, Bevers EM. Compound heterozygosity for 2 novel TMEM16F mutations in a patient with Scott syndrome. *Blood* 2011; **117**: 4399–400.
- 143 Munnix IC, Harmsma M, Giddings JC, Collins PW, Feijge MA, Comfurius P, Heemskerk JW, Bevers EM. Store-mediated calcium entry in the regulation of phosphatidylserine exposure in blood cells from Scott patients. *Thromb Haemost* 2003; **89**: 687–95.
- 144 Yang H, Kim A, David T, Palmer D, Jin T, Tien J, Huang F, Cheng T, Coughlin SR, Jan YN, Jan LY. TMEM16F forms a  $\text{Ca}^{2+}$ -activated cation channel required for lipid scrambling in platelets during blood coagulation. *Cell* 2012; **151**: 111–22.
- 145 Bachelot-Loza C, Badol P, Brohard-Bohn B, Fraiz N, Cano E, Rendu F. Differential regulation of platelet aggregation and aminophospholipid exposure by calpain. *Br J Haematol* 2006; **133**: 419–26.
- 146 Takizawa H, Nishimura S, Takayama N, Oda A, Nishikii H, Morita Y, Kakinuma S, Yamazaki S, Okamura S, Tamura N, Goto S, Sawaguchi A, Manabe I, Takatsu K, Nakauchi H, Takaki S, Eto K. Lnk regulates integrin  $\alpha\text{IIb}\beta 3$  outside-in signaling in mouse platelets, leading to stabilization of thrombus development *in vivo*. *J Clin Invest* 2010; **120**: 179–90.
- 147 Suzuki-Inoue K, Hughes CE, Inoue O, Kaneko M, Cuyun-Lira O, Takafuta T, Watson SP, Ozaki Y. Involvement of Src kinases and PLC $\gamma 2$  in clot retraction. *Thromb Res* 2007; **120**: 251–8.
- 148 Randriamboavonjy V, Isaak J, Fromel T, Viollet B, Fisslthaler B, Preissner KT, Fleming I. AMPK  $\alpha 2$  subunit is involved in platelet signaling, clot retraction and thrombus stability. *Blood* 2010; **116**: 2134–40.

- 149 Martin V, Guillermet-Guibert J, Chicanne G, Cabou C, Jandrot-Perrus M, Plantavid M, Vanhaesebroeck B, Payrastre B, Gratacap MP. Deletion of the p110 $\beta$  isoform of phosphoinositide 3-kinase in platelets reveals its central role in Akt activation and thrombus formation in vitro and in vivo. *Blood* 2010; **115**: 2008–13.
- 150 Ono A, Westein E, Hsiao S, Nesbitt WS, Hamilton JR, Schoenwaelder SM, Jackson SP. Identification of a fibrin-independent platelet contractile mechanism regulating primary hemostasis and thrombus growth. *Blood* 2008; **112**: 90–9.
- 151 Kasahara K, Souri M, Kaneda M, Miki T, Yamamoto N, Ichinose A. Impaired clot retraction in factor XIII A subunit-deficient mice. *Blood* 2010; **115**: 1277–9.
- 152 Schoenwaelder SM, Yuan Y, Cooray P, Salem HH, Jackson SP. Calpain cleavage of focal adhesion proteins regulates the cytoskeletal attachment of integrin  $\alpha$ IIb $\beta$ 3 (platelet glycoprotein IIb/IIIa) and the cellular retraction of fibrin clots. *J Biol Chem* 1997; **272**: 1694–702.
- 153 Verhallen PF, Bevers EM, Comfurius P, Zwaal RF. Correlation between calpain-mediated cytoskeletal degradation and expression of platelet procoagulant activity. A role for the platelet membrane-skeleton in the regulation of membrane lipid asymmetry? *Biochim Biophys Acta* 1987; **903**: 206–17.
- 154 Béguin S, Lindhout T, Hemker HC. The effect of trace amounts of tissue factor on thrombin generation in platelet-rich plasma. *Thromb Haemost* 1989; **61**: 25–9.