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REVIEW

Targeting platelet receptor function in thrombus formation: The risk of bleeding

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ABSTRACT

In this review, we presume that the process of thrombus formation, as assessed in whole blood flow studies and in experimental (murine) thrombosis studies, reflects the platelet responses in human haemostasis and thrombosis. Following this concept, we give an up-to-date overview of the main platelet receptors and signalling pathways that contribute to thrombus formation and are used as targets in (pre)clinical intervention studies to prevent cardiovascular disease. Discussed are receptors for thrombin, thromboxane, ADP, ATP, prostaglandins, von Willebrand factor, collagen, CLEC-2 ligand, fibrinogen and laminin. Sketched are the consequences of receptor deficiency or blockage for haemostasis and thrombosis in mouse and man. Recording of bleeding due to (congenital) platelet dysfunction or (acquired) antiplatelet treatment occurs according to different protocols, while common laboratory methods are used to determine platelet function.

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1. Introduction

Platelets are essential for normal haemostasis by forming a primary plug or thrombus after vascular injury, thus preventing further blood loss. Quantitative or qualitative platelet defects explain a considerable part of spontaneous or induced abnormal bleeding events in the population. Prospective studies suggest that the prevalence of bleeding due to platelet defects is high and comparable to that of von Willebrand disease [1]. Although the most severe platelet disorders are identified at childhood, the majority of patients with milder platelet disorders remain undiagnosed until excessive bleeding occurs after specific challenges, as in surgery or trauma. On the other hand, undesired platelet activation contributes to arterial thrombotic diseases, and antiplatelet medication is the common therapy for secondary prevention, as in cardiovascular disease and stroke. Risk of bleeding is a well-known side effect of this suppression of platelet activation.

The premise of this paper is that the process of thrombus formation, such as assessed in whole blood flow studies and in experimental (murine) thrombosis studies, mirrors the platelet responses that determine haemostasis and thrombosis. In this scenery, we aim to give an up-to-date overview of the main platelet receptors and signalling pathways that contribute to thrombus formation and are used as targets in (pre) clinical intervention studies to attack cardiovascular disease. Since platelet dysfunction and treatment with antiplatelet therapy may both lead to a higher bleeding risk, we also sketch the current views of assessment of normal haemostasis by bleeding scores and discuss current methods to measure platelet function impairment.

2. Platelet receptors, antagonists and thrombus formation

2.1. Thrombin receptors, PAR1, 3 and 4

Thrombin, a short-living proteolytic enzyme generated from prothrombin by coagulation factor Xa, is not only a strong platelet agonist, but also a main effector of the coagulation cascade, inducing fibrin clot formation [2]. Thrombin is generated at phosphatidylserine-exposing membranes from the damaged vessel wall and highly activated platelets [3]. Its formation and inactivation can precisely be measured in platelet-rich plasma or blood by thrombin generation assays [4]. Antithrombin in plasma binds and inactivates thrombin, a process that is enhanced by heparins.

In human platelets, thrombin cleaves and activates the protease-activated receptors (PAR)1 and PAR4. In comparison to PAR4, the former displays a higher affinity to thrombin, transmitting signals at sub-nanomolar thrombin concentrations. Accordingly, PAR1 functions as the key thrombin receptor of human platelets, while PAR4 rather sustains the action of PAR1 [5]. Platelets do not express the factor Xa receptor, PAR2. Both expressed receptors, PAR1 and 4, signal *via* the G-proteins G12/13α and Gqα, which evoke the majority of functional platelet responses (Fig. 1). Current view is that both thrombin receptors only indirectly signal *via* Giα, *i.e.* through ADP secretion and autocrine effects [6]. The PAR-induced activation of G12/13α results in platelet shape change by activation of Rho-associated protein kinase (ROCK) followed by actin cytoskeletal changes, whereas the activation of Gqα

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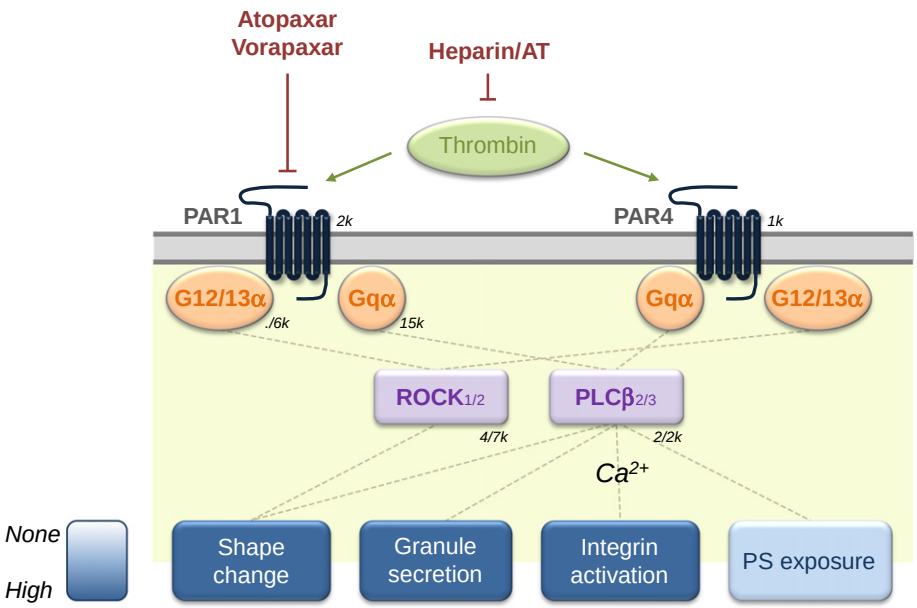

Fig. 1. Signalling and intervention *via* the protease-activated receptors (PARs) for thrombin. Shown are established agonists and receptors on human platelets that are involved in normal haemostasis and are (potential) targets of antithrombotic treatment. Only key signalling proteins are indicated, as far as they associate with the receptors and act as essential molecular switches or second messenger-generating proteins (isoforms in smaller font). Asterisks point to the known presence of dysfunctional mutations in man. Numbers in italic refer to copy numbers (×1000) per platelet, as described in handbooks and proteomic analyses [162,163]. For instance, for G12/13α the indication *./6k* indicates expression levels of 'unknown' (G12α) and ~6000 (G13α) copy numbers per platelet. Dashed lines show networks of signal transmitting proteins linking to the indicated platelet responses (lower boxes). Activation strength of specific responses is represented by a heat map with colour codes from white to blue. Pharmacological inhibitors used in clinic or laboratory are indicated in red, physiological agonists in green. *Abbreviations*: AT, antithrombin; Gq, GTP-binding protein Gq; PLC, phospholipase C; PS, phosphatidylserine; ROCK, Rho-associated protein kinase.

results in Ca^{2+}-dependent integrin $\alpha_{IIb}\beta_3$ activation and secretion *via* the effector enzyme, phospholipase Cβ (PLCβ). Thrombin is a strong platelet agonist, which *via* PAR1/4 evokes maximal shape change, secretion and integrin activation, but thrombin by itself has little effect on platelet procoagulant activity (Fig. 1) [3]. *In vitro*, the various PAR receptors can be activated by specific thrombin receptor-activating peptides (TRAP). Patients with mutations in the genes encoding for PAR receptors have not yet been described, but a patient is reported with platelet Gqα deficiency suffering from mucocutaneous bleeding [7].

Mouse platelets are devoid of PAR1 but express the isoform PAR3, which serves as thrombin-binding co-factor for PAR4 promoting the activity of this receptor [8]. Studies with *Par3*$^{-/-}$ and *Par4*$^{-/-}$ mice revealed marked protection in experimental arterial thrombosis, which was associated with prolonged bleeding times upon challenge [9]. In *Par4*$^{-/-}$ mice, thrombus formation *in vivo* was reduced compared to wildtypes, but knockout platelets showed normal adhesion and normal support of fibrin deposition [10]. These *in vivo* observations suggest a beneficial effect of blocking thrombin receptors in platelets.

In accordance with a key role of PAR1 in human platelet activation, clinical trials have been performed with PAR1 antagonists like Vorapaxar and Atopaxar. Vorapaxar has been evaluated in two phase III clinical trials. The TRACER study did not reveal superiority of Vorapaxar over standard therapy in the primary endpoint, which was a composite of death from cardiovascular causes, myocardial infarction, stroke, recurrent ischemia with rehospitalization, or urgent coronary revascularization [11]. In the TRA-2P TIMI-50 study, where patients with prior stroke were excluded, Vorapaxar was superior to placebo on top of standard care [12,13]. This benefit was at the expense of an increased risk of intracranial bleeding, which was observed in both studies. The other PAR1 antagonist, Atopaxar, has been tested in several phase II trials, showing similar outcomes as Vorapaxar in terms of safety and efficacy [14].

One consideration for the clinical practice, when prescribing PAR1 antagonists in combination with other antiplatelet agents is that, although an extra bleeding risk would be acceptable in comparison to gained antithrombotic protection, the patients need to take even more medication at extra costs, with lower compliance as a side effect [15].

2.2. Thromboxane-prostanoid receptor, TP

The TP receptor (one gene product, previously split into α and β forms) is activated by the fatty acid derivative, thromboxane A_2 [16]. This prostanoid is released from activated platelets as a very unstable metabolite, hence providing a rapid shut-off action mechanism upon stimulation of the TP receptors. Thromboxane A_2 formation requires the release of arachidonic acid from membrane phospholipids, a process catalysed by the Ca^{2+}-dependent cytosolic phospholipase A_2. Arachidonate acts as a substrate for cyclooxygenase 1 (COX1) to produce prostaglandin H_2, which is converted by thromboxane synthase into thromboxane A_2. Signalling *via* the TP receptor takes place *via* G12/13α and Gqα, similarly as for thrombin, but at a lower extent (Fig. 2) [17]. Activation of G12/13α again triggers platelet shape change *via* ROCK activation, while the low activation of Gqα/PLCβ is still sufficient for integrin activation and secretion.

In the laboratory, the stable thromboxane analogue U46619 is used to specifically trigger TP receptors. By itself, U46619 is a weak agonist evoking limited functional responses, but it enhances the effects of other platelet agonists. In agreement with this, collagen-induced platelet activation relies for a considerable extent on the release of thromboxane A_2 and ADP, and ensuing TP and $P2Y_{12}$ receptor activation, respectively [18]. The few patients described with mutations in the thromboxane receptor experience mild bleeding [7]. This agrees with the finding that also in *Tp*$^{-/-}$ mice bleeding times are prolonged [19].

The COX1 complex is irreversibly inhibited by Aspirin and other non-steroid anti-inflammatory drugs (NSAIDs), such as Indomethacin, Diclofenac, Ibuprofen and Naproxen (Fig. 2). The classical test to check for inhibited COX1 activity is measurement of arachidonic acid-induced (*i.e.*, thromboxane-dependent) platelet aggregation. Benefit of Aspirin in the treatment and secondary prevention of cardiovascular disease has clearly been shown in early clinical trials [20,21]. However, Aspirin has side effects experienced by some patients, particularly renal insufficiently, gastrointestinal symptoms and haemorrhagic complications [22]. In accordance with this, also patients with an inherited

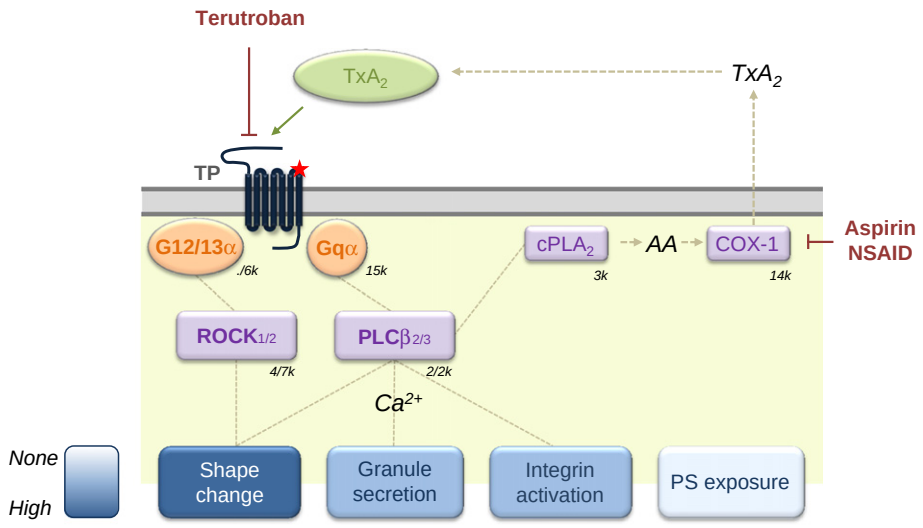

Fig. 2. Signalling and intervention *via* the TP receptor for thromboxane A_2. For explanation, see Fig. 1; AA, arachidonic acid; COX-1, cyclooxygenase 1; $cPLA_2$, cytosolic phospholipase A_2.

'aspirin-like' platelet defect frequently have a mild bleeding phenotype [7]. While biochemical 'resistance' to Aspirin intake (*i.e.*, lack of inhibition of thromboxane A_2 formation) is relatively rare, the drug prevents only part of the thrombotic events [23], likely because thromboxane is a weak platelet agonist. Intake of other NSAIDs may increase the cardiovascular risk, indicating that such drugs also affect other processes than platelet thromboxane formation [24].

One of the developed antagonists for thromboxane receptors, Terutroban, has been tested clinically as an orally active drug. The TAIPAD study indicated that it was as effective as Aspirin in secondary prevention of thrombotic events in cardiovascular disease [25]. On the other hand, superiority of Terutroban over Aspirin could not be demonstrated in the PERFORM trial, in preventing arterial events in patients with stroke [26].

2.3. Purinergic receptors, $P2Y_1$, $P2Y_{12}$ and $P2X_1$

The platelet dense granules (δ-granules) contain high concentrations of the adenosine nucleotides ADP, ATP and adenosine polyphosphate. In response to physiological agonists, these nucleotides are secreted and enforce platelet activation processes in an autocrine manner. Secreted ADP interacts with the purinergic $P2Y_1$ and $P2Y_{12}$ receptors, while ATP binds to $P2X_1$ receptors (Fig. 3). The receptors $P2Y_1$ and $P2Y_{12}$ are coupled to the G-proteins, Gqα and Giα, respectively [27]. Interaction of ADP with $P2Y_1$ results in Ca^{2+} mobilization, shape change and initial platelet aggregation through PLCβ stimulation [28]. Stimulation of the other receptor, $P2Y_{12}$, promotes the formation of large and stable platelet aggregates [29]. The principal signalling mechanism of $P2Y_{12}$ is by Giα-dependent stimulation of phosphoinositide 3-kinase (PI3Kβ/γ), which regulates platelet aggregation *via* actin cytoskeleton-dependent integrin $\alpha_{IIb}\beta_3$ activation [30]. The alternative pathway of Giα-mediated inhibition of adenylate cyclase is nowadays considered to be of lesser importance [31]. Under flow, continuous signalling *via* $P2Y_{12}$ is required to maintain $\alpha_{IIb}\beta_3$ in the active conformation, and to ensure thrombus stability [32].

Patients with $P2Y_1$ deficiencies are not yet known. However, mice lacking this receptor on platelets are protected from collagen/epinephrine-induced thromboembolism and have a bleeding phenotype [28,33]. The $P2Y_1$-deficient platelets show reduced aggregation to all agonists. Conversely, mice with platelets overexpressing $P2Y_1$ are more susceptible to thromboembolism and arterial thrombosis [34].

Thirteen patients, experiencing a mild bleeding diathesis, have been described who either lack $P2Y_{12}$ receptors or have a receptor mutation interfering with ligand binding [29,35]. Platelets from these patients display a lesser and reversible aggregation in response to ADP, but have normal shape change and Ca^{2+} mobilization. Bleeding is also reported for subjects with heterozygous point mutations in the receptor locus [36]. Interestingly, reduced expression in platelets of the $P2Y_{12}$-transmitting protein Giα gives a similar phenotype [7]. Also mice deficient in $P2Y_{12}$ display defective ADP-induced platelet aggregation and increased bleeding times. *In vivo*, their platelets form small and unstable thrombi [37]. Conversely, transgenic mice expressing constitutively active $P2Y_{12}$ exhibit increased platelet activation and arterial thrombosis [38].

As reviewed elsewhere, $P2Y_{12}$ is the pharmacological target of a number of common antiplatelet agents [29]. Thienopyridines derived from the prodrugs Clopidogrel and Prasugrel inhibit $P2Y_{12}$ in an irreversible way, whereas the drugs Ticagrelor and Cangrelor act as reversible $P2Y_{12}$ inhibitors. When taken orally, Clopidogrel and Prasugrel need to be converted by hepatic cytochrome P450 enzymes into active, unstable metabolites. Despite well proven efficacy in multiple clinical trials, the use of Clopidogrel has certain drawbacks [29]. The antiplatelet effect is delayed by the metabolic conversion of the prodrug, while its irreversible mode of action can be a problem if patients acutely need surgery. Furthermore, Clopidogrel has considerable inter-individual variability in responsiveness, which is clinically relevant since 'poor' responders may not be adequately protected from adverse cardiovascular events [39]. Poor responsiveness to Clopidogrel is associated with loss-of-function mutations of cytochrome P450 isoforms [40]. This variability in Clopidogrel responsiveness would argue for routine testing of platelet function for optimal care of patients [29], as suggested in a proof-of-concept trial [41]. The structural analogue Prasugrel is less critically dependent on cytochrome P450 enzymes for conversion into the active metabolite in comparison to Clopidogrel. Prasugrel intake leads to faster appearance of the active metabolite in the blood, and overall greater inhibition of $P2Y_{12}$-dependent platelet functions with less intra-individual variation [29,40]. For both drugs, though, adequate dosing is important, as full $P2Y_{12}$ receptor blockage causes a high bleeding risk.

The PLATO trial demonstrated that the drug Ticagrelor, which acts as an oral and reversible $P2Y_{12}$ antagonist, induces a more consistent platelet inhibition and a greater antithrombotic efficacy, when compared to Clopidogrel, but still at the expense of bleeding [42]. Another reversible $P2Y_{12}$ inhibitor in use is the drug Cangrelor, which is administrated intravenously. A recent meta-analysis suggests that Cangrelor and Clopidogrel are similarly effective in reducing ischemic endpoints after percutaneous coronary intervention [43].

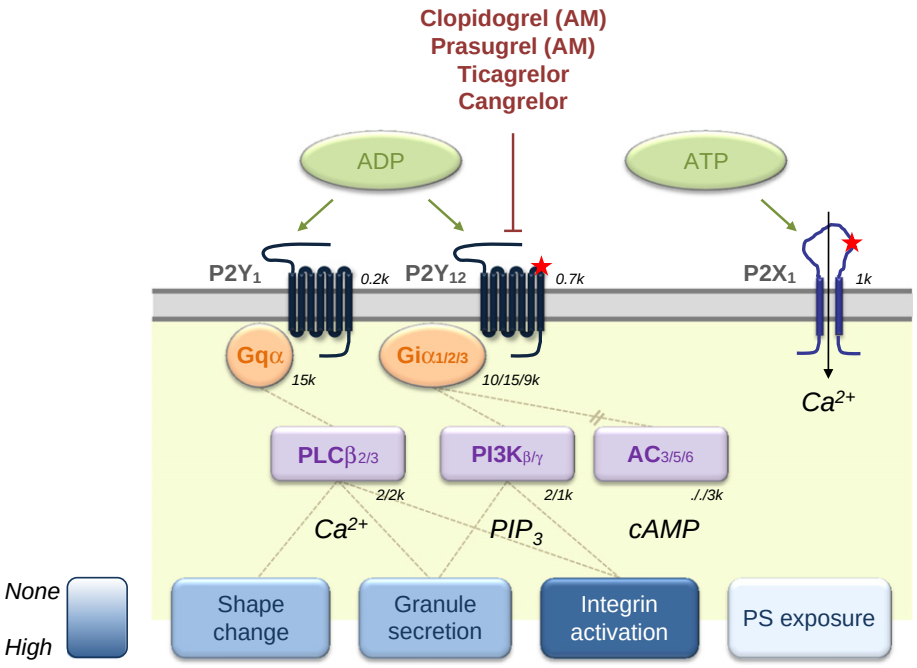

Fig. 3. Signalling and intervention *via* the purinergic receptors for ADP and ATP. For explanation, see Fig. 1; AC, adenylate cyclase; Clopidogrel (AM), Clopidogrel active metabolite; PIP_3, phosphatidylinositol 3-phosphate.

The ATP receptor $P2X_1$ operates as a cation channel that mediates transient Ca^{2+} influx through the plasma membrane and contributes to platelet activation by stimulating shape change (Fig. 3). The Ca^{2+} signal evoked by $P2X_1$ synergizes with that of other receptors, thus explaining why $P2X_1$ stimulation enhances platelet responses evoked by other agonists [44]. One case of a congenital deficiency in $P2X_1$ activation has been presented that was accompanied by a bleeding diathesis [45]. Mice lacking $P2X_1$ are viable, yet males are infertile [46,47]. $P2X_1$-deficient mice show impaired platelet aggregation at low agonist doses, decreased thrombus formation *in vivo* and protection from thromboembolism [47]. No specific drugs for clinical use have been described.

2.4. Prostaglandin receptors, IP and EP1–4

Prostaglandin I_2 (prostacyclin) and prostaglandin E_2 are prostanoids involved in haemostasis by interacting with the platelet IP and EP receptors, respectively. Next to nitric oxide, prostaglandin I_2 is one of the major endothelium-derived platelet inhibitors. It abrogates platelet activation *via* the Gsα-coupled IP receptors [48]. The signal pathway involves activation of the enzyme adenylyl cyclase, which raises the second messenger cyclic AMP and leads to protein phosphorylation events that are strongly inhibitory for platelets (Fig. 4). Several proteins integrate the inhibitory activities of cyclic AMP, in particularly the G-protein regulators CalDAG-GEFI and RGS18 [49,50]. Mice lacking the IP receptor display an increased tendency to arterial thrombosis [51]. For the human IP receptor, dysfunctional mutations are known resulting in a defective function or lack of expression, which were more frequently accompanied by coronary artery obstruction, when compared to silent mutations [52]. Drugs targeting the IP receptor, such as Selexipag, Ilomedine and Teprostinil have primarily been tested for the treatment of pulmonary hypertension, but will also inhibit platelet function.

The vasodilatating prostaglandin E_2 (as drug termed Dinoprostone) is released by various nuclear cells including macrophages. The mechanism by which prostaglandin E_2 influences platelets is complex: it enhances platelet aggregation at low concentrations but inhibits at higher doses. The proaggregatory effect is mediated by prostaglandin E_2 binding to the EP3 receptors and a Giα-linked mechanism (Fig. 4) [53], similarly as described for ADP. It is suggested that EP3 signalling in this way contributes to ADP- and collagen-induced platelet aggregation at low agonist doses. Functional mutations in the human *EP3* gene have not been described. Mice lacking this receptor display a markedly decreased susceptibility to prostaglandin E_2-promoted thrombosis.

At higher concentrations, prostaglandin E_2 binds to the EP1, EP2 and EP4 receptors [54]. All of these are Gsα-coupled receptors, and thus inhibit platelets by increasing cyclic AMP levels [53]. In human platelets, especially the EP4 receptor transmits the inhibitory effect of prostaglandin E_2. A selective EP4 agonist such as ONO AE1–329 thus suppresses human platelet activation on collagen [55]. It was proposed that EP4 agonists can serve as antithrombotics, *e.g.* in cases where Aspirin and ADP antagonists alone are insufficient to prevent thrombotic events.

The EP3 receptor is of some interest as a target, *e.g.* in atherothrombotic disease where prostaglandin E_2 levels are increased at sites of atherosclerotic lesions [56,57]. An EP3 antagonist at the beginning of clinical development (Phase II) is DG-041. *Ex vivo*, this compound inhibits the potentiating effects of prostaglandin E_2 on rat and human platelet aggregation induced by various agonists, in rat without increasing bleeding times [57,58].

2.5. Von Willebrand factor receptor, GPIb–V–IX

Von Willebrand factor (VWF) is a large glycoprotein that is released from endothelial cells in blood plasma as well as from platelets. Ultralarge multimers (strings) of VWF freshly secreted by endothelial cells are most activating for platelets. Under normal conditions these strings are cleaved by the protease ADAMTS13, but when ADAMTS13 activity is absent they become clinically problematic causing thrombotic thrombocytopenia purpurea (TTP) [59]. Bleeding in TTP patients is considered to be a consequence of VWF-induced platelet agglutination and thrombocytopenia.

In healthy subjects VWF circulates as intermediate-size multimers, which capture factor VIII and bind to subendothelial matrix components (collagen, laminin) and platelet aggregates. Under high shear stress conditions as in the arterial circulation, the medium-sized VWF multimers change in conformation and avidly interact with the platelet GPIb–V–IX complex, forming a so-called catch bond [60]. The consequence is

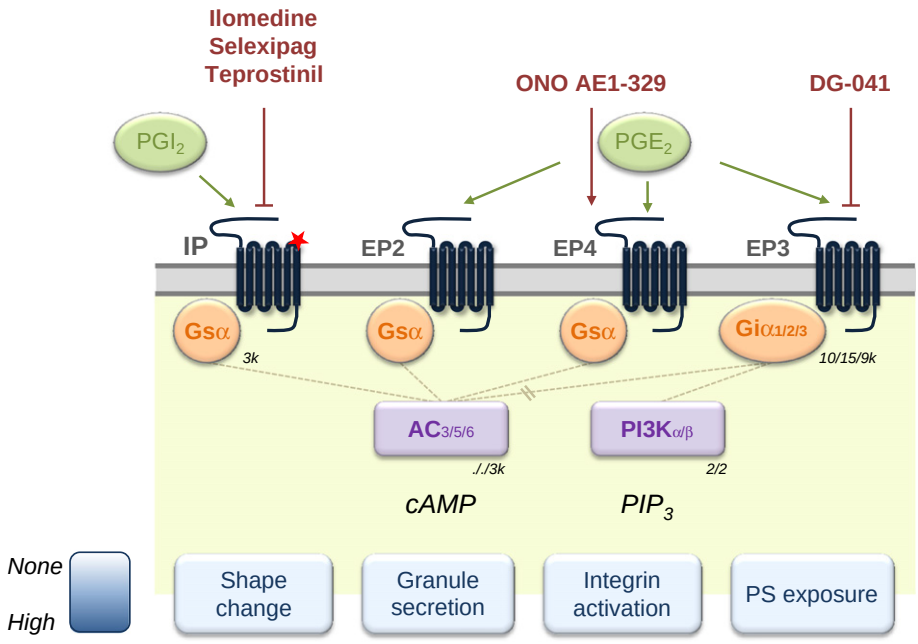

Fig. 4. Signalling and intervention *via* the prostaglandin IP and EP receptors. For explanation, see Fig. 1; PG, prostaglandin.

transient platelet adhesion which, in the presence of other receptor-ligand interactions (*e.g.* collagen), turns into firm adhesion and platelet activation under flow [61,62]. In the presence of high shear gradients, for instance at atherosclerotic geometries, platelets can form thrombi at post-stenotic sites in a GPIb-dependent manner with limited activation [63,64].

In the laboratory, VWF–GPIb interaction is induced by the venom components ristocetin and botrocetin, which force VWF to bind to GPIb–V–IX in the absence of shear. Ristocetin- or shear-induced VWF–GPIb interaction leads to PLCγ and PI3K activity and, thereby, activated $\alpha_{IIb}\beta_3$ [65]. The GPIb-associated adaptor protein 14-3-3ζ plays an intermediary role in assembly of the concerning signal complex [66]. The binding of VWF to GPIb–V–IX (under shear) induces only limited signalling events (Fig. 5). However, it enforces multiple amplification pathways in platelets, including thromboxane A_2, ADP and phospholipase D pathways [66,67].

There is increasing interest in the further roles of VWF and GPIb–V–IX under conditions of coagulation and low shear, as in venous thrombosis. An initial observation was that thrombin binding to GPIb accelerates platelet activation *via* PAR1 [68]. Several other coagulation factors bind to the GPIb–V–IX complex as well, but the functional consequences are not well understood [3]. Under coagulant conditions, VWF binding to GPIb stimulates platelet procoagulant activity and the formation of fibrin fibres on human and mouse platelets [69]. This is compatible with the finding that in mice both VWF and GPIb contribute to venous thrombosis *in vivo*, although the precise mechanisms are not fully understood [70,71]. Together, this points to the existence of synergy pathways of GPIb- and coagulation (thrombin?)-mediated platelet responses.

Genetic deficiency or dysfunction of platelet GPIb–V–IX leads to Bernard–Soulier syndrome, a rare bleeding disorder characterized by thrombocytopenia and giant platelets [7]. A similar phenotype is observed in mice deficient in the GPIbα or GPIbβ chains [72,73]. Interestingly, also in mice, the GPV chain is considered to contribute to thrombus formation by acting as a cleavable substrate for thrombin [74]. The VWF–GPIb axis may also be a suitable target for pharmacological intervention. In baboons, the humanized anti-GPIb antibody h6B4 acts as a safe antithrombotic drug with minimal bleeding [75]. A second approach to interfere with GPIb-mediated thrombus formation may be to target VWF. It has been shown that antibodies against the A3 domain of vWF, through which GPIb interacts, inhibit arterial thrombus formation *in vivo* [76].

Bleeding is a recurrent phenomenon in patients with von Willebrand disease, a disorder characterized by quantitative or qualitative abnormalities in VWF [77]. In most forms of von Willebrand disease, ristocetin-induced platelet clumping *via* VWF (so-called platelet agglutination) is reduced, thus stressing the importance of VWF–GPIb binding for normal haemostasis. In platelet-type 2M and type 2B von Willebrand disease, the VWF–GPIb interaction is increased leading to 'spontaneous' platelet aggregation and even thrombocytopenia [77].

2.6. Collagen receptor, GPVI

The principal signalling receptor for collagen on platelets is glycoprotein VI (GPVI), a member of the immunoglobulin superfamily, which associates with the ITAM-bearing FcRγ-chain [78]. GPVI is only expressed on platelets and megakaryocytes, and it requires dimerization for binding to collagen [79]. Stimulation *via* GPVI leads to full platelet activation, including integrin activation, secretion and procoagulant activity [80]. Established ligands of GPVI are the fibrillar collagen types I or III, collagen-related peptides containing repeats of the sequence, glycine-proline-hydroxyproline, and the snake venom convulxin.

The GPVI-induced signalling mechanism operates through protein tyrosine kinases, and resembles the signalling induced by various other adhesive receptors (Fig. 6) [81,82]. In brief, ligand-occupied GPVI dimers form a large signalling complex, *via* activation of Src-family kinases, phosphorylation of the ITAM motif of the FcRγ-chain, and activation of the central tyrosine kinase, Syk [83]. This results in a cascade of phosphorylation events, with as an end result the activation of key effector enzymes at the plasma membrane, PLCγ and PI3K isoforms [84,85]. The functional consequences are prolonged Ca^{2+} mobilization, secretion, integrin activation, procoagulant activity and membrane blebbing (Fig. 7) [3]. The Ca^{2+}-dependent proteins, protein kinase C and CalDAG-GEFI, regulate many of the downstream responses to secretion and integrin activation (see below) [86,87].

The interest in GPVI as a target for antithrombotic therapy was greatly raised by the recognition that mice lacking the FcRγ-chain showed a greatly impaired thrombus formation in experimental models of arterial thrombosis, that was not accompanied by increased tail bleeding [88]. A similar phenotype was found after depletion of platelet

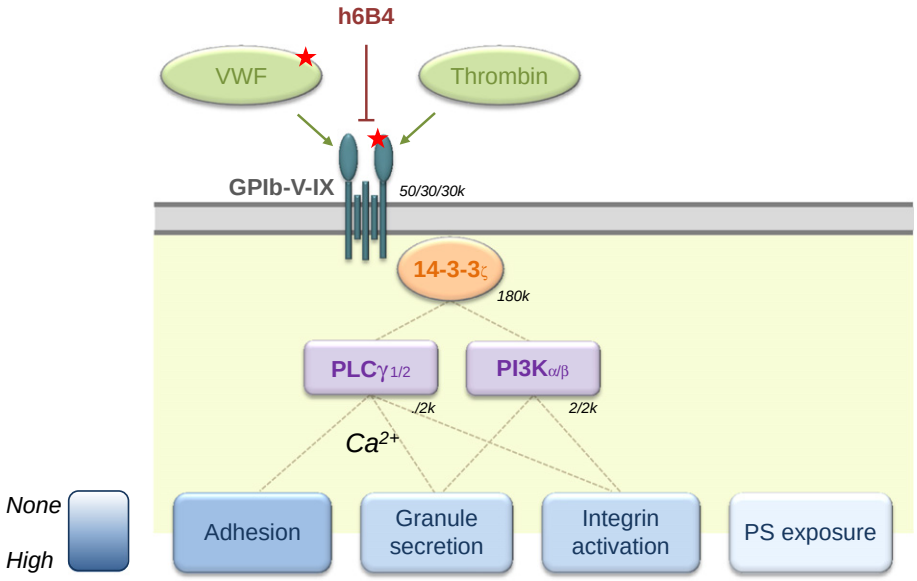

Fig. 5. Signalling and intervention *via* the von Willebrand factor (VWF) receptor, GPIb–V–IX. For explanation, see Fig. 1.

GPVI by injection of antibodies against this receptor [89]. In addition, blocking of GPVI impaired thrombus formation on atherosclerotic plaques, *e.g.* in a mouse model of acute plaque rupturing [90]. On the other hand, deficiency in GPVI did not affect arterial thrombus formation in models of more severe vascular damage with tissue factor exposure, where thrombin generation is considered to be the major driving force of thrombosis [91,92].

Several patients have been described with circulating anti-GPVI antibodies, accompanied by low expression of platelet GPVI, thrombocytopenia and a mild bleeding tendency [78,93]. Mild mucocutaneous bleeding symptoms have been reported for subjects with compound heterozygous mutations or a frame-shift mutation in the *GP6* gene [94]. In addition, large scale genome-wide analyses indicate that variation in the *GP6* locus is one of the major predictors in determining the extent of platelet activation [95,96].

In vitro perfusions with mouse blood indicated that thrombus formation on collagen relies on the presence of both FcRγ-chain and GPVI [97,98]. Thrombus formation on collagen is greatly impaired in mice lacking proteins of the GPVI signalling complex, *i.e.* PLCγ or PI3K isoforms [85,99], whereas animals with constitutively active PLCγ2 show aggravated thrombus formation [100]. These mouse studies jointly suggest that the major functional role of GPVI is the limiting of excessive blood loss upon injury with substantial collagen exposure [101].

Since anti-GPVI agents have the potential to reduce arterial thrombus formation with limited effect on haemostatic activity, there have been several initiatives to develop such substances. Small GPVI-inhibiting m olecules (*e.g.*, Losartan) and blocking anti-GPVI antibodies (10B12, 9O12) have been generated for experimental use with animal and human blood [88,93]. Such anti-GPVI agents strongly inhibit thrombus formation on collagen under flow conditions [102,103]. Injection of the anti-GPVI

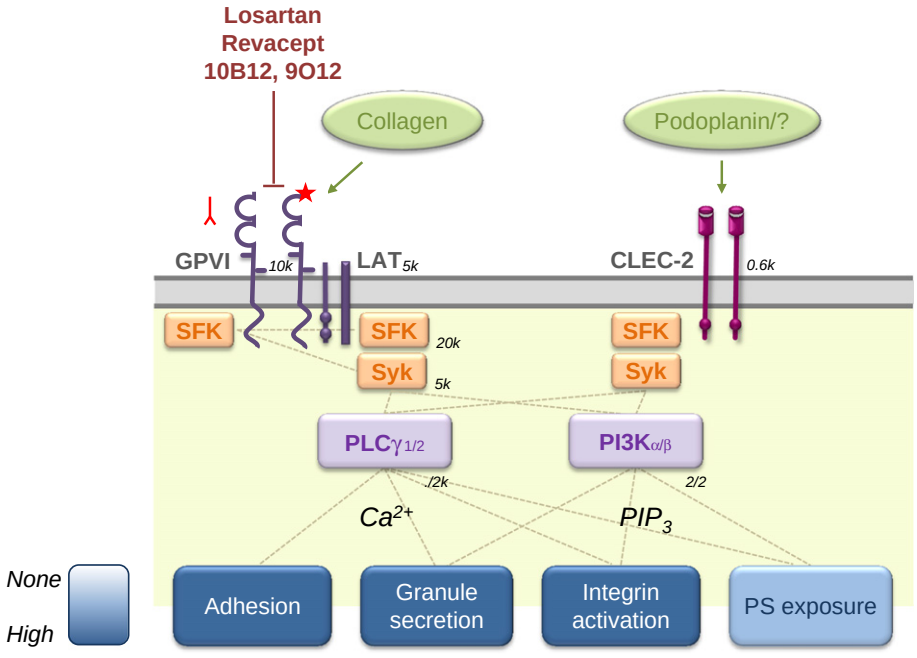

Fig. 6. Signalling and intervention *via* the collagen receptor, GPVI, and the platelet CLEC-2 receptor. For explanation, see Fig. 1; SFK, Src family kinases.

OM2-Fab fragment into cynomolgus monkeys caused inhibition of collagen-induced platelet aggregation [104]. In a different approach, a soluble dimeric GPVI-Fc fusion protein (Revacept) has been generated to block the GPVI-binding sites on exposed collagen, causing suppression of murine arterial thrombosis [105]. This GPVI-Fc fusion protein is presently tested in a phase III trial [106]. Taken together, GPVI is an interesting therapeutic target that deserves further exploration.

2.7. Novel receptor, CLEC-2

The C-type lectin-like type II membrane glycoprotein CLEC-2 has recently been identified as a receptor that also can strongly activate platelets. Ligands of this receptor are the snake venom toxin, rhodocytin and the glycoprotein podoplanin [107,108]. The physiological ligand of CLEC-2 in the vascular system is still unclear. The signalling pathway evoked by CLEC-2 clustering is similar to that of GPVI, *i.e. via* activated protein tyrosine kinases resulting in PLCγ activation, although in this case the mechanism does more rely on Syk than on Src kinases (Fig. 6) [101,107]. Phosphoproteomics analysis of activated platelets also points to a substantial, but not complete overlap of GPVI and CLEC-2 signalling events [109].

In mice, conditional knockout of CLEC-2 or treatment with anti-CLEC-2 antibody caused reduced thrombus stability and prolonged tail bleeding times [107,110]. Strikingly, combined *in vivo* depletion of GPVI and CLEC-2 by antibody treatment, or genetic deficiency of platelet GPVI plus CLEC-2 resulted in severe impairment of arterial thrombus formation, but at the expense of prolonged bleeding times [111]. This points to functional redundancy of the two receptors in thrombosis and haemostasis. Although there are only few human data available, together this suggests that CLEC-2 is an interesting therapeutic target, but bleeding might be an unavoidable side effect.

2.8. Fibrinogen receptor, integrin $\alpha_{IIb}\beta_3$

Integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) is the most abundantly expressed receptor on platelets. On resting platelets $\alpha_{IIb}\beta_3$ assumes a bent form with low ligand affinity. Upon platelet activation by most agonists, the integrin opens by a reversible conformational change, which increases its affinity for fibrinogen, fibronectin, vitronectin and VWF. This inside-out integrin signalling occurs *via* Ca^{2+}-dependent activation of protein kinase C or CalDAG-GEFI, culminating in Rap1b activation and binding of the actin cytoskeletal proteins talin-1 and kindlin-3 to the cytoplasmic $\alpha_{IIb}\beta_3$ domains (Fig. 7A) [112–114]. In the activated state, $\alpha_{IIb}\beta_3$ supports platelet adhesion to fibrinogen and platelet aggregation. Ligand binding to $\alpha_{IIb}\beta_3$ furthermore leads to a train of signalling events, mediated by Src and Syk protein tyrosine kinases, a process known as outside-in signalling (Fig. 7B) [115]. This process drives the spreading of platelets on fibrinogen, the retraction of fibrin clots, and the procoagulant activity of platelets [116].

Mutations in the genes encoding for α_{IIb} and β_3 give rise to one of the most common platelet disorders, Glanzmann's thrombasthenia. Glanzmann patients have platelets lacking $\alpha_{IIb}\beta_3$ or with non-functional $\alpha_{IIb}\beta_3$, and characteristically show mucocutaneous and gastrointestinal bleeding symptoms as well as excessive trauma-related bleeding [117]. Platelets from these patients show impaired aggregation, spreading and clot retraction. Mice lacking expression of the α_{IIb} or β_3-chain are greatly impaired in arterial thrombosis, and have a bleeding phenotype, with prolonged tail bleeding times and spontaneous haemorrhages [118,119].

The autosomal recessive syndrome, leukocyte adhesion deficiency type III (LAD-III or LAD-1/variant), refers to a dysfunction of integrins β_1, β_2 and β_3 in platelets and leukocytes. While the integrin expression levels are normal, LAD-III patients experience severe bleeding and recurrent infections. Recently, it was established that this disorder is caused by mutations in the gene of kindlin-3, which result in abrogated integrin inside-out signalling [120,121]. The bleeding phenotype is explained particularly, but not exclusively, by the inability of $\alpha_{IIb}\beta_3$-mediated platelet aggregation [7].

Three inhibitors of $\alpha_{IIb}\beta_3$ are available for clinical use, namely Abciximab, Eptifibatide and Tirofiban. These drugs are given intravenously to patients undergoing percutaneous coronary intervention

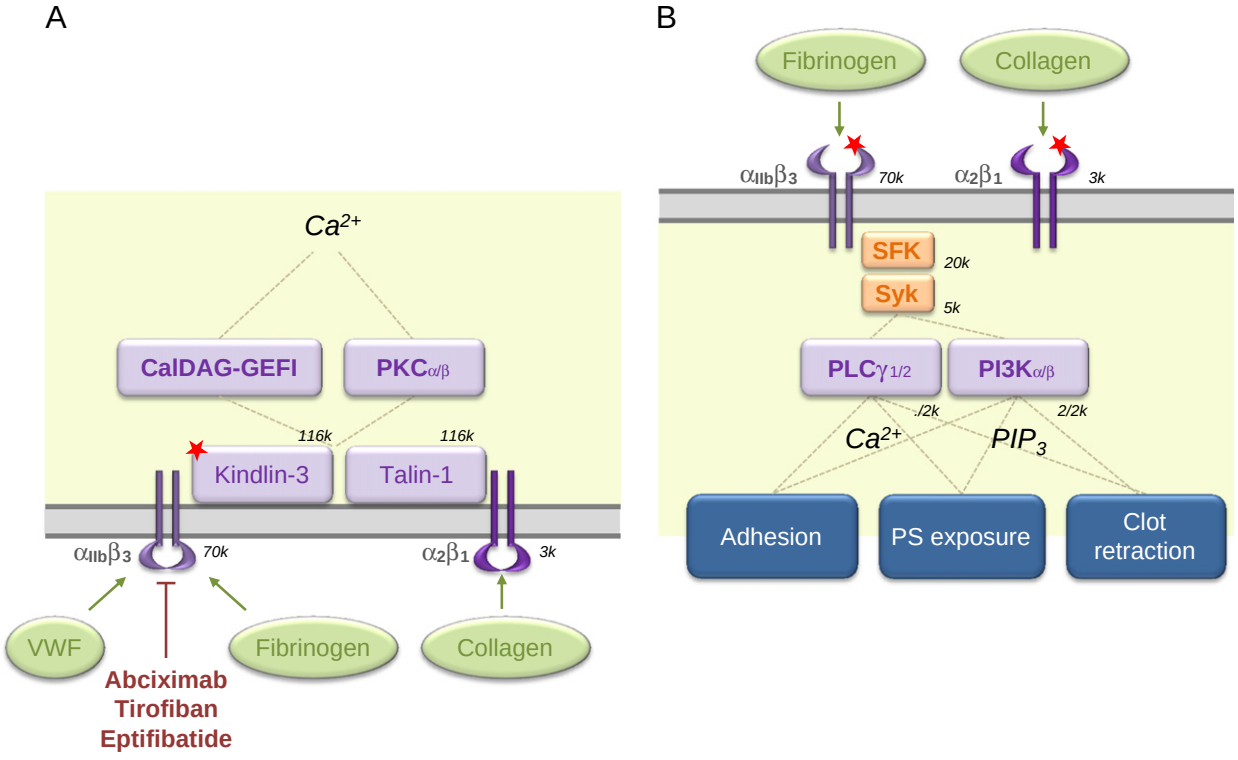

Fig. 7. Signalling and intervention *via* platelet integrins. (A) Inside-out signalling, (B) outside-in signalling. For explanation, see Fig. 1.

after acute coronary syndrome. Integrin inhibitors have been reported to cause more haemorrhagic events in women, but this might be due to improper weight-adjusted dosing [122]. Cardiologists mostly use $\alpha_{IIb}\beta_3$ inhibitors in high-risk patients not pretreated with $P2Y_{12}$ blockers [123]. In the past, also oral $\alpha_{IIb}\beta_3$ inhibitors have been tested, but clinical trials were cancelled due to increased mortality, likely due to a ligand-mimetic function of the compounds, provoking fibrinogen binding to $\alpha_{IIb}\beta_3$ [124]. Summarizing, given the fundamental role of $\alpha_{IIb}\beta_3$ in haemostasis, inhibition of this glycoprotein need to be performed with great care.

2.9. Other platelet integrins

Platelets also express other integrins than $\alpha_{IIb}\beta_3$, which serve as receptors for adhesive proteins. At least for integrin $\alpha_2\beta_1$, receptor function depends on conformational changes and leads to outside-in signalling events. Integrin $\alpha_2\beta_1$ (GPIa/IIa) serves as receptor for fibrillar collagens. Its activation depends on cytoskeleton-mediated kindlin-3 and talin-1 interactions (Fig. 7A) [114]. Likely, lack of activation of $\alpha_2\beta_1$ explains why LAD-III patients (with defective kindlin-3) suffer from a more severe bleeding tendency than Glanzmann patients (missing only $\alpha_{IIb}\beta_3$) [125].

In mice, deficiency in $\alpha_2\beta_1$ results in limited impairment in arterial thrombus formation, which is detected as increased thrombus instability [126]. Mice lacking α_2 or β_1 show unchanged tail bleeding times [97,127]. However, combined deficiency of GPVI and $\alpha_2\beta_1$ leads to a severe bleeding tendency, accompanying major defects in thrombus formation [128]. This suggests that $\alpha_2\beta_1$ plays a supportive rather than essential role in the interaction between platelets and collagen [114], although recent evidence indicates that β_1 integrins do contribute to platelet activation processes [129]. Two patients suffering from minor bleeding are described with reduced expression of $\alpha_2\beta_1$ and impaired platelet adhesion to collagen [130,131]. However, whether the bleeding is due to the altered glycoprotein expression is unclear [78]. In studies of thrombus formation with mouse and human blood, $\alpha_2\beta_1$ enforces the GPVI-dependent platelet adhesion to collagen and ensuing platelet activation [62]. Taken together, $\alpha_2\beta_1$ seems to have a dispensable but still relevant role in haemostasis and thrombosis.

Recently, also the platelet receptor for vascular laminins, integrin $\alpha_6\beta_1$, was found to play an important role in murine platelet activation and arterial thrombosis *in vivo* [132], suggesting that also this integrin could be a target of antithrombotic treatment.

3. Thrombus formation — interactions of multiple platelet receptors

Flow studies have gained considerable insight into the combinatory roles of various platelet ligands and receptors in the process of thrombus formation, reflecting those in haemostasis and arterial thrombosis. Current models of thrombus formation start with shear-dependent platelet adhesion to VWF *via* GPIb–V–IX followed by platelet activation *via* collagen, but also other platelet substrates will contribute to initial platelet adhesion [133]. Platelet activation *via* GPVI, and likely also CLEC-2, initiates responses like shape change (pseudopod formation), α/δ-granule secretion, thromboxane release and integrin activation which, together, evoke a train of autocrine stimulatory events to activate and trap flowing platelets into the growing thrombus [61,82]. Thrombus formation is thus seen as a multifactorial event with amplification loops by receptor interactions of thrombin/PAR isoforms, ADP/P2Y isoforms, ATP/$P2X_1$, thromboxane/TP receptors and fibrinogen/$\alpha_{IIb}\beta_3$ (Fig. 8). However, also many other ligand-receptor interactions – not discussed in this review – contribute to this complexity. These involve, for instance, contact-dependent signalling processes, activation *via* α-granule secretion products (Gas6, thrombospondin), tyrosine phosphatase-linked receptors, and mechanisms controlling fibrin formation, thrombus contraction and thrombus stability [134–136]. Moreover, these processes may occur at different loci in a growing thrombus. Below, we discuss platelet activation processes that are most relevant for haemostasis in man.

The combined involvement of multiple ligand-receptor combinations in thrombus formation becomes especially apparent, when more than one interaction is absent. This is the case for patients lacking α- or δ-granules in platelets, often associated with bleeding symptoms of various severities. Patients with the rare Hermansky–Pudlak syndrome have mutations in one of nine *HPS* genes, resulting in complete δ-granule and lysosome deficiency [7,137]. The functional defect of platelets is at least in part explained by impaired ADP and ATP secretion. More common is a partial impairment of δ-granule secretion with unknown genetic causes [137]. Abnormalities in the α-granules, which store a large variety of proteins, are seen in several syndromes. Well studied is the Gray platelet syndrome, a mild bleeding disorder characterized by absence of α-granules and accompanied with thrombocytopenia, due to mutations in the *NBEAL2* gene [7,137]. Mice deficient in *Nbeal2* phenocopy this syndrome and are protected from arterial thrombus formation; their platelets show diminished adhesion, aggregation and procoagulant activity [138]. It is still unclear which of the α-granule stored proteins mediate these platelet responses. Platelet cytoskeleton defects, such as May–Hegglin anomaly (*MYH9* gene mutation) and Wiskott–Aldrich syndrome (*WAS* mutation) are accompanied by changes in platelet size and thrombocytopenia [7]. Pharmacological interventions to inhibit platelet granule secretion or cytoskeletal changes are not known.

A few intracellular signalling proteins with major roles in platelet functions have been studied as potential targets for therapy. Well advanced are studies on pharmacological inhibition of PI3K, particularly of the β isoform. In flow perfusion studies, PI3Kα/β blockers suppress collagen-induced Ca^{2+} mobilization, secretion, integrin activation and platelet procoagulant activity [85]. A phase I study consisting of intravenous infusion of the PI3Kβ blocker AZD6482 (an analogue of TGX-221) indicated a strong platelet-inhibiting effect *ex vivo* [139].

4. Bleeding and platelet dysfunction or inhibition

4.1. Predicting mild platelet disorders with questionnaires

Typical bleeding symptoms that accompany quantitative or qualitative platelet disorders are excessive bruising, prolonged menses and abnormal bleeding after haemostatic challenges. The magnitude of bleeding depends on the type and severity of the platelet defect [1]. Since patients with a platelet disorder often display only mild bleeding symptoms, which are also features of von Willebrand disease or coagulation deficiencies, proper diagnosis is not always easy [140]. The complexity is well illustrated by the outcome of the UK GAPP study where, in subjects with suspected platelet function deficiencies and abnormal bleeding symptoms, the genetic causes of the signalling defects could only be determined in a minority of the subjects [18].

In the clinic, there is ongoing interest in the development of bleeding assessment tools (BATs) for the diagnostic evaluation of haemorrhagic symptoms by way of a questionnaire and bleeding score. Most questions concern the family history of spontaneous and challenged bleeding events, as well as the frequency and severity of the bleeding symptoms. Specific BATs have been developed for von Willebrand disease, paediatric patients, and women with menorrhagia [141]. In 2010 the ISTH published a consensus BAT to standardize the description of significant bleeding symptoms and the diagnosis of bleeding disorders with main focus on von Willebrand disease [142]. While useful for the documentation of all relevant bleeding symptoms in the prospective GAPP study, the ISTH-BAT score was not predictive for platelet function deficiencies, based on lumi-aggregometry measurements [143]. It should be noted though, that a laboratory defect not always needs to be accompanied by a bleeding phenotype. Since the ISTH-BAT documents on recurrent and mild haemorrhages, which are characteristic for platelet function disorders, further refinement of this BAT may aid in the diagnosis of mild platelet defects.

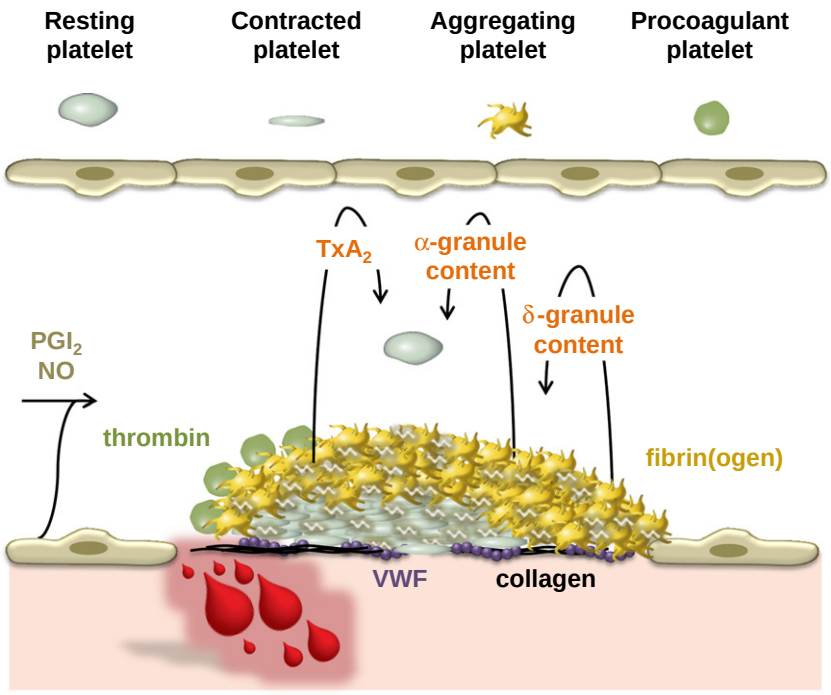

Fig. 8. Simplified mechanism of platelet agonists and adhesive ligands in thrombus formation. See text.

For inherited platelet disorders bleeding symptoms are usually experienced lifelong, so that retrospective evaluation of the bleeding episodes is of particular importance. On the other hand, sudden (acquired) bleeding due to the use of antiplatelet medication requires description of the acute symptoms [142]. Detailed registration of bleeding events by physicians is important not only for prediction, but also for safety reasons. Bleeding events related to the use of antiplatelet therapy, whether or not in combination with invasive procedures, are systematically reported, although this occurs not always according to the same bleeding definitions. Records are made of events during clinical trials and after the prescription of approved drugs (particularly Aspirin and $P2Y_{12}$ inhibitors). Informative BATs to evaluate such acquired bleeding tendencies have not yet been published. At present, listed are only clinically relevant bleedings, requiring transfusion of red blood cells and/or serious adverse events (*e.g.* intracranial haemorrhages). In an attempt to accomplish uniformity in bleeding definitions for cardiovascular clinical trials, a consensus classification was published allowing safety comparisons between studies, which deserves proper attention [144].

4.2. Laboratory testing of platelet dysfunction

Particularly in the last years, efforts have been made to standardize laboratory methods for platelet function testing. Quantitative platelet disorders can be detected by measuring platelet count and mean platelet volume in blood samples. Platelet aggregation in platelet-rich plasma in response to a panel of agonists, determined by light transmission aggregometry (LTA), is the current golden standard for detecting functional abnormalities [1]. Since LTA is relatively insensitive in identifying defects in δ-granule secretion, platelet release of ATP needs to be determined additionally, *e.g.* by lumi-aggregometry. Recommendations have recently been published for the use of standardized LTA tests for UK laboratories [145] and the ISTH [146]. A streamlined, validated panel of platelet agonists (ADP, adrenaline, arachidonic acid, collagen, TRAP, ristocetin) has been developed for diagnosing heritable platelet function disorders by testing with LTA and ATP secretion [18].

Replacing the early method of whole blood impedance aggregometry, the Multiplate and VerifyNow devices are increasingly used for determining platelet aggregation in whole blood, but still require more clinical validation. The PFA-100 is particularly used for testing shear-dependent platelet aggregation in response to collagen/ADP or collagen/adrenaline. The test is capable of detecting severe platelet function defects (Bernard–Soulier, Glanzmann's thrombasthenia) and most types of von Willebrand disease [147]. However, its sensitivity for less severe defects, like δ-granule storage pool deficiencies, is low [148].

Flow cytometry is frequently used for specific platelet function testing or for conditions where platelet counts are too low for LTA [149]. Flow cytometry allows the quantification of platelet receptors, the detection of platelet surface activation markers, and of platelet procoagulant activity. Hence, agonist-induced integrin activation, secretion of α- and δ-granules, and phosphatidylserine expression can easily be determined. This technique has already been used as a readout to determine the genetic determinants of platelet signalling pathways [95]. Flow cytometric tests for assessing (agonist-induced) platelet function in whole blood are in the experimental phase [150,151].

Since platelet activation and coagulation are interdependent processes [3], platelet function – in particular procoagulant activity – can also be tested in platelet-rich plasma by way of thrombin generation assays [4,152]. Recommendations for standardized measurement of platelet-dependent thrombin generation have recently been published by the ISTH [153]. Developments of a CAT-based assay in whole blood can improve clinical applicability of this type of tests [154]. However, their predictive value for platelet-related bleeding still needs to be established. Flow chambers have been used to measure thrombus formation in combination with coagulation (fibrin) at defined shear stress conditions [155]. Efforts are undertaken for better standardization of these flow-based assays and adaption to smaller volumes of blood [156,157].

4.3. Laboratory testing of platelet inhibition

For optimal patient care it is considered to be important to assess the effectiveness of antiplatelet therapy. This is predominantly an issue in cases of dual antiplatelet therapy with Aspirin and $P2Y_{12}$ receptor blockers, as is the standard therapy for patients with acute coronary syndrome or undergoing percutaneous coronary intervention. Due to heterogeneity in individual responses to especially Clopidogrel treatment,

inhibition of platelet function is suboptimal in a considerable part of the treated patients. This so-called high on-treatment platelet reactivity (HPR) associates with an increased risk of secondary atherothrombotic events [42,158]. In this context, also the reported variability in responsiveness to Aspirin requires attention [159]. Platelet function testing in relation to bleeding is also important in patients using the new, potent $P2Y_{12}$ blockers, Prasugrel and Ticagrelor, where low on-treatment platelet reactivity (LPR) is a matter of concern [160]. Together, this would advocate for monitoring platelet reactivity under treatment and concomitant individualized dosing of $P2Y_{12}$ blockers.

A platelet function-tailored approach with Clopidogrel may be most effective for patients with a high risk of stent thrombosis [159]. Further studies, especially with the new $P2Y_{12}$ blockers, are required to support the rationale for personalized treatment [158]. First efforts have been made for defining a therapeutic window for on-treatment platelet reactivity upon intake of $P2Y_{12}$ blockers to minimalize both ischemic and bleeding events [160]. Several platelet function assays are currently available for testing the efficacy and safety of antiplatelet therapy. Although LTA is still the golden standard, currently available point-of-care tests are more convenient for use in daily clinical practice. Of these, the VerifyNow and Multiplate tests may reflect most accurately the variable responsiveness to Aspirin and $P2Y_{12}$ blockers, while the predictive value of the PFA-100 test for $P2Y_{12}$ inhibition is limited [161].

5. Concluding remarks

Recent research on platelet responses in whole blood flow studies and experimental thrombosis studies has greatly contributed to our insight into main platelet receptors in thrombosis and haemostasis. In man, haemorrhagic complications can be caused by defective platelet receptors or by therapeutic inhibition of these receptors. It would be important to register these bleeding events in similar ways by using uniform assessment tools and function tests.

Practice points

- Study of thrombus formation, in whole blood flow studies and experimental murine thrombosis studies, is of relevance to understand the platelet responses in thrombosis and haemostasis in man.
- Main platelet receptors with established or potential clinical relevance are those of thrombin, thromboxane A_2, ADP, ATP prostaglandins, von Willebrand factor, collagen, CLEC-2 ligand, fibrinogen and laminin.
- Platelet dysfunction, either inherited or acquired due to medication, may lead to bleeding, but bleeding symptoms are assessed in different ways.

Research agenda

- Establishment of the therapeutic window for (potential) antiplatelet agents for optimal efficacy in preventing thrombosis with minimal bleeding.
- Development of bleeding assessment tools for identifying platelet function disorders or low on-treatment platelet reactivity; comparison of these tools to outcome of platelet function tests.

Conflicts of interest

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