The effects of arterial flow on platelet activation, thrombus growth, and stabilization

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SPOTLIGHT REVIEW

The effects of arterial flow on platelet activation, thrombus growth, and stabilization

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Abstract Injury of an arterial vessel wall acutely triggers a multifaceted process of thrombus formation, which is dictated by the high-shear flow conditions in the artery. In this overview, we describe how the classical concept of arterial thrombus formation and vascular occlusion, driven by platelet activation and fibrin formation, can be extended and fine-tuned. This has become possible because of recent insight into the mechanisms of: (i) platelet-vessel wall and platelet–platelet communication, (ii) autocrine platelet activation, and (iii) platelet–coagulation interactions, in relation to blood flow dynamics. We list over 40 studies with genetically modified mice showing a role of platelet and plasma proteins in the control of thrombus stability after vascular injury. These include multiple platelet adhesive receptors and other junctional molecules, components of the ADP receptor signalling cascade to integrin activation, proteins controlling platelet shape, and autocrine activation processes, as well as multiple plasma proteins binding to platelets and proteins of the intrinsic coagulation cascade. Regulatory roles herein of the endothelium and other blood cells are recapitulated as well. Patient studies support the contribution of platelet- and coagulation activation in the regulation of thrombus stability. Analysis of the factors determining flow-dependent thrombus stabilization and embolus formation in mice will help to understand the regulation of this process in human arterial disease.

Keywords Platelets • Coagulation • Thrombus • Shear rate • Stabilization

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1. Introduction

At prevalent flow conditions, platelets interact only limitedly with the healthy vessel wall, e.g. to maintain vascular integrity. However, this changes dramatically at conditions of vascular inflammation, damage, or disruption (situations leading to atherogenesis, haemostasis, or arterial thrombosis, respectively), where platelets massively adhere to the activated endothelium or the underlying endothelial matrix.[1–3] In this overview, we discuss shear-dependent mechanisms by which platelets are capable to adhere to activated, damaged or disrupted arterial vessels, and subsequently assemble into a stable or unstable thrombus.[4,5] We describe how the classical concept of arterial thrombus formation, developed almost 20 years ago, is currently extended and fine-tuned due to better insights into the underlying molecular mechanisms in relation to blood flow dynamics. We illustrate how local changes in fluid shear stress can control both platelet and coagulation activation and, thereby, the growth and stabilization of a thrombus. Furthermore, we recapitulate key roles of the vessel wall and leucocytes in the control of thrombus formation. By comparing recently published studies, where the effects of gene knockout have been determined on thrombus stability and embolus formation in mouse, we provide a first encompassing overview of the activation pathways in platelets and blood plasma that may control these processes. Subsequently, we discuss the possible clinical relevance of these findings on flow-dependent thrombus stabilization and embolization. For reasons of space, we only briefly touch the processes of venous thromboembolism and fibrinolysis.

2. Classical concept of flow-dependent platelet adhesion and thrombus formation

How platelets adhere at a site of vascular activation or injury greatly depends on the local blood flow and shear conditions. In the arterial

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system, the mechanical force that is most relevant for platelet adhesion is the shear stress of the blood.[6] Since the velocity of flowing blood is greater in the centre of the artery than near the vessel wall, blood consists of concentric layers through the artery lumen that markedly differ in flow rate. Shear stress is defined as the force per unit area between such layers, and wall shear stress is the force per unit area applied onto the vessel wall. Accordingly, also the shear rate (expressed as shear stress times the viscosity of the blood) varies through the artery lumen from minimal at the centre-line to maximal near the wall. Typical wall shear rates are in the range of 300–800 s^{-1} in large arteries, and of 500–1600 s^{-1} in arterioles of the microcirculation.[6] Especially in stenotic vessels, the wall shear rates can increase up to 10 000 s^{-1} and even higher.[7] Wall shear rates in the venous part of the circulation are in general low.

At a shear rate of >500 s^{-1}, initial tethering of platelets to the vessel wall is primarily mediated by interaction of the receptor complex glycoprotein (GP)Ib-V-IX to the von Willebrand factor (vWF). This multimeric adhesive protein is abundantly present in the plasma and secreted by endothelial cells. It is also bound to locally activated endothelium and deposits at the exposed extracellular matrix, particularly binding to collagen fibres.[8] At high wall shear rates (>5000 s^{-1}) or at sharp gradients of shear rate, the interaction of GPIb-V-IX with the vWF can be sufficient for unstable thrombus formation, albeit it results in no more than weak intracellular signalling in platelets.[9,10] The marked shear gradients around stenotic sites, such as present in arteries with advanced atherosclerosis, stimulate the endothelial release of the vWF and trigger GPIb-V-IX-dependent thrombus formation.[11]

The initial, shear-dependent adhesion of platelets is possible due to unique biomechanical properties of the vWF bond with GPIb-V-IX, as this is characterized by a very rapid on-rate and facilitated by unfolding of vessel wall-adhered vWF multimers.[2] However, the bond between GPIb-V-IX and vWF also has a rapid off-rate, implicating that by itself it is insufficient for stable platelet adhesion, except in situations of quite high-shear rate. The adhesion of platelets to vWF is stabilized by weak activation of integrin $\alpha_{IIb}\beta_3$, which mediates the integrin-dependent binding of platelets to vWF, and also facilitates the binding to platelets of plasma components such as fibrinogen and fibronectin.[12,13] Interestingly, GPIb-V-IX-dependent activation of $\alpha_{IIb}\beta_3$ appears to be impaired in platelets from mice lacking phospholipase D1 by a mechanism that relies on reduced phosphatidic acid production.[14,15]

In both the human and mouse systems, platelet interaction with collagen/vWF provides one of the most potent ways to attain stable adhesion and to trigger platelet activation processes to thrombus formation.[3,12,16] The signalling in platelets occurs by way of interplay between multiple receptors with, next to GPIb-V-IX and $\alpha_{IIb}\beta_3$, the immunoglobulin-family collagen receptor, GPVI, and the adhesive collagen receptor, integrin $\alpha_2\beta_1$.[17,18] Activation of platelets via GPVI, in complex with the Fc receptor γ-chain (FcRγ), is mediated by a 'signalosome' of multiple proteins, including various adapter and scaffold proteins (e.g. LAT, Cbl-b), tyrosine protein kinases (e.g. Syk), phosphatidylinositol 3-kinases (PI 3-kinases), and small GTP-binding proteins and their regulators (like Rac, Rho, CalDAG-GEFI).[19] The GPVI-induced signalling culminates in activation of phospholipase Cγ2 (PLCγ2), which produces second messengers causing an intracellular release of Ca^{2+}, followed by store-regulated influx of extracellular Ca^{2+} via the Ca^{2+}-sensor STIM1, and activation of downstream protein kinases.[20] Integrin $\alpha_2\beta_1$, like other integrins interacting with their substrates, strengthens and stabilizes the adhesion of platelets to collagen.[16,18] Platelets dispose of signalling mechanisms to tightly synchronize the activation state—and thus adhesiveness—of their various integrins.[21] Under shear conditions, platelets can also arrest at other extracellular matrix proteins, like thrombospondin-1, but the ensuing signalling pathways are less intensively studied than for adhesion to collagen/vWF.[8,22]

The shear-dependent adhesion and subsequent intracellular signalling leads to a range of biochemical and morphological platelet responses. Alterations in platelet shape occur via remodelling of the actin-myosin cytoskeleton (via Rac1 and Rho-kinase pathways) and polymerization of microtubules (with Ran-binding protein 10), resulting in the formation of pseudopods and lamellipods after adhesion.[23,24] Other prothrombotic responses include enhanced activation of the various integrins, the release of mediator molecules from platelet dense and alpha granules (such as ADP, ATP, and Gas6), the formation of thromboxane A_2, and the scrambling of membrane phospholipids.[3,15,25] These processes achieve capturing of flowing platelets, thus leading to a growing platelet aggregate, where platelets primarily interact via activated $\alpha_{IIb}\beta_3$ integrins that bridge fibrinogen or fibronectin molecules. At high-shear conditions, platelet–platelet interaction can also be achieved via GPIb-V-IX interacting with vWF, bound itself to the platelet aggregate.[26] Defects in these activation pathways may lead to impaired haemostasis and bleeding, as described in detail elsewhere.[27]

In the classical concept, the consolidation of a growing platelet thrombus is achieved by activation of the coagulation process. Blood coagulation can be initiated by (extrinsic) tissue factor highly expressed at the surface of subendothelial cells and, alternatively, by collagen and platelet-derived polyphosphates, which trigger the (intrinsic) factor XII pathway of coagulation.[28–30] Subendothelial tissue factor, particularly on fibroblasts and smooth muscle cells, needs encryption before it becomes active in coagulation, possibly through disulfide bond changes by protein disulfide isomerase.[3,31] Also tissue factor on microparticles can fulfil such a role.[32] These initial processes can only become effective if enforced in the propagation phase of coagulation, depending on the exposure of pro-coagulant phospholipids, formed at the membrane of platelets and other cells by a Ca^{2+}-dependent phospholipid scramblase.[3,33] Thus, membranes with the surface expression of phosphatidylserine provide an active site for coagulation factor complexes formation and thrombin generation.[2,34] The generated thrombin produces fibrin fibres at the platelet surface, which are considered to stabilize and consolidate the thrombus, and to mediate platelet-dependent clot retraction.[35,36] This fibrin network can trap flowing erythrocytes and leucocytes, ultimately resulting in full-vessel occlusion.

This sequential scheme of arterial (occlusive) thrombus formation explains why such a wide variety of drugs in use or in study can suppress this process, i.e. blockers of: GPIb-V-IX (humanized antibodies), thromboxane A_2 formation (aspirin), $P2Y_{12}$ receptors (clopidogrel, prasugrel, ticagrelor), $\alpha_{IIb}\beta_3$ integrins (abciximab, eptifibatide, tirofiban), thrombin generation (anticoagulants), and protease-activated receptor (PAR)-family thrombin receptors.[37] However, as discussed extensively elsewhere, minor or major bleeding is a known undesired side effect of essentially all these drug types.[15,27] This explains the still continuous search for new targets of antithrombotic drugs, where the risk of bleeding is minimal.

3. Vascular control

Discussed extensively elsewhere are the molecular processes by which the healthy endothelium suppresses the coagulation process.[3] The thrombo-protective potential of vascular endothelial cells also extends to preventing stable platelet adhesion and activation by a variety of

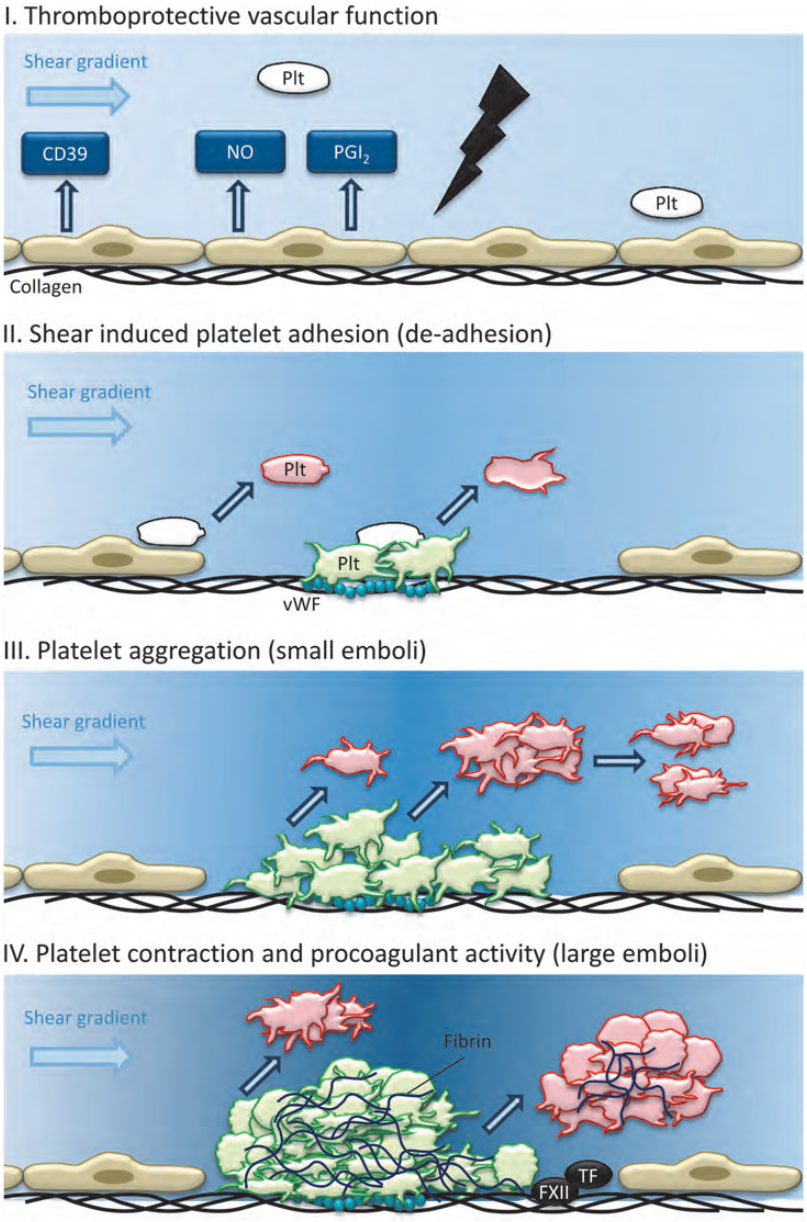

Figure 1 Thrombus growth and instability in a damaged artery. (I) Endothelial activity at the healthy vessel wall prevents platelet (Plt) adhesion. (II) Shear-induced adhesion of platelets at exposed subendothelial collagen/vWF allows platelets to aggregate, partly in a reversible way. (III) During the build-up phase of a thrombus, small emboli are shed. (IV) In a grown thrombus containing contracted and fibrin-anchored platelets, local heterogeneities and blood flow shear gradients allow the shedding of larger emboli.

mechanisms (*Figure 1*). The endothelial products nitric oxide and prostacyclin (prostaglandin I_2) both act to relax vascular smooth muscle cells and inhibit platelet activation.[38] Thus, in healthy endothelium, nitric oxide and prostacyclin maintain a low blood pressure, reduce blood shear forces, and suppress platelet activation. Mouse models confirm the antithrombotic roles of both products. Hence, mice lacking endothelial nitric oxide synthase show an impaired vasodilator response in conductance vessels. Mouse models, in which the COX2-dependent formation of prostacyclin or its action is disrupted, present with a predisposition to thrombotic events.[39] Another platelet-inhibiting protein at the endothelial surface is the ectonucleotidase CD39, which degrades (endothelial-derived) ATP and ADP, and thus prevents platelet activation by these nucleotides. Furthermore, the adenosine produced by CD39 has anti-platelet activity.[40] Clearly, these thrombo-protective effects of the vessel wall are abolished upon local damage or disruption of the endothelium.

A recent topic of interest is the role of vessel wall-adherent leucocytes in thrombus formation. In certain mouse models, neutrophils can adhere even earlier than platelets upon vascular damage, with as a result increased tissue factor-dependent fibrin generation and platelet accumulation.[41] Both neutrophils (forming neutrophil extracellular traps) and monocytes (exposing tissue factor) support the thrombotic process especially under conditions of venous thrombosis.[42,43] The relevance of these processes for the development of arterial thrombosis is still unclear.

4. New concepts: dynamic, fine-tuned regulation of thrombus growth and stabilization

The classical scheme described above considers arterial thrombus formation as a simple progressive process, starting with platelet adhesion and ending with occlusion of the locally activated or damaged vessel. However, many experimental studies, either *in vivo* with experimentally damaged arteries in mice, or *in vitro* with flow chambers perfused with whole-blood, point to a more complex organization of the thrombosis process in time. In the macro-circulation (carotid artery) and the microcirculation (mesenteric and cremaster arterioles), it is often examined that single platelets can adhere and detach during the build-up phase of a thrombus. Furthermore, once a discernible thrombus has been formed, it contracts and tends to shed smaller or larger emboli for a certain period of time (*Figure 1*). Accordingly, flow-dependent dynamics of platelet detachment, embolus shedding, and unstable occlusion seem to be common events during the process of thrombus formation. A large number of studies with genetically modified mice point to the involvement of many platelet-derived and coagulant proteins in the dynamic regulation of the stability of thrombi formed at arterial flow conditions (*Table 1*). Hence, the classical concept of thrombus formation needs adjustments accommodating the flow-dynamic components. Refinements, particularly, explaining the dynamics and heterogeneities of thrombus build-up and fate, are described below. Participation of vascular- or leucocyte-derived proteins in the control of thrombus stability has hardly been described in the literature.

4.1 Integrin $\alpha_{IIb}\beta_3$ activation and reversible platelet aggregation

Impaired or diminished activation of platelet $\alpha_{IIb}\beta_3$ is known to cause instability of thrombi, which are formed under flow *in vivo* or *in vitro*, and to stimulate the detachment of single platelets and small aggregates.[44,45] *In vitro* observations support the notion that $\alpha_{IIb}\beta_3$ activation is a reversible process, and that persistent signalling in platelets is required to keep this integrin in an activated, pro-adhesive conformation.[46] Several autocrine and paracrine (between platelets) signalling processes appear to contribute to persistent integrin activation, and many of these have been shown to be involved in thrombus stabilization. A prominent factor is the release of ADP and its interaction with platelet $P2Y_{12}$ receptors, which stimulates integrin activation via a pathway involving PI 3-kinase, Akt2, Rap1b, and filamin A (*Figure 2*).[46–49] Enforced integrin activation is furthermore achieved by interaction of CD40L with its supposed ligand CD40, both of which are membrane proteins that regulate thrombus stability.[50] Another mechanism for continued integrin activation is provided by interaction of the soluble molecule Gas6 (present in plasma and limitedly stored in platelets) with the platelet TAM receptors, Tyro, Axl, and Mer.[51–53] New data yet suggest that plasma Gas6 may also stimulate the coagulation process by regulating the expression of vascular tissue factor.[54]

Limited integrin activation also explains why, at gradients of shear stress, platelets tend to loosely adhere to a growing thrombus via GPIb-V-IX in an often instable way.[10] Another family of proteins that is considered to modulate platelet integrin function is provided by the tetraspanins, of which TSSC6 and CD151 are abundantly expressed in the platelet membrane. Tetraspanin control of $\alpha_{IIb}\beta_3$ activation may explain why the genetic ablation of TSSC6 or CD151 results in thrombus instability and increased embolus formation.[55,56]

Another mechanism controlling the activation of $\alpha_{IIb}\beta_3$ and other integrins is by redox control of free-cysteine thiols in the extracellular chains, particularly by the protein disulfide isomerase.[57] How the redox control affects thrombus stabilization is still unknown.

4.2 Contact-dependent signalling to tight platelet interactions

Particularly, the work of Brass *et al*. has led to substantial insight into so-called contact-dependent activation pathways, by which platelets can tightly interact with each other in a thrombus.[5,58]

Contact-dependent signalling occurs by pairs of ligands and receptors, such as ephrin B1-EphA4 (which enforces $\alpha_{IIb}\beta_3$ activation and prevents platelet disaggregation), and semaphorin 4D-plexin (which supports Syk-mediated platelet activation); as well as by tight platelet–platelet interactions through JAM- and SLAM-family members.[5,59] Deficiency in several of these proteins has been found to impair the stabilization of mouse thrombi formed *in vivo* (*Table 1*). Jointly, these interaction establish close platelet–platelet contacts, which is considered to be a requirement for the stabilizing contraction of platelets in the thrombus core.[58] Inside platelets, signalling via Rho-kinase to myosin and actin appears to be a key mechanism transmitting the contractile forces from the cell surface to the cytoskeleton.[60] This may explain why arterial thrombi in mice lacking RhoA or myosin heavy chain-9 are characteristically unstable and show frequent embolization.[23,61]

A different set of contact receptors has been identified that negatively regulates platelet activation and thrombus stability. In mice, the absence of the CEACAM1 or ESAM receptors resulted in an increased thrombus growth and reduced embolus shedding.[62,63] It is hence tentative to suggest that, within a thrombus, local balances of the platelet-activating and platelet-inhibiting contact signalling events determine which part of the thrombus can contract to form a stable plug, and which part of the thrombus does not contract and is susceptible for detachment of single of aggregated platelets. Intra-thrombus differences in contact-dependent signalling may also explain the reported heterogeneity within a thrombus with contracted, pro-coagulant, and loosely aggregated platelets.[64] However, as described above, also the partial penetration of thrombin and fibrin into a thrombus may contribute to this heterogeneity.

A negative role in thrombus stability has also been observed for the contact protein, connexin 37.[65]. This is a gap junction protein expressed in platelets, as well as in endothelial cells, smooth muscle cells, monocytes, and macrophages.[65–67] Interestingly, the conclusions from the two publications regarding the role of connexin 37 in platelets are different: one research group concludes that it functions by limiting platelet activation and thrombus stabilization,[65] while the other group finds that it promotes platelet activation.[67] An approach to take this further would be the generation of mice lacking connexin 37 only in platelets.

4.3 Multisided regulation by coagulation

The role of coagulation in thrombus formation and stabilization appears to be more complex than earlier thought. Thrombin that is formed at the thrombus surface contributes to platelet activation by interaction with PARs.[68,69] Recent evidence suggests that the contribution of PARs to platelet activation is dependent on the blood flow rate. Whereas PARs activate platelets at low-shear conditions, their role becomes diminished at pathologically high shear rates.[70] Yet, thrombin-induced signalling contributes to the generation of pro-coagulant PS exposing platelets, which are abundantly formed in arterial thrombi.[71] There is ongoing research to find

Table 1 Reported effects of genetic deficiency in mouse on embolization during arterial thrombus formation *in vivo* or *in vitro*

Gene defect	MGI	Protein defect	Thrombosis	Model Effect on arterial thrombus formation	Emboli	Ref(s)
Platelet receptors and membrane proteins						
Axl	1 347 244	Axl (Gas6 receptor)	Flow device	Increased thrombus disaggregation	+	53
Adra2a	87 934	α2 adrenergic receptor	Mesentery/$FeCl_3$	Increased formation of emboli	+	95
Cd151	1 096 360	Tetraspanin CD151	Carotis/ligation, cremaster/ laser	Increased thrombus instability	++	56
Cd40lg	88 337	CD40L	Mesentery/$FeCl_3$	Delayed occlusion, more unstable thrombi	++	50
Fcer1g	95 496	FcR γ-chain	Carotis/ligation, cremaster/ laser	Smaller thrombi, more emboli formed	+	76,96
Gp1ba	1 333 744	GPIbα	Carotis/ligation	Smaller thrombi, reduced stable platelet adhesion	++	97
Gp5	1 096 363	GPV	Mesentery/$FeCl_3$	Smaller thrombi, increased detachment	++	98,99
Gp6	1 889 810	GPVI	Carotis/$FeCl_3$, ligation	Smaller thrombi, reduced stable platelet adhesion	++	100,101
Itga2	96 600	Integrin α2	Mesentery/$FeCl_3$, flow device	Smaller thrombi, more emboli formed	+	75,102
Lat	1 342 293	LAT	Cremaster/ laser	Smaller thrombi, more emboli formed	++	76
Mertk	96 965	Mer (Gas6 receptor)	Flow device	Increased thrombus disaggregation	+	53
P2yr12	1 918 089	$P2Y_{12}$ receptor	Mesentery/$FlCl_3$, cremaster/ laser	Smaller thrombi, more emboli formed	++	47,103
			Flow device	Increased thrombus embolization	++	103,104
Slamf1	1 351 314	SLAM (CD84)	Mesentery/$FeCl_3$	Delayed occlusion, more emboli formed	+	59
Tspan32	1 350 360	Tetraspanin TSSC6	Mesentery/$FeCl_3$	Increased thrombus instability	+	55
Tyro3	104 294	Sky (Gas6 receptor)	Flow device	Increased thrombus disaggregation	+	53
Ceacam1	1 347 245	CEACAM1	Mesentery/$FeCl_3$	Larger thrombi, less emboli formed	−	62
Esam	1 916 774	ESAM	Cremaster/laser	Larger thrombi, less detachment	−	63
Gja4	95 715	Connexin 37	Mesentery/$FeCl_{3\backslash}$	Increased thrombus formation, less emboli	−	65
Platelet intracellular signalling proteins						
Akt2	104 874	Akt2	Carotis/$FeCl_3$	Smaller thrombi, increased instability	++	105
Cblb	2 146 430	Cbl-b	Carotis/$FeCl_3$	Delayed occlusion, unstable thrombi	+	106
Flna	95 556	Filamin A	Flow device	Increased platelet detachment	+	107
Myh9	107 717	Myosin heavy chain-9	Carotis/$FeCl_3$	Reduced thrombus growth, more emboli formed	+	23
Plcg2	97 616	Phospholipase Cγ2	Cremaster/ laser, flow device	(smaller) thrombi, increased instability	++	76,108
Prkaa2	1 336 173	AMPK-α2	Carotis/$FeCl_3$	Less compact thrombus, more emboli formed	+	109
Pik3cb	1 922 019	PI 3-kinase-β	Flow device	Increased thrombus disaggregation	++	46
Pik3cg	1 353 576	PI 3-kinase-γ	Flow device	Unstable thrombi, increased disassembly	++	46
Rac1	97 845	Rac1	Cremaster/laser, flow device	Increased instability of thrombi	++	110
Ranbp10	1 921 584	Ran-binding protein 10	Mesentery/$FeCl_3$	Reduced occlusion, unstable thrombi	+	24
Rhoa	1 096 342	RhoA	Mesentery/$FeCl_3$	Reduced occlusion, more emboli formed	++	61
Stim1	107 476	STIM1	Mesentery/$FeCl_3$	Delayed occlusion, increased platelet detachment	+	111
Plasma proteins						
C3	88 227	Complement factor 3	Cremaster/photochemical	Delayed thrombus formation, more emboli	+	112
F11	99 481	Factor XI	Mesentery/$FeCl_3$	Increased detachment of thrombi	+	80
F12	1 891 012	Factor XII	Carotis/$FeCl_3$, mesentery/$FeCl_3$	Increased detachment of thrombi	++	80,81
Fgg	95 526	Fibrinogen γ-chain	Carotis/$FeCl_3$, mesentery/$FeCl_3$	Increased detachment of thrombi	++	113,114
Fn	95 566	Fibronectin	Mesentery/$FeCl_3$	Delayed formation of unstable thrombi	++	115
Gas6	95 660	Gas6	Flow device	Increased thrombus disaggregation	+	53
Klk4	1 861 379	Prekallikrein	Mesentery/$FeCl_3$	Reduced thrombus formation, more emboli	+	81
Lep	104 663	Leptin	Carotis/$FeCl_3$	Delayed occlusion, unstable thrombi	+	116
Serpine1	97 608	PAI-1	Carotis/$FeCl_3$	Longer time to occlusion, unstable thrombi	+	117
Thbs1	98 737	Thrombospondin-1	Mesentery/photochemical	Prolonged occlusion, more emboli formed	+	118
Vtn	98 940	Vitronectin	Carotis/$FeCl_3$, mesentery/$FeCl_3$	Longer time to occlusion, unstable thrombi	++	117,119
Wvf	98 941	vWF	Mesentery/$FeCl_3$ (venules)	Reduced thrombus formation, unstable	+	120
Plg	97 620	Plasminogen	Carotis/photochemical	Shortened occlusion, less emboli	−	121

Indicated are the mouse genes, the corresponding proteins in blood platelets or plasma, the murine thrombosis model used (flow device in case of *in vitro* studies), the effect on embolus formation (+, increased; ++, highly increased; −, decreased).
MGI, mouse genome index.

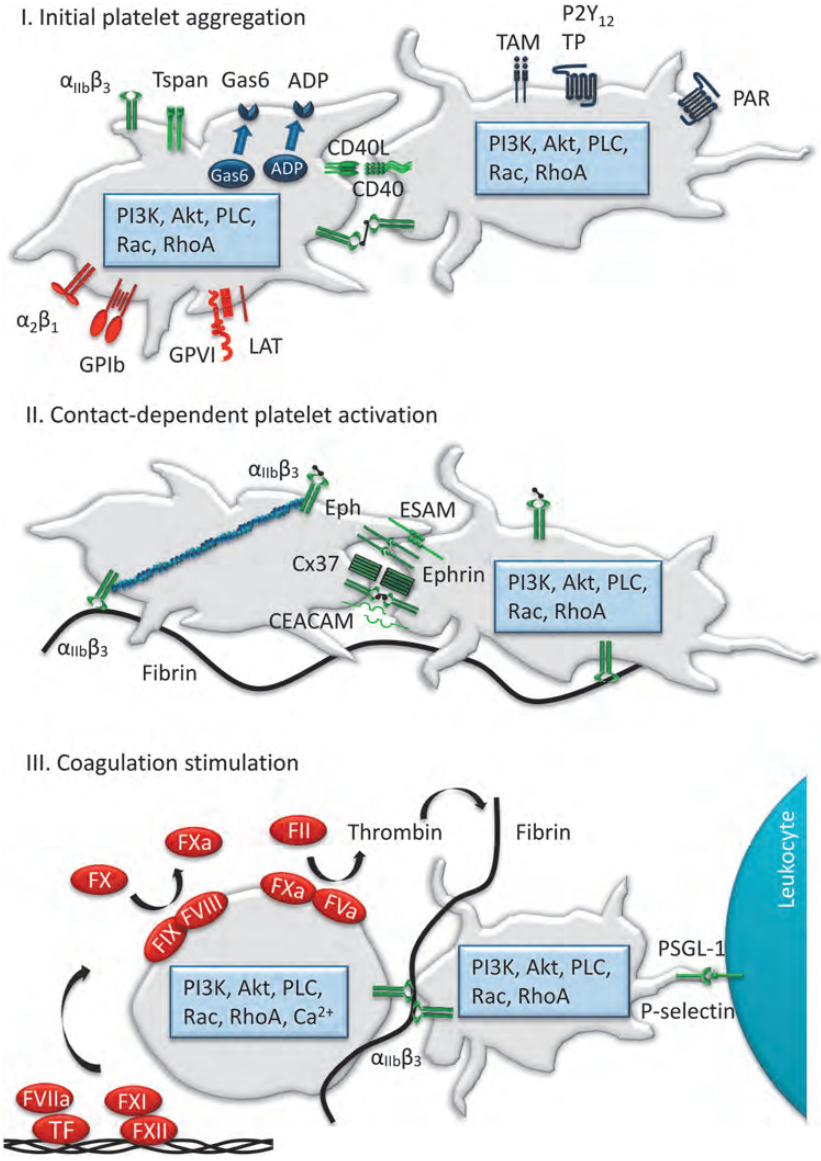

Figure 2 Key platelet and plasma proteins contributing to thrombus stability. (I) Platelet receptors and ligands involved in initial integrin $\alpha_{IIb}\beta_3$ activation and reversible platelet aggregation (see *Table 1*). The absence of these molecules increases thrombus instability. Also indicated is a box with intracellular signalling proteins controlling this process. II. Contact-dependent signalling mechanisms implicated in platelet contraction and irreversible platelet aggregation. Fibrin formed by the coagulation process stabilizes the platelet aggregate. (III) Plasma coagulation factors, via the intrinsic (factor XII, FXII) and extrinsic (tissue factor, TF) pathways, mediating platelet-dependent thrombin and fibrin generation, stabilizing a growing thrombus. Also indicated is a primary mechanism of platelet-leucocyte interaction via P-selectin and PSGL-1. See further Versteeg *et al.*[3].

other strong agonists—besides thrombin—that can support collagen-induced platelet activation (via GPVI). Currently in the spot light are the CLEC-2 receptors, which via an unknown ligand, have been implicated in arterial thrombus formation in mice.[72] Such strong agonists other than thrombin may also stimulate the contraction of platelets, making an aggregate stable.[60] On the other hand, fibrin clot retraction, mediated via activated $\alpha_{IIb}\beta_3$ integrins, is still considered to be a main mechanism for platelet contraction in a stabilising thrombus.[34]

Although it may be obvious that fibrin formation is needed for a stable thrombus, reports on the effects of thrombin inhibitors in arterial models *in vivo* primarily point to a reduced thrombus growth, rather than to thrombus instability.[73–76] A certain amount of fibrin formation yet seems to be important, since in flow devices *in vitro* the inhibition of fibrin polymerization resulted in shear-induced shedding of emboli.[77] Mechanistically, these findings are not easy to explain. At the one hand, the formation rates of thrombin and fibrin decrease at a higher shear rate, as a consequence of thrombin dilution by blood flow, which suggests that thrombin generation is a limiting factor in arterial thrombus formation.[69,78] At the other hand, neither thrombin nor fibrin is uniformly distributed in an arterial thrombus,[73,79] which may imply that a consolidating fibrin network is only present in parts of the thrombus.

Another relevant finding is that especially deficiencies in the intrinsic coagulation pathway (prekallikrein, factor XII, or factor XI) reduce thrombus stability and provoke embolus formation.[80,81] In agreement with this, pharmacological inhibition of the factor XII pathway results

in the formation of large emboli shed from arterial thrombi.[82] These data point to a thrombus-destabilizing effect upon partial—and likely non-uniform—suppression of the clotting process within a thrombus. More research is needed to understand the precise role of the intrinsic coagulation pathway.

Interestingly, reports on the roles of anticoagulant proteins carried by platelets do not describe effects on thrombus stability. For example, tissue-factor pathway inhibitor located in platelets plays a significant role in the control of thrombus growth, but was not reported to influence thrombus stability.[83] The same is true for its cofactor, protein S (Calzavarini, Angelillo-Scherrer, unpublished observations, 2012). More thorough studies to the effect of genetic deficiency restricted to mouse platelet proteins are needed to advance this field.

4.4 Disturbances in blood rheology

An aspect that is discussed extensively elsewhere,[2] is the contribution of blood flow, and in particular of rheological disturbances, on the stabilization or embolization of near-occlusive thrombi. When a stenotic or otherwise vulnerable vessel tends to become occluded, high-shear gradients are generated around the growing thrombus. These flow disturbances will not only accelerate platelet activation and fibrin formation, but also provide the force for embolization of smaller or larger platelet aggregates (*Figure 1*). Flow pulsations by the heart rhythm and vascular distension may further aggravate the extent of embolization and perhaps the size of the emboli, but this has hardly been investigated. Another still poorly studied aspect is how red blood cells—either flowing or when bound to fibrin fibres—contribute to thrombus stability under arterial flow conditions.

5. Genetic mouse models and flow chambers: key pathways of thrombus (in)stability identified?

The above described mechanisms point to involvement of a surprisingly high number of proteins in the vessel wall, platelets, and the coagulating plasma, that contribute to the formation and stabilization of an arterial thrombus. *Table 1* provides a list of experimental thrombosis studies using mice, where effects have been measured of genetic modification on stable platelet adhesion or shedding of emboli, following damage of vessels of the macro-circulation (carotis artery) or microcirculation (mesenteric or cremaster artery). The evidence for thrombus instability comes from either intravital microscopic observations or rapid changes in blood flow, measured with Doppler probes. *Table 2* gives a list of drug interventions that have been shown to influence thrombus stability *in vitro* during the perfusion of the human blood through a flow device at a high arterial shear rate.

As indicated in *Table 1*, for 44 different mouse genes a notable change in stability of arterial thrombi has been reported. This list mostly concerns genes and proteins that also play a role in the overall process of thrombus growth. In short, referring to the mechanisms described above, this concerns genes implicated in: (i) GPIb-V-IX and GPVI-dependent platelet adhesion (also $\alpha_2\beta_1$, FcR γ-chain); (ii) GPVI-mediated platelet signalling to Ca^{2+} rises and beyond (PLCγ2, LAT, Cbl-b, STIM1, Rac1); (iii) integrin activation (PI 3-kinases, Akt2, filamin A, tetraspanins); (iv) autocrine and paracrine regulatory mechanisms supporting integrin activation ($P2Y_{12}$, CD40, CD40L, Axl, Mer, Sky, SLAM); (v) and regulation of platelet contraction (RhoA, myosin). Furthermore relevant are genes of plasma proteins involved in: (vi) adhesion to platelets (vWF, fibrinogen, fibronectin, vitronectin, thrombospondin-1); (vii) activation of platelets (Gas6, leptin); and (viii) activation of the intrinsic coagulation cascade (prekallikrein, factors XI, XII). Interestingly, hardly any reports are available on thrombus instability due to specific platelet secretion defects. A suppressive role is reported for negative regulators of the contact activation (CEACAM1, ESAM, connexin 37).

The studies with human blood and flow devices to a certain extent support involvement of the same platelet activation pathways in thrombus stability and embolization in the human system (*Table 2*). In particular, this concerns a role of the $\alpha_{IIb}\beta_3$ activation pathway, in that inhibition of P2Y receptors, PI 3-kinases, or the integrins themselves results in embolization. Thrombus instability is also examined upon inhibition of platelet contraction (EphA4, ephrinB1), Gas6 activity or fibrin formation. More work is clearly needed to demonstrate the importance of the other proteins identified in mouse for the human system.

Table 2 Reported effects of pharmacological inhibitors on embolization of human thrombi under high-shear flow conditions *in vitro*

Target protein	Inhibitor	Effect on thrombus formation *ex vivo*	Emboli	Ref(s)
Platelet proteins				
EphA4/ephrinB1	Soluble fragments	Increased platelet disaggregation	+	122
Integrin αIIbβ3	Abciximab, eptifibatide	Increased platelet disaggregation	+	123,124
Myosin heave chain-II	Blebbistatin	Increased thrombus instability	+	125
$P2Y_1$ / $P2Y_{12}$ receptors	MRS2179/ticagrelor/2-MeSADP	Increased thrombus instability	+	44,124,126
	AR-C69931MX	Increased platelet disaggregation	+	46
PI 3-kinase-β	TGX-221	Increased platelet disaggregation	+	46
RhoA kinase	Y-27632	Reduced thrombus formation, increased instability	+	60,125
Plasma proteins				
Fibrin polymer	GPRP	More unstable thrombi, releasing platelets	+	77
Gas6	Depleted plasma	Increased thrombus instability	+	53

Perfusion studies of human blood flowed over collagen using flow devices. Indicated are the protein target, the inhibitor(s) use and the effect on embolus formation (+, increased).

6. What can we learn more from patient observations?

In man, thrombosis refers to the pathological condition where thrombi form inopportunely in the lumen of vulnerable vessels, leading to interruption of blood flow, occlusion, and ensuing tissue damage. Antithrombotic drugs, which comprise antiplatelet, anticoagulant, and antifibrinolytic drugs, are commonly used for the treatment and secondary prevention of such thromboses in arteries and veins.[2,37] The common cause is rupture or erosion of an atherosclerotic plaque or a local disturbance in haemodynamic shear forces in the flowing blood. Arterial thrombosis, causing heart attacks, stroke, or limb gangrene, is responsible for almost 50% of mortality in industrialized countries. Next to treatments stimulating vasodilatation, antiplatelet drugs are the first choice for treatment of (secondary) arterial thrombosis.[84] It is relevant to note here that non-steroidal anti-inflammatory drugs, particularly those which inhibit COX2-dependent formation of prostacyclin in the vessel wall, confer a cardiovascular hazard.[39] Such drugs antagonize the capacities of prostacyclin to suppress platelet activation and vasoconstriction. This can predispose to thrombosis, hypertension, and atherosclerosis.

Patients with a transient ischaemic attack/stroke or myocardial infarction mostly suffer from thrombosis of the atherosclerotic carotid or coronary artery. Such patients may present with symptomatic emboli that are shed from the earlier formed thrombi. However, in case of acute stroke or post-operatively after carotid endarterectomy, patients may also develop clinically asymptomatic embolization.[85] Asymptomatic embolization has also been reported following carotid artery stenosis.[86] In such patients, shedding of platelet emboli from the thrombotic carotid artery can be detected using trans-cranial Doppler ultrasound, for the major part without pathological consequences.[87] This indicates that embolization is a frequent phenomenon after a thrombotic event that, although often clinically silent, yet may form an increased risk of becoming symptomatic. More research is clearly needed to ascertain this.

Because of the reduced blood flow in the vein system, venous thrombosis relies more on thrombin and fibrin generation. Patients with venous thrombosis or venous thromboembolism are treated with several types of anticoagulants.[88,89] Vitamin K antagonists produce their anticoagulant effect by interfering with the γ-carboxylation of vitamin K-dependant prothrombin and factors VII, IX, and X. Unfractionated heparins are indirect anticoagulants that bind to antithrombin, enhancing its ability to inhibit activated factor X, thrombin, and other coagulation factors. Low molecular-weight heparins and analogues (danaparoid, fondaparinux) bind to antithrombin, and selectively potentiate its anti-factor Xa activity. Drugs like lepirudin (also bivalirudin, argatroban, dabigatran) are used as direct, selective inhibitors of thrombin, whereas the novel compounds rivaroxaban, apixaban, and edoxaban are direct inhibitors of factor Xa. Whereas all these drugs have proved to be clinically effective, there is hardly any knowledge on how their action is determined by the local flow conditions at the site of the thrombus.

An interesting case is provided by patients with specific coagulation defects in the absence of bleeding. Patients with afibrinogenaemia (complete fibrinogen deficiency) sometimes develop thrombosis. The thrombotic events can be located in either the arterial or venous territories.[90] It is considered that in these patients thrombin that is formed is more active, since it cannot be inactivated by binding to fibrin.[91] One of the consequences is increased platelet activation.[92] The resulting, fibrin-poor thrombi are described as large but loosely packed, confirming that fibrin provides thrombus stability.[93] Interestingly, emboli are frequently observed in these patients. Treatment comprises concomitant infusion of fibrinogen and an anticoagulant, capable of binding fibrin-bound and free thrombin, e.g. a direct thrombin inhibitor.

Severe factor XII deficiency may also provoke pulmonary embolization, e.g. in John Hageman, the index patient with factor XII deficiency. Epidemiological studies show a complex relation between severe factor XII deficiency and increased thrombotic risk.[94] One of the explanations is that complete deficiency in factor XII restricts the formation of fibrin, and facilitates symptomatic embolization in a similar way as observed in murine studies. More translational research is required to link these clinical observations to those of the mouse models.

7. Conclusions: the good and the bad of arterial thrombus stabilization and embolus formation

As described above, mouse experimental thrombosis studies in general indicate that arterial thrombus growth and thrombus stability can be linked processes. Many platelet and plasma proteins that control thrombus growth also appear to play a role in stabilization of the thrombus. This is directly evident from intravital microscopy observations showing that flow-mediated adhesion of platelets can be a reversible event, and that single platelets as well as small or large platelet aggregates regularly detach from a thrombus even in wild-type mice. Hence, some degree of instability may be considered as a natural phenomenon in arterial thrombus formation. This is illustrated in *Figure 1*, schematizing that during thrombus growth smaller and larger emboli are shed. The limited clinical observations so far indicate that such shedding of emboli also occurs in thrombotic human arteries.

On the other hand, thrombus growth and stability do not seem to be controlled in exactly the same ways. For instance, there are surprisingly few reports on a role of platelet secretion products in thrombus stabilization (*Table 1*), whereas platelet secretion is considered to be major determinant of thrombus growth. As schematized in *Figure 2*, key processes controlling the stability of a thrombus are (i) initial platelet integrin activation controlling platelet aggregation, which can be reversible resulting in shedding of platelet emboli; (ii) contact-dependent signalling, stabilizing the platelet aggregates; and (iii) plasma thrombin and fibrin generation via the intrinsic and extrinsic coagulation pathways, which provides the thrombus with a fibrin network, but still allows shedding of platelet-fibrin emboli (microclots).

In the clinical situation, the shedding of relatively large (fibrin-containing) emboli may be most harmful, giving rise to (semi)occlusive thrombus formation downstream in the vasculature. Clinically silent likely are those emboli that are smaller and prone to disintegration. Presently, we can only speculate on the mechanisms that favour the shedding of large emboli with pathological consequences. An interesting hypothesis is that these are due to local or temporal inhomogeneities in a thrombus, e.g. differences in platelet contraction or local incompleteness of fibrin formation. Thus, partial inhibition of 'later' pathways (irreversibly contracted platelets, platelet-fibrin clots) may result in emboli that are not only larger, but also more stable themselves and clinically symptomatic. Another possibility is that such emboli are formed by partial thrombolysis due to restricted fibrinolytic activity. This clearly needs further study. More thorough investigation is also needed to

understand the roles of the natural platelet-inhibiting, coagulation-inhibiting and fibrinolytic pathways in the control of flow-dependent thrombus stability.

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