

Acquired alteration in platelets

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*Acquired alterations in platelets:
Insight into impairment and recovery of
platelet function*

Constance C.F.M.J. Baaten

***Acquired alterations in platelets:
Insight into impairment and recovery of platelet function***

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*Acquired alterations in platelets:
Insight into impairment and recovery in platelet function*

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
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volgens het besluit van het College van Decanen,
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Chapter 1

General Introduction

Platelets play pivotal roles in thrombosis and hemostasis. Upon vascular injury, they acutely respond by forming a plug or aggregate, and thereby prevent further blood loss. Following activation of the coagulation cascade, a fibrin mesh is formed, which stabilizes the platelet plug. This process of clot formation is referred to as hemostasis.¹ When platelet activation or coagulation is impaired due to either genetic or environmental factors, hemostatic clot formation is hampered, which leads to an increased risk of hemorrhage. On the contrary, enhanced platelet activation or coagulation relates to a higher risk of thrombosis, which is the pathological process where aberrant clot formation results in occlusion of a blood vessel. While platelets are considered to have a predominant role in arterial thrombosis, venous thrombosis is more dependent on the coagulation process.^{2,3}

In the present chapter, a general overview is given of platelet functions and of thrombus formation, as far as relevant for the present thesis. Furthermore, a brief description is provided on acquired clinical conditions that are linked to either bleeding or thrombosis.

Regulation of platelet functions and thrombus formation

The formation of new platelets takes place mainly, but not exclusively, in the bone marrow.^{4,5} Proplatelets are shed from the precursor megakaryocytes in a process called thrombopoiesis.⁶ These proplatelets further divide to form the individual platelets that stay in circulation. In man, platelets circulate for 7-10 days on average, meaning that a continuous production of new platelets is needed to maintain a constant platelet count.⁷

Newly formed platelets contain measurable amounts of mRNA that is inherited from the precursor megakaryocytes; hence they are named 'reticulated platelets'.⁸ As the platelet mRNA degrades over time, the older circulating platelets are essentially devoid of these linear ribonucleotides, although they still contain circular RNA forms.⁹ The presence of mRNA is often used as a feature to identify newly formed platelets.¹⁰ In the literature, it has been suggested that reticulated platelets are larger and more reactive than the older ones.^{11,12} However, conclusive evidence for this suggestion is still missing,^{13,14} partly due to the lack of methods that enable a proper detection of reticulated platelets. Characterization of these platelet types in terms of structure and function thus requires more investigation.

Circulating platelets are capable of responding quickly to vascular damage, since they are equipped with a multitude of signaling receptors on the plasma membrane.^{1,15} Under conditions of high shear rate, such as present in the arteries and arterioles, red blood cells normally accumulate in the central part of the vessel lumen, whereby they push the platelets outwards to come in close proximity to the vascular wall.^{16,17} One of the first events, upon vascular damage, is the shear-dependent interaction of these platelets with von Willebrand factor, derived from endothelial cells, that is bound to the exposed collagen fibers.¹⁸ The platelet glycoprotein Ib-V-IX complexes are primarily responsible for the interaction with von Willebrand factor. It causes platelets to roll, which then triggers their firm binding to collagen itself via the receptors glycoprotein VI and integrin $\alpha_2\beta_1$.^{1,19,20} The collagen-attached platelets become activated, primarily through

glycoprotein VI, and respond by granule secretion, in which they release cargo molecules from the α and dense granules. Depending on the strength of the stimulus, platelets will secrete more or less of this granular cargo.²¹ The secretory granules contain many autocrine and paracrine molecules. Large molecules, such as fibrinogen and factor V, are packed in the α granules, while the dense granules are loaded among others with ADP, ATP and polyphosphates.²² Once secreted, ATP and ADP serve as autacoids to activate nearby platelets via the purinergic receptors P2X₁ (for ATP) and P2Y₁ plus P2Y₁₂ (for ADP). Along with secretion, the autacoid thromboxane A₂ is produced via the cyclooxygenase/thromboxane synthase complex, which similarly activates nearby platelets via the TP receptors. Together, these autocrine agents provide a potent amplification loop to enhance effects of initial triggers of platelet activation, *e.g.* provided by collagen or thrombin traces.^{23,24} Another important platelet response is the conformation change of integrin $\alpha_{\text{IIb}}\beta_3$, which is a glycoprotein complex abundantly expressed on the platelet surface. Activation of $\alpha_{\text{IIb}}\beta_3$ integrin enables the binding of fibrinogen, a protein that is necessary for platelet-platelet interactions and thus for stable platelet aggregation.²⁵ The eventual size and activity of a platelet aggregate or a thrombus formed on collagen is furthermore influenced by the presence of other platelet-adhesive proteins in the vessel wall and by local shear stress.²⁶

There is evidence indicating that platelets remain active for a certain time, also after being incorporated into a thrombus. For instance, the platelets in a thrombus formed on collagen secrete matrix metalloproteinases, enzymes which gradually degrade the underlying collagen fibers.²⁷ In addition, these platelets can undergo proteolytic cleavage of several glycoproteins.²⁸ Glycoprotein Iba and glycoprotein VI can be cleaved by members of the A Disintegrin And Metalloprotease family, namely by ADAM17 and ADAM10, respectively, in a process called receptor shedding.²⁹ Both ADAM10 and 17 are membrane-bound proteases, which remove the entire extracellular domain of their target membrane proteins.³⁰ Although shedding of glycoprotein Iba and glycoprotein VI will theoretically impair the ability of platelets to bind von Willebrand factor and collagen, the physiological role of receptor shedding has remained elusive. So far, only the suggestion has been made that shedding is a mechanism to shut down platelet adhesion and activation.³¹ Another suggestion is that the shedding of especially glycoprotein Iba serves to clear platelets from the circulation in the liver.³²

Platelet heterogeneity within thrombi

It has been established in earlier work from our laboratory that the platelets that are incorporated in a thrombus can undergo different types of responses, and thereby show different phenotypes.¹ Accordingly, the composition of an average thrombus is quite heterogeneous, containing multiple platelet phenotypes (Figure 1). How individual platelets decide which phenotype to adapt is largely unknown. The immediate environment of a platelet is considered to be important, but also the composition of a

platelet may contribute to the heterogeneity.^{33,34} It can be hypothesized that the content of cytoplasm and membranes that is inherited from a given precursor megakaryocyte predispose platelets to assume a certain phenotype.

Phenotypes that can be distinguished are adherent platelets – platelets that directly interact with the damaged vessel wall – and aggregating platelets, which in particular interact with each other. The majority of these aggregating platelets, which form the bulk of the thrombus, show high activation of integrin $\alpha_{IIb}\beta_3$, required for platelet aggregation and show significant levels of secretion.^{33,35} Upon strong activation with sustained, high intracellular Ca^{2+} levels, collagen-interacting platelets assume a different, procoagulant phenotype.^{35,36} The procoagulant platelets are characterized by the presence of negatively charged phosphatidylserine at the outer leaflet of the plasma membrane.³⁷ Phosphatidylserine exposure is mediated by the ion channel and phospholipid scramblase anoctamin-6 (previously designated as TMEM16F), causing transbilayer movement of phospholipids over the membrane.^{38,39} The exposed phosphatidylserine supports assembly of the coagulation complexes, tenase and prothrombinase, and thereby catalyzes the formation of thrombin.^{35,40}

Tissue factor that comes into contact with blood upon vascular damage triggers the extrinsic pathway of coagulation, by forming a complex with coagulation factor VII(a) and factor X.⁴¹ Once initial traces of factor Xa and subsequently thrombin are formed, these cleave other coagulation factors, including factor V. In the prothrombinase complexes at the surface of procoagulant platelets, the formed factor Xa assembles with factor Va, which then strongly stimulates the conversion of prothrombin to thrombin.^{42,43} The local generation of thrombin enhances platelet activation, and also converts fibrinogen to fibrin.³⁴ The so-called coated platelets form a specific subset of the procoagulant platelets.⁴⁴ These coated platelets require co-activation with thrombin, and show high levels of fibrin(ogen) binding, despite integrin $\alpha_{IIb}\beta_3$ closure and contain high activity of the transglutaminase factor XIIIa, which assists in platelet-dependent fibrin fiber formation.⁴⁵ Consequently, a fibrin network is formed which extends from the thrombus. *In vivo* models of thrombosis have shown that the fibrin network is distributed in and around a thrombus in a non-homogeneous way.^{46,47} In spite of this knowledge, it is still unclear how the platelet activation state and local shear stress determine the spatial distribution of fibrin.

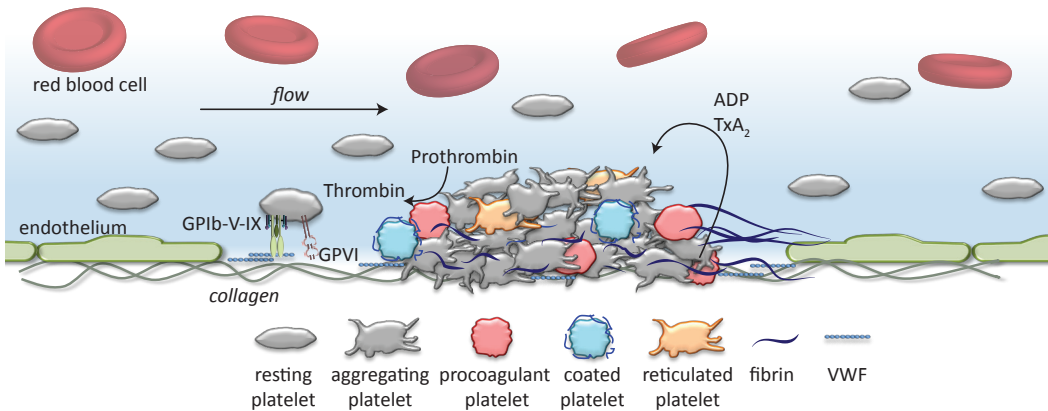


Figure 1: An overview of platelet heterogeneity in thrombus formation. Detailed explanation of the roles of different platelet agonists, receptors and platelet subtypes is given in the text.

Platelets and thrombosis

The most common cause of arterial thrombosis is rupture or erosion of an atherosclerotic plaque.³ For example, in the coronary arteries this results in partial or full occlusion, with myocardial infarction as a consequence. Patients with obstructed coronary arteries are often treated by percutaneous coronary intervention, followed by stent placement. In order to prevent recurrent ischemic events, *e.g.* due to in-stent thrombosis, patients receive dual antiplatelet therapy.⁴⁸ The therapeutic regimen consists of a daily intake of a P2Y₁₂ inhibitor for the period of one year, in combination with aspirin that is recommended to be taken life-long.⁴⁹ The P2Y₁₂ inhibitors that are currently prescribed include the irreversible inhibitors, clopidogrel or prasugrel, or alternatively the reversible inhibitor, ticagrelor.²³ The irreversible inhibitors form an active metabolite in the liver which, once released into the blood stream, specifically binds and inactivates the platelet P2Y₁₂ receptors.⁵⁰ Aspirin, on the other hand, irreversibly abolishes the synthesis of thromboxane A₂, and thereby blocks the reinforcement of platelet response by this autacoid.⁵¹ An important drawback of the dual inhibition of both ADP/P2Y₁₂ and thromboxane A₂ responses is a significantly increased risk of bleeding.²³ Since after one year of treatment, the beneficial antithrombotic effect equalizes the negative effect of bleeding, it is advised to stop administration of the P2Y₁₂ inhibitors at this time point. There are indications that, during the offset period, patients are at increased risk of a recurrent ischemic event.⁵²⁻⁵⁵ Speculations are that this is possibly a consequence of hyperreactivity of the platelets, which are formed after treatment cessation, but this has not been studied in detail.

Bleeding

Under conditions where platelet or coagulation activation is impaired, hemostatic clot formation becomes hampered, leading to an increased risk of bleeding. As of today, the most common treatment option to prevent or treat hemorrhages is the transfusion of blood products.⁵⁶ In the Netherlands, platelet transfusion is commonly performed

with concentrates consisting of platelets pooled from five donors, suspended in plasma to which 'PAS' (platelet-additive solution) medium can be added. These pooled platelet concentrates can be stored for up to 7 days.⁵⁷ Although platelet transfusions temporarily increases the platelet count and hence improves hemostasis, this treatment also has potential risks, such as allergic reactions, infectious diseases and, incidentally, acute lung injury.⁵⁶

Currently, a substantial part of platelet concentrates is administered to patients, who have been diagnosed with a hematological malignancy and who were therefore treated with chemotherapy.⁵⁸ As a consequence of disease and foremost chemotherapeutic treatment, these patients develop severe thrombocytopenia.⁵⁸ Normal platelet count ranges from 150-400 x 10⁹ plts/L, but severely thrombocytopenic patients have a platelet count lower than 50 x 10⁹ plts/L. Besides a low count, it has been reported that the platelets of these patients before onset of chemotherapy can show impairments in P-selectin expression and thromboxane B₂ production, which might explain an increased bleeding risk.^{59,60} However, the severity and etiology of such a platelet dysfunction is unclear.

Another clinical condition in which bleeding is common is that of dilutional coagulopathy. As a consequence of major blood loss due to trauma or surgery (*e.g.* the cardiopulmonary bypass procedure) patients will receive fluid infusion (crystalloids and colloids) to maintain normal fluid and electrolyte homeostasis.⁶¹ As a consequence of both the blood loss and the blood dilution, coagulation factors will become diluted and thrombocytopenia will occur. In order to prevent anemia and preserve hemostasis, transfusion protocols have been developed, in which packed red blood cells, plasma and coagulation factors, and platelet concentrates are given.^{62,63} However, as it is desired to reduce unnecessary use of blood products,⁶³ a better understanding of the individual and combined effects of these blood products is necessary.

Experimental models for thrombosis and hemostasis

To understand the roles of different platelet and coagulation proteins in thrombosis and bleeding, genetic mouse models have been developed, in which a specific platelet or coagulation protein is missing or defective.⁶⁴ Experimentally, arterial damage can be induced in these mice to trigger thrombus formation,⁶⁵ and the tail tip can be cut to monitor the bleeding phenotype.⁶⁴ Taking into account specific differences between the human and mouse hemostatic systems, these genetic mouse models have provided useful insight into the process of thrombus formation, and guided in the development of new antiplatelet and anticoagulant therapies.⁶⁶ As a way to reduce and refine such animal experiments, *in vitro* studies can be performed with isolated whole blood and microfluidic flow chambers.^{67,68} From a translational point of view, using such flow chambers, it is possible to study thrombus formation on multiple thrombogenic surfaces in the absence or presence of coagulation with either mouse or human blood samples.^{26,68}

By systematically comparing the consequences of a genetic deficiency in various *in vivo* and *in vitro* approaches, a broader understanding of the roles of the individual genes in thrombosis and hemostasis can be gained.

Aims and outline of this thesis

The chapters of this thesis have as a common aim to better understand the consequences of an acquired impairment or recovery of platelet functions. In *Chapter 1*, a general introduction is presented in order to provide relevant background information on platelet physiology and mechanisms of platelet activation during thrombus formation. *Chapter 2* provides a new concept regarding the formation of different platelet populations in a physiological environment, and extends this to altered platelet phenotypes in a pathological environment. *Chapter 3* describes how newly formed, juvenile platelets contribute to a recovery of function in patients, who stopped intake of the P2Y₁₂ receptor inhibitor prasugrel at one year after myocardial infarction. This chapter furthermore presents a novel methodology to identify the juvenile platelets, such in comparison to established methods. *Chapter 4* focuses on the dynamic processes of platelet adhesion, aggregation and fibrin clot formation under flow conditions. It is examined how immobilized collagen and tissue factor steer the formation of different types of thrombi with a specific micro-elastic structure. Thrombus phenotype and architecture are also characterized in *Chapter 5*. Here, a new feature of the population of procoagulant platelets is examined, namely the loss of glycoprotein Iba and glycoprotein VI. It is studied how extracellular shedding of these receptors, mediated by the proteases ADAM10 and 17, affects the platelet coagulant activity. This chapter also identifies the main signaling pathways, which regulate ADAM activity in activated platelets. The central theme of *Chapter 6* is identification and characterization of platelet dysfunction, which occurs in leukemia, multiple myeloma and lymphoma patients, who are treated with chemotherapy and develop thrombocytopenia as a consequence. This work focusses on the role of platelet mitochondria in relation to the dysfunction. How platelet transfusion alters the overall platelet responsiveness in these patients is studied as well. *Chapter 7* examines the means for recovery of coagulant activity in patients with dilutional coagulopathy. A central question is how the supplementation of platelets and fibrinogen can alter elastic clot formation and, hence, improve the acquired hemostatic dysfunction. *Chapter 8* presents a comprehensive literature synthesis, aiming to develop a method to systematically compare the consequences of genetic deficiencies in mice for arterial thrombosis and hemostasis. A scoring system is proposed allowing the calibration and comparison of multiple heterogeneous studies using different *in vivo* and *in vitro* models of arterial thrombus formation. These scores are used to construct a network of proteins in thrombosis and hemostasis, allowing the identification of new genes involved in these processes. In the last *Chapter 9*, the principal findings of this thesis are critically discussed in the light of the current literature.

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Chapter 2

Platelet populations and priming in hematological diseases

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Abstract

In healthy subjects and patients with hematological diseases, platelet populations can be distinguished with different response spectra in hemostatic and vascular processes. These populations partly overlap, and are less distinct than those of leukocytes. The platelet heterogeneity is linked to structural properties, and is enforced by inequalities in the environment. Contributing factors are variability between megakaryocytes, platelet ageing, and positive or negative priming of platelets during their time in circulation. Within a hemostatic plug or thrombus, platelet heterogeneity is enhanced by unequal exposure to agonists, with populations of contracted platelets in the thrombus core, discoid platelets at the thrombus surface, patches of ballooned and procoagulant platelets forming thrombin, and coated platelets binding fibrin. Several pathophysiological hematological conditions can positively or negatively prime the responsiveness of platelet populations. As a consequence, *in vivo* and *in vitro* markers of platelet activation can differ in thrombotic and hematological disorders.

1. Introduction

Individual platelets interact in numerous ways with the vessel wall or adherent blood cells. This versatility is fundamental to the role of platelets in a wide range of (patho)physiological processes, ranging from vascular repair, hemostasis and thrombosis, to inflammation progression, innate immunity and tumor metastasis. In the past years, evidence has been accumulating that circulating platelets are markedly heterogeneous in properties, which has led to the suggestion that identifiable populations of platelets with specialized response spectra are best suited for specific roles. In the present paper, we resume the current evidence for heterogeneity in terms of composition and functions of platelets during their formation from megakaryocytes, when circulating over time in the circulation, and once adhered to a vessel wall. We further describe how various physiological and pathophysiological conditions can change or prime the responsiveness of circulating platelets, and hence alter the distribution of platelet populations. We finally define how *in vitro* and *in vivo* markers of platelet activation phenotypes can be judged in relation to thrombotic and hematological disorders.

2. Intrinsic factors of platelet heterogeneity

Platelets from a given subject, healthy or diseased, greatly vary in receptor expression levels and markedly diverge in responsiveness once activated. Different populations of activated platelets can be distinguished, which can differently interact with the inflamed or injured vessel wall and differently support hematological processes.

This heterogeneity is explained by several intrinsic factors, including variability of clonal megakaryocytes, unequal division of megakaryocyte-derived proplatelets, and modifications upon ageing of the newly formed platelets.

2.1. Heterogeneity between megakaryocytes and platelets

Heterogeneity between megakaryocytes, whether or not linked to a specific niche in the bone marrow or lungs, is a likely cause of inter-platelet variability, although there is only limited literature available on this subject. Several authors have described that polyploid megakaryocytes, either cultured from CD34⁺ hematopoietic stem cells or derived from immortalized cell lines, considerably differ from cell to cell, in terms of levels of cytoplasmic and membrane proteins, as well as in agonist-induced Ca²⁺ transients.¹⁻³ Recently, this was demonstrated also for megakaryocytes derived from single-cell clones of forward-programmed human pluripotent stem cells. After re-programming, individual

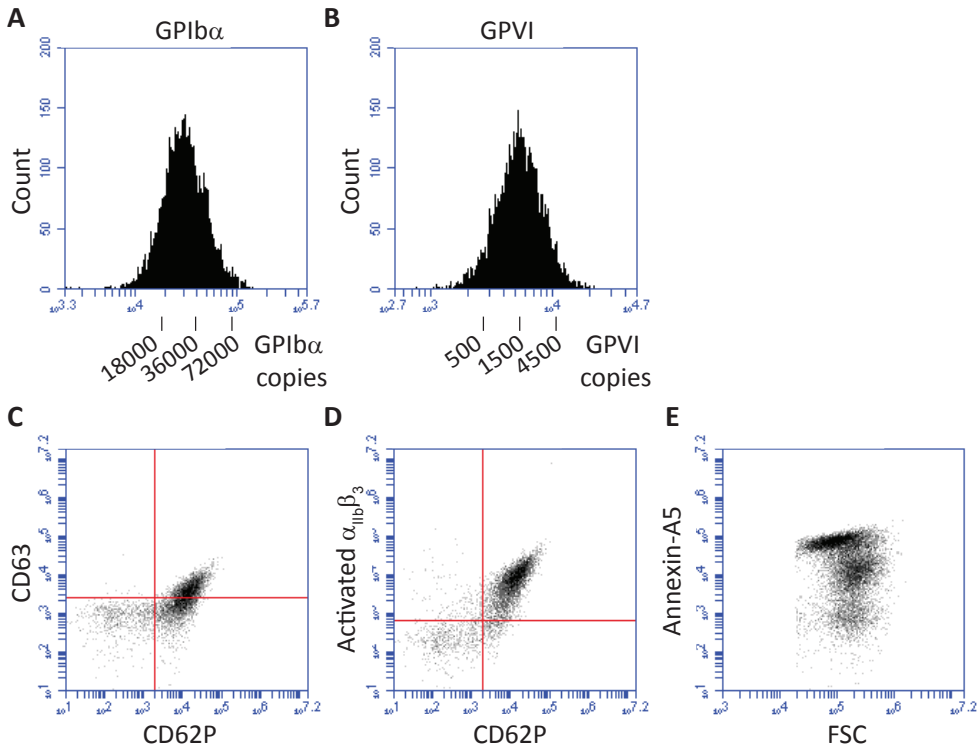


Figure 1. Intrinsic heterogeneity in platelet composition and functions. (A, B) Differential expression of key adhesive receptors in the total platelet population, immunologically stained for GPIb α or GPVI. Indicated in the flow cytometric histograms are the estimated copy numbers, based on proteome analysis.¹²⁸ (C, D) Platelets stimulated with a GPVI agonist immunologically stained for CD62P in combination with CD63; or CD62P in combination with a marker for activated integrin $\alpha_{IIb}\beta_3$. Shown is the presence of different platelet populations with alpha granule secretion (CD62P⁺) with or without dense granule secretion or $\alpha_{IIb}\beta_3$ activation. (E) Two apoptotic platelet populations after stimulation with BH3 mimetic ABT-737, displaying low or high phosphatidylserine (PS) exposure.

megakaryocytes of the same clone showed a 100-fold difference in expression levels of common receptor proteins, such as glycoprotein (GP)Ib α , GPIIb/IIIa and integrin $\alpha_{IIb}\beta_3$, as assessed by flow cytometry.⁴ No studies are yet available to demonstrate whether distinct megakaryocytes (in the bone marrow) also yield different types of platelets. However, one can expect that single platelets ‘inherit’ at least in part their expression profiles of receptors and other signaling molecules from the precursor megakaryocyte. It has been shown that when the environment of megakaryocytes changes (*e.g.* upon inflammation or diabetes), the transcriptome of platelets is influenced as well.^{5,6} For instance, patients diagnosed with the autoimmune disease systemic lupus erythematosus (SLE) may have an altered platelet transcriptome. The changes in platelet mRNA and protein levels were linked to increased procoagulant activity and platelet-monocyte interactions in SLE patients.⁷

Proplatelets are formed from the demarcation compartment of megakaryocytes by pinching off the cytosol containing cell membrane compartment and organelles.⁸ Although studies are lacking, it can be conceived that part of the heterogeneity between platelets may come from unequal retention or re-distribution of the cellular components from the mother cell, including surface receptors, actin and tubulin cytoskeletons, signalosomes, internal membrane vesicles, secretory granules, ribosomes, and mitochondria.

Clear evidence for heterogeneity in platelet size and volume comes from flow cytometric profiles and blood count histograms. Forward side scatter plots (flow cytometry) point to a considerable variation in size, which is in agreement with the relative large distribution width of the platelet volume (blood cell count). Sizable heterogeneity between platelets is also detectable by quantifying the abundantly expressed receptors. Thus, flow cytometric profiles of platelets that are stained with fluorescent antibodies against integrin β_3 (CD61) point to a large inter-platelet variation in expression levels of this integrin.^{9,10} Similarly, staining for GPIIb/IIIa or GPIIb/IIIa gives histograms, which show an about 10-fold difference in fluorescence intensity (indicative of expression level) of platelets in the lower and upper 10% percentiles (Figure 1A-B). Evidence for signaling heterogeneity comes from recordings of the Ca²⁺ responses of single platelets from an arbitrary donor, which appear to vary greatly in the type of transient Ca²⁺ fluxes after stimulation via ADP, thrombin or collagen receptors.¹¹

2.2. Populations of activated platelets

Platelets, upon activation by agonists, are known to form populations with different surface properties.¹⁰ For instance, platelets stimulated with a maximal dose of a GPIIb/IIIa agonist showing CD62P expression (marker of α -granule secretion) only display CD63 expression to a certain degree (marker of δ -granule and lysosome release), while the majority of platelets that express CD62P do have activated integrin $\alpha_{IIb}\beta_3$ (Figure 1C-D). Inter-platelet heterogeneity in granule release has also been observed by electron microscopy.¹² Thus, secretion of granule content can occur in two different ways: single granule secretion (both α and δ granules), or secretion of multiple granules fused into one large compartment (mainly α granules).¹² Dependent on the strength of an activation trigger, secretion of the granule content appears to be regulated differently and can

result in different secretion patterns (Figure 1C-D).^{12, 13} Another consistent observation is that, regardless of the agonist used, a fraction of 10-20% of the platelets fails to stain for CD62P,¹⁴ suggesting that some platelets are refractory to α -granule secretion.

A striking heterogeneity is observed when platelets in suspension are stimulated with combinations of collagen and thrombin receptor agonists. In addition to a population showing integrin $\alpha_{IIb}\beta_3$ activation and granule secretion, another population of swollen platelets appears with exposed phosphatidylserine at their outer membrane surface, inactivated/closed integrins, and capability of coagulation factor binding.^{15, 16} Depending on the activating conditions, the majority of the latter platelets also have a transglutaminase-dependent fibrin coat at their surface,¹⁷ which is in agreement with the early description of coated or COAT platelets.¹⁸ As described below, the various platelet populations are considered to have different roles, in supporting platelet aggregation, procoagulant activity and fibrin formation. The formation of phosphatidylserine-exposing, procoagulant platelets is linked to a prolonged, high cytosolic Ca^{2+} level^{19, 20} *i.e.* a condition required for swelling and phospholipid scrambling via the Ca^{2+} -dependent anoctamin-6 channel (gene *ANO6* or *TMEM16F*).^{21, 22} It can be speculated that, in single platelets, differences in both receptor expression levels and in activity of the Ca^{2+} -flux machinery determine the formation of the platelet populations.

Populations of platelets with different surface properties are also formed after stimulation with the proapoptotic BH3 mimetic ABT-737.²³ Typically, platelets with low and high phosphatidylserine exposure are formed in this case (Figure 1E).

2.3. Changes with platelet maturation, ageing and apoptosis

Early studies supposed that platelet activity correlates with platelet size and, hence, receptor and granule numbers.^{24, 25} Later papers have focused on platelet age, considering that newly formed platelets are larger in size and therefore more active than older platelets.^{26, 27} However, there is still not much support for the idea that platelet size is the only or main factor determining platelet responsiveness to agonists. Association studies do not suggest such a relation. For instance, in a cohort of healthy individuals, mean platelet volume negatively correlated with the amount of newly formed platelets, suggesting that young platelets are not necessarily the largest ones.²⁸

A consistent finding is that newly formed platelets are enriched in mRNA, as observed by specific mRNA-staining and cell counting analyses.²⁹ Young platelets with detectable mRNA in the cytoplasm have been termed as reticulated platelets^{30, 31} or immature platelet fraction.³² The latter fraction, with a normal reference range of 1-5%, is considered to reflect the rate of platelet production in the bone marrow. Given that platelets have overall lost >90% of the mRNA of their progenitor cells - while retaining the stable circular RNAs -,³³ it is clear that the residual mRNA levels in reticulated platelets reflect incomplete mRNA maturation. Interestingly, subject age does not seem to be an important modifier of reticulated platelets, as in older subjects only ~2% of all >6000 identified mRNA and miRNA species were found to be changed in expression levels.³⁴ On the other hand, a Spanish hemophilia study showed that the reticulated platelet fraction has a high heritability component.³⁵ Recent papers describe that certain combinations of mRNA and miRNA correlate with platelet phenotype,

raising the possibility to use these RNA species as biomarkers of platelet function.^{36, 37}

With a circulation time of 7-10 days and a remarkably constant platelet count over time, the daily turnover (production and removal) of platelets is extremely well regulated, being estimated at about 10^{11} platelets per day.^{8, 38} Platelet removal from the circulation occurs via both clearance and “consumption” (at the enormous vascular bed in the human body). Clearance can be triggered by prior platelet activation and an apoptotic process, *e.g.* resulting in the surface exposure of PS,³⁹ or by other still unknown causes.

Evidence for a link between platelet ageing and apoptosis comes from experiments with mice, lacking the pro-apoptotic proteins Bax/Bak, in which platelet survival is greatly extended.⁴⁰ Because aged, Bak-deficient platelets showed a reduced capability of thrombin- and collagen-induced secretion, these were designated as exhausted platelets.⁴¹ On the other hand, in mice lacking the anti-apoptotic protein Bcl-xL, platelet lifetime was shortened and platelet function was also reduced.⁴⁰ The interpretation was that a loss of Bcl-xL leads to activation of Bax/Bak to induce a pro-apoptotic condition. In patients with malignancies, who were treated with the Bcl-xL-degrading drug Navitoclax (a BH-3 mimetic also known as ABT-263), platelet lifetime was also shortened, thus resulting in mild thrombocytopenia.³⁹ Characteristics of apoptotic platelets are mitochondrial dysfunction, caspase proteolytic activity, refractoriness to integrin activation, and surface exposure of phosphatidylserine.^{42, 43}

Another finding was that aged platelets gradually lose sialic acid residues from their surface membrane glycoproteins.³⁸ Desialylated platelets were found to be recognized by Ashwell-Morell receptors in the liver, mediating platelet clearance and, interestingly, also mediating release of thrombopoietin, which is the main cytokine regulating platelet formation from megakaryocytes.⁴⁴ These data are suggestive for a gradual, ongoing formation of the population of (pro)apoptotic platelets.

3. Environmental factors of platelet heterogeneity

Platelets can interact with a large variety of glycoproteins in the inflamed or damaged vessel wall. The adhesive interactions include: activated endothelial cells expressing von Willebrand factor (VWF); subendothelial matrix components such as laminins, collagens, thrombospondins; adhered plasma proteins, such as VWF, fibrin(ogen), fibronectin and vitronectin; leukocytes under inflammatory conditions; lymphatic wall components like podoplanin; tumor cells; and other platelets via fibrinogen bridges. Given that these adhesive ligands all induce different signaling pathways, the response of an adhered platelet will vary with the precise location and substrate of adhesion. Well studied is the environmental directed heterogeneity in responses of platelets adhering to collagen and assembling into a thrombus.^{10, 16} The formation of platelet populations in thrombus formation is further stimulated by the local generation of thrombin.^{45, 46}

3.1. Platelet heterogeneity upon adhesion and activation

Platelets will respond differently, when interacting with specific vascular wall-derived proteins. This response heterogeneity is studied most extensively for platelets adhering to fibrillar collagen via the receptors GPVI and integrin $\alpha_2\beta_1$.^{16, 47} Both receptors

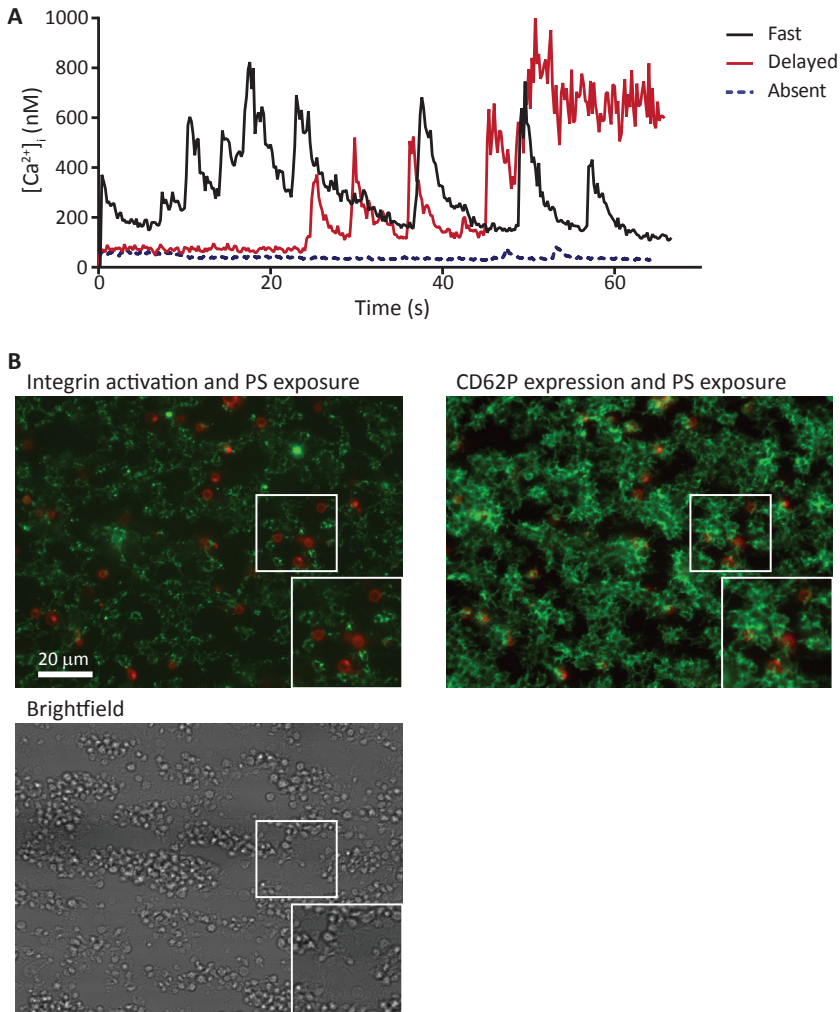


Figure 2. Heterogeneity of platelet responses. (A) Distinct calcium fluxes of single platelets adhered under flow to collagen. Based on the time between adhesion and increase in calcium signal, platelets were classified as having a fast, delayed or absent activation response. (B) Platelet populations in thrombi formed on collagen: aggregating platelets characterized by integrin activation and CD62P expression (green) and procoagulant platelets forming blebs/balloons and exposing PS (red).

act in synergy to immobilize and thereby activate platelets via the GPVI signalosome, resulting in Ca²⁺ elevation, integrin activation and granular secretion.^{48, 49} Collagen fibrils allowing platelet adhesion to both receptors are most strongly activating, and form the largest platelet aggregates. A population of the collagen-adhered platelets – subjected to the highest Ca²⁺ fluxes –, responds by surface exposure of procoagulant phosphatidylserine.⁵⁰ This so-called procoagulant response is accomplished via the ion channel anoctamin-6,²² and accompanied by marked morphological changes involving

swelling, bleb and balloon formation.^{51, 52} Phosphatidylserine exposure facilitates the binding of multiple coagulation factors and promotes thrombin generation.^{19, 20} Remarkably, the majority of collagen-bound platelets is refrained from phosphatidylserine exposure and swelling.

3.2. Platelet heterogeneity within a thrombus

Heterogeneity in platelet activation properties progresses during collagen-dependent thrombus formation in flowing blood, both *in vitro* and *in vivo*. At high, arterial wall-shear rates, platelet adhesion to collagen/VWF is accomplished via GPVI, integrin $\alpha_2\beta_1$ and the VWF receptor, GPIb-V-IX.^{53, 54} Single-cell studies of adhered platelets from flowing blood again show marked differences in Ca^{2+} fluxes (Figure 2A).⁵⁵ Thrombi formed under high-shear flow show clusters of aggregated platelets (staining for activated integrin $\alpha_{IIb}\beta_3$ and P-selectin), alternated with patches of ballooned phosphatidylserine-exposing platelets (binding annexin A5 and coagulation factors) (Figure 2B).^{56, 57} The population of aggregated platelets also undergoes contraction.^{58, 59} It is not quite clear how platelets 'decide' to either aggregate or expose phosphatidylserine. Given that adjacent platelets can form gap junctions,⁶⁰ and communicate with each other,⁶¹ it is possible that cross-communication in the aggregate protects against anoctamin-6-dependent procoagulant activity. In a study where platelet adhesion to multiple surfaces was examined, the response heterogeneity in thrombus formation with different populations of platelets formed was a constant finding, regardless of the surface type and adhesive receptors involved (GPIb-V-IX, GPVI, CLEC-2, $\alpha_6\beta_1$, $\alpha_{IIb}\beta_3$ or CD36).⁶²

Recent *in vivo* studies with mice, where laser-induced injury was applied to cremaster or femoral arterioles, have pointed to another level of platelet heterogeneity within thrombi, *i.e.* an inner core of aggregated and contracted platelets, surrounded by an outer shell of loosely adhered platelets.^{63, 64} Two specific platelet populations were distinguished, *i.e.* platelets in the thrombus core having undergone secretion and CD62P expression, and platelets in the outer shell without secretion.^{63, 64} Several factors may contribute to the heterogeneous thrombus buildup, including local levels of soluble platelet agonists (ADP, thromboxane A_2) and the local wall shear gradients.⁶⁵ Also, this type of platelet heterogeneity in thrombi is likely to be influenced by the type of injury, vascular bed location and the physiological or pathological state of the vessel.

3.3. Coagulation and thrombus heterogeneity

Under conditions favoring coagulation, *e.g.* with tissue factor present, the formation of thrombin and fibrin in a thrombus appear to be highly localized processes. In mouse microvascular thrombosis models, thrombin activity and fibrin deposits appeared to be concentrated at the thrombus base, *i.e.* close to the vascular localization of tissue factor.⁶⁶ *In vitro* models of thrombus formation showed that the population of phosphatidylserine-exposing platelets was responsible for the binding of coagulation factors (prothrombin, factors Va, IXa and Xa), and also for the formation of thrombin and fibrin.^{46, 57} A potent feedback loop was identified, in that the local formation of thrombin enhanced thrombin-mediated phosphatidylserine exposure of nearby platelets, thus increasing the population of procoagulant platelets.⁴⁶ Fibrin formation appeared to be

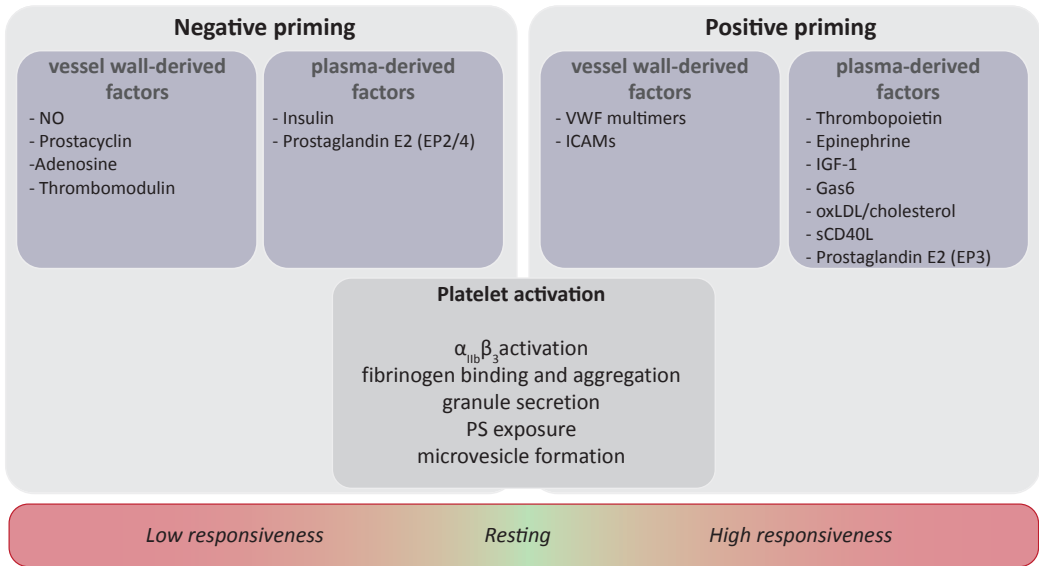


Figure 3. Concept of negative and positive priming of platelets. The indicated factors can cause negative or positive priming of platelet activation processes, either plasma-derived or from the vessel wall.

initially linked to PS exposure, and secondarily dependent on integrin $\alpha_{IIb}\beta_3$ activation and transglutaminase-dependent fibrin cross-linking.^{17, 67} *In vitro* models further indicated that fibrin preferentially localized near the sites of tissue factor and on procoagulant (coated) platelets.⁶⁸ Other identified elements of heterogeneity in a thrombus are the local secretion of polyphosphate clusters (activating factor XII)⁶⁹, and the phosphatidylserine-dependent accumulation of plasmin (activating the fibrinolytic system).⁷⁰ Taken together, during thrombus formation, the coagulation process appears to enhance the formation of distinct platelet populations, and increase the heterogeneity in platelet functions.

4. Positive and negative priming of platelets

Multiple bioactive molecules in the blood can enhance or suppress platelet functionality. In psychological and neurobiological sciences, the implicit memory effect by which exposure to the first stimulus influences the response to the next stimulus, is called 'priming'.⁷¹ By analogy, we can consider the exposure of platelets to enhancing or suppressing agents as positive or negative priming events. Figure 3 gives an illustration of potential priming biomolecules that are either derived from the vascular wall or found in plasma.

4.1. Positive priming: key roles of plasma derived factors

Several hormones enhance platelet activation processes (shape change, secretion, aggregation), when applied in combination with other agonists. Epinephrine, binding to the Gi-coupled α_2 -adrenergic receptor, reduces intracellular cyclic AMP levels which,

e.g. enhances Ca^{2+} fluxes raised by agonists like ADP and thrombin.^{16, 72} Thrombopoietin, a growth factor for megakaryocytes, also sensitizes platelets to agonists at threshold concentrations,^{73, 74} and at shear stress.⁷⁵ Similarly, insulin-like growth factor-1 (IGF1) greatly enhances platelet activation provided that other agonists are present.⁷⁶ *In vitro* studies indicate that positive priming of platelets with thrombopoietin or insulin-like growth factor-1 can overcome the inhibitory effects on platelet function by aspirin or P2Y₁₂ receptor treatment.⁷⁷ This suggests that positive priming of platelets can be a mechanism of resistance to antiplatelet therapy.

Other positive priming molecules are Gas6 (plasma-derived) and soluble CD40L (platelet-derived), both of which stabilize and extend platelet aggregation.^{78, 79} TNF α , a pro-inflammatory cytokine which is synthesized in high amounts during inflammatory conditions, is capable of inducing platelet activation mainly indirectly via endothelial activation,⁸⁰ although some authors report an enhancing effect of TNF α on collagen-induced aggregation.⁸¹

Besides the positive enforcement of such plasma molecules on platelet function, wall shear stress is of great influence as well. Rapid changes in laminar blood flow and shear rate, which occur at sites of stenosis, promote platelet aggregate formation.^{58, 65, 82} This wall-shear effect is highly dependent on VWF activity and autocrine platelet stimulation.⁶⁵ As such, disturbances in blood flow can be considered as a positive priming condition.

Several dietary nutrients are known to positively modulate platelet responsiveness. In addition, gut microbes may contribute to platelet hyperreactivity via the generation of trimethylamine N-oxide (TMAO) from dietary sources of trimethylamine, present in a western diet. The generated TMAO is proposed to prime platelet activation by enhancing Ca^{2+} release from intracellular stores.⁸³

4.2. Negative priming: key roles of endothelial mediators

Not all platelets are freely circulating, as part of the platelets are kept in the spleen and lungs.^{84, 85} In mice, the lungs also significantly contribute to platelet biogenesis.⁸⁶ Whether this also applies to the human situation, remains to be explored. Clearly, these pools of marginated platelets in the lungs and spleen are exposed to different environments. Unclear is at which rate and to what extent the platelet pools exchange, as well as how these different locations alter the activation properties. Adaptions in properties can also arise when platelets are flowing through the micro- and macro-vascular beds lined with specialized endothelial cells.

At least three endothelial-derived substances – likely there are more – are accountable for the major negative priming effect of the vessel wall on platelet activation. These are prostacyclin, nitric oxide and CD39/CD73.⁸⁷ Prostacyclin (prostaglandin I₂), a short-lived eicosanoid produced by endothelial cells, binds to the platelet IP receptor, which couples to the Gs GTP-binding protein, stimulates adenylate cyclase and thereby elevates the inhibitory second messenger cyclic AMP.⁸⁸ Continuous exposure of circulating platelets to prostacyclin leads to cyclic AMP elevation and thereby platelet inhibition. In freshly isolated platelets, supra-basal levels of cyclic AMP have indeed been measured.⁷² However, IP receptors can desensitize and the functional effects of prostacyclin on

platelets may be most prominent locally, *i.e.* after platelet adhesion.⁸⁷ Prostaglandin E₂ (EP receptor agonist), also has an inhibitory though more complex action mechanism.⁸⁸ The effect of PGE₂ depends on the receptor isotype that is activated. Activation of EP3 isoforms by PGE₂ leads to sensitization of platelets, while binding of PGE₂ to EP2 or EP4 receptors causes platelet inhibition. It has been proposed that specifically the blocking of EP3 could be beneficial for the prevention of atherothrombosis.⁸⁹

The unstable free radical nitric oxide is membrane-permeable, and interacts with intracellular guanylate cyclase, raising the inhibitory second messenger cyclic GMP in platelets.⁹⁰ Nitric oxide is produced by endothelial nitric oxide synthase isoforms in response to the pulsatile flow of blood. Together, elevated levels of cyclic AMP and cyclic GMP accomplish strong platelet inhibition via a flood of protein phosphorylation reactions, mediated by the protein kinases A and G, respectively.^{91, 92} The levels of both cyclic nucleotides are balanced via cyclic nucleotide-dependent phosphodiesterases. The hormone insulin can also affect platelet reactivity via cyclic AMP and GMP elevation. In diabetic patients, who are resistant to insulin, a loss of these inhibitory pathways might contribute to platelet hyperreactivity.^{93, 94}

The endothelial-expressed ecto-nucleotidases CD39 and CD73 degrade intravascular ATP/ADP and AMP, respectively, to ultimately produce adenosine.⁸⁷ These activities convert potent platelet-activating autacoids (ATP, ADP) into a platelet inhibitor (adenosine, activating Gs).⁸⁷ Both mouse and human studies point towards a modulating role of ecto-nucleotidases in thrombus formation and thrombosis. In addition to the inhibitory A₂-adenosine receptor, platelets possess receptors linked to protein-tyrosine phosphatases, which also suppress activation processes.⁹⁵ The physiological ligands of these are mostly unknown. Indirectly, also the thrombin-inactivating activity of endothelial thrombomodulin contributes to platelet suppression, *i.e.* by preventing thrombin-induced activation.⁹⁶ Some nutritional components can contribute to negative priming, *e.g.* a mild to moderate alcohol consumption may lower platelet reactivity by inhibiting thromboxane formation.⁹⁷

Given that circulating platelets are exposed to a plethora of positive and negative priming molecules, their net effect must be prevention of platelet adhesion and activation, unless positive stimuli prevail, such as at a damaged vessel wall. The implicit assumption is that a shift or disturbance in the balance of positive and negative priming molecules causes a change in the threshold of platelet activation. This could for example explain why the population of coated platelets increased in a variety of pathophysiological conditions.¹⁸

5. Pathology induced alterations in platelet populations

Considering that multiple bioactive mediators present in the blood or derived from the vessel wall influence the tendency of platelet activation, it can be expected that disease conditions associated with vascular abnormalities or blood changes enhance the positive priming of platelets. An increasing number of reports points to such increased priming events, often ascribed to the formation of different 'types' of platelets, especially in patients with high-burden inflammatory, prothrombotic or hematological diseases (Table 1). Albeit different terms are used to annotate the platelet changes, the majority of

Table 1: Platelet phenotypes and activation markers in hematological and other diseases, induced by negative or positive priming. Representative examples from the literature are given.

Clinical condition	Hemostatic balance	Type of plt	Plt activation markers <i>in vivo</i>	Platelet activation testing <i>in vitro</i>	Refs
Negatively primed (low responsive)					
Congenital defects: Scott syndrome	mild bleeding	procoagulant	n.d.	PS ↓ fibrin formation ↓	22,68
Hematological malignancies	bleeding	hypo-responsive	Plt count ↓ TPO ↑	αIIbβ3, CD62P, CD63, GPIb ↓	100, 101
Renal failure and uremia	bleeding (thrombosis)	dysfunctional	NO ↑	CD62P, Ca2+ ↓ PS, caspase ↑	102, 103
Positively primed (high responsive)					
Diabetes type 2	prothrombotic	angry	sCD62P, sCD40L, PF4, MPV ↑ NO ↓	GPIb, CD41, GPVI ↑ CD63, CD62P, CAT ↑	104, 105, 129, 130
Hypercholesterolemia	prothrombotic	hyper-reactive	PLT turnover, MPV ↑	LTA ↑	108, 109, 131- 133
Myeloproliferative neoplasms (ET, PCV)	prothrombotic	hyper-reactive	PLT count ↑	LTA, CD62P, CAT ↑	110, 111
Positively primed and secondarily inactive (low responsive)					
Cancers (solid tumors)	prothrombotic	hyper-reactive (exhausted)	sCD62P, PEV ↑ PLT count ↑	CD62P, CD63, GPIb ↑ TxB2 ↓	112- 115
Sepsis	thrombosis, bleeding	hyper-reactive/ exhausted	PLT count ↓ sCD62P, sTLT, PEV ↑	CD62P ↑ LTA, fibrinogen binding ↓ (severe sepsis)	116, 119
Stroke	bleeding	exhausted	sCD62P, βTG ↑ TxB2 (urine) ↑	CD62P, CD63 ↓ mepacrine ↓	120- 122
Major trauma (brain)	bleeding	hypo-responsive	PEV ↑ CD62P, PAC mAb ↑ (unstimulated)	LTA ↓	123, 124
Viral hemorrhagic fever	thrombosis (bleeding)	exhausted/ reticulated	sCD62P, sGPVI ↑ TPO, IPF, MPV ↑	αIIbβ3, CD62P, CD63 ↓	125, 126

Abbreviations: CAT, calibrated automated thrombin generation in PRP; ET, essential thrombocytopenia; IPF, immature platelet fraction; LTA, light transmission aggregometry; MPV, mean platelet volume; NO,

nitric oxide; PCV, polycythemia vera; PEV, platelet-derived extracellular vesicles; PF4, platelet factor 4; PLT, platelet; PS, phosphatidylserine; s, soluble; β -TG, β -thromboglobulin; s, soluble; TLT, TREM-like transcript-1; TPO, thrombopoietin.

papers refer to either a gain-of-function (high-responsive platelets) or a loss-of-function (low-responsive platelets). A still unresolved question is how these functional changes relate to the formation of specific platelet populations.

5.1. Negatively primed platelets in disease

The genetic makeup of a subject co-determines platelet responsiveness. Rare genetic modifications of over 25 different platelet proteins have been described that result in low-responsive platelets, and associate with bleeding disorders.^{98, 99} Loss-of-function mutations, often accompanied by thrombocytopenia, in particular concern genes of transcription factors, the actin-myosin and tubulin cytoskeletons, signaling proteins, and granule secretion-regulating proteins.^{98, 99} Genetic polymorphisms in these and other genes may also influence platelet responsiveness.

A clear example how a genetic deficiency can imbalance the formation of platelet populations is the Scott syndrome, a mild bleeding disorder. Herein, mutations in the *ANO6* gene result in the near absence of the procoagulant, phosphatidylserine-exposing platelet population due to inability of phospholipid scrambling,^{21, 22} resulting in a severely compromised fibrin formation.⁶⁸

In addition to genetics, there is accumulating evidence for acquired forms of low-responsive platelets. A primary loss-of-function is observed in patients with hematological malignancies and thrombocytopenia. For instance, platelets from patients with acute leukemia can be impaired in adhesion, aggregation and secretion. These functional defects are likely a consequence of dysfunctional megakaryocytopoiesis.^{100, 101}

In patients with renal failure and associated uremia, it is considered that impaired platelet adhesion to the vessel wall is the main cause of bleeding. Several indicators of low platelet responsiveness are described, including impaired Ca^{2+} fluxes, reduced secretion and low platelet aggregation.¹⁰² On the other hand, also impaired platelet adhesion to the vessel wall due to anemia may explain the hemorrhagic complications in these patients. A low hematocrit limits the margination of platelets towards the vessel wall, and might furthermore restrict the availability of red blood cell-derived ADP and the scavenging effect of hemoglobin on nitric oxide as a platelet inhibitor.¹⁰² Other papers report that renal failure increases the risk of thrombosis, e.g. by increasing the population of apoptotic, phosphatidylserine-exposing platelets.¹⁰³

5.2. Positively primed platelets in disease

Diabetes has often been associated with platelet hyperreactivity, *i.e.* enhanced platelet adhesion, activation, aggregation and platelet-dependent thrombin generation.¹⁰⁴ This increased responsiveness of platelets is explained by high blood-glucose levels, oxidative stress and increased vascular shear stress.¹⁰⁵ Under these hyperglycemic conditions, advanced glycation end products (AGEs) are generated, which

activate platelets via the CD36 pathway.¹⁰⁶ Further, activation of the receptor of AGEs (RAGE) by S100A8/A9 may lead to enhanced production of TPO, thereby increasing platelet production from megakaryocytes upon hyperglycemia.¹⁰⁷ In diabetic patients, antiplatelet treatment strategies were shown to be effective for the secondary prevention of ischemic complications.¹⁰⁴

Plasma cholesterol levels can positively prime platelets, such as manifested by the enhanced activity and larger size of platelets from patients with familial hypercholesterolemia, in whom total lipid and low-density lipoprotein levels are severely elevated. Accumulation of cholesterol in the platelet membrane enhances platelet aggregation by influencing the membrane structure and signaling via surface receptors.^{108, 109} Hypercholesterolemia is also linked to increased platelet production and turnover.¹⁰⁸ Similarly, oxidation of low-density lipoproteins in the intima of atherosclerotic lesions is considered to result in enhanced platelet activation.¹⁰⁸

In the myeloproliferative neoplasms, essential thrombocytosis and polycythemia vera, quantitative as well as qualitative platelet abnormalities have been identified. Particularly in patients carrying the *JAK2*^{V617F} mutation, for whom aspirin treatment is recommended, platelet responsiveness is increased (secretion, aggregation and procoagulant activity), which effect is in part associated with thromboembolic complications.^{110, 111}

Table 2. Markers of the different platelet populations. For abbreviations and explanation, see text and Table 1.

Platelet population	Markers
Reticulated	mRNA (immature platelet fraction)
Secretory	PF4, β TG release CD62P, CD63 expression TxB ₂ formation, serotonin
Aggregating	fibrinogen binding PAC1 mAb binding (activated $\alpha_{IIb}\beta_3$ integrin) platelet-leukocyte aggregate formation
Procoagulant	PS exposure, coagulation factor binding extracellular vesicle formation
Coated	PS exposure, fibrin binding, transglutaminase activity extracellular vesicle formation
Apoptotic	PS exposure, extracellular vesicle formation

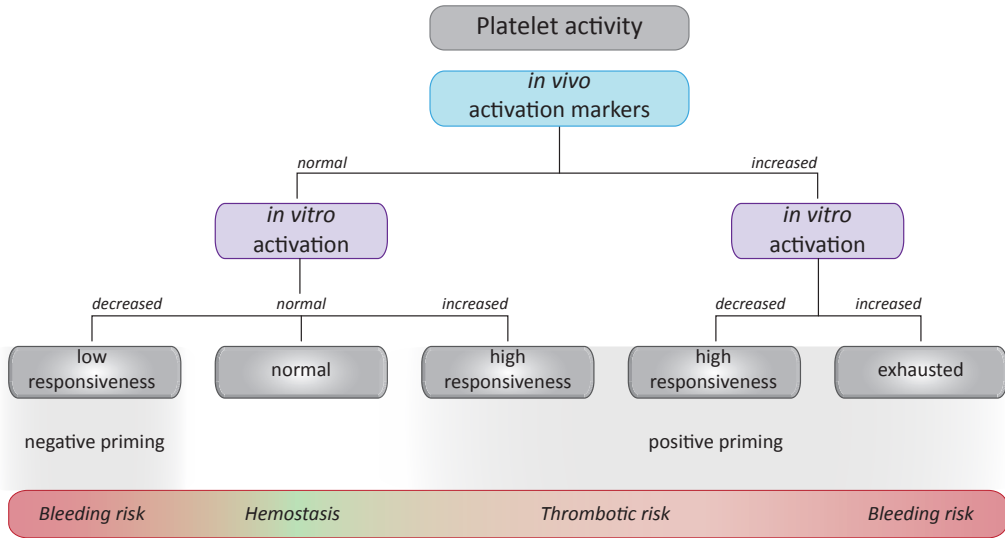


Figure 4. Concept of negative and positive platelet priming resulting in changed platelet activation markers *in vivo* and altered platelet activities *in vitro*. Flow chart for assessing changes in platelet functions in patients with thrombosis or bleeding risk.

5.3. Positively primed and secondarily inactivated platelets in disease

Despite the presence of positive priming molecules *in vivo*, platelet activation *in vitro* may appear to be impaired. This can be a direct consequence of prior activation in the circulation resulting in secondary loss of function. In such cases, authors describe the dysfunctional platelets often as exhausted platelets.

In patients with different types of solid tumors, apart from a high platelet count, platelet activation can be enhanced *in vivo*, as deduced from the presence of platelet-derived soluble (s)CD62P in the blood plasma.¹¹² This associates *in vitro* with a low-level of platelet activation, leading to the assumption that these platelets are 'exhausted', *i.e.* displaying a secondarily down-regulated responsiveness to agonists (CD62P, CD63 expression), although the primarily enhanced activation may predispose patients to an increased risk of thromboembolic complications.^{113, 114} Another marker of ongoing platelet activation in cancer patients is the accumulation of platelet-derived extracellular vesicles, interestingly, with almost no effect on mean platelet volume.¹¹⁵ Strikingly, in those patients a high mean platelet volume associates (though with low odds ratio) with a decreased risk of venous thromboembolism.¹¹³

In sepsis, as a life-threatening condition of systemic inflammation, platelets are positively primed as a result of endothelial damage or activation in combination with enhanced coagulant activity. This leads to increased plasma levels of platelet secretion products as well as platelet-derived extracellular vesicles.¹¹⁶ It has been demonstrated that several pathogens can interact with platelets via Toll-like receptors, FcγRIIIa and/

or integrin $\alpha_{IIb}\beta_3$, thus inducing platelet activation.¹¹⁶⁻¹¹⁸ In severe sepsis, also secondary defects in platelet function have been observed, often concomitantly with a lowering in platelet count.^{116, 119}

Papers studying patients who suffered a stroke, report increased levels in the circulation of platelet activation markers, including sCD62P, β -thromboglobulin and thromboxane B₂, in those patients with a high risk of bleeding.^{120, 121} Since this is accompanied by an impaired platelet activation tendency *in vitro*, the authors type such platelets as exhausted.¹²² A similar platelet phenotype is expected in patients with acute coronary syndrome before the initiation of antiplatelet therapy. An exhausted platelet type has also been observed after major trauma, in particular brain injury, where plasma markers of platelet activation are elevated, while the platelets after isolation are hypo-responsive in terms of aggregation.^{123, 124} The suggestion was made that sub-endothelial tissue exposure upon injury causes a low level of platelet activation, resulting in dysfunctional circulating platelets with an activation history.

Higher fractions (populations) of reticulated platelets have been detected in patients with viral hemorrhagic fever due to hantaviral disease, which was accompanied by increased levels of activation markers such as sCD62P and sGPVI, followed by a secondary loss in platelet activity *in vitro*.¹²⁵ A similar phenotype of exhausted platelets has been described for patients with a severe dengue infection.¹²⁶

It is currently unclear how the different pathologies induce specific changes in platelet function. The pathological environment may alter one specific platelet population or it rather affects all circulating platelets and thereby elicits a shift in the distribution of the different platelet populations. It can be reasonably presumed that this change is specific for the pathological environment. Furthermore, the impact of a pathological condition on platelet function may be multifactorial (*e.g.* diabetes) and therefore even more complex.

6. Platelet heterogeneity and risk assessment for bleeding and thrombosis: recommendations

For diagnosing a known or suspected risk of thrombosis or bleeding, current routine measurements include (i) platelet count and mean volume, (ii) plasma markers of *in vivo* platelet activation, and (iii) *in vitro* platelet activation tests. Particularly, the latter tests are used for the monitoring of anti-platelet therapy. Table 2 presents an outline of how different test outcomes relate to distinct platelet populations, provided that sample processing is up to standards.

The proposed concept of partly overlapping platelet populations that are subjected to positive/negative priming can shed new light on the often paradoxical information obtained from different diagnostic platelet function and activity tests. Figure 4 shows a flow chart aiming to structure the information obtained from such tests for a risk estimate of hemorrhage or thrombosis. From the figure, it will be clear that information on both activation markers *in vivo* and platelet responsiveness *in vitro* is needed to obtain a complete picture. It is noted though that platelets are deprived of endothelial inhibitors when performing *in vitro* measurements, which influences their

responsiveness. Assay measurements should preferably include markers of different platelet populations, given their specific contribution to hemostasis or thrombosis. Understanding of the redistribution of platelet populations in patients with particular hemorrhagic or thrombotic tendencies will also aid in designing an optimized treatment. One such an example is the administration of DDAVP to patients with a mild congenital platelet function defect which selectively augments the formation of coated platelets by enhancing $\text{Na}^+/\text{Ca}^{2+}$ mobilization.¹²⁷

Practice Points

- Platelet heterogeneity can originate from differences between megakaryocytes, variation in proplatelet formation, unequal platelet priming and environmental conditions, as well as platelet ageing. Changes in the physiological environment upon disease can hence influence platelet populations and platelet responses.
- Combining of both in vivo and in vitro markers of platelet activation and activity permits better assessment of a platelet-related thrombotic or hemorrhage risk.

Research agenda

- How the pathophysiological environment affects platelet populations and their responsiveness is incompletely understood and warrants further investigation.
- The causes and consequences of platelet heterogeneity have only partly been resolved. Characterization of the preferential response spectrum of certain platelet populations may contribute to risk factor assessments for prediction of thrombotic or hemorrhagic complications.

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Conflict of interest

The authors report no conflicts of interest.

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Chapter 3

Gradual increase in thrombogenicity of juvenile platelets formed upon offset of prasugrel medication

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Abstract

In patients with acute coronary syndrome, dual antiplatelet therapy with aspirin and a P2Y₁₂ inhibitor like prasugrel is prescribed for one year. Here, we investigated how the hemostatic function of platelets recovers after discontinuation of prasugrel treatment. Therefore, sixteen patients who suffered from ST-elevation myocardial infarction were investigated. Patients were treated with aspirin (100 mg/day, long-term) and stopped taking prasugrel (10 mg/day) after one year. Blood was collected at the last day of prasugrel intake and at 1, 2, 5, 12 and 30 days later. Platelet function in response to ADP was normalized between 5 and 30 days after treatment cessation and *in vitro* addition of the reversible P2Y₁₂ receptor antagonist ticagrelor fully suppressed the regained activation response. Discontinuation of prasugrel resulted in the formation of an emerging subpopulation of ADP-responsive platelets, exhibiting high expression of active integrin $\alpha_{\text{IIb}}\beta_3$. Two different mRNA probes, thiazole orange and the novel 5'Cy5-oligo-dT probe revealed that this subpopulation consisted of juvenile platelets, which progressively contributed to platelet aggregation and thrombus formation under flow. During offset, juvenile platelets were overall more reactive than older platelets. Interestingly, the responsiveness of both juvenile and older platelets increased in time, pointing towards a residual inhibitory effect of prasugrel on the megakaryocyte level. In conclusion, the gradual increase in thrombogenicity after cessation of prasugrel treatment is due the increased activity of juvenile platelets.

Introduction

The autocrine mediator ADP is a moderately strong platelet agonist, stimulating platelet aggregation and thrombus formation and contributing to thrombus stabilization.¹ At present, the ADP receptor P2Y₁₂ is one of the most effective targets for antiplatelet therapy, *i.e.* by clopidogrel, prasugrel, and ticagrelor.^{2, 3} Clopidogrel in combination with aspirin has been the standard therapy over the last decade to prevent recurrent atherothrombotic complications in patients who had a myocardial infarction.⁴ More recently, prescription of prasugrel or ticagrelor instead of clopidogrel is increasingly recommended following publication of the TRITON and PLATO studies, which demonstrated a net clinical benefit over clopidogrel on top of aspirin, due to a higher degree of platelet inhibition.⁴⁻⁹ The thienopyridines clopidogrel and prasugrel are both prodrugs, which require metabolic conversion to form an active metabolite that irreversibly interacts with the P2Y₁₂ receptors of circulating platelets.¹⁰ In contrast, ticagrelor is a reversible, non-competitive P2Y₁₂ antagonist – belonging to the cytopentyl-triazolo class of pyrimidines – interacting with the platelet receptors without metabolic conversion.¹⁰⁻¹²

Due to the irreversible inhibition of the platelet P2Y₁₂ receptors by the active metabolites of clopidogrel and prasugrel, the formation of new platelets is required to recover platelet function.^{7, 9} After ticagrelor treatment, recovery of platelet function is only determined by the elimination time of the drug (half-life of 8 hours).¹⁰ There is limited evidence that during the offset period of irreversible P2Y₁₂ inhibitors (usually

prescribed for one year) patients may have an increased risk of a recurrent myocardial event.¹³⁻¹⁷ This may point to hyperactivity of the newly formed platelets in the offset period, although the mechanism is unclear.

Previous experiments with rats have indicated that stopping clopidogrel treatment resulted in the sudden appearance of a population of fully responsive platelets, whereas recovery from ticagrelor treatment led to a more gradual regain in function of all platelets.¹⁸ The newly formed, juvenile platelets with active P2Y₁₂ receptors seemed to preferentially incorporate into thrombi generated under flow conditions.¹⁸

Juvenile platelets, also described as reticulated platelets because of the presence of reticular-bound mRNA, are those platelets that are shed most recently from the megakaryocytes in the bone marrow.¹⁹⁻²¹ Due to the gradual degradation of mRNA, they form only a small part of the entire platelet population. Only little is known about the properties of reticulated platelets, although incidental studies report on a larger size with more granules,²² and a high reactivity towards platelet agonists.^{23, 24} With a Sysmex analyzer, using cell-permeant fluorescent mRNA dyes containing polymethine and oxazine, or using the mRNA probe thiazole orange, evidence has been obtained that patients with more reticulated platelets respond less effectively to clopidogrel or prasugrel medication.²⁵⁻²⁷

In the present study, we investigated how the hemostatic function of platelets recovers after discontinuation of prasugrel treatment. We hypothesized an immediate recovery of newly formed, juvenile platelets to the level of untreated platelets. Our data provide evidence for a critical role of the juvenile platelets in the regained aggregation of platelets and thrombus formation, and also show that these platelets gradually increase in responsiveness.

Methods

For a detailed description of the methods, please see the supplementary material.

Patients and control subjects

This study was approved by the local medical ethics committee (MEC 12-3-075). All patients and healthy volunteers gave written informed consent for participation according to the Helsinki declaration. Sixteen patients were studied who were treated with prasugrel (10 mg/day) for one year and long-term aspirin (80-100 mg/day) due to a myocardial infarction with ST elevation. After one year of prasugrel treatment, blood was collected on the last treatment day, and at 1, 2, 5 and 30 days later. From two patients, blood samples were also collected after 12 days to better understand the delayed regain of platelet function. Patients with a malignancy, active infection or a known platelet disorder were not included. Blood was obtained by venipuncture into Vacuette tubes, containing K₂-EDTA, for measurement of hemostatic variables and immature platelet fraction (IPF) using a Sysmex XN-9000 analyzer (Sysmex, Chuo-ku Kobe, Japan); 3.2%

(w/v) trisodium citrate for platelet function measurements, or hirudin for whole blood platelet aggregation. Control experiments were performed with blood drawn from healthy volunteers collected in trisodium citrate or acidic citrate dextrose.²⁸

Preparation of platelet-rich plasma, platelets and red cells

Platelet-rich plasma (PRP), platelet-free plasma and washed platelets were prepared as described.²⁹ Platelet counts were determined with a thrombocounter XP300 Sysmex analyzer (Sysmex, Chuo-ku Kobe, Japan). Washed red blood cells were prepared as shown before.³⁰

Irreversible P2Y₁₂ inhibition in vitro

PRP from healthy donors was treated with lysine aspirin.²⁸ The platelets were incubated with the active metabolite of clopidogrel (CAM) or vehicle medium. Mixtures of washed CAM-treated and vehicle-treated platelets were used for measurement of platelet aggregation, integrin $\alpha_{IIb}\beta_3$ activation by flow cytometry and perfusion experiments with reconstituted whole blood.

Platelet aggregation

Aggregation of platelets in PRP was measured using a Chronolog aggregometer (Stago, Asnières sur Seine Cedex, France).³¹ Aggregation of platelets in whole blood was measured by Multiplate impedance aggregometry (Roche Diagnostics, Basel, Switzerland) as described.³² Ticagrelor (1 μ M), being more potent than prasugrel³³, was added *in vitro* to block residual P2Y₁₂ activity, where indicated.

Flow cytometric analysis of platelet subpopulations

Flow cytometry was performed on an Accuri C6 flow cytometer with CFlow Plus software (Becton-Dickinson Bioscience). To check for integrin $\alpha_{IIb}\beta_3$ activation, platelets were activated with 2MeS-ADP in the presence of FITC-conjugated PAC-1 antibody against the activated $\alpha_{IIb}\beta_3$ integrin. Activated platelets were identified as before.³¹ Ticagrelor (1 μ M) was added, where indicated.

Juvenile platelets were identified using two different methods of mRNA staining, *i.e.* with thiazole orange³⁴ or by a novel method using Cy5-labeled oligo-dT, which binds to the poly-A tail of mRNA species. Thiazole orange (15% in filtered PBS) was added to PRP, according to established procedures.³⁴ Samples were activated with 2MeS-ADP in the presence of AF647-fibrinogen. For staining with 5'Cy5-oligo-dT, washed platelets were activated with 2MeS-ADP in the presence of OG488-fibrinogen. Samples were fixed with 0.2% formaldehyde, permeabilized with 0.1% saponin and subsequently incubated with 5'-Cy5-oligo-dT at 37 °C. For all samples, 5'-Cy5-oligo-dA was used as a negative control probe to check for specificity of the staining. Color compensation was not required as fluorescent spectra did not overlap.

The average percentage of juvenile platelets as analyzed by the thiazole orange staining and the oligo-dT staining was 6.7% (\pm 1.9%) and 21.5% (\pm 5.8%) respectively. The discrepancy in the percentage of detected juvenile platelets can be explained by

the higher sensitivity of the oligo-dT staining to detect mRNA in comparison to thiazole orange. In order to use a uniform definition of juvenile platelets, the threshold for juvenile platelets was based on the IPF as determined by the Sysmex XN9000 analyzer, which is an internationally validated method in the clinic. An alternative analysis of juvenile platelets, based on the negative controls of both stainings, is presented in the supplements (Suppl. Fig. 3).

Thrombus formation in whole blood

Whole blood thrombus formation on microspots in a parallel-plate flow chamber was measured, basically as described before.³⁵ Patient blood samples were perfused through the chamber for 4 minutes at a wall-shear rate of 1600 s^{-1} , while 2MeS-ADP ($0.1 \text{ }\mu\text{M}$, f.c.) was co-perfused with a second pump. Ticagrelor was added where indicated. Thrombi were stained with AF647-labelled fibrinogen and, when indicated, with DiOC₆.³⁵ Brightfield and fluorescence microscopic images were captured with an EVOS fluorescence microscope, equipped with a 60x oil objective. Images were analyzed using Metamorph (Molecular Devices, Sunnyvale CA, USA) and ImageJ (open access) software.³⁵

For measurement of thrombus formation of reconstituted blood samples from healthy controls, mixtures of CAM- and vehicle-treated platelets were added to washed red cells and plasma. In these experiments, the CAM-treated and vehicle-treated platelets were pre-labeled with the membrane probes CellVue Maroon and PKH26 respectively. Microscopic DIC and confocal fluorescent images were taken using a Zeiss LSM7 microscope (Oberkochen, Germany).³⁵

Statistical analysis

Statistical analysis was performed using the SPSS Statistics 22 package (Armonk, NY, USA). Statistical analysis was performed using a one-way-repeated-measures-ANOVA or with a Friedman test with a post hoc Wilcoxon signed rank test. Bonferroni correction was applied when comparing multiple groups.

Results

P2Y₁₂-inhibited platelets participate less in thrombus formation

In order to determine how platelets with non-responsive P2Y₁₂ receptors interact with responsive platelets in aggregation and thrombus formation, platelets from control subjects were treated with the clopidogrel active metabolite (CAM) and mixed in various proportions with untreated platelets. All platelets were also treated with aspirin, in order to mimic conditions as in patients (see below). Flow cytometric analysis indicated that, upon stimulation with ADP, these platelet mixtures formed two distinct populations in terms of activation of integrin $\alpha_{\text{IIb}}\beta_3$ and binding of OG488-fibrinogen. The population of fibrinogen-binding platelets decreased with increasing fractions of CAM-treated platelets (Fig. 1A and Suppl. Fig. 1A). Addition of the P2Y₁₂ receptor antagonist ticagrelor decreased the population of fibrinogen-binding platelets to the level of 100%

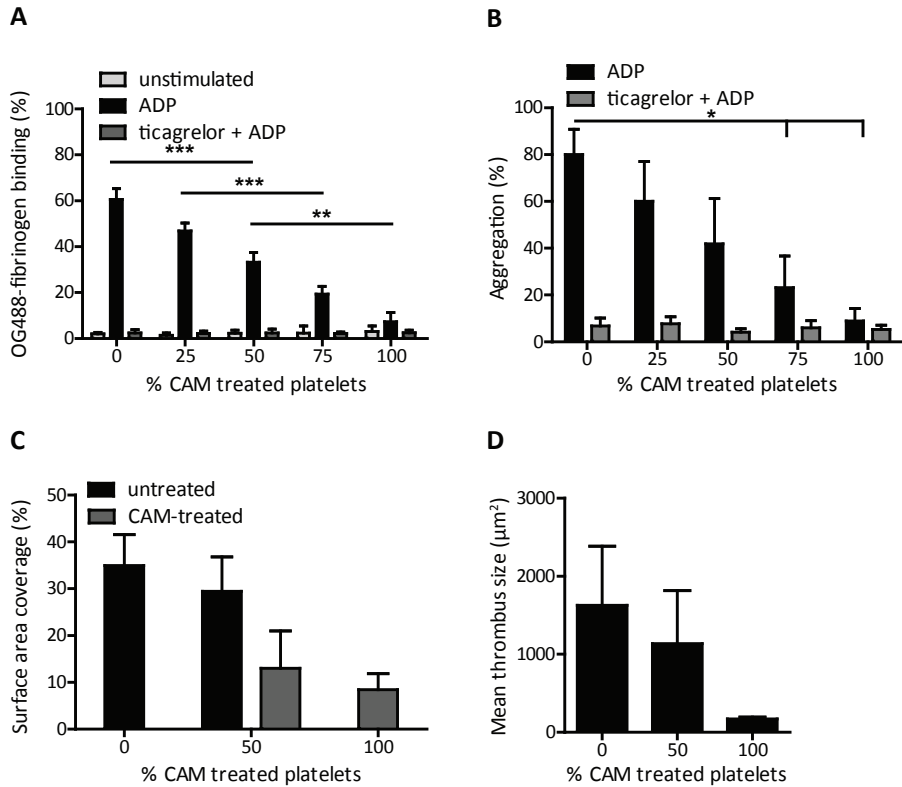


Figure 1. Impaired aggregation and contribution to thrombus formation of P2Y₁₂-inhibited platelets. (A, B) Different mixtures of uninhibited platelets and CAM-treated platelets were preincubated with vehicle or 1 μ M ticagrelor, and stimulated with 20 μ M 2MeS-ADP. (A) Fractions of OG488-fibrinogen binding platelets, representative for uninhibited platelets with activated integrin $\alpha_{IIb}\beta_3$ (flow cytometry). (B) Maximal aggregation (% change in light transmission) within 5 minutes. (C, D) Reconstituted blood with different fractions of CAM-treated platelets was perfused 4 minutes over collagen at 1600 s^{-1} in the presence of 2MeS-ADP. Uninhibited platelets were prelabeled with PKH26 and CAM-treated platelets with CellVue Maroon; for images see Suppl. Figure 2. (C) Quantification of the surface area covered by the populations of labelled platelets. (D) Mean thrombus size. Means \pm SD (n = 3), *p < 0.05, **p < 0.01, ***p < 0.001 (ANOVA with Bonferroni correction).

CAM-treated platelets (Fig. 1A), indicating that the CAM treatment had fully blocked the P2Y₁₂ receptors. Light transmission measurements indicated a gradual decrease in ADP-induced platelet aggregation, when the fraction of CAM-treated platelets increased (Fig. 1B). Addition of ticagrelor again antagonized the remaining aggregation response. Together, these data suggest that the reduced integrin activation of the CAM-treated platelets prevented their incorporation into aggregates.

To investigate this further, we assessed how the CAM-treated platelets participated in thrombus formation on immobilized collagen under high-shear flow conditions. Therefore, the P2Y₁₂-inhibited platelets were labelled with the red-exciting membrane label CellVue Maroon, whereas the untreated platelets were labeled with the green-exciting membrane label PKH26. This labeling did not affect platelet activation responses

(data not shown). Mixtures with 0%, 50% or 100% of CAM-treated platelets were added to red blood cells and plasma from the same donor to obtain reconstituted blood with different proportions of P2Y₁₂-inhibited platelets. In comparison to reconstituted blood with solely uninhibited platelets, increasing proportions of CAM-treated platelets had limited impact on platelet adhesion to the collagen surface, but markedly suppressed the formation of large platelet aggregates (Suppl. Fig. 2). As a result, with CAM-treated platelets, surface area coverage (Fig. 1C) and mean thrombus size (Fig. 1D) on collagen progressively decreased. Strikingly, with 50% of CAM-treated platelets, about 30% of the

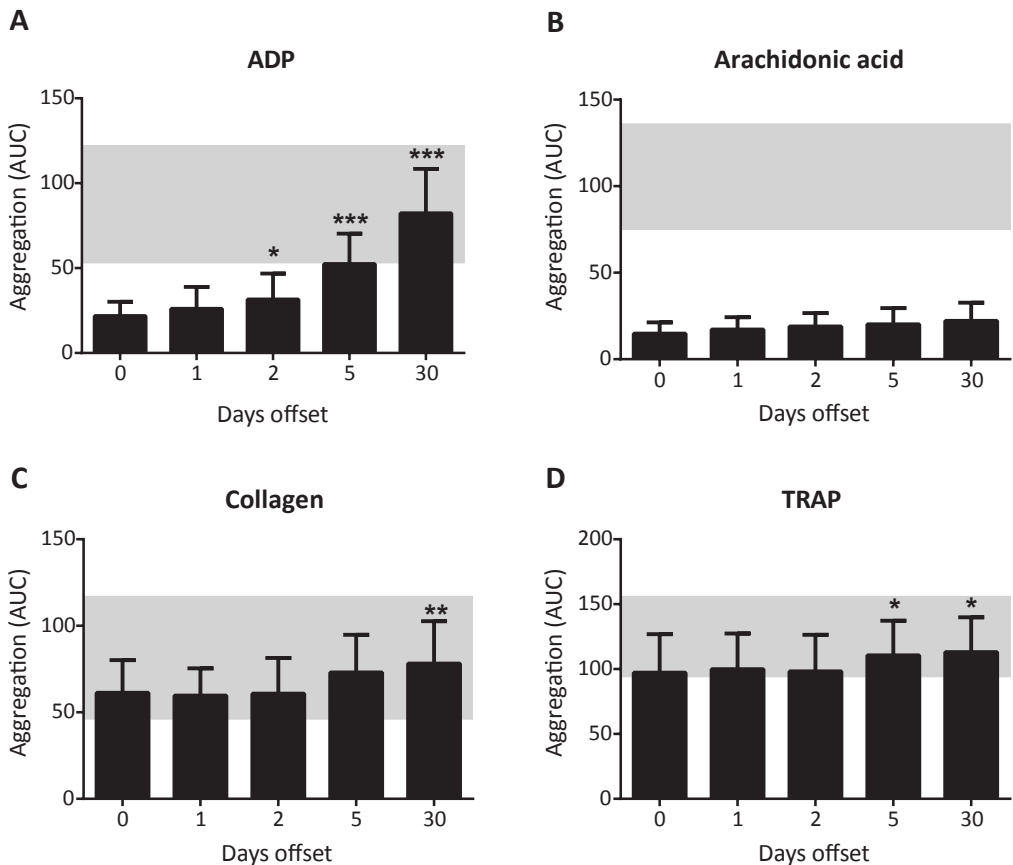


Figure 2. Partial restoration of platelet aggregation in whole blood upon prasugrel offset. Whole blood samples from patients (at indicated days after stopping prasugrel intake) were stimulated with 6.4 μ M ADP (A), 0.5 mM arachidonic acid (B), 3.2 μ g/ml collagen (C), or 32 μ M TRAP (D). Measurements by Multiplate impedance aggregometry; outcome expressed as the area under the impedance curve (AUC). Grey blocks indicate normal ranges, established for healthy subjects. Means \pm SD ($n = 15-16$), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. day 0 (ANOVA with Bonferroni correction).

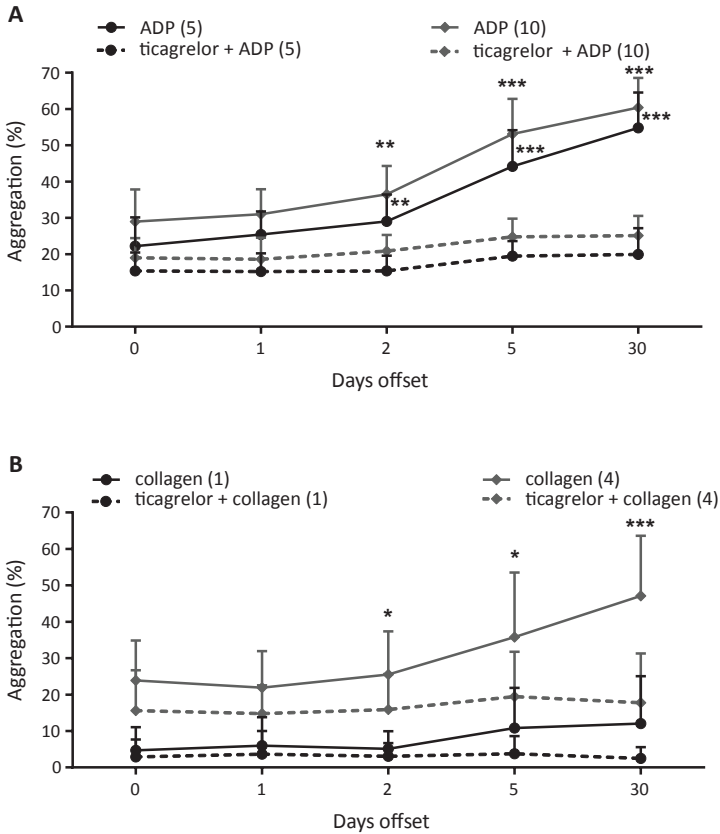


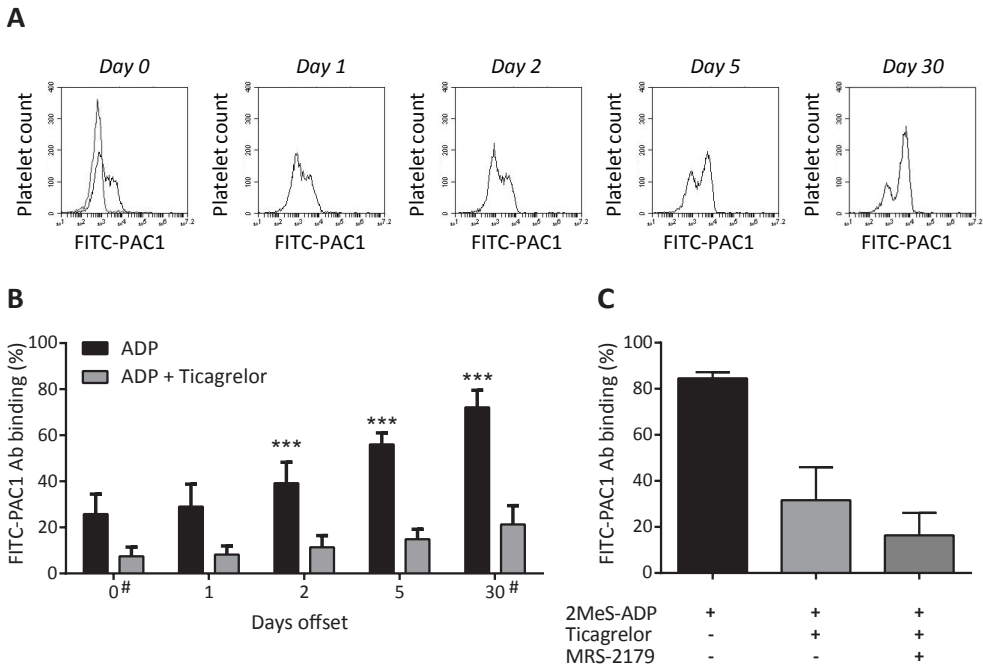
Figure 3. Restored ADP- and collagen-induced aggregation of platelets upon prasugrel offset. Patient PRP (at indicated days after stopping prasugrel intake) activated with 5-10 μ M ADP (A) or 1-4 μ g/ml collagen (B), in the presence or absence of 1 μ M ticagrelor (blocking P2Y₁₂ receptors). Platelet aggregation was assessed by light transmission aggregometry (% change in maximal light transmission). Means \pm SD (n = 16), *p < 0.05, **p < 0.01 and ***p < 0.001 vs. day 0 (ANOVA with Bonferroni correction).

surface area coverage was occupied by the P2Y₁₂-inhibited platelets, while the remaining 70% was occupied by the P2Y₁₂-responsive platelets. Together, these results indicate that P2Y₁₂-inhibited platelets participate less in platelet aggregation and thrombus formation.

Gradual restoration of platelet aggregation in patients upon prasugrel offset

The offset phase of prasugrel medication was studied in 16 patients. The patients had a mean age of 59 ± 9 years (mean \pm SD); three patients were diagnosed with type II diabetes mellitus (Suppl. Table 1). Blood samples taken at day 0 (*i.e.* last day of prasugrel intake) showed a normal hematocrit of 0.435 ± 0.035 l/l and platelet count of $239 \pm 81 \times 10^9$ /l. Subsequent blood samples were taken at days 1, 2, 5, and 30, and no noticeable changes in hematocrit or platelet count were observed.

Measurements of whole blood aggregation (Multiplate assay) showed a gradual increase in ADP-induced aggregation upon offset from days 2 to 30 (Fig. 2A). Interestingly, the aggregation response further increased at day 30 in comparison to day 5. Whole



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Figure 4. Reappearance of ADP-responsive platelets upon prasugrel offset. Whole blood from patients or healthy controls was preincubated with vehicle, 1 μ M ticagrelor or 50 μ M MRS-2179 (as indicated), and then stimulated with 1 μ M 2MeS-ADP. Activation of $\alpha_{IIb}\beta_3$ was assessed by flow cytometric analysis using FITC-labelled PAC-1 mAb. (A) Representative histograms of PAC-1 mAb binding to patient platelets stimulated with ADP (black) or unstimulated (grey) during offset. (B) Quantification of positive platelets. (C) Flow cytometric analysis of healthy control platelets. Means \pm SD (n = 16), ***p < 0.001 vs. day 0 ADP (ANOVA with Bonferroni correction); #n = 15.

blood aggregation in response to arachidonic acid remained below the normal range (Fig. 2B), thus confirming that all patients still used aspirin during the offset period. The aggregation responses to collagen and thrombin receptor-activating peptide (TRAP) were within the normal ranges, but slightly increased at later sampling points (Fig. 2C-D). Similarly, light transmission aggregometry in PRP indicated a restoration in ADP-induced platelet aggregation from day 2 onward (Fig. 3A). In this test, the aggregation response with two ADP concentrations was near maximal already at day 5. Collagen-induced platelet aggregation also significantly improved, but only at later time points (Fig. 3B). Control experiments in the presence of ticagrelor showed that the increase in aggregation during prasugrel offset was fully antagonized, confirming that the regained platelet reactivity was fully due to increased P2Y₁₂ receptor function (Fig. 3A-B).

Formation of a highly reactive population of juvenile platelets upon prasugrel offset

Based on earlier experiments with rats,¹⁸ we expected during the offset phase of prasugrel medication the rapid formation of a population of newly formed, fully P2Y₁₂-responsive platelets. A pertinent question was how the regained response in

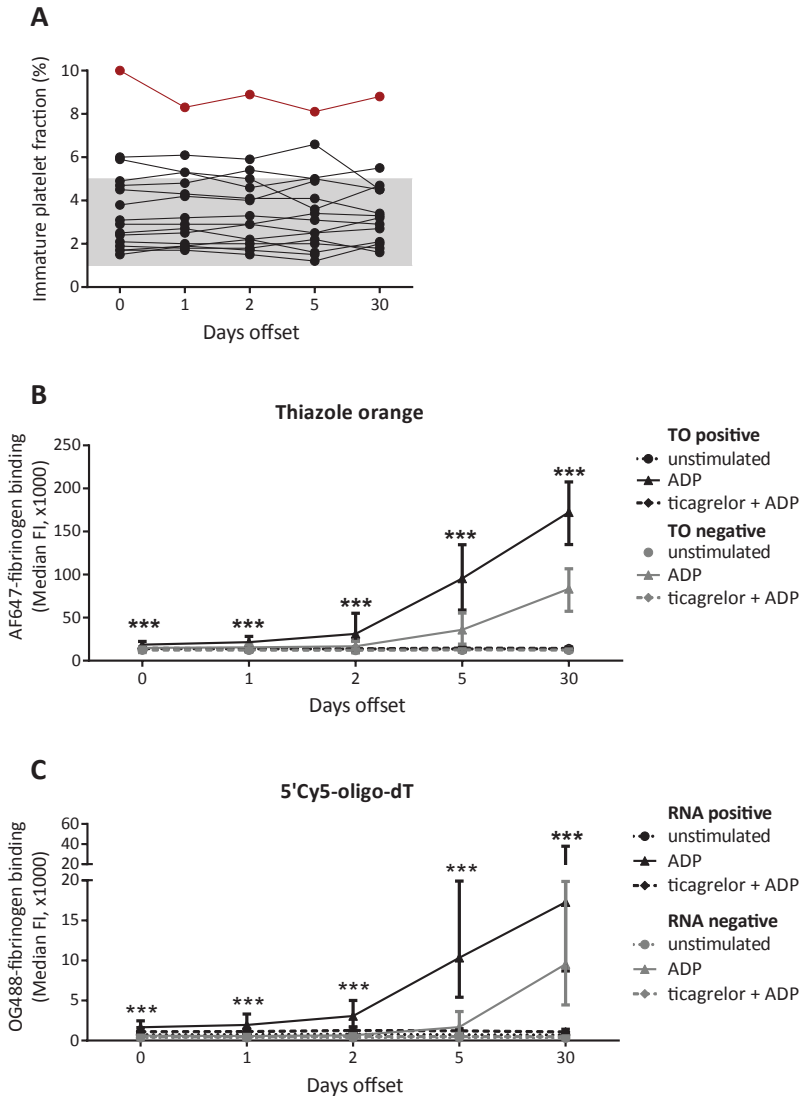


Figure 5. Increased ADP-induced $\alpha_{IIb}\beta_3$ activation of juvenile platelets formed upon prasugrel offset. Platelets from 16 patients during offset from prasugrel were activated and analyzed by flow cytometry, with per sample a gating for juvenile platelets based on the immature platelet fraction (Sysmex XN-9000 analyzer). **(A)** Immature platelet fraction for each of the patients during offset, as determined with a Sysmex XN-9000 analyzer. Red dots are from patient with a high immature platelet fraction (IPF = 8.8%). Juvenile platelets were identified by staining with thiazole orange or 5'Cy5-oligo-dT. **(B)** PRP was stained with thiazole orange and activated with 1 μ M 2MeS-ADP in the presence of AF647-fibrinogen. Shown is extent of activated $\alpha_{IIb}\beta_3$ of thiazole-positive and -negative platelets, as assessed from fibrinogen binding. **(C)** Washed platelets were activated with 1 μ M 2MeS-ADP in the presence of OG488-fibrinogen. The cells were subsequently fixed and permeabilized with saponin to allow staining of mRNA by incubation with 5'Cy5-oligo-dT. Negative control stains were performed with 5'Cy5-oligo-dA. Shown is extent of $\alpha_{IIb}\beta_3$ activation of mRNA-positive and -negative platelets, as determined from fibrinogen binding. Medians \pm IQR (n = 16), ***p < 0.001 vs. corresponding mature platelet fraction (Friedman test).

P2Y₁₂ receptor activity was linked to the appearance of this new platelet population. To investigate this, flow cytometry was used to analyze platelets stimulated with the stable (nucleotidase-resistant) ADP analogue, 2MeS-ADP, for binding of FITC-labeled PAC-1 antibody, indicative of integrin $\alpha_{IIb}\beta_3$ activation. At day 0, a limited fraction of $26 \pm 9\%$ of the platelets showed activated $\alpha_{IIb}\beta_3$, and this fraction (recognized as a separate peak in the histograms) gradually increased to $56 \pm 5\%$ at day 5 and $72 \pm 7\%$ at day 30 (Fig. 4A-B). In comparison, activation of aspirin-treated platelets from healthy control subjects with 2MeS-ADP resulted in a similar percentage of platelets with activated $\alpha_{IIb}\beta_3$ as platelets collected at 30 days after prasugrel cessation (Fig. 4C). In platelets from patients (Fig. 4B) and healthy controls (Fig. 4C), the fraction of platelets binding FITC-PAC1 antibody was

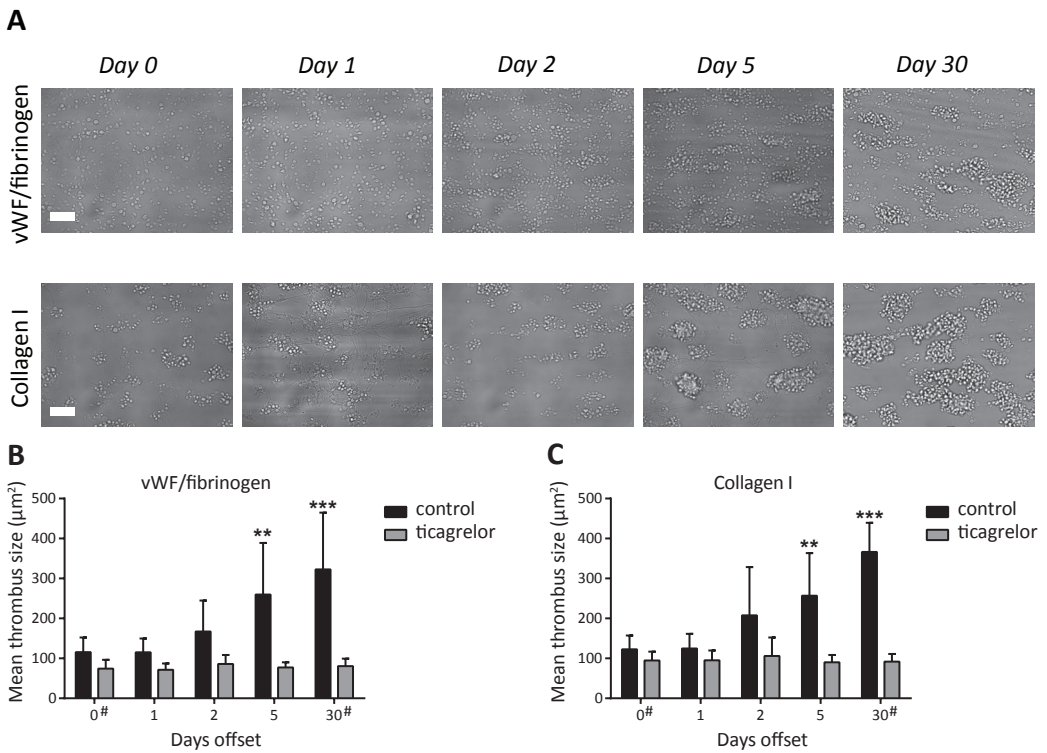


Figure 6. Restored thrombus formation under flow upon prasugrel offset. Whole blood samples from patients (at indicated days after stopping prasugrel intake) were co-perfused with $0.1 \mu\text{M}$ 2MeS-ADP over microspots containing vWF/fibrinogen or collagen type I, at a shear rate of 1600 s^{-1} for 4 minutes. Vehicle (control) or $10 \mu\text{M}$ ticagrelor was added to the blood. (A) Brightfield images of thrombi from a representative patient formed on the two microspots. Bars = $25 \mu\text{m}$. Assessment of mean thrombus size for surfaces with vWF/fibrinogen (B) or collagen I (C). Means \pm SD ($n = 16$), ** $p < 0.01$, *** $p < 0.001$ vs. day 0 (ANOVA with Bonferroni correction); # $n = 15$.

greatly, but incompletely reduced by the addition of ticagrelor. Co-incubations with the P2Y₁ receptor antagonist MRS-2179 indicated that the residual $\alpha_{\text{IIb}}\beta_3$ activation was most likely due to activation via P2Y₁ receptors.

Several assays were performed to determine whether the accumulating platelets with activated $\alpha_{\text{IIb}}\beta_3$ indeed consisted of newly formed, juvenile platelets. Therefore, platelet mRNA was quantified using two different mRNA probes: thiazole orange as an established, but weak fluorescent mRNA dye²²; and 5'Cy5-labeled oligo-dT, binding to the platelet mRNA poly-A tails²¹, which was added to pre-activated and permeabilized platelets. Supplemental figure 3 shows typical dotplots of unstimulated and ADP-activated platelets stained with the 5'Cy5-oligo-dT (Suppl. Fig. 3A-B) or thiazole orange (Suppl. Fig. 3C-D). Juvenile and old platelets were discriminated per blood sample, based on the negative control and the immature platelet fraction determined by Sysmex XN-9000 (Fig 5A). For the majority of the patients, with one noticeable exception, the immature platelet fraction was in the normal range, with mean values of $3.6 \pm 1.9\%$, and remaining constant per patient during the study (Fig. 5A). Markedly, with either mRNA probe (thiazole orange or 5'Cy5-oligo-dT), the fraction of platelets with positive staining showed increased fibrinogen binding following ADP stimulation from day 2 on (Fig. 5B-C). Fibrinogen binding ($\alpha_{\text{IIb}}\beta_3$ activation) of this juvenile platelet population continued to increase from day 5 to day 30. The difference between 5 and 30 days was significant for thiazole orange ($p = 0.003$), and was borderline significant for 5'Cy5-oligo-dT ($p = 0.063$), thus suggesting partial inhibition of these platelets even after 5 days offset. With either mRNA probe, the fraction of (older) platelets with negative staining was substantially lower in fibrinogen binding, with noticeable increase of activated integrins only from day 5 onwards (Fig. 5B-C). The difference in fibrinogen binding between juvenile and older platelets was significant at all days ($p < 0.001$). These results were confirmed using an alternative analysis based on the appropriate negative controls (Suppl. Fig 3.) Here again, the reactivity of juvenile platelets, expressed as activation ratio, was significantly higher in comparison to mature platelets at all days. Moreover, this analysis also shows the increasing reactivity of juvenile platelets over time for both stainings (Suppl. Fig 4A). Additional platelet measurements with a limited number of patients showed that, after 12 days of prasugrel discontinuation, the reactivity of juvenile platelets was in between the day 5 and 30 values (Suppl. Fig. 4B). Interestingly, in patients with a high immature platelet fraction (IPF $\geq 7.0\%$), the population of juvenile platelets showed a faster increase in fibrinogen binding, being near maximal already at day 5 of prasugrel offset (Suppl. Fig. 4A-B).

Gradual increase in thrombus size upon prasugrel offset

To investigate whether the increased reactivity of juvenile platelets after prasugrel cessation translates into enhanced thrombus formation, whole blood was perfused over microspots containing vWF/fibrinogen or type I collagen.³⁵ Given the major role of P2Y₁₂ signaling in thrombus build-up³⁶, we determined thrombus size at the different time points. Regardless of the surface, at day 0 many single platelets and small aggregates were detected, whilst at later offset days larger aggregates were formed. From the recorded brightfield images it was apparent that the thrombi at days 5 and 30 displayed

a more contracted morphology (Fig. 6A). Quantification of the feature size showed on both microspots a progressive increase in mean thrombus size during offset (Fig. 6B-C). On the other hand, platelet adhesion to vWF/fibrinogen or collagen was not changed between day 0 and 30, as surface area coverage (by single platelets and aggregates) remained similar (Suppl. Fig. 5A-B).

Staining of thrombi with AF647-labeled fibrinogen allowed assessment of integrin $\alpha_{\text{IIb}}\beta_3$ activation. On both the vWF/fibrinogen and collagen microspots, a marked increase in fibrinogen binding to the aggregated platelets was detected for the later blood samples (Suppl. Fig. 6A-B). Addition of ticagrelor to the blood resulted in a nearly complete abolition of platelet aggregation at all offset days, but did not block platelet adhesion. With blood samples from a limited number of patients, staining with DiOC₆ made it possible to record z-stacks by confocal microscopy for 3D visualization of the thrombi. This illustrated the gradual increase in thrombus size from day 0 to day 5 and 30 (Suppl. Fig. 6C). Taken together, these results indicate that the accumulation of highly P2Y₁₂-responsive, juvenile platelets upon prasugrel offset led to a gradual increase in formation of large-size thrombi.

Discussion

In this paper, we confirm earlier findings^{7, 9, 15, 16, 37} that when treatment with an irreversible P2Y₁₂ antagonist is stopped, platelet aggregation in response to ADP gradually recovers in time, when measured by light transmission aggregometry. Near maximal aggregation after stopping prasugrel intake was already reached at day 5, which is in line with results from earlier trials, such as Recovery showing recuperation of this platelet response after 7-9 days of prasugrel cessation.⁹ However, we also found that other platelet function tests, such as ADP-induced whole blood aggregation and integrin $\alpha_{\text{IIb}}\beta_3$ activation were incompletely recovered at day 5 in comparison to day 30. *In vitro* addition of ticagrelor completely antagonized the time-dependent increase in platelet responses, thereby proving that the recuperation was due to regained P2Y₁₂ signaling.

Detailed flow cytometric analysis indicated that the functional recovery during prasugrel offset was caused by the appearance of a population of juvenile platelets that was increasingly responsive towards ADP. Separation of newly formed and older platelets with two mRNA probes, thiazole orange and a new Cy5-conjugated oligo-dT probe, revealed increased responsiveness to ADP of the positively stained platelet population in terms of integrin $\alpha_{\text{IIb}}\beta_3$ activation and fibrinogen binding. However, both probes also gave unexpected results. First, we observed a marked increase in ADP responsiveness of the juvenile platelet population after only two days of offset, and for the older platelet population after five days. This suggested that the majority of juvenile platelets formed during the first days still had inhibited P2Y₁₂ receptors, taking into account the presence of the prasugrel active metabolite in the circulation for 7-8 hours post prasugrel administration.³⁸ Second, we found a steady rise in the responsiveness of juvenile platelets up to days 12 and 30 of offset. This also points towards residual ADP receptor inhibition of new platelets, likely at the megakaryocytic level, for more than 5 days. In agreement with this hypothesis, experimental animal models have shown that prasugrel is present in the bone marrow.³⁹ An alternative explanation for this phenomenon might be a long-

term increase in autocrine platelet-stimulating effects due to the larger population of P2Y₁₂-responsive platelets.^{3,40} However, flow cytometric analysis did not point to a higher extent of integrin $\alpha_{IIb}\beta_3$ activation of the ADP-responsive platelet population as a whole.

To determine how the increased reactivity of juvenile platelets translates into hemostasis, we studied thrombus formation under flow on two different adhesive surfaces using whole blood. Platelet deposition and aggregate formation on the vWF/fibrinogen and the collagen surfaces restored during the offset and was only maximal at day 30. The regained P2Y₁₂ activity was most apparent from thrombus size, with larger thrombi towards the end phase of the offset. This is in agreement with earlier work showing that signaling via P2Y₁₂ is crucial for thrombus formation and stabilization.^{1,36} Also others found a relative preponderance of thiazole orange-stained platelets in a thrombus.⁴¹

In recent years, it has been debated whether the termination of clopidogrel or prasugrel intake leads to a rebound effect of recurrent cardiovascular events, perhaps related to platelet hyper-reactivity. Several research groups did report a rebound effect within 90 days after cessation of clopidogrel,¹³⁻¹⁷ while other researchers could not confirm this.^{37, 42-44} This discussion has led to clinical trials investigating the effect of tapering clopidogrel medication with the idea to prevent platelet hyper-reactivity, but with no beneficial effect so far.^{42, 43, 45} The present work may explain this ambiguity in clinical offset effects. On the one hand, cessation of clopidogrel or prasugrel medication will lead to the appearance of the newly formed platelets with uninhibited P2Y₁₂ receptors, which preferentially partake in thrombus formation. On the other hand, as shown in this study, at least during the first few days these juvenile platelets do not appear to be hyperactive, possibly due to residual receptor blockage at the megakaryocyte level. When tapering the medication, the prolonged time interval between two consecutive dosages will result in the alternative formation of uninhibited and inhibited platelets.

The present study has potential limitations, as we have investigated a relative small number of patients. Further, in our initial *ex vivo* studies we used the active metabolite of clopidogrel. Although prasugrel is a more potent P2Y₁₂ antagonist in comparison to clopidogrel,⁵ we added the active metabolite of clopidogrel at concentrations high enough for maximal inhibition.

Patients on dual antiplatelet therapy who require surgery are recommended to stop prasugrel intake 7 days beforehand.⁹ Our findings that prasugrel can still affect the reactivity of juvenile platelets during several days after treatment cessation does not plea for a shortening of this period. The compromised reactivity of juvenile platelets during the initial days of offset can contribute to a risk of bleeding upon surgery. When urgent surgery is required or when bleeding has to be controlled, platelet transfusions have shown to be effective in restoring haemostasis at 6 hours after a loading dose of prasugrel.⁴⁶ Altogether, the present study provides clinically relevant detailed insights into the mechanisms of prasugrel offset, and thereby provides better insight into the optimal treatment regimen of P2Y₁₂ inhibitors.

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Authorship and disclosures

Authorship: CCFMJB performed experiments, analyzed data and drafted the paper; RW, JpVG, FS, and SMdW performed experiments; LFV and HC recruited the patients; YMCH contributed analytical tools; SN and JJJvG designed research and drafted the paper; JMWH and PEJvdM designed research and drafted and finalized the paper.

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Supplemental data

Supplemental methods

Materials

2MeS-ADP and D-Phe-Pro-Arg chloromethyl ketone (PPACK) were from SantaCruz Biotechnology (Santa Cruz, CA, USA); fluorescein isothiocyanate (FITC)-conjugated PAC-1 antibody against active integrin $\alpha_{IIb}\beta_3$ and thiazole orange from Becton-Dickinson Bioscience (Franklin Lakes NJ, USA), and aspirin was from Sanofi (Paris, France). Ticagrelor and the clopidogrel active metabolite (CAM) were kindly provided by AstraZeneca R&D (Mölnådal, Sweden). 5'-Cy5-conjugated oligo-dA20 and oligo-dT20 were obtained from Eurogentec (Maastricht, The Netherlands). Alexa Fluor (AF)-647 and Oregon Green (OG)-488 conjugated human fibrinogen were from Invitrogen (Bleiswijk, The Netherlands). Collagen type I came from Nycomed Pharma (Munich, Germany); 3,3'-dihexyloxa carbocyanine iodide (DiOC₆) from Anaspec (Fremont CA, USA); CellVue Maroon from eBioscience (San Diego CA, USA), and iloprost from Bayer Pharma (Berlin, Germany). Multiplate test kits were from Roche Diagnostics (Basel, Switzerland). Other compounds came from Sigma (St. Louis, MO, USA).

Patients and control subjects

This study was approved by the local medical ethics committee (MEC 12-3-075). All patients and healthy volunteers gave written informed consent for participation according to the Helsinki declaration. Sixteen patients were studied who were treated with prasugrel (10 mg/day) for one year and long-term aspirin (80-100 mg/day) due to a myocardial infarction with ST elevation. After one year of prasugrel treatment, blood was collected on the last day of prasugrel intake, and at 1, 2, 5 and 30 days after the last dose. From two patients, blood samples were also collected after 12 days to better understand the delayed regain of platelet function. Patients with a malignancy, active infection or a known platelet disorder were not included. Blood was obtained by venipuncture with a Vacutainer 21-gauge needle (Becton-Dickinson Bioscience). Blood collection was into Vacuette tubes, containing K₂-EDTA, for measurement of hemostatic variables and immature platelet fraction using a Sysmex XN-9000 analyzer, according to protocols of the supplier (Sysmex, Chuo-ku Kobe, Japan). Blood samples were also collected into 3.2% (w/v) trisodium citrate for platelet function measurements, and into hirudin for whole-blood platelet aggregation. Control experiments were performed with blood drawn from healthy volunteers. Collection was into trisodium citrate, or into acidic citrate dextrose (ACD, 80 mM trisodium citrate, 52 mM citric acid and 180 mM glucose) for the preparation of washed platelets.¹

Preparation of platelet-rich plasma, platelets and red cells

Platelet-rich plasma (PRP) was prepared by centrifuging citrate-anticoagulated blood at 240 g for 15 minutes at room temperature. Platelet-free plasma was obtained by centrifuging citrate-anticoagulated blood twice at 2630 g for 10 minutes. Washed platelets were prepared from ACD-anticoagulated blood, as described², and were suspended in HEPES buffer pH 7.45 (10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 0.1% glucose and 0.1% bovine serum albumin). Platelet counts were determined with a thrombocounter XP300 Sysmex analyzer (Sysmex, Chuo-ku Kobe, Japan).

Washed red blood cells were prepared by centrifuging citrate-anticoagulated blood at 240 g for 15 minutes at room temperature.³ After removal of PRP, HEPES buffer pH 7.45 was added in a 1:2 volume ratio. The red blood cells were centrifuged twice at 2630 g for 10 minutes, with resuspension in HEPES buffer pH 7.45. This resulted in a preparation of >99.9% red cells.

Irreversible P2Y₁₂ inhibition in vitro

PRP from healthy donors was treated with 100 μM lysine aspirin for 30 minutes, and platelets were pelleted by centrifugation.¹ The resuspended cells were incubated for 15 minutes with 10 μM CAM or vehicle medium. Residual unbound CAM was removed by a second final centrifugation step, where 5 nM iloprost was added to prevent platelet activation. Mixtures of the washed CAM-treated and vehicle-treated platelets in HEPES buffer pH 7.45 were used for measurement of: platelet aggregation (light transmission aggregometry in the presence of 2 mM CaCl₂ and 0.1 mg/ml fibrinogen); integrin α_{IIb}β₃ activation by flow cytometry in the presence of 75 μg/ml OG488-fibrinogen; and perfusion experiments with reconstituted whole blood.

Platelet aggregation

Aggregation of platelets in PRP was measured using a Chronolog aggregometer (Stago, Asnières sur Seine Cedex, France).⁴ Preincubation with ticagrelor or vehicle medium was for 5 minutes at 37 °C. Aggregation of platelets in whole blood was measured by Multiplate impedance aggregometry (Roche Diagnostics, Basel, Switzerland) as described.⁵ Aggregation was measured in response to ADP (6.4 μM), arachidonic acid (0.5 mM), collagen (3.2 μg/ml) or thrombin receptor-activating peptide SFLLRN (TRAP, 32 μM) at 37 °C during 6 minutes. Ticagrelor (1 μM) was added in vitro to block residual P2Y₁₂ activity, where indicated. Extent of platelet aggregation was assessed from the area under the impedance curve.

Flow cytometric analysis of platelet subpopulations

Flow cytometric measurements were performed on an Accuri C6 flow cytometer with CFlow Plus software (Becton-Dickinson Bioscience). To check for integrin α_{IIb}β₃ activation, samples of citrated patient blood were diluted (1:20) into HEPES buffer pH 7.45, and incubated with 1 μM ticagrelor or vehicle control for 15 minutes at room temperature.

Platelets were activated with 1 μM 2MeS-ADP in the presence of FITC-conjugated PAC-1 antibody (1.25 $\mu\text{g}/\text{ml}$) against the activated $\alpha_{\text{IIb}}\beta_3$ integrin for 10 minutes. Ticagrelor (1 μM) was added, where indicated. Activated platelets were identified as before.⁴

Juvenile platelets were identified using two different methods of mRNA staining, i.e. with thiazole orange⁶ or by a novel method using Cy5-labelled oligo-dT, which binds to the poly-A tail of mRNA species. Thiazole orange (15% in filtered PBS: 136 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4 , 6.46 mM Na_2HPO_4) was added to PRP for 30 minutes at room temperature, according to established procedures.⁶ Platelets were then centrifuged at 5550 g for 3 minutes to remove excess and unbound thiazole orange, and resuspended in Hepes buffer pH 7.45. Samples were activated with 1 μM 2MeS-ADP in the presence of AF647-fibrinogen (75 $\mu\text{g}/\text{ml}$) for 15 minutes. For staining with 5'Cy5-oligo-dT, washed platelets in suspension ($1 \times 10^8/\text{ml}$) were activated with 1 μM 2MeS-ADP in the presence of OG488-fibrinogen (75 $\mu\text{g}/\text{ml}$) for 15 minutes. Samples were fixed (15 minutes) with 0.2% formaldehyde and permeabilized (10 minutes) with 0.1% saponin. 5'-Cy5-oligo-dT (1 μM) was incubated for 15 minutes at 37 °C. For all samples, 5'-Cy5-oligo-dA (1 μM) was used as a negative control probe to check for specificity of the staining with 5'-Cy5-oligo-dT. Color compensation was not required as fluorescent spectra did not overlap.

The average percentage of juvenile platelets as analyzed by the thiazole orange staining and the oligo-dT staining was 6.7% ($\pm 1.9\%$) and 21.5% ($\pm 5.8\%$) respectively. The discrepancy in the percentage of detected juvenile platelets can be explained by the higher sensitivity of the oligo-dT staining to detect mRNA in comparison to thiazole orange. In order to use a uniform definition of juvenile platelets, the threshold for juvenile platelets was based on the IPF as determined by the Sysmex XN9000 analyzer, which is an internationally validated method in the clinic. An alternative analysis of juvenile platelets, based on the negative controls of both stainings, is presented in the supplements (Supplemental Fig.3).

Thrombus formation in whole blood

Whole-blood thrombus formation on microspots in a parallel-plate flow chamber was measured, basically as described before.⁷ Briefly, 0.5 μl microspots containing either collagen I (100 $\mu\text{g}/\text{ml}$) or fibrinogen (250 $\mu\text{g}/\text{ml}$) plus vWF (50 $\mu\text{g}/\text{ml}$) were perfused with citrate-anticoagulated whole blood, which was recalcified with 7.5 mM CaCl_2 and 3.75 mM MgCl_2 in the presence of 40 μM PPACK immediately before the experiment. Patient blood samples were perfused through the chamber for 4 minutes at a wall-shear rate of 1600 s^{-1} , while 2MeS-ADP (0.1 μM , f.c.) was co-perfused with a second pump. Ticagrelor was added where indicated. Thrombi were stained with AF647-labelled fibrinogen and, when indicated, DiOC₆, as described elsewhere.⁷ Brightfield and fluorescence microscopic images were captured with an EVOS fluorescence microscope, equipped with a 60x oil objective. Images were analysed using Metamorph (Molecular Devices, Sunnyvale CA, USA) and ImageJ (open access) software.⁷

For measurement of thrombus formation of reconstituted blood samples from healthy controls, mixtures of CAM- and vehicle-treated platelets ($2.5 \times 10^8/\text{ml}$, final count) were added to washed red cells (45% hematocrit) and plasma (30-35% of total volume). In these experiments, the CAM-treated platelets were pre-labeled with the membrane

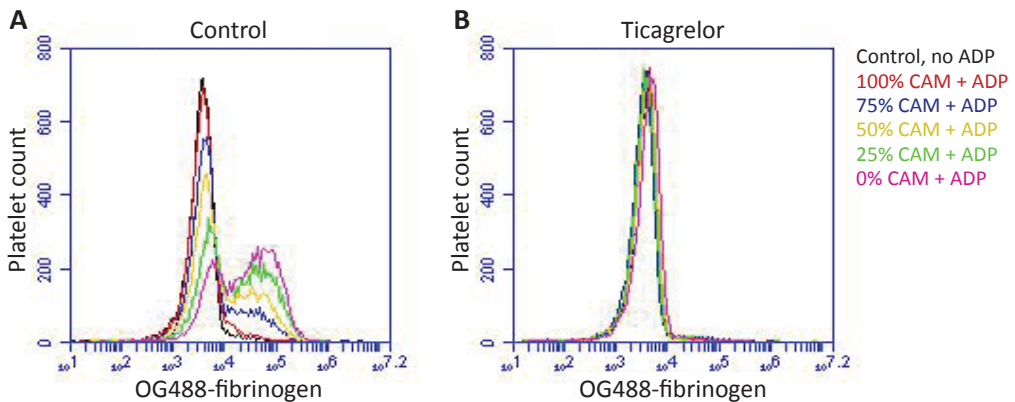
probe CellVue Maroon (1.6 μM), whereas the vehicle-treated platelets were pre-labeled with the membrane probe PKH26 (0.8 μM). Both probes were not transferrable from cell to cell (data not shown). The reconstituted whole blood was again co-perfused with 2MeS-ADP over collagen. Microscopic DIC and confocal fluorescent images were taken using a Zeiss LSM7 microscope (Oberkochen, Germany).⁷

Statistical analysis

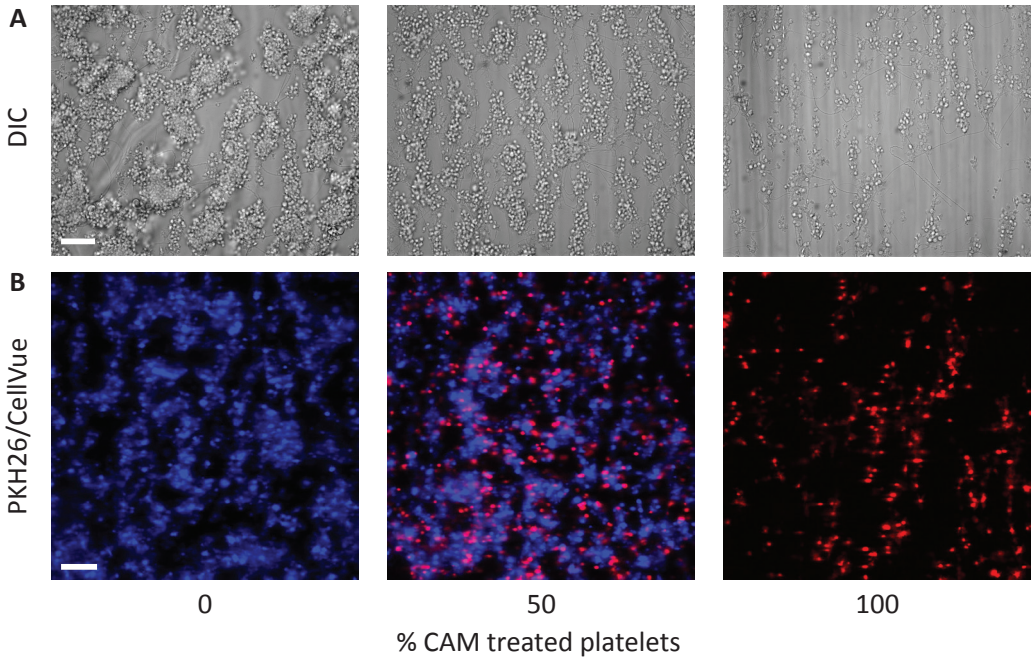
Statistical analysis was performed using the SPSS Statistics 22 package (Armonk, NY, USA). Statistical analysis was performed using a one-way-repeated-measures-ANOVA or with a Friedman test with a post hoc Wilcoxon signed rank test. Bonferroni correction was applied when comparing multiple groups.

Supplemental Table 1. Study characteristics. Means \pm SD.

Variables	
<i>Patient characteristics</i>	
Age (years)	59 \pm 9
Female/male (n)	3/13
Diabetes Mellitus (n)	3
Aspirin use (n)	16
Statins use (n)	13
<i>Hematological variables</i>	
Hematocrit (L/L)	0.435 \pm 0.035
Platelet count ($10^9/L$)	239 \pm 81
Mean platelet volume (fL)	10.8 \pm 0.7
Immature platelet fraction (IPF) Sysmex (%)	3.6 \pm 1.9

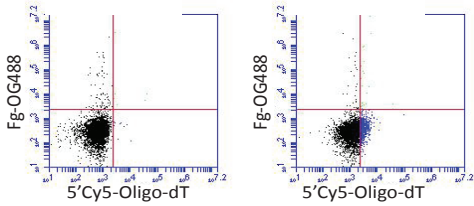


Supplemental Figure 1. Impaired fibrinogen binding of populations of P2Y₁₂-inhibited platelets. Mixtures of control platelets and CAM-treated platelets were preincubated with 1 μ M ticagrelor or vehicle, and stimulated for 15 minutes with 20 μ M 2MeS-ADP in the presence of OG488-fibrinogen. Fibrinogen binding was assessed by flow cytometry. Shown are representative histograms of fibrinogen binding after stimulation in the presence of vehicle (**A**) or ticagrelor (**B**).

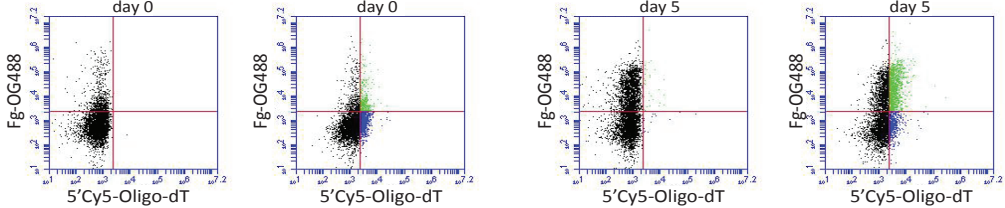


Supplemental Figure 2. Impaired contribution to thrombus formation of P2Y₁₂-inhibited platelets. Reconstituted blood with different fractions of CAM-treated platelets was perfused 4 minutes over collagen at 1600 s⁻¹ in the presence of 2MeS-ADP. Uninhibited platelets were prelabeled with PKH26 (blue) and CAM-treated platelets with CellVue Maroon (red). Shown are representative DIC (**A**) and dual-color fluorescence (**B**) images. Bars = 25 μm. See further, Figure 1.

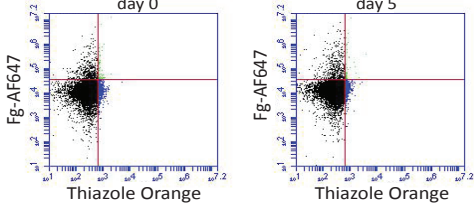
A Oligo-dT staining, unstimulated



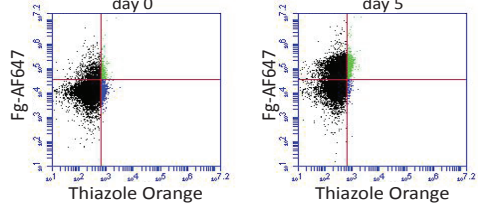
B Oligo-dT staining, ADP activated



C Thiazole Orange staining, unstimulated



D Thiazole Orange staining, ADP activated

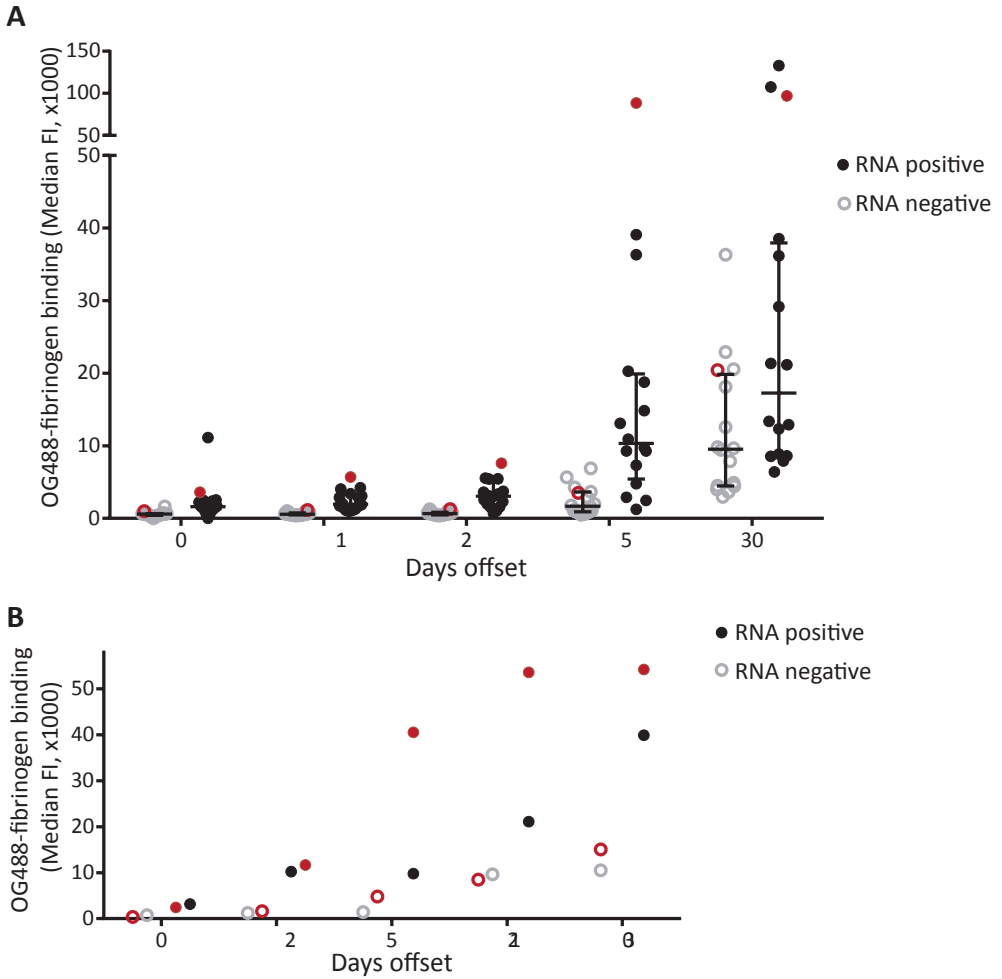


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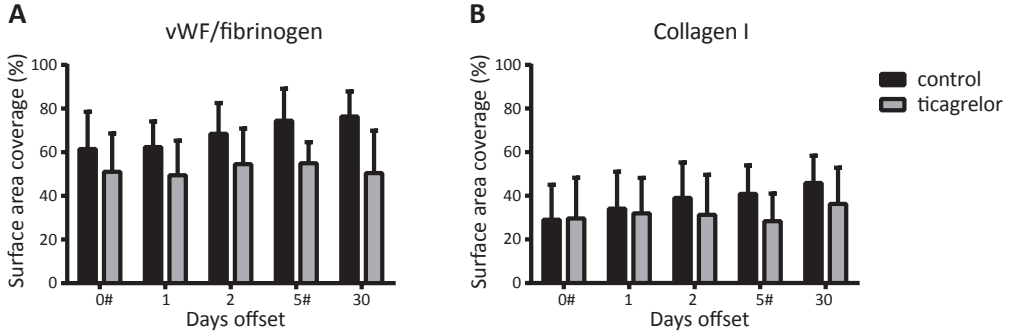
Activation ratio		day 0	day 1	day 2	day 5	day 30
Oligo-dT staining	Mature	0.17 (±0.03)	0.24 (±0.03)	0.33 (±0.03)	0.82 (±0.10)	2.70 (±0.26)
	Juvenile	0.44 (±0.08)*	0.66 (±0.11)*	0.84 (±0.09)***	1.81 (±0.22)**	6.30 (±0.99)*
Thiazole Orange	Mature	0.18 (±0.04)	0.24 (±0.04)	0.41 (±0.06)	1.02 (±0.12)	3.35 (±0.59)
	Juvenile	0.38 (±0.08)*	0.50 (±0.08)***	0.92 (±0.13)***	2.42 (±0.27)***	8.18 (±1.32)**

Supplemental Figure 3. Alternative analysis confirms higher reactivity of juvenile platelets and increased activation in time upon prasugrel offset Juvenile platelets were detected using the oligo-dT staining or the thiazole orange staining, as described for Figure 5. Shown are representative dot plots of oligo-dA/dT or thiazole orange vs. Fg-OG488/AF647. Thresholds for juvenile and mature platelets were based on the negative controls oligo-dA and PBS for the oligo-dT and thiazole orange staining respectively. Thresholds for activated platelets were based on unstimulated samples. Unstimulated juvenile platelets (blue) and activated juvenile platelets (green) are depicted. Representative dot plots of unstimulated (**A**) or ADP-activated (**B**) mature and juvenile platelets as identified by the oligo-dT staining. Representative dot plots of unstimulated (**C**) or ADP-activated (**D**) mature and juvenile platelets using the thiazole orange staining.

Table. Using the percentage of platelets in each quadrant of the dot plots, the activation ratio was calculated for both mature (Q-UL/Q-LL) and juvenile platelets (Q-UR/Q-LR) using either staining. Mean ± SEM (n =16), *p < 0.05, **p < 0.01, ***p < 0.001 vs. corresponding mature ratio (ANOVA with Bonferroni correction).

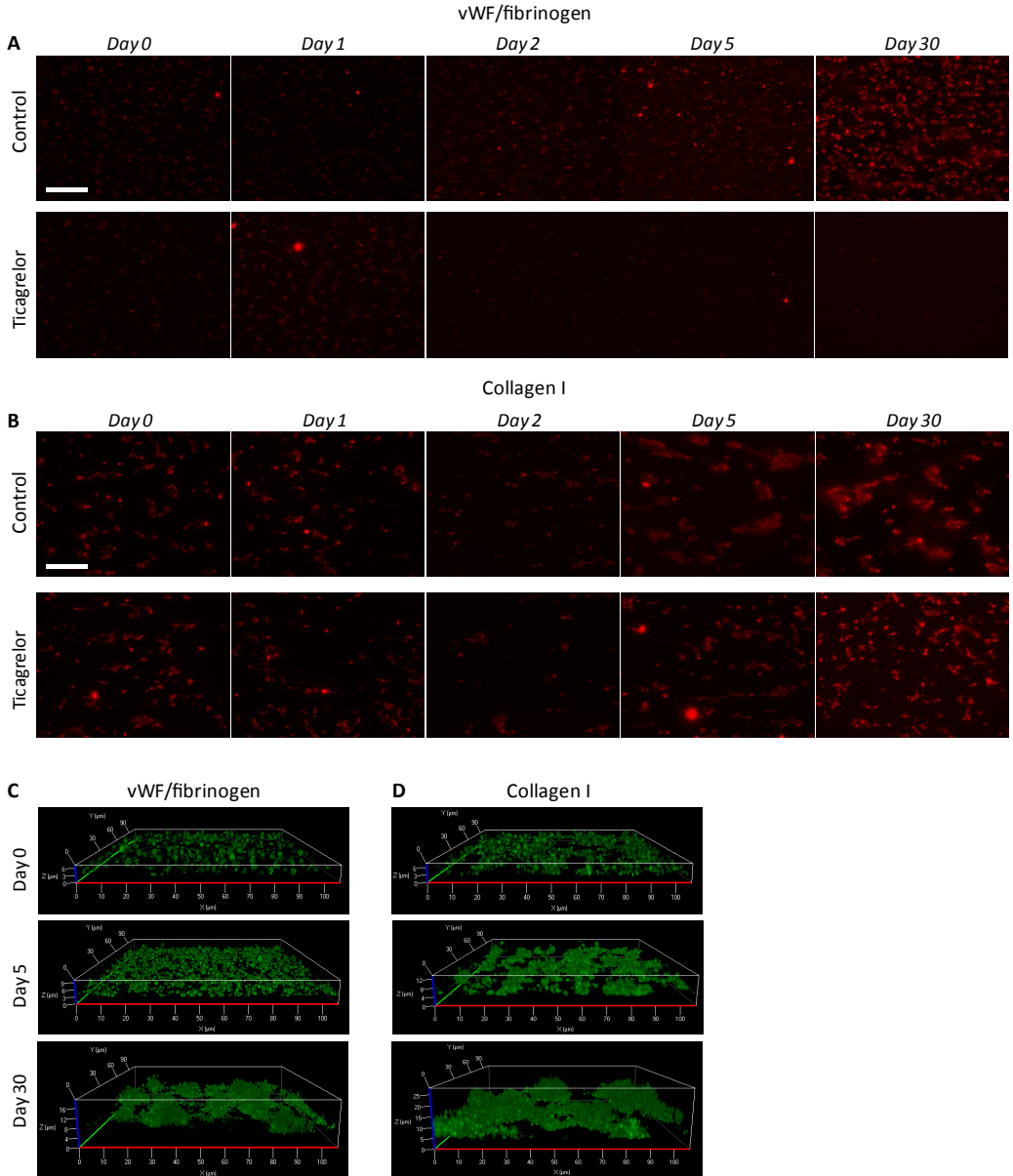


Supplemental Figure 4. Increased ADP-induced $\alpha_{IIb}\beta_3$ activation of juvenile platelets formed upon prasugrel offset. Platelets from patients during offset from prasugrel were activated and analyzed by flow cytometry. Juvenile platelets were identified by staining with the mRNA probe, 5'Cy5-oligo-dT. See further Figure 5. Washed platelets were activated with 1 μ M 2MeS-ADP in the presence of OG488-fibrinogen. The cells were fixed and permeabilized with saponin to allow staining of mRNA by incubation with 5'Cy5-oligo-dT. Shown is extent of $\alpha_{IIb}\beta_3$ activation of mRNA-positive and -negative platelets, per subject. **(A)** Data from 16 patients, medians \pm IQR. **(B)** Data from additional patients including day 12 time points. Red dots are from patients with a high immature platelet fraction (IPF \geq 7.0%).



Supplemental Figure 5. Unchanged platelet adhesion in thrombus formation under flow upon prasugrel offset. Whole blood samples from patients (at indicated days after stopping prasugrel intake) were co-perfused with 2MeS-ADP over microspots containing vWF/fibrinogen (A) or collagen type I (B), as in Figure 6. Brightfield images were analyzed for surface area coverage of all platelets (single platelets and aggregates) at different offset days. Means \pm SD (n = 16), ***p < 0.001 vs. day 0 (ANOVA with Bonferroni correction); #n = 15.

3



Supplemental Figure 6. Restored thrombus formation under flow upon prasugrel offset. Whole blood samples from patients (at indicated days after stopping prasugrel intake) were co-perfused with 2MeS-ADP over microspots containing vWF/fibrinogen or collagen type I, at a shear rate of 1600 s^{-1} for 4 minutes. See also Figure 6. Staining of thrombi was with AF647-fibrinogen and the platelet membrane label DiOC₆. Vehicle (control) or 10 μM ticagrelor was added to the blood. **(A, B)** Shown are (for a representative patient) fluorescence images of fibrinogen binding (red) of the thrombi formed on vWF/fibrinogen or collagen I. Bars = 25 μm . **(C, D)** Representative confocal z-stacks of thrombi on vWF/fibrinogen or collagen I stained with DiOC₆ (green).

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Chapter 4

Platelet control of fibrin distribution and microelasticity in thrombus formation under flow

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Abstract

Platelet- and fibrin-dependent thrombus formation is regulated by blood flow and exposure of collagen and tissue factor. However, interactions between these blood-borne and vascular components are not well understood. Here, we developed a method to assess whole blood thrombus formation on microspots with defined amounts of collagen and tissue factor, allowing determination of the mechanical properties and intrathrombus composition. Confining the collagen content resulted in diminished platelet deposition and fibrin formation at high shear flow conditions, but this effect was compensated by a larger thrombus size and increased accumulation of fibrin in the luminal regions of the thrombi at the expense of the base regions. These thrombi were more dependent on tissue factor-triggered thrombin generation. Microforce nanoindentation analysis revealed a significantly increased microelasticity of thrombi with luminal-oriented fibrin. At a low shear rate, fibrin fibers tended to lumenally cover the thrombi, again resulting in a higher microelasticity. Studies with blood from patients with distinct hemostatic insufficiencies indicated an impairment in formation of a platelet-fibrin thrombus in the cases of dilutional coagulopathy, thrombocytopenia, Scott syndrome and hemophilia B. Taken together, our data indicate that: (i) thrombin increases the platelet thrombus volume; (ii) tissue factor drives formation of fibrin outside of the platelet thrombus; (iii) limitation of platelet adhesion redirects fibrin from bottom to top of the thrombus; (iv) a lower shear rate promotes thrombus coverage with fibrin; (v) the fibrin distribution pattern determines thrombus microelasticity; and (vi) the thrombus-forming process is reduced in patients with diverse hemostatic defects.

Introduction

During hemostasis, blood flow and platelet activation in combined action with the coagulation system determine the formation of platelet-fibrin thrombi at the injured vessel wall. Multiple components of the damaged endothelial and subendothelial layers are known to influence the dynamics of thrombus growth and fibrin polymerization.¹ Key vascular constituents triggering this process are collagen fibers and tissue factor (TF), serving as potent platelet- and coagulation-stimulating agents, respectively.² Thrombin that is formed on the surface of activated platelets and on injured vascular cells is a central player in the hemostatic process by enhancing platelet activation, producing activated coagulation factors and converting fibrinogen into fibrin.³ Yet, the complex role of thrombin in formation of a platelet-fibrin thrombus under flow is at best incompletely studied.

Murine *in vivo* models of arterial thrombus formation have shown that the type and severity of vascular injury are determinative for the precise triggering of this process. Exposure of the vasculature to FeCl₃, resulting in endothelial denudation, leads to collagen-dependent platelet activation with an additional role of TF, particularly upon milder FeCl₃ injury.⁴⁻⁷ Thrombin and fibrin generation are elicited on procoagulant platelets exposing phosphatidylserine.^{8,9} Platelets, furthermore, control the coagulation process by secreting pro- and anticoagulant proteins, whereas there is ample evidence for a role of platelet-exposed TF.^{10, 11} On the other hand, in most laser-induced injury

models, thrombus formation is primarily triggered by thrombin that is generated via vascular TF.^{12, 13} Severe laser injury, however, also leads to collagen exposure and collagen-dependent platelet activation.^{14, 15}

Together, these *in vivo* findings indicate that the relative availability of platelet-adhesive (like collagen) and coagulation-triggering (like TF) substances determines the buildup of a thrombus and, by implication, the roles of thrombin and fibrin herein. In support of this idea, different patterns of fibrin deposition have been detected in injury models, such as fibrin structures around a thrombus,¹⁶ and thrombin/fibrin-rich areas in the thrombus core region.^{17, 18} How the platelet- and coagulant-activities of the thrombogenic surface, for example, the relative abundance of collagen and TF, influence the buildup and composition of a platelet-fibrin thrombus has not been examined so far.

Viscoelasticity is considered to be an important hemostatic property of a fibrin-containing thrombus. For instance, in patients with severe factor XI deficiency, a low density fibrin network is associated with impaired hemostasis.^{19, 20} However, the conditions that favor high elasticity of a platelet-fibrin thrombus or clot are largely unknown. The limited evidence available suggests that a local, high concentration of thrombin enforces the fibrin network,²¹ and that blood flow supports the alignment of stiff fibrin fibers.^{22, 23} Macroscopic thromboelastic studies have not come much further than demonstrating that, in patients with dilutional coagulopathy, the overall clot strength is predictive for the risk of perioperative bleeding.²⁴

In the present article, we investigated how the type of thrombogenic surface - with defined relative amounts of collagen and TF - affects the formation and composition of platelet-fibrin thrombi that are formed under high and low shear flow conditions. We developed standardized protocols, in which citrate-anticoagulated blood was flowed over defined collagen/TF microspots, and the deposition of platelets and fibrin(ogen) were assessed simultaneously. We then determined the distribution of fibrin within and outside the thrombus by confocal microscopy, as well as the microelasticity of the formed thrombi by a novel method of nanoindentation. The data reveal a surface- and flow-dependent thrombus buildup with a different location of fibrin.

Materials and Methods

Materials and methods are available in the Supplemental data.

Results

Collagen-TF surface determines buildup of platelet-fibrin thrombus and extent of fibrin formation under flow

To study the roles of platelets and coagulation in whole blood thrombus formation under flow, we applied a previously developed microspot method of thrombus formation in parallel-plate flow chambers,²⁵ which was adapted to operate under strictly controlled coagulant conditions. The changes included: (i) preparation of microspots with defined amounts of collagen and tissue factor (TF), (ii) co-infusion of citrate-anticoagulated

blood with Mg^{2+}/Ca^{2+} recalcification medium to obtain physiological concentrations of divalent cations; (iii) adequate mixing of blood with medium by pushing through a tube-slit converter in the flow chamber, and (iv) real-time confocal recording of both platelet

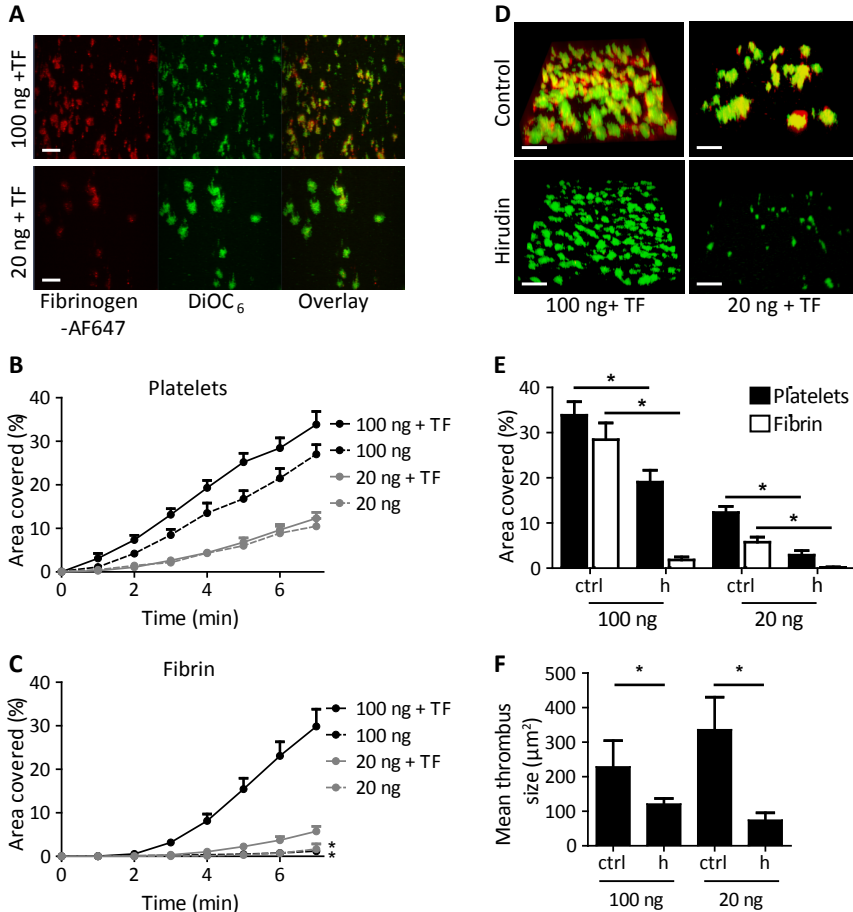


Figure 1. Collagen-tissue factor (TF) coating determines the buildup of platelet-fibrin thrombus formed under flow. Citrated whole blood was perfused during recalcification over microspots with collagen (20 or 100 ng) in the absence or presence of TF (10 pg) for 7 minutes at a wall shear rate of 1000 s^{-1} . Blood samples were prelabeled with DiOC₆ to label platelets (green) and with AF647-fibrin(ogen) to monitor fibrin formation (red). Control samples were pretreated with hirudin (h, 3 $\mu\text{g}/\text{mL}$), where indicated. Two-color microscopic confocal images were recorded in real time at frequency of 1 Hz. (A) Representative fluorescence images from collagen/TF microspots after 7 minutes of flow (bars, 50 μm). (B, C) Time-dependent accumulation of (B) DiOC₆-labeled platelets and (C) AF647-fibrin(ogen) during blood flow. (D) Representative stacks of thrombi in 3D (z-step 1 μm) of platelets (green) and fibrin(ogen) (red) on collagen/TF microspots after 7 minutes of flow (bars, 50 μm). (E) Fluorescence area covered by platelets and fibrin on collagen/TF microspots after 7 minutes of flow. (F) Average thrombus size on spot as determined by morphometric image analysis. Means \pm SEM ($n = 6-15$), * $p < 0.05$.

deposition and fibrin generated on the microspots using 3,3'-dihexyloxycarbocyanine (DiOC₆) and Alexa Fluor (AF)647-fibrinogen as probes, which were pre-added to the blood samples (Supplemental Fig. 1).

Pilot data indicated that co-coating of 10 pg TF per microspot resulted in optimal shortening of time to fibrin formation, as checked with bright-field image recording, such as in agreement with published findings.²⁶ Perfusion of blood samples at a shear rate of 1000 s⁻¹ over microspots enriched in collagen (100 ng per microspot) resulted in a rapid formation of platelet thrombi that were rich in fibrin (Fig. 1A-C). Lowering the collagen content (20 ng per microspot) significantly decreased platelet deposition after 7 minutes of perfusion (area covered, 12.3 ± 1.4 % vs. 33.8 ± 3.0 %; *p* = 0.005) and delayed the onset of fibrin formation (3 vs. 5 minutes), as compared to the high collagen microspots (Fig. 1A-C). In the absence of TF, when coagulation is triggered via the intrinsic pathway,²⁷ platelet deposition was decreased on high collagen but unaltered on low collagen, whereas fibrin formation was greatly delayed in either case (Supplemental Table 1). Pretreatment of the

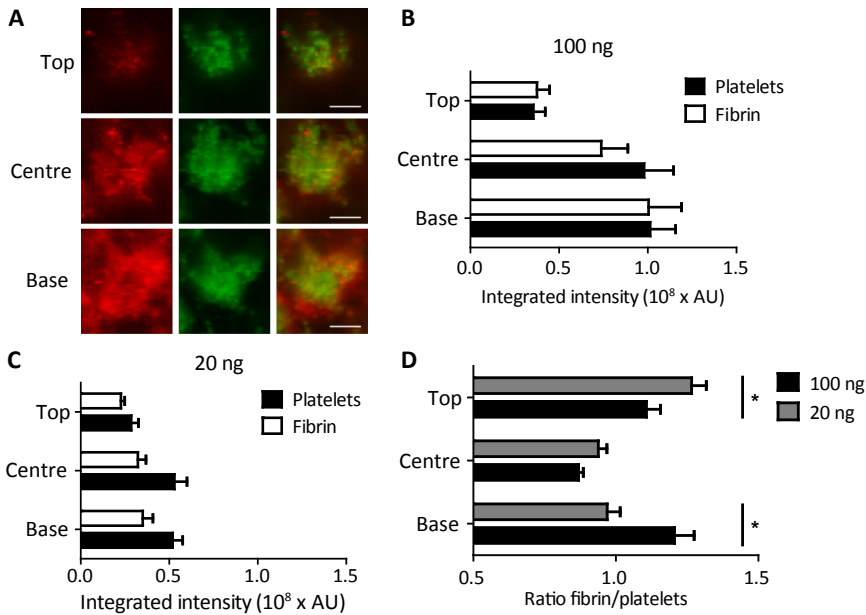


Figure 2. Collagen-tissue factor (TF) coating determines distribution of fibrin through a thrombus. Platelet-fibrin thrombi were formed by blood flow over microspots of collagen (20 or 100 ng) plus TF (10 pg) for 7 minutes, as described in Figure 1. Confocal images of DiOC₆ (green) and AF647-fibrin(ogen) (red) fluorescence in 3D were captured. (A) Representative fluorescence images of optical slices at base, center and top of thrombus on collagen (100 ng)/TF microspots (bars, 20 μm). (B, C) Integrated fluorescence intensity (arbitrary units, AU) of platelet (green) and fibrin (red) fluorescence from z stacks at base (<10 μm), center (10-20 μm) and top (>20 μm) of thrombi formed. (D) Ratio of platelet/fibrin fluorescence intensity at base, center and top of thrombi. Means \pm SEM (*n* = 6-7), **p* < 0.05.

blood with corn trypsin inhibitor to block the intrinsic coagulation pathway resulted in a delayed formation of fibrin only for microspots not containing TF (data not shown). In the absence of collagen (only TF), neither platelets nor fibrin deposited on the surface.

Reconstruction of stacks of confocal images in 3D indicated that the platelet-fibrin thrombi formed on high collagen microspots were relatively small and dense, when compared with the more dispersed thrombi formed on low collagen microspots (Fig. 1A, D). Persistent thrombin activity on each type of microspot was confirmed by the addition of a fluorogenic thrombin substrate, which was continuously cleaved (data not shown). Markedly, with hirudin added to the blood, platelet deposition decreased on high collagen microspots (surface area coverage from 33.8 to 19.1 %, $p = 0.001$), whereas on low collagen hardly any platelets were left (from 12.3 to 2.9 %, $p < 0.0001$). As expected, with hirudin present, fibrin formation was completely suppressed on either surface (Fig. 1E). Quantification also showed that hirudin significantly reduced the size of individual thrombi on the high collagen surface (from 228 ± 76 to $121 \pm 16 \mu\text{m}^2$, $p = 0.007$), a reduction that was even more pronounced on the low collagen surface (from 335 ± 94 to $74 \pm 21 \mu\text{m}^2$, $p < 0.001$) (Fig. 1F).

As microscopic images showed a gradual contraction of platelets in fibrin-containing thrombi, we aimed to quantify this process by addition of 3 % DiOC₆-labeled washed platelets to the blood, and then monitoring the movement of adhered platelets in time. It seemed that the overall displacement rate of platelets was high on microspots with collagen/TF during the time of fibrin formation ($1.06 \pm 0.11 \times 10^{-3} \mu\text{m/s}$), whereas it was non-significantly changed ($0.87 \pm 0.10 \times 10^{-3} \mu\text{m/s}$) in the presence of Gly-Pro-Arg-Pro, inhibiting fibrin polymerization, and significantly lowered in the presence of hirudin ($0.64 \pm 0.05 \times 10^{-3} \mu\text{m/s}$, $p = 0.0064$). Hence, under the present flow conditions, platelet contraction did occur in the absence of thrombin or fibrin but was enhanced by the formation of either. This resolves a dispute in the literature that platelet-fibrin retraction is considered a thrombin-dependent event²⁸ but can still occur under conditions where thrombin is blocked.²⁹

By lowering the wall shear rate from 1000 to 150 s^{-1} , platelet deposition on high or low collagen was reduced (Supplemental Table 1), as expected due to a diminished role of von Willebrand factor.²⁵ On the other hand, total fibrin formation did not reduce at lower shear rate (Supplemental Fig. 2). Co-localization analysis of fluorescence from platelets and fibrin indicated major extension of the fibrin fibers outside of platelet aggregates especially at low shear conditions (150 s^{-1}), and to a lesser extent at the higher shear rate (Supplemental Fig. 2). Jointly, these data indicate that limitation of the platelet-adhesive collagen surface resulted in an overall reduced platelet deposition, which was partly compensated by a larger thrombus size because of the activity of TF-triggered thrombin generation. Lowering of the shear rate also reduced platelet accumulation but in a relative way promoted fibrin formation.

Collagen-TF surface and shear rate define distribution of fibrin in a thrombus

To assess the amount and localization of thrombus-associated fibrin on different microspots, z stacks of confocal images of the fluorescent-labeled platelets and fibrin were captured at the end of high shear flow experiments (Fig. 2A). On microspots with

high collagen, the majority of platelet and fibrin fluorescence was located near the base (0-10 μm) and center regions (10-20 μm) of the thrombi. On microspots with low collagen, overall thrombus volume was reduced from 1.10 ± 0.20 to $0.46 \pm 0.10 \times 10^6 \mu\text{m}^3$ ($p < 0.001$; Supplemental Table 1). In this case, fluorescence from platelets and fibrin was more evenly distributed among the base, center and top regions of the thrombus (Fig. 2B, C). Markedly, the top regions of thrombi formed on low collagen showed a significantly higher ratio of fibrin to platelet fluorescence ($p = 0.022$), compared with those on high collagen (Fig. 2D). In contrast, the base regions of thrombi formed on low collagen had a lower fibrin/platelet ratio ($p = 0.013$), compared to the high collagen microspots.

The images were also analyzed for co-localization of platelets and fibrin, in order to assess the appearance of fibrin outside the aggregated platelets. On high collagen microspots, more fibrin fibers extended outside the platelet area at the base of the thrombi (28.8 ± 1.9 % of fibrin-positive pixels) than at the center and top regions of the thrombus (8.9 ± 2.1 % and 5.3 ± 1.8 % fibrin-positive pixels, respectively; $p < 0.001$). Fibrin outgrowth was further restricted on low collagen microspots, where only 9.7 ± 2.8 %, 2.3 ± 0.6 % and 5.2 ± 1.1 % of the fibrin fluorescence were detected outside the platelet aggregates, at the base, center or top regions of thrombi, respectively.

Interestingly, when compared with a high shear rate, at a low shear rate of 150 s^{-1} , overall thrombus volume reduced from 1.10 ± 0.20 to $0.78 \pm 0.02 \times 10^6 \mu\text{m}^3$ ($p = 0.032$) on high collagen and from 0.46 ± 0.10 to $0.29 \pm 0.04 \times 10^6 \mu\text{m}^3$ ($p = 0.026$) on low collagen. At low shear rate, the majority of the fibrin was located outside of the platelet regions, which were low in height, such as apparent from analysis of z stacks of confocal images (Supplemental Table 1).

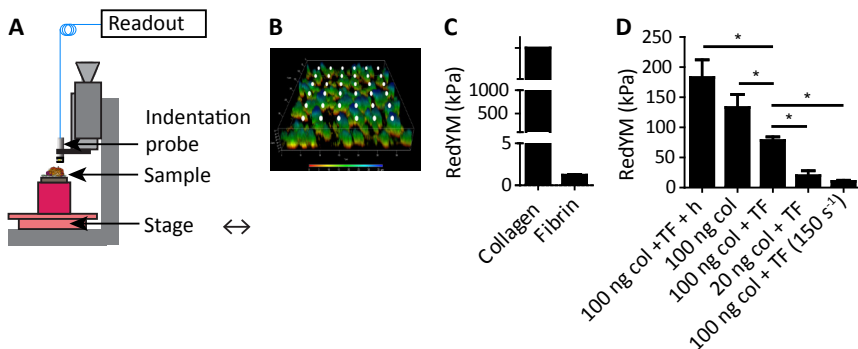


Figure 3. Collagen-tissue factor (TF) coating determines microelasticity of platelet-fibrin thrombus formed under flow. Platelet-fibrin thrombi were formed on microspots with collagen \pm TF, as described in Figure 1. Flow experiments were carried out at shear rate of 1000 s^{-1} , except where indicated otherwise (150 s^{-1}). After careful rinse with HEPES buffer pH 7.5, coverslips with thrombi were subjected to nanoindentation (6 \times 6 indentations/microspot). Control coverslips were used for scanning electron microscopy. (A) Schematic presentation of nanoindenter, featuring the indentation probe, sample and automated x-y stage. (B) Schematic grid pattern of preset 6 \times 6 indentations per microspot with height gradient. (C) Reduced Young's modulus (RedYM, kPa), a parameter inversely related to the microelasticity, in control measurements with a collagen-coated surface or with fibrinated plasma ($n = 16$). (D) RedYM assessed for thrombi formed on indicated collagen/TF surfaces ($n = 36-72$). Means \pm SEM, * $p < 0.05$, h indicates hirudin.

Collagen-TF surface and shear rate influence microelasticity of a thrombus

A novel method of ferrule-top nanoindentation, related to atomic force microscopy, was used to determine how the quantity and localization of fibrin fibers affected the mechanical and microelastic properties of the thrombi formed on microspots. This method is schematized in Fig. 3A, and described in more detail in the Supplement data. By gradually indenting and retracting a nanoindentation tip at multiple spots per coverslip, loading and unloading curves could be obtained, wherein slopes of the unloading curves informed on the reduced Young's modulus (RedYM), as an inverse measure for the microelasticity of the sample (Supplemental Fig. 3). By applying multiple nanoindentations per microspot surface in a grid-like pattern, high-resolution information on the microelasticity of the thrombi on the surface could be obtained (Fig. 3B). Uncoated (not shown) or collagen-coated coverslips without thrombi gave a high RedYM of >1000 kPa (Fig. 3C). In contrast, coverslips covered with coagulated, fibrin-containing plasma produced a low RedYM of 1.22 ± 0.1 kPa, thus pointing to high microelasticity of the fibrin network. Nanoindentation measurements of thrombi formed on high collagen (78.2 ± 5.9 kPa) and low collagen (20.0 ± 8.1 kPa) microspots resulted in different RedYM values ($p = 0.019$), indicating that the latter had a higher microelasticity (Fig. 3D). Similarly, for thrombi formed on high collagen, lowering of the shear rate from 1000 s^{-1} to 150 s^{-1} resulted in a lower RedYM ($p < 0.0001$) and hence a higher microelasticity (Fig. 3D). This suggested that intrathrombus fibrin increased the microelasticity of the sample. This was confirmed by nanoindentation analysis of fibrin-poor thrombi, formed on collagen without TF, giving high RedYM values of 133.1 ± 21.6 kPa. In the presence of hirudin to block thrombin activity, and hence fibrin formation, the RedYM even further increased to 182.9 ± 29.3 kPa (Fig. 3D). Together, this indicates that the thrombi with fibrin in the top or with outside coverage of fibrin, being formed on low density collagen microspots or at low shear rate, display a higher microelasticity, when compared with thrombi in which fibrin is accumulated at the base.

Formation of platelet-fibrin thrombus under conditions of impaired hemostasis

Using several approaches, we further assessed the roles of platelets and other blood components in the formation of fibrin-containing thrombi. Considering the diminished clotting activity of patients with perioperative dilutional coagulopathy,^{30, 31} we first determined the consequences of blood dilution *in vitro*. Blood samples were flowed at a shear rate of 1000 s^{-1} over high collagen/TF microspots. In the absence of dilution, this resulted in a high platelet deposition and fibrin formation (see above). However, dilution of the blood to 80, 60 and 40 % resulted in a gradual decrease in platelet deposition after 7 minutes of perfusion, amounting to 14 ± 2 % ($p = 0.046$), 10 ± 3 % ($p = 0.004$) and 8 ± 3 % ($p < 0.001$), respectively, in comparison with the undiluted sample. In contrast, amounts of fibrin only reduced at dilution to 60 and 40 % blood, which reduction was accompanied by a prolonged time to onset of fibrin formation (Fig. 4A, B). Comparable results were obtained under conditions of a low, venous shear rate of 150 s^{-1} (Supplemental Fig. 4). Reconstitution of the 40 % diluted blood with either washed platelets or red blood cells

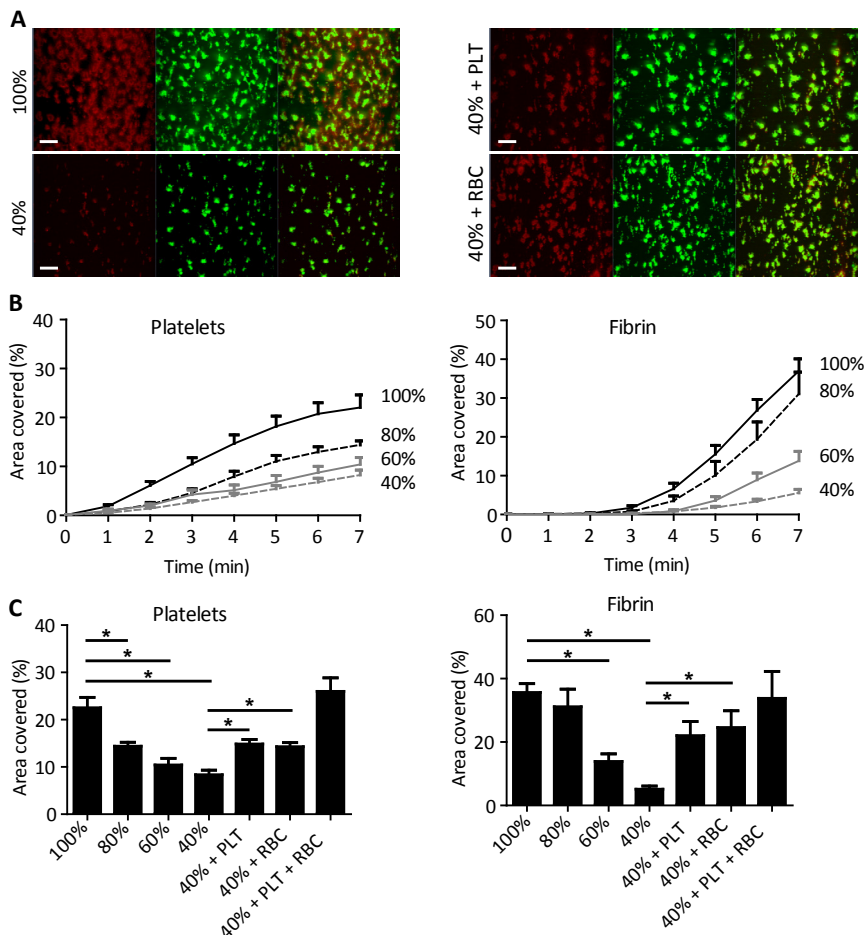


Figure 4. Restoration of platelet-fibrin thrombus formation by reconstitution of diluted blood. Platelet-fibrin thrombi were formed on microspots with collagen (100 ng) and tissue factor (TF; 10 pg), as described in Figure 1. Blood samples were diluted to indicated percentages with saline (keeping fixed concentrations of citrate, MgCl₂ and CaCl₂). Specific samples were reconstituted with autologous washed platelets (PLT) or red blood cells (RBC), added to the original counts. **(A)** Representative fluorescence images of DiOC₆-labeled platelets (green) and AF647-fibrin (red) after 7 minutes of flow using 100 % or 40 % blood with(out) reconstitution (bars, 50 μm). **(B)** Time-dependent accumulation of fluorescence from platelets (green) and fibrin (red) during perfusion. **(C)** Fluorescence area covered after 7 minutes of flow by platelets and fibrin. Means ± SEM (*n* = 5-12), **p* < 0.05.

caused partial improvement of both platelet deposition and fibrin formation (Fig. 4A, C). Interestingly, complete recovery of the thrombus-forming process was obtained by addition of platelets in combination with red blood cells (Fig. 4C). These data pointed to

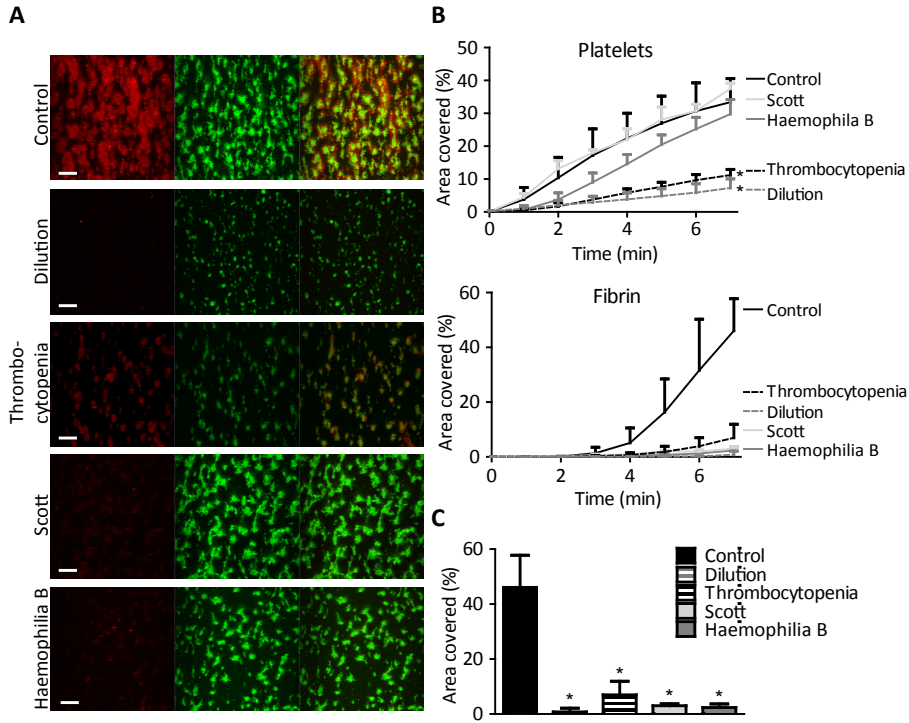


Figure 5. Impaired platelet-fibrin thrombus formation using blood from patients with impaired hemostasis. Blood was obtained from 7 healthy control subjects, 3 patients with dilutional coagulopathy because of massive fluid infusion during cardiothoracic surgery, 2 patients with acquired thrombocytopenia, 1 Scott syndrome, or 2 patients with hemophilia B. At least 3 blood samples were analyzed per condition. Platelet-fibrin thrombi were formed in a standard way by blood flow at 1000 s^{-1} over microspots with collagen (100 ng) and TF (10 pg), as described in Figure 1. (A) Representative images of DiOC₆-labeled platelets (green) and AF647-fibrin(ogen) (red) after 7 minutes (bars, 50 μm). (B) Time-dependent accumulation of platelet and fibrin fluorescence during blood perfusion. (C) Fluorescence area covered by fibrin after 7 minutes of flow. Means \pm SD ($n = 6-9$), *, significantly different from reference range of control blood samples.

a limiting role of platelet adhesion (enforced by marginalization with erythrocytes) not only for platelet aggregation but also for fibrin formation under conditions of high shear flow. This was confirmed with scanning electron microscopy (Supplemental Fig. 5).

As a second approach, blood was used from various patients with hemostatic insufficiencies and a bleeding risk. Blood samples were obtained from 3 patients with dilutional coagulopathy because of massive fluid infusion during surgery. In this case, only few platelet aggregates of small size were formed and no fibrin was generated during the measured time of 7 minutes (Fig. 5A). With blood samples from 2 thrombocytopenic patients, platelet deposition was comparably low, although measurable amounts of fibrin were formed (Fig. 5A, C), such in accordance with normal coagulation activity in this condition. A requirement for platelet procoagulant activity (phosphatidylserine exposure) was investigated with blood samples from a Scott patient, whose platelets lack this property.³² Whereas platelet deposition and aggregate size were not affected,

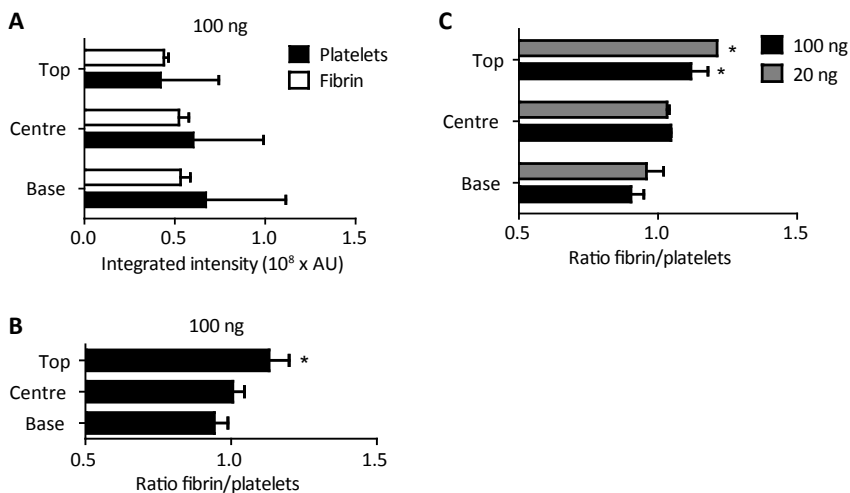


Figure 6. Altered fibrin distribution within thrombus formed by thrombocytopenic blood. Platelet-fibrin thrombi were formed by blood flow over microspots with collagen (20 or 100 ng) and tissue factor (TF; 10 pg), as described in Figure 1. Confocal images of DiOC₆ (green) and AF647-fibrin (red) staining in 3D were captured. **(A)** Integrated fluorescence intensity (arbitrary units, AU) of platelet (green) and fibrin (red) fluorescence from z stacks at base (>10 μm), center (10–20 μm) and top (>20 μm) regions of thrombi after 7 minutes of blood perfusion on high collagen/TF. **(B, C)** Ratio of platelet/fibrin staining at base, center and top of thrombi after 7 minutes on high collagen **(B)** or after 12 minutes **(C)** of blood perfusion on low and high collagen. Means \pm SD ($n = 3$), * $p < 0.05$ vs. base.

fibrin formation was essentially absent in the Scott blood samples. Similarly, impaired coagulation, as in blood from 2 patients with hemophilia B (~5 % factor IX), led to normal platelet aggregation under flow, but an impaired fibrin formation (Fig. 5A, C).

Because the flow of thrombocytopenic blood over high collagen microspots still gave limited fibrin formation, the intrathrombus localization of fibrin could be analyzed in more detail. Analysis of z stacks of confocal images indicated that fibrin and platelets were similarly distributed throughout the thrombi (Fig. 6A). Yet, the ratio of fibrin to platelets was higher in the top regions of thrombi (Fig. 6B), with only limited fibrin extending from the platelet aggregates at base areas ($12 \pm 8\%$ of fibrin-positive pixels). This fibrin distribution pattern, concentrated in platelet regions, persisted even after prolongation of the perfusion time to 12 minutes (Fig. 6C). After 12 minutes, at high and low collagen microspots, fibrin deposition outside the platelet regions at the thrombus base gradually increased to $34 \pm 14\%$ and $6 \pm 1\%$, respectively. Jointly, these results pointed to platelet control of fibrin formation and distribution in thrombi formed on collagen/TF surfaces, relying on phosphatidylserine exposure.

Discussion

This study shows that, under conditions of high shear blood flow and coagulation, platelet deposition and platelet-dependent fibrin formation are decreased when collagen as a platelet-activating substrate becomes limited. Although fewer thrombi are formed

on microspots with low collagen, we see a partly compensating effect in that thrombus size is increased and the contribution of thrombin to thrombus growth is relatively more important. Nanoindentation measuring viscoelastic properties of the sample indicates that the thrombi formed on low collagen possess enhanced microelasticity because of fibrin accumulation in the luminal region of thrombi. Interestingly, redistribution of fibrin from the base to the luminal region of thrombi is also observed under conditions of thrombocytopenia. Hence, a limitation of either the platelet-activating surface or the platelet number leads to reductions in platelet deposition and fibrin accumulation, which effects are accompanied by an altered fibrin distribution throughout the thrombus. Blood flow at low shear rate similarly enhances microelasticity by reducing platelet deposition and increasing the formation of a fibrin coat surrounding the thrombi.

Model of thrombus buildup, determining fibrin distribution and microelasticity

Platelet adhesion to collagen is known to result in a sustained rise in cytosolic calcium and phosphatidylserine exposure. This procoagulant surface greatly promotes the assembly of coagulation factors culminating in the formation of factor Xa and thrombin.² Earlier, in flowing mouse blood, we have established that in the presence of collagen and TF this platelet-dependent factor Xa formation is essential for the propagation of fibrin formation and for boosting platelet phosphatidylserine exposure.³ The present results allow to extend this coagulation-platelet activation cross talk to the human system, in that we find that human deficiency in factor IX (hemophilia B patient) or deficiency in platelet phosphatidylserine exposure (Scott syndrome patient) results in almost complete abolition of fibrin formation under flow conditions.

Although in the past years progress has been made in determining the mechanical properties of fibrin fibers formed under static conditions, little is known about the elasticity of platelet-fibrin thrombi formed under flow. Recently, a new method of nanoindentation has been used to assess the microelasticity of clotted murine platelet-rich plasma.³³ In this study, we have used this technique to measure the physical characteristics of human thrombi formed at high and low shear rates and find that thrombi with a high fibrin content in the thrombus top region are highest in microelasticity. Jointly, our results indicate that not so much the fibrin content, but rather the fibrin distribution through a thrombus determines its elastic properties. We hypothesize that the high microelasticity of thrombi with luminal-oriented fibrin ameliorates the hemostatic process. In agreement with this, thromboelastometry studies have indicated that a high clot elasticity associates with less bleeding in patients with hemostatic insufficiencies.^{20,34} If platelet adhesion is limited (low collagen), we see a relatively high fibrin content in the top region of an apparently 'loose' thrombus. On the other hand, at high platelet adhesion (high collagen), fibrin appears to be primarily formed at the base region of thrombi. Summarizing these data as in Figure 7, we conclude that: (i) the presence of thrombin increases platelet-thrombus volume independent of the shear rate; (ii) the presence of TF drives fibrin formation outside of the thrombus; (iii) limitation of platelet adhesion redirects fibrin from the bottom to the

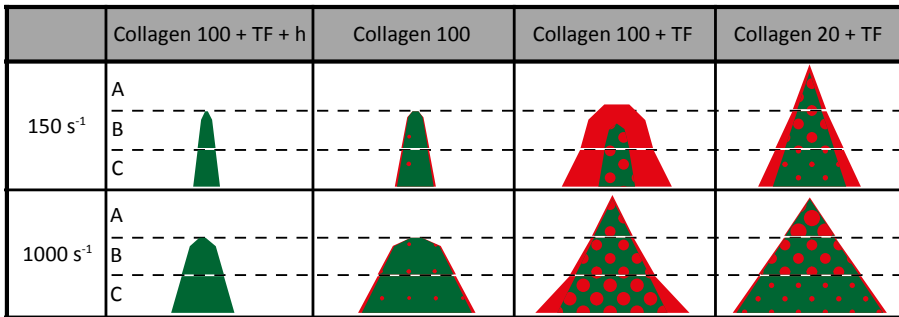


Figure 7. Schematic representation of thrombus buildup on collagen/TF surfaces. Platelet-fibrin thrombi were formed by blood perfusion over microspots with indicated amounts of collagen and TF at indicated shear rates, as described in Figure 1. In specific blood samples, thrombin was inhibited with hirudin (+ h). Multiple parameters of thrombus formation were assessed; for full data see Supplemental Table 1. Cartoons illustrate key characteristics of thrombi in terms of: width (scale 1-5), height (scale 1-3), fibrin inside platelet thrombus (sphere size: 1-4), and fibrin outside platelet thrombus (size of shell). Presentation is for three thrombus zones: **A**, <10 μm from microspot surface; **B**, 10-20 μm ; **C**, >20 μm .

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top of the thrombus; (iv) lowering of the shear rate results in a more fibrin rich thrombus; and (v) the thrombus microelasticity is determined by the distribution pattern of fibrin.

Comparison to *in vivo* models

These findings provide an extension of the hemostatic thrombus model, based on *in vivo* observations, presented by the groups of Brass and Diamond, in which thrombin activity is confined to the dense thrombus core.^{18,35,36} Previous *in vivo* studies have shown that, upon laser-induced injury of arterioles, where exposed TF drives the thrombotic process, fibrin concentrates near the vessel wall and in the vascular-oriented part of the thrombus.^{6,12,14} Our findings suggest that this vascular-oriented fibrin distribution pattern points to a relatively high abundance of platelet-adhesive substrates. These substrates can be collagens, as well as other vascular components, such as laminins and von Willebrand factor. In other words, it seems that the 'strength' of the platelet-adhesive surface controls the platelet packing density during thrombus buildup, and thereby the thrombin retention and fibrin distribution pattern. Confirmative evidence for this hypothesis comes from *in vivo* studies using PAR4-deficient mice, in which fibrin was found to redistribute through the whole thrombus as a consequence of diminished platelet activation.¹⁶

A different pattern of fibrin distribution occurs under low shear flow conditions, where relatively more (microelastic) fibrin is formed, appearing as a coat that covers the thrombi. This might be explained by a reduction in the flow-dependent removal of thrombin and fibrin monomers.³⁷ In accordance with our results, it has been shown that the

thrombi formed in mesenteric venules after laser injury are surrounded by a fibrin cap.³⁸

Effects of hemostatic insufficiencies

Under conditions of (perioperative) dilution, we find that platelet deposition is more sensitive to blood dilution than fibrin formation, which is impaired already at 60 % blood. This contrasts to static measurements of macroscopic clot strength by thromboelastometry, which seemed to be less sensitive to dilution effects.³⁰ Single-pass flow perfusion likely makes the difference here because under flow the deposited platelets provide a rate-limiting surface for the formation of thrombin and subsequent fibrin, whereas the same flow removes procoagulant factors and hence restricts fibrin formation.³⁷ Remarkably, in reconstitution experiments, the addition of both platelets and red blood cells was required for complete reversal of dilution effects. By implication, this indicates that under conditions of flow and sufficient margination of platelets by red blood cells, a coagulant activity of >60 % is required for unrestricted generation of fibrin. These findings are supported by hydrodynamic simulations indicating that platelet interactions with the vessel wall are promoted by an increasing hematocrit.³⁹

A limitation of the present study is the absence of vascular cells such as endothelial cells, which can also provide a procoagulant surface for the formation of thrombin.⁴⁰ On the other hand, current overviews indicate that collagen (TF)-based *in vitro* flow studies compare well with *in vivo* arterial thrombosis models, when evaluating the functional effects of genetic knockout in mice.^{41, 42} Hence, we consider the present test of formation of platelet-fibrin thrombi under flow as a valid method to determine the hemostatic capacity of a blood sample. For fibrin localization we used fluorescently labeled fibrinogen and therefore the distinction between fibrinogen and fibrin cannot be made unambiguously. We minimized the contribution of fibrinogen to the fibrin signal by threshold settings and the presence of fluorescent fibrin fibers was always confirmed from bright-field images. The advantage is that this method prevents underestimation of the fibrin signal as a consequence of poor permeation in the thrombus when using a fibrin antibody.

In summary, we conclude that under conditions of flow and coagulation the amount and localization of fibrin are regulated by the relative abundance of triggers for platelet adhesion/activation (*e.g.* collagen) and coagulation (TF), and that the fibrin distribution is determinative for the clot microelasticity. Thrombin herein influences thrombus growth as well as fibrin formation, depending on its local concentration.

Sources of funding

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Disclosures

NR, EJB and KL are employees of Optics11, Amsterdam, The Netherlands. Other authors have nothing to disclose.

Significance

Activation of blood platelets as well as the coagulation system is required for a normal hemostatic response. While it is known that platelet- and fibrin-dependent thrombus formation is regulated by blood flow, the blood composition and vascular triggers such as collagen and tissue factor, the interactions between these blood-borne and vascular components are not well understood. Our data imply that the relative abundance of vascular triggers of platelet adhesion/activation (collagen) at one side and of coagulation (tissue factor) at the other side is determining for both thrombus growth and thrombus composition, in particular regarding the distribution of fibrin. Furthermore, we show that the fibrin distribution is determinative for the thrombus microelasticity as assessed by nanoindentation.

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Supplemental data

Methods

Healthy volunteers and patients

Blood was taken from healthy volunteers and patients after informed consent was obtained in accordance with the Declaration of Helsinki. Studies were approved by the local medical ethics committee (METC 10-3-023). All donors were free from antiplatelet and anticoagulant medication for at least 2 weeks. Blood was taken from healthy control donors and from two patients with hemophilia B (both 5 % factor IX), a patient with Scott syndrome (deficient anoctamin 6 expression),¹ two patients with immune-induced thrombocytopenia (platelet count $85 \times 10^9/L$ and $22 \times 10^9/L$), or from three patients with dilutional coagulopathy due to massive fluid infusion during cardiothoracic surgery (4.5-5 L), collected in the operating theatre. At least three blood samples were analyzed for all conditions.

Blood collection and preparation

Blood was collected in 1:10 (v/v) 3.2 % trisodium citrate for whole blood perfusion experiments and the preparation of washed red blood cells, or in 1:6 (v/v) acidic citrate dextrose (ACD, 80 mM trisodium citrate, 52 mM citric acid and 180 mM glucose) for the isolation of washed platelets. Platelets and red blood cells were isolated as described.² Cell count was determined using a thrombocounter (Coulter Electronics, High Wycombe, United Kingdom).

Formation of platelet-fibrin thrombi under conditions of coagulation and flow

Thrombus formation under flow conditions was determined by perfusion of citrated whole blood over a collagen/tissue factor (TF) coated glass coverslip in a transparent parallel-plate perfusion chamber (width 3 mm, depth 50 μ m, length 300 mm).³ Coverslips were coated with microspots containing 20 or 100 ng Horm type I collagen (Nycomed Pharma, Munich, Germany) in the absence or presence of 10 pg recombinant human TF (Innovin; Dade Behring, Deerfield IL, USA). After coating, surfaces were blocked with 1 % bovine serum albumin (BSA; Sigma, St. Louis MO, USA) in Hepes buffer pH 7.45 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$), and used within 4 hours. Immediately before use, blood samples were pre-labeled with the membrane probe DiOC₆ (f.c. 0.5 μ g/mL, Anaspec, Reeuwijk, The Netherlands) to identify platelets (green) and with Alexa fluor (AF)647-fibrinogen (f.c. 16.5 μ g/mL, Molecular Probes, Life Technologies, New York NY, USA) to detect fibrinogen and fibrin (red). Using two pulse-free micro-pumps (Model 11 Plus, 70-2212, Harvard apparatus, Holliston MA, USA) and a Y-shaped flattened mixing tube, blood samples in 1 mL plastic syringe (Becton Dickinson, Franklin Lakes NJ, USA) were mixed with coagulation medium (Hepes buffer pH 7.45 supplemented with 32 mM $MgCl_2$ and 63 mM $CaCl_2$) also in 1 mL syringe at a volume ratio of 10:1 (push mode). This resulted in complete mixing and physiological (mM) concentrations of Ca^{2+} and Mg^{2+} .⁴

Where indicated, blood samples were pre-treated with 3 $\mu\text{g}/\text{mL}$ hirudin (Refludan; Schering, Berlin, Germany) to block formed thrombin. Blood perfusion over microspots in the flow chamber was at a wall shear rate of 150 or 1000 s^{-1} for at least 7 minutes.

For assessing platelet contraction in a thrombus, citrated blood samples were spiked with 3% of autologous, washed DiOC₆-labeled platelets. Where indicated, spiked blood samples were pre-treated with Gly-Pro-Arg-Pro (GPRP) or hirudin.

Two-color confocal fluorescence microscopy and image analysis

During or after blood perfusion, two-colored images were recorded using a fast line-scanning Zeiss LSM7 system, equipped with OPSS lasers and a 40 \times oil-immersion objective (numerical aperture 1.30) (Carl Zeiss, Oberkochen, Germany). Confocal fluorescence images (16 bit, 1024 \times 1024 pixels, single channel plus overlay) were obtained at both 488 and 635 nm excitation. Fluorescence was detected with an ultra-sensitive CCD line camera after selection by optical emission filters of 495-555 nm and 665-750 nm (pinhole 1 AU). For time series, fluorescence images were taken at 1 s intervals; for z stacks, fluorescence images were taken at 1 μm z-steps.

Image analysis of morphometry and fluorescence was performed with Live7 Metamorph software, version 7.5.0.0 (MDS Analytical Technologies, Sunnyvale, Canada) and ImageJ (version 1.48g; Rasband, NIH, Bethesda, MD, USA), as described elsewhere.⁵ For the quantification of fibrin and platelet fluorescence, images were thresholded to eliminate background. Integrated fluorescence intensity as well as percentage area covered were calculated per image. For co-localization of two-color fluorescence, binary images were created based on threshold to determine overlap percentages. As a standard, integrated fluorescence intensity was compared to surface area coverage of fluorescence features. Considering the overall similarity of these two analyses (Supplemental Fig. 1), for most conditions data of surface area coverage are given only.

For the quantification of (fluorescent) fibrin formation, threshold levels of fibrinogen binding to platelets was determined under conditions where coagulation was inhibited. Image intensities above this threshold were considered as originating from fibrin fibers. The presence of fluorescent fibrin fibers was always confirmed from brightfield images. Times to onset of fibrin formation were the moments at which the fluorescence threshold was passed, as confirmed by visual inspection of brightfield images.

For measurement of platelet contraction in a thrombus, time series of DiOC₆-labeled images were collected during flow. Movement towards each other of labeled platelets within thrombi was analyzed off-line via tracking analysis, as described.⁶ In brief, individual images of time-lapse series were thresholded to a binary images. Using the Mtrack2 plugin displacement was measured by including objects (platelets) of <5

pixels which were present >60 seconds. Objects were rejected when the displacement was >25 pixels s⁻¹ (indicating noise or platelets not part of one thrombus). Calculated was per condition the average movement of platelets in time (µm/s).

Dilution and reconstitution of whole blood samples

Citrated blood was diluted in vitro with saline medium, consisting of 154 mM NaCl, 10.8 mM trisodium citrate, 2 mM CaCl₂ and 2 mM MgCl₂, in order to keep equal concentrations of free Ca²⁺ and Mg²⁺ in all diluted samples.² Washed platelets and/or red cells were added to diluted blood samples to restore original cell counts, as described before.² Amounts of fluorescent labels, DiOC₆ and AF647-fibrinogen, were adjusted according to the extent of dilution and reconstitution.

Measurement of thrombin generation on microspots in flow chamber

Fluorogenic thrombin substrate Z-Gly-Gly-Arg-AMC (f.c. 0.5 mM, Thromboscope, Maastricht, The Netherlands) was added to blood samples where indicated, and used to measure thrombin generation on preformed thrombi under static conditions.⁷ Real-time fluorescence accumulation due to substrate cleavage was recorded at 447/30 nm emission every 30 seconds for at least 5 minutes, using an inverted fluorescence microscope equipped with an Olympus 60 × oil-immersion objective (numerical aperture 1.35), equipped with a 360 nm led cube (EVOS, Life Technologies, Bleiswijk, The Netherlands).⁵ Temporal analysis of fluorescence was performed using ImageJ.

Determination of microelasticity of thrombi on microspots by nanoindentation technology

Microelasticity of formed thrombi on coverslips was analyzed with a recently developed Piuma nanoindenter (Optics11, Amsterdam, The Netherlands), which features a calibrated nanoindentation probe consisting of a cantilever fabricated on top of a ferruled optical fibre. Recent developments in indentation probe fabrication have resulted in probes that can measure soft (biological) materials, e.g. cartilage and vascular models. These probes measure the material hardness in the Pascal to kilo-Pascal range.⁸ In atomic force microscopy a cantilever tip is used that is repelled from the surface by minute forces. In contrast, in nanoindentation methods a spherical, cylinder or Berkovich shaped tip is used that is forced into the sample. The employed Optics11 Piuma Nanoindenter system has been validated before with biological samples.⁹⁻¹¹

In brief, a probe with a defined cantilever of 1.39 N/m and calibrated spherical tip of 56.5 µm was gradually brought into contact with the sample surface, up to a depth of maximum 10 % of the sample thickness.¹² The counter-force of the sample on the tip results in cantilever bending, which is linear with the load on the cantilever. The result is a load-displacement curve of both loading (indenting) and unloading (retraction) of the probe. The probe, fixed on a closed-loop z-piezoelectric translator, was equipped with an integrated strain gage sensor and controlled by a closed-loop controller. From the slope of the unloading curve, the maximum load and the indentation area (tip surface area), a reduced Young's modulus (RedYM) can be calculated as a proxy measure of microelasticity.¹³ The modulus is the amount of force per unit area (stress), needed to

reach a certain amount of deformation (unit = Pa). Hence, a higher RedYM indicates that the given material is harder to deform. This measurement outcome is similar to atomic force microscopy.¹⁴

As a conservative rule of thumb, the indentation value is independent of the height and size of a sample, provided that the indentation depth is smaller than the sample radius and <10 % of the sample height.^{12, 15} With these criteria met for the present conditions, the RedYM reflects the elastic modulus of platelet-fibrin thrombi. The attractiveness of the nanoindentation technique is that the mechanoelastic properties of a microscopic sample can directly be determined without any further sample knowledge. A drawback is the lack of visual feedback of the probe location above the thrombus sample. To overcome this, we have indented all samples (old and new conditions) 36 times in a grid-like pattern. With an optical magnifier, we further confirmed that the right spots on the sample were indented.

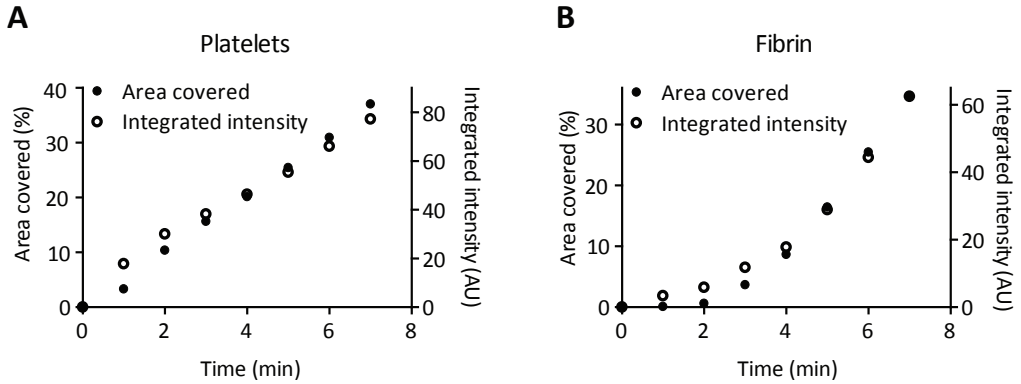
Coverslips with thrombi were carefully washed and incubated with Hepes buffer pH 7.45. The diameter of the contact area of indentation tip with thrombi was $\sim 20 \mu\text{m}^2$. Per microspot, 6 x 6 indentations were performed following a gridding pattern with a step-size of 50 μm . All indents were depth controlled (1 μm to a maximum of 5 μm). Loading and unloading times were set at 3 seconds, data were collected for 1 second per step. The unloading curves were used to calculate slopes using the compliance method of Oliver and Pharr in order to calculate the RedYM.^{13, 16} This is a measure of the microrigidity of the indented spot, which is inversely related to the microelasticity. A high RedYM reflects high microrigidity or stiffness of the indented sample and, hence, low microelasticity. Of the grid nanoindentations, only curves reflecting thrombi on glass were included. As negative and positive controls bare collagen surfaces and macroscopic fibrin clots were used, which showed a low and high microelasticity, respectively. Furthermore, scanning electron microscopy pictures were obtained for direct comparison of the dimensions of tip and thrombi.

Scanning electron microscopy

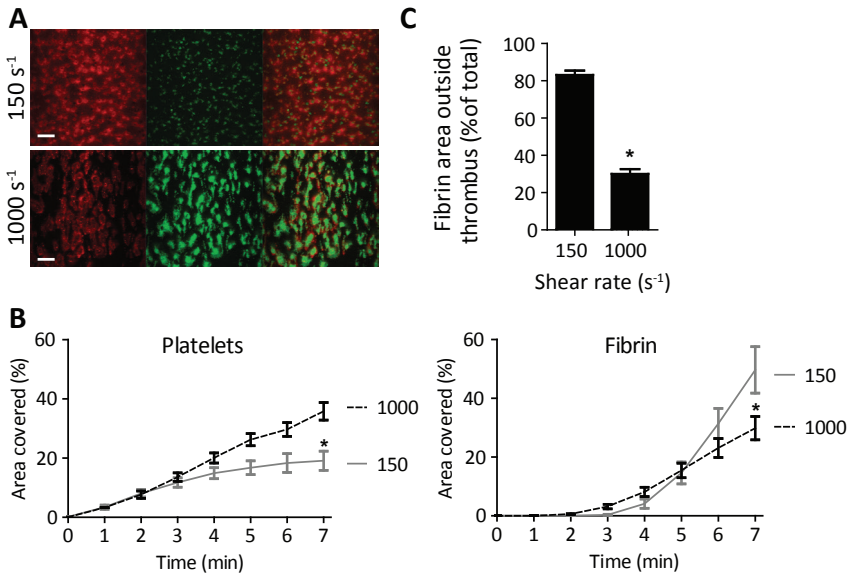
Thrombi were formed on microspots in a perfusion chamber, as described above, and prepared for scanning electron microscopy. In short, after disassembly of the perfusion chamber, coverslips were removed and gently washed with Hepes buffer pH 7.45. Fixation was with 2.5 % glutaraldehyde in 0.1 M K-phosphate buffer pH 7.4 at 4 °C. Following a rinsing step with 0.1 M phosphate buffer, samples were dehydrated in graded ethanol series, then critical point dried, mounted with silver paint on specimen stubs, and coated with gold. Thrombi were visualised with a Philips XL30 scanning electron microscope at 10 kV (Eindhoven, The Netherlands).

Statistics

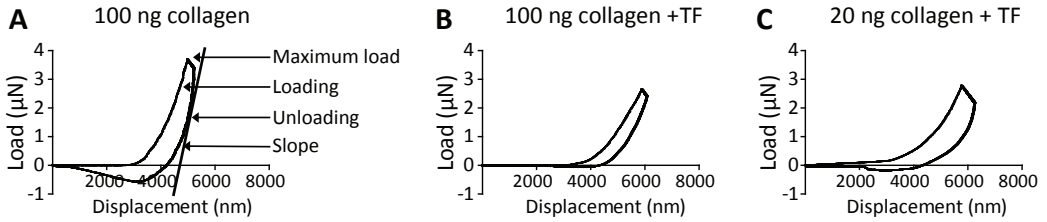
Statistical significance of differences between two independent groups was determined using the independent samples *t* test. Differences with *p* values <0.05 were considered significant. Error bars indicate inter-individual variability, except for patient data, where the variation of independent measurements is shown; values outside reference ranges of controls (mean \pm 2SD) were considered to be different.



Supplemental Figure 1. Quantification of platelet-fibrin thrombus formation on collagen/TF surfaces. Citrated whole blood was perfused during recalcification over microspots with collagen (100 ng) and TF (10 pg) for 7 minutes at a wall shear rate of 1000 s⁻¹. Blood samples were pretreated with DiOC₆ to label platelets (green) and with AF647-fibrinogen (red). **(A, B)** Analysis of integrated fluorescence intensity (open) and surface area coverage above background (close) from deposited platelets **(A)** and fibrin **(B)**. Note high similarity of either analysis parameter.

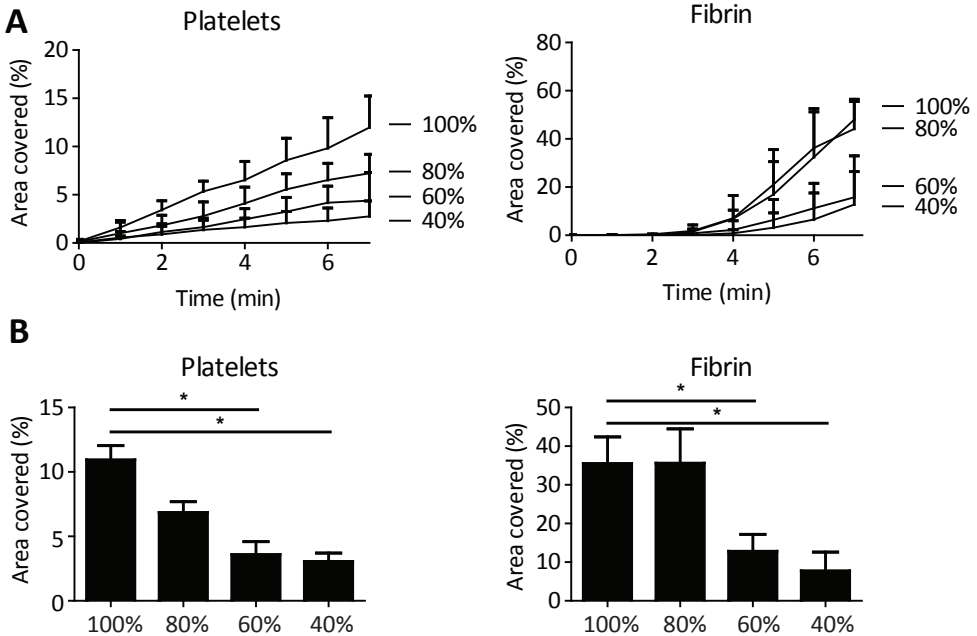


Supplemental Figure 2. Effect of wall shear rate in platelet-fibrin thrombus formation on collagen-tissue factor surfaces. Platelet-fibrin thrombi were formed by blood perfusion over microspots with collagen (100 ng) and TF (10 pg), as in Figure 1. Wall shear rate was 150 or 1000 s⁻¹. **(A)** Representative images after 7 minutes of flow of DiOC₆-labeled platelets (green) and AF647-fibrinogen (red) (bars, 50 μm). **(B)** Time-dependent deposition of fluorescent-labeled platelets and fibrin during blood perfusion. **(C)** Quantification of platelet-fibrin co-localization at indicated shear rates. Means ± SEM (*n* = 5-10), **p* < 0.05.

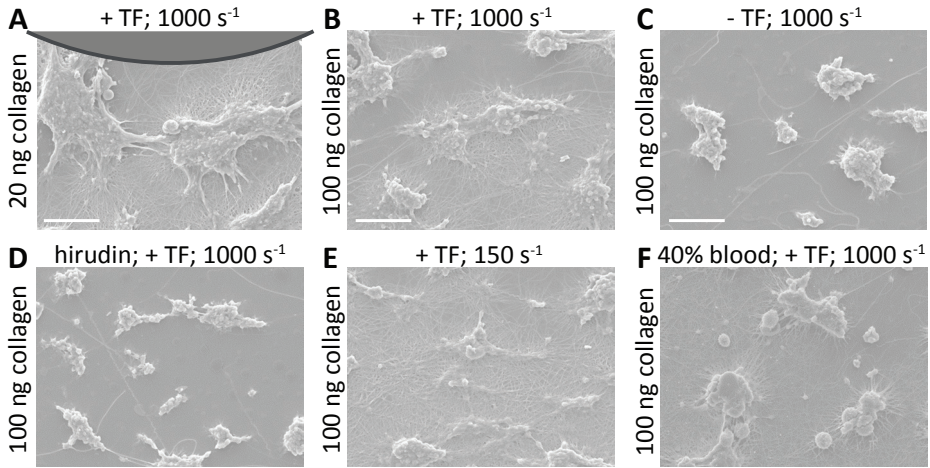


Supplemental Figure 3. Nanoindentation load-displacement curves for the assessment of microelasticity of platelet-fibrin thrombi. Platelet-fibrin thrombi were formed by blood perfusion over microspots with collagen ± TF, as in Figure 1. Microspot surfaces were subjected to nanoindentation, as in Figure 4. Shown are representative load-displacement curves for thrombi formed on (A) 100 ng collagen, (B) 100 ng collagen plus 10 pg TF, or (C) 20 ng collagen plus 10 pg TF. Indicated is the loading curve during indentation, the maximum load and the unloading curve with slope (= RedYM). The RedYM represents a measure of the microrigidity of the sample, which is inversely related to microelasticity.

4



Supplemental Figure 4. Effects of blood dilution on formation of platelet-fibrin thrombus at low shear rate. Platelet-fibrin thrombi were formed by perfusion of blood over microspots with collagen (100 ng) and TF (10 pg) at low shear rate of 150 s^{-1} for 7 minutes. Whole blood samples were undiluted or diluted to indicated % with saline (see Figure 5). (A) Time-dependent accumulation of fluorescence from platelets (green) and fibrin (red) during blood perfusion. (B) Fluorescence area covered by platelets and fibrin after 7 minutes of flow. Means ± SEM ($n = 6$), * $p < 0.05$.



Supplemental Figure 5. Scanning electron microscopy of platelet-fibrin thrombus formed on different surfaces. Platelet-fibrin thrombi were formed by perfusion of blood over microspots with collagen \pm TF at shear of 150 or 1000 s^{-1} . Thrombi on microspots were fixed, stained and visualised with a scanning electron microscope at 10 kV. Representative ($n = 3$) images are shown from thrombi formed on: (A) 20 ng collagen + TF; (B) 100 ng collagen + TF; (C) 100 ng collagen; (D) 100 ng collagen + TF, with hirudin in blood; (E) 100 ng collagen + TF, perfusion at 150 s^{-1} ; (F) 100 ng collagen + TF, 40 % whole blood (bars, 20 μm). Upper part shows schematic representation of the spherical nanoindentation cantilever to scale.

Supplemental Table 1. Collagen-tissue factor surface determines platelet-fibrin thrombus buildup under flow. Citrated whole blood was perfused during recalcification over microspots with collagen (20 or 100 ng) with(out) TF (10 pg) for 7 minutes at a wall shear rate of 150 or 1000 s⁻¹. Blood samples were pretreated with DiOC₆ to label platelets and with AF647-fibrinogen to monitor fibrin formation. Two-color confocal microscopic images were recorded in real time at a frequency of 1 Hz. For determination of thrombus volume, confocal z stacks were recorded after 7 minutes of perfusion (z-step 1 μm). *SAC, surface area covered; NA, not applicable due to relatively low thrombus height. Means ± SEM (n = 6-15).

		Collagen 100		Collagen 100 +TF		Collagen 20 + TF	
		150 s ⁻¹	1000 s ⁻¹	150 s ⁻¹	1000 s ⁻¹	150 s ⁻¹	1000 s ⁻¹
SAC* (%)	platelets	9.04 ± 1.7	27.0 ± 2.2	19.1 ± 3.2	33.8 ± 3.0	5.62 ± 0.7	12.3 ± 1.4
	fibrin	0.73 ± 0.7	1.19 ± 0.1	49.9 ± 6.8	29.8 ± 4.0	4.30 ± 1.7	5.76 ± 1.1
Fibrin area outside thrombus (% of total)	0-10 μm (base)	48.8 ± 9.7	36.3 ± 8.4	83.1 ± 0.3	28.8 ± 1.9	69.6 ± 3.0	9.7 ± 2.8
	10-20 μm (center)	54.0 ± 18.0	55.5 ± 8.2	91.4 ± 2.1	8.9 ± 2.1	55.8 ± 5.4	2.3 ± 0.6
	>20 μm (top)	54.1 ± 13.9	75.5 ± 14.0	76.9 ± 4.8	5.3 ± 1.8	57.5 ± 1.3	5.2 ± 1.1
Thrombus volume (x 10 ⁶ μm ³)	platelets	0.15 ± 0.02	0.28 ± 0.04	0.16 ± 0.02	0.79 ± 0.1	0.15 ± 0.02	0.40 ± 0.1
	fibrin	0.03 ± 0.007	0.02 ± 0.01	0.72 ± 0.03	0.76 ± 0.2	0.20 ± 0.03	0.24 ± 0.07
	total	0.16 ± 0.02	0.28 ± 0.04	0.78 ± 0.02	1.10 ± 0.2	0.29 ± 0.04	0.46 ± 0.1
Ratio fibrin/platelets	0-10 μm (base)	0.86 ± 0.07	0.84 ± 0.02	0.97 ± 0.02	1.21 ± 0.1	0.88 ± 0.01	0.97 ± 0.1
	10-20 μm (center)	1.09 ± 0.1	1.20 ± 0.1	1.07 ± 0.02	0.87 ± 0.04	0.98 ± 0.01	0.93 ± 0.08
	>20 μm (top)	NA	NA	NA	1.07 ± 0.1	1.18 ± 0.01	1.27 ± 0.1

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This chapter is embargoed at request

Chapter 5

Platelet heterogeneity in glycoprotein shedding: consequence for procoagulant responsiveness

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Blood Advances. 2018; major revision

This chapter is embargoed at request

Chapter 6

Impaired mitochondrial activity explains platelet dysfunction in thrombocytopenic cancer patients undergoing chemotherapy

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Haematologica. 2018; major revision

Chapter 7

Additive roles of platelets and fibrinogen in whole blood fibrin clot formation upon dilution as assessed by thromboelastometry

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Abstract

Background and aim: Blood dilution after transfusion fluids leads to diminished coagulant activity monitored by rotational thromboelastometry, assessing elastic fibrin clot formation, or by thrombin generation testing. We aimed to determine the contributions of blood cells (platelets, red blood cells) and plasma factors (fibrinogen, prothrombin complex concentrate) to fibrin clot formation under conditions of hemodilution in vitro or in vivo. **Methods:** Whole blood or plasma diluted in vitro was supplemented with platelets, red cells, fibrinogen or prothrombin complex concentrate (PCC). Thromboelastometry was measured in whole blood as well as plasma; thrombin generation was determined in parallel. Similar tests were performed with blood from 48 patients, obtained before and after massive fluid infusion during cardiothoracic surgery. **Results:** Addition of platelets or fibrinogen, in additive and independent ways, reversed the impaired fibrin clot formation (thromboelastometry) in diluted whole blood. In contrast, supplementation of red blood cells or prothrombin complex concentrate was ineffective. Platelets and fibrinogen independently restored clot formation in diluted plasma, resulting in thromboelastometry curves approaching those in whole blood. In whole-blood from patients undergoing dilution during surgery, elastic clot formation was determined by both the platelet count and the fibrinogen level. Thrombin generation in diluted (patient) plasma was not changed by fibrinogen, but improved markedly by prothrombin complex concentrate. **Conclusions:** In dilutional coagulopathy, platelets and fibrinogen independently, but not red blood cells or vitamin K-dependent coagulation factors, determine thromboelastometry parameters measured in whole-blood and plasma. Clinical decisions for transfusion based on thromboelastometry should take into account the platelet concentration.

Introduction

It is still a matter of debate how insufficiencies of the hemostatic system lead to bleeding under conditions of massive fluid infusion during cardiothoracic surgery. Fluid infusion diminishes a variety of processes, including platelet activity, coagulation factor levels, thrombin generation and fibrin clot formation. The latter process is monitored by rotational thromboelastometry or thromboelastography, which are frequently used to monitor patients during surgery.¹⁻³ Elastic fibrin clot formation can be measured in whole blood samples, thus providing a rapid point-of-care method for goal-directed coagulation management of surgery or trauma patients with, for example, fibrinogen concentrate.⁴

Recently, we have shown that combined plasma measurement of thrombin generation and fibrin clot formation results in a better prediction of the bleeding risk after major surgery than the use of either test alone.⁵ The rationale is that thrombin generation monitors the rate and extent of formed thrombin, as a central controlling enzyme of the coagulation cascade.⁶ On the other hand, thromboelastometry, being more sensitive for fibrinogen^{7,8} and platelets,^{8,9} reports on the more advanced stage of elastic fibrin clot formation. In patients with bleeding during major surgery or experiencing trauma, impaired hemostasis can be restored by administration of fibrinogen¹⁰⁻¹² or vitamin K-dependent coagulation factors (prothrombin complex concentrate, PCC).^{10,13,14}

Both types of concentrates are also effective in large animal models of injury-induced bleeding,¹⁵⁻¹⁷ suggesting that normalization of part of the coagulation factors helps to stop bleeding after dilution.

Vitamin K-dependent coagulation factors, platelets and fibrinogen all contribute to the process of elastic clot formation by providing enzymatic activity, strength and mass of a clot, respectively. However, it is still unresolved how, under conditions of dilutional coagulopathy, reduced levels of these blood components interact to limit fibrin clot formation, nor is it clear how red blood cells are involved in this process. In the present paper we aimed to determine these interactions. Using thromboelastometry, we measured fibrin clot formation in whole blood and plasma under conditions of dilution *in vitro* or *in vivo*, and assessed the effects of reconstitution of blood cells (platelets, red blood cells) and plasma coagulation factors (fibrinogen, PCC). The results show that the clot-forming process is controlled by platelets and fibrinogen in additive and independent ways, implicating that the platelet count is an important functional variable, independently of fibrinogen, in whole-blood thromboelastometry under condition of *in vivo* dilution in patients undergoing cardiothoracic surgery.

Materials and Methods

Materials

Bovine serum albumin (BSA) and apyrase were obtained from Sigma (St. Louis MO, USA). Human thrombin calibrator and thrombogram software were from Thrombinoscope (Maastricht, The Netherlands), thrombin substrate Z-Gly-Gly-Arg aminomethyl coumarin (Z-GGR-AMC) was from Bachem (Bubendorf, Switzerland). Thromboelastometry International (Munich, Germany) supplied the corresponding hardware, software and cuvettes.

Fibrinogen concentrate (Haemocomplettan P) and prothrombin complex concentrate (PCC, Beriplex) were from CSL-Behring (Marburg, Germany). PCC contains the vitamin K-dependent coagulation factors, prothrombin and factor VII, IX and X, and the anticoagulant factors, protein C and S, and antithrombin/heparin; 1 U/mL PCC refers to 100% prothrombin. Recombinant tissue factor (Innovin) was from Dade Behring (Deerfield IL, USA). Phospholipid vesicles containing phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine (20:60:20) were purchased from Avanti (Alabaster AL, USA) and prepared by ultrasonication.¹⁸ All other reagents came from sources described before.⁵

Blood donors

The studies were approved by the local medical ethics committee (MEC 07-2-114). Healthy donors and patients gave full informed consent for blood donation, according to the Helsinki declaration. Healthy subjects had not taken antithrombotic medication for at least two weeks. For *in vitro* dilution experiments, blood was obtained from 15 healthy subjects by venipuncture using a 1.2 mm needle, allowing the blood to drip freely into open tubes (first 2-3 mL were discarded). The collection tubes contained either 1/10

volume of trisodium citrate (0.129 M) for the preparation of platelet-free plasma; or 1/6 volume of acidic citrate dextrose (ACD: 80 mM trisodium citrate, 52 mM citric acid and 180 mM glucose) for the isolation of washed platelets.

Blood samples were obtained from 48 patients before and after a cardiopulmonary bypass (CPB) procedure. This sample size is based on the results of an earlier study, where the effects of *in vivo* dilution on elastic fibrin clot formation in only plasma were determined.¹⁹ Patients were admitted in the hospital in the period of October to December 2010. Patients had stopped taking anticoagulant drugs at least 1 week before the procedure. Blood samples were collected into 0.129 M trisodium citrate (1:10) Vacuette tubes (Greiner, Alphen a/d Rijn, The Netherlands) at two time points: (i) after induction of anesthesia, but prior to the CPB procedure and heparin administration; (ii) after surgery and infusion of protamine to neutralize heparin, when the activated clotting time (ACT) was normalized. In samples from 4 patients coagulation times pointed to the presence of residual traces of heparin. In these cases, assays were repeated in the presence of 10 µg/mL polybrene, i.e. a concentration not influencing the assay parameters (data not shown). None of the patients developed surgical complications other than bleeding during the time span of blood sampling. Transfusion of blood products during surgery was guided on the basis of low blood cell counts, prolonged aPTT, and/or clinical observation of bleeding. Whole blood thromboelastometry was measured immediately after collection; plasma samples were stored for later measurements.

Preparation of plasma, washed platelets and red blood cells

Platelet-free plasma was obtained from citrate-anticoagulated blood, by centrifuging twice at 2630 g for 10 min.²⁰ Plasma samples were immediately snap-frozen into liquid nitrogen, and stored at -80° C until further use. Washed platelets were prepared from ACD-anticoagulated blood, and suspended in Hepes buffer pH 7.45 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 0.1% glucose and 0.1% BSA), as described.¹⁹ Platelet count was determined with a thrombocounter (Coulter Electronics, Luton, UK). For the isolation of red blood cells, citrate-anticoagulated blood was centrifuged at 240 g for 15 min, after which the red cell layer was supplemented with Hepes buffer pH 7.45 in a 1:2 volume ratio. Red cells were then centrifuged at 2630 g for 10 min, suspended with Hepes buffer pH 7.45 (2:1), and washed again, which yielded a highly purified suspension of red cells (99.9%).

Dilution of whole blood or plasma and reconstitution experiments

Whole blood or plasma was diluted *in vitro* with saline medium, consisting of 137 mM NaCl, 12.9 mM trisodium citrate, 2 mM CaCl₂ and 2 mM MgCl₂, in order to keep equal concentrations of free Ca²⁺ and Mg²⁺ in all diluted samples. Coagulation factor levels in plasma were determined, as described.⁵ Percentage values of plasma are given as final concentrations (relative to citrate-anticoagulated plasma taken as 100%). In all reconstitution experiments, replacement of plasma by added supplements was taken into account when calculating the final extent of dilution. Red blood cells were added at

1:10 volume ratio. Where indicated, factor concentrates in saline were added to plasma samples from healthy subjects or patients, and incubated for 10 min before starting the experiment.

Rotational thromboelastometry

In samples from the same donors, elastic fibrin clot formation was measured in whole blood and plasma (citrate-anticoagulated) by thromboelastometry, using equipment and cuvettes from TEM International. Coagulation was triggered with 10 pM tissue factor and a surplus of CaCl_2 .¹⁹ Plasma samples were supplemented with phospholipid vesicles (4 μM , final concentration) or washed platelets from one healthy donor ($100\text{-}250 \times 10^9/\text{L}$, final count). Runs were performed in duplicate or, when >5% variation was observed, in triplicate. Curves were analyzed for slope of elastic clot formation (α -angle), maximum strength of the clot (maximal clot firmness, MCF) and the time to the onset of clot formation (clotting time, CT).

Thrombin generation

Thrombin generation in plasma was measured, using the calibrated automated thrombogram (CAT) method.¹⁹ Plasma samples were supplemented with either phospholipid vesicles (4 μM) or washed platelets from a single healthy donor ($100\text{-}250 \times 10^9/\text{L}$). Assays were run in 96-well U-bottom plates (Milford, MA, USA) in the presence of fluorogenic substrate Z-GGR-AMC (2.5 mM) and CaCl_2 (16.7 mM, final concentrations). Coagulation was triggered with 10 pM tissue factor. Measurements were performed in triplicate. First-derivate curves of thrombin generation were obtained by using Thrombogram software.²⁰ Curves were analyzed for maximal rate of thrombin generation (thrombin peak height), thrombin-generating capacity (endogenous thrombin potential, ETP) and the time until thrombin formation (lag time). Samples containing added PCC were analyzed for 10 min after triggering coagulation, to ensure adequate curve calibration.

Statistical analysis

Data are given as means \pm SD. The Shapiro-Wilk test was used to test for normal distribution of the data. Statistical analysis was performed, as appropriate, using the Mann-Whitney *U* test. Patient data were not normally distributed and are given as medians with interquartile ranges. The Wilcoxon matched pairs signed ranked test was used for comparing pre- and post-surgical values. Determinants of thromboelastometry curves were identified by multiple linear regression analysis. P-values <0.05 were considered statistically significant. The program GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA) was used for graphical purposes and relevant statistics; the SPSS 20 (IBM, Armonk, NY, USA) package was used for regression analysis and statistical testing.

Results

Determinants of thromboelastometry and thrombin generation curves for whole blood and plasma upon in vitro dilution

Blood samples from healthy subjects were diluted *in vitro*, reconstituted with various blood components, and analyzed for elastic fibrin clot formation by thromboelastometry. Coagulation was triggered via the extrinsic pathway with recombinant human tissue factor at an optimal concentration of 10 pM.¹⁹ To keep cationic concentrations constant, citrate-anticoagulated blood samples were diluted with saline medium, containing equimolar concentrations of citrate, CaCl₂ and MgCl₂. The MCF, as a curve parameter determining the overall clotting capacity, was half-maximal at 30% blood, while the α -angle, detecting the rate of elastic clot formation, was half-maximal at 20% blood (Fig. 1A). The clotting time (CT) was prolonged from <40% blood (Fig. 1B). Strikingly,

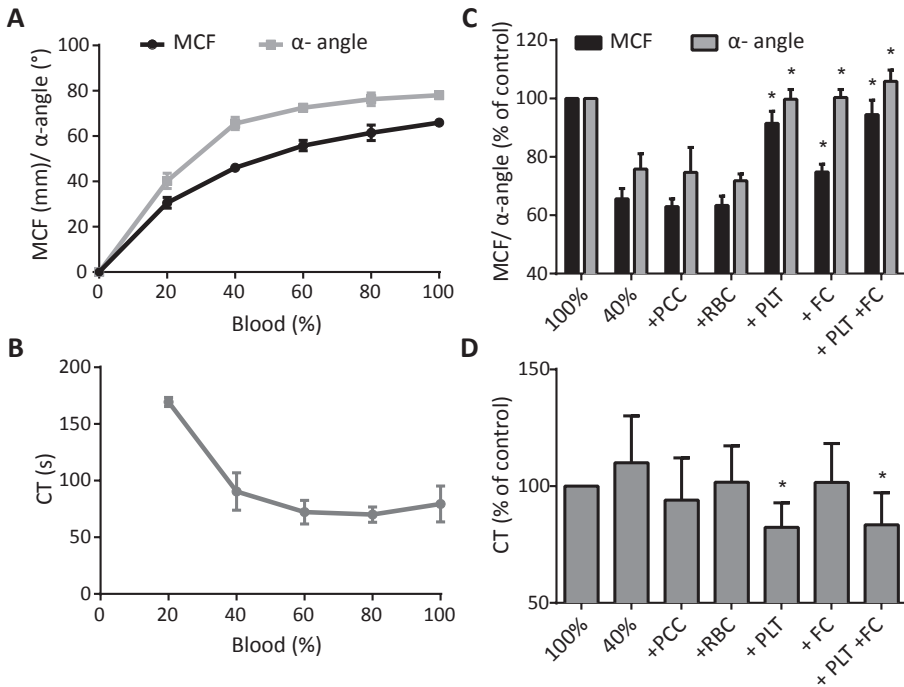
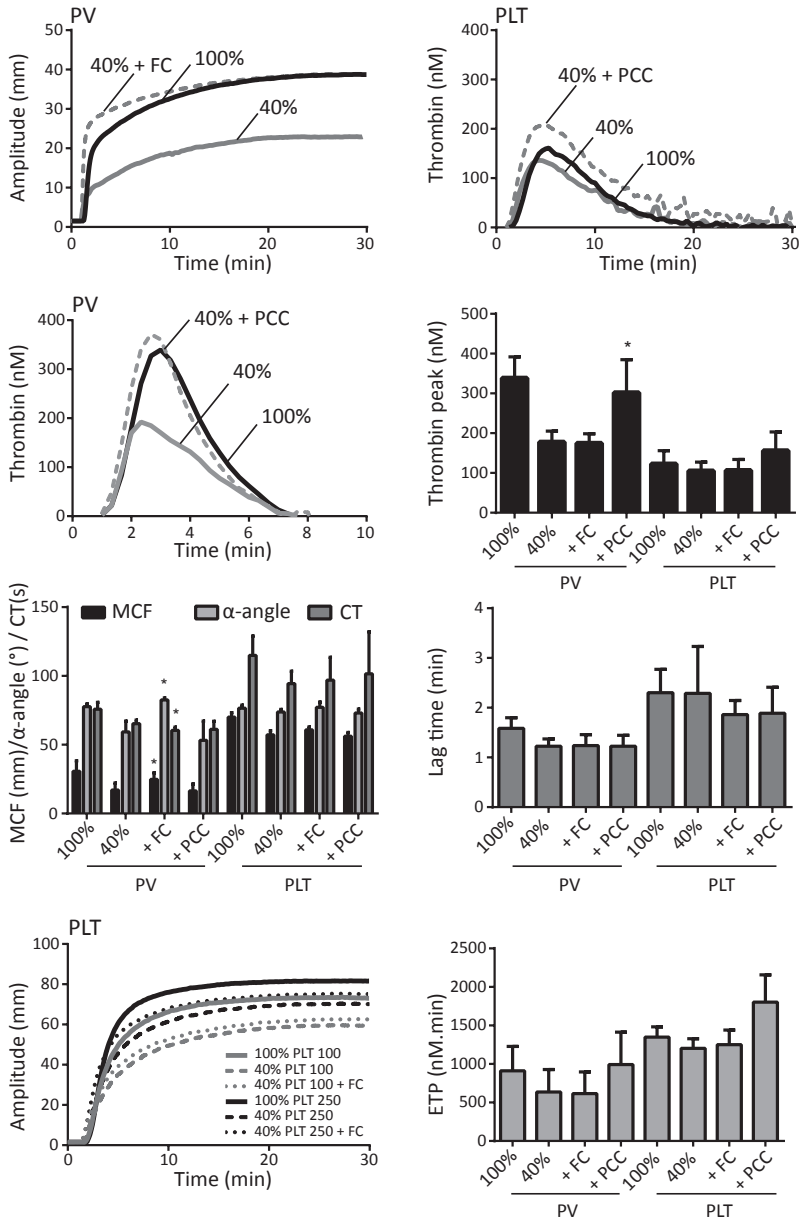


Figure 1: Determinants of thromboelastometry in diluted whole blood. Whole blood samples from healthy subjects were diluted with saline medium (see methods) to the indicated percentage. Levels of hemostatic factors in the undiluted blood (plasma) were: platelets 220-250 $\times 10^9/L$; red blood cells 33-37% hematocrit; fibrinogen 3.0-3.5 g/L, prothrombin 100%. **(A, B)** Effect of blood dilution on maximal clot firmness (MCF), α -angle and clotting time (CT) of thromboelastometry curves. **(C, D)** Whole blood diluted to 40% was supplemented (final concentrations) with prothrombin complex concentrate (PCC 1 U/mL, equivalent to 100% prothrombin), red blood cells (RBC, 37% hematocrit), autologous platelets (PLT, 250 $\times 10^9/L$) and/or fibrinogen concentrate (FC, 3.0 g/L.). Values of MCF, α -angle and CT are given in comparison to the control condition with undiluted blood (100%). Means \pm SD ($n = 5-8$), * $p < 0.05$ vs. no addition.



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Figure 2: Determinants of thromboelastometry and thrombin generation in diluted plasma. Normal plasma (fibrinogen 3.2 g/L, prothrombin 100%) was diluted with saline medium. Samples were replenished (final concentrations) with fibrinogen concentrate (FC, 3.0 g/L) or prothrombin complex concentrate (PCC, 100% prothrombin). Final plasma dilution of all samples was 40%. Coagulation was triggered with 10 pM tissue factor in the presence of either procoagulant phospholipid vesicles (PV, 4 μ M) or platelets (PLT, 100 $\times 10^9$ /L). **(A, C)** Representative thromboelastometry curves with phospholipid vesicles or platelets. **(B, D)** Representative thrombin generation curves with phospholipid vesicles or platelets. **(E-H)** Effect of added fibrinogen or PCC on parameters of thromboelastometry **(E)** and thrombin generation curves **(F-H)**. Means \pm SD ($n = 5-6$), * $p < 0.05$ vs. no addition.

after dilution of blood to 40% (as in patients undergoing major surgery with bleeding), neither the MCF nor the α -angle changed by addition of PCC or red blood cells (Fig. 1C). Addition of red blood cells to raise the hematocrit from 10 to 35% (at 40% plasma) did not influence thromboelastometry curve parameters (data not shown, but see below). In contrast, re-addition of either platelets (to $250 \times 10^9/L$) or fibrinogen concentrate (to 3.0 g/L) to the diluted blood markedly restored parameters of fibrin clot formation (Fig. 1C). Only addition of platelets normalized the CT (Fig. 1D).

Given the inability of red cells to affect thromboelastometry curves, further experiments were performed with isolated plasma, as this allowed simultaneous measurements of elastic clot formation and thrombin generation. Coagulation was triggered again with 10 pM tissue factor, while phospholipid vesicles (4 μM) or autologous platelets ($100\text{--}250 \times 10^9/L$) were added to provide a phospholipid surface for coagulation activity.¹⁹ In the presence of phospholipid vesicles, thromboelastometry curves remained low in slope (α -angle) and amplitude (MCF). Plasma dilution to 40% resulted in further lowering of the curves (Fig. 2A), and in diminished thrombin generation (Fig. 2B). Supplementation of fibrinogen concentrate, but not of PCC, antagonized the dilution effect on thromboelastometry (Fig. 2A, E). In contrast, only PCC markedly antagonized the dilution effect on thrombin generation (Fig. 2B, F-H). This analysis underscored the fibrinogen level as a major variable (independently of platelets) in elastic clot formation.

In the presence of platelets ($100 \times 10^9/L$), the rate and maximal amplitude of elastic clot formation were markedly higher and reached the maximal levels detected in whole blood (Fig. 2C, E), thus pointing to an additive effect of platelets to the thromboelastometry curves. In this case, plasma dilution to 40% only moderately diminished the thromboelastometry curves. In measurements of thrombin generation with platelets, plasma dilution was of limited effect on thrombin peak height, total amount of thrombin (ETP) or lag time (Fig. 2F-H).

Dose-response studies were performed to better define the effects of fibrinogen and PCC in diluted plasma. For thromboelastometry curves, added fibrinogen up to 3-4 g/L (final concentration) caused a dose-dependent increase in MCF and α -angle, regardless of whether PCC was added or not (Fig. 3A, B). Added fibrinogen did not affect the CT, while only the highest concentration of PCC caused a slight prolongation in CT (Fig. 3C). Addition of PCC resulted in a dose-dependent increase in thrombin generation, reaching 300% of the normal thrombin peak height and ETP level. At none of the doses, fibrinogen influenced parameters of thrombin generation (Fig. 3D, E). However, PCC at high doses prolonged the lag time to thrombin formation (Fig. 3F), which can be explained by the presence of anticoagulant proteins in this concentrate. Together, these data indicate that, in diluted whole blood and plasma, fibrin clot formation relies on the amounts of platelets and fibrinogen, whereas the thrombin generation process is regulated by the levels of vitamin K-dependent coagulation factors, as present in PCC.

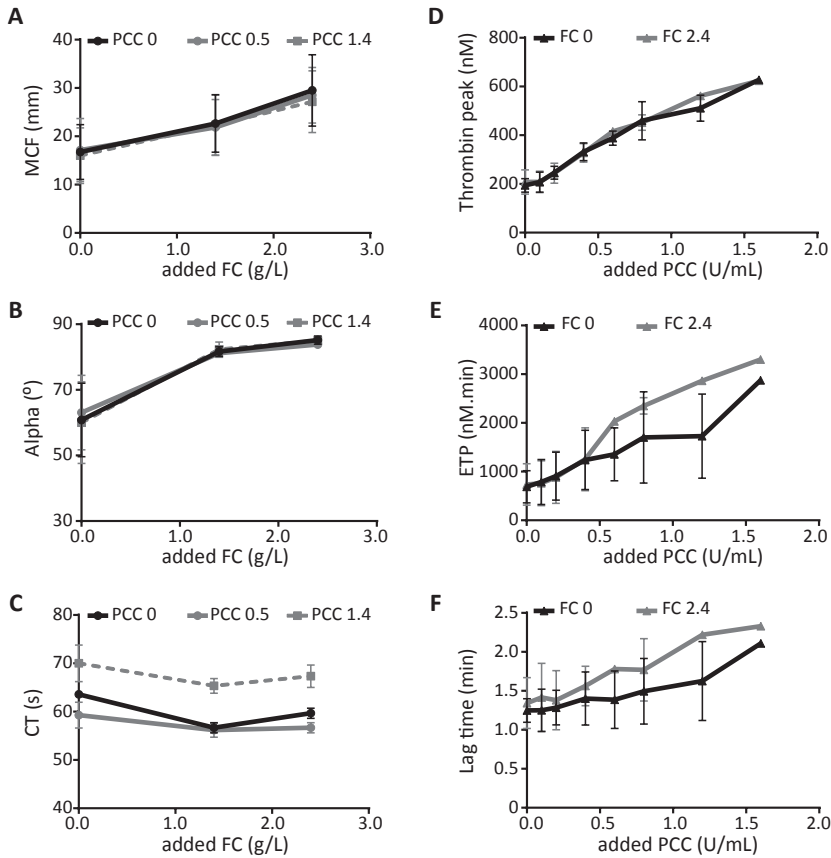


Figure 3: Dissimilar effects of fibrinogen and prothrombin complex concentrate on thromboelastometry and thrombin generation in diluted plasma. Normal plasma was diluted with saline medium and replenished with fibrinogen concentrate (FC, 1.4-2.4 g/L) and/or prothrombin complex concentrate (PCC, 0.1-1.6 U/mL). Final plasma dilution in all samples was 40%. Coagulation was triggered with 10 pM tissue factor in the presence of phospholipid vesicles. Effects of addition of different amounts of FC with or without PCC (0.5/1.4 U/mL) on MCF (A), α -angle (B) and CT (C) of the thromboelastometry curves. Effects of addition of different amounts of PCC with or without FC (2.4 g/L) on peak height (D), ETP (E) and lag time (F) of the thrombin generation curves. Means \pm SD ($n = 3-6$).

In thromboelastometry, effects of varying the levels of both platelets and fibrinogen were then compared. These blood components were added in various combinations to diluted plasma, while keeping the final extent of dilution constant. The three-dimensional plot of Fig. 4A indicates that, up to 250×10^9 platelets/L and 2.7 g fibrinogen/L, the MCF increased with higher levels of both platelets and fibrinogen (Fig. 4A). Even at the highest fibrinogen concentrations, the MCF raised with the platelet count. This is also apparent from a plot of the α -angle, although this parameter reached a maximal value at lower fibrinogen and platelet levels (Fig. 4B). Regression analysis revealed that the platelet count contributed more strongly to the MCF than the fibrinogen level; with a standardized regression coefficient β of 0.760 and 0.498, respectively (Suppl. Table 1).



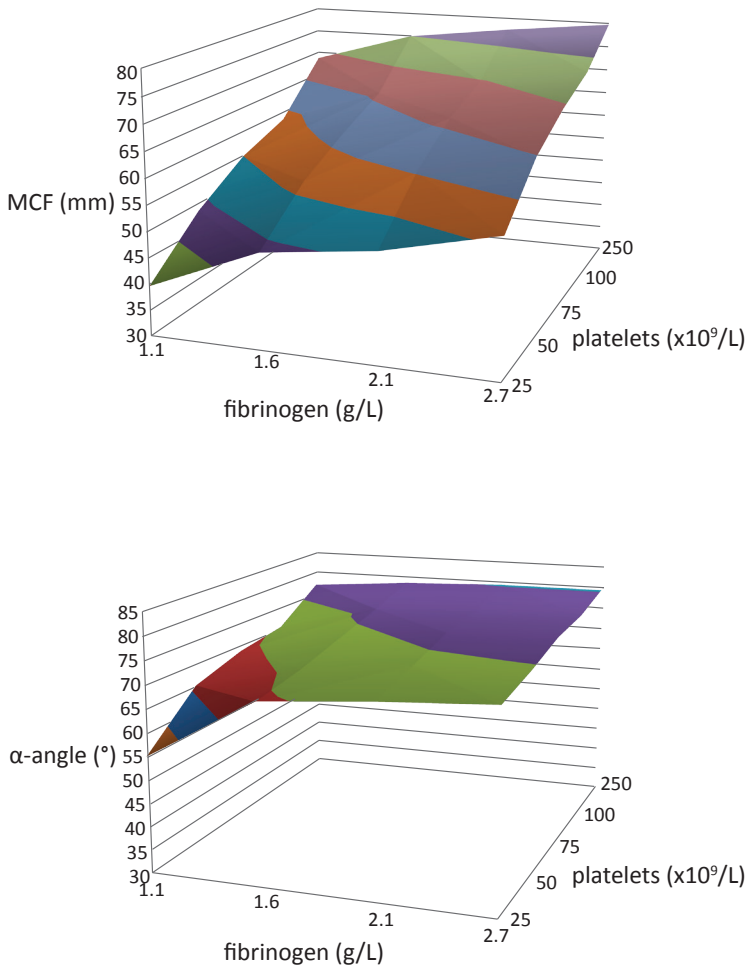


Figure 4: Combined contribution of fibrinogen and platelets to thromboelastometry. Plasma was diluted as in Fig. 3; fibrinogen levels were adjusted to 1.1-2.7 g/L, while platelets were added at 25-250 $\times 10^9$ /L. Thromboelastometry curves were analyzed, as described. Graphs show effect of fibrinogen and platelet variation on MCF **(A)** and α -angle **(B)**. See also Suppl. Table 1.

Furthermore, the relative contribution of fibrinogen to MCF appeared to be independent of the platelet count, *i.e.* standardized regression coefficients for fibrinogen were >0.89 . This was also true for the α -angle.

Predictive variables of whole blood thromboelastometry and thrombin generation in patients undergoing hemodilution during surgery

To assess the clinical relevance of these different roles of platelets and fibrinogen in thromboelastometry, blood and plasma samples were studied from patients undergoing cardiothoracic surgery and *in vivo* dilution by massive fluid infusion. Blood samples were analyzed from 48 patients, with mean age of 67 (range 51-75) years, of whom 33 underwent coronary artery bypass grafting and 10 replacement of the aortic or mitral valve (Suppl. Table 2). Total fluid volume transfused during the surgery procedure was 4.6 ± 1.4 L (mean \pm SD). A minority (12 patients) needed transfusion with 2.2 ± 1.5 units packed red cells. Blood samples were obtained from the patients before and after the surgical procedure. All patients received heparin (344 ± 77 mg) after collection of the first blood sample, and the heparin was antagonized with protamine (269 ± 51 mg) before the second blood collection. Plasma samples were checked for absence of residual heparin activity.

In the group of patients, the platelet count dropped with 45%, the hematocrit level reduced with 25%, and the aPTT significantly prolonged from 30.0 (median) to 35.0 sec (Table 1). Plasma levels of prothrombin, antithrombin and factor X reduced with 41-46%, while fibrinogen decreased from 3.50 (median) to 1.90 g/L. In remaining plasma samples from a subgroup of 7 patients, levels of other coagulation factors were determined; factors VII, VIII and IX were reduced by 46%, 32% and 38%, respectively. Overall, this pointed to a dilution of ~40% of most blood components due to fluid infusion.

Whole blood samples pre- and post-surgery from all patients were analyzed by thromboelastometry upon triggering with tissue factor (Table 2). As expected, curves of clot formation narrowed significantly, in that the MCF decreased from median 66.0 (62.0-70.0) to 55.0 (48.0-60.0) mm, while the α -angle decreased from 76.0 (74.0-79.0)

Table 1: Hematological parameters of patients before and after cardiothoracic surgery. Blood was obtained from 48 patients before and after a CPB procedure. Inactivation of heparin was checked in all post-CPB samples. Mean changes (Δ) due to the surgery procedure are indicated in percentages. Medians with interquartile ranges ($n = 48$). ** $p < 0.001$ vs. pre-surgery.

Parameter	Pre-surgery	Post-surgery	Δ
Platelets ($\times 10^9/L$)	199 (159-227)	110 (83.3-140)**	-44.7%
Hematocrit (ml/ml)	0.32 (0.30-0.36)	0.24 (0.23-0.26)**	-25.0%
Hemoglobin (mM)	6.70 (6.10-7.50)	5.00 (4.68-5.53)**	-25.4%
aPTT (s)	30.0 (28.0-30.0)	35.0 (32.0-38.0)**	16.7%
Prothrombin (%)	103 (87.0-118)	55.5 (43.5-64.0)**	-46.1%
Antithrombin (%)	98.0 (90.5-109)	57.5 (52.0-65.8)**	-41.3%
Factor X (%)	86.0 (74.5-109)	47.5 (42.3-58.0)**	-44.8%
Fibrinogen (g/L)	3.50 (2.90-4.30)	1.90 (1.50-2.30)**	-45.7%



Table 2. Parameters of thromboelastometry in whole blood from patients before and after surgery. Thromboelastometry was determined in whole blood from 48 patients pre- and post-surgery, as described in the methods section. Data are medians with interquartile ranges. * $p < 0.05$, ** $p < 0.001$ vs. corresponding pre-surgery.

Parameter	Pre-surgery	Post-surgery	Δ
MCF (mm)	66.0 (62.0-70.0)	55.0 (48.0-60.0)**	-16.7%
α -angle (°)	76.0 (74.0-79.0)	70.5 (63.4-72.8)**	-7.2%
CT (s)	112 (98.0-143)	135 (117-156)*	20.5%
CFT (s)	70.0 (57.0-85.0)	104 (88.0-138)**	48.6%

to 70.5 (63.4-71.80) degrees. Both the CT and clot formation time (CFT) significantly prolonged. During the 30 minutes of measurement, no signs of fibrin degradation were observed (not shown).

Multiple regression analysis demonstrated that in pre-surgery as well as in post-surgery samples, the platelet count and fibrinogen level were significant predictors of whole blood thromboelastometry. Standardized regression coefficients indicated that platelet count and fibrinogen level contributed similarly to the variation in MFC (Table 3). In contrast, variation in red blood cell count (hematocrit) or prothrombin level did not significantly add to this variation in either pre-surgery or post-surgery samples ($p > 0.1$).

Thromboelastometry and thrombin generation were also determined in plasma samples prepared from pre- and post-surgery blood (Table 4). Post-surgery thromboelastometry curves, obtained with plasma containing phospholipid vesicles, showed a consistent reduction in MCF (-46%) and α -angle (-11%) and a prolongation of the CT (+30%). These parameters also changed with platelets present. Analysis of thrombin generation measurements indicated a marked dilution effect post-surgery on thrombin peak height (-28%) and ETP (-7%), particularly when assessed with phospholipids. Exclusion of the data from patients, who had received red blood cells ($n=12$), resulted in similar changes of thromboelastometry and thrombin generation data (not shown).

To determine the potential for normalization, post-surgery plasma samples were supplemented with fibrinogen concentrate and PCC. These concentrates were added at amounts corresponding to the expected effect of transfusion of 2 units fresh frozen plasma,⁵ *i.e.*, 0.4 g/L fibrinogen plus 0.11 U/mL PCC. This addition significantly increased MCF in the presence of phospholipids from 16.5 (12.5-20.0) to 21.0 (17.0-25.0) mm, but left the parameter unchanged in the presence of platelets (Table 4). The same addition caused a 25-33% increase in thrombin generation (thrombin peak height and ETP), both with phospholipids and platelets.

Table 3. Predicting variables of thromboelastometry in whole blood from patients before and after surgery. Fibrin clot formation was measured in whole blood from 48 patients pre- and post-surgery. Predicting variables of thromboelastometry MCF were obtained by linear, multiple regression analysis. Abbreviations: b indicates unstandardized regression coefficient; SE b, standard error of b; β , standardized regression coefficient.

	b	SE b	β	p-value	partial correlation
Pre-surgery					
<i>Step 1</i>					
Constant	45.59	3.985		<0.001	
Fibrinogen	2.655	0.986	0.382	0.011	0.424
Platelets	0.054	0.018	0.422	0.005	0.460
<i>Step 2</i>					
Constant	44.07	9.627		<0.001	
Fibrinogen	3.031	1.079	0.436	0.009	0.450
Platelets	0.062	0.021	0.485	0.006	0.471
Hematocrit	7.989	23.92	0.047	0.741	0.060
Prothrombin	-0.042	0.044	-0.152	0.348	-0.169
Post-surgery					
<i>Step 1</i>					
Constant	33.805	2.505		<0.001	
Fibrinogen	5.783	1.296	0.507	<0.001	0.608
Platelets	0.080	0.021	0.439	<0.001	0.552
<i>Step 2</i>					
Constant	27.95	7.122		<0.001	
Fibrinogen	5.491	1.502	0.481	0.001	0.543
Platelets	0.080	0.025	0.440	0.003	0.498
Hematocrit	21.06	25.14	0.084	0.408	0.147
Prothrombin	0.024	0.073	0.048	0.749	0.057



Table 4: Comparison of thromboelastometry and thrombin generation tests in patient plasmas before and after surgery. Plasma samples from patients, before and after surgery, were supplemented with either 4 μM phospholipid vesicles or 100×10^9 platelets/L, as indicated. Coagulation was triggered with 10 pM tissue factor. Parallel plasma samples post-surgery were supplemented with fibrinogen concentrate (FC, 0.4 g/L, f.c.) and prothrombin complex concentrate (PCC, 0.11 U/mL, f.c.). Medians with interquartile ranges ($n = 48$). * $p < 0.05$, ** $p < 0.001$ vs. pre-surgery samples, or vs. no addition.

Parameter	Pre-surgery	Post-surgery	Δ	Post + FC/PCC
Thromboelastometry				
<i>phospholipid vesicles</i>				
MCF (mm)	30.5 (26.0-37.0)	16.5 (12.5-20.0)**	-46%	21.0 (17.0-25.0)**
α -angle ($^\circ$)	79.0 (77.0-81.0)	70.0 (63.0-74.0)**	-11%	74.0 (71.0-78.0)**
CT (s)	89.0 (83.0-103)	116 (100-132)**	30%	90.5 (77.0-105)**
<i>Platelets</i>				
MCF (mm)	73.0 (71.0-76.0)	65.0 (62.0-69.0)**	-11%	63.5 (59.0-66.0)
α -angle ($^\circ$)	79.0 (77.0-80.0)	77.0 (75.0-78.0)**	-2.5%	76.5 (74.0-79.0)
CT (s)	104 (93.0-135)	111 (97.0-127)	6.7%	95.5 (85.0-112)**
Thrombin generation in plasma				
<i>phospholipid vesicles</i>				
Peak height (nM)	297 (249-325)	213.6 (179-232)**	-28%	269.5 (230-295)**
ETP (nM x min)	1159 (997-1313)	1083 (922-1252)**	-6.6%	1415 (1291-1563)**
<i>Platelets</i>				
Peak height (nM)	144.5 (114-168)	133.6 (113-151)*	-7.5%	167.1 (147-191)**
ETP (nM x min)	1282 (1094-1412)	1159 (1041-1358)*	-9.6%	1536 (1423-1805)**

The MCF of whole blood thromboelastometry with pre- and post-surgery samples was dependent on the fibrinogen level (partial correlation coefficients 0.917 and 0.890, respectively, $p < 0.001$). Notably, the MCF from curves obtained with whole blood samples correlated significantly with the MCF measured with plasma plus phospholipids ($R = 0.83$, $p < 0.001$) or with plasma plus platelets ($R = 0.75$, $p < 0.001$) (Fig. 5). On the other hand, the various parameters of thromboelastometry curves (whole blood or plasma) did not correlate with those of thrombin generation ($p > 0.2$). Together, this indicated that the predictive variables of thromboelastometry curves of patient whole blood and plasma were similar, but differed from those predicting thrombin generation curves.

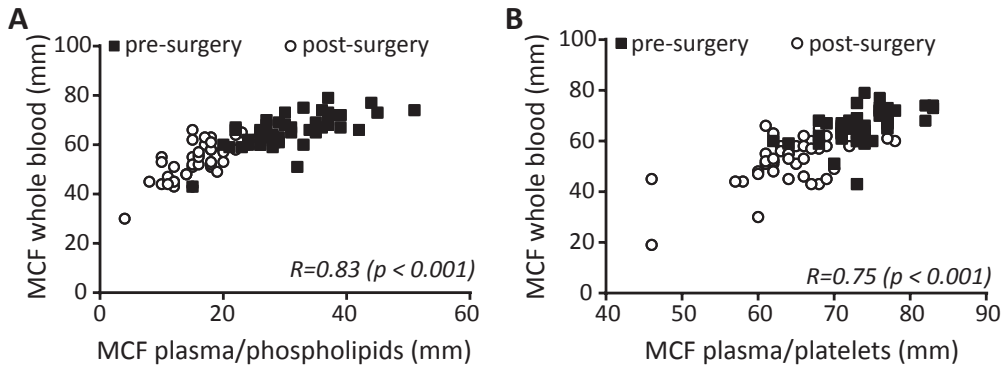


Figure 5: High correlation of thromboelastometry MCF values in whole blood and plasma samples. Thromboelastometry was measured in whole blood and plasma with phospholipids or platelets ($100 \times 10^9/L$) from 48 patients. MCF was derived from the thromboelastometry curves. **(A)** MCF in whole blood vs. plasma/phospholipids, **(B)** MCF in whole blood vs. plasma/platelets. Correlation coefficients and p-values are given of combined pre- and post-surgery data.

Discussion

In this paper, we studied principal sources of variation of the processes of elastic fibrin clot formation (thromboelastometry) and thrombin generation under conditions of *in vitro* or *in vivo* dilution. Coagulation in all cases was fully activated with tissue factor.²¹ It appeared that, in whole blood or plasma diluted *in vitro* to 40%, both the platelet count and fibrinogen concentration determine the kinetics and extent of fibrin clot formation in additive ways, with no more than limited contributions of red blood cells and vitamin K-dependent coagulation factors. In other words, certain (threshold) values of thromboelastometry parameters could be obtained by supplementation of platelets and fibrinogen at different relative amounts, but not by red blood cells. On the other hand, the best way to restore impaired thrombin generation in diluted plasma was by increasing the levels of vitamin K-dependent coagulation factors.

In the present paper we find that, under conditions of hemodilution, thromboelastometry parameters in whole blood are not improved by raising the hematocrit. A limited contribution of red blood cells is also apparent from the observation that curve parameters were similar in whole blood and in platelet-containing plasma (without red blood cells). Other authors, using undiluted blood have reported a negative contribution of the hematocrit on thromboelastometry parameters.²²⁻²⁴ Comparing with the present results, this suggests that under high viscous conditions, as in undiluted blood, red blood cells interfere with the formation of an elastic platelet-fibrin clot, while this interference becomes smaller under less viscous conditions.

The recognition that platelets and fibrinogen in additive ways contribute to thromboelastometry parameters sheds new light on our earlier conclusion¹⁹ that platelets can partly compensate for the dilutional effect on elastic clot formation. The apparently independent contribution of platelets and fibrinogen most likely reflects differences in

function in this process, *i.e.* providing elasticity to the fibrin clot by contraction (platelets) and by determining the mass of a fibrin clot (fibrinogen).²⁵ This suggestion is supported by the finding that also in blood samples from patients subjected to *in vivo* dilution, the platelet count and fibrinogen level are independent variables of the clot-forming process.

Marked differences were found, when comparing the effects of factor concentrates on thromboelastometry and thrombin generation under conditions of dilution *in vitro* or *in vivo*. Supplementation of fibrinogen concentrate, but not of PCC, in a dose-dependent way restored elastic fibrin clot formation, as detected by an increased MCF and α -angle, without affecting thrombin generation. Interestingly, the CT was hardly changed by PCC addition to whole blood or plasma, which agrees with published findings that the CT prolongs when coagulation factors fall below 35% of normal.²⁶ Conversely, supplementation of PCC, but not of fibrinogen concentrate, restored thrombin generation. These data indicate that, although thromboelastometry is often viewed as an integrated global measure of coagulation, its sensitivity to reduced levels of vitamin K-dependent coagulation factor is limited.

Human and large animal studies indicate that both types of factor concentrates can improve hemostasis and reduce blood loss after major trauma.^{4,15-17} In porcine models, infusion of a factor concentrate, not containing anticoagulant proteins, in contrast to fibrinogen concentrate, promoted disseminated intravascular coagulation and thus caused a prothrombotic condition.^{27,28} *In vitro* analysis indeed indicates that the absence of anticoagulant factors in such concentrates promotes thrombin generation.²⁹ In the present study, we find a prolonged CT (thromboelastometry) and lag time (thrombin generation) in the presence of a high concentration of PCC, which hence reflects the presence of anticoagulant factors that delay the formation of thrombin.

In patient plasmas after surgery, parameters of thrombin generation were less strongly reduced than those of thromboelastometry. Impairment in thrombin generation is demonstrated by the reduction in thrombin peak level as well as by the prolongation of the CT. Comparison of the curve parameters learned that the thrombin peak height was a better responsive indicator for dilution than the ETP. The latter agrees with other papers reporting on effects of *in vitro* dilution in thrombin generation.^{21,30}

Taken together, we conclude that the platelet count and fibrinogen level, in additive ways, but not the amount of red blood cells, determine the extent of elastic fibrin clot formation in diluted whole blood or plasma. Thrombin generation in diluted plasma, on the contrary, is dependent on vitamin K-dependent coagulation factors. By implication, clinical decisions for transfusion with fibrinogen or plasma that are based on thromboelastometry should take into account the platelet concentration.

Acknowledgments

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What is known on this topic

- Fibrinogen is a key determinant of elastic fibrin clot formation, assessed by whole-blood thromboelastometry or thromboelastography

What this paper adds

- Platelets and fibrinogen contribute in additive and independent ways to elastic fibrin clot formation under conditions of *in vitro* or *in vivo* dilution.
- In dilutional coagulopathy, platelets and fibrinogen, but not red blood cells or vitamin K-dependent coagulation factors, determine thromboelastometry parameters measured in whole-blood and plasma.
- Clinical decisions for transfusion of fibrinogen or plasma based on thromboelastometry should take into account the platelet concentration.
- In diluted plasma, thrombin generation is more dependent on vitamin K-dependent coagulation factors than elastic clot formation.

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Supplemental data

Supplemental Table 1: Relative contribution of platelets and fibrinogen to thromboelastometry curves.

PRP was reconstituted to the indicated platelet number ($\times 10^9/L$) and the relative contribution of fibrinogen to the MCF and alpha angle was analyzed by linear regression analysis. Abbreviations: *b*, unstandardized regression coefficient, SE *b*, standard error of *b*; β , standardized regression coefficient (n=5).

		b	SE b	β	p-value
<i>MCF</i>					
Plt 25	constant	30.82	1.850		<0.001
	fibrinogen	9.700	0.943	0.924	<0.001
Plt 50	constant	39.54	2.087		<0.001
	fibrinogen	9.663	1.064	0.906	<0.001
Plt 75	constant	46.16	1.451		<0.001
	fibrinogen	9.064	0.739	0.945	<0.001
Plt 100	constant	50.28	1.608		<0.001
	fibrinogen	8.839	0.820	0.931	<0.001
Plt 250	constant	62.84	1.562		<0.001
	fibrinogen	6.667	0.796	0.892	<0.001
<i>Alpha</i>					
Plt 25	constant	48.80	2.961		<0.001
	fibrinogen	9.925	1.509	0.840	<0.001
Plt 50	constant	61.20	1.300		<0.001
	fibrinogen	5.431	0.662	0.888	<0.001
Plt 75	constant	64.74	1.096		<0.001
	fibrinogen	4.981	0.559	0.903	<0.001
Plt 100	constant	67.64	1.288		<0.001
	fibrinogen	4.232	0.656	0.835	<0.001
Plt 250	constant	76.34	1.040		<0.001
	fibrinogen	1.610	0.530	0.582	0.007

Supplemental Table 2: Patient and surgery characteristics. Means \pm SD (n=48).

Characteristic	
Male/female (n)	32/16
Mean age in years (range)	67 (51-75)
Medication prior to surgery (n)	
• aspirin	28
• coumarin	2
• fraxiparine	5
Type of surgery (n)	
• coronary artery bypass graft	33
• valve replacement	10
• other	5
Heparin during surgery + protamine (n)	48
Perfusion time (min)	91 \pm 31
Type of transfusion (n)	
• red blood cells	12
• fresh frozen plasma	0
• fluids	48
Transfused fluids:	
• priming fluid (L)	1.4 \pm 0.4
• cardioplegia (L) (n=11)	1.9 \pm 0.8
• colloids/crystalloids (L)	1.8 \pm 0.8
• total (L)	4.6 \pm 1.4

This chapter is embargoed at request

Chapter 8

A synthesis approach of mouse studies to identify new genes and proteins in arterial thrombosis and bleeding

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Submitted

Chapter 9

General Discussion

As stated in *Chapter 1*, the overall goal of the studies was to better understand the consequences of an acquired impairment or recovery in platelet function in various physiological and clinical settings. This required further investigation on the activation processes of platelets, either isolated or in whole blood, during the process of thrombus formation. In the next paragraphs, I will discuss how the findings of this thesis have led to new insights into the causes of structural and functional heterogeneity between platelets. Subsequently, I will confer mechanisms by which an acquired low platelet count or platelet dysfunction can lead to impaired hemostasis, and how these can be overcome. In the last section, I will describe new insights arising from the synthesis study describing the hemostasis and thrombosis balance in genetically modified mice.

Heterogeneity in platelet structure and function

It has been recognized for more than a decade that substantial differences exist among platelets.¹⁻³ The review *Chapter 2* describes in detail how and why platelets from a given subject are heterogeneous in terms of structural characteristics (*e.g.*, platelet size and receptor densities) and functional features (*e.g.*, forming populations of aggregating, procoagulant and coated platelets). Given the life span of human platelets of 7-10 days,⁴ both younger (juvenile or reticulated) and older platelets are circulating. It has been proposed that the juvenile platelets are larger in size and are more reactive towards agonists than older platelets.^{5,6} Most of the evidence has come from studies in which juvenile platelets were (weakly) labeled with thiazole orange, an mRNA probe with a debated specificity.⁷ In *Chapter 3*, we introduced a new method to identify juvenile platelets, namely by staining with fluorescently labeled oligo-dT, which directly binds to the poly-A tail of mRNA species. Using this probe we were able to demonstrate a regain of function in the newly formed platelets from patients, who stopped taking the irreversible P2Y₁₂ receptor antagonist prasugrel. Hence, in this specific setting we found a higher reactivity of (unblocked) juvenile platelets than their older counterparts. Whether these juvenile platelets are larger was not apparent from the flow cytometric analysis.

Although juvenile platelets were more reactive, by themselves they were unable to compensate for the impairment in thrombus and fibrin formation due to a low platelet count despite a relatively high number of juvenile platelets (immune thrombocytopenia, *Chapter 4*). While we find that during antiplatelet therapy and the offset period, irreversible P2Y₁₂ inhibition impairs the reactivity of the juvenile platelets, several other studies have shown a positive correlation between the percentage of juvenile platelets and on-treatment platelet reactivity.⁸⁻¹⁰ Thereby, it was suggested that, due to a high platelet turnover, juvenile platelets are incompletely inhibited by antiplatelet therapy and, thus, contribute to high on-treatment platelet reactivity.^{8,10} However, our data in *Chapter 3* are not supportive of this hypothesis, in that the reactivity of juvenile platelets during the initial days of the offset period was still low and only gradually increased in

time. This observation agrees with the evidence from other authors that the intrinsic structural properties of juvenile platelets, rather than a high turnover, explain a high on-treatment activity.¹¹

According to their presumed functions, activated platelets can be separated into the - partly overlapping - populations of adhesive, aggregating, procoagulant and coated platelets. Each of these populations is characterized by a specific set of surface markers and can be distinguished in a thrombus (Figure 1).^{1,12,13} How individual platelets transform into one of these populations is not fully clear. In *Chapter 2* we reviewed the current understanding of platelet heterogeneity and the characteristics of platelet populations. We postulate that both the genetic make-up (inherited from megakaryocytes) and the vascular environment of a platelet determine its activation profile, and hence direct them to a specific population.

A population with unique properties is that of procoagulant platelets.¹⁴ These platelets are characterized by surface exposure of negatively charged phosphatidylserine (PS) at the outer leaflet of the plasma membrane, which property greatly enhances their interaction with vitamin K-dependent coagulation factors.^{15,16} In *Chapter 5* we investigated how in particular the PS-exposing platelets are implicated in the extracellular shedding of glycoprotein (GP) receptors, mediated by the membrane-linked proteases ADAM10 and ADAM17.^{17,18} We discovered that shedding of both GPIIb α (by ADAM17) and GPVI (by ADAM10) is predominantly confined to the PS-exposing population (Figure 1), regardless of the type of stimulus, *i.e.* the Ca²⁺-elevating receptor ligands CRP-XL/thrombin or the apoptosis-inducing compound ABT-737.¹⁹ Kinetic experiments furthermore indicated that PS exposure can precede glycoprotein shedding. However, PS exposure (or platelet swelling) is not required for platelet ADAM activity, as shedding of GPIIb α in Scott platelets, which are devoid of PS exposure, was unaltered. The Scott platelets are deficient in both membrane phospholipid scrambling and swelling, because of a lack of the Ca²⁺-dependent ion channel anoctamin-6 (gene *ANO6* or *TMEM16F*).^{20,21} Together, these findings indicate that ADAM activation in platelets occurs via regulatory mechanisms that are similar but not identical to those of PS exposure. Thus, glycoprotein shedding and PS exposure appear to be two independent events. This is in contrast to a recent report demonstrating a necessity for PS exposure to induce ADAM activity in lymphocytes.²²

In *Chapter 5*, we also studied the signaling pathways leading to ADAM-mediated glycoprotein shedding. Inhibitor experiments indicated that both caspase activity (with ABT-737) and prolonged elevation in cytosolic Ca²⁺ (with CRP-XL/thrombin or Ca²⁺-ionophore) with a secondary role for protein kinase C activation could trigger shedding of GPIIb α and GPVI. However, these pathways were not mutually exclusive, as the chelation of Ca²⁺ in ABT-737-stimulated platelets partly diminished GPIIb α shedding. This is in line with an earlier publication from our laboratory, showing the existence of a population of apoptotic platelets that shows increased levels of cytosolic Ca²⁺.²³

Another relevant finding was that cleavage of GPIIb α and GPVI lowers the adhesion tendency of the platelets and, conversely, increases their procoagulant activity. It appeared that those platelets that had lost their GPIIb α and GPVI receptors showed a *decreased* binding of VWF, but an *increased* binding of prothrombin and factor Xa. As a consequence, the platelets with cleaved receptors mediated a faster onset of

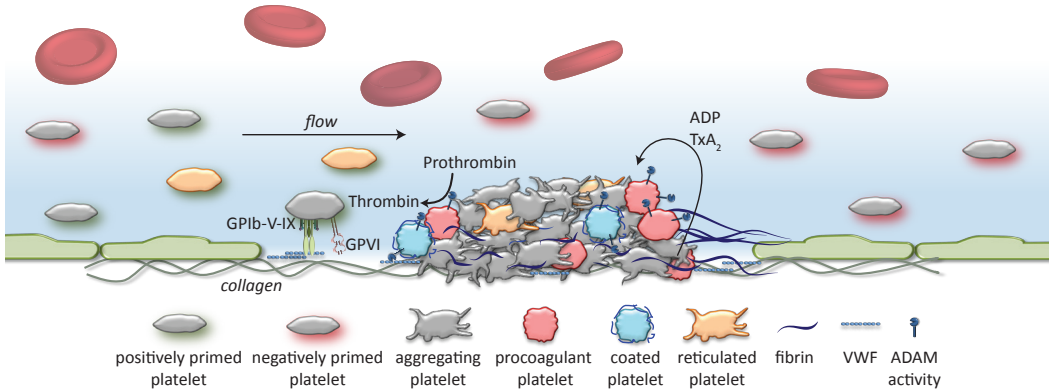


Figure 1. Proposed model of platelet priming and heterogeneity in thrombus formation. Platelets are positively or negatively primed by genetic and environmental factors and as such can show differential responses to vascular injury. This translates into the formation of a heterogenous thrombus with a distinct localization of the different platelet types and their specific responses (e.g. receptor shedding located specifically at the procoagulant platelets) within the thrombus. As such also the pattern of fibrin formation and thereby, the elasticity of the thrombus is affected.

fibrin formation under flow. Specific blockade of GPIIb/IIIa shedding also increased the procoagulant activity, suggesting a predominant role of GPIIb/IIIa in the control of this process.

At first sight a loss of glycoprotein receptors in procoagulant platelets is counterintuitive, because the literature reports that both GPIIb/IIIa and GPIIb/IIIa can act as receptors for thrombin and fibrin, respectively, *i.e.* two major players of the coagulation cascade.²⁴⁻²⁶ Our data thus implicate that the platelet population that is most supportive of coagulation cannot steer this process via GPIIb/IIIa and GPIIb/IIIa. Accordingly, other populations must account for the GPIIb/IIIa- and GPIIb/IIIa-dependent effects of thrombin and fibrin, respectively: for instance, the populations of adhesive and aggregated platelets. In *Chapter 4* we find that, at conditions where platelet adhesion is limited, thrombus formation is highly dependent on thrombin, whilst fibrin accumulates in the thrombus top. One might speculate that in such situations the relative importance of GPIIb/IIIa and GPIIb/IIIa as coagulant receptors increases. Glycoprotein-dependent binding of thrombin and fibrin may also facilitate the formation of procoagulant and coated platelet elsewhere in the thrombus.

Acquired impairment of hemostasis: effects of reduced platelet count or dysfunction

Given the central role of platelets as mediators of hemostasis, both an adequate platelet count (platelet mass) and platelet function is needed to prevent bleeding.^{27,28} In *Chapter 2* we present the concept that platelets can be negatively or positively primed by their physiological environment, with positive priming becoming more important at conditions where the thrombosis risk increases. By changing the environment, not only

anti-platelet but also anticoagulant therapies will affect the priming of platelets, and thereby alter their responsiveness. As a result, platelets can become hypo- or hyper-responsive (Figure 2).

Chapter 6 provides an illustration of how disease and treatment can affect platelet mass, function and priming. In this chapter, we investigated platelet and coagulant functions in patients, who had been diagnosed with a hematological malignancy and were treated with chemotherapy. As a consequence of disease and chemotherapy, these patients develop severe thrombocytopenia and are hence at increased risk for bleeding, ultimately with a necessity of platelet transfusion.²⁹ Our data indicate that, after a couple of weeks of chemotherapy treatment, the remaining platelets of the patients are dysfunctional in multiple aspects. To a varying extent, these platelets display defective integrin activation, granule secretion and Ca^{2+} signaling. As the direct effect of chemotherapeutic exposure to the circulating platelets was minimal, it is concluded that the impairment in platelet function likely originates at the level of megakaryocytes. The high variability in platelet responses among patients could not be explained by the diagnosis or type of chemotherapy. This would argue for a variable susceptibility of the megakaryocytes. From patient to patient, the anti-proliferative effects of chemotherapeutics, *e.g.* inhibition of DNA replication and cell cycle progression, might differently affect the process of megakaryopoiesis.³⁰ Furthermore, even megakaryocytic precursor cells have a different sensitivity to chemotherapeutics.³¹ This implies that the extent of damage inflicted to the megakaryocytes and derived platelets is determined by the chemotherapeutic agents administered and the stage of megakaryopoiesis.

Given the cytotoxic nature of chemotherapy, we investigated whether the dysfunction of the patient platelets was due to apoptosis. This was also driven by data that integrin activation and secretion are impaired in apoptotic platelets.³² However, we could not find indications of ongoing apoptosis in platelets from the patients. For instance, no caspase activity was detected, and caspase inhibition did not affect PS exposure. Rather, we found strong evidence that mitochondrial dysfunction could account for the diminished platelet responsiveness.

Under normal conditions, the energy demand increases upon platelet activation. As platelets rely on glycolysis and mitochondrial respiration for sufficient ATP production, an impairment in mitochondrial respiration can result in decreased platelet reactivity.³³ Our data suggest that, in the patient platelets, the mitochondrial function is inadequate to support the higher energy demand needed for activation, with as a result lower platelet responsiveness. Mitochondrial dysfunction can also explain the increased PS exposure in the patient platelets, given the observation that mitochondria normally have a suppressive function in PS exposure.³⁴ Along the same line, a deficiency in glucose metabolism can enhance PS exposure.³⁵

Impaired platelet responsiveness appeared to be a main determinant of disturbed hemostasis in patients treated by chemotherapy (*Chapter 6*). On the other hand, the combined lowering of coagulation factors and platelets accounts for the increased bleeding risk in patients with dilutional coagulopathy during major surgery (*Chapter 7*). Here, we investigated the contribution of the plasmatic and cellular components of blood to the hemostatic potential, as measured by elastic fibrin clot formation (rotational thromboelastometry) and thrombin generation (assessed by the calibrated

automated thrombin generation test). The method of rotational thromboelastometry or thromboelastography is frequently used to monitor the hemostatic capacity of the blood from patients during major surgery.³⁶ Advantages of this point-of-care technique are that only small blood volumes are needed and that the measurement time is short.^{37,38} Our *in vitro* data suggest that upon dilutional coagulopathy – dilution of whole blood or plasma with crystalloids to 40% – fibrin clot formation could be normalized by reconstitution with either platelets or fibrinogen. On the other hand, administration of prothrombin complex concentrate (containing a mixture of pro- and anti-coagulation factors) or red blood cell concentrate was ineffective. In blood samples from patients taken before and after hemodilution, we found that only the platelet count and the fibrinogen level were relevant predictors of clot strength. Thrombin generation though could be restored by the addition of prothrombin complex concentrate, in which case addition of fibrinogen or platelets was without effect.

In *Chapter 4*, we used a whole blood perfusion assay to investigate thrombus and fibrin formation in blood obtained from patients with dilutional coagulopathy. Both thrombus and fibrin formation were severely impaired in these blood samples. In this test system – in contrast to rotational thromboelastometry – it appeared that reconstitution of both platelets and red blood cells was needed to achieve normal thrombus and fibrin formation at 40% dilution. The role of red blood cells herein, likely, is to improve platelet marginalization and adhesion to the microfluidic chamber surface.³⁹ Importantly, this platelet marginalization effect of (transfused) red blood cells, considered to be relevant for hemostasis (see *Chapter 1*), is not detected in other, static assays. Taken together, this work clearly indicates that the ‘hemostatic benefit’ of various blood products is often dependent on the type of test used. Accordingly, not every test outcome necessarily detects or reflects the actual bleeding risk of a patient.

Normalization of hemostasis: recovery of platelet count and function

Given that platelets, as anucleate cellular fragments, have only a low ability to synthesize proteins,^{40,41} the production of new platelets will be needed to repair any dysfunction (Figure 2). In *Chapter 3*, we investigated platelet function during one month after cessation of the irreversible P2Y₁₂ antagonist prasugrel. We could demonstrate that the formation of juvenile platelets was needed for a recovery of P2Y₁₂ signaling and platelet responses. However, surprisingly, this recovery was only partial during the first days after prasugrel discontinuation, and it only gradually increased during the offset period. These findings point to the presence of a ‘reservoir’ of inhibited P2Y₁₂ receptors in the megakaryocytes, which during the initial days of offset still produce P2Y₁₂-inhibited platelets.

This resembles the situation of chemotherapy-treated cancer patients, who also produce platelets with a megakaryocyte-inherited dysfunction (*Chapter 6*). Usually, the thrombocytopenia lasts for two to three weeks and then platelet count reverts to normal when chemotherapeutic treatments are stopped.²⁹ Markedly, before the platelet count restores, the megakaryocyte ploidy increases,⁴² and the fraction of reticulated platelets rises.^{43,44} Our data indicate that an increase in platelet count – after discontinuation

of chemotherapy – is accompanied by a normalization of function of the (reticulated) platelets. This suggests that the restoration process starts with a normalization of megakaryopoiesis.

In case of active or expected bleeding, the common treatment protocol in the Netherlands is a transfusion with pooled platelet concentrate.^{45,46} In *Chapter 6*, we found that platelet transfusion to patients receiving chemotherapy resulted in an improved platelet activity in thrombus and fibrin formation. These results are in agreement with other reports.^{47,48} Since the platelet concentrates used for transfusion purposes are stored for several days (median six days), this might be accompanied by a loss of mitochondrial function.⁴⁹ However, no sign of this was found, since the mitochondrial membrane potential – a sensitive indicator for platelet quality⁵⁰ – was comparable to that of freshly isolated platelets from healthy volunteers.

Inactivation in circulation Inactivation in megakaryocytes Formation of new active platelets Transfusion of blood components

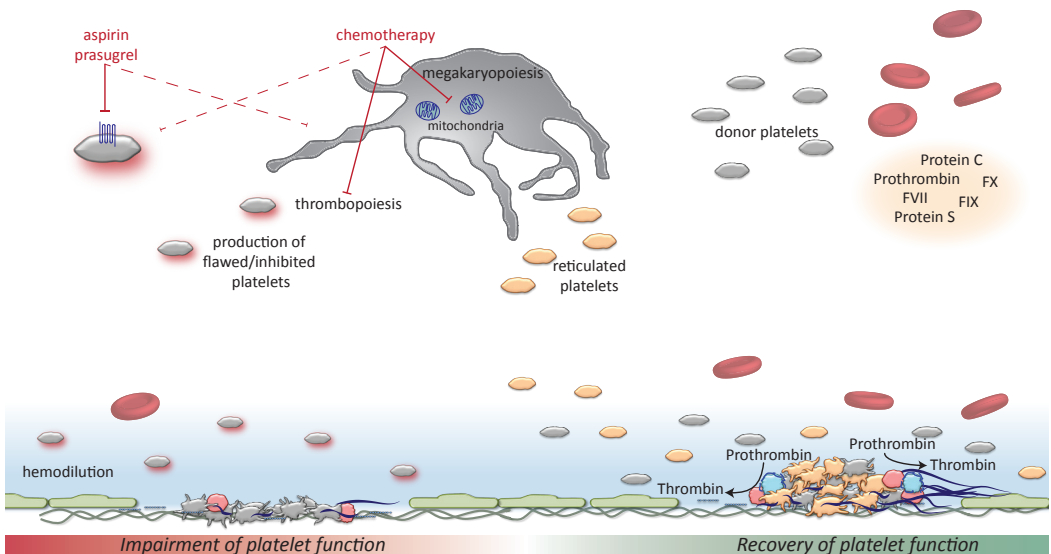


Figure 2. Mechanisms of impairment and recovery of platelet function. Impairment of platelet function can result from a direct inhibition of the platelets themselves during circulation and/or an indirect inhibition at the level of the megakaryocytes. Further, dilution of plasmatic and cellular components can affect platelet functionality as well. As platelets are anucleate cells, recovery of platelet function is dependent on the production of new platelets by megakaryocytes. When immediate improvement of hemostasis is required, transfusion of blood components can aid in the recovery of thrombus and fibrin formation.

Genetic alterations in hemostasis and thrombosis

Chapter 8 describes a synthesis paper to compile the outcomes of published *in vivo* and *in vitro* studies on effects of gene depletion in mouse on arterial thrombus formation. This paper provides insight into the roles of 401 mouse genes in experimentally induced thrombosis with or without prolonged bleeding times. In particular, the synthesis approach enabled us to compare various methods employed to study *in vivo* and *in vitro* thrombus formation. Given that a conventional meta-analysis showed major limitations in numbers of studies to be included, we developed a three- and a five-point scoring scale in order to evaluate and combine results from individual published studies. The score normalization was used for a newly developed consistency analysis in order to determine the strength of the data from literature. This led to the identification of 19 genes, which regulated murine arterial thrombus formation, but were not implicated in tail bleeding. Remarkably, genes (proteins) that are currently targeted in the clinic by anti-platelet or anti-coagulant drugs are not included in this list, which is in agreement with increased incidence of bleeding seen with all these drugs. There is high interest in the development of new antithrombotic drugs that have a better antithrombotic efficacy without enhancing bleeding, *e.g.* by targeting receptors like GPVI (one of the 19 genes identified) or PAR1 (not expressed on mouse platelets).^{51,52}

Another relevant outcome was that, in general, published results from *in vivo* thrombosis models (including thromboembolism) correlated well with those of *in vitro* flow chamber models, in particular for the collagen-dependent methods. This is of translational interest, since also with human blood, flow chamber tests are considered as a suitable initial screening tool for evaluating the efficacy of established and novel antithrombotic drugs.^{53,54}

Altogether, the findings of this thesis point to multiple interacting roles of the different blood components – platelets, red blood cells and plasma – in hemostatic reactions from platelet aggregation and thrombus formation to fibrin clot formation. Regarding platelets, it has become clear that different populations are formed with distinct properties and distinct interactions with other blood components. Although thrombocytopenia and platelet dysfunction often coincide, both conditions are relevant in a hemostatic imbalance.

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Chapter 10

Summary

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Summary

Platelets play crucial roles in thrombosis and hemostasis. In an injured vessel wall they rapidly adhere, become activated and aggregate into a thrombus clot to halt bleeding. Similarly, following rupture of an atherosclerotic plaque, platelets activate, aggregate and form an intra-arterial thrombus. Alterations in platelet activation and/or count can hence disturb the processes of thrombus and clot formation, with consequences for thrombosis or hemostasis. In this thesis, my emphasis is on acquired modes of platelet dysfunction and the underlying mechanisms of these.

Chapter 1 provides relevant background information on the regulation of platelet activation and thrombus formation. Highlighted are key platelet receptors responsible for adhesion to the vessel wall and, furthermore, the evidence for response heterogeneity between platelets. Also introduced are the clinical concepts how thrombosis is prevented by dual antiplatelet therapy, with bleeding as a side effect; as well as the conditions for platelet transfusion. In the review *Chapter 2*, a novel concept is presented how inter-platelet heterogeneity can link to priming of platelets towards increased or decreased activation properties. Platelet heterogeneity can be observed at multiple levels. Structural heterogeneity can result from variability between individual megakaryocytes and from ageing of platelets in the circulation. Within a thrombus, response heterogeneity is enhanced by the specific location of the platelet in the thrombus and unequal distribution of receptor agonists, such collagen, ADP and thrombin. Regarding priming, during their time in circulation platelets will be exposed to a wide range of molecules that either increase or reduce the responsiveness. It is considered that, in pathological conditions, where the physiological balance between positive and negative priming factors is disturbed, platelets can become either hyper- or hypo-responsive. This can lead to an increased risk of thrombosis or bleeding, respectively. As a consequence of an underlying pathology, platelets can even be activated in the circulation, and subsequently appear as non-responsive, when assayed with *in vitro* tests. Therefore, we conclude that measurement is needed of both *in vivo* markers of platelet activation and *in vitro* activity of platelets, for a better understanding of the possible risks for thrombosis or bleeding.

In order to prevent recurrent thrombotic events, patients who suffered a myocardial infarction will receive dual antiplatelet therapy for one year, consisting of the cyclo-oxygenase inhibitor aspirin and an irreversible inhibitor of the ADP receptor P2Y₁₂, prasugrel. In *Chapter 3* we investigated how the platelet activation properties recovered in patients, who stopped prasugrel intake after one year of treatment. Platelet responsiveness to ADP was hence tested at 0, 1, 2, 5 and 30 days after the cessation of drug intake. We found that after 5 days platelet responses to ADP were markedly improved, but still in part inhibited. *In vitro* addition of ticagrelor - a reversible P2Y₁₂ antagonist - suppressed the regained ADP responsiveness after >2 days, thus providing evidence for a gradual recovery of P2Y₁₂-induced signaling. Interestingly, during the first days after discontinuation of prasugrel, we identified a separate population of platelets with fully regained responsiveness to ADP. Staining with the mRNA probes, thiazole orange or 5'-Cy-oligo-dT, showed that this population consisted of so-called juvenile platelets, *i.e.* newly released from the megakaryocytes residing in the bone marrow. Although the reactivity of the juvenile platelets was higher than that of older platelets,

interestingly, the reactivity of both populations continued to increase during the whole offset period, suggesting a prolonged inhibitory effect of prasugrel intake on P2Y₁₂ receptors of megakaryocytes.

In *Chapter 4* we determined the interactions between immobilized collagen and tissue factor for platelet- and fibrin-dependent thrombus formation under flow conditions. It appeared that, alongside collagen that is required for platelet adhesion, the tissue factor-triggered generation of thrombin is a controlling factor determining the platelet-thrombus volume. In particular at low flow rates, the thrombin generated on procoagulant platelets drives the formation of fibrin fibres outside of the thrombus area. Furthermore, under conditions where platelet adhesion is a limiting factor, we find fibrin fibres redistributed from the bottom to the top of a thrombus. This chapter also elucidates the effects of the flow shear rate on the localization of fibrin and on the micro-elasticity of the fibrin-thrombus.

The glycoproteins (GP), GPIb α and GPVI, are key surface receptors for platelet adhesion to von Willebrand factor and collagen, respectively. Both receptors are known to be susceptible to cleavage of their extracellular domains by the platelet membrane-bound extracellular proteases, ADAM10/17, in a process called receptor shedding. In *Chapter 5* we investigated the signaling pathways underlying ADAM10/17 mediated receptor shedding as well as the implications of this process for platelet functions. Interestingly, we found heterogeneity in extent of shedding between populations of (activated) platelets. Platelets with phosphatidylserine exposure due to a high intracellular calcium level or a high caspase activity appeared to preferentially cleave the glycoproteins GPIb α and GPVI. Phosphatidylserine exposure also coincided with glycoprotein shedding in platelets of a thrombus. However, as the platelets from a Scott syndrome patient, which are unable to expose phosphatidylserine, still showed unchanged ADAM mediated cleavage of GPIb α we concluded that phosphatidylserine exposure was not a prerequisite for shedding. Given the procoagulant effect of phosphatidylserine exposure, we subsequently investigated how receptor shedding affected the procoagulant potential of platelets. Remarkably, we found that this was increased by enhancing the binding of prothrombin to platelets and by accelerating fibrin formation.

In *Chapter 6* we identified and characterized the dysfunction of platelets acquired by chemotherapy treatment in patients, who were diagnosed with a hematological malignancy and developed thrombocytopenia. We found that platelets from these patients show impaired integrin activation, granule secretion and spreading, when stimulated via their ADP, thrombin or collagen receptors. Markedly, the severity of the platelet dysfunction was not related to the disease type, the type of chemotherapy, or the platelet count. Moreover, platelet dysfunction was not explained by apoptosis, nor was the spontaneous phosphatidylserine exposure in the absence of agonists, since no caspase activity could be detected. Rather, mitochondrial impairments were associated with the compromised platelet responses as patient platelets showed a lower

mitochondrial O₂ consumption and a decreased mitochondrial membrane potential. Transfusion of the patients with platelet concentrates led to an overall recovery of responsiveness, thrombus formation and platelet-dependent fibrin formation.

As a consequence of substantial blood loss due to trauma or major surgery, infusion of crystalloids and/or colloid fluids is necessary for patients to maintain fluid and electrolyte homeostasis. However, the infusion also leads to dilution of the hemostatic factors and ultimately to an increased risk of bleeding, a condition known as dilutional coagulopathy. In *Chapter 7* we investigated how addition of fibrinogen, platelets, red blood cells or prothrombin complex concentrate altered clot formation and thrombin generation in plasma samples resembling those of dilutional coagulopathy. We demonstrate that, in this case, fibrinogen as well as platelets can normalize the elastic clot formation assessed by thromboelastometry. In contrast, the impaired thrombin generation was particularly normalized by the addition of prothrombin complex concentrate. These results could be confirmed with blood obtained from patients before and after major fluid infusion. Here, again, it appeared that the platelet count and fibrinogen level were main predictors of the whole blood thromboelastometry outcome, in contrast to the hematocrit and prothrombin level. Clinical decisions on transfusion with fibrinogen or plasma, based on thromboelastometry measurements alone, should therefore take into account the platelet count.

In *Chapter 8*, a synthesis paper is presented of published *in vivo* and *in vitro* studies of murine arterial thrombosis and tail bleeding. In total 1407 studies were included, resulting in a comprehensive analysis of the roles of 401 mouse genes in thrombosis and hemostasis. By developing a scoring system of the pro- and antithrombotic effects of genetic modification, we found high correlations between gene effects of the most commonly used *in vivo* and *in vitro* models. Moreover, this synthesis identified 19 genes that solely affect arterial thrombosis without a role in bleeding. By constructing a network of human orthologues of murine genes that affect thrombosis and/or bleeding, we could identify multiple novel genes with a possible role in thrombosis and hemostasis. A role of several of these new genes was validated by studying thrombus formation *in vitro* using blood from eight genetically modified mouse models. This synthesis approach can provide a guide for the identification of new antithrombotic targets in the future.

In *Chapter 9*, the most important findings of this thesis are discussed in view of the current literature. It is reasoned that knowledge is needed of not only the platelet count, but also of the activation properties of the different platelet populations, in order to make an appropriate assessment of the hemostatic imbalance of patients with an (acquired) risk of thrombosis or bleeding.



Samenvatting

Bloedplaatjes spelen een cruciale rol bij zowel hemostase als trombose. Wanneer een bloedvatwand beschadigd raakt, leidt dat tot snelle aanhechting en activering van de bloedplaatjes. Het gevolg hiervan is een trombus van geaggregeerde plaatjes, die een bloeding kan stelpen. Ook na het scheuren van een arteriële atherosclerotische plaque worden plaatjes geactiveerd, en zij vormen dan een trombus die de arterie kan afsluiten. Als gevolg hiervan kunnen dus veranderingen in ofwel plaatjesactivering ofwel plaatjesaantal leiden tot een abnormale trombusvorming, en daarmee tot een verstoorde hemostase of verhoogde trombosegeïgning. In dit proefschrift zijn dergelijke veranderingen onderzocht, met een nadruk op relevante verworven vormen van plaatjesdysfunctie en de mechanismen die daarvoor verantwoordelijk kunnen zijn.

Hoofdstuk 1 biedt relevante achtergrondinformatie over de regulatie van plaatjesactivering en trombusvorming. Uitgelicht zijn de voornaamste plaatjesreceptoren, zoals de receptoren die zorgen voor adhesie aan de vaatwand. Verder zijn de belangrijkste aanwijzingen in kaart gebracht voor heterogeniteit tussen verschillende plaatjestypen. Om het klinische belang te benadrukken van een adequate onderdrukking van plaatjesactiviteit bij (arteriële) trombose beschrijft dit hoofdstuk ook de mogelijkheden tot preventie middels duale antiplaatjetherapie, die echter regelmatig bloedingen als bijwerking hebben. In *Hoofdstuk 2* is een nieuw concept gepresenteerd over hoe de heterogeniteit tussen plaatjes van invloed kan zijn op het (on)gevoeliger maken (*priming*) van deze cellen voor stimulerende of inhiberende signalen. Deze heterogeniteit kan meerdere oorzaken hebben. Heterogeniteit in de samenstelling van plaatjes kan het gevolg zijn van verschillen tussen individuele megakaryocyten in het beenmerg, maar ook van een geleidelijke plaatjesveroudering. Binnen een trombus wordt de heterogeniteit tussen plaatjes ook bepaald door hun precieze locatie en daarmee samenhangend de ongelijke verdeling van plaatjesagonisten zoals collageen, ADP en trombine. Wat betreft het begrip *priming* worden plaatjes in de circulatie blootgesteld aan een scala van moleculen die hun responsiviteit stimuleren of juist remmen. Beargumenteerd is dat onder pathologische omstandigheden, waarin de fysiologische balans tussen positieve en negatieve *priming*-factoren verstoord is, de plaatjes van een patiënt hyper- danwel hypo-responsief kunnen worden. Dit kan leiden tot een verhoogd risico op trombose of bloeding. Als gevolg van een aandoening kunnen plaatjes in de circulatie zelfs geactiveerd worden en vervolgens dysfunctioneel raken, zoals dan blijkt uit *in vitro* testen. Op grond hiervan concluderen wij dat voor een goed begrip van de mogelijke risico's op trombose of bloedingen er metingen nodig zijn van zowel markers van de plaatjesactivering *in vivo* als de activiteit van geïsoleerde plaatjes *in vitro*.

Om een herhaalde trombose te voorkomen krijgen patiënten die een myocardinfarct hebben gehad een jaar lang duale antiplaatjetherapie, die bestaat uit de cyclo-oxygenase remmer aspirine en een irreversibele remmer van de P2Y₁₂ receptor voor ADP, prasugrel. In *Hoofdstuk 3* hebben we onderzocht hoe de plaatjesactivering herstelt in patiënten, die stoppen met prasugrel-behandeling. Daarvoor zijn de plaatjesresponsen op ADP gemeten op 0, 1, 2, 5 en 30 dagen na de laatste prasugrel-inname. Duidelijk werd dat op 5 dagen na stoppen de plaatjesrespons op ADP sterk verbeterd is, maar toch nog gedeeltelijk geremd. De herwonnen respons op ADP kon onderdrukt worden met ticagrelor – een reversibele P2Y₁₂ antagonist. Dit bevestigde dus een gradueel herstel van de signalering via P2Y₁₂ receptoren. Al op enige dagen na het stoppen van prasugrel-inname konden we

een afzonderlijke populatie van plaatjes identificeren, waarvan hun responsiviteit op ADP grotendeels hersteld was. Door plaatjes te labelen met een mRNA marker zoals thiazole-oranje of 5'-Cy-oligo-dT, konden we aantonen dat de hoog-reactieve populatie bestond uit zogenaamde juveniele plaatjes, dat wil zeggen plaatjes die recent uit het beenmerg zijn vrijgekomen. Hoewel de reactiviteit van de juveniele plaatjes hoger was dan die van de oude (geremde) plaatjes, bleef de reactiviteit van beide populaties toenemen tijdens de periode na het stoppen van de inname van prasugrel. Dit suggereert dat het plaatjes-onderdrukkend effect van prasugrel verlengd wordt door remming van de P2Y₁₂ receptoren van megakaryocyten in het beenmerg.

In *Hoofdstuk 4* is nagegaan hoe een oppervlak met collageen met/zonder weefselfactor bepalend is voor plaatjesadhesie, trombusvorming en ontstaan van een fibrinestolsel onder stromingscondities. Uit het onderzoek bleek dat naast collageen dat de plaatjesadhesie initieert, de trombine die ontstaat middels weefselfactor bepalend is voor de grootte van de gevormde plaatjes-fibrinestolsels. Met name bij lage stromingscondities stimuleerde trombinegeneratie op het oppervlak van procoagulante plaatjes de vorming van fibrinevezels buiten de trombus. Daarnaast bleek dat onder omstandigheden, waarbij de plaatjesadhesie beperkt is, de fibrinevezels herverdelen van de onderkant naar de top van de trombus. Dit hoofdstuk beschrijft verder hoe de stromingssnelheid en de lokalisatie van fibrine bepalend zijn voor de microelasticiteit van een trombus.

De glycoproteïnen (GP) GPIIb/IIIa en GPVI zijn belangrijke receptoren op het oppervlak van plaatjes voor hechting aan respectievelijk von Willebrand factor en collageen. Bekend is dat beide receptoren gevoelig zijn voor het knippen van hun extracellulaire domein door de extracellulaire proteasen ADAM10/17, welke zich ook op het plaatjesoppervlak bevinden. Dit knipproces wordt receptor *shedding* genoemd. In *Hoofdstuk 5* hebben wij onderzocht welke signaleringspaden in plaatjes ten grondslag liggen aan deze ADAM10/17-gemedieerde receptor-*shedding*. Ook hebben we de gevolgen bepaald van het *shedding*-proces voor de functionaliteit van plaatjes. Een belangrijke bevinding was dat de *shedding* van zowel GPIIb/IIIa als GPVI heterogeen verloopt, namelijk op bepaalde populaties van (geactiveerde) plaatjes. Vooral geactiveerde plaatjes met expositie van fosfatidylserine als gevolg van een hoge intracellulaire calciumconcentratie of een hoge caspase-activiteit bleken actief in het ADAM-gemedieerde knippen van GPIIb/IIIa en GPVI. Ook voor plaatjes in een trombus viel de glycoproteïne *shedding* samen met fosfatidylserine-expositie. Echter gezien de onveranderde *shedding* van GPIIb/IIIa in plaatjes van een patiënt met Scott syndroom, die dysfunctioneel zijn in fosfatidylserine-expositie, kon de conclusie getrokken worden dat fosfatidylserine-expositie geen vereiste is voor receptor-*shedding*. Gezien het procoagulante effect van fosfatidylserine-expositie, hebben we vervolgens onderzocht hoe *shedding* het stollingsvermogen van plaatjes beïnvloedt. Opmerkelijk vonden we een verhoogd stollingspotentiaal, omdat bij plaatjes met *shedding* de binding van stollingsfactoren toeneemt en de fibrinevorming versnelt.

Hoofdstuk 6 had als doel identificatie van de verworven dysfunctie van plaatjes in patiënten, die gediagnostiseerd zijn met een hematologische maligniteit, en na chemotherapie behandeling een trombocytopenie ontwikkelen. Aangevoerd kon worden dat de plaatjes van deze patiënten een verminderde activering van integrines, een lage secretie van granula, en een defecte spreiding vertoonden, wanneer ze gestimuleerd

werden met ADP, trombine of collageen. Typisch was dat de mate van plaatjesdysfunctie niet gerelateerd was aan de diagnose, het type chemotherapie of het plaatjesaantal. Bovendien kon noch de plaatjes-dysfunctie noch de spontane fosfatidylserine-expositie van plaatjes verklaard worden door actieve apoptose, aangezien geen activiteit van de apoptose-marker caspase gedetecteerd kon worden. Echter de lage plaatjesfunctie was geassocieerd met een verminderde activiteit van de mitochondriën. De patiënten-plaatjes vertoonden namelijk een verminderde zuurstofconsumptie en een verlaagd membraanpotentiaal in de mitochondriën. Transfusie van de patiënten met plaatjesconcentraat leidde tot een algeheel verbeterde plaatjesresponsiviteit, trombusvorming en plaatjes-afhankelijke fibrinevorming.

Toediening van kristalloïde en colloïde vloeistoffen is noodzakelijk om een goede vocht- en elektrolytenbalans te behouden, met name wanneer er bij een patiënt substantieel bloedverlies optreedt als gevolg van trauma of ingrijpende chirurgie. Echter deze vloeistof-infusie leidt ook tot een verdunning van hemostatische factoren in het bloed, en kan daarmee aanleiding geven tot een verhoogd bloedingsrisico. Deze conditie staat bekend als verdunningscoagulopathie. In *Hoofdstuk 7* hebben wij onderzocht hoe in verdunde plasmamonsters de extra additie van fibrinogeen, bloedplaatjes, rode bloedcellen of *protrombine complex concentraat* (PCC) leidt tot een verbeterde trombinegeneratie en stolselvorming. Dit onderzoek werd uitgevoerd met plasmamonsters, die qua samenstelling vergelijkbaar waren met die van patiënten met verdunningscoagulopathie. In dit hoofdstuk konden we aantonen dat zowel fibrinogeen als plaatjesconcentraat een normaliserend effect heeft op de fibrinestolselvorming, zoals gemeten met tromboelastometrie. Anderzijds werd de lage trombinegeneratie het beste genormaliseerd door toevoeging van PCC. Deze conclusies werden bevestigd voor bloedmonsters verkregen van operatiepatiënten, bij wie het bloed verdund was door vloeistofinfusie tijdens de ingreep. Ook in deze metingen bleek dat het plaatjesaantal en de fibrinogeenconcentratie de belangrijkste voorspellende variabelen waren van de volbloed tromboelastometrie-test. Ook nu waren hematocriet en het protrombine-niveau veel minder relevant. In de klinische beslisregels omtrent transfusie van ofwel fibrinogeen ofwel plasma, die tegenwoordig vaak gebaseerd worden op tromboelastometrie-metingen, zou daarom ook het plaatjesaantal opgenomen moeten worden.

In *Hoofdstuk 8* is een vergelijkende kwantitatieve analyse beschreven van een groot aantal gepubliceerde studies naar arteriële trombose en staartbloeding in genetisch gemodificeerde muizen. In totaal zijn 1407 studies vergeleken, waarmee inzicht verkregen werd in de rol van 401 muizengenen bij arteriële trombose, trombo-embolisme en hemostase. Middels een scoringsprocedure voor de pro- en antitrombotische effecten van genetische modificatie konden we een hoge correlatie vaststellen van de uitkomst van de meest gebruikte *in vivo* en *in vitro* trombosemodellen. Sterkte-analyse resulteerde in 19 muizengenen die een positieve rol spelen bij arteriële trombose, maar geen effect hebben op bloeding. Na samenstellen van een netwerk van humane orthologe eiwitten van die muizengenen die trombose en/of hemostase beïnvloeden, konden we meerdere nieuwe eiwitten en genen identificeren die mogelijk betrokken zijn bij deze processen.

Van een aantal van deze konden we een betrokkenheid valideren, door bestudering van de trombusvorming *in vitro*. Deze synthese-aanpak kan een toekomstige leidraad vormen voor de identificatie van nieuwe antitrombotische targets.

In *Hoofdstuk 9* zijn de meest belangrijkste bevindingen van dit proefschrift bediscussieerd in het licht van de huidige literatuur. Verder is beredeneerd dat zowel het plaatjesaantal als de activeringseigenschappen van de verschillende plaatjespopulaties bekend dient te zijn om tot een beoordeling te komen van de hemostatische disbalans van patiënten met een (verworven) risico op trombose of bloedingen.

Valorization

To valorize: to assign value or merit to

Recent years more emphasis has been put on assigning social and/or economical value to scientific findings.¹ However, the direct contribution to society is not as clear-cut for all scientific developments. To be able to improve the patient's quality of life or to ameliorate treatment, first a better understanding of the underlying physiological and pathological mechanisms has to be gained. Here, basic research is essential.

Platelets are an integral part of the hemostatic system. Upon vascular damage, platelets quickly respond by forming a platelet plug, which is stabilized by the formation of fibrin. Aberrant functionality of platelets can result in an imbalance in the hemostatic system and thereby contribute to thrombosis or hemorrhage. Arterial thrombosis underlies the development of cardiovascular disease, the number one cause of death globally, according to the World Health Organization.² Here, antiplatelet drugs are an essential part in the treatment of cardiovascular disease.³ A downfall of antiplatelet therapy is the increased risk of hemorrhage.⁴ The high occurrence of bleeding complications as a result of antithrombotic therapy again underlines the delicate hemostatic balance, indicating the need for more insight into the processes of hemostasis and thrombosis to provide optimal treatment.

In this thesis two novel techniques are presented. In *Chapter 3*, we describe a new approach for the detection of juvenile platelets, *i.e.* the oligo-dT staining. The number of juvenile platelets is an indication of the platelet turnover, *i.e.* production of new platelets and degradation/consumption of circulating platelets. In different pathological conditions one or both processes can be affected. Knowledge of merely platelet count is insufficient to distinguish between a defect in platelet production or in degradation/consumption. Here, the measurement of the number of juvenile platelets can be of great use. The current approach to detect juvenile platelets relies on the usage of thiazole orange to label mRNA, which is especially detectable in newly formed platelets.⁵ Since thiazole orange shows a specific staining of the dense granules, results can be confounded by granular content.⁶ Although the procedures for oligo-dT staining are more laborious, the oligo-dT staining might be a suitable replacement for thiazole orange due to the higher specificity. The second novel technique is described in *Chapter 4*, in which a new integrative whole blood perfusion assay is presented that allows the simultaneous assessment of both platelet and coagulant functions under flow conditions. Most current assays only study either platelet function or coagulant activity under static conditions. However, platelets promote coagulation by providing a procoagulant surface for the assembly and activation of coagulation factors, which leads to enhanced generation of thrombin. Thrombin in turn is an important platelet activator. Using this novel whole blood perfusion assay, reciprocal effects of thrombus and fibrin formation (coagulation) can be studied, providing a better reflection of the patients' hemostatic potential.

In case immediate improvement of hemostasis is required, such as in conditions with a high risk of bleeding, transfusion of blood components is one of few treatment options available.⁷ However, transfusion of blood products entails several severe

side effects such as allergic reactions, infections and incidentally acute lung injury.⁷ Furthermore, transfusion of blood products is associated with high costs. Currently, a substantial part of platelet concentrates (67%) is administered to patients with severe thrombocytopenia as a consequence of hematological malignancies and chemotherapy treatment.^{8,9} For the prevention of bleeding these patients receive prophylactic platelet transfusions on guidance of the platelet count, i.e. when the platelet count drops below $10 \times 10^9/L$.⁸ However, in this patient population platelet count shows only a weak correlation with bleeding.¹⁰ In *Chapter 6* we demonstrated that platelets from severely thrombocytopenic cancer patients treated with chemotherapy are highly dysfunctional in multiple aspects. We also showed variability in platelet responsiveness between patients, which was independent of platelet count. The next step would be to study whether platelet responsiveness is linked to the occurrence of bleeding. This may not only lead to better prevention of bleeding but also to a more efficient transfusion practice wherein only those patients will receive platelet concentrates that are likely to benefit. Ultimately this will reduce the number of platelet transfusions and thereby the negative side effects and high costs associated with it.

Patients, who suffer from severe blood loss due to trauma or surgery, may require fluid infusion to maintain electrolyte and fluid homeostasis. However, due to the large volumes of fluid infused, hemostatic factors are diluted as well, resulting in a condition better known as dilutional coagulopathy. In order to correct the dilution of the hemostatic factors, transfusion of blood products is necessary. In *Chapter 4 and 7*, the contribution of multiple blood components to thrombus and fibrin formation was investigated. We demonstrated that the addition of fibrinogen and platelets ameliorated clot formation, while addition of prothrombin complex concentrate improved thrombin generation. The addition of red blood cells only proved to be beneficial for thrombus formation when thrombus formation was studied under flow using the whole blood perfusion assay. This highlights the importance of the incorporation of flow when studying thrombus and fibrin formation. Assays that are currently used in the clinic to monitor clot formation (e.g. thromboelastometry) measure platelet function and/or coagulant capacity under static conditions. Where thromboelastometry assesses hemostasis globally¹¹ and thrombin generation determines the coagulant capacity in the absence or presence of platelets¹², both assays lack the incorporation of shear stress. Here, whole blood perfusion assays such as described in *Chapter 4*, can be of great added value to determine the need and effect of transfusion in a clinical setting.

In order to gain more insight into thrombosis and hemostasis, *in vivo* studies are of great importance. *Chapter 8* presents a synthesis of all published studies on thrombosis and hemostasis in genetically altered mice. By combining the results of all studies, several important observations could be confirmed and made. Firstly, *in vitro* models for thrombus formation correlated well to *in vivo* models for thrombosis. Thereby, *in vitro* whole blood perfusion models can partly replace and refine animal models. Secondly, by constructing a network of all genes, new genes that are highly likely to affect thrombosis and hemostasis, could be detected. Using this network, also new genes could be identified that affected thrombosis but not bleeding and are thereby, possible new targets for antithrombotic therapy.

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Curriculum Vitae

Constance Baaten was born on December 5th 1988 in Maasbracht, The Netherlands. In 2001 she started secondary school at the Trevianum Scholengroep in Sittard, where she received her gymnasium degree in 2007. The same year she started the bachelor study of Molecular Life Sciences at Maastricht University. Here she received twice the Top 3% Award of the Faculty of Health Medicine and Life Science. After finishing her bachelor degree, she obtained her Master's degree with the highest distinction (summa) cum laude at Maastricht University in Molecular Life Sciences – Oncology and Developmental Biology in 2012. Subsequently, she started as a PhD student at the Cardiovascular Research Institute Maastricht (CARIM) at the department of Biochemistry (Maastricht University, the Netherlands). Under supervision of Prof. Dr. J.W.M. Heemskerk and Dr. P.E.J. van der Meijden, she performed research in the field of thrombosis and hemostasis as described in this thesis. During this period, she worked in the laboratories of Prof. Dr. K. Kunzelmann (Regensburg, Germany), Prof. Dr. B. Nieswandt (Würzburg, Germany) and Prof. Dr. W. Ouwehand (Cambridge, United Kingdom). She presented her research at various international conferences in Bergamo (Italy), Leeds (United Kingdom) and Basel (Switzerland). Currently, she works as a researcher at the department of Biochemistry.

Constance Baaten werd geboren op 5 december 1988 te Maasbracht. In 2001 begon ze haar middelbare schoolopleiding aan de Trevianum Scholengroep te Sittard. Aldaar behaalde ze haar Gymnasiumdiploma (profiel Natuur en Gezondheid) in 2007. Aan de Universiteit Maastricht startte ze met de bacheloropleiding Moleculaire Levenswetenschappen. In deze periode kon ze tweemaal de top 3% onderscheiding van de Faculty of Health Medicine and Life Science in ontvangst nemen. Na het behalen van haar diploma in 2010, volgde ze aan dezelfde universiteit de masteropleiding Molecular Life Sciences met als specialisatie Oncology and Developmental Biology. In 2012 ontving ze haar masterdiploma met de hoogste onderscheiding, cum laude. Op 1 september 2012 begon ze als PhD-student bij het Cardiovascular Research Institute Maastricht (CARIM), binnen de Vakgroep Biochemie van de Universiteit Maastricht. Onder begeleiding van Prof. Dr. J.W.M. Heemskerk en Dr. P.E.J. van der Meijden voerde ze wetenschappelijk onderzoek uit in het domein van Trombose en Hemostase, zoals beschreven in dit proefschrift. Tijdens haar promotieperiode werkte ze in de laboratoria van Prof. Dr. K. Kunzelmann te Regensburg (Duitsland), Prof. Dr. B. Nieswandt te Würzburg (Duitsland) en Prof. Dr. W. Ouwehand in Cambridge (Verenigd Koninkrijk). Tevens presenteerde ze haar onderzoek op diverse internationale congressen in Bergamo (Italië), Leeds (Verenigd Koninkrijk) en Basel (Zwitserland). Momenteel is zij werkzaam als onderzoeker binnen de Vakgroep Biochemie.

Publications

Full papers

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6. **Baaten CC**, Swieringa F, Misztal T, Mastenbroek TG, Feijge MA, Donners MM, Collins PW, van der Meijden PE, Heemskerk JW. Heterogeneous regulation of platelet adhesive-receptor shedding in thrombus formation. Joint BSHT, AiP & UK Platelet Group Meeting, Leeds, United Kingdom; November 2016.
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 10. **Baaten CC**, Swieringa F, Misztal T, Mastenbroek TM, Feijge MA, Bock PE, Donners MM, Collins PW, Li R, van der Meijden PE, Heemskerk JW. Mechanisms of platelet adhesive-receptor shedding in platelet populations in thrombus formation. XXVI Congress of the International Society on Thrombosis and Haemostasis (ISTH), Berlin, Germany; July 2017. RPTH. 2017, 1: Suppl s1.
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Oral and poster presentations

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2. Kuijpers MJ, **Baaten CC**, Sabrkhany S, Moenen FC, Beckers EA, Henskens YM, Aarts MJ, Griffioen AW, oude Egbrink MG, Heemskerk JW, van der Meijden PE. Anti-cancer therapy inhibits platelet function. CARIM Symposium, Maastricht, The Netherlands, November 2016. (poster)
3. **Baaten CC**, Swieringa F, Misztal T, Mastenbroek TG, Feijge MA, Donners MM, Collins PW, van der Meijden PE, Heemskerk JW. Heterogeneous regulation of platelet adhesive-receptor shedding in thrombus formation. Joint BSHT, AiP & UK Platelet Group Meeting, Leeds, United Kingdom, November 2016. (poster)
4. **Baaten CC**, Swieringa F, Misztal T, Mastenbroek TG, Feijge MA, Donners MM, Collins PW, van der Meijden PE, Heemskerk JW. Heterogeneous regulation of platelet adhesive-receptor shedding in thrombus formation. GTH Annual Meeting, Basel, Switzerland, February 2017. (oral)
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as a consequence of mitochondrial dysfunction. XXVI Congress of the International Society on Thrombosis and Haemostasis (ISTH), Berlin, Germany; July 2017. RPTH. 2017, 1: Suppl s1. (poster)

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Awards

Best oral presentation, GTH, Basel, Switzerland, 2017.

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Constance



