Molecular mechanisms underlying the platelet procoagulant response: back to basics

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Promotor

Prof. Dr. Johan W. M. Heemskerk

Copromotor

Dr. Judith M.E.M. Cosemans

Beoordelingscommissie

Prof. Dr. C.P.M. Reutelingsperger (voorzitter)

Prof. Dr. F.C.S. Ramaekers

Prof. Dr. E. Lutgens (Academisch Medisch Centrum, UvA, Amsterdam)

Prof. Dr. J. de Mey (University of Southern Denmark, Odense, Denmark)

Dr. I.M.L.W. Körver-Keularts

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Chapter 1

General introduction

Platelet activation and blood coagulation in arterial thrombosis

Arterial thrombosis refers to the formation of a partially or fully occlusive thrombus in the blood at the site of a damaged, often atherosclerotic artery. Current understanding is that two major processes in the blood contribute to arterial thrombus formation, i.e. aggregation of platelets, which make up the core of the thrombus, and assembly of a fibrin clot, which consolidates the thrombus and traps red cells.¹⁻³ As such, arterial thrombus formation can be a clinically silent event, when non-essential arteries are occluded or when blood flow is only partly impaired. However, when the thrombus obstructs the blood flow to parts of the heart or brains, it becomes symptomatic and dangerous. Hence, depending on the site and size of a thrombus, arterial thrombosis can lead to heart ischemia, stroke or peripheral arterial disease.²

In general, the processes of platelet activation and blood coagulation are highly interdependent.^{4,5} An initial step in the activation of platelets upon vascular damage is the adhesion of flowing platelets to exposed collagen and von Willebrand factor via the two major collagen receptors, i.e. glycoprotein VI (GPVI) and integrin $\alpha_2\beta_1$, and the von Willebrand factor receptor complex, glycoprotein Ib-V-IX.^{6,7} The collagen-activated platelets produce a range of

paracrine mediators, which attract other flowing platelets to form a platelet aggregate. Some of the highly activated platelets become procoagulant and by allowing the assembly of coagulation factors at their surface can stimulate thrombin formation. Thrombin cleaves fibrinogen into monomeric fibrin, which polymerizes into fibrin fibers that become additionally cross-linked via the transglutaminase factor XIII, and stabilizes the growing thrombus.⁸

The complexity of the mechanism of thrombus formation is addressed in further detail in the review Chapter 2. In the paragraphs below, I will primarily focus on background information of those aspects of the thrombus-forming process that are most relevant for this thesis: platelet heterogeneity, characteristics of procoagulant platelets, and the regulation of membrane phospholipid scrambling.

Heterogeneity in platelets upon thrombus formation: differential role for intracellular calcium

In the classical concept, it is considered that two types of platelets are formed in a growing thrombus: aggregating and procoagulant platelets.⁷ Discriminative in the formation of these platelet populations is the rise in intracellular calcium concentration. Whereas a transient rise in intracellular calcium is sufficient for platelet integrin $\alpha_{IIb}\beta_3$

activation and secretion – two processes that are implicated in platelet aggregation –, a high, sustained rise in calcium is required for the exposure of phosphatidylserine, which enables platelet to become procoagulant.^{9,10} Platelet aggregation is triggered by a conformational change of integrin $\alpha_{IIb}\beta_3$, enabling the binding of fibrinogen to this receptor and allowing platelet-platelet interactions via fibrinogen cross-bridges.¹¹ Aggregated platelets have a relatively minor role in coagulation.¹² In contrast, platelets with negatively charged phosphatidylserine exposed at the outer membrane actively stimulate the coagulation process. In particular, they provide a surface for the calcium-dependent assembly of the tenase and prothrombinase complexes that are responsible for the formation of activated factor X and thrombin, respectively.¹³ Interestingly, phosphatidylserine-exposing platelets characteristically contain *inactive* $\alpha_{IIb}\beta_3$ integrins and form large blebbing structures, which may serve to enhance the accessibility to coagulation factors.¹⁴ How intracellular calcium signaling regulates the formation of these platelet populations is described in more detail in Chapter 3. Whereas the heterogeneity in platelet activation processes is already known for several years,^{15,16} the discriminative mechanisms causing platelet procoagulant response –

including integrin inactivation and bleb formation – are not well understood.

Under *in vitro* conditions, an effective way to stimulate phosphatidylserine exposure, and thus the formation of procoagulant platelets, is by co-stimulation of the collagen receptor GPVI and thrombin receptors.^{15,17} However, in papers of the Dale group platelets stimulated by collagen and thrombin are defined as a distinctive subpopulation of so-called 'coated' platelets, *i.e.* containing an extracellular coat of serotonin-derivatized proteins.¹⁸⁻²⁰

Interestingly, the definition of coated platelets is divergent and was subject to change over the years. Whereas coated platelets were initially recognized by the presence of serotonin-derivatized proteins at the cell surface¹⁸⁻²⁰, Jobe et al. redefined coated platelets as those platelets containing a high surface level of bound fibrinogen, cross-linked via the transglutaminase factor XIII.²¹ In recent years, it is starting to become common to consider coated platelets as more or less equivalent to phosphatidylserine-exposing platelets.²² However, this immediately raises the question whether the presence of a protein coat would interfere with coagulation factor binding. If and how coated platelets are different from procoagulant platelets is presently unclear.

Membrane phospholipid scrambling and anoctamins

In resting platelets, the membrane phospholipids are asymmetrically distributed over the two leaflets of the plasma membrane. The choline-containing phospholipids, phosphatidylcholine and sphingomyelin, dominate the outer leaflet, while the aminophospholipids, phosphatidylserine and phosphatidylethanolamine, are largely concentrated in the inner leaflet.^{23,24} The traditional view is that this asymmetry is maintained by an ATP-dependent aminophospholipid uptake mechanism, directing these phospholipids to the inner membrane leaflet.^{25,26} Triggering of platelets with potent Ca^{2+} -mobilizing agents abolishes this asymmetry by randomization of the phospholipids, a process driven by Ca^{2+} -influx channels.^{27,28} Alternatively, phospholipid randomization can be achieved in a calcium-independent way by apoptosis.²⁹ For long, it has been speculated that the externalization of phosphatidylserine is mediated by specific enzymes, designated as phospholipid scramblases.⁸ Early evidence suggests that the calcium-activated scramblase differs from the protein involved in apoptotic phospholipid scrambling.²³ Recently, a critical role has been reported for the transmembrane protein anoctamin-6 in Ca^{2+} -dependent

phospholipid scrambling in platelets.^{30,31}

Anoctamin-6 is a member of the anoctamin family of calcium-activated chloride channels. This name is derived from the previously supposed selectivity for anions and the presence of eight membrane-spanning domains.^{32,33} Several recent studies have shown that anoctamin-6 has the capacity to function as both a chloride channel and a cation channel.^{31,34,35} In addition to anoctamin-6, also other members of the anoctamin family may act as regulators of phosphatidylserine exposure, in particular anoctamin-1.^{32,36,37} In which way the anion and cation channel activities relate to the process of phospholipid scrambling in platelets is unknown.

Of great interest in this regard is that for patients identified with the Scott syndrome, critical mutations in the gene *ANO6* (previously named *TMEM16F*), encoding for anoctamin-6, could be established.^{30,38} The Scott syndrome is an rare inherited, moderately severe bleeding disorder, which is characterized by defective calcium-dependent phospholipid scrambling in hematopoietic cells.^{39,40} As a consequence of this defect, platelets and other blood cells from Scott syndrome patients are deficient in phosphatidylserine externalization after triggering with potent calcium-mobilizing agonists. Laboratory tests show normal

plasma levels of coagulation factors and normal platelet aggregation, but some whole-blood tests are reduced such as prothrombin consumption and thromboplastin inactivation.³⁹ This is in line with the mild clinical symptoms seen in Scott syndrome patients, such as excessive bleeding after trauma, but absence of spontaneous hemorrhages like in hemophilia. Initial experiments with patient cells and anoctamin-6 deficient mice have indicated that the defective calcium-dependent phosphatidylserine externalization in platelets from Scott syndrome patients can be attributed to reduced expression of anoctamin-6.^{30,38} However, whether anoctamin-6 is also involved in other characteristics of procoagulant platelets such as integrin inactivation and membrane blebbing is unknown. One of the novel omics tools to potentially determine if and how platelets from Scott syndrome patients are different from platelets from control subjects is the use of quantitative proteomics and phosphoproteomics.⁴¹

Aims and outline of the thesis

The studies described in this thesis have been directed to better understand the multiple mechanisms that determine the heterogeneity in platelet activation processes leading to aggregating and procoagulant platelets, with a special focus on the role of anoctamin-6.

Chapter 2 provides an extensive overview on the platelet responses implicated in thrombin generation and fibrin formation within the context of arterial thrombus formation and vascular occlusion. The subsequent **Chapter 3** gives an overview of the different populations of activated platelets that can be distinguished in a growing thrombus, with emphasis on their surface properties and their likely distinct roles in coagulation processes, *i.e.* thrombin generation, fibrin formation and fibrin clot retraction. Herein, a main distinction is made between, at one side, platelets with highly elevated cytosolic Ca^{2+} and exposed phosphatidylserine that control thrombin generation, and at the other site, platelets with activated integrin $\alpha_{\text{IIb}}\beta_3$ that regulate clot retraction.

Chapter 4 studies the mechanism underlying the process of integrin $\alpha_{\text{IIb}}\beta_3$ inactivation or closure in procoagulant platelets. Novel data are provided that calpain-induced cleavage of integrin-associated proteins as well as anoctamin-6-dependent phospholipid scrambling are principal determinants of integrin $\alpha_{\text{IIb}}\beta_3$ inactivation. Detailed studies how coated platelets (activated by collagen/thrombin receptors) differ from phosphatidylserine-exposing platelets are given in **Chapter 5**. This chapter furthermore reveals a novel function of coated platelets in the regulation of

platelet-dependent fibrin formation, and identifies redundant roles of integrin $\alpha_{IIb}\beta_3$ and factor XIII in this process.

In **Chapter 6**, the role is investigated of anoctamin-6 in phosphatidylserine exposure in platelets stimulated with calcium-mobilizing agonists or apoptotic agents using platelets from a patient with Scott syndrome with dysfunctional mutations in the *ANO6* gene. In the ensuing **Chapter 7**, additional studies are performed with platelets from Scott patients and anoctamin-deficient mice to determine whether the deficiency in anoctamin-6 can account for the multiple responses of procoagulant platelets. A pertinent question is whether the formation of membrane blebs, accompanying phosphatidylserine

exposure, is a consequence of anoctamin-6 expression. The studies to further characterize the altered properties of Scott platelets are pursued in **Chapter 8**. Here, quantitative (phospho)proteomics is used to compare the expression levels and the phosphorylation status of the platelet proteome from a Scott syndrome patient with the platelet proteome from a healthy control. In addition, this chapter describes a new developed tool to detect changes in calpain-dependent protein cleavage between Scott and control platelets. In the general discussion of **Chapter 9**, the principle findings of this thesis are considered in relation to the current literature.

References

1. Brass LF, Wannemacher KM, Ma P, Stalker TJ. Regulating thrombus growth and stability to achieve an optimal response to injury. *J Thromb Haemost* 2011;**9 Suppl** 1:66-75.
2. Jackson SP. Arterial thrombosis--insidious, unpredictable and deadly. *Nat Med* 2011;**17**:1423-1436.
3. Mastenbroek TG, van Geffen JP, Heemskerk JW, Cosemans JM. Acute and persistent platelet and coagulant activities in atherothrombosis. *J Thromb Haemost* 2015;**13 Suppl** 1:S272-80.
4. Munnix IC, Cosemans JM, Auger JM, Heemskerk JW. Platelet response heterogeneity in thrombus formation. *Thromb Haemost* 2009;**102**:1149-1156.
5. Badimon L, Vilahur G. Thrombosis formation on atherosclerotic lesions and plaque rupture. *Journal of Internal Medicine* 2014;**276**:618-632.
6. Ruggeri ZM. The role of von Willebrand factor in thrombus formation. *Thromb Res* 2007;**120 Suppl** 1:S5-9.
7. Versteeg HH, Heemskerk JW, Levi M, Reitsma PH. New fundamentals in hemostasis. *Physiol Rev* 2013;**93**:327-358.
8. Bevers EM, Comfurius P, Zwaal RF. Regulatory mechanisms in maintenance and modulation of transmembrane lipid asymmetry: pathophysiological implications. *Lupus* 1996;**5**:480-487.
9. Heemskerk JW, Vuist WM, Feijge MA, Reutelingsperger CP, Lindhout T. Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine, and procoagulant activity of adherent platelets: evidence for regulation by protein tyrosine kinase-dependent Ca^{2+} responses. *Blood* 1997;**90**:2615-2625.
10. Varga-Szabo D, Braun A, Nieswandt B. Calcium signaling in platelets. *J Thromb Haemost* 2009;**7**:1057-1066.
11. Shattil SJ, Kim C, Ginsberg MH. The final steps of integrin activation: the end game. *Nat Rev Mol Cell Biol* 2010;**11**:288-300.

12. Briede JJ, Wielders SJ, Heemskerk JW, Baruch D, Hemker HC, Lindhout T. von Willebrand factor stimulates thrombin-induced exposure of procoagulant phospholipids on the surface of fibrin-adherent platelets. *J Thromb Haemost* 2003;**1**:559-565.
13. Munnix IC, Kuijpers MJ, Auger J, Thomassen CM, Panizzi P, van Zandvoort MA, Rosing J, Bock PE, Watson SP, Heemskerk JW. Segregation of platelet aggregatory and procoagulant microdomains in thrombus formation: regulation by transient integrin activation. *Arterioscler Thromb Vasc Biol* 2007;**27**:2484-2490.
14. Cosemans JM, Iserbyt BF, Deckmyn H, Heemskerk JW. Multiple ways to switch platelet integrins on and off. *J Thromb Haemost* 2008;**6**:1253-1261.
15. Siljander PR, Munnix IC, Smethurst PA, Deckmyn H, Lindhout T, Ouwehand WH, Farndale RW, Heemskerk JW. Platelet receptor interplay regulates collagen-induced thrombus formation in flowing human blood. *Blood* 2004;**103**:1333-1341.
16. Kempton CL, Hoffman M, Roberts HR, Monroe DM. Platelet heterogeneity: variation in coagulation complexes on platelet subpopulations. *Arterioscler Thromb Vasc Biol* 2005;**25**:861-866.
17. Briede JJ, Heemskerk JW, van't Veer C, Hemker HC, Lindhout T. Contribution of platelet-derived factor Va to thrombin generation on immobilized collagen- and fibrinogen-adherent platelets. *Thromb Haemost* 2001;**85**:509-513.
18. Alberio LJ, Clemetson KJ. All platelets are not equal: COAT platelets. *Curr Hematol Rep* 2004;**3**:338-343.
19. Dale GL. Coated-platelets: an emerging component of the procoagulant response. *J Thromb Haemost* 2005;**3**:2185-2192.
20. Szasz R, Dale GL. COAT platelets. *Curr Opin Hematol* 2003;**10**:351-355.
21. Jobe SM, Leo L, Eastvold JS, Dickneite G, Ratliff TL, Lentz SR, Di Paola J. Role of FcRγ and factor XIIIa in coated platelet formation. *Blood* 2005;**106**:4146-4151.
22. Colucci G, Stutz M, Rochat S, Conte T, Pavicic M, Reusser M, Giabbani E, Huynh A, Thurlmann C, Keller P, Alberio L. The effect of desmopressin on platelet function: a selective enhancement of procoagulant COAT platelets in patients with primary platelet function defects. *Blood* 2014;**123**:1905-1916.
23. Bevers EM, Williamson PL. Phospholipid scramblase: an update. *FEBS Lett* 2010;**584**:2724-2730.
24. Zwaal RF, Comfurius P, Bevers EM. Mechanism and function of changes in membrane-phospholipid asymmetry in platelets and erythrocytes. *Biochem Soc Trans* 1993;**21**:248-253.
25. Tang X, Halleck MS, Schlegel RA, Williamson P. A subfamily of P-type ATPases with aminophospholipid transporting activity. *Science* 1996;**272**:1495-1497.
26. Schroit AJ, Zwaal RF. Transbilayer movement of phospholipids in red cell and platelet membranes. *Biochim Biophys Acta* 1991;**1071**:313-329.
27. Harper MT, Londono JE, Quick K, Londono JC, Flockerzi V, Philipp SE, Birnbaumer L, Freichel M, Poole AW. Transient receptor potential channels function as a coincidence signal detector mediating phosphatidylserine exposure. *Sci Signal* 2013;**6**:ra50.
28. van Kruchten R, Braun A, Feijge MA, Kuijpers MJ, Rivera-Galdos R, Kraft P, Stoll G, Kleinschnitz C, Bevers EM, Nieswandt B, Heemskerk JW. Antithrombotic potential of blockers of store-operated calcium channels in platelets. *Arterioscler Thromb Vasc Biol* 2012;**32**:1717-1723.
29. Schoenwaelder SM, Yuan Y, Josefsson EC, White MJ, Yao Y, Mason KD, O'Reilly LA, Henley KJ, Ono A, Hsiao S, Willcox A, Roberts AW, Huang DC, Salem HH, Kile BT, Jackson SP. Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function. *Blood* 2009;**114**:663-666.
30. Suzuki J, Umeda M, Sims PJ, Nagata S. Calcium-dependent phospholipid scrambling by TMEM16F. *Nature* 2010;**468**:834-838.
31. Yang H, Kim A, David T, Palmer D, Jin T, Tien J, Huang F, Cheng T, Coughlin SR, Jan YN, Jan LY. TMEM16F forms a Ca²⁺-activated cation channel required for lipid scrambling in platelets during blood coagulation. *Cell* 2012;**151**:111-122.
32. Pedemonte N, Galletta LJ. Structure and function of TMEM16 proteins (anoctamins). *Physiol Rev* 2014;**94**:419-459.
33. Picollo A, Malvezzi M, Accardi A. TMEM16 proteins: unknown structure and confusing functions. *J Mol Biol* 2015;**427**:94-105.
34. Kunzelmann K, Nilius B, Owsianik G, Schreiber R, Ousingsawat J, Sirianant L, Wanitchakool P, Bevers EM, Heemskerk JW. Molecular functions of anoctamin 6 (TMEM16F): a chloride channel,

- cation channel, or phospholipid scramblase? *Pflugers Arch* 2014;**466**:407-414.
35. Shimizu T, Iehara T, Sato K, Fujii T, Sakai H, Okada Y. TMEM16F is a component of a Ca^{2+} -activated Cl^- channel but not a volume-sensitive outwardly rectifying Cl^- channel. *Am J Physiol Cell Physiol* 2013;**304**:C748-759.
 36. Fagerberg SK, Skals M, Leipziger J, Praetorius HA. P2X receptor-dependent erythrocyte damage by α -hemolysin from *Escherichia coli* triggers phagocytosis by THP-1 cells. *Toxins* 2013;**5**:472-487.
 37. Suzuki J, Fujii T, Imao T, Ishihara K, Kuba H, Nagata S. Calcium-dependent phospholipid scramblase activity of TMEM16 protein family members. *J Biol Chem* 2013;**288**:13305-13316.
 38. Castoldi E, Collins PW, Williamson PL, Bevers EM. Compound heterozygosity for 2 novel TMEM16F mutations in a patient with Scott syndrome. *Blood* 2011;**117**:4399-4400.
 39. Weiss HJ, Vivic WJ, Lages BA, Rogers J. Isolated deficiency of platelet procoagulant activity. *Am J Med* 1979;**67**:206-213.
 40. Zwaal RF, Comfurius P, Bevers EM. Scott syndrome, a bleeding disorder caused by defective scrambling of membrane phospholipids. *Biochim Biophys Acta* 2004;**1636**:119-128.
 41. Burkhardt JM, Gambaryan S, Watson SP, Jurk K, Walter U, Sickmann A, Heemskerk JW, Zahedi RP. What can proteomics tell us about platelets? *Circ Res* 2014;**114**:1204-1219.

Chapter 2

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

Cosemans JM¹, Angelillo-Scherrer A², Mattheij NJ¹,
Heemskerk JW¹

¹Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands; ²Service and Central Laboratory of Hematology, Lausanne University Hospital, Lausanne, Switzerland

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Abstract

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

1. Introduction

At prevalent flow conditions, platelets interact only limitedly with the healthy vessel wall, e.g. to maintain vascular integrity. However, this changes dramatically at conditions of vascular inflammation, damage, or disruption (situations leading to atherogenesis, haemostasis, or arterial thrombosis, respectively), where platelets massively adhere to the activated endothelium or the underlying endothelial matrix.^{1–3} In this overview, we discuss shear-dependent mechanisms by which platelets are capable to adhere to activated, damaged or disrupted arterial

vessels, and subsequently assemble into a stable or unstable thrombus.^{4,5} We describe how the classical concept of arterial thrombus formation, developed almost 20 years ago, is currently extended and fine-tuned due to better insights into the underlying molecular mechanisms in relation to blood flow dynamics. We illustrate how local changes in fluid shear stress can control both platelet and coagulation activation and, thereby, the growth and stabilization of a thrombus. Furthermore, we recapitulate key roles of the vessel wall and leucocytes in the control of thrombus formation. By comparing

recently published studies, where the effects of gene knockout have been determined on thrombus stability and embolus formation in mouse, we provide a first encompassing overview of the activation pathways in platelets and blood plasma that may control these processes. Subsequently, we discuss the possible clinical relevance of these findings on flow-dependent thrombus stabilization and embolization. For reasons of space, we only briefly touch the processes of venous thromboembolism and fibrinolysis.

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

How platelets adhere at a site of vascular activation or injury greatly depends on the local blood flow and shear conditions. In the arterial system, the mechanical force that is most relevant for platelet adhesion is the shear stress of the blood.⁶ Since the velocity of flowing blood is greater in the centre of the artery than near the vessel wall, blood consists of concentric layers through the artery lumen that markedly differ in flow rate. Shear stress is defined as the force per unit area between such layers, and wall shear stress is the force per unit area applied onto the vessel wall. Accordingly, also the shear rate (expressed as shear stress times the viscosity of the blood) varies through the

artery lumen from minimal at the centre-line to maximal near the wall. Typical wall shear rates are in the range of 300–800 s^{-1} in large arteries, and of 500–1600 s^{-1} in arterioles of the microcirculation.⁶ Especially in stenotic vessels, the wall shear rates can increase up to 10 000 s^{-1} and even higher.⁷ Wall shear rates in the venous part of the circulation are in general low.

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

The initial, shear-dependent adhesion of platelets is possible due to unique biomechanical properties of the vWF

bond with GPIb-V-IX, as this is characterized by a very rapid on-rate and facilitated by unfolding of vessel wall-adhered vWF multimers.² However, the bond between GPIb-V-IX and vWF also has a rapid off-rate, implicating that by itself it is insufficient for stable platelet adhesion, except in situations of quite high-shear rate. The adhesion of platelets to vWF is stabilized by weak activation of integrin $\alpha_{IIb}\beta_3$, which mediates the integrin-dependent binding of platelets to vWF, and also facilitates the binding to platelets of plasma components such as fibrinogen and fibronectin.^{12,13} Interestingly, GPIb-V-IX-dependent activation of $\alpha_{IIb}\beta_3$ appears to be impaired in platelets from mice lacking phospholipase D1 by a mechanism that relies on reduced phosphatidic acid production.^{14,15} In both the human and mouse systems, platelet interaction with collagen/vWF provides one of the most potent ways to attain stable adhesion and to trigger platelet activation processes to thrombus formation.^{3,12,16} The signalling in platelets occurs by way of interplay between multiple receptors with, next to GPIb-V-IX and $\alpha_{IIb}\beta_3$, the immunoglobulin-family collagen receptor, GPVI, and the adhesive collagen receptor, integrin $\alpha_2\beta_1$.^{17,18} Activation of platelets via GPVI, in complex with the Fc receptor γ -chain (FcR γ), is mediated by a 'signalosome' of multiple proteins,

including various adapter and scaffold proteins (e.g. LAT, Cbl-b), tyrosine protein kinases (e.g. Syk), phosphatidylinositol 3-kinases (PI 3-kinases), and small GTP-binding proteins and their regulators (like Rac, Rho, CalDAG-GEFI).¹⁹ The GPVI-induced signalling culminates in activation of phospholipase $\text{C}\gamma_2$ (PLC γ_2), which produces second messengers causing an intracellular release of Ca^{2+} , followed by store-regulated influx of extracellular Ca^{2+} via the Ca^{2+} -sensor STIM1, and activation of downstream protein kinases.²⁰ Integrin $\alpha_2\beta_1$, like other integrins interacting with their substrates, strengthens and stabilizes the adhesion of platelets to collagen.^{16,18} Platelets dispose of signalling mechanisms to tightly synchronize the activation state—and thus adhesiveness—of their various integrins.²¹ Under shear conditions, platelets can also arrest at other extracellular matrix proteins, like thrombospondin-1, but the ensuing signalling pathways are less intensively studied than for adhesion to collagen/vWF.^{8,22}

The shear-dependent adhesion and subsequent intracellular signalling leads to a range of biochemical and morphological platelet responses. Alterations in platelet shape occur via remodelling of the actin-myosin cytoskeleton (via Rac1 and Rho-kinase pathways) and polymerization of

microtubules (with Ran-binding protein 10), resulting in the formation of pseudopods and lamellipods after adhesion.^{23,24} Other prothrombotic responses include enhanced activation of the various integrins, the release of mediator molecules from platelet dense and alpha granules (such as ADP, ATP, and Gas6), the formation of thromboxane A₂, and the scrambling of membrane phospholipids.^{3,15,25} These processes achieve capturing of flowing platelets, thus leading to a growing platelet aggregate, where platelets primarily interact via activated $\alpha_{IIb}\beta_3$ integrins that bridge fibrinogen or fibronectin molecules. At high-shear conditions, platelet–platelet interaction can also be achieved via GPIb-V-IX interacting with vWF, bound itself to the platelet aggregate.²⁶ Defects in these activation pathways may lead to impaired haemostasis and bleeding, as described in detail elsewhere.²⁷

In the classical concept, the consolidation of a growing platelet thrombus is achieved by activation of the coagulation process. Blood coagulation can be initiated by (extrinsic) tissue factor highly expressed at the surface of subendothelial cells and, alternatively, by collagen and platelet-derived polyphosphates, which trigger the (intrinsic) factor XII pathway of coagulation.^{28–30} Subendothelial tissue factor, particularly on fibroblasts and

smooth muscle cells, needs encryption before it becomes active in coagulation, possibly through disulfide bond changes by protein disulfide isomerase.^{3,31} Also tissue factor on microparticles can fulfil such a role.³² These initial processes can only become effective if enforced in the propagation phase of coagulation, depending on the exposure of pro-coagulant phospholipids, formed at the membrane of platelets and other cells by a Ca^{2+} -dependent phospholipid scramblase.^{3,33} Thus, membranes with the surface expression of phosphatidylserine provide an active site for coagulation factor complexes formation and thrombin generation.^{2,34} The generated thrombin produces fibrin fibres at the platelet surface, which are considered to stabilize and consolidate the thrombus, and to mediate platelet-dependent clot retraction.^{35,36} This fibrin network can trap flowing erythrocytes and leucocytes, ultimately resulting in full-vessel occlusion.

This sequential scheme of arterial (occlusive) thrombus formation explains why such a wide variety of drugs in use or in study can suppress this process, i.e. blockers of: GPIb-V-IX (humanized antibodies), thromboxane A₂ formation (aspirin), P2Y₁₂ receptors (clopidogrel, prasugrel, ticagrelor), $\alpha_{IIb}\beta_3$ integrins (abciximab, eptifibatide, tirofiban), thrombin generation (anticoagulants), and protease-activated receptor (PAR)-

family thrombin receptors.³⁷ However, as discussed extensively elsewhere, minor or major bleeding is a known undesired side effect of essentially all these drug types.^{15,27} This explains the still continuous search for new targets of antithrombotic drugs, where the risk of bleeding is minimal.

3. Vascular control

Discussed extensively elsewhere are the molecular processes by which the healthy endothelium suppresses the coagulation process.³ The thrombo-protective potential of vascular endothelial cells also extends to preventing stable platelet adhesion and activation by a variety of mechanisms (Figure 1). The endothelial products nitric oxide and prostacyclin (prostaglandin I₂) both act to relax vascular smooth muscle cells and inhibit platelet activation.³⁸ Thus, in healthy endothelium, nitric oxide and prostacyclin maintain a low blood pressure, reduce blood shear forces, and suppress platelet activation. Mouse models confirm the antithrombotic roles of both products. Hence, mice lacking endothelial nitric oxide synthase show an impaired vasodilator response in conductance vessels. Mouse models, in which the COX2-dependent formation of prostacyclin or its action is disrupted, present with a predisposition to thrombotic events.³⁹ Another platelet-

inhibiting protein at the endothelial surface is the ectonucleotidase CD39, which degrades (endothelial-derived) ATP and ADP, and thus prevents platelet activation by these nucleotides. Furthermore, the adenosine produced by CD39 has anti-platelet activity.⁴⁰ Clearly, these thrombo-protective effects of the vessel wall are abolished upon local damage or disruption of the endothelium. A recent topic of interest is the role of vessel wall-adherent leucocytes in thrombus formation. In certain mouse models, neutrophils can adhere even earlier than platelets upon vascular damage, with as a result increased tissue factor-dependent fibrin generation and platelet accumulation.⁴¹ Both neutrophils (forming neutrophil extracellular traps) and monocytes (exposing tissue factor) support the thrombotic process especially under conditions of venous thrombosis.^{42,43} The relevance of these processes for the development of arterial thrombosis is still unclear.

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

The classical scheme described above considers arterial thrombus formation as a simple progressive process, starting with platelet adhesion and ending with occlusion of the locally activated or damaged vessel. However, many experimental studies, either *in vivo* with

I. Thromboprotective vascular function

II. Shear induced platelet adhesion (de-adhesion)

III. Platelet aggregation (small emboli)

IV. Platelet contraction and procoagulant activity (large emboli)

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

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

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

Impaired or diminished activation of platelet $\alpha_{IIb}\beta_3$ is known to cause instability of thrombi, which are formed under flow *in vivo* or *in vitro*, and to stimulate the detachment of single platelets and small aggregates.^{44,45} *In vitro* observations support the notion that $\alpha_{IIb}\beta_3$ activation is a reversible process, and that persistent signalling in platelets is required to keep this integrin in an activated, pro-adhesive conformation.⁴⁶ Several autocrine and paracrine (between platelets) signalling processes appear to contribute to persistent integrin activation, and many of these have been shown to be involved in thrombus stabilization. A prominent factor is the release of ADP and its interaction with platelet P2Y₁₂ receptors, which stimulates integrin activation via a pathway involving PI3-kinase, Akt2, Rap1b, and filamin A (*Figure 2*).^{46–49} Enforced integrin activation is furthermore achieved by interaction of CD40L with its supposed ligand CD40, both of which are membrane proteins that regulate thrombus stability.⁵⁰ Another mechanism for continued integrin activation is provided by interaction of the soluble molecule Gas6 (present in plasma and limitedly stored in platelets) with the platelet TAM receptors, Tyro, Axl, and Mer.^{51–53} New

Table 1. Reported effects of genetic deficiency in mouse on embolization during arterial thrombus formation *in vivo* or *in vitro*. Indicated are the mouse genes, the corresponding proteins in blood platelets or plasma, the murine thrombosis model used (flow device in case of *in vitro* studies), the effect on embolus formation (+, increased; ++ highly increased, -, decreased). Abbreviation: MGI, mouse genome index.

Gene defect	MGI	Protein defect	Thrombosis model	Effect on arterial thrombus formation	Emboli	Ref(s).
Platelet receptors and membrane proteins						
Axl	1347244	Axl (Gas6 receptor)	flow device	increased thrombus disaggregation	+	53
Adra2a	87934	a2 adrenergic receptor	mesentery/FeCl ₃	increased formation of emboli	+	95
Cd151	1096360	tetraspanin CD151	carotis/ligation, cremaster/laser	increased thrombus instability	++	56
Cd40lg	88337	CD40L	mesentery/FeCl ₃	delayed occlusion, more unstable thrombi	++	50
Fcgr1g	95496	FcR g-chain	carotis/ligation, cremaster/laser	smaller thrombi, more emboli formed	+	76,96
Gp1ba	1333744	GP1ba	carotis/ligation	smaller thrombi, reduced stable platelet adhesion	++	97
Gp5	1096363	GPV	mesentery/FeCl ₃	smaller thrombi, increased detachment	++	98,99
Gp6	1889810	GPVI	carotis/FeCl ₃ , ligation	smaller thrombi, reduced stable platelet adhesion	++	100,101
Itga2	96600	integrin a2	mesentery/FeCl ₃ , flow device	smaller thrombi, more emboli formed	+	75,102
Lat	1342293	LAT	cremaster/ laser	smaller thrombi, more emboli formed	++	76
Mertk	96965	Mer (Gas6 receptor)	flow device	increased thrombus disaggregation	+	53
P2Yr12	1918089	P2Y ₁₂ receptor	mesentery/FeCl ₃ , cremaster/laser	smaller thrombi, more emboli formed	++	47,103
			flow device	increased thrombus embolization	++	103,104
Slamf1	1351314	SLAM (CD84)	mesentery/FeCl ₃	delayed occlusion, more emboli formed	+	59
Tspan32	1350360	tetraspanin TSSC6	mesentery/FeCl ₃	increased thrombus instability	+	55
Tyro3	104294	Sky (Gas6 receptor)	flow device	increased thrombus disaggregation	+	53
Ceacam1	1347245	CEACAM1	mesentery/FeCl ₃	larger thrombi, less emboli formed	-	62
Esam	1916774	ESAM	cremaster/laser	larger thrombi, less detachment	-	63
Gja4	95715	connexin 37	mesentery/FeCl ₃	increased thrombus formation, less emboli	-	65
Platelet intracellular signaling proteins						
Akt2	104874	Akt2	carotis/FeCl ₃	smaller thrombi, increased instability	++	105
Cblb	2146430	Cbl-b	carotis/FeCl ₃	delayed occlusion, unstable thrombi	+	106
Flna	95556	filamin A	flow device	increased platelet detachment	+	107
Myh9	107717	myosin heavy chain-9	carotis/FeCl ₃	reduced thrombus growth, more emboli formed	+	23
Plcg2	97616	phospholipase Cg2	cremaster/ laser, flow device	(smaller) thrombi, increased instability	++	76,108
Prkaa2	1336173	AMPK-a2	carotis/FeCl ₃	less compact thrombus, more emboli formed	+	109
Plk3cb	1922019	PI 3-kinase-b	flow device	increased thrombus disaggregation	++	46
Pik3cg	1353576	PI 3-kinase-g	flow device	unstable thrombi, increased disassembly	++	46
Rac1	97845	Rac1	cremaster/laser, flow device	increased instability of thrombi	++	110
Ranbp10	1921584	Ran-binding protein 10	mesentery/FeCl ₃	reduced occlusion, unstable thrombi	+	24
Rhoa	1096342	RhoA	mesentery/FeCl ₃	reduced occlusion, more emboli formed	++	61
Stim1	107476	STIM1	mesentery/FeCl ₃	delayed occlusion, increased platelet detachment	+	111
Plasma proteins						
C3	88227	complement factor 3	cremaster/photochemical	delayed thrombus formation, more emboli	+	112
F11	99481	factor XI	mesentery/FeCl ₃	increased detachment of thrombi	+	80
F12	1891012	factor XII	carotis/FeCl ₃ , mesentery/FeCl ₃	increased detachment of thrombi	++	80,81
Fgg	95526	fibrinogen g-chain	carotis/FeCl ₃ , mesentery/FeCl ₃	increased detachment of thrombi	++	113,114
Fn	95566	fibrinectin	mesentery/FeCl ₃	delayed formation of unstable thrombi	++	115
Gas6	95660	Gas6	flow device	increased thrombus disaggregation	+	53
Klk4	1861379	prekallikrein	mesentery/FeCl ₃	reduced thrombus formation, more emboli	+	81
Lep	104663	leptin	carotis/FeCl ₃	delayed occlusion, unstable thrombi	+	116
Serpine1	97608	PAI-1	carotis/FeCl ₃	longer time to occlusion, unstable thrombi	+	117
Thbs1	98737	thrombospondin-1	mesentery/photochemical	prolonged occlusion, more emboli formed	+	118
Vtn	98940	vitronectin	carotis/FeCl ₃ , mesentery/FeCl ₃	longer time to occlusion, unstable thrombi	++	117,119
Vwf	98941	vWF	mesentery/FeCl ₃ (venules)	reduced thrombus formation, unstable	+	120
Plg	97620	plasminogen	carotis/photochemical	shortened occlusion, less emboli	-	121

data yet suggest that plasma Gas6 may also stimulate the coagulation process by regulating the expression of vascular tissue factor.⁵⁴

Limited integrin activation also explains why, at gradients of shear stress, platelets tend to loosely adhere to a growing thrombus via GPIb-V-IX in an often unstable way.¹⁰ Another family of proteins that is considered to modulate platelet integrin function is provided by

the tetraspanins, of which TSSC6 and CD151 are abundantly expressed in the platelet membrane. Tetraspanin control of $\alpha_{IIb}\beta_3$ activation may explain why the genetic ablation of TSSC6 or CD151 results in thrombus instability and increased embolus formation.^{55,56} Another mechanism controlling the activation of $\alpha_{IIb}\beta_3$ and other integrins is by redox control of free-cysteine thiols in the extracellular chains, particularly by

the protein disulfide isomerase.⁵⁷ How the redox control affects thrombus stabilization is still unknown.

Contact-dependent signalling to tight platelet interactions

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

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

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

A negative role in thrombus stability has also been observed for the contact protein, connexin 37.⁶⁵ This is a gap junction protein expressed in platelets, as well as in endothelial cells, smooth muscle cells, monocytes, and macrophages.^{65–67} Interestingly, the conclusions from the two publications regarding the role of connexin 37 in platelets are different: one research group concludes that it functions by limiting platelet activation and thrombus

I. Initial platelet aggregation

II. Contact-dependent platelet activation

III. Coagulation factor stimulation

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

stabilization,⁶⁵ while the other group finds that it promotes platelet activation.⁶⁷ An approach to take this further would be the generation of mice lacking connexin 37 only in platelets.

Multisided regulation by coagulation

The role of coagulation in thrombus formation and stabilization appears to be more complex than earlier thought. Thrombin that is formed at the thrombus surface contributes to platelet activation by interaction with PARs.^{68,69} Recent evidence suggests that the contribution of PARs to platelet activation is dependent on the blood flow rate. Whereas PARs activate platelets at low-shear conditions, their role becomes diminished at pathologically high shear rates.⁷⁰ Yet, thrombin-induced signalling contributes to the generation of pro-coagulant PS exposing platelets, which are abundantly formed in arterial thrombi.⁷¹ There is ongoing research to find other strong agonists—besides thrombin—that can support collagen-induced platelet activation (via GPVI). Currently in the spot light are the CLEC-2 receptors, which via an unknown ligand, have been implicated in arterial thrombus formation in mice.⁷² Such strong agonists other than thrombin may also stimulate the contraction of platelets, making an aggregate stable.⁶⁰ On the other hand, fibrin clot retraction, mediated via activated $\alpha_{IIb}\beta_3$ integrins, is

still considered to be a main mechanism for platelet contraction in a stabilising thrombus.³⁴

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

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

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

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

Disturbances in blood rheology

An aspect that is discussed extensively elsewhere,² is the contribution of blood flow, and in particular of rheological disturbances, on the stabilization or embolization of near-occlusive thrombi. When a stenotic or otherwise vulnerable vessel tends to become occluded, high-shear gradients are generated around the growing thrombus. These flow disturbances will not only accelerate platelet activation and fibrin formation,

but also provide the force for embolization of smaller or larger platelet aggregates (*Figure 1*). Flow pulsations by the heart rhythm and vascular distension may further aggravate the extent of embolization and perhaps the size of the emboli, but this has hardly been investigated. Another still poorly studied aspect is how red blood cells—either flowing or when bound to fibrin fibres—contribute to thrombus stability under arterial flow conditions.

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

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

thrombus stability *in vitro* during the perfusion of the human blood through a flow device at a high arterial shear rate. As indicated in *Table 1*, for 44 different mouse genes a notable change in stability of arterial thrombi has been reported. This list mostly concerns genes and proteins that also play a role in the overall process of thrombus growth. In short, referring to the mechanisms described above, this concerns genes implicated in: (i) GPIb-V-IX and GPVI-dependent platelet adhesion (also $\alpha_2\beta_1$, FcR γ -chain); (ii) GPVI-mediated platelet signalling to Ca^{2+} rises and beyond (PLCy2, LAT, Cbl-b, STIM1, Rac1); (iii) integrin activation (PI 3-kinases, Akt2, filamin A, tetraspanins); (iv) autocrine and paracrine regulatory mechanisms supporting integrin activation (P2Y₁₂, CD40, CD40L, Axl, Mer, Sky, SLAM); (v) and regulation of platelet contraction (RhoA, myosin). Furthermore relevant are genes of plasma proteins involved in: (vi) adhesion to platelets (vWF, fibrinogen, fibronectin, vitronectin, thrombospondin-1); (vii) activation of platelets (Gas6, leptin); and (viii) activation of the intrinsic coagulation cascade (prekallikrein, factors XI, XII). Interestingly, hardly any reports are available on thrombus instability due to specific platelet secretion defects. A suppressive role is reported for negative regulators of the contact activation (CEACAM1, ESAM, connexin 37).

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

6. What can we learn more from patient observations?

In man, thrombosis refers to the pathological condition where thrombi form inopportunistically in the lumen of vulnerable vessels, leading to interruption of blood flow, occlusion, and ensuing tissue damage. Antithrombotic drugs, which comprise antiplatelet, anticoagulant, and antifibrinolytic drugs, are commonly used for the treatment and secondary prevention of such thromboses in arteries and veins.^{2,37} The common cause is rupture or erosion of an atherosclerotic plaque or a local disturbance in haemodynamic shear forces in the flowing blood. Arterial

thrombosis, causing heart attacks, stroke, or limb gangrene, is responsible for almost 50% of mortality in industrialized countries. Next to treatments stimulating vasodilatation, antiplatelet drugs are the first choice for treatment of (secondary) arterial thrombosis.⁸⁴ It is relevant to note here that non-steroidal anti-inflammatory drugs, particularly those which inhibit COX2-dependent formation of prostacyclin in the vessel wall, confer a cardiovascular hazard.³⁹ Such drugs antagonize the capacities of prostacyclin to suppress platelet activation and vasoconstriction. This can predispose to thrombosis, hypertension, and atherosclerosis.

Patients with a transient ischaemic attack/stroke or myocardial infarction mostly suffer from thrombosis of the atherosclerotic carotid or coronary artery. Such patients may present with symptomatic emboli that are shed from the earlier formed thrombi. However, in case of acute stroke or post-operatively after carotid endarterectomy, patients may also develop clinically asymptomatic embolization.⁸⁵ Asymptomatic embolization has also been reported following carotid artery stenosis.⁸⁶ In such patients, shedding of platelet emboli from the thrombotic carotid artery can be detected using transcranial Doppler ultrasound, for the major part without pathological consequences.⁸⁷ This indicates that

embolization is a frequent phenomenon after a thrombotic event that, although often clinically silent, yet may form an increased risk of becoming symptomatic. More research is clearly needed to ascertain this.

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

Table 2. Reported effects of pharmacological inhibitors on embolization of human thrombi under high-shear flow conditions *in vitro*. Perfusion studies of human blood flowed over collagen using flow devices. Indicated are the protein target, the inhibitor(s) use and the effect on embolus formation (+, increased).

Target protein	Inhibitor	Effect on arterial thrombus formation	Emboli	Ref(s).
Platelet proteins				
EphA4/ephrinB1	[soluble fragments]	increased platelet disaggregation	+	122
integrin α IIb β 3	abciximab, eptifibatide	increased platelet disaggregation	+	123,124
myosin heavy chain-II	blebbistatin	increased thrombus instability	+	125
P2Y ₁ / P2Y ₁₂ receptors	MRS2179 / ticagrelor / 2-MeSADP)	increased thrombus instability	+	44,124,126
	AR-C69931MX	increased platelet disaggregation	+	46
PI 3-kinase-b	TGX-221	increased platelet disaggregation	+	46
RhoA kinase	Y-27632	reduced thrombus formation, increased instability	+	60,125
Plasma proteins				
fibrin polymer	GPRP	more unstable thrombi, releasing platelets	+	77
Gas6	[depleted plasma]	increased thrombus instability	+	53

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

studies show a complex relation between severe factor XII deficiency and increased thrombotic risk.⁹⁴ One of the explanations is that complete deficiency in factor XII restricts the formation of fibrin, and facilitates symptomatic embolization in a similar way as observed in murine studies. More translational research is required to link these clinical observations to those of the mouse models.

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

As described above, mouse experimental thrombosis studies in general indicate that arterial thrombus growth and thrombus stability can be linked processes. Many platelet and plasma proteins that control thrombus growth also appear to play a role in stabilization of the thrombus. This is directly evident from intravital microscopy observations showing that flow-mediated adhesion of platelets can be a reversible event, and

that single platelets as well as small or large platelet aggregates regularly detach from a thrombus even in wild-type mice. Hence, some degree of instability may be considered as a natural phenomenon in arterial thrombus formation. This is illustrated in *Figure 1*, schematizing that during thrombus growth smaller and larger emboli are shed. The limited clinical observations so far indicate that such shedding of emboli also occurs in thrombotic human arteries. On the other hand, thrombus growth and stability do not seem to be controlled in exactly the same ways. For instance, there are surprisingly few reports on a role of platelet secretion products in thrombus stabilization (*Table 1*), whereas platelet secretion is considered to be major determinant of thrombus growth. As schematized in *Figure 2*, key processes controlling the stability of a thrombus are (i) initial platelet integrin activation controlling platelet aggregation, which can be reversible resulting in shedding of platelet emboli; (ii) contact-dependent signalling, stabilizing the platelet aggregates; and (iii) plasma thrombin and fibrin generation via the intrinsic and extrinsic coagulation pathways, which provides the thrombus with a fibrin network, but still allows shedding of platelet-fibrin emboli (microclots). In the clinical situation, the shedding of relatively large (fibrin-containing) emboli

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

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References

1. Ruggeri ZM, Mendolicchio GL. Adhesion mechanisms in platelet function. *Circ Res* 2007;**100**:1673-1685.
2. Jackson SP. Arterial thrombosis: insidious, unpredictable and deadly. *Nat Med* 2011;**17**:1423-1436.
3. Versteeg HH, Heemskerk JW, Levi M, Reitsma PS. New fundamentals in hemostasis. *Physiol Rev* 2013;**93**:327-358.
4. Jackson SP. The growing complexity of platelet aggregation. *Blood* 2007;**109**:5087-5095.
5. Brass LF, Wannemacher KM, Ma P, Stalker TJ. Regulating thrombus growth and stability to achieve an optimal response to injury. *J Thromb Haemost* 2011;**9** (Suppl. 1):66-75.
6. Kroll MH, Hellums JD, McIntire LV, Schafer AI, Moake JL. Platelets and shear stress. *Blood* 1996;**88**:1525-1541.
7. Sakariassen KS. Thrombus formation on apex of arterial stenoses: the need for a fluid high shear stenosis diagnostic device. *Future Cardiol* 2007;**3**:193-201.
8. Ruggeri ZM. Platelet-vessel wall interactions in flowing blood. In: *Hemostasis and Thrombosis* 2001:683-698.
9. Ruggeri ZM, Orje JN, Habermann R, Federici AB, Reininger AJ. Activation-independent platelet adhesion and aggregation under elevated shear stress. *Blood* 2006;**108**:1903-1910.
10. Nesbitt WS, Westein E, Tovar-Lopez FJ, Tolouei E, Mitchell A, Fu J, Carberry J, Fouras A, Jackson SP. A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. *Nat Med* 2009;**15**:665-673.
11. Westein E, van der Meer AD, Kuijpers MJ, Frimat JP, van den Berg A, Heemskerk JW. Atherosclerotic geometries spatially confine and exacerbate pathological thrombus formation poststenosis in a von Willebrand factor-dependent manner. *Proc Natl Acad Sci USA* 2013;**110**:1357-1362.
12. Ruggeri ZM, Dent JA, Saldívar E. Contribution of distinct adhesive interactions to platelet aggregation in flowing blood. *Blood* 1999;**94**:172-178.
13. Wu YP, Vink T, Schiphorst M, van Zanten GH, IJsseldijk MJ, de Groot PG, Sixma JJ. Platelet thrombus formation on collagen at high shear rates is mediated by von Willebrand factor-glycoprotein Ib interaction and inhibited by von Willebrand factor-glycoprotein IIb/IIIa interaction. *Arterioscler Thromb Vasc Biol* 2000;**20**:1661-1667.
14. Elvers M, Stegner D, Hagedorn I, Kleinschnitz G, Braun A, Kuijpers MJ, Boesl M, Chen Q, Heemskerk JW, Stoll G, Frohman MA, Nieswandt B. Impaired integrin $\alpha_{IIb}\beta_3$ activation and shear-dependent thrombus formation in mice lacking phospholipase D1. *Science Signal* 2010;**3**:103 ra101.
15. Stegner D, Nieswandt B. Platelet receptor signaling in thrombus formation. *J Mol Med* 2011;**89**:109-121.
16. Farndale RW, Sixma JJ, Barnes MJ, de Groot PG. The role of collagen in thrombosis and haemostasis. *J Thromb Haemost* 2004;**2**:561-573.
17. Siljander PR, Munnix IC, Smethurst PA, Deckmyn H, Lindhout T, Ouwehand WH, Farndale RW, Heemskerk JW. Platelet receptor interplay regulates collagen-induced thrombus formation in flowing human blood. *Blood* 2004;**103**:1333-1341.
18. Auger JM, Kuijpers MJ, Senis YA, Watson SP, Heemskerk JW. Adhesion of human and mouse platelets to collagen under shear: a unifying model. *FASEB J* 2005;**19**:825-827.
19. Watson SP, Auger JM, McCarty OJ, Pearce AC. GPVI and integrin $\alpha_{IIb}\beta_3$ signaling in platelets. *J Thromb Haemost* 2005;**3**:1752-1762.
20. Varga-Szabo D, Braun A, Nieswandt B. STIM1 and Orai1 in platelet function. *Cell Calcium* 2011;**50**:70-78.
21. Van de Walle G, Schoolmeester A, Iserbyt BF, Cosemans JM, Heemskerk JW, Hoylaerts MF, Nurden A, Vanhoorelbeke K, Deckmyn H. Activation of $\alpha_{IIb}\beta_3$ is sufficient but also an imperative prerequisite to activate $\alpha_2\beta_1$ on platelets. *Blood* 2007;**109**:595-602.
22. Jurk K, Clemetson KJ, de Groot PG, Brodde MF, Steiner M, Savion N, Varon D, Sixma JJ, van Aken H, Kehrel BE. Thrombospondin-1 mediates

- platelet adhesion at high shear via glycoprotein Ib (GPIb): an alternative/backup mechanism to von Willebrand factor. *FASEB J* 2003;**17**:1490-1492.
23. Léon C, Eckly A, Hechler B, Aleil B, Freund M, Ravanat C, Jourdain M, Nonne C, Weber J, Tiedt R, Gratacap MP, Severin S, Cazenave JP, Lanza F, Skoda R, Gachet C. Megakaryocyte-restricted MYH9 inactivation dramatically affects hemostasis while preserving platelet aggregation and secretion. *Blood* 2007;**110**:3183-3191.
 24. Meyer I, Kunert S, Schwiebert S, Hagedorn I, Italiano JE, Dutting S, Nieswandt B, Bachmann S, Schulze H. Altered microtubule equilibrium and impaired thrombus stability in mice lacking RanBP10. *Blood* 2012;**120**:3594-3602.
 25. Gibbins JM. Platelet adhesion signalling and the regulation of thrombus formation. *J Cell Sci* 2004;**117**:3415-3425.
 26. Nesbitt WS, Mangin P, Salem HH, Jackson SP. The impact of blood rheology on the molecular and cellular events underlying arterial thrombosis. *J Mol Med* 2006;**84**:989-995.
 27. Wei AH, Schoenwaelder SM, Andrews RK, Jackson SP. New insights into the haemostatic function of platelets. *Br J Haematol* 2009;**147**:415-430.
 28. Mackman N, Tilley RE, Key NS. Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis. *Arterioscler Thromb Vasc Biol* 2007;**27**:1687-1693.
 29. Müller F, Mutch NJ, Schenk WA, Smith SA, Esterl L, Spronk HM, Schmidbauer S, Gahl WA, Morrissey JH, Renné T. Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. *Cell* 2009;**139**:1143-1156.
 30. Van der Meijden PE, Munnix IC, Auger JM, Govers-Riemslog JW, Cosemans JM, Kuijpers MJ, Spronk HM, Watson SP, Renné T, Heemskerk JW. Dual role of collagen in factor XII-dependent thrombus and clot formation. *Blood* 2009;**114**:881-890.
 31. Chen VM, Ahamed J, Versteeg HH, Berndt MC, Ruf W, Hogg PJ. Evidence for activation of tissue factor by an allosteric disulfide bond. *Biochemistry* 2006;**45**:12020-12028.
 32. Owens AP, Mackman N. Role of tissue factor in atherothrombosis. *Curr Atheroscler Rep* 2012;**14**:394-401.
 33. Monroe DM, Hoffman M. What does it take to make the perfect clot? *Arterioscler Thromb Vasc Biol* 2006;**26**:41-48.
 34. Heemskerk JW, Mattheij N, Cosemans JM. Platelet-based coagulation: different populations, different functions. *J Thromb Haemost* 2013;**11**:2-16.
 35. Reininger AJ, Bernlochner I, Penz SM, Ravanat C, Smethurst P, Farndale RW, Gachet C, Brandl R, Siess W. A 2-step mechanism of arterial thrombus formation induced by human atherosclerotic plaques. *J Am Coll Cardiol* 2010;**55**:1147-1158.
 36. Cosemans JM, Schols SE, Stefanini L, de Witt S, Feijge MA, Hamulyak K, Deckmyn H, Bergmeier W, Heemskerk JW. Key role of glycoprotein Ib/V/IX and von Willebrand factor in platelet activation-dependent fibrin formation at low shear flow. *Blood* 2011;**117**:651-660.
 37. Borissoff JI, Spronk HM, ten Cate H. The hemostatic system as a modulator of atherosclerosis. *N Engl J Med* 2011;**364**:1746-1760.
 38. Moncada S, Higgs EA. Nitric oxide and the vascular endothelium. *Handb Exp Pharmacol* 2006;**176**:213-254.
 39. Funk CD, FitzGerald GA. COX-2 inhibitors and cardiovascular risk. *J Cardiovasc Pharmacol* 2007;**50**:470-479.
 40. Johnston-Cox HA, Ravid K. Adenosine and blood platelets. *Purinergic Signal* 2011;**7**:357-365.
 41. Darbousset R, Thomas GM, Mezouar S, Frère C, Bonier R, Mackman N, Renné T, Dignat-George F, Dubois C, Panicot-Dubois L. Tissue factor-positive neutrophils bind to injured endothelial wall and initiate thrombus formation. *Blood* 2012;**120**:2133-2143.
 42. Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers DD, Wroblewski SK, Wakefield TW, Hartwig JH, Wagner DD. Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci USA* 2010;**107**:15880-15885.
 43. Von Brühl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorentz M, Khandoga

- A, Tirniceriu A, Coletti R, Köllnberger M, Byrne RA, Laitinen I, Walch A, Brill A, Pfeiler S, Manukyan D, Braun S, Lange P, Riegger J, Ware J, Eckart A, Haidari S, Rudelius M, Schultz C, Echtler K, Brinkmann V, Schwaiger M, Pressner KT, Wagner DD, Mackman N, Engelmann B, Massberg S. Monocytes, neutrophils and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J Exp Med* 2012;**209**:819-835.
44. Goto S, Tamura N, Ishida H, Ruggeri ZM. Dependence of platelet thrombus stability on sustained glycoprotein IIb/IIIa activation through adenosine 5-diphosphate receptor stimulation and cyclic calcium signaling. *J Am Coll Cardiol* 2006;**47**:155-162.
45. Cosemans JM, Iserbyt BF, Deckmyn H, Heemskerk JW. Multiple pathways to switch platelet integrins on and off. *J Thromb Haemost* 2008;**6**:1253-1261.
46. Cosemans JM, Munnix IC, Wetzker R, Heller R, Jackson SP, Heemskerk JW. Continuous signaling via phosphoinositide 3-kinase isoforms β and γ is required for platelet ADP receptor function in dynamic thrombus stabilization. *Blood* 2006;**108**:3045-3052.
47. André P, Delaney SM, LaRocca T, Vincent D, DeGuzman F, Jurek M, Koller B, Phillips DR, Conley PB. P2Y₁₂ regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. *J Clin Invest* 2003;**112**:398-406.
48. Canobbio I, Stefanini L, Cipolla L, Ciraolo E, Gruppi C, Balduini C, Hirsch E, Torti M. Genetic evidence for a predominant role of PI3K β catalytic activity in ITAM- and integrin-mediated signaling in platelets. *Blood* 2009;**114**:2193-2196.
49. Nieswandt B, Varga-Szabo D, Elvers M. Integrins in platelets. *J Thromb Haemost* 2009;**7** S1:206-209.
50. André P, Srinivasa Prasad KS, Denis CV, He M, Papalia JM, Hynes RO, Phillips DR, Wagner DD. CD40L stabilizes arterial thrombi by a β_3 integrin-dependent mechanism. *Nat Med* 2002;**8**:247-252.
51. Angelillo-Scherrer A, Garcia de Frutos P, Aparicio C, Melis E, Savi P, Lupu F, Arnout J, Dewerchin M, Hoylaerts MF, Herbert JM, Collen D, Dahlbäck B, Carmeliet P. Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. *Nat Med* 2001;**7**:215-221.
52. Angelillo-Scherrer A, Burnier L, Flores N, Savi P, DeMol M, Schaeffer P, Herbert J-M, Lemke G, Goff SP, Matsushima GK, Earp HS, Vesin C, Hoylaerts MF, Plaisance S, Collen D, Conway EM, Wehrle-Haller B, Carmeliet P. Role of Gas6 receptors in platelet signaling during thrombus stabilization and implications for antithrombotic therapy. *J Clin Invest* 2005;**115**:237-246.
53. Cosemans JM, van Kruchten R, Olieslagers S, Schurgers LJ, Verheyen FK, Munnix ICA, Waltenberger J, Angelillo-Scherrer A, Hoylaerts MF, Carmeliet P, Heemskerk JW. Potentiating roles for Gas6 and Tyro, Axl and Mer (TAM) receptors in human and murine platelet activation and thrombus stabilization. *J Thromb Haemost* 2010;**8**:1797-1808.
54. Foley JH, Conway EM. Gas6 gains entry into the coagulation cascade. *Blood* 2013;**121**:570-571.
55. Goschnick MW, Lau LM, Wee JL, Liu YS, Hogarth PM, Robb LM, Hickey MJ, Wright MD, Jackson DE. Impaired outside-in integrin $\alpha_{IIb}\beta_3$ signaling and thrombus stability in TSSC6-deficient mice. *Blood* 2006;**108**:1911-1918.
56. Orłowski E, Chand R, Yip J, Wong C, Goschnick MW, Wright MD, Ashman LK, Jackson DE. A platelet tetraspannin superfamily member, CD515, is required for regulation of thrombus growth and stability in vivo. *J Thromb Haemost* 2009;**7**:2074-2084.
57. Essex DW. Redox control of platelet function. *Antioxid Redox Signal* 2009;**11**:1191-1225.
58. Brass LF, Zhu L, Stalker TJ. Minding the gaps to promote thrombus growth and stability. *J Clin Invest* 2005;**115**:3385-3392.
59. Nanda N, Andre P, Bao M, Clauser K, Deguzman F, Howie D, Conley PB, Terhorst C, Phillips DR. Platelet aggregation induces platelet aggregate state stability via SLAM family receptor signalling. *Blood* 2005;**106**:3028-3034.
60. Ono A, Westein E, Hsiao S, Nesbitt WS, Hamilton JR, Schoenwaelder SM, Jackson SP. Identification of a fibrin-independent platelet contractile

- mechanism regulating primary hemostasis and thrombus growth. *Blood* 2008;**112**:90-99.
61. Pleines I, Hagedorn I, Gupta S, May F, Chakarova L, van Hengel J, Offermanns S, Krohne G, Kleinschnitz C, Brakebusch C, Nieswandt B. Megakaryocyte-specific RhoA deficiency causes macrothrombocytopenia and defective platelet activation in hemostasis and thrombosis. *Blood* 2012;**119**:1054-1063.
 62. Wong C, Liu Y, Chand R, Wee JL, Oates L, Nieswandt B, Reheman A, Ni H, Beauchmin N, Jackson DE. CEACAM1 negatively regulates platelet-collagen interactions and thrombus growth in vitro and in vivo. *Blood* 2009;**113**:1818-1828.
 63. Stalker TJ, Wu J, Morgans A, Traxler EA, Wang L, Chatterjee MS, Lee D, Quertermouse T, Hall RA, Hammer DA, Diamond SL, Brass LF. Endothelial cell specific adhesion molecule (ESAM) localizes to platelet-platelet contacts and regulates thrombus formation in vivo. *J Thromb Haemost* 2009;**7**:1886-1896.
 64. Munnix IC, Kuijpers MJ, Auger JM, Thomassen CM, Panizzi P, van Zandvoort MA, Rosing J, Bock PE, Watson SP, Heemskerk JW. Segregation of platelet aggregatory and procoagulant microdomains in thrombus formation. Regulation by transient integrin activation. *Arterioscler Thromb Vasc Biol* 2007;**27**:2484-2490.
 65. Angelillo-Scherrer A, Fontana P, Burnier L, Roth I, Sugamele R, Brisset A, Morel S, Nolli S, Sutter E, Chassot A, Capron C, Borgel D, Saller F, Chanson M, Kwak BR. Connexin 37 limits thrombus propensity by downregulating platelet reactivity. *Circulation* 2011;**124**:930-939.
 66. Chanson M, Kwak BR. Connexin37: a potential modifier gene of inflammatory disease. *J Mol Med* 2007;**85**:787-795.
 67. Vaiyapuri S, Jones CI, Sasikumar P, Moraes LA, Munger SJ, Wright JR, Ali MS, Sage T, Kaiser WJ, Tucker KL, Stain CJ, Bye AP, Jones S, Oviedo-Orta E, Simon AM, Mahaut-Smith MP, Gibbins JM. Gap junctions and connexin hemichannels underpin hemostasis and thrombosis. *Circulation* 2012;**125**:2479-2491.
 68. Siljander P, Farndale RW, Feijge MA, Comfurius P, Kos S, Bevers EM, Heemskerk JW. Platelet adhesion enhances the glycoprotein VI-dependent procoagulant response: involvement of p38 MAP kinase and calpain. *Arterioscler Thromb Vasc Biol* 2001;**21**:618-627.
 69. Berny MA, Munnix IC, Auger JM, Schols SE, Cosemans JM, Panizzi P, Bock PE, Watson SP, McCarty OJ, Heemskerk JW. Spatial distribution of factor Xa, thrombin, and fibrin(ogen) on thrombi at venous shear. *Plos One* 2010;**5**:e10415.
 70. Lee H, Sturgeon SA, Jackson SP, Hamilton JR. The contribution of thrombin-induced platelet activation to thrombus growth is diminished under pathological blood shear conditions. *Thromb Haemost* 2012;**107**:328-337.
 71. Munnix IC, Strehl A, Kuijpers MJ, Auger JM, van der Meijden PE, van Zandvoort MA, oude Egbrink M, Nieswandt B, Heemskerk JW. The glycoprotein VI-phospholipase Cy2 signaling pathway controls thrombus formation induced by collagen and tissue factor in vitro and in vivo. *Arterioscler Thromb Vasc Biol* 2005;**25**:2673-2678.
 72. May F, Hagedorn I, Pleines I, Bender M, Vögtle T, Eble J, Elvers M, Nieswandt B. CLEC-2 is an essential platelet-activating receptor in hemostasis and thrombosis. *Blood* 2009;**114**:3464-3472.
 73. Furie B, Furie BC. Thrombus formation in vivo. *J Clin Invest* 2005;**115**:3355-3362.
 74. Mangin P, Yap CL, Nonne C, Sturgeon SA, Goncalves I, Yuan Y, Schoenwaelder SM, Wright CE, Lanza F, Jackson SP. Thrombin overcomes the thrombosis defect associated with platelet GPVI/FcRg deficiency. *Blood* 2006;**107**:4346-4353.
 75. Kuijpers MJ, Pozgajova M, Cosemans JM, Munnix IC, Eckes B, Nieswandt B, Heemskerk JW. Role of murine integrin $\alpha_2\beta_1$ in thrombus stabilization and embolization: contribution of thromboxane A_2 . *Thromb Haemost* 2007;**98**:1072-1080.
 76. Kalia N, Auger JM, Atkinson B, Watson SP. Critical role of FcR g-chain, LAT, PLCg2 and thrombin in arteriolar thrombus formation upon

- mild, laser-induced endothelial injury in vivo. *Microcirculation* 2008;**15**:325-335.
77. Colace TV, Muthard RW, Diamond SL. Thrombus growth and embolism on tissue factor-bearing collagen surfaces under flow. Role of thrombin with and without fibrin. *Arterioscler Thromb Vasc Biol* 2012;**32**:1466-1476.
78. Okorie UM, Denney WS, Chatterjee MS, Neeves KB, Diamond SL. Determination of tissue factor thresholds that trigger coagulation versus venous and arterial shear rates: amplification of 100 fM circulating tissue factor requires flow. *Blood* 2008;**111**:3507-3513.
79. Welsh JD, Colace TV, Muthard RW, Stalker TJ, Brass LF, Diamond SL. Platelet-targeting sensor reveals thrombin gradient within blood clots forming in microfluidic assay in mouse. *J Thromb Haemost* 2012;**10**:2344-2353.
80. Renné T, Pozgajova M, Grüner S, Schuh K, Pauer HU, Burfeind P, Gailani D, Nieswandt B. Defective thrombus formation in mice lacking coagulation factor XII. *J Exp Med* 2005;**202**:271-281.
81. Revenko AS, Gao D, Crosby JR, Bhattacharjee G, Zhao C, May C, Gailani D, Monia BP, MacLeod AR. Selective depletion of plasma prekallikrein or coagulation factor XII inhibits thrombosis in mice without increased risk of bleeding. *Blood* 2011;**118**:5302-5311.
82. Hagedorn I, Schmidbauer S, Pleines I, Kleinschnitz C, Kronthaler U, Stoll G, Dickneite G, Nieswandt B. Factor XIIa inhibitor recombinant human albumin infestin-4 abolishes occlusive arterial thrombus formation without affecting bleeding. *Circulation* 2010;**121**:1510-1517.
83. Maroney SA, Cooley BC, Ferrel JP, Bonesho CE, Mast AE. Murine hematopoietic cell tissue factor pathway inhibitor limits thrombus growth. *Arterioscler Thromb Vasc Biol* 2011;**31**:821-826.
84. Ruggeri ZM. Platelets in atherothrombosis. *Nat Med* 2002;**8**:1227-1234.
85. King A, Markus HS. Doppler embolic signals in cerebrovascular disease and prediction of stroke risk: a systematic review and meta-analysis. *Stroke* 2009;**40**:3711-3717.
86. Markus HS, King A, Shipley M, Topakian R, Cullinane M, Reihill S, Bornstein NM, Schaafsma A. Asymptomatic embolisation for prediction of stroke in the Asymptomatic Carotid Emboli Study (ACES): a prospective observational study. *Lancet Neurol* 2010;**9**:663-671.
87. Kruis RW, Vlasveld FA, van Dijk D. The (un)importance of cerebral microemboli. *Semin Cardiothorac Vasc Anesth* 2010;**14**:111-118.
88. Maan A, Padmanabhan R, Shaikh AY, Mansour M, Ruskin JN, Heist EK. Newer anticoagulants in cardiovascular disease: a systematic review of the literature. *Cardiol Rev* 2012;**20**:209-221.
89. Schulman S. Advances in the management of venous thromboembolism. *Best Pract Res Clin Haematol* 2012;**25**:361-377.
90. Girolami A, de Marinis GB, Bonamigo E, Lombardi AM. Recombinant FVIIa concentrate-associated thrombotic events in congenital bleeding disorders other than hemophilias. *Hematology* 2012;**17**:346-349.
91. De Bosch NB, Mosesson MW, Ruiz-Sáez A, Echenagucia M, Rodriguez-Lemoin A. Inhibition of thrombin generation in plasma by fibrin formation (antithrombin I). *Thromb Haemost* 2002;**88**:253-258.
92. Korte W, Feldges A. Increased prothrombin activation in a patient with congenital afibrinogenemia is reversible by fibrinogen substitution. *Clin Invest* 1994;**72**:396-398.
93. Remijn JA, Wu YP, IJsseldijk MJ, Zwaginga JJ, Sixma JJ, de Groot PG. Absence of fibrinogen in afibrinogenemia results in large but loosely packed thrombi under flow conditions. *Thromb Haemost* 2001;**85**:736-742.
94. Zeerleder S, Schloesser M, Redondo M, Willemin WA, Engel W, Furlan M, Lämmle B. Reevaluation of the incidence of thromboembolic complications in congenital factor XII deficiency: a study on 73 subjects from 14 Swiss families. *Thromb Haemost* 1999;**82**:1240-1246.
95. Pozgajova M, Sachs UJ, Hein L, Nieswandt B. Reduced thrombus stability in mice lacking the α 2A-adrenergic receptor. *Blood* 2006;**108**:510-514.
96. Dubois C, Panicot-Dubois L, Merrill-Skoloff G, Furie B, Furie BC. Glycoprotein VI-dependent

- and -independent pathways of thrombus formation in vivo. *Blood* 2006;**107**:3902-3906.
97. Massberg S, Gawaz M, Grüner S, Schulte V, Konrad I, Zöhlhöfer D, Heinzmann U, Nieswandt B. A crucial role of glycoprotein VI for platelet recruitment to the injured arterial wall in vivo. *J Exp Med* 2003;**197**:41-49.
 98. Moog S, Mangin P, Lenain N, Strassel C, Ravanat C, Schuhler S, Freund M, Santer M, Kahn M, Nieswandt B, Gachet C, Cazenave JP, Lanza F. Platelet glycoprotein V binds to collagen and participates in platelet adhesion and aggregation. *Blood* 2001;**98**:1038-1046.
 99. Ni H, Ramakrishnan V, Ruggeri ZM, Papalia JM, Phillips DR, Wagner DD. Increased thrombogenesis and embolus formation in mice lacking glycoprotein V. *Blood* 2001;**98**:368-373.
 100. Bender M, Hagedorn I, Nieswandt B. Genetic and antibody-induced glycoprotein VI deficiency equally protects mice from mechanically and FeCl₃-induced thrombosis. *J Thromb Haemost* 2011;**9**:1423-1426.
 101. Grüner S, Prostredna M, Koch M, Miura Y, Schulte V, Jung SM, Moroi M, Nieswandt B. Relative antithrombotic effect of soluble GPVI dimer compared with anti-GPVI antibodies in mice. *Blood* 2005;**105**:1492-1499.
 102. Neeves KB, Maloney SF, Fong KP, Schmaier AA, Kahn ML, Brass LF, Diamond SL. Microfluidic focal thrombosis model for measuring murine platelet deposition and stability: PAR4 signaling enhances shear-resistance of platelet aggregates. *J Thromb Haemost* 2008;**6**:2193-2201.
 103. Stolla M, Stefanini L, Roden RC, Chavez M, Hirsch J, Greene T, Ouellette TD, Maloney SF, Diamond SL, Poncz M, Woulfe DS, Bergmeier W. The kinetics of $\alpha_{IIb}\beta_3$ activation determines the size and stability of thrombi in mice: implications for antiplatelet therapy. *Blood* 2011;**117**:1005-1013.
 104. Nergiz-Unal R, Cosemans JM, Feijge MA, van der Meijden PE, Storey RF, van Giezen JJ, oude Egbrink MG, Heemskerk JW, Kuijpers MJ. Stabilizing role of platelet P2Y₁₂ receptors in shear-dependent thrombus formation on ruptured plaques. *Plos One* 2010;**5**:e10130.
 105. Woulfe D, Jiang H, Morgans A, Monks R, Birnbaum M, Brass LF. Defects in secretion, aggregation and thrombus formation in platelets from mice lacking Akt2. *J Clin Invest* 2004;**113**:441-450.
 106. Daniel JL, Dangelmaier CA, Mada S, Buitrago L, Jin J, Langdon WY, Tsygankov AY, Kunapuli SP, Sanjay A. Cbl-b is a novel physiological regulator of glycoprotein VI-dependent platelet activation. *J Biol Chem* 2010;**285**:17282-17291.
 107. Falet H, Pollitt AY, Begonja AJ, Weber SE, Duerschmied D, Wagner DD, Watson SP, Hartwig JH. A novel interaction between FlnA and Syk regulates platelet ITAM-mediated receptor signaling and function. *J Exp Med* 2011;**207**:1967-1979.
 108. Rathore V, Wang D, Newman DK, Newman PJ. Phospholipase Cy2 contributes to stable thrombus formation on VWF. *FEBS Lett* 2004;**27**:26-30.
 109. Randriamboavonjy V, Isaak J, Fromel T, Viollet B, Fisslthaler B, Preissner KT, Fleming I. AMPK α 2 subunit is involved in platelet signaling, clot retraction and thrombus stability. *Blood* 2010;**116**:2134-2140.
 110. McCarty OJ, Larson MK, Auger JM, Kalia N, Atkinson BT, Pearce AC, Ruf S, Henderson RB, Tybulewicz VL, Machesky LM, Watson SP. Rac1 is essential for platelet lamellipodia formation and aggregate stability under flow. *J Biol Chem* 2005;**280**:39474-39484.
 111. Varga-Szabo D, Braun A, Kleinschnitz C, Bender M, Pleines I, Pham M, Renné T, Stoll G, Nieswandt B. The calcium sensor STIM1 is an essential mediator of arterial thrombosis and ischemic brain infarction. *J Exp Med* 2008;**205**:1583-1591.
 112. Gushiken FC, Ha H, Li J, Rumbaut RE, Afshar-Kharghan V. Abnormal platelet function in C3-deficient mice. *J Thromb Haemost* 2009;**7**:865-870.
 113. Ni H, Denis CV, Subbarao S, Degen JL, Sato TN, Hynes RO, Wagner DD. Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. *J Clin Invest* 2000;**106**:385-392.

114. Jirouskova M, Chereshnev I, Vaananan H, Degen JL, Collier BS. Antibody blockade or mutation of the fibrinogen gamma-chain C-terminus is more effective in inhibiting murine arterial thrombus formation than complete absence of fibrinogen. *Blood* 2004;**103**:1995-2002.
115. Ni H, Yuen PTS, Papalia JM, Trevithick JE, Sakai T, Fässler R, Hynes RO, Wagner DD. Plasma fibronectin promotes thrombus growth and stability in injured arterioles. *Proc Natl Acad Sci USA* 2003;**100**:2415-2419.
116. Konstantinides S, Schafer K, Neels JG, Dellas C, Loskutoff DJ. Inhibition of endogenous leptin protects mice from arterial and venous thrombosis. *Arterioscler Thromb Vasc Biol* 2004;**24**:2196-2201.
117. Koschnick S, Konstantinides S, Schafer K, Crain K, Loskutoff DJ. Thrombotic phenotype of mice with a combined deficiency in plasminogen activator inhibitor 1 and vitronectin. *J Thromb Haemost* 2005;**3**:2290-2295.
118. Bonnefoy A, Daenens K, Feys HB, De Vos R, Vandervoort P, Vermeylen J, Lawler J, Hoylaerts MF. Thrombospondin-1 controls vascular platelet recruitment and thrombus adherence in mice by protecting (sub)endothelial vWF from cleavage by ADAMTS-13. *Blood* 2006;**107**:955-964.
119. Reheman A, Gross P, Yang H, Chen P, Allen D, Leytin V, Freedman J, Ni H. Vitronectin stabilizes thrombi and vessel occlusion but plays a dual role in platelet aggregation. *J Thromb Haemost* 2005;**3**:875-883.
120. Chauhan AK, Kisucka J, Lamb CB, Bergmeier W, Wagner DD. Von Willebrand factor and factor VIII are independently required to form stable occlusive thrombi in injured veins. *Blood* 2007;**109**:2424-2429.
121. Matsuno H, Kozawa O, Okada K, Ueshima S, Matsuo O, Uematsu T. Plasmin generation plays different roles in the formation and removal of arterial and venous thrombus in mice. *Thromb Haemost* 2002;**87**:98-104.
122. Prévost N, Woulfe DS, Jiang H, Stalker TJ, Marchese P, Ruggeri ZM, Brass LF. Eph kinases and ephrins support thrombus growth and stability by regulating integrin outside-in signaling in platelets. *Proc Natl Acad Sci USA* 2005;**102**:9820-9825.
123. Speich HE, Furman RR, Lands LT, Moodie GD, Jennings LK. Elevating local concentrations of GPIIb-IIIa antagonists counteracts platelet thrombus stability. *J Thromb Thrombolysis* 2012;in press.
124. Hosokawa K, Ohnishi T, Fukasawa M, Kondo T, Sameshima H, Koide T, Tanaka KA, Maruyama I. A microchip flow-chamber system for quantitative assessment of the platelet thrombus formation process. *Microvasc Res* 2012;**83**:154-161.
125. Calaminus SD, Auger JM, McCarty OJ, Wakelam MJ, Mecheskys LM, Watson SP. MyosinIIa contractility is required for maintenance of platelet structure during spreading on collagen and contributes to thrombus stability. *J Thromb Haemost* 2007;**5**:2136-2145.
126. Stephens G, He M, Wong C, Jurek M, Luedemann HC, Shapurian G, Munnelly K, Muir C, Conley PB, Phillips DR, André P. Development of a perfusion chamber assay to study in real time the kinetics of thrombosis and the antithrombotic characteristics of antiplatelet drugs. *Thromb J* 2012;**10**:11.

Chapter 3

Platelet-based coagulation: different populations, different functions

Heemskerk JW, Mattheij NJ, Cosemans JM

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

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Abstract

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

1. Introduction

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

activated protein C, antithrombin and C1 inhibitor.

It is widely accepted that phospholipid membranes containing the negatively charged lipid, phosphatidylserine (PS), are required to propagate and enhance the coagulation reactions. This membrane dependency implies ultimate cellular control of the coagulation process. In the blood system, activated platelets with surface-exposed PS cleave high amounts of FX and prothrombin into the proteolytically active forms, FXa and thrombin.^{2,3} Yet, platelets also have other roles in coagulation. They provide a scaffold for the formation of fibrin fibres³ and, once fibrin clots are formed, they regulate the process of clot retraction.⁴

The interwoven nature of platelet activation and the coagulation system has been observed in numerous studies.

For instance, in the most common *in vivo* thrombosis models, *i.e.* ferric chloride-induced damage of arteries or arterioles, the thrombotic process is similarly sensitive to defects in platelet activation as to inhibition of coagulation.⁵ This *in vivo* work has shown that: thrombus formation is triggered by collagen, next to tissue factor and thrombin;⁶⁻⁹ platelets as well as coagulation contribute to arterial and venous thrombus formation;^{10,11} and fibrin formation occurs already at initial stages of thrombus formation.¹²

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

2. Platelet interactions with (anti)coagulation factors

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

Fibrinogen and fibrin. The fibrinogen receptor, integrin $\alpha_{IIb}\beta_3$, is the most abundantly expressed platelet glycoprotein (GP). Platelet-fibrinogen binding requires activation of $\alpha_{IIb}\beta_3$ via configurational changes, a response that is induced by most platelet agonists.¹³ This integrin is also supposed to bind fibrin. However, whether integrin-bound fibrinogen can be converted into fibrin is unclear. Specific fibrin receptors have not been reported, although a role for platelet GPIb in fibrin binding has been proposed.¹⁴

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

serve as assembly sites for components of the tenase complex (FVIIIa, FIXa, FX) and the prothrombinase complex (FVa, FXa, prothrombin).

Investigations by fluorescence microscopy have shown that labelled FX and prothrombin only bind to PS-exposing platelets in a thrombus.^{16,17} However, once cleaved into thrombin, the prothrombin label appeared to redirect to the sites of fibrin fibres,¹⁷ which is in agreement with indications that fibrin acts as a site for thrombin. So far, specific protein receptors on platelets for vitamin K-dependent factors like FIX or FX have not been identified.^{2,3} A recent proposal, though, is that these factors may stay bound to the platelet surface after formation of cysteine bridges, catalysed by protein disulfide isomerase isoforms.¹⁸ Unlike some other cell types, platelets lack the signalling FXa receptor, protease-activated receptor 2 (PAR2).¹⁹ An unexpected finding was that FXa, next to ADAM10/17, influences platelet activation by cleaving the collagen receptor GPVI.^{20,21}

Thrombin. Thrombin is the only vitamin K-dependent factor known to bind to specific receptor proteins on platelets. Thrombin interacts with high specificity with protease-activated receptors, *i.e.* the PAR1 and PAR4 isoforms in human platelets, and the PAR3 and PAR4 isoforms in mouse platelets. Of these,

only PAR1 and 4 are cleavable by thrombin. In addition, high-affinity thrombin binding sites are present on GPIb, while GPV serves as a thrombin cleavage substrate.²²

Tissue factor. Similarly to megakaryocytes, platelets express limited amounts of tissue factor *e.g.* upon sepsis.^{23,24} It is doubted whether the levels of tissue factor present in platelets are physiologically relevant.²⁵ Furthermore, activated platelets release considerable amounts of tissue factor pathway inhibitor (TFPI), which restricts thrombus growth.²⁶ Hence, platelet-derived TFPI is expected to rapidly inactivate any tissue factor present at the platelet surface.

Factor V. Platelet α -granules store about 20% of the blood content of FV. Once released, platelet FV becomes activated by traces of thrombin. It is reported by one group that platelet-derived FVa is more resistant to protease inactivation than the FVa in plasma,²⁷ but this needs confirmation. FVa binds to PS-exposing (platelet) membranes via its C2 domain. Once bound to PS-exposing platelets, it joins FXa to form local prothrombinase complexes.^{17,28} Using flow cytometry, a subpopulation of (thrombin-activated) platelets was detected with intermediate FVa binding.²⁹ This is in agreement with the observation that FVa is incorporated

into the membrane of coated platelets,³⁰ and may point to a gradual accumulation of FVa at the platelet surface.

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

Factors VIII and IX. While not a vitamin K-dependent factor, FVIIIa is able to bind to PS-exposing membranes via its C-2 domain, likely in a similar manner as FVa.³³ This binding of FVIIIa enhances FIXa binding and thus stimulates tenase complex formation and FXa generation on PS-exposing platelets. Since FVIII in plasma is carried by von Willebrand factor (VWF), FVIII can also interact with platelets via VWF and GPIb-V-IX. However, in kinetic studies of fibrin formation, a role for VWF-bound FVIII in platelet coagulant activity could not be detected.³⁴ The binding of FIXa appears to be confined to PS-exposing platelets,

as determined by annexin A5-binding studies, and relies on prior elevation of cytosolic Ca^{2+} .^{35,36}

Factors XI and XII. Decades ago, it was already proposed that platelets promote fibrin formation in a FXII-dependent manner.³⁷ Recent observations point to marked roles of FXI and FXII in arterial thrombus formation in mice, suggesting that platelets in some way stimulate the intrinsic pathway of coagulation.³⁸ One explanation for the thrombus-stimulating effect of FXI and FXII is the binding of FXII to collagen, and its subsequent activation in the presence of prekallikrein and high molecular weight kininogen.³⁹ There is however also some evidence that FXI and FXII can bind to the platelet surface via GPIb α .⁴⁰ However, an early suggestion that platelet-bound FXIa is active and cleaves FIX could not be confirmed. More recent studies with human and mouse platelets point to FXI binding with the platelet apolipoprotein E receptor 2 (ApoER2, LRP8).⁴¹ It is still not quite clear whether platelets directly activate FXII. Some studies suggested that polyphosphates released from platelets may fulfil such a role,⁴² but recent data indicate that the size of platelet-derived polyphosphates (60-100 phosphate residues) is too small for this action.⁴³ On the other hand, platelet-size polyphosphates do accelerate the activation of FXI by thrombin.⁴⁴

Factor XIII. The coagulation factor FXIIIa acts as a transglutaminase in the cross-linking of fibrin fibers. Experiments with immobilised FXIIIa have shown that platelets bind to this protein via the integrins $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$.⁴⁵ FXIIIa has also been implicated in the chemical cross-linking of secretory proteins from the α -granules on the surface of so-called coated platelets (see below).⁴⁶ However, also other tissue-type transglutaminases may fulfil the latter role.⁴⁷

Anticoagulation factors. Platelets secrete significant amounts of the anticoagulant proteins TFPI and protease nexin-1 (PN-1, gene *SERPINE2*). Binding of TFPI has been detected at the surface of coated platelets.⁴⁸ The protein PN-1 inactivates thrombin, tissue plasminogen activator (tPA) and plasmin, thereby acting as a platelet-dependent, negative regulator of coagulation and fibrinolysis.⁴⁹ The anticoagulant vitamin K-dependent factors, protein S and protein C, both of which bind to PS membranes, have been shown to suppress thrombin generation at the platelet surface. It is also reported that activated protein C binds to the platelet LRP8 receptor,⁵⁰ but the importance of this interaction for the regulation of anticoagulation is unknown. The anticoagulant protein Z acts as a FXa inhibitor,^{51,52} but binding to platelets is unclear. Similarly, binding sites have not been described for anticoagulant

proteins like antithrombin and C1 inhibitor, which irreversibly bind to and inactivate the active sites of coagulation factors.

As summarized in Table 1, most coagulation factors appear to interact preferentially with PS-exposing platelets. Thus, especially these highly activated platelets provide assembly sites for coagulation factor complex formation, resulting in a greatly enhanced generation of FXa and thrombin. Precisely how the anticoagulation factors act at the platelet surface is not clear. Another unresolved issue is whether all these factors bind to PS-exposing platelets at the same time, or whether fibrin binding or formation perhaps prevents platelet interaction with other factors.

3. Different platelet populations controlling distinct coagulation steps

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

Procoagulant platelets (agonist-induced PS exposure). Within a thrombus, formed in a damaged vessel *in vivo* or on a

Table 1. Reported interactions of (anti)coagulation factors with platelets. For explanation and references, see text.

(Anti)coagulation factor	Binding site on platelets and result	Platelet population
Fibrin(ogen)	Fibrinogen: binding to $\alpha IIb\beta 3$. Precursor for fibrin formation. Fibrin receptor unknown.	Aggregating and clot-retracting platelets
Factor II	FII (prothrombin): binding to PS (via Gla domain)	FII: PS-exposing platelets.
Tissue factor	FIIa (thrombin): binding to PAR1,3,4, GPIb-V-IX.	FIIa: all platelets
Factor V	Weakly expressed. Physiological role unclear	N.a.
Factor VII	Released by platelets. Binding to PS (via C2 domain), other receptors unknown. Activation of prothrombin.	PS-exposing and coated platelets
Factor VIII	Binding to PS (via Gla domain), GPIb-V-IX.	PS-exposing platelets
Factor IX	Activation of FIX and FX	PS-exposing platelets
Factor X	Binding to PS (via C2-domain), indirectly via GPIb-V-IX. Activation of FX	PS-exposing platelets
Factor XI	Binding to PS (via Gla domain). Activation of FX	PS-exposing platelets
Factor XII	Binding to PS (via Gla domain), other receptors unknown.	PS-exposing and coated platelets
TFPI	Activation of prothrombin	Coated platelets
Protease nexin-1	Binding to LRP8, GPIb-V-IX	Not described
Protein C	Binding site unclear	Not described
Protein S	Binding to integrins $\alpha IIb\beta 3$ and $\alpha v\beta 3$	PS-exposing and coated platelets
	Released by platelets, binding site unclear	Coated platelets
	Released by platelets, binding site unclear	N.a.
	Binding to PS (via Gla domain), LRP8	PS-exposing platelets
	Binding to PS (via Gla domain)	PS-exposing platelets

collagen matrix *in vitro*, platelets with different surface properties are clearly distinguishable.^{53,54} Aggregated platelets in the inner thrombus core, close to activating components of the vessel wall, have formed pseudopods, undergone secretion and bind fibrinogen (see below). Discoid platelets at the outer part of growing thrombi may easily detach and lack these activation markers. A distinct population of highly activated platelets is present close to collagen fibres and as patches around a thrombus. These platelets are characterised by surface-exposed PS (detected with the probes annexin A5 and lactadherin), prolonged cytosolic Ca^{2+} rises, a rounded structure, and capability to bind coagulation factors such as FV and

FX.^{16,17} They furthermore produce procoagulant microparticles. Patches of PS-exposing platelets are particularly formed under coagulant conditions, thereby implying a role of thrombin as platelet agonist. The population of PS-exposing platelets has also been described as procoagulant platelets and platelets with a sustained calcium-induced platelet (SCIP) morphology.^{55,56} Several studies have shown that *in vivo* injection of annexin A5 or lactadherin (chelating exposed PS) significantly impaired thrombus formation in mouse vessels,^{10,57} thus pointing to a functional importance of this platelet population. Studies with isolated coagulation factors have shown that PS-exposing platelets enhance the activities of tenase and

prothrombinase complexes by almost 1000-fold.^{2,58} Recent *ex vivo* flow studies have furthermore indicated that platelets with prolonged high Ca^{2+} and PS exposure also catalyze fibrin network formation, although fibrin formation can also occur at distant sites.³⁴ Jointly, this indicates that PS exposure is a prerequisite for platelet-dependent thrombin generation, with fibrin formation is not directly linked to this.

Coated platelets. Dale and co-workers have identified a population of platelets, formed after stimulation with collagen plus thrombin, which has been termed coated platelets.^{30,59} Characteristic of these platelets is their ability to irreversibly bind several α -granular proteins, including FV, thrombospondin, fibrinogen, fibronectin and VWF.⁶⁰ It is considered that these proteins cluster together at the platelet surface and form an immobilised coat. First reports proposed that FXIII activity is required for chemical cross-linking of these granular proteins to serotonin residues on the platelet membrane.⁴⁶ However, in studies using mice deficient in FXIII was shown that at least fibrinogen and VWF bind to coated platelets in the absence of FXIIIa, suggesting (additional) involvement of the tissue-type transglutaminase expressed by platelets.⁴⁷ Considering that coated platelets also expose PS,³⁰ the question

raises whether these two populations are identical. This is also suggested by the findings that coated and PS-exposing platelets are both formed in response to strong agonists (e.g. collagen and thrombin), through the same cellular processes (e.g. mitochondrial permeability transition pore formation), and both shed microparticles.^{59,61-64} Partial overlap of these two populations was indeed observed in dual labelling studies of thrombi with annexin A5 and a serotonin probe.¹⁶ On the other hand, stimulation of washed platelets with the Ca^{2+} ionophore A23187 results in the formation of PS-exposing platelets, which do not appear to have a coat of FVa³⁰ or high fibrin(ogen).⁴⁷ Together, this suggests that coat formation occurs secondarily to PS exposure, most likely as a consequence of the assembly of platelet secretion products and plasma factors at the outer membrane. Whether the formation of a protein coat affects the binding and activation of coagulation factors is unknown.

Aggregating and clot-retracting platelets. The aggregate-forming platelets in a thrombus are characterised by fibrinogen binding and the presence of active integrin $\alpha_{\text{IIb}}\beta_3$ at their surface (determined with PAC-1 antibody in case of human platelets and JON/A for mouse platelets). As indicated above, this population of platelets differs from the

PS-exposing platelets, also because the latter tend to close their integrins.^{53,55} Interestingly, under coagulant conditions, the aggregated platelets with activated $\alpha_{IIb}\beta_3$ integrins are known to regulate fibrin clot retraction. This process, dependent on platelet fibrin(ogen) binding and integrin outside-in signalling, translates the contractile forces generated in the platelet actin-myosin cytoskeleton to connecting fibrin fibres around platelets.⁶⁵ A diffuse fibrin network is thereby converted into a small and dense platelet-fibrin clot. Under flow conditions, this process is observed as contraction of platelet aggregates and reflects the initial phases of coagulation (see Suppl. Movie). It is also seen as a mechanism for narrowing the gaps between platelets to allow contact-dependent signalling.⁶⁶ Because PS-exposing platelets are non-adhesive, have closed integrins, and a disturbed actin cytoskeleton,⁵⁶ it is quite unlikely that they will participate in clot retraction. This points to a major difference between the aggregating and PS-exposing platelet populations with respect to the regulation of coagulation. While the PS-exposing platelets produce FXa and thrombin outside the platelet plug, the aggregated platelets consolidate the plug by clot retraction.

Apoptotic and necrotic platelets. In a pioneering paper, Schoenwaelder and

colleagues describe that the anti-tumour drugs ABT-737 and ABT-263 induce platelet PS exposure at relatively minor rises in cytosolic Ca^{2+} .⁶⁷ These compounds inhibit the anti-apoptotic proteins Bcl-2/Bcl-x_L, and cause activation of the Bak/Bax pathway to start a mitochondrial-dependent route of apoptosis and subsequent PS exposure. The mechanism differs from that of agonist-induced PS exposure, since apoptotic PS exposure appears to rely on caspase activation. Other studies indicate that the ABT compounds disrupt normal platelet activation responses, such as secretion, integrin activation and platelet aggregation.⁶⁸ Interestingly, the same or a similar apoptotic pathway is triggered in aging or stored platelets, which show a gradual decline in Bcl-x_L expression and also liberate the pro-apoptotic Bak/Bax proteins.^{69,70} The aging process is slow, requiring a time span of several days to yield PS-positive platelets, as examined with the probes annexin A5 and lactadherin.⁷¹⁻⁷³ Although *in vitro* tests have shown that aging, apoptotic platelets have a coagulant potential and support thrombin generation, it is questionable whether these PS-exposing platelets fulfil a role in coagulation under physiological conditions, since they will be rapidly taken up by scavenging cells in the circulation.

Because of the distinctions between apoptotic and agonist-stimulated PS-exposing platelets, e.g. with respect to caspase activation, it has been suggested that the latter are activated by a necrotic cell death pathway.⁷⁴ Given the common definition of necrosis as premature death of cells due to external sources (e.g., hypoxia or injury), we do not prefer this terminology. Instead, we propose the term 'procoagulant platelets', thus referring to their capability to support thrombin and fibrin generation.

Platelet microparticles. Both procoagulant, PS-exposing⁷⁵ and aging⁷⁶ platelets can release microparticles. As these microparticles in part expose PS they have a thrombin-generating potential. This seems to be relevant in particular at pathophysiological conditions, when elevated levels of platelet-derived microparticles have been measured.⁷⁷ Under normal conditions, their role may be limited, because of rapid interaction with leukocytes and other scavenging cells.⁷⁸ Jointly, this overview points to the presence of two types of activated platelets with fundamentally different roles in the coagulation process. At the one hand, the PS-exposing and coated platelets (with partial overlap) that are characterized by a high activation state, binding of multiple coagulation factors, and the capability to stimulate thrombin

generation. These platelets also actively form fibrin, with the coated platelets having a fibrin layer at their surface. On the other hand, aggregating platelets, which characteristically express activated integrins and have a role in fibrin clot retraction, which implies they bind fibrin as well. Although apoptotic (aging) platelets also expose PS, it is unclear whether these have a physiological role in coagulation. As reviewed elsewhere, the mechanisms for this heterogeneity in platelet fate is still incompletely understood.⁵³ It seems that intrinsic platelet factors such as platelet size and structure, protein composition, genetic factors and platelet age account only for part of the response heterogeneity. Platelet environmental factors, such as the local rheology, exposure to agonists, surrounding cells and plasma, likely are at least as important. This is further discussed below.

4. Ligands and receptors mediating platelet PS exposure and fibrin formation

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

VWF and GPIb-V-IX. A contribution of the GPIb-V-IX complex to platelet PS exposure, thrombin generation and subsequent fibrin formation has been reported by several groups, that indicated that this stringently relies on the interaction of GPIb with VWF.^{34,79,80} Particularly under coagulant conditions and low shear rate (implying the presence of thrombin), VWF binding to GPIb can lead to prolonged Ca^{2+} responses, PS exposure, assembly of coagulation factors and formation of fibrin at the platelet surface.³⁴ The signalling mechanism downstream of GPIb may involve the actin cytoskeleton. Initially, platelet procoagulant activity via GPIb was considered to rely on the presence of fibrin, interacting with GPIb via VWF.¹⁴ However, studies with platelets from Bernard-Soulier patients pointed to a fibrin-independent signalling role of GPIb-V-IX to enhance thrombin-induced Ca^{2+} mobilisation.⁸¹ In mouse platelets, GPIb β was found to potentiate the Ca^{2+} rises and PS exposure induced by either thrombin- or collagen-receptor agonists. This procoagulant effect was independent of the *N*-terminal part of GPIb α , as it was not affected by endopeptidase cleavage of this glycoprotein.⁸² Interestingly, deficiency in GPV, which is also part of the GPIb-V-IX complex, increased rather than decreased responses of mouse

platelets to thrombin by a still undisclosed mechanism.⁸³

Thrombin and receptors. The thrombin receptors of human platelets, PAR1 and PAR4, belong to the class of G-protein coupled receptors (GPCR), and elevate cytosolic Ca^{2+} via Gq and phospholipase C β (PLC β) stimulation.⁸⁴ The consequence is a oscillatory (spiking) Ca^{2+} signal, which by itself is insufficient for evoking PS exposure.⁸⁵ However, thrombin markedly enhances collagen-induced PS exposure, in which case PAR1 appears to be the primarily acting receptor,^{2,86} rather than the binding of thrombin to GPIb.⁸⁷ Some authors, though, report a role for GPIb in PS exposure, for instance in thrombin-stimulated gel-filtered platelets where residual VWF may be present.⁷⁹

An explanation for the primary role of PAR1 in thrombin-mediated PS exposure comes from the recent observation that inhibition of protein kinase C (PKC) negatively regulates Ca^{2+} rises and PS exposure elicited by PAR1, but not by the other thrombin receptor PAR4.⁸⁸ Yet, PAR4 activation has an assistant role in potentiating Ca^{2+} rises and PS exposure.⁸⁹ In mouse platelets, where only PAR4 - and not the other receptor PAR3 - is cleaved by thrombin,⁹⁰ thrombin-dependent PS exposure seems to rely on the presence of both isoforms.

The literature contains some evidence that the PAR isoforms on platelets can be cleaved by other proteases than thrombin. For example, PAR1 cleavage has been reported by ADAM17, a related plasma protease,⁹¹ or by matrix metalloproteinase-1.⁹² At present, the physiological relevance of these alternative ways of thrombin receptor cleavage is unclear. The same holds for the reported cleavage of PAR4 by the fibrinolysis protease plasmin⁹³ or the neutrophil-derived cathepsin G.⁹⁴

Thromboxane and TP receptors. In spite of the prominent role of thromboxane A₂ in collagen-induced platelet aggregation, this autocrine agonist is no more than limitedly effective in stimulating PS exposure. In fact, blockade of thromboxane production by aspirin affected PS exposure of collagen-adhered platelets only in combination with ADP receptor blockers.⁹⁵ Along the same line, aspirin treatment only slightly affected thrombin generation in coagulating platelet-rich plasma.⁹⁶ In agreement with these findings, stimulation of the platelet TP receptors is known to cause only limited Ca²⁺ mobilisation.

ADP, fibrinogen and receptors. The receptors for ADP (in particular P2Y₁₂) and fibrinogen (integrin $\alpha_{IIb}\beta_3$) appear to have supporting and partially redundant

roles in PS exposure and platelet-dependent thrombin generation. This was most clearly observed under coagulant conditions, *e.g.* in tissue factor-activated platelet-rich plasma. In this case, autocrine produced ADP was found to prolong Ca²⁺ rises and to increase PS exposure via P2Y₁₂ mediated signalling towards phosphoinositide 3-kinase (PI3K).^{97,98} Blockage of P2Y₁₂ by intake of the prodrug, clopidogrel, significantly suppressed the procoagulant effect of platelets in coagulating plasma.⁹⁹ Similarly, blockage of this receptor by the active metabolite of prasugrel impaired ADP/collagen induced Ca²⁺ rises, PS exposure and thrombin generation in whole blood.^{100,101}

Recent work indicated that also integrin $\alpha_{IIb}\beta_3$ outside-in signalling via PLC and the tyrosine kinase Syk supports thrombin-induced Ca²⁺ rises and PS exposure, along with platelet-dependent thrombin generation.¹⁰² The contributing role of $\alpha_{IIb}\beta_3$ partly overlapped with that of autocrine mechanisms via P2Y₁₂, which is not a surprise given the prominent role of P2Y₁₂ in integrin activation. These findings explain why integrin antagonists cause marked suppression of tissue factor-induced thrombin generation, and prolong the clotting times in platelet-rich plasma.^{103,104}

Immune receptor FcγRIIA. There is limited evidence that stimulation of the immune receptor FcγRIIA on human platelets induces PS exposure and microparticle formation, *e.g.* via antibodies against the heparin-platelet factor 4 complex.¹⁰⁵ Platelet activation via this receptor, which likely signals via the Syk-PLCγ2 pathway, is considered to contribute to the pathology of heparin-induced thrombocytopenia.

Collagen and GPVI. Circulating platelets will encounter vascular collagen only after endothelial damage and luminal exposure of the extracellular matrix. Under *in vitro* conditions, immobilised fibres of collagen type I provide relatively effective triggers for PS exposure, once platelets are stably adhered via the immunoglobulin-type collagen receptor GPVI.¹⁰⁶ Studies of platelet interaction with immobilised collagen under flow have indicated that all key constituents of the GPVI signalosome contribute to GPVI-induced PS exposure. In line with this, platelets from mice lacking the FcR γ-chain, LAT, Syk or PLCγ2 or with blocked GPVI showed marked reduction in collagen-dependent PS exposure and thrombin generation.^{107,108} On the other hand, mouse platelets with a gain-of-function mutation in PLCγ2 were more active in collagen-dependent PS exposure and thrombus formation.¹⁰⁹ The adhesive collagen receptor, integrin

$\alpha_2\beta_1$, was found to support procoagulant activity in an indirect way, by enforcing collagen-platelet interactions via GPVI.¹¹⁰ Inhibition of the protein tyrosine kinase Syk, which together with Src-family kinases controls PLCγ2 activity, suppressed GPVI-induced PS exposure, whereas inhibition of protein phosphatases increased this response.^{16,111} The responsible phosphatase has been unknown for long, but recent data point to involvement of the phosphatase TULA2, which associates to Syk and can negatively regulate GPVI-induced signalling events.¹¹² This phosphatase might also control PS exposure.

Even though platelet adhesion to immobilised collagen causes appreciable PS exposure, platelets need co-stimulation of collagen with other agonists like thrombin to obtain substantial fractions of PS-exposing platelets.^{2,113} This co-stimulation likely has a dual effect: it potentiates the Ca^{2+} rises in platelets, and also activates the integrins ($\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$) needed for stable platelet adhesion to collagen fibres and for signalling via the low-affinity GPVI receptors. This is in contrast to the process of collagen-induced platelet aggregation, which is less dependent on high Ca^{2+} rises, and only requires $\alpha_{IIb}\beta_3$ activation via autocrine agonists like ADP and thromboxane. Other ligands of GPVI, such as cross-

linked collagen-related peptide (low-affinity) and particularly the snake venom convulxin (high affinity) also provoke PS exposure, yet again this response is markedly enhanced by co-stimulation with thrombin.¹⁰⁶

Other ligands and receptors. A newly described platelet receptor acting via a similar pathway of LAT-Syk-PLC γ 2 is the protein CLEC2, which can be activated by

the snake venom rhodocytin and the lymphatic protein podoplanin.¹¹⁴ There are no reports yet on platelet procoagulant activity induced by CLEC2 activation.

In addition to collagen, a number of other adhesive proteins have been found to promote PS exposure especially after immobilisation at a surface. For instance, immobilised thrombospondin-1 and oxidised LDL can stimulate PS exposure

Figure 1. Platelet-based coagulation: signalling towards prolonged high Ca^{2+} rises, required for PS exposure. Gradient colour bar indicates range of receptors with low Ca^{2+} -mobilising potency due to PLC γ activation, causing spiking Ca^{2+} signals (blue), to receptors with high Ca^{2+} -mobilising potency due to PLC γ 2 activation, causing prolonged Ca^{2+} signals (red). Isoforms of PI3K produce the phosphoinositide PIP₃, which can stimulate PLC γ 2 activity. Co-signalling via multiple receptors will result in accumulated Ca^{2+} signals, to reach threshold levels of prolonged high Ca^{2+} required for PS exposure. The DAG and Ca^{2+} -dependent effector proteins, PKC and CALDAG-GEFI, act as modulators of the Ca^{2+} signal. See also text.

of adhered platelets at limited extent via the receptor CD36.¹¹⁵ Furthermore, under coagulant conditions with thrombin present, surface-immobilised fibrinogen¹⁷ and laminin¹¹⁶ can stimulate PS exposure of adhered platelets via $\alpha_{IIb}\beta_3$ and FXII, respectively. Findings that platelet adhesion favours procoagulant activity are clearly relevant, not only because stable adhesion permits more continued signalling, but also because this provides a way to locally confine the coagulation process to sites of platelet adhesion and activation. In summary, only few physiological agonists, particularly those stimulating GPVI, are capable of inducing PS exposure by themselves. On the other hand, a larger number of agonists, including VWF, fibrinogen, thrombin, ADP, antibodies and thrombospondin, appear to enhance the process of (GPVI-induced) PS exposure by stimulating their respective receptors. In most cases the co-agonists act by increasing the Ca^{2+} signal towards a prolonged high Ca^{2+} rise (see Fig. 1). Apparently, in order to generate a sufficient Ca^{2+} signal for PS exposure a certain degree of co-stimulation is needed of receptors linked to PLC γ isoforms ($\alpha_{IIb}\beta_3$, Fc γ RIIA, GPVI) and receptors linked to PLC γ isoforms (GPIb, PAR1/4). This is supported by the observation that there is an assistant role of PI3K isoforms that produce the phospholipid, phosphatidylinositol

trisphosphate (PIP₃), which acts as membrane binding site and enhancer of PLC γ isoforms.¹¹⁷ Regarding the control of fibrin formation, contribution of the VWF-GPIb axis has been clarified so far. Given the high activation conditions required for PS exposure, it is reasonable to assume that not all platelets in a thrombus encounter sufficient levels of agonists, thus explaining part of the response heterogeneity. On the other hand, intrinsic platelet factors may contribute as well, e.g. different numbers of receptors or signalling molecules.⁵³

5. Platelet signalling to PS exposure and fibrin formation: central role of Ca^{2+}

Since the early finding that Ca^{2+} ionophores (A23187 or ionomycin) cause rapid and full PS exposure in essentially all platelets due to phospholipid membrane scrambling, it is clear that a prolonged high Ca^{2+} is a sufficient trigger for this platelet response.^{2,58} This is confirmed by the observation that most physiological agonists or combinations provoking PS exposure also induce sustained Ca^{2+} rises. The recent literature provides novel insight into the signalling pathways involved (see also Fig. 2).

Store-operated Ca^{2+} entry (SOCE). An initial finding was that Ca^{2+} entry via SOCE not only prolongs the platelet Ca^{2+} signal, but also promotes PS exposure.¹¹⁸

In the last few years, by using murine knockout and pharmacological approaches, a central role of two channel proteins in SOCE has been demonstrated.⁹ Stromal interaction molecule 1 (STIM1) was identified as central Ca^{2+} sensor in the platelet reticular membrane, monitoring Ca^{2+} store depletion, while Orai1 was identified as Ca^{2+} entry channel that is activated through coupling to STIM1.^{119,120} The chaperone protein cyclophilin A appeared to act as a positive Ca^{2+} modulator of STIM1.¹²¹ With mouse platelets lacking STIM1 or Orai1, collagen-dependent thrombus

formation was greatly impaired along with GPVI-induced PS exposure.^{119,120,122,123} In contrast, no role was found for the paralogues STIM2 and Orai3, which are both present in platelets. Interestingly, it was found that Orai1 contributes only little to Ca^{2+} entry in platelets activated by thrombin and $\text{PLC}\gamma$. This may explain why in the presence of high thrombin concentrations, PS exposure is only limitedly dependent on STIM1 and Orai1.¹²² Observations like these have raised the question whether another Ca^{2+} entry mechanism (receptor-operated Ca^{2+} entry) can operate

Procoagulant response

Figure 2. Platelet-based coagulation: central role of elevated Ca^{2+} in PS exposure. Mechanisms for Ca^{2+} elevation contributing to PS exposure include: Ca^{2+} mobilisation from the endoplasmic reticulum via IP_3 receptors (IP_3R); Ca^{2+} entry by SOCE via STIM1 and Orai1 channels; and release of mitochondrial Ca^{2+} via MPTP formation. Elevated Ca^{2+} activates the protease calpain, triggers phospholipid scrambling via anoctamin 6, thereby resulting in PS exposure. The apoptotic pathway of PS exposure via Bak/Bax-induced caspase activation is relatively independent of Ca^{2+} .

downstream of thrombin receptors to facilitate PS exposure.¹²⁴ Recent findings with human and mouse platelets suggest that the cation channel TRPC6 can play such a role.^{125,126} Earlier reports on a function of the isoform TRPC1 in Ca^{2+} entry¹²⁷ were ruled out because of a complete lack of a phenotype in mouse *Trpc1*^{-/-} platelets.^{126,128}

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

Mitochondrial collapse. Mitochondrial collapse by MPTP formation can be induced by various forms of cellular stress, *e.g.* by free oxygen radicals. Several groups have examined a role for this mitochondrial process in platelet procoagulant activity. In Ca^{2+} ionophore-stimulated platelets, inhibition of MPTP formation with cyclosporin A was found to prevent depolarization of the mitochondrial inner membrane potential as well as microparticle formation.

However, PS exposure was only moderately inhibited, indicating that mitochondrial collapse per se is not a strict requirement for PS exposure.¹³² In agreement with this, Ca^{2+} ionophore-induced PS exposure was unchanged in platelets from mice deficient in cyclophilin D, which is a component of the MPTP.⁶² Yet, in the cyclophilin D deficient platelets, PS exposure and thrombin generation induced by the receptor agonists convulxin and thrombin was strongly diminished. Along the same line, cyclosporin A was found to suppress the formation of coated platelets in response to convulxin/thrombin.⁶³ This implies a role of mitochondrial collapse also in fibrin (coat) formation. In other cells, MPTP formation abolishes the sequestering of Ca^{2+} in mitochondria.¹³³ Hence, also in platelets, mitochondrial collapse may lead to a loss of Ca^{2+} flux control, thus provoking agonist (convulxin/thrombin)-induced PS exposure. In contrast, the apoptosis-induced PS exposure, while also involving mitochondria, is essentially Ca^{2+} -independent and specifically relies on Bak/Bax and caspase activation.⁷⁴

Signalling downstream of Ca^{2+} . The majority of platelet agonists signal via $\text{PLC}\beta$ or γ isoforms, which produce diacylglycerol (DAG) and the Ca^{2+} -mobilising second messenger inositol 1,4,5-trisphosphate (IP_3), resulting in

activation of protein kinase C (PKC) and CalDAG-GEFI (Fig. 1). Detailed studies using knockout mice and specific inhibitors point to a two-sided role for PKC isoforms in platelet activation and PS exposure.¹³⁴ The conventional isoforms PKC α and PKC β , which are activated by DAG and Ca²⁺, positively contribute to platelet secretion, Ca²⁺ signalling and PS exposure.¹³⁵⁻¹³⁷ The novel isoform PKC θ , being activated by DAG alone, acts differently, in that it suppresses GPVI-induced Ca²⁺ mobilisation and PS exposure.¹³⁶ This negative role of PKC θ can explain why general PKC stimulation with phorbol ester down-regulates these platelet responses.¹³⁴ Yet, some authors report a slight stimulation of platelet function by PKC θ .¹³⁸ In sum, the role of PKC isoforms in platelet PS exposure (procoagulant response) can be understood in terms of PKC-dependent fine-tuning of the Ca²⁺ signal.

Mouse experiments furthermore point to a separate role in procoagulant activity of the regulatory protein CalDAG-GEFI.¹³⁹ It activates the small GTPase Rap1b, thereby supporting integrin activation and other platelet functions. Platelets from CalDAG-GEFI-deficient mice were found to be substantially impaired in PS exposure during thrombus formation on collagen, by a mechanism that is not yet understood.¹⁴⁰ It was concluded that CalDAG-GEFI, like STIM1, contributes to

the first wave of thrombin generation catalysed by PS-exposing procoagulant platelets. Furthermore, platelet CalDAG-GEFI appeared to regulate fibrin clot formation.

With the recent discovery of the platelet protein anoctamin 6 (*TMEM16F* gene) as key regulator of Ca²⁺-dependent phospholipid scrambling and hence PS exposure,¹⁴¹ other speculations on proteinous PS translocators have been overruled.⁷⁷ In platelets from patients with Scott syndrome, a moderate bleeding disorder, anoctamin 6 is lacking, while ionomycin- and in agonist-induced PS exposure are greatly impaired,^{141,142} in spite of normal Ca²⁺ rises.¹⁴³ Anoctamin 6 is a multiple membrane-spanning protein with supposed Ca²⁺ binding site, but whether it operates alone in phospholipid scrambling and PS exposure is unclear. Another Ca²⁺-dependent protein contributing to microparticle formation is the protease calpain, but inhibitor studies have indicated that its role in PS exposure is moderate at best.¹⁴⁴

Taken together, the available evidence indicates that PS exposure in response to agonist stimulation is accomplished by multiple pathways synergising to elevated Ca²⁺ (Fig. 2). In other words, platelet stimulation with multiple agonists that cause massive Ca²⁺ store depletion and SOCE, due to STIM1/Orai1 interaction, seems to result in a loss of

the normal control of Ca^{2+} handling by Ca^{2+} pumps, PKC θ and MPTP formation, so that cytosolic Ca^{2+} becomes prolonged high, and the Ca^{2+} -dependent phospholipid scrambling via anoctamin 6 becomes activated. How this facilitates extracellular fibrin formation is unclear.

6. Platelet signalling to clot retraction: central role of integrins

The regulation of clot retraction by platelets has been less extensively studied than the process of PS exposure. In the majority of the literature clot retraction is considered as an integrin $\alpha_{\text{IIb}}\beta_3$ -dependent event. Despite the only sparse mechanistic studies, clot retraction is generally used as a marker of $\alpha_{\text{IIb}}\beta_3$ outside-in signalling. As platelet

integrin signalling is reviewed elsewhere,¹³ we here summarize what is known of the signalling processes implicated in platelet-dependent clot retraction.

Signalling downstream of integrin $\alpha_{\text{IIb}}\beta_3$.

Several investigations, mostly using mouse blood, have indicated that clot retraction is mediated by signalling proteins that also contribute to $\alpha_{\text{IIb}}\beta_3$ integrin outside-in signalling. Platelet proteins implicated in clot retraction include the Src-family tyrosine kinases, Src and Fyn, the adapter protein Lnk, AMP-activated protein kinase, and the effector proteins PLC γ 2 and PI3K β .¹⁴⁵⁻¹⁴⁷ Recent studies point to two phases in platelet-dependent clot retraction upon

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

stimulation with thrombin: an initial formation of high-adhesion integrin $\alpha_{IIb}\beta_3$ contacts with fibrin, followed by phase where the platelet contractile forces are transmitted to the fibrin clot.^{65,148} The force generation appears to occur via the actin cytoskeleton and depends on activation of myosin IIA via Rho kinase and myosin light-chain kinase.¹⁴⁹ An extracellular process required for clot retraction is the cross-linking of platelet-associated fibrin fibres by the transglutaminase FXIIIa.¹⁵⁰ The Ca^{2+} -dependent protease calpain suppresses clot retraction, likely due to the cleavage of cytoskeletal proteins.¹⁵¹ In this context, it is relevant to note that calpain accomplishes considerable degradation of cytoskeletal proteins in PS-exposing platelets,¹⁵² thus providing another explanation why the PS-exposing platelet population is not involved in clot retraction.

Hence, the signalling processes contributing to platelet-regulated clot retraction, as far as unravelled, rely on integrin $\alpha_{IIb}\beta_3$ -dependent signalling processes controlling actin-myosin interactions, which are strikingly different from those leading to PS exposure.

References

- 1 Mann KG, Brummel K, Butenas S. What is all that thrombin for? *J Thromb Haemost* 2003;**1**:1504-14.

7. Conclusion

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

Disclosure of conflict of interests

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- 2 Heemskerk JW, Bevers EM, Lindhout T. Platelet activation and blood coagulation. *Thromb Haemost* 2002;**88**:186-93.

- 3 Monroe DM, Hoffman M, Roberts HR. Platelets and thrombin generation. *Arterioscler Thromb Vasc Biol* 2002;**22**:1381-9.
- 4 Jurk K, Kehrel BE. Platelets: physiology and biochemistry. *Semin Thromb Hemost* 2005;**31**:381-92.
- 5 Jackson SP. Arterial thrombosis: insidious, unpredictable and deadly. *Nat Med* 2011;**17**:1423-36.
- 6 Chou J, Mackman N, Merrill-Skoloff G, Pedersen B, Furie BC, Furie B. Hematopoietic cell-derived microparticle tissue factor contributes to fibrin formation during thrombus propagation. *Blood* 2004;**104**:3190-7.
- 7 Dubois C, Panicot-Dubois L, Merrill-Skoloff G, Furie B, Furie BC. Glycoprotein VI-dependent and -independent pathways of thrombus formation in vivo. *Blood* 2006;**107**:3902-6.
- 8 Kalia N, Auger JM, Atkinson B, Watson SP. Critical role of FcR γ -chain, LAT, PLC γ 2 and thrombin in arteriolar thrombus formation upon mild, laser-induced endothelial injury in vivo. *Microcirculation* 2008;**15**:325-35.
- 9 Stegner D, Nieswandt B. Platelet receptor signaling in thrombus formation. *J Mol Med* 2011;**89**:109-21.
- 10 Kuijpers MJ, Munnix IC, Cosemans JM, van Vlijmen BJ, Reutelingsperger CP, oude Egbrink MG, Heemskerk JW. Key role of platelet procoagulant activity in tissue factor- and collagen-dependent thrombus formation in arterioles and venules in vivo. Differential sensitivity to thrombin inhibition. *Microcirculation* 2008;**15**:269-82.
- 11 Brill A, Fuchs TA, Chauhan AK, Yang JJ, De Meyer SF, Köllnberger M, Wakefield TW, Lämmle B, Massberg S, Wagner DD. Von Willebrand factor-mediated platelet adhesion is critical for deep vein thrombosis in mouse models. *Blood* 2011;**117**:1400-7.
- 12 Furie B, Furie BC. Thrombus formation in vivo. *J Clin Invest* 2005;**115**:3355-62.
- 13 Shattil SJ, Kim C, Ginsberg MH. The final steps of integrin activation: the end game. *Nat Rev Mol Cell Biol* 2010;**11**:288-300.
- 14 Béguin S, Kumar R, Keularts I, Seligsohn U, Collier BS, Hemker HC. Fibrin-dependent platelet procoagulant activity requires GPIb receptors and von Willebrand factor. *Blood* 1999;**93**:564-70.
- 15 Zwaal RF, Schroit AJ. Pathophysiological implications of membrane phospholipid asymmetry in blood cells. *Blood* 1997;**89**:1121-32.
- 16 Munnix IC, Kuijpers MJ, Auger JM, Thomassen CM, Panizzi P, van Zandvoort MA, Rosing J, Bock PE, Watson SP, Heemskerk JW. Segregation of platelet aggregatory and procoagulant microdomains in thrombus formation. Regulation by transient integrin activation. *Arterioscler Thromb Vasc Biol* 2007; **27**: 2484-90.
- 17 Berny MA, Munnix IC, Auger JM, Schols SE, Cosemans JM, Panizzi P, Bock PE, Watson SP, McCarty OJ, Heemskerk JW. Spatial distribution of factor Xa, thrombin, and fibrin(ogen) on thrombi at venous shear. *Plos One* 2010; **5**: e10415.
- 18 Jurk K, Lahav J, van Aken H, Brodde MF, Nofer JR, Kehrel BE. Extracellular protein disulfide isomerase regulates feedback activation of platelet thrombin generation via modulation of coagulation factor binding. *J Thromb Haemost* 2011; **9**: 2278-90.
- 19 Daubie V, Cauwenberghs S, Senden NHM, Pochet R, Lindhout T, Buurman WA, Heemskerk JW. Factor Xa and thrombin evoke additive calcium and proinflammatory responses in endothelial cells subjected to coagulation. *Biochim Biophys Acta* 2006; **1763**: 860-9.
- 20 Gardiner EE, Karunakaran D, Shen Y, Arthur JF, Andrews RK, Bernd MC. Controlled shedding of platelet glycoprotein (GP)VI and GPIb/IX/V by ADAM family metalloproteinases. *J Thromb Haemost* 2007; **7**: 1530-7.
- 21 Al-Tamimi M, Grigoriadis G, Tran H, Paul E, Servadei P, Berndt MC, Gardiner EE, Andrews RK. Coagulation-induced shedding of platelet glycoprotein VI mediated by factor Xa. *Blood* 2011; **117**: 3912-20.
- 22 Ravanat C, Freund M, Mangin P, Azorsa DO, Schwartz C, Moog S, Schuhler S, Dambach J, Cazenave JP, Lanza F. GPV is a marker of in vivo platelet activation: study in a rat thrombosis model. *Thromb Haemost* 2000; **83**: 327-33.

- 23 Panes O, Matus V, Saez CG, Quiroga T, Pereira J, Mezzano D. Human platelets synthesize and express tissue factor. *Blood* 2007; **109**: 5242-50.
- 24 Rondina MT, Schwertz H, Harris ES, Kraemer BF, Campbell RA, Mackman N, Grissom CK, Weyrich AS, Zimmerman GA. The septic milieu triggers expression of spliced tissue factor mRNA in human platelets. *J Thromb Haemost* 2011; **9**: 748-58.
- 25 Kretz CA, Vaezzadeh N, Gross PL. Tissue factor and thrombosis models. *Arterioscler Thromb Vasc Biol* 2010; **30**: 900-8.
- 26 Maroney SA, Cooley BC, Ferrel JP, Bonesho CE, Mast AE. Murine hematopoietic cell tissue factor pathway inhibitor limits thrombus growth. *Arterioscler Thromb Vasc Biol* 2011; **31**: 821-6.
- 27 Gould WR, Silveira JR, Tracy PB. Unique in vivo modifications of coagulation factor V produce a physically and functionally distinct platelet-derived cofactor. *J Biol Chem* 2004; **279**: 2383-93.
- 28 Majumder R, Quinn-Allen MA, Kane WH, Lentz BR. A phosphatidylserine binding site in factor Va C1 domain regulates both assembly and activity of the prothrombinase complex. *Blood* 2008; **112**: 2795-802.
- 29 Fager AM, Wood JP, Bouchard BA, Feng P, Tracy PB. Properties of procoagulant platelets: defining and characterization the subpopulation binding a functional prothrombinase *Arterioscler Thromb Vasc Biol* 2010; **30**: 2400-7.
- 30 Alberio L, Safa O, Clemetson KJ, Esmon CT, Dale GL. Surface expression and functional characterization of α -granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin. *Blood* 2000; **95**: 1694-702.
- 31 Weeterings C, de Groot PG, Adelmeijer J, Lisman T. The glycoprotein Ib-V-IX complex contributes to tissue factor-independent thrombin generation by recombinant factor VIIa on the activated platelet surface. *Blood* 2008; **112**: 3227-33.
- 32 Lisman T, Adelmeijer J, Cauwenberghs S, van Pampus EC, Heemskerk JW, de Groot PG. Recombinant factor VIIa enhances platelet adhesion and activation under flow conditions at normal and reduced platelet count. *J Thromb Haemost* 2005; **3**: 742-51.
- 33 Gilbert GE, Novakovic VA, Kaufman RJ, Miao H, Pipe SW. Conservative mutations in the C2 domains of factor VIII and factor V alter phospholipid binding and cofactor activity. *Blood* 2012; **120**: 1923-32.
- 34 Cosemans JM, Schols SE, Stefanini L, de Witt S, Feijge MA, Hamulyak K, Deckmyn H, Bergmeier W, Heemskerk JW. Key role of glycoprotein Ib/V/IX and von Willebrand factor in platelet activation-dependent fibrin formation at low shear flow. *Blood* 2011; **117**: 651-60.
- 35 London FS, Marcinkiewicz M, Walsh PN. A subpopulation of platelets responds to thrombin- or SFLLRN-stimulation with binding sites for factor IXa. *J Biol Chem* 2004; **279**: 19854-9.
- 36 London FS, Marcinkiewicz M, Walsh PN. PAR1-stimulated factor IXa binding to a small platelet subpopulation requires a pronounced and sustained increase of cytoplasmic calcium. *Biochemistry* 2006; **45**: 7289-98.
- 37 Walsh PN. Platelet coagulant activities in thrombasthenia. *Br J Haematol* 1972; **23**: 553-69.
- 38 Renné T, Pozgajova M, Grüner S, Schuh K, Pauer HU, Burfeind P, Gailani D, Nieswandt B. Defective thrombus formation in mice lacking coagulation factor XII. *J Exp Med* 2005; **202**: 271-81.
- 39 Van der Meijden PE, Munnix IC, Auger JM, Govers-Riemslog JW, Cosemans JM, Kuijpers MJ, Spronk HM, Watson SP, Renné T, Heemskerk JW. Dual role of collagen in factor XII-dependent thrombus and clot formation. *Blood* 2009; **114**: 881-90.
- 40 Baglia FA, Shrimpton CN, Emsley J, Kitagawa K, Ruggeri ZM, Lopez JA, Walsh PN. Factor XI interacts with the leucine-rich repeats of glycoprotein Iba on the activated platelet. *J Biol Chem* 2004; **279**: 49323-9.
- 41 White-Adams TC, Beryn MA, Tucker EI, Gertz JM, Gailani D, Urbanus RT, de Groot PG, Gruber A, McCarty OJ. Identification of coagulation factor XI as a ligand for platelet apolipoprotein E receptor 2 (ApoER2). *Arterioscler Thromb Vasc Biol* 2009; **29**: 1602-7.

- 42 Müller F, Mutch NJ, Schenk WA, Smith SA, Esterl L, Spronk HM, Schmidbauer S, Gahl WA, Morrissey JH, Renné T. Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. *Cell* 2009; **139**: 1143-56.
- 43 Smith SA, Choi SH, Davis-Harrison R, Huyck J, Boettcher J, Reinstra CM, Morrissey JH. Polyphosphate exerts differential effects on blood clotting, depending on the polymer size. *Blood* 2010; **116**: 4353-9.
- 44 Choi SH, Smith SA, Morrissey JH. Polyphosphate is a cofactor for the activation of factor XI by thrombin. *Blood* 2011; **118**: 6963-70.
- 45 Magwenzi SG, Ajjan RA, Standeven KF, Parapia LA, Naseem KM. Factor XIII supports platelet activation and enhances thrombus formation by matrix proteins under flow conditions. *J Thromb Haemost* 2011; **9**: 820-33.
- 46 Dale GL, Friese P, Batar P, Hamilton SF, Reed GL, Jackson KW, Clemetson KJ, Alberio L. Stimulated platelets use serotonin to enhance their retention of procoagulant proteins on the cell surface. *Nature* 2002; **415**: 175-9.
- 47 Jobe SM, Leo L, Eastvold JS, Dickneite G, Ratliff TL, Lentz SR, Di Paola J. Role of FcRγ and factor XIIIa in coated platelet formation. *Blood* 2005; **106**: 4146-51.
- 48 Maroney SA, Haberichter SL, Friese P, Collins ML, Ferrel JP, Dale GL, Mast AE. Active tissue factor pathway inhibitor is expressed on the surface of coated platelets. *Blood* 2007; **109**: 1931-7.
- 49 Boulaftali Y, Ho-Tin-Noe B, Pena A, Loyau S, Venisse L, Francois D, Richard B, Arocas V, Collet JP, Jandrot-Perrus M, Bouton MC. Platelet protease nexin-1, a serpin that strongly influences fibrinolysis and thrombolysis. *Circulation* 2011; **12**: 1326-34.
- 50 White TC, Berny MA, Tucker EI, Urbanus RT, de Groot PG, Fernandez JA, Griffin JH, Gruber A, McCarty OJ. Protein C supports platelet binding and activation under flow: role of glycoprotein Ib and apolipoprotein E receptor 2. *J Thromb Haemost* 2008; **6**: 995-1002.
- 51 Van 't Veer C, Butenas S, Golden NJ, Mann KG. Regulation of prothrombinase activity by protein S. *Thromb Haemost* 1999; **82**: 80-7.
- 52 Dayer MR, Ghayour O, Dayer MS. Mechanism of protein Z-mediated inhibition of coagulation factor Xa by Z-protein-dependent inhibitor: a molecular dynamic approach. *ISRN Hematol* 2012; **2012**: 762728.
- 53 Munnix IC, Cosemans JM, Auger JM, Heemskerk JW. Platelet response heterogeneity in thrombus formation. *Thromb Haemost* 2009; **102**: 1149-56.
- 54 Nesbitt WS, Westein E, Tovar-Lopez FJ, Tolouei E, Mitchell A, Fu J, Carberry J, Fouras A, Jackson SP. A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. *Nat Med* 2009; **15**: 665-73.
- 55 Kulkarni S, Jackson SP. Platelet factor XIII and calpain negatively regulate integrin $\alpha_{IIb}\beta_3$ adhesive function and thrombus growth. *J Biol Chem*. **279**; 2004:30697-706.
- 56 Heemskerk JW, Kuijpers MJ, Munnix IC, Siljander PR. Platelet collagen receptors and coagulation. A characteristic platelet response as possible target for antithrombotic treatment. *Trends Cardiovasc Med* 2005; **15**: 86-92.
- 57 Shi J, Pipe JT, Heegaard CW, Gilbert GE. Lactadherin blocks thrombosis and hemostasis in vivo: correlation with platelet phosphatidylserine exposure. *J Thromb Haemost* 2008; **6**: 1167-74.
- 58 Bevers EM, Comfurius P, Zwaal RF. Platelet procoagulant activity, physiological significance and mechanisms of exposure. *Blood Rev* 1991; **5**: 146-54.
- 59 Dale GL. Coated-platelets: an emergent component of the procoagulant response. *J Thromb Haemost* 2005; **3**: 2185-92.
- 60 Szasz R, Dale GL. Thrombospondin and fibrinogen bind serotonin-derivatized proteins on COAT platelets. *Blood* 2002; **100**: 2827-31.
- 61 Dale GL, Remenyi G, Friese P. Quantitation of microparticles released from coated-platelets. *J Thromb Haemost* 2005; **3**: 2081-8.
- 62 Jobe SM, Wilson KM, Leo L, Raimondi A, Molkenin JD, Lentz SR, DiPaola J. Critical role for the mitochondrial permeability transition pore and cyclophilin D in platelet activation and thrombosis. *Blood* 2008; **111**: 1257-65.

- 63 Remenyi G, Szasz R, Frieze P, Dale GL. Role of mitochondrial permeability transition pore in coated-platelet formation. *Arterioscler Thromb Vasc Biol* 2005; **25**: 467-71.
- 64 Yakimenko AO, Verholomova FY, Kotova YN, Ataullakhanov FI, Panteleev MA. Identification of different procoagulatory. *Biophys J* 2012; **102**: 2261-9.
- 65 Schoenwaelder SM, Ono A, Nesbitt WS, Lim J, Jarman K, Jackson SP. Phosphoinositide 3-kinase p110 β regulates integrin $\alpha_{IIb}\beta_3$ avidity and the cellular transmission of contractile forces. *J Biol Chem* 2010; **285**: 2886-96.
- 66 Brass LF, Zhu L, Stalker TJ. Minding the gaps to promote thrombus growth and stability. *J Clin Invest* 2005; **115**: 3385-92.
- 67 Schoenwaelder SM, Yuan Y, Josefsson EC, White MJ, Tao Y, Mason KD, O'Reilly LA, Henley KJ, Ono A, Hsiao S, Willcox A, Roberts AW, Huang DCS, Salem HH, Kile BT, Jackson SP. Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function. *Blood* 2009; **114**: 663-6.
- 68 Vogler M, Hamali HA, Sun XM, Bampton ET, Dinsdale D, Snowden RT, Dyer MJS, Goodall AH, Cohen GM. BCL-2/BCL-XL inhibition induces apoptosis, disrupts cellular calcium homeostasis and prevents platelet activation. *Blood* 2011; **117**: 7145-54.
- 69 Kodama T, Takehara T, Hikita H, Shimizu S, Shigekawa M, Li W, Miyagi T, Hosui A, Tatsumi T, Ishida H, Kanto T, Hiramatsu N, Yin XM, Hayashi N. BH3-only activator proteins Bid and Bim are dispensable for Bak/Bax-dependent thrombocyte apoptosis induced by Bcl-xl deficiency. Molecular requisites for the mitochondrial pathway to apoptosis in platelets. *J Biol Chem* 2011; **286**: 13905-13.
- 70 Mason KD, Carpinelli MR, Fletcher JI, Collinga JE, Hilton AA, Ellis S, Kelly PN, Ekert PG, Metcalf D, Roberts AW, Huang DCS, Kile BT. Programmed anuclear cell death delimits platelet life span. *Cell* 2007; **128**: 1173-86.
- 71 Curvers J, van Pampus EC, Feijge MA, Rombout-Sestrirenkova E, Giesen PL, Heemskerk JW. Decreased responsiveness and development of activation markers of platelets stored in plasma. *Transfusion* 2004; **44**: 49-58.
- 72 Rand ML, Wang HH, Bang KW, Poon KS, Packham MA, Freedman J. Procoagulant surface exposure and apoptosis in rabbit platelets: association with shortened survival and steady-state senescence. *J Thromb Haemost* 2004; **2**: 651-9.
- 73 Albanyan AM, Murphy FM, Rasmussen JT, Heegaard CW, Harrison P. Measurement of phosphatidylserine exposure during storage of platelet concentrates using the novel probe lactadherin: a comparison study with annexin V. *Transfusion* 2009; **49**: 99-107.
- 74 Jackson SP, Schoenwaelder SM. Procoagulant platelets: are they necrotic? *Blood* 2010; **116**: 2011-8.
- 75 Sims J, Wiedmer T, Esmen CT, Weiss HJ, Shattil SJ. Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome, an isolated defect in platelet procoagulant activity. *J Biol Chem* 1989; **264**: 17049-57.
- 76 Cauwenberghs S, Feijge MA, Harper AG, Sage SO, Curvers J, Heemskerk JW. Shedding of procoagulant microparticles from unstimulated platelets by integrin-mediated destabilization of actin cytoskeleton. *FEBS Lett* 2006; **580**: 5313-20.
- 77 Morel O, Jesel L, Freyssinet JM, Toti F. Cellular mechanisms underlying the formation of circulating microparticles. *Arterioscler Thromb Vasc Biol* 2010; **31**: 15-26.
- 78 Vasina E, Heemskerk JW, Weber C, Koenen RR. Platelets and platelet-derived microparticles in vascular inflammatory disease. *Inflamm Allergy Drug Targets* 2010; **9**: 346-54.
- 79 Dörmann D, Clemetson KJ, Kehrel B. The GPIb thrombin-binding site is essential for thrombin-induced platelet procoagulant activity. *Blood* 2000; **86**: 2469-78.
- 80 Béguin S, Keularts I, Al Dieri R, Belluci S, Caen J, Hemker HC. Fibrin polymerization is crucial for thrombin generation in platelet-rich plasma in a vWF-GPIb-dependent process, defective in Bernard-Soulier syndrome. *J Thromb Haemost* 2004; **2**: 170-6.

- 81 McNicol A, Sutherland M, Zou R, Drouin J. Defective thrombin-induced calcium changes and aggregation of Bernard-Soulier platelets are not associated with deficient moderate-affinity receptors. *Arterioscler Thromb Vasc Biol* 1996; **16**: 628-32.
- 82 Ravanat C, Strassel C, Hechler B, Schuhler S, Chicanne G, Payrastre B, Gachet C, Lanza F. A central role of GPIb-IX in the procoagulant function of platelets that is independent of the 45-kDa GPIb α N-terminal extracellular domain. *Blood* 2010; **116**: 1157-64.
- 83 Ramakrishnan V, Reeves PS, DeGuzman F, Deshpande U, Ministri Madrid K, DuBridge RB, Phillips DR. Increased thrombin responsiveness in platelets from mice lacking glycoprotein V. *Proc Natl Acad Sci USA* 1999; **96**: 13336-41.
- 84 Offermanns S. Activation of platelet function through G protein-coupled receptors. *Circ Res* 2006; **99**: 1293-304.
- 85 Heemskerk JW, Feijge MA, Henneman L, Rosing J, Hemker HC. The Ca²⁺-mobilizing potency of a-thrombin and thrombin-receptor-activating peptide on human platelets. Concentration and time effects of thrombin-induced Ca²⁺ signaling. *Eur J Biochem* 1997; **249**: 547-55.
- 86 Andersen H, Greenberg DL, Fujikawa K, Xu WF, Chung DW, Davie EW. Protease-activated receptor 1 is the primary mediator of thrombin-stimulated platelet procoagulant activity. *Proc Natl Acad Sci USA* 1999; **96**: 11189-93.
- 87 Keuren JF, Wiolders SJ, Ulrichs H, Hackeng T, Deckmyn H, Heemskerk JW, Bevers E, Lindhout T. Synergistic effect of thrombin on collagen-induced platelet procoagulant activity is mediated through protease-activated receptor-1. *Arterioscler Thromb Vasc Biol* 2005; **25**: 1499-505.
- 88 Harper MT, Poole AW. PKC inhibition markedly enhances Ca²⁺ signaling and phosphatidylserine exposure downstream of protease-activated receptor-1 but not protease-activated receptor-4 in human platelets. *J Thromb Haemost* 2011; **9**: 1599-607.
- 89 Dorsam RT, Tuluc M, Kunapuli SP. Role of protease-activated and ADP receptor subtypes in thrombin generation on human platelets. *J Thromb Haemost* 2004; **2**: 804-12.
- 90 Coughlin SR. Thrombin signalling and protease-activated receptors. *Nature* 2000; **407**: 258-64.
- 91 Andrews RK, Karunakaran D, Gardiner EE, Berndt MC. Platelet receptor proteolysis. A mechanism for downregulating platelet reactivity. *Arterioscler Thromb Vasc Biol* 2007; **27**: 1511-20.
- 92 Trivedi V, Boire A, Tchernychev B, Kaneider NC, Leger AJ, O'Callaghan K, Covic L, Kuliopulos A. Platelet matrix metalloprotease-1 mediates thrombogenesis by activating PAR1 at a cryptic site. *Cell* 2009; **137**: 332-43.
- 93 Quinton TM, Kim S, Derian CK, Jin J, Kunapuli SP. Plasmin-mediated activation of platelets occurs by cleavage of protease-activated receptor 4. *J Biol Chem* 2004; **279**: 18434-9.
- 94 Sambrano GR, Huang W, Faruqi T, Mahrus S, Craik C, Coughlin SR. Cathepsin G activates protease-activated receptor-4 in human platelets. *J Biol Chem* 2000; **275**: 6819-23.
- 95 Lecut C, Schoolmeester A, Kuijpers MJ, Broers JLV, van Zandvoort MA, Vanhoorelbeke K, Deckmyn H, Jandrot-Perrus M, Heemskerk JW. Principal role of glycoprotein VI in $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ activation during collagen-induced thrombus formation. *Arterioscler Thromb Vasc Biol* 2004; **24**: 1727-33.
- 96 Vanschoonbeek K, Feijge MA, van Kampen RJW, Kenis H, Hemker HC, Giesen PL, Heemskerk JW. Initiating and potentiating roles of platelets in tissue factor-induced thrombin generation in the presence of plasma: subject-dependent variation in thrombogram characteristics. *J Thromb Haemost* 2004; **2**: 476-84.
- 97 Léon C, Alex M, Klocke A, Morgenstern E, Moosbauer C, Eckly A, Spannagl M, Gachet C, Engelmann B. Platelet ADP receptors contribute to the initiation of intravascular coagulation. *Blood* 2004; **103**: 594-600.
- 98 Van der Meijden PE, Schoenwaelder SM, Cosemans JM, Wetzker R, Heller R, Jackson SP, Heemskerk JW. Dual P2Y₁₂ receptor signaling in thrombin-stimulated platelets: involvement of phosphoinositide 3-kinase β but not γ isoforms

- in Ca^{2+} mobilization and procoagulant activity. *FEBS J* 2008; **275**: 371-85.
- 99 Van der Meijden PE, Feijge MA, Giesen PL, Huijberts M, van Raak EP, Heemskerk JW. Platelet P2Y_{12} receptors enhance signalling towards procoagulant activity and thrombin generation: a study with healthy subjects and patients at thrombotic risk. *Thromb Haemost* 2005; **93**: 1128-37.
- 100 Frelinger AL, Jakubowski JA, Li Y, Barnard MR, Linden MD, Rarnow I, Fox ML, Sugidachi A, Winters KJ, Furman MI, Michelson AD. The active metabolite of prasugrel inhibits adenosine diphosphate- and collagen-stimulated platelet procoagulant activities. *J Thromb Haemost* 2008; **6**: 359-65.
- 101 Judge HM, Buckland RJ, Sugidachi A, Jakubowski JA, Storey RF. The active metabolite of prasugrel effectively blocks the platelet P2Y_{12} receptor and inhibits procoagulant and pro-inflammatory reactions. *Platelets* 2008; **19**: 125-33.
- 102 Van der Meijden PE, Swieringa S, Feijge MA, Gilio K, Hamulyák K, Heemskerk JW. Integrin $\alpha\text{IIb}\beta 3$ outside-in signaling causes platelet procoagulant activity and thrombin generation. *Cell Mol Life Sci* 2012; **in press**.
- 103 Ilveskero S, Lassila R. Abciximab inhibits procoagulant activity but not the release reaction upon collagen- or clot-adherent platelets. *J Thromb Haemost* 2003; **1**: 805-13.
- 104 Goto S, Tamura N, Li M, Handa M, Ikeda Y, Handa S, Ruggeri ZM. Differential effects of various anti-GPIIb/IIIa agents on shear-induced platelet activation and expression of procoagulant activity. *J Thromb Haemost* 2003; **1**: 2022-30.
- 105 Lhermusier T, van Rottem J, Garcia C, Xuereb JM, Ragab A, Martin V, Gratacap MP, Sie P, Payrastra B. The Syk-kinase inhibitor R406 impairs platelet activation and monocyte tissue factor expression triggered by heparin-PF4 complex directed antibodies. *J Thromb Haemost* 2011; **8**: 2067-76.
- 106 Siljander P, Farndale RW, Feijge MA, Comfurius P, Kos S, Bevers EM, Heemskerk JW. Platelet adhesion enhances the glycoprotein VI-dependent procoagulant response: involvement of p38 MAP kinase and calpain. *Arterioscler Thromb Vasc Biol* 2001; **21**: 618-27.
- 107 Munnix IC, Strehl A, Kuijpers MJ, Auger JM, van der Meijden PE, van Zandvoort MA, oude Egbrink M, Nieswandt B, Heemskerk JW. The glycoprotein VI-phospholipase $\text{Cy}2$ signaling pathway controls thrombus formation induced by collagen and tissue factor in vitro and in vivo. *Arterioscler Thromb Vasc Biol* 2005; **25**: 2673-8.
- 108 Auger JM, Kuijpers MJ, Senis YA, Watson SP, Heemskerk JW. Adhesion of human and mouse platelets to collagen under shear: a unifying model. *FASEB J* 2005; **19**: 825-7.
- 109 Elvers M, Pozgai R, Varga-Szabo D, May F, Pleines I, Kuijpers MJ, Heemskerk JW, Nieswandt B. Platelet hyperactivity and a prothrombotic phenotype in mice with a gain-of-function mutation in phospholipase $\text{Cy}2$. *J Thromb Haemost* 2010; **8**: 1353-63.
- 110 Siljander PR, Munnix IC, Smethurst PA, Deckmyn H, Lindhout T, Ouwehand WH, Farndale RW, Heemskerk JW. Platelet receptor interplay regulates collagen-induced thrombus formation in flowing human blood. *Blood* 2004; **103**: 1333-41.
- 111 Heemskerk JW, Vuist WM, Feijge MA, Reutelingsperger CP, Lindhout T. Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine and procoagulant activity of adherent platelets. Evidence for regulation by protein tyrosine kinase-dependent Ca^{2+} responses. *Blood* 1997; **90**: 2615-25.
- 112 Thomas DH, Getz TM, Newman TN, Dangelmaier CA, Carpino N, Kunapuli SP, Tsygankov AY, Daniel JL. A novel histidine tyrosine phosphatase, TULA-2, associates with Syk and negatively regulates GPVI signaling in platelets. *Blood* 2010; **116**: 2570-8.
- 113 Bevers EM, Comfurius P, van Rijn JL, Hemker HC, Zwaal RF. Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. *Eur J Biochem* 1982; **122**: 429-36.
- 114 Séverin S, Pollitt AY, Navarro-Nunez L, Nash CA, Mourao-Sa D, Eble JA, Senis YA, Watson SP. Syk-dependent phosphorylation of CLEC-2. A novel

- mechanism of hem-immunoreceptor tyrosine-based activation motif signaling *J Biol Chem* 2011; **286**: 4107-16.
- 115 Nergiz-Unal R, Lamers MM, van Kruchten R, Luiken JJ, Cosemans JM, Glatz JF, Kuijpers MJ, Heemskerk JW. Signaling role of CD36 in platelet activation and thrombus formation on immobilized thrombospondin or oxidized low density lipoprotein. *J Thromb Haemost* 2011; **9**: 1835-46.
- 116 White-Adams TC, Berny MA, Patel IA, Tucker EI, Gailani D, Gruber A, McCarty OJ. Laminin promotes coagulation and thrombus formation in a factor XII-dependent manner. *J Thromb Haemost* 2010; **8**: 1295-301.
- 117 Gilio K, Munnix IC, Mangin P, Cosemans JM, Feijge MA, van der Meijden PE, Olieslagers S, Chrzanowska-Wodnicka MB, Lillian R, Schoenwaelder S, Koyasu S, Sage SO, Jackson SP, Heemskerk JW. Non-redundant roles of phosphoinositide 3-kinase isoforms α and β in glycoprotein VI-induced platelet signaling and thrombus formation. *J Biol Chem* 2009; **285**: 33750-62.
- 118 Smeets EF, Heemskerk JW, Comfurius P, Bevers EM, Zwaal RF. Thapsigargin amplifies the platelet procoagulant response caused by thrombin. *Thromb Haemost* 1993; **70**: 1024-9.
- 119 Varga-Szabo D, Braun A, Kleinschnitz C, Bender M, Pleines I, Pham M, Renné T, Stoll G, Nieswandt B. The calcium sensor STIM1 is an essential mediator of arterial thrombosis and ischemic brain infarction. *J Exp Med* 2008; **205**: 1583-91.
- 120 Braun A, Varga-Szabo D, Kleinschnitz C, Pleines I, Bender M, Austinat M, Bösi M, Stoll G, Nieswandt B. Orai1 (CRACM1) is the platelet SOC channel and essential for pathological thrombus formation. *Blood* 2009; **113**: 2056-63.
- 121 Elvers M, Herrmann A, Seizer P, Münzer P, Beck S, Schönberger T, Borst O, Martin-Romero FJ, Lang F, May AE, Gawaz M. Intracellular cyclophilin A is an important Ca^{2+} regulator in platelets and critically involved in arterial thrombus formation. *Blood* 2012; **120**: 1317-26.
- 122 Gilio K, van Kruchten R, Braun A, Berna-Erro A, Feijge MA, Stegner D, van der Meijden PE, Kuijpers MJ, Varga-Szabo D, Heemskerk JW, Nieswandt B. Roles of STIM1 and Orai1 in glycoprotein VI- and thrombin-dependent procoagulant activity and thrombus formation. *J Biol Chem* 2010; **285**: 23629-38.
- 123 Bergmeier W, Oh-hora M, McCarl CA, Roden RC, Bray PF, Feske S. R93W mutation in Orai1 causes impaired calcium influx in platelets. *Blood* 2009; **109**: 6875-8.
- 124 Harper MT, Poole AW. Store-operated calcium entry and non-capacitative calcium entry have distinct roles in thrombin-induced calcium signaling in human platelets. *Cell Calcium* 2011; **50**: 351-8.
- 125 Ramanathan G, Gupta S, Thielmann I, Pleines I, Varga-Szabo D, May F, Mannhalter C, Dietrich A, Nieswandt B, Braun A. Defective diacylglycerol-induced Ca^{2+} entry but normal agonist-induced activation responses in TRPC6-deficient mouse platelets. *J Thromb Haemost* 2012; **10**: 419-29.
- 126 Hassock SR, Zhu MX, Trost C, Flockerzi V, Authi KS. Expression and role of Trpc proteins in human platelets: evidence that Trpc6 forms the store-independent calcium entry channel. *Blood* 2002; **100**: 2801-11.
- 127 Lopez JJ, Salido GM, Pariente JA, Rosado JA. Interaction of Stim1 with endogenously expressed human canonical Trp1 upon depletion of intracellular Ca^{2+} stores. *J Biol Chem* 2006; **281**: 28254-64.
- 128 Varga-Szabo D, Authi KS, Braun A, Bender M, Ambily A, Hassock SR, Gudermann T, Dietrich A, Nieswandt B. Store-operated Ca^{2+} entry in platelets occurs independently of transient receptor potential (TRP) C1. *Pflügers Arch* 2008; **457**: 377-87.
- 129 Tomasiak M, Stelmach H, Rusak T, Ciborowski M, Radziwon P. The involvement of Na^+/K^+ -ATPase in the development of platelet procoagulant response. *Acta Biochimica Polonica* 2007; **54**: 625-39.
- 130 Bucki R, Pastore JL, Giraud F, Janmey PA, Sulpice JC. Involvement of the Na^+/H^+ exchanger in membrane phosphatidylserine exposure during human platelet activation. *Biochim Biophys Acta* 2006; **1761**: 195-204.

- 131 Wolfs JL, Wielders SJ, Comfurius P, Lindhout T, Giddings JC, Zwaal RF, Bevers EM. Reversible inhibition of the platelet procoagulant response through manipulation of the Gardos channel. *Blood* 2006; **108**: 2223-8.
- 132 Leytin V, Allen DJ, Mutlu A, Gyulkhandanyan AV, Mykhalov S, Freedman J. Mitochondrial control of platelet apoptosis: effect of cyclosporin A, an inhibitor of the mitochondrial permeability transition pore. *Lab Invest* 2009; **89**: 374-84.
- 133 Starkov AA. The molecular identity of the mitochondrial Ca^{2+} sequestration system. *FEBS J* 2010; **277**: 3652-63.
- 134 Strehl A, Munnix IC, Kuijpers MJ, van der Meijden PE, Cosemans JM, Feijge MA, Nieswandt B, Heemskerk JW. Dual role of platelet protein kinase C in thrombus formation: stimulation of pro-aggregatory and suppression of procoagulant activity in platelets. *J Biol Chem* 2007; **282**: 7046-55.
- 135 Konopatskaya O, Gilio K, Harper MT, Zhao Y, Cosemans JM, Karim AZ, Whiteheart SW, Molkentin JD, Verkade P, Watson SP, Heemskerk JW, Poole AW. PKC α regulates platelet granule secretion and thrombus formation in mice. *J Clin Invest* 2009; **119**: 399-407.
- 136 Gilio K, Harper MT, Cosemans JM, Konopatskaya O, Munnix IC, Prinzen L, Leitges M, Liu Q, Molkentin JD, Heemskerk JW, Poole AW. Functional divergence of platelet protein kinase C (PKC) isoforms in thrombus formation on collagen. *J Biol Chem* 2010; **285**: 23410-9.
- 137 Harper MT, Poole AW. Diverse functions of protein kinase C isoforms in platelet activation and thrombus formation. *J Thromb Haemost* 2010; **8**: 454-62.
- 138 Nagy B, Bhavaraju K, Getz T, Bynagari YS, Kim S, Kunapuli SP. Impaired activation of platelets lacking protein kinase C- θ isoform. *Blood* 2009; **113**: 2557-67.
- 139 Stefanini L, Roden RC, Bergmeier W. CalDAG-GEFI is at the nexus of calcium-dependent platelet activation. *Blood* 2009; **114**: 2506-14.
- 140 Ahmad F, Boulaftali Y, Greene TK, Ouelette TD, Poncz M, Feske S, Bergmeier W. Relative contributions of stromal interaction molecule 1 and CalDAG-GEFI to calcium-dependent platelet activation and thrombosis. *J Thromb Haemost* 2011; **9**: 2077-86.
- 141 Suzuki J, Umeda M, Sims PJ, Nagata S. Calcium-dependent phospholipid scrambling by TMEM16F. *Nature* 2010; **468**: 834-8.
- 142 Castoldi E, Collins PW, Williamson PL, Bevers EM. Compound heterozygosity for 2 novel TMEM16F mutations in a patient with Scott syndrome. *Blood* 2011; **117**: 4399-400.
- 143 Munnix IC, Harmsma M, Giddings JC, Collins PW, Feijge MA, Comfurius P, Heemskerk JW, Bevers EM. Store-mediated calcium entry in the regulation of phosphatidylserine exposure in blood cells from Scott patients. *Thromb Haemost* 2003; **89**: 687-95.
- 144 Bachelot-Loza C, Badol P, Brohard-Bohn B, Fraiz N, Cano E, Rendu F. Differential regulation of platelet aggregation and aminophospholipid exposure by calpain. *Br J Haematol* 2006; **133**: 419-26.
- 145 Takizawa H, Nishimura S, Takayama N, Oda A, Nishikii H, Morita Y, Kakinuma S, Yamazaki S, Okamura S, Tamura N, Goto S, Sawaguchi A, Manabe I, Takatsu K, Nakauchi H, Takaki S, Eto K. Lnk regulates integrin $\alpha_{IIb}\beta_3$ outside-in signaling in mouse platelets, leading to stabilization of thrombus development in vivo. *J Clin Invest* 2010; **120**: 179-90.
- 146 Suzuki-Inoue K, Hughes CE, Inoue O, Kaneko M, Cuyun-Lira O, Takafuta T, Watson SP, Ozaki Y. Involvement of Src kinases and PLC γ 2 in clot retraction. *Thromb Res* 2007; **120**: 251-8.
- 147 Randriamboavonjy V, Isaak J, Fromel T, Viollet B, Fisslthaler B, Preissner KT, Fleming I. AMPK α 2 subunit is involved in platelet signaling, clot retraction and thrombus stability. *Blood* 2010; **116**: 2134-40.
- 148 Martin V, Guillermet-Guibert J, Chicanne G, Cabou C, Jandrot-Perrus M, Plantavid M, Vanhaesebroeck B, Payraastre B, Gratacap MP. Deletion of the p110 β isoform of phosphoinositide 3-kinase in platelets reveals its central role in Akt activation and thrombus formation in vitro and in vivo. *Blood* 2010; **115**: 2008-13.
- 149 Ono A, Westein E, Hsiao S, Nesbitt WS, Hamilton JR, Schoenwaelder SM, Jackson SP. Identification

- of a fibrin-independent platelet contractile mechanism regulating primary hemostasis and thrombus growth. *Blood* 2008; **112**: 90-9.
- 150 Kasahara K, Sourì M, Kaneda M, Miki T, Yamamoto N, Ichinose A. Impaired clot retraction in factor XIII A subunit-deficient mice. *Blood* 2010; **115**: 1277-9.
 - 151 Schoenwaelder SM, Yuan Y, Cooray P, Salem HH, Jackson SP. Calpain cleavage of focal adhesion proteins regulates the cytoskeletal attachment of integrin $\alpha_{IIb}\beta_3$ (platelet glycoprotein IIb/IIIa) and the cellular retraction of fibrin clots. *J Biol Chem* 1997; **272**: 1694-702.
 - 152 Verhallen PF, Bevers EM, Comfurius P, Zwaal RF. Correlation between calpain-mediated cytoskeletal degradation and expression of platelet procoagulant activity. A role for the platelet membrane-skeleton in the regulation of membrane lipid asymmetry? *Biochim Biophys Acta* 1987; **903**: 206-17.

Chapter 4

Dual mechanism of integrin $\alpha_{\text{IIb}}\beta_3$ closure in procoagulant platelets

Mattheij NJ¹, Gilio K¹, van Kruchten R¹, Jobe SM², Wieschhaus AJ³,
Chishti AH⁴, Collins P⁵, Heemskerk JW¹, Cosemans JM¹

¹Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, The Netherlands; ²Department of Pediatrics, Emory University, Atlanta, United States; ³Department of Pharmacology, University of Illinois, Chicago, United States; ⁴Department of Molecular Physiology and Pharmacology, Tufts University School of Medicine, Boston, United States; ⁵Arthur Bloom Haemophilia Centre, Department of Haematology, Medical School of Cardiff University, Cardiff, United Kingdom

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Abstract

Aggregation of platelets via activated integrin $\alpha_{IIb}\beta_3$ is a prerequisite for thrombus formation. Phosphatidylserine-exposing platelets with a key role in the coagulation process disconnect from a thrombus by integrin inactivation via an unknown mechanism. Here we show that $\alpha_{IIb}\beta_3$ inactivation in procoagulant platelets relies on a sustained high intracellular Ca^{2+} , stimulating intracellular cleavage of the β_3 chain, talin and Src kinase. Inhibition of calpain activity abolished protein cleavage, but only partly suppressed $\alpha_{IIb}\beta_3$ inactivation. Integrin $\alpha_{IIb}\beta_3$ inactivation was unchanged in platelets from *Capn1*^{-/-} mice, suggesting a role of the calpain-2 isoform. Scott syndrome platelets, lacking the transmembrane protein TMEM16F and having low phosphatidylserine exposure, displayed reduced $\alpha_{IIb}\beta_3$ inactivation with the remaining activity fully dependent on calpain. In platelets from *Ppif*^{-/-} mice, lacking mitochondrial permeability transition pore (mPTP) formation, agonist-induced phosphatidylserine exposure and $\alpha_{IIb}\beta_3$ inactivation were reduced. Treatment of human platelets with cyclosporin A gave a similar phenotype. Together, these data point to a dual mechanism of $\alpha_{IIb}\beta_3$ inactivation via calpain(-2) cleavage of integrin-associated proteins and via TMEM16F-dependent phospholipid scrambling with an assistant role of mPTP formation.

Introduction

Integrin $\alpha_{IIb}\beta_3$ plays a crucial role in platelet aggregation in response to physiological agonists. Once in its activated conformation, $\alpha_{IIb}\beta_3$ accomplishes platelet-platelet interactions via bridges of fibrinogen and von Willebrand factor. Under thrombotic conditions in flowing blood, $\alpha_{IIb}\beta_3$ -dependent platelet aggregation mediates thrombus formation and, finally, occlusion of a damaged cardiac or carotid artery.¹ Microscopic observations have shown that, both *in vivo* and in parallel-plate flow chambers, the aggregated platelets in a thrombus are surrounded by patches of procoagulant

platelets with quite distinct properties.²

The latter platelets characteristically are elevated in cytosolic Ca^{2+} , have a rounded morphology with attached microparticles, and exposed the negatively charged lipid, phosphatidylserine (PS). This contrasts to the classical pseudopod-containing structure of aggregated platelets in a thrombus, which do not expose PS.³ Time-lapse videos show that the rounded morphology arises during platelet disconnection from the thrombus core, suggesting a well-controlled mechanism of platelet detachment.⁴ This population of PS-exposing platelets was found to bind

multiple coagulation factors, greatly promoting the process of thrombin generation.^{3,5} It has been argued that the platelet detachment is mediated by inactivation or closure of previously activated $\alpha_{IIb}\beta_3$ integrins.⁶ However, the regulation of such a process is not well understood.

Activation of $\alpha_{IIb}\beta_3$ leads to the appearance of high-affinity binding sites for fibrinogen and von Willebrand factor at the platelet surface. The signaling mechanism to $\alpha_{IIb}\beta_3$ activation, unraveled in considerable detail, involves several pathways, *i.e.*, via a phospholipase C and protein kinase C route, resulting in transient Ca^{2+} fluctuations, and via phosphoinositide 3-kinase route. The consequence is activation of a chain of regulatory proteins CalDAG-GEFI, Rap1b and Rap1-GTP-interacting adapter molecule (RIAM);^{7,8} and these establish interaction of the cytoskeleton proteins, kindlin-3 and talin-1, with the β_3 chain of the $\alpha_{IIb}\beta_3$ complex, with as a result unclasping of the α_{IIb} and β_3 chains.^{9,10} Another proposed mechanism of $\alpha_{IIb}\beta_3$ activation is that talin-1 modulates the integrin conformation by a tilting effect on the β_3 chain.¹¹

In response to ADP and ADP-releasing platelet agonists, the conformational change of $\alpha_{IIb}\beta_3$ is considered to be an intrinsically reversible process.⁶ The active integrin itself can evoke multiple

signaling events in platelets.¹² A key role in this so-called outside-in signaling pathway is provided by the protein tyrosine kinase Src, which is constitutively associated with the β_3 cytoplasmic tail.¹³ Src, for instance, activates other tyrosine kinases such as Syk.⁸ In addition, a functional role of the Ca^{2+} -dependent thiol protease, calpain, has been suggested in integrin $\alpha_{IIb}\beta_3$ activation and signaling, but this has remained controversial. Calpain may either activate $\alpha_{IIb}\beta_3$ by cleaving talin,¹⁴ or counteract the high-affinity conformational state of $\alpha_{IIb}\beta_3$ by cleaving the cytoplasmic tail of the β_3 chain.¹⁵

While essentially all platelet agonists cause $\alpha_{IIb}\beta_3$ activation, only strong agonists are capable of inducing the formation of procoagulant, PS-exposing platelets. Effective inducers of the latter response are the combination of the glycoprotein VI agonist convulxin (Cvx) with thrombin (Thr), or otherwise Ca^{2+} ionophores such as A23187 and ionomycin.^{16,17} Common to these agonists is that they induce a high and sustained rise in cytosolic Ca^{2+} , which is considered to trigger the Ca^{2+} -operated membrane protein, TMEM16F, which regulates the scrambling of phospholipids and exposure of PS.^{18,19} In platelets from Scott syndrome patients, who lack a functional TMEM16F, agonist-induced PS exposure is greatly diminished.^{20,21} Also a role in TMEM16F-

mediated phospholipid scrambling is the Ca^{2+} -dependent depolarization of the inner mitochondrial membrane and formation of a mitochondrial permeability transition pore (mPTP).¹⁹ This is consistent with the observation

that mouse platelets lacking cyclophilin D (*Ppif* gene), an mPTP component, are defective in PS exposure.²² Another platelet population often described in the literature is that of coated platelets.^{23,24} These are formed

Figure 1. Increase in light transmission, but loss of aggregating potential and inactivation of $\alpha_{IIb}\beta_3$ after platelet stimulation with Cvx/Thr or ionomycin. Washed human platelets were stimulated with Thr (4 nM), Cvx (100 ng/ml), Cvx/Thr or ionomycin (10 μ M) $\pm$ tirofiban (5 μ g/ml) in the presence of 2 mM CaCl_2 , as indicated. (A) Representative light transmission traces from 5 independent experiments. (B) Single platelet count after 30 min of stimulation. (C, D) Two-color flow cytometry after stimulation at indicated times with Cvx/Thr; labeling was with FITC-PAC1 mAb plus AF647-annexin A5. (C) Representative dot plots of FL1 (FITC-PAC1 mAb) versus FL4 (AF647-annexin A5) after 5 or 25 min of activation. (D) Percentages of platelet populations: no staining (white bars), staining with PAC1 mAb (light gray bars), double staining (dark gray bars), and annexin A5 positive (black bars). (E, F) Two-color flow cytometry after stimulation with ionomycin; further as for panels C and D. Means $\pm$ S.D. ($n = 5$), $*p < 0.05$ vs. Thr (paired T-test).

by dual stimulation with collagen and thrombin (but not Ca^{2+} ionophore), and can be characterized by high and stable surface retention of labeled fibrinogen. Fibrinogen as well as other platelet secretion products (e.g. factor V, thrombospondin, fibronectin, von Willebrand factor), bind to these platelets in a $\alpha_{\text{IIb}}\beta_3$ -independent way via transglutaminase activity. Procoagulant and coated platelets represent two not completely overlapping populations, as not all PS-exposing platelets display high fibrinogen binding.²

In the present study, we investigated the mechanism of integrin $\alpha_{\text{IIb}}\beta_3$ inactivation in procoagulant platelets, by determining the functional roles of calpain, Src-dependent signaling, TMEM16F and mPTP formation.

Experimental procedures

Materials

Horm type-I collagen was purchased from Nycomed. Convulxin (Cvx) was purified to homogeneity from the venom of *Crotalus durissus terrificus* (Latoxan). Annexin A5 labeled with Alexa Fluor-647 (AF647) and Fura-2 acetoxymethyl ester were from Invitrogen. Annexin A5 labeled with fluorescein isothiocyanate (FITC) was from Pharmatarget; FITC-labeled PAC1 monoclonal antibody (mAb) against activated human integrin $\alpha_{\text{IIb}}\beta_3$ from BD bioscience; phycoerythrin (PE)-labeled JON/A mAb against activated

mouse $\alpha_{\text{IIb}}\beta_3$ from Emfret Analytics. Mouse anti-talin mAb and anti-v-Src were from Sigma; mAb anti-Tyr⁷⁷³ β_3 chain (Ab38460) was from Abcam. Rabbit antibodies Ab762, Ab754, and Ab759, directed against (calpain-dependent) cleavage sites of human/mouse integrin β_3 , were kindly provided by Dr. X. Du (University of Illinois, Chicago).²⁵ Tirofiban came from MSD, human α -thrombin from Kordia, and MDL-28170 from Tocris. Pre-stained SDS-PAGE standards and Laemmli sample buffer were from Biorad. Other reagents were purchased from Calbiochem.

Blood Collection and Platelet Preparation

Blood from healthy volunteers and a Scott syndrome patient was collected in acid-citrate-dextrose anticoagulant after full informed consent (Helsinki declaration). Experiments were approved by the local Medical Ethics Committees. Platelet-rich plasma (PRP) and washed platelets were prepared, as described.²⁶ Washed platelets were suspended into Hepes buffer pH 7.45 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl_2 , 5 mg/ml glucose and 1 mg/ml bovine serum albumin). Final concentration of platelets in plasma or buffer medium was $1 \times 10^8/\text{ml}$, unless indicated otherwise.

Animal studies were approved by the local Animal Experimental Committees. Mice homozygous for a targeted deletion

of the cyclophilin D (*Ppif* gene) were generated, as described.²² The mice were bred on a Sv129 background, and compared to *Ppif*^{+/+} animals of the same breeding. Calpain-1 deficient mice (*Capn1*^{-/-}) were generated, as previously reported.^{27,28} These mice were bred on a C57Bl/6 background and were compared to *Capn1*^{+/+} mice of the same genetic background. Mouse platelets were isolated as described before,²⁹ and suspended in modified Hepes buffer pH 7.45 (5 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 0.42 mM NaH₂PO₄, 5 mg/ml glucose and 1 mg/ml bovine serum albumin). Final concentration was 1 × 10⁸/ml, unless indicated otherwise.

Western Blot Analysis

Washed platelets in (modified) Hepes buffer pH 7.45 were incubated with the calpain inhibitors, calpeptin (200 μM) or MDL28170 (200 μM), or vehicle for 15 min, as desired. Platelets were

stimulated with Cvx (100 ng/ml), Thr (8 nM) or ionomycin (20 μM). Samples (5 × 10⁷ platelets) were taken at indicated time points, and lysed in ice-cold 4× lysis buffer (NaCl 600 mM, Tris 10 mM, EGTA 4 mM, EDTA 4 mM, NP-40 4%). Lysed samples were separated on 8% SDS-PAGE gels, proteins were transferred to PVDF blotting membranes by semi-dry transfer. Immunostaining was with antibodies against talin (1:500), integrin β₃ (Ab762, 1:10000; Ab754, 1:1000), phospho-Tyr⁷⁷⁴ β₃-chain (1:500), or Src (2.5 μg/ml) for 1 h, followed by overnight incubation with horse radish peroxidase-coupled secondary antibody at 4 °C. Stained blots were visualized with an ECL system. Blot quantification was by densitometric analysis, as before.³⁰

Platelet Aggregation and Activation

Light transmission traces, reflecting platelet aggregation, were measured using a Chronolog aggregometer under

MFI ± SD	CD61	CD41a	CD62P	CD42b
Control IgG	336 ± 19	337 ± 19	337 ± 19	337 ± 19
Vehicle	7616 ± 1105	25485 ± 134	766 ± 50	17154 ± 883
Thrombin	17188 ± 1397	45995 ± 344	7282 ± 943	11338 ± 3895
Convulxin	13811 ± 828	38928 ± 3408	5903 ± 255	13987 ± 1491
Thrombin/convulxin	12327 ± 1607	40906 ± 1863	5874 ± 417	18979 ± 469
Ionomycin	8465 ± 340	24235 ± 1586	3148 ± 82	10899 ± 304

Supplemental Figure S1. Glycoprotein expression levels of platelets stimulated with different agonists. Washed human platelets were left unstimulated or were stimulated with convulxin (Cvx, 100 ng/ml) and/or thrombin (Thr, 4 nM) or ionomycin (10 μM) in the presence of 2 mM CaCl₂ for 30 min. Glycoprotein expression levels were examined by flow cytometry after staining with FITC-labeled mAbs. Unstimulated platelets were also incubated with FITC-labeled control IgG. Data are presented as mean fluorescence intensities (MFI) ± S.D. (n = 3).

constant stirring (37 °C). Platelets (1×10^8 /ml) in Hepes buffer containing 2 mM CaCl_2 were stimulated with Thr (4 nM) and/or Cvx (100 ng/ml), or with ionomycin (10 μM); experiments were in the presence or absence of tirofiban (5 $\mu\text{g}/\text{ml}$). Aggregation of platelets was also assessed by single cell count analysis using a Coulter counter (Coulter Electronics).

For flow cytometry, washed human or mouse platelets (1×10^8 /ml) were preincubated with indicated inhibitors or Me_2SO vehicle for 10 min, and stimulated in the presence of 2 mM CaCl_2 with thrombin (4 nM) and/or convulxin (100 ng/ml), or with ionomycin (10 μM). In samples taken after 5-30 min, surface expression of PS was detected with AF647-labeled annexin A5. In addition, activated integrin $\alpha_{\text{IIb}}\beta_3$ was detected using FITC-labeled PAC1 mAb for human platelets or PE-labeled JON/A mAb for mouse platelets. Samples were analyzed with a FACScan flow cytometer (BD Accuri Cytometers).

Platelet samples were fixed and stained for transmission electron microscopy, as described before.³⁰ Calpain activity in platelets was assessed using a calpain activity assay kit according to the manufacturer's instructions (Abcam, Cambridge, MA). Results are expressed as relative fluorescence units per mg lysate protein.

Thrombus Formation on Collagen under Flow

Collagen-induced thrombus formation was assayed, as described before.^{20,31} In brief, PPACK/fragmin-anticoagulated mouse blood was flowed over a coverslip coated with collagen type-I in a transparent parallel-plate perfusion chamber, at shear rate of 1000 s^{-1} for 4 min. Thrombi formed on the collagen surface were post-stained with AF647-annexin A5 and PE-JON/A mAb in modified Hepes buffer pH 7.45, containing CaCl_2 (2 mM) and heparin (1 U/ml). Phase-contrast and fluorescence images were captured for analysis of surface area coverage of adherent platelets, and of platelets with active integrins or exposed PS.³² Image analysis was performed with Metamorph software Version 7.5.0.0 (MDS Analytical Technologies).

Platelet Ca^{2+} Responses

Cytosolic Ca^{2+} was measured in human platelets, preloaded with fluorescent Fura-2 acetoxymethyl ester (2.5 μM) during 45 min at ambient temperature under gentle rotation.³³ Washed platelets were stimulated, while recording changes in fluorescence by calibrated ratio fluorometry. Calcium responses were expressed as time-integrals over baseline $[\text{Ca}^{2+}]_i$.

Supplemental Figure S1. Transmission electron microscopic images of unstimulated and various types of stimulated platelets. Washed human platelets were left unstimulated or were stimulated with thrombin (4 nM) or ionomycin (10 μ M) in the presence of 2 mM CaCl_2 for 30 min (no stirring to prevent aggregation). Platelets were then fixed, pelleted and prepared for transmission electron microscopic observation, as described before (Cosemans, *J. Thromb. Haemost.* 2010; 8:1797). Shown are representative images (bars = 2 μ m).

Statistics

Significance of differences between control and experimental groups as well as changes between groups over time were determined by 1-way or 2-way analysis of variance followed by a Bonferroni post-hoc test. A Student's t-test was performed to compare paired samples. Data are expressed as means $\pm$ S.D. *P* values <0.05 were considered to be significant.

Results

Closure of Activated Integrin $\alpha_{IIb}\beta_3$ in PS-Exposing Platelets

Earlier work demonstrated the appearance of two distinct populations of platelets during thrombus formation, *i.e.* co-aggregated platelets with activated $\alpha_{IIb}\beta_3$ integrins (binding PAC1 mAb) and loosely attached platelets showing PS exposure (binding

coagulation factors Va and Xa and annexin A5).^{3,20} The impaired adhesion of PS-exposing platelets seemingly contrasts to the observation that, in platelet suspensions, the Ca^{2+} -ionophore ionomycin (causing full PS exposure) produces changes in light transmission that are suggestive of platelet aggregation.^{34,35}

We reinvestigated this by first stimulating washed human platelets with Thr (4 nM) or Cvx (100 ng/ml). As shown in Fig. 1A, this resulted in a rapid increase of light transmission, which was almost fully suppressed by the $\alpha_{IIb}\beta_3$ antagonist tirofiban (inhibition at 25 min $87 \pm 3\%$ and $72 \pm 4\%$, respectively; mean $\pm$ S.D., $n=4$). Integrin-dependent platelet aggregation with Thr or Cvx was confirmed by a major reduction in single platelet count (Fig. 1B).

Figure 2. Intracellular cleavage of β_3 chain, talin and Src after platelet stimulation with Cvx/Thr or ionomycin. Platelets were left unstimulated or stimulated with Cvx/Thr or ionomycin, as described for Fig. 1. Samples (5×10^7 platelets) were lysed after 5 or 30 min, and subjected to electrophoresis and Western blotting. Blots were stained for: (A) full-length β_3 -chain with Ab762, (B) calpain-cleaved β_3 -chain with Ab754, anti-talin mAb (C), or anti-Src mAb (D). Shown are representative blots and bar graphs of densitometric analysis of stained bands, corrected for loading control (anti-tubulin mAb). Means $\pm$ S.D. ($n = 3-4$), * $p < 0.05$ (1-way ANOVA, Bonferroni correction).

Supplemental Figure S2. Effect of platelet concentration on appearance of platelet population dual labeled with PAC1 mAb and annexin A5. Washed human platelets at indicated concentrations were stimulated for 5-30 min with convulxin (100 ng/ml) plus thrombin (4 nM) in the presence of 2 mM CaCl₂ with or without the Rho kinase inhibitor HA-1077 (40 μM). Two-color flow cytometry was performed after labeling with FITC-PAC1 mAb and AF647-annexin A5. Shown are dot plots of FL1 (FITC-PAC1 mAb) versus FL4 fluorescence (AF647-annexin A5), representative of at least 3 experiments.

On the other hand, the combined application of Cvx/Thr or addition of ionomycin (10 μM) provoked similar rapid increases in light transmission (Fig. 1A). However, the Cvx/Thr-induced light transmission increase was only partly suppressed with tirofiban (inhibition 60 ± 7% at 25 min). In comparison, tirofiban did not affect the light transmission increase induced by ionomycin (-9 ± 8%). Platelet lysis could be excluded, because

single cell count analysis indicated that ionomycin treatment resulted in a platelet count that remained at 90% of the original count (Fig. 1B). Electron microscopic analysis indicated that, unlike the pseudopod-containing platelets formed by thrombin, ionomycin treatment resulted in single, rounded platelets with a translucent appearance (supplemental Fig. S1). Together, these data indicate that the pseudo

Figure 3. Role of calpain in cleavage of β_3 chain, talin and Src induced by Cvx/Thr or ionomycin. Platelets were stimulated as described for Fig. 2, but preincubated for 10 min with either vehicle or calpain inhibitors, calpeptin (200 μ M) or MDL-28170 (200 μ M). Western blots from protein-separated lysates were stained for: (A) full-length β_3 -chain with Ab762, (B) calpain-cleaved β_3 -chain with Ab754, anti-talin mAb (C), or anti-Src mAb (D). Shown are representative blots and bar graphs of densitometric analysis of stained protein bands, corrected for loading control (anti-tubulin mAb). Means $\pm$ S.D. ($n = 4$), $*p < 0.05$ (1-way ANOVA, Bonferroni correction).

aggregation observed after Cvx/Thr or ionomycin stimulation is a consequence of the morphological change with a rounded and translucent platelet structure, reducing light transmission, rather than a consequence of integrin-dependent platelet aggregate formation. Absence of integrin activation with these agonists was further demonstrated by dual color flow cytometry, using FITC-PAC1 mAb and AF647-annexin A5, probing activated $\alpha_{IIb}\beta_3$ and PS exposure, respectively. Upon stimulation with Cvx/Thr, initially a population of platelets only binding PAC1 mAb was formed, which was gradually replaced by a population of PAC1 negative platelets which only bound annexin A5 (Fig. 1C,D). Within the time interval of 5 to 30 min, $40 \pm 12\%$ ($n = 4$) of platelets lost the capability to bind PAC1 mAb. Similar results were found when fluorescent lactadherin was used instead of annexin A5 (data not shown). Integrin closure could not be explained by a decrease in expression levels of α_{IIb} and β_3 chains,

since these levels were even increased after stimulation with Thr, Cvx or Cvx/Thr. This increase in expression levels is explained by an increase membrane surface due to granule secretion, as detected by CD62P expression (supplemental Table S1). In response to ionomycin ($10 \mu\text{M}$), the majority of platelets bound annexin A5 but not PAC1 mAb (Fig. 1E,F). However, at low ionomycin doses ($<5 \mu\text{M}$), transient PAC1 mAb binding was detected, accompanied by a corresponding reduction in single platelet count (data not shown). Expression levels of α_{IIb} and β_3 remained unchanged after high ionomycin stimulation (supplemental Table S1), thus indicating that this treatment precludes or antagonizes integrin activation. Increased expression levels of α_{IIb} and β_3 were not observed, which was explained by a diminished secretion after ionomycin stimulation in comparison to Cvx/Thr stimulation.

Supplemental Figure S3. Absence of integrin β_3 -chain phosphorylation at Tyr⁷⁷³ in platelets stimulated with agonists causing PS exposure. Washed human platelets were left unstimulated or were stimulated with thrombin (4 nM) and/or convulxin (100 ng/ml) or ionomycin ($10 \mu\text{M}$) in the presence of 2 mM CaCl_2 , as indicated. Western blots of gel-separated samples, taken after 5 or 30 min, were immuno-stained for phospho-Tyr⁷⁷³ β_3 chain or for tubulin as a loading control. Shown are representative Western blots.

Taken together, these results suggest that PS exposure induced by these strong agents is accompanied by either closure of activated integrin or by lack of integrin activation, which prevents the formation of large platelet aggregates.

Interestingly, Fig. 1C-D points to a small population of Cvx/Thr-stimulated platelets, capable to bind both PAC1 mAb and annexin A5. Flow-cytometric studies were performed to characterize

this dual-labeled platelet population. It remained unchanged in size upon stimulation with Cvx/SFLLRN (thrombin receptor-activating peptide) or in the presence of transglutaminase inhibitor, cadaverine (data not shown). This population for the major part (<4%) did not exhibit high fibrinogen binding. Hence, these platelets could not be identified as 'coated', *i.e.* containing a transglutaminase-dependent fibrin coat.

Other experiments showed that the population of dual-labeled platelets reduced in size, when incubations were performed at lower platelet count ($\leq 1 \times 10^8/\text{ml}$), or contained a Rho kinase inhibitor, which antagonizes platelet contraction (supplemental Fig. S2). It was thus concluded that it consisted of micro-aggregates of – perhaps contracted – platelets with either active integrins or exposed PS.

Role of Calpain-Mediated Protein Cleavage in Integrin $\alpha_{IIb}\beta_3$ Closure

Considering the described role of calpain in $\alpha_{IIb}\beta_3$ -dependent cleavage of cytoskeletal-associated proteins,²⁵ we examined whether relevant targets of

this protease were cleaved under conditions of PS exposure. Western blot analysis was performed of gel-separated platelet lysates, using antibodies against the full length intracellular β_3 chain (Ab762) and against a calpain cleavage site in the β_3 chain at residue 754 (Ab754).¹⁵ Blots were also analyzed for the degradation of talin-1 and Src kinase, using suitable antibodies. After 5 min of stimulation with ionomycin, we noted major cleavage of the β_3 chain, talin and Src into smaller protein fragments (Fig. 2A-D). After stimulation with Cvx/Thr, cleavage of the β_3 chain, talin and Src required ~ 20 min to complete. Flow cytometry experiments, performed in parallel samples, confirmed the presence

Supplemental Figure S4. Unchanged intracellular cleavage of integrin β_3 chain in activated calpain-1 deficient platelets. Platelets from *Capn1*^{-/-} and *Capn1*^{+/+} mice were left unstimulated or were stimulated with convulxin (Cvx, 100 ng/ml) and/or thrombin (Thr, 4 nM) or ionomycin (10 μM) for 5-30 min. Western blots of samples taken at indicated time points were stained for full-length integrin β_3 chain using Ab762 (A), or for cytosolic cleaved β_3 -chain using Ab754 (B). Staining for α -tubulin was used as loading control. Shown are representative Western blots and bar graphs of densitometric analysis of stained protein bands, normalized for the loading control. Means $\pm$ S.D. ($n = 3-4$).

of PS exposure and absence of integrin activation with ionomycin and a gradual PS exposure and integrin closure with Cvx/Thr (compare Fig. 1). Together, these results suggested that phospholipid scrambling is one of the mechanisms contributing to intracellular integrin cleavage and inability to activation.

Considering that calpain may regulate tyrosine phosphorylation of the β_3 chain,²⁷ we checked how platelet stimulation with Cvx/Thr or ionomycin influenced the β_3 phosphorylation at Tyr⁷³³, *i.e.* a phosphorylation site indicative for $\alpha_{IIb}\beta_3$ outside-in signaling.³⁶ Whereas platelet stimulation with Thr alone resulted in persistent Tyr⁷⁷³ phosphorylation, stimulation with Cvx/Thr resulted in a loss of phosphorylation at 30 min, whereas ionomycin did not give any phosphorylation at all (supplementary Fig. S3). Hence, this phosphorylation site appears to be lost in platelets stimulated with the PS-exposing agonists.

To investigate a functional role of calpain activity in this protein degradation, two structurally different pharmacological inhibitors were used, *i.e.* calpeptin and MDL-28170, both of which are established inhibitors of Ca²⁺-dependent proteases.²⁸ Either compound fully inhibited the degradation of β_3 chain, talin and Src in platelets that were stimulated with Cvx/Thr or ionomycin

(Fig. 3A-D).

We then determined effects of these inhibitors on the process of integrin inactivation. In the time frame of 5 to 30 min, calpeptin as well as MDL-28170 significantly, but incompletely affected the integrin closure in response to Cvx/Thr (Fig. 4A,B). Measurement of calpain activity indicated that both calpeptin and MDL-28170 nearly completely blocked this proteolytic activity in the stimulated platelets (Fig. 4C). Neither of the inhibitors affected initial PS exposure ($78 \pm 13\%$ and $107 \pm 12\%$ of control, respectively). In Fura-2-loaded platelets, neither inhibitor changed the Cvx/Thr-induced intracellular Ca²⁺ rises (Fig. 4D).

Subsequent experiments were performed with mice lacking the major Ca²⁺-dependent cysteine protease, calpain-1 (μ -calpain, *Capn1*^{-/-} gene), that accounts for ~80% of the calpain activity in mouse and human platelets.³⁷ Assessment of collagen-dependent thrombus formation indicated that the *Capn1*^{-/-} blood formed enlarged thrombi, which displayed increased integrin $\alpha_{IIb}\beta_3$ activation (JON/A mAb binding), but decreased PS exposure (AF647-annexin A5 binding) in comparison to wild type *Capn1*^{+/+} blood (Fig. 5A). This suggests a negative role of calpain-1 in murine $\alpha_{IIb}\beta_3$ activation under these conditions. In platelet suspensions stimulated with Cvx/Thr, *Capn1*^{-/-} and *Capn1*^{+/+} platelets

Figure 5. Unchanged agonist-induced PS exposure and $\alpha_{IIb}\beta_3$ closure in calpain-1 deficient platelets. (A) Blood from *Capn1*^{+/+} or *Capn1*^{-/-} mice was perfused over collagen for 4 min at 1000 s⁻¹. Thrombi were labeled with PE-JON/A mAb and AF647-annexin A5. Shown are representative brightfield and fluorescence images (bars = 20 μ m), as well as quantitative analyses of (fluorescent) platelet deposition. (B-E) Washed mouse platelets were stimulated with Cvx/Thr for 5-30 min, and analyzed by flow cytometry. Shown are percentages of platelets binding PE-JON/A (B) or AF647-annexin A5 (C). Platelets were pretreated with vehicle or MDL-28170 (200 μ M), as indicated. Treatment effects are shown of time-dependent changes in $\alpha_{IIb}\beta_3$ closure (D) or PS exposure (E). Means $\pm$ S.D. ($n = 3-4$), * $p < 0.05$ vs. vehicle (2-way ANOVA, Bonferroni correction).

displayed similar $\alpha_{IIb}\beta_3$ activation and closure, while also PS exposure was comparable (Fig. 5B,C). Treatment of *Capn1*^{-/-} and *Capn1*^{+/+} platelets with MDL-28170 partly suppressed integrin closure, but did not result in a significant decrease in PS exposure (Fig. 5D,E).

After stimulation with Cvx/Thr or ionomycin, the knockout and wild type platelets gave similar cleavage patterns of the integrin β_3 chain (supplemental Fig. S4). Furthermore, total calpain inhibition with MDL-28170 fully blocked the cleavage of β_3 -chain in *Capn1*^{+/+} and *Capn1*^{-/-} platelets (supplemental Fig. S5). In conclusion, while the pharmacological data suggest a partial role for calpain activity in the process of integrin $\alpha_{IIb}\beta_3$ closure of PS-exposing mouse and human platelets, calpain-1 (at least in mice) does not appear to be the main β_3 -chain/talin-degrading protease or target of the calpain inhibitors.

Role of Phospholipid Scrambling in Integrin $\alpha_{IIb}\beta_3$ Closure

Inactivation of integrin $\alpha_{IIb}\beta_3$ under conditions of PS exposure may point to a causal link between $\alpha_{IIb}\beta_3$ closure and scrambling of membrane phospholipids. This possibility was investigated using platelets from a Scott syndrome patient lacking the transmembrane protein TMEM16F, whose platelets are impaired in phospholipid scrambling in response to Cvx/Thr or Ionomycin.³⁸ Defective PS

exposure in response to Cvx/Thr in the patient's platelets was accompanied by a more persistent integrin $\alpha_{IIb}\beta_3$ activation (Fig. 6A,B). In the patient, ionomycin caused partial and transient $\alpha_{IIb}\beta_3$ activation, indicating that a mechanism of integrin closure was still operating. Interestingly, treatment of the Scott platelets with MDL-28170 fully blocked the time-dependent closure of integrin $\alpha_{IIb}\beta_3$, in contrast to the partial blockage observed in control platelets (Fig. 6C). Western blots indicated that the cleavage pattern of the β_3 -chain was not affected in Scott platelets after stimulation with ionomycin (Fig. 7) or Cvx/Thr (not shown). Furthermore, the cleavage was fully inhibited by MDL-21870. Together, these results suggest a dual mechanism of integrin $\alpha_{IIb}\beta_3$ closure in procoagulant platelets partly via TMEM16F-dependent scrambling of the plasma membrane phospholipids, and partly via calpain-dependent cleavage of intracellular proteins among which the integrin β_3 chain.

Involvement of Mitochondrial Transition Pore Formation in $\alpha_{IIb}\beta_3$ Closure

Considering that mPTP formation plays an important role in Cvx/Thr-stimulated PS exposure in platelets,²² we investigated whether this mitochondrial process is also involved in integrin $\alpha_{IIb}\beta_3$ closure. Experiments were performed with platelets harvested from mice

Supplemental Figure S5. Calpain and mPTP inhibitors suppress cleavage of integrin β_3 chain in activated calpain-1 deficient platelets. Platelets from *Capn1*^{+/+} and *Capn1*^{-/-} mice were left unstimulated or were stimulated for 30 min with convulxin (Cvx, 100 ng/ml) and thrombin (Thr, 4 nM) or ionomycin (10 μ M) for 30 min in the presence or absence of vehicle (DMSO), MDL-28170 (200 μ M) or cyclosporin A (5 μ M). Western blots of samples taken at indicated time points were stained for full-length integrin β_3 chain using Ab762 (A), or for cytosolic cleaved β_3 -chain using Ab754 (B). Staining for α -tubulin was used as loading control. Shown are representative Western blots and bar graphs of densitometric analysis of stained protein bands, normalized for the loading control. Means $\pm$ S.D. ($n = 3$).

deficient in cyclophilin D (*Ppif* gene), which forms part of the mPTP. Hence, *Ppif*^{-/-} mouse platelets allow evaluating integrin closure in a second model, next to Scott platelets, in which PS exposure is blunted. Perfusion of *Ppif*^{-/-} blood over collagen resulted in decreased platelet deposition and PS exposure (AF647-annexin A5 binding), when compared to wild type *Ppif*^{+/+} blood (Fig. 8A). Also in suspensions of *Ppif*^{-/-} platelets stimulated with Cvx/Thr, PS exposure was markedly reduced, while $\alpha_{IIb}\beta_3$ activation and closure were suppressed in a similar way (Fig. 8B). Treatment of wild type *Ppif*^{+/+} platelets with cyclosporin A, a compound

known to block mPTP formation,³⁹ suppressed PS exposure and integrin closure to the level observed in the knockout platelets, whereas cyclosporin A treatment of *Ppif*^{-/-} platelets was without any effect. Intracellular Ca^{2+} rises were not affected in *Ppif*^{-/-} platelets as compared to controls (unpublished observation, Jobe *et al.* 2012).

Similar experiments were performed with human platelets treated with cyclosporin A. Again, cyclosporin A suppressed PS exposure as well as integrin closure in response to Cvx/Thr (Fig. 8C). In Fura-2-loaded platelets, cyclosporin A did not affect Cvx/Thr-

Figure 6. Partial role of TMEM16F in agonist-induced PS exposure and $\alpha_{IIb}\beta_3$ closure. Platelets from control subjects or a Scott patient (deficient in TMEM16F) were stimulated with Cvx/Thr (100 ng/ml, 4 nM) or ionomycin (19 μ M) for 5-30 min, as in Fig. 1. Samples were analyzed by flow cytometry for PS exposure (AF647-annexin A5 binding) or $\alpha_{IIb}\beta_3$ activation (FITC-PAC1 binding), as indicated. (A) Shown are percentages of PS-exposing platelets and increase in PS exposure between 5-30 min; (B) Also, percentages of PAC1-binding platelets and percentages of $\alpha_{IIb}\beta_3$ closure between 5-30 min. (C) Effect of platelet treatment with MDL-28170 (200 μ M) on PAC1-binding and $\alpha_{IIb}\beta_3$ closure. Means $\pm$ S.D. ($n = 4$), * $p < 0.05$ vs. vehicle (2-way ANOVA, Bonferroni correction).

Figure 7. Unchanged agonist-induced cleavage of β_3 chain in the absence of TMEM16F. Platelets from the Scott patient (deficient in TMEM16F) were stimulated with ionomycin for 5-30 min, as in Fig. 1. Western blots from samples were stained for full-length β_3 -chain (Ab762) or calpain-cleaved β_3 -chain (Ab754). Blots were reprobed with anti-tubulin mAb as loading control. Shown are representative blots.

induced intracellular Ca^{2+} rises (data not shown). In addition, Src cleavage observed after prolonged Cvx/Thr stimulation was prevented by cyclosporin A treatment (data not shown). Together, these observations point to a central role of mPTP formation in PS exposure and integrin closure.

Discussion

In this study, we demonstrate a dual mechanism responsible for integrin $\alpha_{IIb}\beta_3$ closure (inactivation) in PS-exposing platelets being dependent on Ca^{2+} and calpain activity (but not calpain-1), and relying on Ca^{2+} -dependent phospholipid scrambling mediated by the transmembrane protein TMEM16F. The calpain-dependent proteolysis under conditions of PS exposure involves intracellular cleavage of the integrin β_3 chain, talin and Src kinase. These findings implicate that PS-exposing, procoagulant platelets lose not only their capability of integrin-dependent adhesion and aggregate formation, but also the

competence for integrin-dependent outside-in signaling and downstream responses including formation of tight platelet-platelet contacts. This model provides an explanation for the loose association of PS-exposing platelets in a thrombus, and begins to outline a basis for bound coagulation factors and thrombin generation. The recognized mechanisms of integrin closure or inability to activation thus form the basis of earlier recognized heterogeneity of platelets in thrombus formation.^{5,20}

High and sustained elevation in cytosolic Ca^{2+} , a requirement for PS exposure,⁴⁰ appears to be required for achieving optimal integrin closure, most likely calpain activity and TMEM16F. Mouse platelets deficient in calpain-1 showed a moderate increase in integrin activation and reduced PS exposure upon collagen-dependent thrombus formation, which is consistent with a moderate reduction in $\alpha_{IIb}\beta_3$ closure under these mild activation conditions. This result seemingly contrasts with earlier findings that

Figure 8. Partial role of mPTP formation in agonist-induced PS exposure and $\alpha_{IIb}\beta_3$ closure. (A) Blood from *Ppif*^{+/+} or *Ppif*^{-/-} mice was perfused over collagen for 4 min at 1000 s⁻¹. Thrombi were labeled with PE-JON/A mAb and AF647-annexin A5. Shown are representative brightfield and fluorescence images (bars = 20 μ m), as well as quantitative analyses of (fluorescent) platelet deposition. (B,C) Washed mouse platelets were pretreated with vehicle (0.2% Me₂SO) or cyclosporin A (4 μ M), stimulated for 5-30 min with Cvx/Thr, and analyzed by flow cytometry. Bars indicate percentages of platelets binding PE-JON/A (B) or AF647-annexin A5 (C). Means $\pm$ S.D. (n = 3-4), * p < 0.05 (2-way ANOVA, Bonferroni correction). (D,E) Human platelets, preincubated with vehicle (0.2% Me₂SO) or cyclosporin A (4 μ M), were stimulated for 5-30 min with Cvx/Thr. Shown are time-dependent changes in percentages of platelets binding FITC-PAC1 (D) or AF647-annexin A5 (E). Means $\pm$ S.D. (n = 4), * p < 0.05 (1-way ANOVA, Bonferroni correction).

Capn1^{-/-} platelets show reduced aggregation at low thrombin concentrations,²⁷ but stipulates that a direct role of calpain-1 in integrin activation remains subtle. In contrast, we found that the Capn1^{-/-} platelets show nearly normal integrin closure as well as cleavage of β_3 , talin and Src, when stimulated with strong agonists such as Cvx/Thr. Given the complete abolishment of protein cleavage with two calpain inhibitors (calpeptin and MDL-28170), we concluded that another cysteine protease is involved under conditions of high Ca²⁺ concentration. It is likely that this protease is calpain-2, which accounts for ~20% of the platelet calpain activity, and is known to require millimolar Ca²⁺ concentrations for activation.⁴¹ Given the heterogeneity in intracellular Ca²⁺ concentrations these high levels can be reached locally in procoagulant platelets. Unfortunately, a role for calpain-2 could not be directly tested in platelets as calpain-2 deficiency in mice is lethal.⁴² The mechanistic reason for this lethality is still unknown, but a general role for this calpain isoform in cell proliferation and cell cycle progression has been suggested.

Regarding the integrin closure of Cvx/Thr-stimulated platelets, inhibitor studies demonstrated clear, but partial, inhibitory effects of MDL-28170 and calpeptin. In suspensions of platelets where micro-aggregate formation was

prevented, both compounds suppressed integrin closure of control platelets by ~50% (under conditions where the cleavage of β_3 chain, talin and Src were blocked); whereas it fully abrogated the partly impaired and delayed integrin closure in Scott syndrome platelets, which do not show PS exposure. This clearly supports a model of dual mechanism of integrin closure that is in part calpain-dependent (likely through the degradation of proteins implicated in $\alpha_{IIb}\beta_3$ activation), and in part mediated by TMEM16F (via Ca²⁺-mediated phospholipid scrambling). The latter pathway is absent in the Scott syndrome platelets. It is conceivable that the profound intra-membrane changes caused by phospholipid scrambling affect interactions of the integrin α_{IIb} and β_3 chains or of integrin-associated proteins even in the absence of calpain cleavage. However, the precise mechanism remains to be investigated.

While the calpain-mediated cleavage of specific proteins in PS-exposing platelets has been reported before,⁴ we now show that the protease responsible is not calpain-1, but likely calpain-2. Moreover, this cleavage occurs at a much faster and extensive scale than anticipated, including complete degradation of several proteins that are known to play a key role in integrin activation and signaling, *i.e.* the β_3 chain, talin and Src kinase. The eventual result will be that

these PS-exposing platelets (with a prominent role in coagulation) are no longer capable to carry out the Src/Syk- and talin-dependent responses of aggregated platelets using active integrins, including clot retraction and tight platelet-platelet contact formation. Earlier studies have shown a critical role of the mPTP and cyclophilin D in Cvx/Thr-induced but not ionomycin-induced PS externalization.²² Using platelets from *Ppif*^{-/-} mice, this was confirmed in the present study. In whole-blood flow assay over collagen, the cyclophilin D-deficient platelets showed a consistent reduction in thrombus formation and PS exposure. Moreover, the reduced PS exposure was accompanied by a similar reduction in integrin closure, thus suggesting a common, mitochondrial-dependent

denominator for the impairment of PS exposure and $\alpha_{IIb}\beta_3$ inactivation.

Taken together, our findings reveal a novel, dual mechanism of $\alpha_{IIb}\beta_3$ inactivation via calpain (not calpain-1) dependent cleavage of integrin-associated proteins and via TMEM16F-dependent phospholipid scrambling, with an assistant role of mPTP formation.

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References

1. Jackson SP. Arterial thrombosis: insidious, unpredictable and deadly. *Nat. Med.* 2011;**17**:1423-1436.
2. Heemskerk JW, Matthij NJ, Cosemans JM. Platelet-based coagulation: different populations, different functions. *J. Thromb. Haemost.* 2013;**11**:2-11.
3. Munnix IC, Kuijpers MJ, Auger J, Thomassen CM, Panizzi P, van Zandvoort MA, Rosing J, Bock PE, Watson SP, Heemskerk JW. Segregation of platelet aggregatory and procoagulant microdomains in thrombus formation: regulation by transient integrin activation. *Arterioscler. Thromb. Vasc. Biol.* 2007;**27**:2484-2490.
4. Kulkarni S, Jackson SP. Platelet factor XIII and calpain negatively regulate integrin $\alpha_{IIb}\beta_3$ adhesive function and thrombus growth. *J. Biol. Chem.* 2004;**279**:30697-30706.
5. Berny MA, Munnix IC, Auger JM, Schols SE, Cosemans JM, Panizzi P, Bock PE, Watson SP, McCarty OJ, Heemskerk JW. Spatial distribution of factor Xa, thrombin, and fibrin(ogen) on thrombi at venous shear. *Plos One* 2010;**5**:e10415.
6. Cosemans JM, Iserbyt BF, Deckmyn H, Heemskerk JW. Multiple ways to switch platelet integrins on and off. *J. Thromb. Haemost.* 2008;**6**:1253-1261.
7. Shattil SJ, Kim C, Ginsberg MH. The final steps of integrin activation: the end game. *Nat. Rev. Mol. Cell Biol.* 2010;**11**:288-300.
8. Watson SP, Auger JM, McCarty OJ, Pearce AC. GPVI and integrin $\alpha_{IIb}\beta_3$ signaling in platelets. *J. Thromb. Haemost.* 2005;**3**:1752-1762.
9. Ma YQ, Qin J, Plow EF. Platelet integrin $\alpha_{IIb}\beta_3$: activation mechanisms. *J. Thromb. Haemost.* 2007;**5**:1345-1352.

10. Nieswandt B, Varga-Szabo D, Elvers M. Integrins in platelet activation. *J. Thromb. Haemost.* 2009;**7** Suppl 1:206-209.
11. Kim C, Ye F, Hu X, Ginsberg MH. Talin activates integrins by altering the topology of the beta transmembrane domain. *J. Cell Biol.* 2012;**197**:605-611.
12. Shattil SJ, Newman PJ. Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood* 2004;**104**:1606-1615.
13. Arias-Salgado EG, Lizano S, Sarkar S, Brugge JS, Ginsberg MH, Shattil SJ. Src kinase activation by direct interaction with the integrin β cytoplasmic domain. *Proc. Natl. Acad. Sci. USA* 2003;**100**:13298-13302.
14. Yan B, Calderwood DA, Yaspan B, Ginsberg MH. Calpain cleavage promotes talin binding to the β_3 integrin cytoplasmic domain. *J. Biol. Chem.* 2001;**276**:28164-28170.
15. Pfaff M, Du X, Ginsberg M. H. Calpain cleavage of integrin β cytoplasmic domains. *FEBS Lett.* 1999;**460**:17-22.
16. Arachiche A, Kerbiriou-Nabias D, Garcin I, Letellier T, Dachary-Prigent J. Rapid procoagulant phosphatidylserine exposure relies on high cytosolic calcium rather than on mitochondrial depolarization. *Arterioscler. Thromb. Vasc. Biol.* 2009;**29**:1883-1889.
17. Siljander PR, Munnix IC, Smethurst PA, Deckmyn H, Lindhout T, Ouwehand WH, Farndale RW, Heemskerk JW. Platelet receptor interplay regulates collagen-induced thrombus formation in flowing human blood. *Blood* 2004;**103**:1333-1341.
18. Suzuki J, Umeda M, Sims PJ, Nagata S. Calcium-dependent phospholipid scrambling by TMEM16F. *Nature* 2010;**468**:834-838.
19. Van Kruchten R, Mattheij NJ, Saunders C, Feijge MA, Swieringa F, Wolfs JL, Collins PW, Heemskerk JW, Bevers EM. Both TMEM16F-dependent and TMEM16F-independent pathways contribute to phosphatidylserine exposure in platelet apoptosis and platelet activation. *Blood* 2013;**10**:1850-7.
20. Munnix IC, Cosemans JM, Auger JM, Heemskerk JW. Platelet response heterogeneity in thrombus formation. *Thromb. Haemost.* 2009;**102**:1149-1156.
21. Castoldi E, Collins PW, Williamson PL, Bevers EM. Compound heterozygosity for two novel TMEM16F mutations in a patient with Scott syndrome. *Blood* 2011;**117**:4399-4400.
22. Jobe SM, Wilson KM, Leo L, Raimondi A, Molkentin JD, Lentz SR, Di Paola J. Critical role for the mitochondrial permeability transition pore and cyclophilin D in platelet activation and thrombosis. *Blood* 2008;**111**:1257-1265.
23. Dale GL. Coated-platelets: an emergent component of the procoagulant response. *J. Thromb. Haemost.* 2005;**3**:2185-2192.
24. Jobe SM, Leo L, Eastvold JS, Dickneite G, Ratliff TL, Lentz SR, Di Paola J. Role of FcR γ and factor XIIIa in coated platelet formation. *Blood* 2005;**106**:4146-4151.
25. Du X, Saido TC, Tsubuki S, Indig FE, Williams MJ, Ginsberg MH. Calpain cleavage of the cytoplasmic domain of the integrin β_3 subunit. *J. Biol. Chem.* 1995;**270**:26146-26151.
26. Van der Meijden PE, Schoenwaelder SM, Feijge MA, Cosemans JM, Munnix IC, Wetzker R, Heller R, Jackson SP, Heemskerk JW. Dual P2Y₁₂ receptor signaling in thrombin-stimulated platelets: involvement of phosphoinositide 3-kinase β but not γ isoform in Ca²⁺ mobilization and procoagulant activity. *FEBS J.* 2008;**275**:371-385.
27. Kuchay SM, Kim N, Grunz EA, Fay WP, Chishti AH. Double knockouts reveal that protein tyrosine phosphatase 1B is a physiological target of calpain-1 in platelets. *Mol. Cell Biol.* 2007;**27**:6038-6052.
28. Kuchay SM, Wieschhaus AJ, Marinkovic M, Herman IM, Chishti AH. Targeted gene inactivation reveals a functional role of calpain-1 in platelet spreading. *J. Thromb. Haemost.* 2012;**10**:1120-1132.
29. Gilio K, van Kruchten R, Braun A, Berna-Erro A, Feijge MA, Stegner D, van der Meijden PE, Kuijpers MJ, Varga-Szabo D, Heemskerk JW, Nieswandt B. Roles of platelet STIM1 and Orai1 in glycoprotein VI- and thrombin-dependent procoagulant activity and thrombus formation. *J. Biol. Chem.* 2010;**285**:23629-23638.

30. Cosemans JM, Van Kruchten R, Olieslagers S, Schurgers LJ, Verheyen FK, Munnix IC, Waltenberger J, Angelillo-Scherrer A, Hoylaerts MF, Carmeliet P, Heemskerk JW. Potentiating role of Gas6 and Tyro3, Axl and Mer (TAM) receptors in human and murine platelet activation and thrombus stabilization. *J. Thromb. Haemost.* 2010;**8**:1797-1808.
31. Gilio K, Harper MT, Cosemans JM, Konopatskaya O, Munnix IC, Prinzen L, Leitges M, Liu Q, Molkentin JD, Heemskerk JW, Poole AW. Functional divergence of platelet protein kinase C (PKC) isoforms in thrombus formation on collagen. *J. Biol. Chem.* 2010;**285**:23410-23419.
32. Cosemans JM, Schols SE, Stefanini L, de Witt S, Feijge MA, Hamulyak K, Deckmyn H, Bergmeier W, Heemskerk JW. Key role of glycoprotein Ib/V/IX and von Willebrand factor in platelet activation-dependent fibrin formation at low shear flow. *Blood* 2011;**117**:651-660.
33. Heemskerk JW, Feijge MA, Henneman L, Rosing J, Hemker HC. The Ca^{2+} -mobilizing potency of α -thrombin and thrombin-receptor-activating peptide on human platelets: concentration and time effects of thrombin-induced Ca^{2+} signaling. *Eur. J. Biochem.* 1997;**249**:547-555.
34. Wolfs JL, Comfurius P, Rasmussen JT, Keuren JF, Lindhout T, Zwaal RF, Bevers EM. Activated scramblase and inhibited aminophospholipid translocase cause phosphatidylserine exposure in a distinct platelet fraction. *Cell Mol. Life Sci.* 2005;**62**:1514-1525.
35. Fuse I, Higuchi W, Uesugi Y, Aizawa Y. Pathogenetic analysis of three cases with a bleeding disorder characterized by defective platelet aggregation induced by Ca^{2+} ionophores. *Br. J. Haematol.* 2001;**112**:603-608.
36. Naik MU, Stalker TJ, Brass LF, Naik UP. JAM-A protects from thrombosis by suppressing integrin $\alpha_{\text{IIb}}\beta_3$ -dependent outside-in signaling in platelets. *Blood* 2012;**119**:3352-3360.
37. Kuchay SM, Chishti AH. Calpain-mediated regulation of platelet signaling pathways. *Curr. Opin. Hematol.* 2007;**14**:249-254.
38. Munnix IC, Harmsma M, Giddings JC, Collins PW, Feijge MA, Comfurius P, Heemskerk JW, Bevers EM. Store-mediated calcium entry in the regulation of phosphatidylserine exposure in blood cells from Scott patients. *Thromb. Haemost.* 2003;**89**:687-695.
39. Leytin V, Allen DJ, Mutlu A, Gyulkhandanyan AV, Mykhaylov S, Freedman J. Mitochondrial control of platelet apoptosis: effect of cyclosporin A, an inhibitor of the mitochondrial permeability transition pore. *Lab. Invest.* 2009;**89**:374-384.
40. Heemskerk JW, Bevers EM, Lindhout T. Platelet activation and blood coagulation. *Thromb. Haemost.* 2002;**88**:186-193.
41. Goll DE, Thompson VF, Li H, Wei W, Cong J. The calpain system. *Physiol. Rev.* 2003;**83**:731-801.
42. Dutt P, Croall DE, Arthur JS, Veyra TD, Williams K, Elce JS, Greer PA. m-Calpain is required for preimplantation embryonic development in mice. *BMC Dev. Biol.* 2006; **6**:3.

Chapter 5

Coated platelets are a subpopulation of phosphatidylserine-exposing platelets and function in platelet-dependent fibrin formation via factor XIII and integrin $\alpha_{IIb}\beta_3$

Mattheij NJ¹, Swieringa F¹, Berny-Lang MA², May F³, van der Meijden PEJ¹, Suylen DPL¹, Nolte MW³, Hackeng TM¹, McCarty OJ², Heemskerk JWM¹, Cosemans JMEM¹

¹Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands; ²Department of Biomedical Engineering, Oregon Health & Science University, Portland OR, USA;

³CSL Behring GmbH, Marburg, Germany

Resubmitted

Abstract

Coated platelets, formed by collagen (convulxin) and thrombin activation, have been characterized in different ways, such as by the formation of a protein coat of α -granular proteins or by exposure of procoagulant phosphatidylserine. Accordingly, their functional role has remained unclear. Here, we used a novel transglutaminase probe, Rhod-A14, to identify subpopulations of platelets with a cross-linked protein coat, and compared these with other platelet subpopulations. Platelet stimulation with convulxin/thrombin resulted in initial integrin $\alpha_{IIb}\beta_3$ activation, the appearance of a platelet population with high fibrinogen binding - independently of active integrins -, followed by phosphatidylserine exposure and binding of factors Va and Xa. Transglutaminase-active platelets, detected with Rhod-A14, formed as a subpopulation of the phosphatidylserine-exposing platelets. The high fibrin(ogen) binding relied on the presence of thrombin. This staining pattern was confirmed in tissue-factor triggered platelet-rich plasma. The high fibrin(ogen) and Rhod-A14 binding were antagonized by combined inhibition of transglutaminase activity and $\alpha_{IIb}\beta_3$. In thrombi formed on collagen, Rhod-A14 again bound to a subpopulation of phosphatidylserine-exposing platelets via transglutaminase activity and later, under coagulant conditions, to fibrin fibers. Markedly, in thrombi from mice deficient in factor XIII, but not in wild-type thrombi, platelet-driven fibrin formation and Rhod-A14 binding were abolished by blockage of integrin $\alpha_{IIb}\beta_3$. In conclusion, transglutaminase-active 'coated' platelets, with initial $\alpha_{IIb}\beta_3$ activation and high fibrinogen binding, form a subpopulation of phosphatidylserine-exposing platelets, and function in platelet-dependent fibrin fiber formation via factor XIII and integrin $\alpha_{IIb}\beta_3$.

Introduction

Platelet activation and blood coagulation are highly reciprocally interacting processes and both essential for thrombosis and hemostasis. Activated platelets support and steer the coagulation process by at least four mechanisms, *i.e.* by release of coagulation factors like factor V and XIII; by exposing the procoagulant phospholipid phosphatidylserine (PS) at

their outer surface to support thrombin generation; by providing a scaffold for the formation of fibrin fibers; and by causing retraction of the fibrin clot.^{1,2} In a growing thrombus, the aggregated and single, procoagulant platelets form two distinct populations,^{3,4} which is at least partly explained by the high Ca^{2+} response required for PS exposure and coagulation factor binding, and by the calpain-dependent closure of active

$\alpha_{IIb}\beta_3$ integrins after PS exposure, thus antagonizing inclusion into a platelet aggregate.^{5,6}

However, also another platelet class has been identified, usually referred to as coated platelets,⁷ which may partly overlap with the two other platelet populations.³ In the initial paper, Dale and co-workers describe COAT platelets (later renamed as coated platelets) as a population of platelets arising after combined stimulation with collagen and thrombin, which bind α -granule proteins, including factor V, fibrinogen, von Willebrand factor, thrombospondin, fibronectin and α_2 -antiplasmin, in a transglutaminase-dependent way via the formation of covalent serotonin conjugations.⁸

Since the first description, coated platelets are invariably considered as platelets formed after combined stimulation of collagen receptors (*e.g.* with collagen, convulxin or collagen-related peptide) and thrombin receptors (*e.g.* with thrombin or thrombin receptor-activating peptides), but the definition of this population may differ from paper to paper. The Dale group has been using as a characteristic the retention on the platelet surface of secreted proteins, including platelet-derived serotonin-derivatized proteins, factor V and tissue factor pathway inhibitor.⁹⁻¹¹ Another definition has been used by the Jobe group, *i.e.* platelets

containing high surface levels of fibrinogen, likely through cross-linking via the transglutaminase, factor XIII.¹² However, in recent years, it is becoming common to consider coated platelets more or less equivalent to PS-exposing platelets, binding the coagulation factors V(a) and Xa. For instance, platelet subpopulations in patient studies have been characterized using the PS-binding probe, annexin A5.¹³⁻¹⁶ This ambiguity in definition and described properties raises the question, if 'coated' platelets form a platelet subpopulation – after collagen/thrombin receptor stimulation – that is distinguishable from that of PS-exposing platelets with perhaps a different function.

In the present paper, we used a specific transglutaminase substrate, *i.e.* the α_2 -antiplasmin-derived peptide Rhod-A14, as a tool to identify transglutaminase-active platelets. We compared the binding of Rhod-A14 to platelets, stimulated via the collagen and thrombin receptors, with other makers of platelet activation. The results indicate that transglutaminase activity partly overlaps but is not identical to PS exposure. We furthermore provide evidence that the transglutaminase activity along with integrin $\alpha_{IIb}\beta_3$ activation is required for fibrin anchoring at the platelet surface and platelet-dependent fibrin formation.

Experimental procedure

Materials

Horm type-I collagen was purchased from Nycomed. The glycoprotein VI agonist, convulxin came from Stago BNL. Human α -thrombin was from Kordia; human factor XIII was from CSL Behring GmbH, and PAR1-activating hexapeptide SFLLRN from Bachem. Annexin A5 labeled with Alexa Fluor-647 (AF647) or

fluorescein isothiocyanate (FITC) came from Invitrogen; FITC-labeled thrombospondin from Abcam and HiLyte-488 (HL488)-labeled fibronectin from Tebu-bio. Active-site OG488-labeled human factor Xa and prothrombin as well as AF488-labeled bovine factor V(a) were prepared, as described.¹⁷ FITC-labeled PAC1 mAb was from Becton Dickinson; FITC-labeled anti-

Figure 1. Transglutaminase activity confined to a subpopulation of PS-exposing platelets. (A) Washed human platelets (5×10^7 /ml) were stimulated with convulxin (Cvx, 100 ng/ml) plus thrombin (Thr, 4 nM) in the presence of 0.2 mM GPRP and 2 mM CaCl_2 . Samples, taken at given time points, were labeled for 5 minutes with Rhod-A14 and AF647-annexin A5. Platelets were pretreated with K9-DON (100 μM), where indicated. Shown are representative histograms obtained by flow cytometry. (B) Platelet subpopulations staining with Rhod-A14 (white bars) or AF647-annexin A5 (black bars) after 60 minutes stimulation with Cvx (100 ng/ml), Thr (4 nM), SFLLRN (15 μM) and/or ionomycin (10 μM), as indicated. (C-E) Effects of factor XIII and transglutaminase inhibitors on platelet subpopulations staining with Rhod-A14 (C) or AF647-annexin A5 (E) after stimulation with Cvx (100 ng/ml) plus Thr (4 nM). Platelets were preincubated with vehicle (○, solid line), 100 unit/ml factor XIII (●, dotted line), 100 μM K9-DON (■, dotted line), or 200 μM Boc-DON (◆, dotted line). Solid grey line, unstimulated platelets. (D) Dose-dependent inhibition of Rhod-A14 binding (60 minutes) by K9-DON or Boc-DON. Mean $\pm$ SEM ($n=3-6$), * $p<0.05$.

integrin β_3 mAb from BD BioScience; FITC-labeled WAK mAb against platelet-bound fibrinogen from WAK Chemie; fibrin-specific mAb (anti-fibrin II β chain, clone T2G1) from Accurate Chemical & Scientific Corporation.

The 14-amino acid transglutaminase peptide substrate, GNQEQVSPLTLK(C-rhodamine)W (Rhod-A14), derived from the N-terminal peptide sequence of α_2 -antiplasmin, was synthesized as described elsewhere.¹⁸ The substrate contains the 14 amino acids of α_2 -antiplasmin, implicated in its conjugation to fibrin.¹⁹ The tissue-type transglutaminase specific inhibitor, *tert*-butyloxycarbonyl-(6-_____diazot-5-oxonorleucyl)-QIV-OMe (Boc-DON) and the general transglutaminase inhibitor, Ac-LGPG-DON-SLVIG-NH₂ (K9-DON) came from Zedira. Other chemicals were from sources described before.²⁰

Blood Collection and Platelet Preparation

Experiments were approved by the local Medical Ethics Committees. Healthy subjects donated blood after full informed consent according to the Helsinki declaration. For coagulation experiments, venous blood was collected into 1/10 volume of 129 mM trisodium citrate. Platelet-rich plasma (PRP) and washed platelets were prepared from acid-citrate-dextrose anticoagulated blood, as described before.²¹ Washed platelets were suspended into Hepes

buffer A (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 5 mg/ml glucose, 1 mg/ml BSA, pH 7.45). Final concentration of platelets in buffer medium was 5×10^7 /ml, unless indicated otherwise.

Animal studies were approved by the local Animal Experimental Committees. Mice deficient in factor XIII A1 subunit (*F13a1*^{tm1Gdi}, abbreviated as *F13a1*^{-/-}) were generated, as previously reported.²² The mice were bred on a mixed 129Ola/CBACa background and were compared to *F13a1*^{+/+} mice of the same backgrounds (Harlan). Murine blood was taken on trisodium citrate for whole-blood flow experiments; other blood samples were taken on acid-citrate-dextrose anticoagulant to isolate washed platelets, as described.²⁰ Isolated mouse platelets were resuspended in modified Hepes buffer B (5 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 0.42 mM NaH₂PO₄, 5 mg/ml glucose, 1 mg/ml BSA, pH 7.45).⁶

Flow Cytometric Platelet Analyses

Washed human or mouse platelets (5×10^7 /ml) were preincubated with indicated inhibitors or Me₂SO vehicle for 10 minutes, and stimulated with thrombin (4 nM) and/or convulxin (100 ng/ml) in the presence of 2 mM CaCl₂. In the activations, 0.2 mM GPRP was added to prevent formation of large fibrin fibres.²¹ Platelet sub-populations were

Supplemental Figure 1. Transglutaminase-positive platelets with inactive $\alpha_{IIb}\beta_3$ integrin appear after high fibrinogen binding. (A) Washed platelets were stimulated with convulxin and thrombin, as indicated for Figure 1. At given time points, samples were labeled for 5 minutes with Rhod-A14 in combination with AF647-annexin A5, FITC-PAC-1 mAb or AF647-fibrinogen. (A, B, C) Representative dot plots at indicated time points. (D) Time courses of fractions of FITC-PAC-1 (■), AF647-fibrinogen (●), AF647-annexin A5 (▼) and Rhod-A14 (▲) positive platelets. Mean $\pm$ SEM (n=3).

distinguished by probing with AF647-annexin A5 (1:200), AF488-factor V (20 nM), OG488-factor Xa (100 nM), AF647-fibrinogen (100 μ g/ml), FITC-thrombospondin (20 μ g/ml) and HL488-fibronectin (50 μ g/ml). After staining for 5 minutes, samples were analyzed with a FACScan flow cytometer (BD Accuri Cytometers).⁶ AF647-annexin A5 and AF647-fibrinogen were measured in the FL-4 channel, Rhod-A14 was measured in the FL-2 channel, while other fluorescent probes were measured in the FL-1 channel. Citrate-anticoagulated human PRP was obtained by centrifuging blood at 240 g for 15 minutes; platelet-free

plasma (PFP) then by centrifuging twice at 2630 g for 10 minutes. Platelet count in PRP was determined with a Thrombocounter (Coulter Electronics), and normalized with autologous PFP. Citrate-anticoagulated human PFP was defibrinated by 30 minutes treatment with anrod protease (1.3 units/ml).²¹ The formed fibrin clots were removed from the plasma by centrifugation. Reconstituted PRP was activated with tissue factor (2 pM, f.c.) and CaCl_2 (16.7 mM) in the presence of GPRP (2 mM) at 37 °C, after which samples were taken for fluorescent labeling. Analysis was by flow cytometry as above.

Figure 2. Transglutaminase-positive platelets are not identical to procoagulant platelets. (A-B) Washed platelets were stimulated with convulxin plus thrombin, as indicated for Figure 1. At given time points, samples were taken and labeled with AF647-fibrinogen (○), OG488-factor Xa (▲), AF647-annexin A5 (●), AF488-factor V(a) (■), or Rhod-A14 (◆). **(A)** Fractions of platelets binding the indicated probes. **(B)** Effect of platelet treatment with K9-DON (100 μ M) (grey bars) on fractions of positive platelets. **(C-D)** Citrate-anticoagulated PRP (1×10^8 platelets/ml) was preincubated with vehicle or K9-DON (100 μ M), and used for measurement of thrombin generation; coagulation was triggered with tissue factor/ CaCl_2 . Representative thrombin generation curves **(C)** and quantification of peak height **(D)**. Mean $\pm$ SEM ($n=3-4$), $*p<0.05$.

Thrombin Generation

Thrombin generation was measured in citrate-anticoagulated human PRP.²³ The PRP was diluted with autologous PFP to a count of 1.5×10^8 platelets/ml. Samples were activated with convulxin (100 ng/ml) plus thrombin (4 nM) or vehicle for 15 minutes. Aliquots (4 volumes) were transferred to a polystyrene 96-wells plate (Immulon 2HB, Dynex Technologies), already containing 1 volume of buffer C (20 mM Hepes, 140 mM NaCl, 5 mg/ml BSA and 6 pM tissue factor). Coagulation was started by

adding 1 volume of buffer D (2.5 mM Z-GGR-AMC, 20 mM Hepes, 140 mM NaCl, 100 mM CaCl_2 and 60 mg/ml BSA). First-derivative curves were converted into curves of nanomolar thrombin concentrations using a calibrator for human α -thrombin.²⁴ All analyses were in triplicate.

Thrombus Formation on Collagen under Flow

Whole blood thrombus formation on collagen was assayed in the absence or presence of coagulation, as described

before.²⁵ In brief, if no coagulation was required, citrate-anticoagulated mouse blood was recalcified in the presence of hirudin (3 μ M) and PPACK (40 μ M). The blood was recalcified and flowed over a coverslip coated with collagen type-I in a transparent parallel-plate perfusion chamber, at wall-shear rate of 1000 s^{-1} for 4 minutes.²⁶ If desired, coagulation was introduced by co-perfusion of mouse blood with 0.5 volume of Ca-Hepes buffer (15 mM $CaCl_2$, 7.5 mM $MgCl_2$ and 0.42 mM NaH_2PO_4 , pH 7.45) at 1000 s^{-1} . Coagulation of human blood was introduced by co-perfusion with 0.1 volume of adapted Ca-Hepes buffer (75 mM $CaCl_2$, 37.5 mM $MgCl_2$, 136 mM NaCl, 5 mM Hepes, 2.7 mM KCl, 1 mg/ml BSA, pH 7.45) at 1000 s^{-1} . Thrombi formed on collagen were post-stained with Rhod-A14 and AF647-annexin A5 in Hepes buffer A or B, supplemented with $CaCl_2$ (2 mM) and heparin (1 unit/ml). Phase-contrast and fluorescence images were captured and analyzed with Metamorph software version 7.5.0.0 (MDS Analytical Technologies), as detailed elsewhere.²⁶ Quantitative statistical co-localization on two-color confocal images was performed using the ZEN software 2010 B SP1.

Statistics

Significance of differences between control and experimental groups as well as changes between groups over time

were determined by 1-way or 2-way analysis of variance followed by a Bonferroni post-hoc test. The Student's t-test was performed to compare paired samples. Distribution of fibrin fibers was evaluated by χ^2 analysis.²⁷ p values <0.05 were considered to be significant.

Results

'Coated' platelets as transglutaminase active platelets

First, we assessed the appearance of a coated platelet population following the initial description of formation of a transglutaminase-dependent protein 'coat' at the platelet surface.⁸ Therefore, we synthesized a fluorescent-labeled 14-amino acid transglutaminase peptide substrate, GNQEQVSPLTLK(C-tetramethylrhodamine)W (Rhod-A14), derived from the N -terminal peptide sequence of α_2 -antiplasmin. This peptide contains a glutamine at Q³, which serves as a transglutamination site for cross-linking to available ϵ -amino lysine residues.¹⁸ The Rhod-A14 peptide was site-specifically labeled with a tetramethylrhodamine group, which did not interfere with its substrate properties. Control experiments indicated that the peptide could act as a substrate for both tissue-type transglutaminase and factor XIIIa (not shown, but see below).

Flow cytometric analysis indicated that the labeled Rhod-A14 bound to a

Supplemental Figure 2. High fibrinogen binding precedes PS exposure independently of transglutaminase activity. (A) Washed platelets were stimulated with convulxin (Cvx) plus thrombin (Thr), as indicated for Figure 1. At given time points, samples were taken and stained with AF647-fibrinogen and FITC-annexin A5. Shown are representative histograms obtained by flow cytometry. (B-C) Staining patterns with AF647-fibrinogen (B) and FITC-annexin A5 (C) after platelet stimulation with Cvx/Thr (100 ng/ml, 4 nM) (○), Cvx/SFLLRN (100 ng/ml, 15 μ M) (■), Thr alone (4 nM) (●) or ionomycin (10 μ M) (▲). (D) Platelets were activated with Cvx/Thr, and pretreated with K9-DON (50 μ M) or Boc-DON (200 μ M), as indicated. Limited inhibiting effect on high fibrinogen binding by K9-DON and Boc-DON. Mean $\pm$ SEM (n=3-6), * p <0.05

population of convulxin/thrombin activated platelets, which was gradually formed after about 15 minutes and increased to about 50% until 60 minutes (Figure 1A). This markedly differed from the appearance of PS-exposing platelets, which was already maximal after 15 minutes. Two-color flow cytometry indicated that the Rhod-A14 positive platelets represented a discrete subpopulation: these platelets were in majority PS positive (binding annexin A5),

but had inactive $\alpha_{IIb}\beta_3$ integrins (no binding of PAC-1 mAb) (Suppl. Figure 1). When comparing various strong platelet agonists, we found that the Ca^{2+} -ionophore ionomycin, whilst causing nearly complete PS exposure, resulted in Rhod-A14 labeling of a subpopulation of $\sim 25\%$ platelets (Figure 1B). Platelet stimulation with convulxin/SFLLRN resulted in a similar Rhod-A14 positive fraction, whereas stimulation with thrombin alone led to neither Rhod-A14

incorporation nor PS exposure.

To further characterize the Rhod-A14 positive population, washed platelets were pretreated with purified factor XIII prior to stimulation with convulxin/thrombin. This resulted in an enhanced appearance of the Rhod-A14 binding cells (Figure 1C). Control experiments confirmed the involvement of transglutaminase activity, since all Rhod-A14 binding was antagonized in a dose-dependent way by the general transglutaminase inhibitor K9-DON and the tissue transglutaminase inhibitor Boc-DON (Figure 1D). In sharp contrast, neither the addition of factor XIII nor

these inhibitors affected the PS exposure evoked by convulxin/thrombin (Figure 1E). Taken together, these results indicate that the transglutaminase-active, Rhod-A14 positive platelets form as a subpopulation of PS-exposing platelets after collagen/thrombin receptor stimulation.

'Coated' platelets as high fibrinogen-binding platelets

Platelet activation with convulxin/thrombin was found to result in a fraction of platelets displaying high fibrinogen binding, which have also been referred to as coated platelets.¹² In our

Figure 3. High fibrinogen binding of platelets is independent of $\alpha_{IIb}\beta_3$ activation and PS exposure in tissue factor-activated plasma. Defibrinated PFP was reconstituted with platelets and triggered with tissue factor (2 pM) and CaCl_2 (16.7 mM). Samples were taken for fluorescent labeling at indicated time points. (A-B) Populations of platelets with high binding of OG488-fibrinogen and binding AF647-annexin A5. Shown are total fractions (A), and sub-fractions (B) of high-fibrinogen and annexin A5 binding platelets. (C-D) Populations of platelets binding FITC-PAC-1 mAb and AF647-annexin A5. Shown are total fractions (C) and sub-fractions (D) of platelets positive for FITC-PAC-1 mAb and AF647-annexin A5. Mean $\pm$ SEM (n=3).

Supplemental Figure 3. Determinants of high fibrinogen binding of platelets in tissue factor-activated plasma. Defibrinated PPP was reconstituted with platelets and activated with tissue factor (2 pM) and CaCl_2 (16.7 mM). Samples were taken for fluorescent labeling after 0 and 60 minutes. Shown are representative dot plots obtained by flow cytometry. (A-B) Labeling with AF646-annexin A5 plus FITC-PAC-1 mAb, (C-D) labeling with AF646-annexin A5 plus either OG488-fibrinogen or (E) FITC-WAK Ab. Mean $\pm$ SEM (n=3-4).

hands, after 5 minutes of stimulation with convulxin/thrombin, the binding of fluorescently labeled fibrinogen reached a maximal high level, which was faster than the appearance of PS-exposing platelets (Suppl. Figure 2A-C). Other strong platelet agonists, ionomycin and convulxin/SFLLRN induced only low fibrinogen binding. However, thrombin alone induced similarly high fibrinogen binding as in the presence of convulxin. Notably, the transglutaminase inhibitors K9-DON or Boc-DON affected neither fibrinogen binding nor PS exposure (Suppl. Figure 2D). Interestingly, the extent of fibrinogen binding per platelets slightly declined upon prolonged incubation with convulxin/thrombin,

whilst the fraction of fibrinogen-positive platelets remained stable. Dual color flow cytometry indicated that Rhod-A14 binding platelet fraction was a subpopulation of the high fibrinogen-binding cells (Suppl. Figure 1C). Furthermore, a considerable fraction of the high fibrinogen-binding platelets also exposes PS, but these fractions were not identical (Suppl. Figure 2A). Hence, the high fibrinogen binding appears to rely on the presence of thrombin, but not on PS exposure.

'Coated' platelets as procoagulant, PS-exposing platelets

In several recent papers, coated platelets have been proposed to be equivalent to

Figure 4. High fibrinogen binding of platelets requires both transglutaminase and $\alpha_{IIb}\beta_3$ activities. Washed platelets were stimulated with convulxin (100 ng/ml) and thrombin (4 nM), as indicated for Figure 1. Preincubation was with vehicle, K9-DON (100 μ M) and/or tirofiban (5 μ g/ml). Flow cytometric data were assessed in the presence of Rhod-A14 and AF647-fibrinogen. Shown are percentages of mean fluorescence intensity of fibrinogen binding (A) and platelets positive for Rhod-A14 (B). Mean $\pm$ SEM (n=3), * p <0.05.

coagulation factor binding platelets or PS- exposing platelets.^{7,13,28} To investigate this in more detail, we compared the fractions of convulxin/thrombin-stimulated platelets that bind AF647-annexin A5, OG488-factor Xa, FITC-factor V or Rhod-A14. As shown in Figure 2A, the appearance of a platelet population with binding sites for annexin A5, factor V and factor Xa occurred fast with similar kinetics, whereas the formation of Rhod-A14-binding platelets was considerably slower. Pre-incubation with transglutaminase inhibitor K9-DON did not influence the binding of annexin A5 nor the coagulation factors, but abolished the binding of Rhod-A14 (Figure 2B). In agreement with this result,

the presence of K9-DON did not influence the process of thrombin generation in PRP (Figure 2C-D). This indicated that the transglutaminase activity of platelets was independent of the interaction of factor Va and Xa, and of subsequent platelet-dependent thrombin generation, which relies on this interaction.²³

'Coated' platelets as fibrin-forming platelets

Earlier work demonstrated that platelet stimulation with strong agonists leads to integrin $\alpha_{IIb}\beta_3$ activation, followed by PS exposure and calpain-dependent closure of the integrin.⁶ Furthermore, we found that PS exposure instigates the formation of a fibrin network linked to the platelet

surface.²⁹ Here, we reinvestigated this by assessing the determinants of high fibrin(ogen) binding and PS exposure of platelets in tissue factor-activated blood plasma. For this purpose, plasma samples were defibrinated to prevent massive clot formation,²¹ and then reconstituted with washed platelets. After triggering coagulation with tissue factor and calcium, the majority of platelets showed rapid and sustained high fibrinogen binding, followed by the appearance of a platelet subpopulation with PS exposure, as determined by dual-

labeling with AF647-annexin A5 and OG488-fibrinogen (Figure 3A). Detailed analysis indicated that only part of the high fibrinogen-binding platelets exposed PS, whereas the majority of PS-exposing platelets showed high fibrinogen binding (Figure 3B). High fibrinogen binding again was dependent of the presence of thrombin, since these platelets stained with an anti-fibrin Ab and the thrombin inhibitor hirudin substantially suppressed fibrinogen binding (data not shown). High fibrinogen binding of a subpopulation of

Supplemental Figure 4. Impaired transglutaminase activity and fibrinogen binding of factor XIII-A deficient platelets. Washed platelets from wild-type 129Ola or CBACa mice or $F13a1^{-/-}$ mice of mixed 129SV/CBA background were stimulated with convulxin (100 ng/ml) and thrombin (4 nM). After 60 minutes, samples were taken and labeled with Rhod-A14 (A), AF647-annexin A5 (B), OG488-factor Xa (C) or AF647-fibrinogen (D). Shown are fractions of platelets binding the indicated probes. Mean $\pm$ SEM (n=3), * p <0.05.

Figure 5. Subpopulation of PS-exposing platelets in thrombi is transglutaminase positive. Human whole blood was perfused over collagen under anti-coagulated (A) or coagulated (B) conditions at shear rate of 1000 s^{-1} . Blood samples were preincubated with vehicle (control) or K9-DON (100 μM). (A-B) Representative microscopic images of differential interference contrast (DIC), and confocal images of AF647-annexin A5 or Rhod-A14 fluorescence (bars, 20 μm). Right bar graphs indicate co-localization coefficient of Chl1 and Chl2 (Chl1-T1), and co-localization coefficient of Chl2 and Chl1 (Chl2-T1), as assessed with Zen software. Mean $\pm$ SEM (n=4), * p <0.05.

the PS-exposing cells was observed not only with labeled fibrinogen, but also with an antibody (FITC-WAK) recognizing platelet-bound fibrin(ogen) (Suppl. Figure 3). This is in agreement with the results with washed platelets described above that, in the presence of thrombin, the high fibrin(ogen) binding is regulated differently from PS exposure.

Staining of the tissue factor-triggered platelets in plasma with FITC-PAC-1 mAb, recognizing activated integrin $\alpha_{\text{IIb}}\beta_3$,

pointed to rapid integrin activation that subsequently declined (Figure 3C). However, when these platelets were stained for the integrin β_3 -chain, fluorescence remained high (not shown), indicating that the integrin was still present and accessible at the platelet surface. In comparison to integrin activation, exposure of PS rose slower in time (Figure 3C). Similarly as reported before,⁶ the majority of PAC-1 mAb positive platelets were annexin A5

negative, while the annexin A5 positive platelets were mostly PAC-1 mAb negative (Figure 3D). Dot plots of platelets in plasma, double labeled with AF647-annexin A5 and FITC-PAC-1 mAb, confirmed that most PS-exposing cells were unable to bind PAC-1 mAb (Suppl. Figure 3C). In this condition, again a population of PS-positive platelets showed a mean 2.8-fold increase in Rhod-A14 fluorescence above background. Taken together, these results suggests that the high fibrin(ogen)-binding platelet fraction contains a fibrin coat, which precedes PS exposure and primes for binding or Rhod-A14.

Roles of transglutaminases and integrin $\alpha_{IIb}\beta_3$ in platelet fibrin formation under flow conditions

Suspecting an involvement of transglutaminases and integrin $\alpha_{IIb}\beta_3$ in the high fibrin(ogen) binding, we investigated the effects of transglutaminase inhibitor K9-DON alone or together with the $\alpha_{IIb}\beta_3$ inhibitor, tirofiban. In washed platelets stimulated with convulxin/thrombin, the combined presence of the K9-DON and tirofiban blocked the binding of fibrin(ogen) and Rhod-A14 (Figure 4A, B).

In flow experiments, where whole blood was perfused over a collagen surface, we determined the localization of Rhod-A14 binding in thrombi formed on the

collagen. Double staining with Rhod-A14 and AF657-annexin A5 indicated that, under non-coagulant conditions (with thrombin inhibitor present), Rhod-A14 binding was restricted to a sub-population of PS-exposing platelets (Figure 5A). Co-localization analysis demonstrated that the majority of Rhod-A14 positive pixels also stained with AF647-annexin A5. Addition of K9-DON completely blocked the Rhod-A14 staining, but did not affect PS exposure. Zoomed images indicated that the Rhod-A14 labeling was confined to balloon-shaped, PS-exposing platelets, but often did not label the complete platelet (Figure 5A, right panel).

When thrombi were allowed to form on collagen under coagulant conditions (recalcification without thrombin inhibitor), this resulted in increased Rhod-A14 staining, which again was antagonized by K9-DON, as well as in higher AF637-annexin A5 staining, which was not affected by K9-DON (Figure 5B). In this case, PS-exposing balloon-shaped platelets still bound Rhod-A14, but the majority of the label was located at sites distinct from PS exposure at fibrin fibers. To specifically test the contribution of the transglutaminase factor XIII in Rhod-A14 binding to platelets, we analyzed platelets from *F13a1*^{-/-} mice, deficient in the A-subunit of factor XIII. Washed platelets from *F13a1*^{-/-} mice, when stimulated with convulxin/thrombin,

showed greatly reduced Rhod-A14 binding in comparison to wild-type platelets (Suppl. Figure 4A). However, in the *F13a1*^{-/-} platelets, PS exposure and the binding of factor Xa were unchanged (Suppl. Figure 4B, C), while the binding of fibrin(ogen) was reduced but not significant in comparison to *F13a1*^{+/+} platelets (Suppl. Figure 4D).

Flow experiments with *F13a1*^{+/+} or *F13a1*^{-/-} mouse blood over collagen under non-coagulant conditions resulted in thrombi of similar size with no more than low Rhod-A14 labeling (Figure 6A, B). This can be explained by the absence of tissue-type transglutaminase in mouse

platelets.³⁰ Under coagulant conditions, large fibrin-containing thrombi were formed with both *F13a1*^{+/+} and *F13a1*^{-/-} blood (Figure 6C, D). However, Rhod-A14 incorporated only into the fibrin fibers of wild-type thrombi and not of factor XIII-deficient thrombi. The Rhod-A14 staining again was antagonized by K9-DON, but not by tirofiban. These results hence indicated that the process of fibrin fiber formation can occur in the absence of transglutaminase activity.

In subsequent experiments, *F13a1*^{+/+} and *F13a1*^{-/-} blood was prelabeled with AF647-fibrinogen to investigate in more detail the structure and orientation of

Figure 6. Absence of factor XIII results in fibrin formation without transglutaminase activity. Whole blood from *F13a1*^{+/+} or *F13a1*^{-/-} mice was perfused over collagen under non-coagulant (A, B) or coagulant (C, D) conditions during 4 minutes at shear rate of 1000 s⁻¹. Blood samples were preincubated with vehicle (control) or K9-DON (100 μM), and post-stained with Rhod-A14. Shown are microscopic images of brightfield and Rhod-A14 fluorescence (bars, 20 μm); also quantification of thrombus size and Rhod-A14 fluorescence. Mean ± SEM (n=4) **p*<0.05.

the fibrin fibers by confocal fluorescence microscopy. Detailed examination indicated that, for wild-type thrombi, fibrin fibers were connected to the platelet surface and oriented to all directions (Figure 7A, B). This staining pattern resembled the star-like formation of fibrin at the platelet surface observed before.²⁹ In wild-type thrombi treated with integrin blocker tirofiban and also in factor XIII-deficient thrombi, the orientation of fibrin fibers from platelets was typically more frequent in the direction of the blood flow (Figure 7B). Markedly, the addition of tirofiban to factor XIII-deficient blood nearly completely blocked the star-like orientation of fibrin fibers, but still allowed the plasmatic formation of fibrin fibers rectilinear with the blood flow. These data thus indicate a non-redundancy of the transglutaminase factor XIII and integrin $\alpha_{IIb}\beta_3$ in platelet-dependent fibrin formation.

Discussion

The original paper by Dale *et al.* describes COAT (later renamed coated) platelets as a population of platelets, arising upon combined stimulation with collagen and thrombin, that can bind secreted α -granule proteins in a transglutaminase-dependent way.⁸ In the present paper, we used a novel fluorescent-labeled transglutaminase peptide substrate, Rhod-A14, to identify

transglutaminase-active platelets and hence further characterize the formation and responses of coated platelets. In particular, we evaluated how transglutaminase-active platelets compared to other platelet populations, earlier proposed as coated platelets, *i.e.* platelets with PS exposure, binding of coagulation factors Va and Xa, and high fibrinogen binding.

In washed human platelets, stimulated by convulxin/thrombin, we identified a slowly formed subpopulation of PS-exposing platelets capable to bind Rhod-A14. The Rhod-A14 incorporation was prevented by the general transglutaminase inhibitor K9-DON and the tissue-type transglutaminase inhibitor Boc-DON, while it was enhanced by addition of factor XIII. Factor XIII is also stored into platelets and needs to be externalized and cleaved by thrombin in order to deencrypt its transglutaminase activity.¹² Markedly, we also observed K9-DON/Boc-DON inhibitable Rhod-A14 binding in the absence of thrombin, *e.g.* with PS-exposing platelets stimulated by ionomycin. These findings thus suggest a role of both factor XIII and tissue-type transglutaminase activities in the binding of the probe to platelets. Indeed, human platelets are known to contain both factor XIII and tissue-type transglutaminases, which can be present at the platelet surface.^{8,30}

Figure 7. Absence of factor XIII and blockage of integrin $\alpha_{IIb}\beta_3$ impairs platelet-derived fibrin formation. (A) Blood from *F13a1*^{+/+} or *F13a1*^{-/-} mice was flowed over collagen under coagulant conditions (4 minutes, shear rate 500 s⁻¹). Tirofiban (5 μ g/ml) was added, where indicated. Confocal images of AF647-fibrinogen fluorescence (bars, 20 μ m). (B) Graphically display of the directions (tangents) of all fibrin fibers, traversing an arbitrary line perpendicular to the flow direction (dotted white line). Right panel: quantified octagonal distribution of tangent direction of fibrin fibers. Mean $\pm$ SEM (n=4) **p*<0.0001.

In flow cytometric dot plots, convulxin/thrombin-stimulated Rhod-A14-positive platelets appeared as a discrete population of platelets with high fibrin(ogen) binding and PS exposure, while only a subpopulation of PS-positive and high fibrin(ogen)-binding platelets bound Rhod-A14. We further found that PS exposure, but not Rhod-A14 binding, was accompanied by the binding to platelets of the coagulation factors Va and Xa. This is in agreement with the results from other groups, also reporting

high similarity of the annexin A5- and the coagulation factor-binding platelet populations.^{31,32} Furthermore, both in time of appearance and in subpopulation, the high fibrin(ogen)-binding platelets differed from the Rhod-A14-binding platelets. Hence, in contrast to the habit of others,^{13,14} we consider (transglutaminase-active) coated platelets as non-identical to fibrinogen-binding platelets.

Whereas the formation of a transglutaminase coat appeared to be a

relatively slow process following stimulation of isolated platelets with potent agonists like convulxin/thrombin, Rhod-A14 binding platelets were much faster formed (minutes) during whole-blood thrombus formation on collagen. This can be explained by a rapid availability of transglutaminases like factor XIII in the blood plasma, but it may also point to a relatively slow externalization of platelet-derived transglutaminases in the absence of plasma. Given that transglutaminases are broad-spectrum enzymes, cross-linking ϵ -(γ -glutamyl)lysine isopeptide bonds of multiple proteins,^{33,34} it can safely be assumed that Rhod-A14-binding platelets are covered (coated) with a large set of cross-linked proteins at their surface.

Whereas convulxin/thrombin stimulated platelets showed persistently high fibrin(ogen) binding, PAC-1 mAb binding gradually decreased after an initial peak. This is indicative of secondary inactivation of integrin $\alpha_{IIb}\beta_3$, such in agreement with earlier results.^{6,35} One explanation for the persistent fibrin(ogen) binding to platelets with apparently inactive integrins, is that the activated $\alpha_{IIb}\beta_3$ displays distinct binding sites for fibrinogen and fibrin, allowing interaction with fibrin independently of the classical integrin activation domains.³⁶ On the other hand, the high fibrin(ogen) binding of washed platelets was antagonized by combined inhibition

of $\alpha_{IIb}\beta_3$ and transglutaminases. This suggested initial binding of fibrinogen to the activated integrin, followed by secondary binding of a growing fibrin fiber to platelet surface proteins via transglutaminase activity.

The experiments where thrombus formation was studied in mouse blood clearly indicated that murine factor XIII is required for Rhod-A14 binding, but is dispensable for the formation of fibrin fibers, apparently in the absence of transglutaminase cross-linking. These data are in agreement with those of others, reporting that deficiency in murine factor XIII is dispensable for high fibrinogen binding and PS exposure.¹² Strikingly, however, we found that the combined absence of factor XIII and blockage of $\alpha_{IIb}\beta_3$ resulted in abolition of the formation of star-like fibrin patterns at the platelets surface, thus pointing to a combined role of factor XIII and integrin $\alpha_{IIb}\beta_3$ in platelet-driven fibrin fiber formation.

In sum we report the following mechanism for formation of coated, Rhod-A14-binding platelets: initial collagen/thrombin receptor-induced $\alpha_{IIb}\beta_3$ activation leads to fibrin(ogen) binding, which on PS-exposing platelets can be consolidated by transglutaminase activity, and act as a scaffold for platelet-driven fibrin fiber formation. Since many of these coated platelets still bind coagulation factors, they can in addition

have a role in stimulating prothrombinase activity and thrombin generation.

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Conflicts of interest.

F.M. and M.W.N. are employees of CSL Behring GmbH. All other co-authors declare no relevant conflict of interest.

References

1. Cosemans JM, Angelillo-Scherrer A, Mattheij NJ, Heemskerk JW. The effects of arterial flow on platelet activation, thrombus growth, and stabilization. *Cardiovasc Res*. 2013;**99**:342-352.
2. de Witt SM, Swieringa F, Cavill R, Lamers MM, van Kruchten R, Mastenbroek T, Baaten C, Coort S, Pugh N, Schulz A, Scharrer I, Jurk K, Zieger B, Clemetson KJ, Farndale RW, Heemskerk JW, Cosemans JM. Identification of platelet function defects by multi-parameter assessment of thrombus formation. *Nat Commun*. 2014;**5**:4257.
3. Munnix IC, Cosemans JM, Auger JM, Heemskerk JW. Platelet response heterogeneity in thrombus formation. *Thromb Haemost*. 2009;**102**:1149-1156.
4. Versteeg HH, Heemskerk JW, Levi M, Reitsma PH. New fundamentals in hemostasis. *Physiol Rev*. 2013;**93**:327-358.
5. Heemskerk JW, Mattheij NJ, Cosemans JM. Platelet-based coagulation: different populations, different functions. *J Thromb Haemost*. 2013;**11**:2-16.
6. Mattheij NJ, Gilio K, van Kruchten R, Jobe SM, Wieschhaus AJ, Chishti AH, Collins P, Heemskerk JW, Cosemans JM. Dual mechanism of integrin $\alpha_{IIb}\beta_3$ closure in procoagulant platelets. *J Biol Chem*. 2013;**288**:13325-13336.
7. Dale GL. Coated-platelets: an emerging component of the procoagulant response. *J Thromb Haemost*. 2005;**3**:2185-2192.
8. Dale GL, Friesse P, Batar P, Hamilton SF, Reed GL, Jackson KW, Clemetson KJ, Alberio L. Stimulated platelets use serotonin to enhance their retention of procoagulant proteins on the cell surface. *Nature*. 2002;**415**:175-179.
9. Alberio LJ, Clemetson KJ. All platelets are not equal: COAT platelets. *Curr Hematol Rep*. 2004;**3**:338-343.
10. Szasz R, Dale GL. Thrombospondin and fibrinogen bind serotonin-derivatized proteins on COAT-platelets. *Blood*. 2002;**100**:2827-2831.
11. Maroney SA, Habichter SL, Friesse P, Collins ML, Ferrel JP, Dale GL, Mast AE. Active tissue factor pathway inhibitor is expressed on the surface of coated platelets. *Blood*. 2007;**109**:1931-1937.
12. Jobe SM, Leo L, Eastvold JS, Dickneite G, Ratliff TL, Lentz SR, Di Paola J. Role of FcRgamma and factor XIIIa in coated platelet formation. *Blood*. 2005;**106**:4146-4151.
13. Kirkpatrick AC, Tafur AJ, Vincent AS, Dale GL, Prodan CI. Coated-platelets improve prediction of stroke and transient ischemic attack in asymptomatic internal carotid artery stenosis. *Stroke*. 2014;**45**:2995-3001.
14. Prodan CI, Stoner JA, Cowan LD, Dale GL. Higher coated-platelet levels are associated with stroke recurrence following nonlacunar brain infarction. *J Cereb Blood Flow Metab*. 2013;**33**:287-292.
15. Prodan CI, Vincent AS, Dale GL. Coated-platelet levels are persistently elevated in patients with mild traumatic brain injury. *J Head Trauma Rehabil*. 2014;in press.
16. Prodan CI, Szasz R, Vincent AS, Ross ED, Dale GL. Coated-platelets retain amyloid precursor protein on their surface. *Platelets*. 2006;**17**:56-60.

17. Munnix IC, Kuijpers MJ, Auger J, Thomassen CM, Panizzi P, van Zandvoort MA, Rosing J, Bock PE, Watson SP, Heemskerk JW. Segregation of platelet aggregatory and procoagulant microdomains in thrombus formation. Regulation by transient integrin activation. *Arterioscler Thromb Vasc Biol.* 2007;**27**:2484-2490.
18. Miserus RJ, Herías MV, Prinzen L, Lobbes MB, Van Suylen RJ, Dirksen A, Hackeng TM, Heemskerk JW, van Engelshoven JM, Daemen MJ, van Zandvoort MA, Heeneman S, Kooi ME. Molecular MRI of early thrombus formation using a bimodal $\alpha 2$ -antiplasmin-based contrast agent. *JACC Cardiovasc Imaging.* 2009;**2**:987-996.
19. Hara T, Bhayana B, Thompson B, Kessinger CW, Khatri A, McCarthy JR, Weissleder R, Lin CP, Tearney GJ, Jaffer FA. Molecular imaging of fibrin deposition in deep vein thrombosis using fibrin-targeted near-infrared fluorescence. *JACC Cardiovasc Imaging.* 2012;**5**:607-615.
20. Gilio K, Harper MT, Cosemans JM, Konopatskaya O, Munnix IC, Prinzen L, Leitges M, Liu Q, Molkentin JD, Heemskerk JW, Poole AW. Functional divergence of platelet protein kinase C (PKC) isoforms in thrombus formation on collagen. *J Biol Chem.* 2010;**285**:23410-23419.
21. van der Meijden PE, Feijge MA, Swieringa F, Gilio K, Nergiz-Unal R, Hamulyák K, Heemskerk JW. Key role of integrin $\alpha_{IIb}\beta_3$ signaling to Syk kinase in tissue factor-induced thrombin generation. *Cell Mol Life Sci.* 2012;**69**:3481-3492.
22. Lauer P, Metzner HJ, Zettlmeissl G, Li M, Smith AG, Lathe R, Dickneite G. Targeted inactivation of the mouse locus encoding coagulation factor XIII-A: hemostatic abnormalities in mutant mice and characterization of the coagulation deficit. *Thromb Haemost.* 2002;**88**:967-974.
23. Vanschoonbeek K, Wouters K, van der Meijden PE, van Gorp PJ, Feijge MA, Herfs M, Schurgers LJ, Hofker MH, de Maat MP, Heemskerk JW. Anticoagulant effect of dietary fish oil in hyperlipidemia: a study of hepatic gene expression in APOE2 knock-in mice. *Arterioscler Thromb Vasc Biol.* 2008;**28**:2023-2029.
24. Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E, Wagenvoort R, Lecompte T, Béguin S. Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb.* 2003;**33**:4-15.
25. Gilio K, van Kruchten R, Braun A, Berna-Erro A, Feijge MA, Stegner D, van der Meijden PE, Kuijpers MJ, Varga-Szabo D, Heemskerk JW, Nieswandt B. Roles of STIM1 and Orai1 in glycoprotein VI- and thrombin-dependent procoagulant activity and thrombus formation. *J Biol Chem.* 2010;**285**:23629-23638.
26. Van Kruchten R, Cosemans JM, Heemskerk JWM. Measurement of whole blood thrombus formation using parallel-plate flow chambers: a practical guide. *Platelets.* 2012;**23**:229-242.
27. Cosemans JM, Munnix IC, Wetzker R, Heller R, Jackson SP, Heemskerk JW. Continuous signaling via PI3K isoforms beta and gamma is required for platelet ADP receptor function in dynamic thrombus stabilization. *Blood.* 2006;**108**:3045-3052.
28. Abaeva AA, Canault M, Kotova YN, Obydeny SI, Yakimenko AO, Podoplelova NA, Kolyadko VN, Chambost H, Mazurov AV, Ataullakhanov FI, Nurden AT, Alessi MC, Panteleev MA. Procoagulant platelets form an alpha-granule protein-covered "cap" on their surface that promotes their attachment to aggregates. *J Biol Chem.* 2013;**288**:29621-29632.
29. Cosemans JM, Schols SE, Stefanini L, de Witt S, Feijge MA, Hamulyák K, Deckmyn H, Bergmeier W, Heemskerk JW. Key role of glycoprotein Ib/V/IX and von Willebrand factor in platelet activation-dependent fibrin formation at low shear flow. *Blood.* 2011;**117**:651-660.
30. Puszkin EG, Raghuraman V. Catalytic properties of a calmodulin-regulated transglutaminase from human platelet and chicken gizzard. *J Biol Chem.* 1985;**260**:16012-16020.
31. London FS, Marcinkiewicz M, Walsh PN. A subpopulation of platelets responds to thrombin- or SFLRN-stimulation with binding sites for factor IXa. *J Biol Chem.* 2004;**279**:19854-19859.
32. Panteleev MA, Ananyeva NM, Greco NJ, Ataullakhanov FI, Saenko EL. Two subpopulations of thrombin-activated platelets differ in their binding of the components of the intrinsic factor X-activating complex. *J Thromb Haemost.* 2005;**3**:2545-2553.

33. Nikolajsen CL, Dyrland TF, Poulsen ET, Enghild JJ, Scavenius C. Coagulation factor XIIIa substrates in human plasma: identification and incorporation into the clot. *J Biol Chem.* 2014;**289**:6526-6534.
34. Siebenlist KR, Meh DA, Mosesson MW. Protransglutaminase (factor XIII) mediated crosslinking of fibrinogen and fibrin. *Thromb Haemost.* 2001;**86**:1221-1228.
35. Kulkarni S, Jackson SP. Platelet factor XIII and calpain negatively regulate integrin $\alpha_{IIb}\beta_3$ adhesive function and thrombus growth. *J Biol Chem.* 2004;**279**:30697-30706.
36. Podolnikova NP, Yakovlev S, Yakubenko VP, Wang X, Gorkun OV, Ugarova TP. The interaction of integrin $\alpha_{IIb}\beta_3$ with fibrin occurs through multiple binding sites in the α_{IIb} β -propeller domain. *J Biol Chem.* 2014;**289**:2371-2783.

Chapter 6

Both TMEM16F-dependent and TMEM16F-independent pathways contribute to phosphatidylserine exposure in platelet apoptosis and platelet activation

van Kruchten R¹, Mattheij NJ¹, Saunders C², Feijge MA¹, Swieringa F¹, Wolfs JL¹, Collins PW³, Heemskerk JW¹, Bevers EM¹

¹Department of Biochemistry, Cardiovascular Research Institute Maastricht, University of Maastricht, Maastricht, The Netherlands; ²Welsh Blood Service, Pontyclun, United Kingdom; ³Arthur Bloom Haemophilia Centre, School of Medicine, Cardiff University, Cardiff, United Kingdom

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Abstract

Scott syndrome, a bleeding disorder caused by defective phospholipid scrambling, has been associated with mutations in the TMEM16F gene. The role of TMEM16F in apoptosis- or agonist-induced phosphatidylserine (PS) exposure was studied in platelets from a Scott syndrome patient and control subjects. Whereas stimulation of control platelets with the BH3-mimetic ABT737 resulted in 2 distinct fractions with moderate and high PS exposure, the high PS-exposing fraction was markedly delayed in Scott platelets. High, but not moderate, PS exposure in platelets was suppressed by chelation of intracellular Ca^{2+} , whereas caspase inhibition completely abolished ABT737-induced PS exposure in both Scott and control platelets. On the other hand, high PS exposure induced by the Ca^{2+} -mobilizing agonists convulxin/thrombin fully relied on mitochondrial depolarization and was virtually absent in Scott platelets. Finally, PS exposure induced by collagen/thrombin was partly affected in Scott platelets, and the residual PS positive fraction was insensitive to inhibition of caspases or mitochondrial depolarization. In conclusion, TMEM16F is not required for, but enhances, caspase-dependent PS exposure; convulxin-/thrombin-induced PS exposure is entirely dependent on TMEM16F, whereas collagen/thrombin-induced PS exposure results from 2 distinct pathways, one of which involves mitochondrial depolarization and is mediated by TMEM16F.

Introduction

Transbilayer lipid asymmetry in platelets and other cells is maintained by an aminophospholipid translocase, an active transporter pumping aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine from the outer to the inner leaflet of the plasma membrane. Under particular conditions, phospholipid asymmetry is disrupted by a “scrambling” process, in which the phospholipids become increasingly randomized over both plasma membrane leaflets.¹ As a consequence, PS, which in resting cells is exclusively located in the inner leaflet,

becomes exposed at the membrane outer leaflet and can be probed with the PS-binding proteins annexin A5 or lactadherin. For apoptotic cells, externalized PS provides a signal for removal by the mononuclear phagocytic system.² In blood cells, PS-exposing membranes strongly promote the process of thrombin generation and fibrin clot formation.^{3,4} In platelets, phospholipid scrambling and PS externalization are induced by activation with strong agonists like convulxin/thrombin and collagen/thrombin, stimulating glycoprotein VI, and the platelet

thrombin receptors.^{5,6} Known requirements for agonist-induced phospholipid scrambling are a sustained high intracellular Ca^{2+} concentration^{1,3} and loss of the mitochondrial membrane potential.^{7,8} Hence, platelets lacking cyclophilin D, a positive modulator of the mitochondrial permeability transition pore (MPTP), are protected against PS externalization upon convulxin/thrombin stimulation.⁹

Scott syndrome is a rare bleeding disorder in which the Ca^{2+} -dependent phospholipid scrambling of platelets and other blood cells is impaired.¹⁰ Although

agonist-induced Ca^{2+} responses were found to be unchanged, Scott syndrome platelets show diminished PS exposure upon stimulation with convulxin/thrombin, collagen/thrombin, or Ca^{2+} -ionophore ionomycin compared with healthy subjects.¹¹ Recently, TMEM16F was recognized as a protein critically involved in Ca^{2+} -induced phospholipid scrambling. A homozygous mutation in TMEM16F (IVS12-1G→T, causing premature termination of translation) was found in the genome of the proband Scott patient.¹² Another Scott syndrome patient appeared to be

Figure 1. Proapoptotic agent ABT737 causes PS exposure in platelets from a patient with Scott syndrome. Washed platelets from healthy control subjects or a patient with Scott syndrome were treated with ABT737 (10 mM) in the presence of 1 mM CaCl_2 . (A) Representative histograms (black lines) for AF647-annexin A5 binding after 1- or 3-h stimulation with ABT737. Markers M1, M2, and M3 indicate fractions of platelets with no, moderate, or high annexin A5 binding, respectively. Gray curves show unstimulated platelets treated with vehicle. (B) Corresponding dot plots (forward scatter vs AF647 fluorescence) of platelets treated with ABT737 or vehicle. (C) Distribution of M1, M2, and M3 platelet populations upon ABT737 treatment (10 mM).

compound heterozygous in TMEM16F,¹³ suggesting that a defective TMEM16F is the cause of the Scott syndrome phenotype.

Besides the agonist-induced pathway, platelets possess a distinct pathway to PS exposure, which was shown to be elicited with ABT737 or ABT263 (Navitoclax), both of which are BH-3 mimetics that activate caspase-dependent apoptosis in a Bax/Bakdependent manner.^{14,15} This apoptotic pathway may play a role in the clearance of aging platelets.¹⁶ As Bcl-xL is gradually degraded in such platelets, its restraint of the proapoptotic function of Bax and Bak will gradually diminish in time and hence prime platelets for apoptosis.^{17,18} This explains why BH-3 mimetics like ABT737 and ABT263 induce thrombocytopenia and affect the platelet hemostatic function.^{19,20} Whether TMEM16F is involved in PS exposure upon apoptosis has not been investigated. In this study, we aimed to elucidate the roles of TMEM16F in agonist- and apoptosis-induced PS exposure using platelets from a Scott patient. We provide evidence for TMEM16F-independent phospholipid scrambling in the apoptotic pathway that leads to moderate PS exposure in a caspase-dependent way. The data furthermore point to a TMEM16F-independent process of caspase/mitochondrial-independent PS

exposure in response to collagen/thrombin.

Methods

Materials

ABT737 and Ac-DEVD-AFC were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), ABT-263 from Selleckchem (Houston, TX), and annexin A5-labeled with fluorescein isothiocyanate (FITC) from PharmaTarget (Maastricht, The Netherlands). Acetylsalicylic acid (aspirin) was from Genfarma (Maarssen, The Netherlands). Fibrillar type I collagen (Horm) was from Nycomed (Munich, Germany). Fluo-4 acetoxymethyl ester, Alexa Fluor-647 (AF647)-labeled annexin A5, dimethyl BAPTA acetoxymethyl ester, ionomycin, and pluronic F-127 were from Invitrogen (Carlsbad, CA). Apyrase (grade V), dimethylsulfoxide, bovine serum albumin, human fibrinogen, MRS2179, 2-APB, and thrombin were from Sigma-Aldrich (St. Louis, MO). FITC-lactadherin was from Haematologic Technologies (Essex Junction, VT). Tetramethyl rhodamine methyl ester (TMRE) was from Anaspec (San Jose, CA). Q-VD-Oph and cyclosporin A were from Calbiochem (San Diego, CA). A fluorometric assay for caspase 3 activity (BF1100) came from R&D Systems (Minneapolis, MN). Convulxin was purified as described before.²¹ Cangrelor was kindly provided by AstraZeneca (Möln Dahl, Sweden).

Blood collection and platelet preparation

Human blood was obtained from healthy volunteers and a patient from the UK with Scott syndrome after they provided informed consent in accordance with the Declaration of Helsinki under protocols reviewed by the local ethics committees. The UK Scott patient was described earlier as compound heterozygous in TMEM16F with one mutation, IVS6 1 1G→A, resulting in exon 6 skipping and another mutation (c.1219insT) leading to a premature stop of translation, causing defective TMEM16F expression.¹³ Blood was collected into acid citrate dextrose to prepare washed platelets in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 7.45 (136 mM NaCl, 10 mM HEPES, 2.7 mM KCl, 2 mM MgCl₂, 0.1% glucose, and 0.1% bovine serum albumin) as described.²² Platelet counts were determined using a Coulter counter (Beckman).

Measurement of PS exposure

Washed platelets (1×10^8 /mL) were preincubated with inhibitor or vehicle control (dimethylsulfoxide) and activated with indicated agents under nonstirring conditions at 37°C. For aging experiments, platelets were stored at room temperature in sterile HEPES buffer, pH 7.45, supplemented with 0.2% penicillin/streptomycin. For experiments performed in the absence of extracellular

Ca²⁺, HEPES buffer was supplied with 1 mM EGTA. Where indicated, intracellular Ca²⁺ was chelated by platelet treatment with 20 mM dimethyl BAPTA acetoxymethyl ester. Phospholipid scrambling was measured as PS exposure by labeling in the presence of 2 mM CaCl₂ with fluorescent annexin A5 (0.25 mg/mL) or fluorescent lactadherin (16 nM). Labeling was done by transferring 2 mL platelet suspension into 60 mL measurement buffer with 2 mM CaCl₂. After 2 min of labeling, samples were analyzed with an Accuri C6 flow cytometer and Flow C6 software. Threshold forward scatter and side scatter gates were set to exclude microparticles. The range M3 was defined as the annexin A5 fluorescence comprising 95% of maximally activated platelets using ionomycin. The range M1 was set to include 95% of all unstimulated platelets, and M2 was the range between M1 and M3.

Calcium measurements

For measurements of [Ca²⁺]_i by flow cytometry, washed platelets (3×10^8 /mL) were loaded for 45 min with 8 mM Fluo-4 AM in the presence of 0.4 mg/mL pluronic F-127. Following a wash step, the platelets were resuspended in HEPES buffer, pH 7.45, containing 2 mM CaCl₂ and activated by convulxin/thrombin (70 ng/mL, 4 nM) at 2×10^6 platelets/mL.

Supplemental Figure 1. Absence of high PS-exposing fraction in Scott syndrome platelets treated with ABT737

(A) Washed platelets from healthy control subjects or a patient with Scott syndrome were stimulated by ABT737 (0.33-10 μM) in the presence of 1 mM CaCl₂. Representative flow cytometry histograms and dot plots of AF647-annexin A5 binding after 1 hour stimulation with ABT737 of control (black) and Scott platelets (grey curves). (B) Fluo-4-loaded platelets were stimulated with ABT737 (1 μM) for 1 hour in the presence of 1 mM CaCl₂. Fluo-4 fluorescence was determined of the M1, M2 and M3 fractions. Mean ± S.E.M. (n=4), * p<0.05.

Detection of mitochondrial depolarization

Platelets were loaded with TMRE (50 nM) for 30 min prior to stimulation for assessment of mitochondrial membrane depolarization by flow cytometry. In combination with confocal microscopy, platelets were allowed to adhere to fibrinogen-coated coverslips before stimulation with indicated agonist. Slides were precoated with 500 mg/mL fibrinogen for 10 min. After stimulation with the indicated agonists, confocal images were taken with a Live-7 Zeiss line-scanning microscope system.

Caspase activity assay

Washed platelets (1 × 10⁸/mL) were preincubated and activated as indicated. At fixed time points, 100-mL samples were centrifuged (2 min at 2300 g), and platelet pellets were resolved into 100 mL lysis buffer (caspase-3 fluorometric assay, R&D Systems). Samples in lysis buffer were mixed 1:1 with 50 mL caspase substrate mix containing 9.8 mM dithiothreitol and 49 mM Ac-DEVD-AFC. Fluorescence was measured in time using a Spectramax M2. Caspase-3 activity was assessed as fluorescence units per minute.

Figure 2. High PS exposure induced by ABT737 depends on elevated intracellular Ca^{2+} . (A-F) Washed platelets were stimulated by 10 mM ABT737 in the absence of CaCl_2 or after loading with dimethyl-BAPTA (1 BAPTA) in the presence of 2 mM CaCl_2 . Representative flow cytometry histograms (A-B) and dot plots (C-D) of AF647-annexin (Anx) A5 binding after 1-h treatment with ABT737. Markers M1, M2, and M3 indicate fractions of platelets with no, moderate, or high Anx A5 binding, respectively. (E-F) Distribution of platelet populations over M1, M2, and M3 during treatment in time. (G) Platelets were loaded with the intracellular Ca^{2+} probe Fluo-4 and stimulated with ABT737 (10 mM, 1 h) in the presence of 1 mM CaCl_2 . Fluo-4 fluorescence was determined for the M1, M2, and M3 fractions. Dotted line represents maximal Fluo-4 fluorescence obtained with 10 mM ionomycin, as positive control. (H) Anx A5 binding of platelets stimulated by ABT737 in the presence of 1 mM CaCl_2 or 1 mM EGTA after 1 to 3 h. (I) Caspase activity (arbitrary fluorescence units/min) of ABT737-stimulated platelets in the presence of 1 mM CaCl_2 or 1 mM EGTA. Mean $\pm$ SEM (n 5 3-6); * $P < .05$.

Statistical analysis

Data are presented as means $\pm$ SEM. As appropriate, the significance of differences was determined by analysis

of variance analysis with Bonferroni posttesting, paired t test, or the independent samples t test, using GraphPad Prism software.

Results

BH3-mimetics induce delayed PS exposure in Scott syndrome platelets

To investigate the role of TMEM16F in apoptosis-induced PS exposure, platelets from a patient with Scott syndrome and from healthy control subjects were treated with the BH3-mimetic ABT737. As shown in Figure 1A, a 1-h treatment with 10 mM ABT737 caused ~70% of the platelets to expose PS for both control and Scott platelets. However, whereas for control platelets the histogram of the annexin A5-positive fraction gave a bimodal distribution with moderate (marker region M2) and high (marker region M3) PS exposure, the Scott platelets exhibited a fraction with mainly moderate PS exposure. Similar results were found at lower concentrations of ABT737, ranging from 3.3 to 0.33 mM (supplemental Figure 1A). Note that the extent of annexin A5 binding in the M3 fraction of the ABT737-treated control platelets was comparable with that of platelets stimulated with ionomycin or convulxin/thrombin (see below). A similar bimodal labeling pattern was found with the PS-selective probe, FITC-lactadherin (data not shown). Prolonged incubation with ABT737 up to 3 h increased the fraction with the highest (M3) annexin A5 binding at the expense of the fraction with moderate (M2) annexin A5 binding for both Scott and control platelets (Figure 1A-C). Similar

results were obtained with the related compound ABT263 (data not shown).

Supplemental Figure 2. ABT737-induced PS exposure in Scott syndrome platelets is caspase-dependent. Washed platelets from healthy control subjects or a patient with Scott syndrome were stimulated by ABT737 (10 μ M) in the presence of 1 mM CaCl_2 . (A) Caspase activity after 1 hour of ABT737 treatment (arbitrary fluorescence units/min). (B) Effects of caspase inhibition (20 μ M Q-VD-Oph, 10 minutes) on percentage of annexin A5-positive platelets (M2 plus M3 fractions). Mean $\pm$ S.E.M. (n=3), * p < 0.05.

Figure 3. Prolonged storage causes PS exposure in Scott syndrome platelets. Percentage of annexin (Anx) A5-positive platelets (M2 and M3 fractions) from healthy controls and Scott patient during 1- to 4-h storage at room temperature in sterile buffer medium. Mean $\pm$ SEM (n = 3).

The ABT737-induced PS exposure in platelets was previously shown to be dependent on caspase proteases.¹⁴ This was confirmed by the measurement of caspase activity in platelets. Comparable caspase activity was detected in Scott syndrome platelets and platelets from controls (supplemental Figure 2A), ruling out the possibility that a difference in caspase activity attributed to the observed effects. Furthermore, the pan-caspase inhibitor Q-VD-Oph completely abolished ABT737-induced PS exposure in both Scott syndrome and control platelets (supplemental Figure 2B). These data suggest that the proapoptotic agent ABT737 activates 2 mechanisms leading to PS exposure, one of which causes a gradual increase in PS exposure that is independent of TMEM16F and one resulting in high PS exposure that requires the presence of a functional TMEM16F.

Because TMEM16F is considered to be essential for Ca^{2+} -dependent phospholipid scrambling, we questioned to what extent the scrambling process observed after ABT737 treatment is regulated by intracellular Ca^{2+} levels. Indeed, chelation of intracellular Ca^{2+} levels in platelets loaded with dm-BAPTA resulted in strong suppression of the high annexin A5-binding population, with little effect on the moderate PS-exposing fraction (Figure 2A-F). Furthermore, Fluo-4 measurements revealed that the high

annexin A5-binding fraction had increased Ca^{2+} levels compared with the moderate PS-exposing fractions (Figure 2G). However, chelation of extracellular Ca^{2+} by 1 mM EGTA resulted in a significant suppression of ABT737-induced PS-exposure in both the moderate and high annexin A5-binding populations (Figure 2H). The defective PS exposure in the presence of EGTA was not due to a reduction in caspase activity (Figure 2I). Further experiments with aspirin (100 mM), P2Y₁ blocker MRS-2179 (50 mM), and P2Y₁₂ blocker cangrelor (10 mM) indicated that the fractions of moderate and high annexin A5-binding platelets changed with <2% by blocking of the secondary mediator release.

Normal PS exposure in Scott syndrome platelets during prolonged storage

A gradual increase in surface-exposed PS can be observed in platelets that are stored for a prolonged time (C.S., unpublished data, 2012). To determine whether Scott syndrome platelets possess a similar mechanism to expose PS under aging conditions, Scott and control platelets were stored up to 4 d at room temperature. As shown in Figure 3, the patient's platelets were equally able to expose PS upon storage as controls, indicating that in vitro aging-related PS exposure occurs independently of TMEM16F activity.

Convulxin-/thrombin-stimulated Scott platelets do not expose PS despite normal cyclophilin D-mediated mitochondrial depolarization and intracellular Ca²⁺ response

Mitochondrial depolarization through cyclophilin D-dependent formation of the MPTP has been shown to be crucial for convulxin-/thrombin-induced PS exposure in platelets.⁹ Involvement of cyclophilin D-mediated mitochondrial depolarization was assessed by measuring TMRE fluorescence and by investigating the effect of the MPTP inhibitor cyclosporin A. Stimulation of Scott syndrome platelets by convulxin/thrombin did not cause significant PS exposure (Figure 4A), whereas in healthy controls, 40% of the platelets became annexin A5 positive. This response was almost completely suppressed by cyclosporin A. The lack of PS exposure in convulxin-/thrombin-stimulated Scott platelets was not due to the absence of mitochondrial depolarization, as demonstrated by flow cytometry (Figure 4B). The loss of TMRE from mitochondria in Scott platelets stimulated with convulxin/thrombin was confirmed by confocal microscopy (Figure 4E-F). No differences in intracellular Ca²⁺ levels were found between Scott syndrome platelets and healthy controls after convulxin/thrombin stimulation (Figure 5A-C), which is in line with previous

results showing normal store-operated Ca²⁺ entry with such agonists.¹¹

Analysis of the flow cytometry histograms revealed a marked difference between ABT737 and convulxin/thrombin stimulation. Whereas ABT737 treatment resulted in 2 populations of platelets with moderate and high PS exposure, after convulxin/thrombin stimulation, even at early time points, only high PS-exposing platelets residing in marker region M3 were found (Figure 4C-D). Because this response was virtually absent in Scott platelets, we concluded that convulxin-/thrombin-induced PS exposure depends on a functional TMEM16F.

Collagen/thrombin stimulation induces an additional pathway to platelet PS exposure, which is independent of caspases, cyclophilin D, or TMEM16F

In previous studies, it was found that collagen-/thrombin-induced PS exposure is only partially impaired in platelets from patients with Scott syndrome.^{10,11} We questioned whether the residual PS exposure observed after collagen/thrombin stimulation in Scott platelets results from activation of the apoptotic pathway. Figure 6A shows that caspase inhibition by Q-VD-Oph did not affect the fraction of PS-exposing platelets induced by collagen/thrombin in either Scott platelets or healthy control platelets, indicating no

Figure 4. Cvx-/Thr-induced PS exposure is absent in Scott syndrome platelets despite normal cyclophilin D-dependent mitochondrial depolarization. (A-D) Platelets from healthy controls or a Scott patient were loaded with TMRE and preincubated with vehicle (dimethylsulfoxide) or cyclosporin A (CsA) (4 μ M) for 10 min prior to stimulation by Cvx/Thr (70 ng/mL, 4 nM, for 15 min) in the presence of 2 mM CaCl_2 . Graphs show annexin (Anx) A5 binding (A) and loss of TMRE (B) of washed control and Scott syndrome platelets. (C-D) Representative histogram and bar graph showing the distribution of Cvx-/Thr-stimulated control platelets (15 min) over the fractions M1, M2, and M3. (E-F) TMRE-loaded Scott platelets adhered to fibrinogen (10 min) were stimulated with Cvx/Thr (70 ng/mL, 4 nM) or ionomycin (10 μ M) in the presence of 2 mM CaCl_2 . (E) Representative images after 15-min stimulation, (F) The percentage of TMRE-negative platelets. Mean $\pm$ SEM ($n = 3-6$); * $P < .05$.

appreciable contribution of the apoptotic pathway in collagen-/thrombin-induced PS exposure.

We then asked whether the residual PS-exposing activity is cyclophilin D-mediated. The collagen-/thrombin-induced PS exposure in Scott syndrome appeared insensitive to inhibition with cyclosporine A, whereas this compound effectively reduced mitochondrial depolarization (Figure 6A-B). Treatment

of control platelets resulted in a reduction of collagen-/thrombin-induced PS exposure from 53% to 19% and a correspondingly reduced mitochondrial depolarization. These data suggest that collagen/thrombin activates 2 different pathways leading to PS exposure, one that requires MPTP formation and is dependent on TMEM16F and one that does not involve MPTP and functions independently of TMEM16F. Control

studies showed that both collagen-/thrombin- and convulxin-/thrombin-induced PS exposure are entirely dependent on intracellular Ca^{2+} , as platelet treatment with dm-BAPTA virtually abolished PS exposure (<4% PS-positive platelets).

Discussion

In this study, we show that Scott syndrome platelets have retained the capacity to expose PS upon ABT737-induced apoptosis. Remarkably, though, Scott syndrome platelets show only moderate levels of annexin A5 binding after 1 h of ABT737 treatment, whereas

healthy controls have an additional platelet fraction with high annexin A5 binding despite the fact that the total fraction of annexin-positive platelets is virtually the same for the patient and healthy controls. This argues for a partial role of TMEM16F in apoptotic PS exposure. Because TMEM16F is reported to be crucial for Ca^{2+} -induced phospholipid scrambling¹² and the fraction of high annexin A5-binding platelets correlates with platelets high in intracellular Ca^{2+} , we propose that next to a TMEM16F-independent mechanism, a TMEM16F-dependent mechanism is activated during platelet apoptosis,

Figure 5. Scott syndrome platelets display normal convulxin (Cvx)-/thrombin (Thr)-induced Ca^{2+} responses. (A-B) Time drive of fluorescence increase of washed control (A) and Scott syndrome (B) platelets loaded with Fluo-4 and activated by Cvx/Thr (70 ng/mL, 4 nM) in the presence of 2 mM CaCl_2 . (C) Average change in mean fluorescence intensity (MFI), representing cytosolic Ca^{2+} , after 10 min stimulation. n.s., not significant.

which accelerates the appearance of PS-exposing platelets. The high annexin A5-binding population was effectively suppressed by chelation of intracellular Ca^{2+} , which is in concordance with TMEM16F being a Ca^{2+} -dependent scramblase.¹² Interestingly, the onset of the TMEM16F-dependent scrambling process (Figure 2E platelets in the region M3) appears to be delayed compared with the initiation of the TMEM16F-independent scrambling (region M2). Because the apoptotic process compromises Ca^{2+} homeostasis,^{23,24} it is not unlikely that a certain time span of increased cytosolic Ca^{2+} concentration is required to activate the TMEM16F-dependent scrambling mechanism. We do not know whether the actual rates of phospholipid scrambling differ between M3 and M2 platelets. It is tempting to speculate that the scrambling process in the M3 platelets is similar to that of ionomycin-induced scrambling, although it should be noted that apoptosis-induced PS exposure is caspase dependent, whereas ionomycin-induced PS exposure is not. Previously, induction of apoptosis in Scott syndrome lymphoid cells resulted in phospholipid scrambling similar to that in control lymphoid cells.²⁵ In that study, the rate of lipid scrambling did not increase when apoptotic control B-lymphocytes were treated with Ca^{2+} ionophore, suggesting different pathways leading to lipid scrambling

rather than the existence of more than one scramblase protein.

Although the role of high cytosolic Ca^{2+} in agonist-induced PS exposure is well recognized,¹ it is unclear how Ca^{2+} affects phospholipid scrambling in apoptotic cells. In this paper, we show an inhibitory effect of chelating extracellular Ca^{2+} on ABT737-induced PS exposure. The lack of response upon removal of extracellular Ca^{2+} cannot be explained by decreased activity of the apoptotic machinery itself, as caspase activity is not affected by removal of extracellular Ca^{2+} . Previously, Schoenwaelder et al¹⁴ have shown that ABT737-induced filamin and gelsolin cleavage was not affected by chelation of extracellular Ca^{2+} . Our data indicate that, while the high PS-exposing platelet fraction M3 is absent upon chelation of intracellular Ca^{2+} , the moderate PS-exposing fraction M2 also has a Ca^{2+} -sensitive component. At present, we cannot distinguish whether this Ca^{2+} dependency is located at the level of signaling events downstream of caspase activity or at the level of the scramblase activity.

The most pronounced defect in Scott syndrome platelets was found upon activation with convulxin/thrombin; whereas these agonists induced ~40% PS-positive platelets in healthy control platelets, PS exposure was virtually absent in the patient platelets. However, Ca^{2+} responses and mitochondrial

depolarization were not affected in the patient platelets, indicating that the defect is downstream of Ca^{2+} signaling and MPTP formation. This finding is supported by a report showing that mitochondrial depolarization is normal in a canine model of impaired phospholipid scrambling.²⁶ Inhibition of MPTP formation by cyclosporin A almost completely abrogated agonist-induced PS exposure in healthy control platelets (Figure 4A), in line with observations by Jobe et al.⁹ Together, these data suggest that MPTP formation is required but not

sufficient for agonist-induced platelet PS exposure via TMEM16F. PS exposure induced by collagen/thrombin was only partially impaired in Scott syndrome platelets, confirming previous observations.^{11,27} The residual PS exposure typically was insensitive to inhibition by cyclosporin A (MPTP formation) or inhibition of caspases. In platelets from healthy controls, cyclosporin A was unable to completely suppress collagen-/thrombin-induced PS exposure. Earlier, Dasgupta and colleagues²⁸ reported a partial

Figure 6. Residual collagen-/thrombin-induced PS exposure in Scott syndrome platelets is insensitive to Q-VD-Oph or cyclosporin A (CsA). Annexin (Anx) A5 binding (A) and TMRE loss (B) of washed control and Scott syndrome platelets stimulated by collagen (Col)/thrombin (Thr) (10 $\mu\text{g}/\text{mL}$, 4 nM) for 15 min in the presence of 2 mM CaCl_2 preincubated with vehicle (dimethylsulfoxide), Q-VD-Oph (20 μM), or CsA (4 μM) for 10 min. Mean $\pm$ SEM (n = 3-6); * $P < .05$.

impairment in collagen-/thrombin-induced PS exposure of cyclophilin D-deficient murine platelets. Taken together, these data suggest that 2 distinct pathways to PS exposure become activated by combined collagen/thrombin stimulation: a pathway sensitive to cyclosporin A inhibition and dependent on TMEM16F, and another pathway, which is cyclosporin A insensitive and operational in the absence of TMEM16F. This may explain the complete deficiency of PS exposure in Scott syndrome platelets activated by convulxin/thrombin, as the latter is more dependent on MPTP formation.⁹ The difference in PS exposure between stimulation with collagen/thrombin and convulxin/thrombin is interesting, because both collagen and convulxin are well-recognized GPVI agonists.²¹ We emphasize, however, that this difference has been repeatedly observed on different occasions. The distinct effects observed with collagen, compared with convulxin, may indicate the involvement of platelet collagen receptors other than GPVI (eg, integrin $\alpha 2\beta 1$, GPIb via von Willebrand factor) in collagen-/thrombin-induced PS exposure.²⁹

References

1. Bevers EM, Williamson PL. Phospholipid scramblase: an update. *FEBS Lett.* 2010;**584**:2724-2730.
2. Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of

Acknowledging the inherent limitations of in vitro experimentation, this study demonstrates that TMEM16F-dependent and -independent phospholipid scrambling can be induced in platelets upon apoptosis, aging, and activation. However, it is still unclear whether TMEM16F by itself can act as a phospholipid scramblase or whether this protein has a regulatory function. If TMEM16F proves to be a scramblase itself, this will imply the existence of at least one additional and perhaps more proteins with scramblase activity in platelets. The identification of novel proteins with scramblase activity or the identification of novel regulators of scramblase activity will create new opportunities for interfering with phospholipid scrambling, with consequences for platelet procoagulant activity, life span, and storage.

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phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol.* 1992;**14**:2207-2216.

3. Heemskerk JW, Bevers EM, Lindhout T. Platelet activation and blood coagulation. *Thromb Haemost.* 2002;**88**:186-193.
4. Lentz BR. Exposure of platelet membrane phosphatidylserine regulates blood coagulation. *Prog Lipid Res.* 2003;**42**:423-438.
5. Heemskerk JW, Kuijpers MJ, Munnix IC, Siljander PR. Platelet collagen receptors and coagulation. A characteristic platelet response as possible target for antithrombotic treatment. *Trends Cardiovasc Med.* 2005;**15**:86-92.
6. Rosing J, van Rijn JL, Bevers EM, van Dieijen G, Comfurius P, Zwaal RF. The role of activated human platelets in prothrombin and factor X activation. *Blood.* 1985;**65**:319-332.
7. Arachiche A, Kerbirou-Nabias D, Garcin I, Letellier T, Dachary-Prigent J. Rapid procoagulant phosphatidylserine exposure relies on high cytosolic calcium rather than on mitochondrial depolarization. *Arterioscler Thromb Vasc Biol.* 2009;**29**:1883-1889.
8. Dale GL, Friesse P. Bax activators potentiate coated-platelet formation. *J Thromb Haemost.* 2006;**4**:2664-2669.
9. Jobe SM, Wilson KM, Leo L, Raimondi A, Molkentin JD, Lentz SR, Di Paola J. Critical role for the mitochondrial permeability transition pore and cyclophilin D in platelet activation and thrombosis. *Blood.* 2008;**111**:1257-1265.
10. Zwaal RF, Comfurius P, Bevers EM. Scott syndrome, a bleeding disorder caused by defective scrambling of membrane phospholipids. *Biochim Biophys Acta.* 2004;**1636**:119-128.
11. Munnix IC, Harmsma M, Giddings JC, Collins PW, Feijge MA, Comfurius P, Heemskerk JW, Bevers EM. Storemediated calcium entry in the regulation of phosphatidylserine exposure in blood cells from Scott patients. *Thromb Haemost.* 2003;**89**:687-695.
12. Suzuki J, Umeda M, Sims PJ, Nagata S. Calciumdependent phospholipid scrambling by TMEM16F. *Nature.* 2010;**468**:834-838.
13. Castoldi E, Collins PW, Williamson PL, Bevers EM. Compound heterozygosity for 2 novel TMEM16F mutations in a patient with Scott syndrome. *Blood.* 2011;**117**:4399-4400.
14. Schoenwaelder SM, Yuan Y, Josefsson EC, White MJ, Yao Y, Mason KD, O'Reilly LA, Henley KJ, Ono A, Hsiao S, Willcox A, Roberts AW, Huang DC, Salem HH, Kile BT, Jackson SP. Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function. *Blood.* 2009;**114**:663-666.
15. Zhang H, Nimmer PM, Tahir SK, Chen J, Fryer RM, Hahn KR, Iciek LA, Morgan SJ, Nasarre MC, Nelson R, Preusser LC, Reinhart GA, Smith ML, Rosenberg SH, Elmore SW, Tse C. Bcl-2 family proteins are essential for platelet survival. *Cell Death Differ.* 2007;**14**:943-951.
16. Kile BT. The role of the intrinsic apoptosis pathway in platelet life and death. *J Thromb Haemost.* 2009;**7** Suppl 12:14-217.
17. Kodama T, Takehara T, Hikita H, Shimizu S, Shigekawa M, Li W, Miyagi T, Hosui A, Tatsumi T, Ishida H, Kanto T, Hiramatsu N, Yin XM, Hayashi N. BH3-only activator proteins Bid and Bim are dispensable for Bak/Bax-dependent thrombocyte apoptosis induced by Bcl-xL deficiency: molecular requisites for the mitochondrial pathway to apoptosis in platelets. *J Biol Chem.* 2011;**286**:13905-13913.
18. Mason KD, Carpinelli MR, Fletcher JI, Collinge JE, Hilton AA, Ellis S, Kelly PN, Ekert PG, Metcalf D, Roberts AW, Huang DC, Kile BT. Programmed anuclear cell death delimits platelet life span. *Cell.* 2007;**128**:1173-1186.
19. Schoenwaelder SM¹, Jarman KE, Gardiner EE, Hua M, Qiao J, White MJ, Josefsson EC, Alwis I, Ono A, Willcox A, Andrews RK, Mason KD, Salem HH, Huang DC, Kile BT, Roberts AW, Jackson SP. Bcl-xL-inhibitory BH3 mimetics can induce a transient thrombocytopathy that undermines the hemostatic function of platelets. *Blood.* 2011;**118**:1663-1674.
20. Vogler M, Hamali HA, Sun XM, Bampton ET, Dinsdale D, Snowden RT, Dyer MJ, Goodall AH, Cohen GM. BCL2/BCL-X(L) inhibition induces apoptosis, disrupts cellular calcium homeostasis, and prevents platelet activation. *Blood.* 2011;**117**:7145-7154.
21. Siljander P, Farndale RW, Feijge MA, Comfurius P, Kos S, Bevers EM, Heemskerk JW. Platelet adhesion enhances the glycoprotein VI

- dependent procoagulant response: Involvement of p38 MAP kinase and calpain. *Arterioscler Thromb Vasc Biol.* 2001;**21**:618-627.
22. van der Meijden PE, Feijge MA, Giesen PL, Huijberts M, van Raak LP, Heemskerk JW. Platelet P2Y₁₂ receptors enhance signaling towards procoagulant activity and thrombin generation. A study with healthy subjects and patients at thrombotic risk. *Thromb Haemost.* 2005;**93**:1128-1136.
23. Jackson SP, Schoenwaelder SM. Procoagulant platelets: are they necrotic? *Blood.* 2010;**116**:2011-2018.
24. Zong WX, Thompson CB. Necrotic death as a cell fate. *Genes Dev.* 2006;**20**:1-15.
25. Williamson P, Christie A, Kohlin T, Schlegel RA, Comfurius P, Harmsma M, Zwaal RF, Bevers EM. Phospholipid scramblase activation pathways in lymphocytes. *Biochemistry.* 2001;**40**:8065-8072.
26. Brooks MB, Catalfamo JL, Friesse P, Dale GL. Scott syndrome dogs have impaired coated-platelet formation and calcein-release but normal mitochondrial depolarization. *J Thromb Haemost.* 2007;**5**:1972-1974.
27. Rosing J, Bevers EM, Comfurius P, Hemker HC, van Dieijen G, Weiss HJ, Zwaal RF. Impaired factor X and prothrombin activation associated with decreased phospholipid exposure in platelets from a patient with a bleeding disorder. *Blood.* 1985;**65**:1557-1561.
28. Dasgupta SK, Argaiz ER, Mercado JE, Maul HO, Garza J, Enriquez AB, Abdel-Monem H, Prakasam A, Andreeff M, Thiagarajan P. Platelet senescence and phosphatidylserine exposure. *Transfusion.* 2010;**50**:2167-2175.
29. Nieswandt B, Watson SP. Platelet-collagen interaction: is GPVI the central receptor? *Blood.* 2003;**102**:449-461.

Chapter 7

Survival protein anoctamin-6 controls multiple platelet responses including phospholipid scrambling, swelling and protein cleavage

Mattheij NJ¹, Braun A², van Kruchten R¹, Castoldi E¹, Pircher J³,
Baaten CC¹, Wülling M⁴, Kuijpers MJ¹, Köhler R⁵, Poole AW⁶,
Schreiber R⁷, Vortkamp A⁴, Collins PW⁸, Nieswandt B²,
Kunzelmann K⁷, Cosemans JM¹, Heemskerk JW¹

¹Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), University of Maastricht, Maastricht, the Netherlands; ²Rudolf Virchow Centre & DFG Research Centre for Experimental Biomedicine, University of Würzburg, Würzburg, Germany; ³Walter Brendel Centre of Experimental Medicine and German Centre of Cardiovascular Research, partner site Munich Heart Alliance, Ludwig-Maximilians-Universität München, Germany; ⁴Department of Developmental Biology, Centre for Medical Biotechnology, University of Duisburg-Essen, Germany; ⁵Aragon Institute of Health Sciences I+CS/IIS and ARAID, Zaragoza, Spain; ⁶School of Physiology and Pharmacology, University of Bristol, Bristol, United Kingdom; ⁷Institute of Physiology, University of Regensburg, Regensburg, Germany; ⁸Arthur Bloom Haemophilia Centre, School of Medicine, Cardiff University, Cardiff, United Kingdom

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Abstract

Purpose: The Scott syndrome is a rare bleeding disorder, characterized by altered Ca^{2+} -dependent platelet signaling with defective phosphatidylserine (PS) exposure and microparticle formation, and is linked to mutations in the ANO6 gene, encoding anoctamin-6 (Ano6). We investigated how the complex platelet phenotype of this syndrome is linked to defective expression of anoctamins or other ion channels. **Procedures:** Mice were generated with heterozygous or homozygous deficiency in Ano6, Ano1 or Ca^{2+} -dependent $\text{K}_{\text{Ca}3.1}$ Gardos channel. Platelets from these mice were extensively analyzed on molecular functions, and compared to platelets from a Scott patient. **Findings:** Deficiency in Ano1 or Gardos channel did not reduce platelet responses compared to control mice ($P>0.1$). In two mouse strains, deficiency in Ano6 resulted in reduced viability with increased bleeding time to 28.6 minutes (control 6.4 minutes, $P<0.05$). Platelets from the surviving Ano6-deficient mice resembled Scott patient platelets in: (i) normal collagen-induced aggregate formation ($P>0.05$) with reduced PS exposure (-65 to 90%); (ii) lowered Ca^{2+} -dependent swelling (-80%) and membrane blebbing (-90%); (iii) reduced calpain-dependent protein cleavage (-60%); and (iv) moderately affected apoptosis-dependent PS exposure. **Conclusions:** Mouse deficiency of Ano6 but not of other channels, affects viability and phenocopies the complex changes in platelets from hemostatically impaired Scott patients.

Introduction

The uncommon Scott syndrome is characterized as a mild bleeding disorder associated with a low prothrombin consumption in blood serum. Platelets from Scott patients show a defect in Ca^{2+} -induced phosphatidylserine (PS) exposure and microparticle formation, but unchanged Ca^{2+} signaling and aggregation.¹⁻⁴ For long it has been recognized that the defective PS exposure in blood cells from Scott patients results from impaired phospholipid scrambling, a process that normally abolishes the asymmetric distribution of PS and

phosphatidylethanolamine over the plasma membrane upon persistent elevation of cytosolic Ca^{2+} .⁵⁻⁷ Consequence of the defective PS exposure is a markedly impaired procoagulant activity of platelets, which agrees with the bleeding phenotype.⁸ On the other hand, it is described that PS exposure induced by apoptosis⁹ is no more than little affected in the blood cells from Scott patients.^{10,11}

Recently, in two unrelated Scott syndrome patients, dysfunctional mutations have been identified in the ANO6 gene (alternatively named TMEM16F), which codes for the integral

membrane protein anoctamin 6 (Ano6).^{12,13} In a murine B-cell line model, the silencing of *Ano6* resulted in an impairment of Ca^{2+} -dependent PS exposure.¹² A role of anoctamins as transporters for the negatively charged PS was also confirmed by structural analysis.¹⁴ In addition, there is substantial electrophysiological evidence that Ano6, like other members of the anoctamin family, can operate as a Ca^{2+} -induced ion channel that is permeable to chloride ions, monovalent cations and calcium ions.^{10,15-17} We and others have reported that in blood cells from Scott patients¹⁰ and Ano6-deficient mice¹⁷ this ion conductance is markedly reduced. Hence, the question arises which other (patho)physiological relevant functions are regulated by Ano6 than only Ca^{2+} -dependent phospholipid scrambling. Several observations in the literature are compatible with a broader role of Ano6. For instance, platelets from Scott syndrome patients show reduced Ca^{2+} -dependent microvesiculation,^{1,3} impaired inactivation of the integrin $\alpha_{\text{IIb}}\beta_3$,¹⁸ and defective aging-dependent PS exposure.¹⁹ On the other hand, Scott platelets are not completely devoid of PS exposure and procoagulant activity, when stimulated with Ca^{2+} -mobilizing agonists such as thrombin plus collagen.^{6,11} This suggests involvement of other proteins beside Ano6 in the regulation of Ca^{2+} -

induced phospholipid scrambling. In platelets as well as erythrocytes, a role in PS exposure of has been proposed for volume-sensitive Cl^- channels;²⁰ and for intermediate conductance Ca^{2+} -activated K^+ channels, known as $\text{K}_{\text{Ca}3.1}$ or Gardos channels.^{21,22} Furthermore, overexpression studies suggest that also other members of the anoctamin family can contribute to PS exposure.²³ In particular the isoform Ano1 is of interest, since in erythrocytes it regulates α -hemolysin-induced phospholipid scrambling.²⁴ Hence, the question can be raised if a gene defect in *ANO6* alone is sufficient for the altered blood cell properties in the Scott syndrome. In the present paper, we used several molecular and functional approaches to unravel the apparently multiple and non-redundant functions of Ano6 in platelets and erythrocytes. We compared the alterations in platelet properties of a Scott patient in comparison to platelets from healthy control subjects; and furthermore compared the platelet properties of several new strains of Ano6-deficient mice, as well as from mice lacking Ano1 or Gardos channels. Our data point to a remarkable set of phenotypic changes linked to defective Ano6 expression in both man and mouse, including low residual Ca^{2+} -dependent PS exposure, absence of swelling morphological changes, presence of

alternative Ano6 splice variants, and a moderate bleeding tendency.

Methods

Materials

ABT-737 was obtained from Santa Cruz Biotechnology, annexin A5 labeled with fluorescein isothiocyanate (FITC) was from PharmaTarget; Alexa Fluor-647 (AF647)-labeled annexin A5 and ionomycin were purchased from Invitrogen. The glycoprotein VI agonist convulxin was purified, as described (25). FITC-labeled anti-mouse CD62P (P-selectin) monoclonal antibody (mAb) as

well as phycoerythrin (PE)-conjugated JON/A mAb directed against the active conformation of mouse integrin $\alpha_{IIb}\beta_3$ were from Emfret Analytics. Human α -thrombin was from Sigma-Aldrich. Affinity-purified polyclonal rabbit antibodies directed against human and mouse Ano1 (DOG-1) and Ano6 (G-14 against extracellular domain) were from Santa Cruz, anti- α -tubulin mAb came from Abcam. Rabbit antibody directed against mouse $K_{Ca}3.1$ was obtained as described.²⁶ Sources of other materials were as reported before.¹⁸

Figure 1. Normal thrombus formation with residual PS exposure and abolished ballooning in Scott syndrome. Blood from healthy control subjects or a Scott patient was perfused over a collagen surface for 4 min at 1000 s^{-1} . Formed thrombi were stained with FITC- α CD62P (P-selectin) mAb and AF647-annexin A5 (PS exposure). **A)** Representative phase-contrast and fluorescence images (bars $25\text{ }\mu\text{m}$), zoomed in as indicated (inserts $25 \times 25\text{ }\mu\text{m}$). Arrow indicates non-ballooning platelet with small patches of PS exposure. **B)** Surface-area-coverage (SAC%) of aggregated platelets, P-selectin expression, PS exposure and ballooning. For PS exposure, dotted line indicates background fluorescence (no platelet thrombi). **C)** Western blots of control and Scott platelet lysates, stained for Ano6, and reprobbed with anti- α -tubulin mAb as lane loading control. Means $\pm$ SE ($n = 3$), $*P < 0.05$ (2-way ANOVA).

Blood donors

Blood was obtained from healthy volunteers and an accessible patient with Scott syndrome, after full informed consent (Helsinki declaration), under protocols reviewed by the local ethics committees. The Scott patient has been genotyped as compound heterozygous for two different mutations in the *ANO6* gene (alias *TMEM16F*), with one splicing mutation (IVS6 + 1G→A) causing exon 6 skipping, and another mutation in exon 11 (c.1219insT) leading to a premature stop of translation.¹³ An analysis of splice variants is described in the Supplemental Materials.

Animals

Mouse experiments were approved by the local animal care and use committees. Experiments were performed using genetically modified and corresponding wild-type animals from the same breeding, simultaneously at the same location. Mice with a homozygous deficiency of the Gardos channel $K_{Ca}3.1$ (*Kcnn4*^{-/-}) and wild-type littermates (*Kcnn4*^{+/+}) had a mixed 129Sv/C57BL/6 background, and were generated and genotyped, as described.²⁷ Homozygous *Kcnn4*^{-/-} mice suffer from hypertension, but survive normally.^{27,28} Mice with megakaryocytes and platelets homozygously deficient in anoctamin 1 (*Ano1* gene, alias *Tmem16a*) were generated by crossing *Ano1*^{fl/fl} mice

with transgenic mice expressing a Cre-recombinase after the PF4 promoter (C57BL/6 background). The mice were genotyped using the following nucleotide primers: wild-type *Ano1*^{+/+} (229 bp, 2165 bp in flox) GCAGAAAAGT GCCAGAGACC (forward), TTTCCAATGG CCTAGACCTG (reverse); for *Ano1*^{-/-} (462 bp in flox) ATAGCAGCTT TGCTCCTTCG (forward), CTCGTCCTGC AGTTCATTCA (reverse).

In two laboratories (Würzburg, Regensburg), the AW-382 embryonic stem (ES) cell clone *Ano6*^{gt(AW0382)} (*Ano6*^{AW}, Wellcome Trust Sanger Institute, MGI: 3857206, stock number 020701-UCD) was used to generate mice with genetic deficiency of *Ano6* (alias *Tmem16f*). The ES cell clone contains a β -geo reporter gene trap in intron 3 of *Ano6* which disturbs mRNA transcription and synthesis of the protein. Male chimeras derived from the embryonic stem cell line were bred with C57BL/6 females to generate heterozygous *Ano6*^{AW+/-} mice. The latter were cross-bred at C57BL/6 background to obtain homozygous deficient mice. Presence of the gene trap cassette was detected by PCR using the following primers: TTATCGATGA GCGTGGTGGT TATGC (forward); GCGCGTACAT CGGGCAAATA ATATC (reverse).

In addition, mice from the *Ano6*^{tm1Avor} (*Ano6*^{Avor}) strain were used, generated as described.²⁹ To reduce postnatal lethality of homozygous mutants, *Ano6*^{Avor+/-} mice

were crossed on a C57B6L background to obtain *Ano6^{Avor/-}* animals. Mice were genotyped for heterozygous or homozygous deficiency in *Ano6* expression by PCR.²⁹ Per strain, wild-types were used from the same breeding. Analysis of murine *Ano6* splice variants is described in the Supplemental Materials.

Embryonic development

Inbred, pregnant female *Ano6^{+/-}* mice were sacrificed at gestational days 10.5, 12.5 or 16.5. Viable embryos and death bodies were counted, and embryos were checked for macroscopic abnormalities.

Blood collection and isolation of platelets and erythrocytes

Human blood was collected into PPACK/fragmin for flow perfusion studies, as described before.³⁰ Other samples were collected into trisodium citrate for preparation of PRP³¹ or into acid-citrate-dextrose anticoagulant for preparation of isolated platelets.¹⁸ Washed cells were suspended into HEPES buffer pH 7.45 (10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 5 mM glucose and 0.1% bovine serum albumin); 2 mM CaCl₂ was added directly before the experiments. Cell concentrations were determined with a Beckman Coulter counter.

Blood was obtained from adult mice via orbital puncture under anesthesia, according to the local ethical permissions. Mouse blood was collected into

PPACK/heparin/fragmin for flow perfusion studies, as described before.³² Blood was also collected into trisodium citrate for preparation of PRP.¹⁸ For platelet isolation, mouse blood was taken into 1/6 volume of acid-citrate-dextrose anticoagulant (ACD: 85 mM sodium citrate, 78 mM citric acid, and 11 mM D-glucose). Platelets¹⁸ and erythrocytes²² were separated by centrifugation, washed, and suspended in modified HEPES buffer pH 7.45 (5 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 0.42 mM Na₂HPO₄, 5 mM glucose, and 0.1% bovine serum albumin); 2 mM CaCl₂ was added directly before the experiments. Cell concentrations were determined with a Coulter counter.

Flow cytometric analyses

Suspensions of washed human platelets were activated, as described.¹¹ Washed mouse platelets in suspension (1 x 10¹¹/l)

	<i>Ano1^{+/+}</i>	<i>Ano1^{+/-}</i>	<i>Ano6^{+/+}</i> (AW)	<i>Ano6^{+/+}</i> (AW)
Count (10 ⁶ /μL)	<i>n.d.</i>	<i>n.d.</i>	582 ± 51	590 ± 21
Size (AU)	<i>n.d.</i>	<i>n.d.</i>	324 ± 14	326 ± 12
GPIb	319 ± 4	327 ± 14	343 ± 9	344 ± 10
GPV	279 ± 7	257 ± 2	245 ± 5	251 ± 3
GPVI	54 ± 4	47 ± 5	37 ± 0.7	37 ± 1
GPIX	370 ± 10	351 ± 4	351 ± 5	360 ± 6
α _{IIb} β ₃	487 ± 17	504 ± 12*	391 ± 20	401 ± 11
α ₂	44 ± 2	42 ± 3	42 ± 1	42 ± 1
CD9	1155 ± 27	1116 ± 18	1114 ± 9	1144 ± 18
β ₁	134 ± 4	133 ± 7	113 ± 4	119 ± 3
CLEC2	111 ± 6	119 ± 9	100 ± 3	107 ± 9

Suppl. Table S1. Surface expression of glycoproteins on mouse platelets with homo- or heterozygous deficiency in *Ano1* or *Ano6*. Data are from mouse strain *Ano6^{AW}* and corresponding wild-types. Shown are mean fluorescence intensities, unless indicated otherwise. Means ± SE (*n* = 4).

Supplement Figure S1. Screening for alternative transcripts in the Scott patient. Blood (2.5 ml) was collected from the Scott patient and two normal controls. Total blood cell RNA was isolated using Trizol Reagent and quantified spectrophotometrically. Alternatively spliced *ANO6* transcripts were amplified using transcript-specific primers based on the transcript information available from www.ensembl.org. A) Schematic representation of the human *ANO6* pre-mRNA and mature mRNAs, including alternative transcript 1 (ENST00000441606) and alternative transcript 2 (ENST00000423947). Main transcript corresponds to (ENST00000320560). In alternative transcript 1, exon 1 is replaced by exon 1b (light grey), which contains a viable start codon for initiation of translation. Alternative transcript 2 contains an extra exon 1a (dark grey) between exons 1 and 2. Note that the reading frame from exon 2 onwards is the same as in the main transcript. B) Amplification of different portions of the *Ano6* cDNA in the Scott patient (lanes P), in the two normal control subjects (lanes C1-C2), and in a blank sample (lane B).

were stimulated in the presence of 2 mM CaCl_2 with indicated agonists or vehicle at room temperature. Platelet samples were analyzed for PS after 30 min of activation and labeling with AF647-annexin A5 (1:200).¹⁸ Other labels used were FITC-anti-P-selectin mAb (1:10) to detect P-selectin expression and PE-JON/A mAb (1:10) to detect integrin $\alpha_{IIb}\beta_3$ activation. For apoptosis induction, platelets were stimulated with the BH3 mimetic ABT-737 (1 μM) for 1 h at 37 °C.¹⁸ Washed mouse erythrocytes (1 x 10¹¹/l) were activated with ionomycin in

the presence of 1 mM CaCl_2 at 37 °C. Samples were analyzed with a BD FACS Calibur or BD Accuri C6 flow cytometer. Glycoprotein expression levels on platelets were determined by flow cytometry as described elsewhere.³³ Data analysis was performed using the programs WinMDI or CFlow Plus.

Light transmission measurements

Changes in light transmission were monitored in suspensions of washed platelets (1 x 10¹¹/l), activated with agonists in the presence of CaCl_2 (2 mM)

under constant stirring at 37 °C, using a Chronolog aggregometer. Tirofiban (5 µg/ml) was present if indicated. Hypotonic shock response³⁴ was induced by adding 125 µl of distilled water with(out) CaCl₂ (4 mM) and/or ionomycin (10 µM) to 250 µl PRP. Transmission changes were recorded with a Chronolog aggregometer under stirring at 37 °C.

Whole blood thrombus formation

Collagen-induced thrombus formation was assayed using PPACK/fragmin-anticoagulated human³⁵ or mouse³⁶ blood, basically as described. In brief, blood samples were flowed over a coverslip coated with collagen type-I in a transparent parallel-plate perfusion chamber, at a shear rate of 1000 s⁻¹ for 4

Figure 2. Integrin $\alpha_{IIb}\beta_3$ -independent turbidity changes and reduced integrin cleavage by Scott syndrome platelets. A) Suspensions of washed platelets from control subjects or Scott patient in CaCl₂-containing medium were stimulated with convulxin (Cvx, 100 ng/ml) plus thrombin (Thr, 4 nM) or with ionomycin (Iono, 10 µM) in the presence or absence of tirofiban (2 µg/ml). Shown are representative changes in light transmission ($n = 3$). B) Hypotonic shock response of PRP induced by dilution (33%) in the presence or absence of CaCl₂ (4 mM). Shown are representative traces of changes in light transmission. C) Western blots probed for the intact and cleaved β_3 chain (detected with Ab762 and Ab754, respectively) of platelets stimulated with Cvx/Thr or Iono in the presence of CaCl₂. Blots (representative for 3 performed) were reprobed with anti- α -tubulin mAb as lane loading control. D) Quantification of β_3 chain cleavage (5 min) from densitometric profiles. Means $\pm$ SE ($n = 4$ controls).

	Ano1 ^{+/+}	Ano1 ^{-/-}	Ano6 ^{+/+} (AW)	Ano6 ^{-/-} (AW)	Ano6 ^{+/+} (PAC)	Ano6 ^{-/-} (PAC)	Ano6 ^{-/-} (PAC)
Integrin activat. (%)							
ADP	<i>n.d.</i>	<i>n.d.</i>	42 ± 10	26 ± 5	45 ± 7	44 ± 10	50 ± 9
Thr	80 ± 10	81 ± 9	78 ± 3	69 ± 5	86 ± 5	73 ± 14	80 ± 5
Cvx/Thr	76 ± 11	73 ± 15	72 ± 5	66 ± 5	55 ± 10	56 ± 11	59 ± 1
Ionomycin	<i>n.d.</i>	<i>n.d.</i>	7 ± 2	3 ± 1	6 ± 2	6 ± 4	4 ± 2
P-selectin expr. (%)							
ADP	<i>n.d.</i>	<i>n.d.</i>	10 ± 3	9 ± 2	14 ± 6	3 ± 0.6	2 ± 1
Thr	84 ± 7	81 ± 9	85 ± 4	88 ± 3	76 ± 9	86 ± 8	60 ± 11
Cvx/Thr	82 ± 7	82 ± 8	86 ± 3	85 ± 4	90 ± 5	83 ± 8	84 ± 3
Ionomycin	64 ± 6	61 ± 7	67 ± 4	57 ± 4	70 ± 3	71 ± 6	60 ± 2
PS exposure (%)							
ADP	<i>n.d.</i>	<i>n.d.</i>	0.7 ± 0.1	1 ± 0.3	1 ± 0.2	2 ± 1	1 ± 0.4
Thr	18 ± 3	15 ± 1	15 ± 2	15 ± 6	17 ± 3	11 ± 4	7 ± 3*
Cvx/Thr	53 ± 5	55 ± 3	76 ± 12	63 ± 5	72 ± 5	64 ± 5	15 ± 2*
Ionomycin	58 ± 10	42 ± 9	87 ± 6	92 ± 2	85 ± 2	88 ± 4	5 ± 1*

Suppl. Table S2. Activation properties of mouse platelets homo- or heterozygously deficient in *Ano1* or *Ano6*. Washed platelets from indicated mice were stimulated with ADP (10 μ M), thrombin (Thr 4 nM), convulxin/thrombin (Cvx 100 ng/ml, Thr 4 nM) or ionomycin (iono 10 μ M) plus 2 mM CaCl₂, as indicated. Fractions of platelets binding PE-JON/A mAb (integrin activation), FITC- α CD62P mAb (P-selectin) or AF647-annexin A5 (PS exposure) were assessed after 15 min. Data are from mouse strains *Ano6*^{AW} and *Ano6*^{AWor} and corresponding wild-types. Means $\pm$ SE ($n = 4-6$); * $P < 0.05$ compared to corresponding wild-type.

min. Thrombi formed on the collagen surface were post-stained with AF647-annexin A5, FITC-anti-CD62P mAb and PE-JON/A mAb in (modified) HEPES buffer pH 7.45 supplemented with CaCl₂ (2 mM) and heparin (1 U/ml). Phase-contrast and fluorescence images were captured for analysis of surface area coverage of adherent platelets, and of platelets with active integrins, P-selectin exposure or PS exposure.³⁰ Image analysis was performed using Metamorph software Version 7.5.0.0 (MDS Analytical Technologies).³⁷

Western blot analyses

Washed platelet or erythrocyte suspensions were lysed with ice-cold 4x

lysis buffer (600 mM NaCl, 10 mM Tris, 4 mM EGTA, 4 mM EDTA, 4% NP40). Samples (5 $\times 10^7$ human platelets, 1 $\times 10^8$ mouse platelets) were separated on 8% SDS-PAGE gels, transferred to blotting membranes by semi-dry transfer.¹⁸ Membranes were immunostained with antibodies against K_{Ca}3.1, Ano1, Ano6 (all 1:1000) or integrin β_3 (Ab762, 1:10000; Ab754, 1:1000), and later reprobed with α -tubulin (1:1000). Incubation with secondary horseradish peroxidase-coupled antibody was overnight at 4 °C, and visualization with an ECL system. Quantification was performed by densitometric analysis of stained blots, as described.¹⁸

Statistics

Intervention effects were statistically compared with a paired sample *t* test, using the statistical package for social sciences (SPSS, version 11.0). Groups were compared by 2-way ANOVA (Bonferroni correction, if required).

Results

Residual PS exposure and abolished swelling of Scott platelets during thrombus formation

Platelet aggregation and PS exposure (procoagulant activity) in response to the main collagen receptor, glycoprotein VI (GPVI), can simultaneously be quantified in thrombi formed during whole-blood perfusion over a collagen surface.^{35,38} In flow studies with blood from a Scott patient,¹⁹ we established that platelet adhesion, aggregate formation and α -granule secretion (expression of P-selectin) were similar to the platelet responses of healthy control subjects (Fig. 1A-B). In contrast, collagen-induced PS exposure was greatly reduced, but not completely abolished with the patient blood. Interestingly, the residual PS exposure appeared as patches on platelets with a normal, non-swollen shape (Fig. 1A, *inserts*). This contrasted to the large, ballooning morphology (diameter $\sim 10 \mu\text{m}$) of platelets from control subjects with high PS exposure. Quantification of the recorded microscopic images indicated that

essentially no ballooning platelets were formed with Scott blood (Fig. 1A, *right panel*). Hence, the major defect of GPVI-dependent PS exposure in Scott platelets is accompanied by absence of platelet ballooning.

Identification of human ANO6 splice variants

An explanation for the low PS exposure in patient's platelets may be residual expression of the *ANO6* gene. However, no Ano6 protein could be detected on western blots from Scott platelets (Fig. 1C). This was confirmed by quantitative proteomic analysis,³⁹ showing a >160 -fold reduction of identified Ano6 peptides in the Scott platelets (F. Solari, N. Mattheij, R Zahedi, unpublished data, 2015). On the other hand, in line with a previous report,¹³ Ano6 mRNA was readily detectable in the patient's blood cells. RT-PCR analysis indicated expression of the default transcript as well as two alternatively spliced transcripts 1 and 2 (Supplemental Fig. S1). Due to the nature of the two mutations in the patient's cells (a splicing mutation in intron 6 and a frameshift mutation in exon 11), it is unlikely that these alternative transcripts are translated into a functional Ano6 protein, except for some residual correctly spliced pre-mRNA transcribed from the IVS6+1G>A allele.

Supplement Figure S2. Hypertonic media induced delayed PS exposure and absence of ballooning structures. A)

Control and Scott platelets were activated by ionomycin (10 μ M) for indicated time-points in isotonic (black lines) or hypertonic (grey lines) HEPES buffer and labeled with FITC-annexin A5. Means $\pm$ SE ($n = 4$) B) Representative phase contrast images of platelets from control and Scott platelets stimulated by ionomycin for 15 min in isotonic or hypertonic HEPES buffer (bars = 5 μ m).

Reduced aggregation-independent light-transmission changes and integrin inactivation of Scott platelets

In platelets from control subjects, stimulation with convulxin/thrombin (stimulating GPVI and thrombin receptors) or ionomycin (Ca^{2+} ionophore, conditions as in Fig. 2A) resulted in $51 \pm 3\%$ and $85 \pm 11\%$ of PS-exposing platelets, respectively (means $\pm$ SE, $n = 8$). These values were not influenced by the integrin $\alpha_{\text{IIb}}\beta_3$ inhibitor, tirofiban ($P > 0.30$). In Scott platelets, the same agonists evoked severely reduced but residual PS exposure of $1.7 \pm 0.5\%$ and $2.0 \pm 0.6\%$ platelets ($n = 3$). Light transmission recording with control platelets indicated that convulxin/thrombin and ionomycin provoked rapid clearance of the cell suspensions, which effect was only partly reduced by tirofiban, inhibiting platelet aggregation (Fig. 2A, left panels). In line with earlier data,¹⁸ this suggested that the residual increase in light transmission

was due to shape change from discoid platelets to translucent, ballooned structures. Such ballooned platelets could indeed be observed by microscopy (Suppl. Fig S2). In contrast, Scott platelets showed only limited changes in light transmission after stimulation in the presence of tirofiban (Fig. 2A, right panels). In this case, no ballooned structures were seen (Suppl. Fig. S2). Light transmission changes due to platelet swelling can also be assessed in the hypotonic shock response, which measures the reaction of platelets in plasma to dilution with water.³⁴ Water-induced light transmission changes were comparable for control and Scott platelets, even in the presence of CaCl_2 to stimulate Ca^{2+} -dependent ion fluxes (Fig. 2B). With CaCl_2 present, the late increase in light transmission accompanying balloon formation however was markedly delayed with Scott platelets. To determine a role of

ion influxes, platelets were then stimulated with ionomycin in hypertonic medium. Hypertonia resulted in delayed PS exposure and residual ballooning in control platelets, but failed to induce PS exposure or ballooning in Scott platelets (Suppl. Fig. S2). In control cells, Ca^{2+} -induced PS exposure is accompanied by calpain-mediated

cleavage of the integrin β_3 -chain, which results in inactivation of $\alpha_{IIb}\beta_3$.¹⁸ Similarly, in Scott platelets stimulation with ionomycin resulted in rapid β_3 -chain cleavage which was complete within 5 min (Fig. 2C-D).

However, stimulation with convulxin/thrombin led to a markedly delayed cleavage in the patient's

Figure 3. Unchanged thrombus formation and PS exposure of mice lacking K_{Ca}3.1 Gardos channels. A) Expression of K_{Ca}3.1 protein in platelets from corresponding *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice; control lane refers to murine fibroblasts. Western blots are from cell lysates probed with anti-murine K_{Ca}3.1 mAb, and reprobed with anti- β -actin mAb as loading control. B) Washed platelets from *Kcnn4*^{+/+} or *Kcnn4*^{-/-} mice in CaCl_2 -containing medium were stimulated with convulxin (Cvx, 100 ng/ml) plus thrombin (Thr, 4 nM) or with ionomycin (Iono, 10 μ M), as indicated. Flow cytometric measurement of platelets binding A647-annexin A5, determined after 15 min. C-D) Blood from *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice was perfused over collagen for 4 min at 1000 s^{-1} . Thrombi were stained with PE-JON/A mAb (integrin activation) FITC- α CD62P mAb (P-selectin expression) and AF647-annexin A5 (PS exposure). C) Representative phase-contrast and fluorescence images (bars 25 μ m); D) quantitative analysis of surface-area-coverage (SAC%) of thrombi or fluorescence. Dotted line indicates background fluorescence for PS exposure (no platelet thrombi). Means $\pm$ SE ($n = 5-7$), * $P < 0.05$ (2-way ANOVA).

platelets in comparison to control platelets. Together, these data point to a major defect in Ca^{2+} - and tonic-dependent light transmission clearance (balloon formation) and a partial defect in integrin β_3 -chain cleavage in Ca^{2+} -activated Scott platelets.

Subtle role of murine Gardos channels in platelet PS exposure and thrombus formation

Pharmacological studies with human platelets suggest a role for Gardos ($\text{K}_{\text{Ca}3.1}$) K^+ channels in agonist-induced PS exposure (22). We reinvestigated a role of these channels in *Kcnn4*^{-/-} mice lacking the $\text{K}_{\text{Ca}3.1}$ protein. Western blot analysis indicated weak expression of the 47-kDa channel protein in platelets (Fig.

3A) and erythrocytes (not shown) of wild-type, but not of *Kcnn4*^{-/-} mice. After stimulation with convulxin/thrombin or ionomycin, *Kcnn4*^{+/+} and *Kcnn4*^{-/-} platelets were equally high in PS exposure (Fig. 3B). Whole-blood perfusion over collagen resulted in subtle changes in thrombus formation (Fig. 3C). Whereas the overall deposition of *Kcnn4*-deficient platelets was unchanged ($P>0.1$), these platelets were slightly, but significantly more increased in $\alpha_{\text{IIb}}\beta_3$ activation and PS exposure (Fig. 3D). Jointly, these data point to a minor negative, rather than a positive role for the murine $\text{K}_{\text{Ca}3.1}$ channel in collagen-dependent PS exposure.

Supplement Figure S3. Unchanged thrombus formation by *Ano1*-deficient platelets. Blood from *Ano1*-deficient and corresponding wild-type mice was perfused over collagen for 4 minutes at 1000 s⁻¹. Thrombi were stained with FITC- α CD62P mAb (P-selectin) and AF647-annexin A5 (PS exposure). **A)** Representative phase contrast and fluorescence images (bars = 25 μ m). **B)** Results of quantitative image analysis. Means $\pm$ SE ($n = 7-8$).

Figure 4. Embryonic lethality after breeding of *Ano6*-deficient mice. A) Viability of embryos generated by in-breeding of heterozygous *Ano6^{-/-}* mice (*Ano6^{AW}* strain) at gestational days (E) 10.5, 12.5 or 16.5. B) Embryo presenting with exencephaly (left, E16.5), embryos presenting with major abdominal and intracranial bleeding (E12.5). C-D) Westerns blots of *Ano6* protein expression in platelet lysates from (C) heterozygous *Ano6^{-/-}* mice (*Ano6^{AW}* strain) and (D) homozygous *Ano6^{-/-}* mice (*Ano6^{Avor}* strain).

*No role for murine *Ano1* in platelet PS exposure or thrombus formation*

The isoform *Ano1* is an ubiquitously expressed ion channel displaying Ca^{2+} -dependent Cl^- conductance, which has been implicated in hemolysis-induced PS exposure of erythrocytes.^{24,40} Evaluation of platelets from mice with genetic deficiency in *Ano1* showed normal expression of the main surface glycoproteins (Suppl. Table S1) and normal activation in comparison to wild-type platelets with respect to agonist-induced integrin $\alpha_{\text{IIb}}\beta_3$ activation, P-selectin expression and PS exposure

(Suppl. Table S2). Measurements of whole-blood thrombus formation on collagen demonstrated that platelet deposition, P-selectin expression and PS exposure were similar for *Ano1*-deficient and corresponding wild-type mice (Suppl. Fig. 3, $P > 0.1$).

*Lethality of *Ano6*-deficient mice and identification of *Ano6* splice variants*

Several approaches were followed to obtain mice with genetic deficiency of *Ano6*. In two laboratories, we generated heterozygous *Ano6^{-/-}* mice using the stem cell clone AW-382,

carrying a β -geo reporter gene trap in the *Ano6* gene (*Ano6^{AW}* strain). These mice produced alive offspring with the gene trap insertion (data not shown), although at a lower number than expected. We hence investigated the embryos in heterozygous females at days (E) 10.5, 12.5 and 16.5 of gestation. Morphological examination indicated that, at all time points, most embryos developed normally. However, several embryos were detected as death bodies, with exencephaly, or with signs of abdominal or intracranial bleeding (Fig. 4A-B). Western blot analysis indicated that platelets from surviving *Ano6^{AW/+}* mice with gene trap insertion had significant expression of Ano6 protein

(Fig. 4C), and hence were not homozygously deficient in this gene. In addition, we used heterozygous *Ano6*-deficient mice from the *Ano6^{Avor}* strain, described before.²⁹ Inbreeding resulted in offspring with ~30% of the expected number of *Ano6^{Avor/-}* sucklings. In contrast to the *Ano6^{AW}* strain, no lethality or obvious bleeding was seen in fetuses at days E14.5-18.5. As described,²⁹ several deficient fetuses died around birth, *e.g.* due to asphyxia. However, ~30% of *Ano6^{-/-}* mice survived longer than 2 months (*Ano6^{Avor}* strain), and these were used for phenotypic analysis. RT-PCR analyses of tissues from the surviving *Ano6^{Avor/-}* mice (C57BL/6 background) showed residual Ano6

Supplement Figure S4. Screening for alternative transcripts in *Ano6* deficient mice. A) Schematic representation of the mouse *Ano6* pre-mRNA, including main transcript (ENSMUSG00000064210) and alternative transcript 1 (unpublished). Alternative transcript 1 contains exon 1b as a starting codon. B) (Left panel) cDNA was isolated from the heart, lung and spleen from wild-type and *Ano6^{Avor/-}* mice. Amplification of a *Ano6* cDNA amplicon spanning exons 1-4 yielded expected 282 bp fragment in heart, lung and spleen from control mice (lane 1-3), while *Ano6* deficient mice lack this fragment (lane 4-6). Amplification of a *Ano6* cDNA amplicon spanning exons 2-4 (middle panel) and exon 3-4 (right panel) yielded the expected 217 bp and 142 bp fragments, respectively.

Figure 5. Normal thrombus formation, residual PS exposure and abolished ballooning in the absence of *Ano6*. Blood from corresponding *Ano6*^{+/+} and *Ano6*^{-/-} mice (*Ano6*^{Avor} strain) was perfused over collagen for 4 min at 1000 s⁻¹. Thrombi on the surface were stained as indicated for Fig. 3. **A)** Representative phase-contrast and fluorescence images (bars 25 μ m; inserts 25 x 25 μ m). **B)** Surface area coverage (SAC%) of thrombi or fluorescence from integrin activation, P-selectin expression, PS exposure and ballooning. Dotted line indicates background fluorescence for PS exposure (no platelet thrombi). Means $\pm$ SE ($n = 3$), * $P < 0.05$ (2-way ANOVA).

mRNA expression, which was explained by the presence of alternative transcript 1 (Suppl. Fig. S4). Western blot analysis indicated that in platelets from *Ano6*^{Avor/-} mice the 106 kDa *Ano6* protein was absent (Fig. 4D), thus indicating that the alternative transcript was not efficiently transcribed. Markedly, tail bleed time was prolonged in adult *Ano6*^{Avor/-} mice (28.6 ± 7.5 min) in comparison to *Ano6*^{Avor/+} (4.9 ± 1.8 min) and *Ano6*^{Avor/+} (6.4 ± 1.6 min) animals (means $\pm$ SE, $n = 5-7$, $P < 0.05$).

*Residual PS exposure and abolished swelling of *Ano6*-deficient mouse platelets in thrombus formation*

We then compared activation characteristics of platelets from mice of the *Ano6*^{AW} and *Ano6*^{Avor} strains. Platelets from both types of heterozygous mice expressed normal levels of surface glycoproteins (Suppl. Table S1), and were unchanged in $\alpha_{IIb}\beta_3$ activation, P-selectin expression and PS exposure, when compared to corresponding wild-type platelets (Suppl. Table S2). Flow perfusion experiments, performed with

Ano6^{AW+/-} or *Ano6*^{Avor+/-} blood did not reveal significant changes in parameters of thrombus formation, including PS exposure, in comparison to the wild-type blood (Suppl. Fig. 5-6). On the other hand, isolated erythrocytes from heterozygous *Ano6*^{AW+/-} or *Ano6*^{Avor+/-} mice displayed a significant lower ionomycin-induced PS exposure by $34.0 \pm 6.6\%$ or $31.6 \pm 8.1\%$, respectively, compared to corresponding wild-type erythrocytes (means $\pm$ SE, $n = 4-5$, $P < 0.05$).

We then assessed thrombus formation on collagen using blood from surviving adult homozygous deficient *Ano6*^{-/-} mice (*Ano6*^{Avor} strain). Thrombus formation as

such was unchanged, with similar platelet adhesion, integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression, in comparison to thrombi from wild-type mice (Fig. 5A-B). However, while wild-type thrombi showed 1-2% of the surface as PS exposure, as before,¹⁸ the *Ano6*^{Avor-/-} thrombi displayed a substantial $76.2 \pm 2.6\%$ ($n = 4$, $P < 0.05$) reduction of this parameter (Fig. 5B). High-magnification images indicated residual patches of PS exposure in some of the collagen-bound *Ano6*^{Avor-/-} platelets (Fig. 5A, insert). Notably, these platelets with residual PS exposure were smaller in size than the 8 μ m-ballooning structures seen with wild-type platelets. Image quantification

A *Ano6*^{AW}

B *Ano6*^{AW}

Supplement Figure S5. Unchanged thrombus formation by heterozygous *Ano6*^{AW+/-} platelets. Blood from *Ano6*^{AW+/-} or corresponding wild-type mice was perfused over collagen for 4 min at 1000 s^{-1} . Thrombi were stained with PE-JON/A mAb (integrin activation), FITC- α CD62P mAb (P-selectin) and AF647- annexin A5 (PS exposure). A) Representative microscopic images (bars = $25 \mu\text{m}$). B) Results of quantitative image analysis. Means $\pm$ SE ($n = 5-6$).

Figure 6. Residual Ca^{2+} -dependent PS exposure by *Ano6*-deficient platelets. A-B) Washed platelets from corresponding *Ano6^{+/+}* and *Ano6^{-/-}* mice (*Ano6^{Avor}* strain) were stimulated in CaCl_2 -containing medium with convulxin (Cvx 100 ng/ml) plus thrombin (Thr, 4 nM) for 30 min. C-D) Washed platelets were stimulated with the BH3 mimetic ABT-737 (10 μM) for 1 h. Shown are representative histograms of AF647-annexin A5 binding after stimulation (black lines) or vehicle controls (grey lines). Markers M1, M2 and M3 indicate fractions of platelets with no, moderate or high annexin A5 binding, respectively. Means $\pm$ SE ($n = 3-4$), * $P < 0.05$ vs. wild-type (2-way ANOVA).

further learned that platelet ballooning was essentially abolished in *Ano6^{Avor-/-}* blood samples (Fig. 5B).

Activation studies indicated that ADP-, thrombin- and convulxin-induced integrin activation and P-selectin expression were unaffected in washed *Ano6^{Avor-/-}* platelets (Suppl. Table S2). On the other hand, in these knockout platelets PS exposure in response to ionomycin (data not shown) or convulxin/thrombin (Fig. 6A-B) was strongly impaired. In ~10% of the platelets residual, low PS exposure was

observed after stimulation with convulxin/thrombin.

Human Scott platelets display a partly reduced PS exposure in response to the apoptosis-stimulating agent ABT-737.¹¹ In particular, low cytometric analysis of ABT-737-treated patient's platelets indicated a reduced fraction with high PS exposure (M3 fraction), and an increased fraction with moderate PS exposure (M2 fraction), which can form at minimal or low Ca^{2+} rises. We studied this ABT-737-induced response in *Ano6^{Avor-/-}* platelets, and noted that, similarly, the M2 fraction

(moderate PS exposure) was increased at the expense of the M3 fraction (high PS exposure), when compared to wildtype control platelets (Fig. 6C-D).

Impaired aggregation-independent light transmission changes and integrin inactivation of Ano6-deficient mouse platelets

Similarly to human platelets, wild-type mouse platelets stimulated with convulxin/thrombin or ionomycin, showed light transmission changes in the presence of tirofiban to block platelet aggregation due to clearance of the platelet suspension (Fig. 7A). This clearance was substantially lower (-80%) for Ano6-deficient platelets. In PRP, we monitored the dilution-induced hypotonic shock response, which was similar for Ano6-deficient and wild-type platelets (Fig. 7B). Yet, with CaCl₂ present, the later increase in light transmission accompanying balloon formation was greatly delayed in the deficient platelets. Finally, we determined the cleavage of the integrin β_3 -chain in response to convulxin/thrombin, which remained partial for 30 min in *Ano6^{Avor-/-}* platelets (reduction to -60%), whereas it was complete in this time frame in *Ano6^{Avor+/+}* platelets (Fig. 7C-D). Ionomycin-induced β_3 -chain cleavage was not different in the presence or absence of Ano6. In control experiments, we established that the β_3 -chain cleavage was annulled by

the calpain inhibitor calpeptin, as described before,¹⁸ indicating it was due to calpain-dependent proteolytic activity. Together, these findings demonstrate a major deficiency in Ca²⁺-dependent light transmission clearance (balloon formation) and a partial defect in integrin β_3 -chain cleavage in platelets from the Ano6-deficient mice.

Discussion

In the present paper, we compared the activation properties of human Scott platelets with those of mouse platelets lacking one of the transmembrane proteins previously linked to PS exposure, the K_{Ca}3.1 Gardos channels or the anoctamins 1 or 6. Although pharmacological evidence (using clotrimazol and charybdotoxin) has suggested a positive role of the K_{Ca}3.1 channels in PS exposure in human platelets,²² we could not confirm this in *Kcnn4^{-/-}* mice. Instead, upon thrombus formation on collagen, K_{Ca}3.1 deficiency increased PS exposure along with integrin activation, suggesting that this channel suppresses rather than stimulates platelet activation. Assuming that the K_{Ca}3.1 channels act similarly in human and mouse platelets, the difference with previously published data,^{21,22} might be explained by off-target effects of the pharmacological inhibitors used.

The anoctamin Ano1 can function as a Ca^{2+} -activated Cl^- channel,^{16,23} and has been implicated in PS exposure in erythrocytes treated with α -hemolysin.²⁴ In smooth muscle cells, Ano1 can regulate cell proliferation.⁴⁰ In the present experiments, we could not confirm a role of Ano1 in Ca^{2+} -dependent PS exposure, given that *Ano1*^{-/-} mouse platelets responded normally to collagen, convulxin/thrombin or ionomycin. Earlier studies indicated that murine deficiency in *Ano6* affects mineral deposition in skeletal tissue²⁹ and arterial thrombus formation *in vivo*.¹⁷ By cross-breeding we could obtain viable and adult *Ano6*^{-/-}

offspring from mice of the *Ano6*^{Avor}, but not of the *Ano6*^{AW} strain. Although this remains to be confirmed, the survival of the *Ano6*^{Avor/-} mice might be related to the expression of alternative *Ano6* mRNA transcripts in key tissues. On the other hand, heterozygous *Ano6*^{AW+/-} and *Ano6*^{Avor+/-} mice survived normally without obvious phenotypic defects. In the heterozygous mice, thrombus formation and platelet activation properties were unchanged in our hands, but heterozygous erythrocytes from both lines showed a reduced ionomycin-induced PS exposure. Using blood from the surviving *Ano6*^{Avor/-} mice, we found

Supplement Figure S6. Unchanged thrombus formation by heterozygous *Ano6*^{Avor} platelets. Blood from heterozygous *Ano6*^{Avor} or corresponding wild-type mice was perfused over collagen for 4 min at 1000 s-1. Thrombi were stained with PE-JON/A mAb (integrin activation), FITC- α CD62P mAb (P-selectin) and AF647-annexin A5 (PS exposure). A) Representative microscopic images (bars = 25 μ m). B) Results of quantitative image analysis. Mean $\pm$ SE ($n = 4$).

that collagen-dependent thrombus formation was unchanged in terms of platelet deposition, aggregation, integrin activation and secretion. On the other hand, collagen-dependent PS exposure in platelets was greatly but not completely reduced, and morphological changes to a ballooning structure were abolished. A similar, combined defect in platelet PS exposure and ballooning was observed with blood from the Scott patient.

Phenotypic resemblance of the *Ano6*^{Avor} and patient platelets was also noticed

from flow cytometric studies. In both *Ano6*^{-/-} and Scott platelets, PS exposure was mostly, yet incompletely reduced after convulxin/thrombin treatment, and was slightly diminished upon apoptosis stimulation with ABT-737. Furthermore, the platelets from *Ano6*-deficient mice and the Scott patient displayed a reduction in convulxin/thrombin-induced integrin β_3 cleavage, likely due to reduced calpain activation.¹⁸ Together, these data indicate that the functional and morphological alterations found in

Figure 7. Integrin $\alpha_{IIb}\beta_3$ -independent turbidity changes and reduced integrin cleavage by *Ano6*-deficient platelets. A) Suspensions of washed platelets from corresponding *Ano6*^{+/+} and *Ano6*^{-/-} mice (*Ano6*^{Avor} strain) in CaCl₂-containing medium were stimulated with convulxin (Cvx, 100 ng/ml) plus thrombin (Thr, 4 nM) or with ionomycin (Iono, 10 μ M) in the presence or absence of tirofiban (2 μ g/ml). Shown are representative changes in light transmission ($n = 3$). B) Hypotonic shock response of PRP by water dilution (33%) in the presence or absence of CaCl₂ (4 mM). Shown are representative traces of changes in light transmission. C-D) Western blots probed for the intact and cleaved integrin β_3 chain (detected with Ab762 and Ab754, respectively) of platelets stimulated with Cvx/Thr or Iono (concentrations as above) in the presence of CaCl₂. Blots (representative for 3 performed) were reprobed with anti- α -tubulin mAb as lane loading control. D) Quantification of β_3 chain cleavage from densitometric profiles. Means $\pm$ SE ($n = 3$), * $P < 0.05$.

platelets from the Scott patient are quite well phenocopied in platelets from *Ano6*-deficient mice.

As summarized elsewhere,^{17,23,41,42} *Ano6* can function not only as a Ca^{2+} -dependent phospholipid scramblase, but also as a chloride and cation channel. The inability of *Ano6*-deficient platelets to form balloons - like Scott platelets - suggests a defect in Ca^{2+} -dependent ion influx and swelling. This idea is confirmed by our finding that a hypertonic environment markedly delays the PS exposure and balloon formation. The swelling seems to be a consequence of multiple ion influxes, as we found that specific depletion of Cl^- or Na^+ ions was without effect (N. Mattheij, unpublished 2014). On the other hand, it should be noted that also other Ca^{2+} channels, in particular *Orai1* and *TRPC* isoforms, can contribute to PS exposure in platelets.^{43,44}

The Scott syndrome has been described as a moderate bleeding disorder, with hemorrhagic episodes only after trauma or childbirth.^{2,3} Although the syndrome is rare (with 3 patients characterized so far), it is likely underdiagnosed. The present data with *Ano6^{Avor/-}* mice - in spite of a reduced survival - point to limited hemostatic insufficiency, as mouse tail bleed times were increased. The residual platelet PS exposure, and delayed thrombin and fibrin generation in addition, may offer an explanation for

the limited severity of bleeding. Interestingly, we have identified in both mouse and human samples alternative splice variants of *Ano6*. In agreement with this finding, also other authors describe the expression of *Ano6* splice variants - which differ in their N-terminal cytoplasmic domains, but are similar in PS scrambling activities -, in different human tissues.⁴⁵ In man, the nature of the two *ANO6* gene mutations in the Scott patient leads to impaired translation of all identified splice variants. In mouse, the tissue distribution of the splice variants is still unclear. These variants might be of functional importance during embryonic development and at later life. On the other hand, we could not detect *Ano6* protein expression, and hence find no evidence for a role of these splice variants, in the residual PS exposure in human Scott syndrome or mouse *Ano6^{Avor/-}* platelets. Not unlikely, other Ca^{2+} -dependent anoctamin isoforms,²³ enforced by *Orai1* and *TRPC* Ca^{2+} entry channels,^{43,44} may account for the low PS exposure in the absence of *Ano6* channels.

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References

1. Sims PJ, Wiedmer T, Esmon CT, Weiss HJ, Shattil SJ. Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome: an isolated defect in platelet procoagulant activity. *J. Biol. Chem* 1989;**264**:17049-17057.
2. Toti F, Satta N, Fressinaud E, Meyer D, Freyssinet JM. Scott syndrome, characterized by impaired transmembrane migration of procoagulant phosphatidylserine and haemorrhagic complications, is an inherited disorder. *Blood* 1996;**87**:1409-1415.
3. Satta N, Toti F, Fressinaud E, Meyer D, Freyssinet JM. Scott syndrome: an inherited defect of the procoagulant activity of platelets. *Platelets* 1997;**8**:117-124.
4. Munnix IC, Harmsma M, Giddings JC, Collins PW, Feijge MA, Comfurius P, Heemskerk JW, Bevers EM. Store-mediated calcium entry in the regulation of phosphatidylserine exposure in blood cells from Scott patients. *Thromb. Haemost.* 2003;**89**:687-695.
5. Bevers EM, Comfurius P, Zwaal RF. Changes in membrane phospholipid distribution during platelet activation. *Biochim. Biophys. Acta*. 1983;**736**:57-66.
6. Zwaal RF, Schroit AJ. Pathophysiological implications of membrane phospholipid asymmetry in blood cells. *Blood* 1997;**89**:1121-1132.
7. Williamson P, Christie A, Kohlin T, Schlegel RA, Comfurius P, Harmsma M, Zwaal RF, Bevers EM. Phospholipid scramblase activation pathways in lymphocytes. *Biochemistry* 2001;**40**:8065-8072.
8. Heemskerk JW, Mattheij NJ, Cosemans JM. Platelet-based coagulation: different populations, different functions. *J. Thromb. Haemost.* 2013;**11**:2-11.
9. Schoenwaelder SM, Yuan Y, Josefsson EC, White MJ, Yao Y, Mason KD, O'Reilly LA, Henley KJ, Ono A, Hsiao S, Willcox A, Roberts AW, Huang DC, Salem HH, Kile BT, Jackson SP. Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function. *Blood* 2009;**114**:663-666.
10. Kmit A, van Kruchten R, Ousingsawat J, Mattheij NJ, Senden-Gijsbers B, Heemskerk JW, Schreiber R, Bevers EM, Kunzelmann K. Calcium-activated and apoptotic phospholipid scrambling induced by Ano6 can occur independently of Ano6 ion currents. *Cell Death Dis.* 2013;**4**:e611.
11. Van Kruchten R, Mattheij NJ, Saunders C, Feijge MA, Swieringa F, Wolfs JL, Collins PW, Heemskerk JW, Bevers EM. Both TMEM16F-dependent and TMEM16F-independent pathways contribute to phosphatidylserine exposure in platelet apoptosis and platelet activation. *Blood* 2013;**121**:1850-1857.
12. Suzuki J, Umeda M, Sims PJ, Nagata S. Calcium-dependent phospholipid scrambling by TMEM16F. *Nature* 2010;**468**:834-838.
13. Castoldi E, Collins PW, Williamson PL, Bevers EM. Compound heterozygosity for 2 novel TMEM16F mutations in a patient with Scott syndrome. *Blood* 2011;**117**:4399-4400.
14. Brunner JD, Lim NK, Schenck S, Duerst A, Dutzler R. X-ray structure of a calcium-activated TMEM16 lipid scramblase. *Nature* 2014;**516**:207-212.
15. Martins JR, Faria D, Kongsuphol P, Reisch B, Schreiber R, Kunzelmann K. Anoctamin 6 is an essential component of the outwardly rectifying chloride channel. *Proc. Natl. Acad. Sci. U.S.A.* 2011;**108**:18168-18172.
16. Tian Y, Schreiber R, Kunzelmann K. Anoctamins are a family of Ca²⁺-activated Cl⁻ channels. *J. Cell Sci.* 2012;**125**:4991-4998.
17. Yang H, Kim A, David T, Palmer D, Jin T, Tien J, Huang F, Cheng T, Coughlin SR, Jan YN, Jan LY. TMEM16F forms a Ca²⁺-activated cation channel required for lipid scrambling in platelets during blood coagulation. *Cell* 2012;**151**:111-122.
18. Mattheij NJ, Gilio K, van Kruchten R, Jobe SM, Wieschhaus AJ, Chishti AH, Collins P, Heemskerk JW, Cosemans JM. Dual mechanism of integrin $\alpha_{IIb}\beta_3$ closure in procoagulant platelets. *J. Biol. Chem.* 2013;**288**:13325-13336.
19. Clark SR, Thomas CP, Hammond VJ, Aldrovandi M, Wilkinson GW, Hart KW, Murphy RC, Collins PW, O'Donnell VB. Characterization of platelet aminophospholipid externalization reveals fatty acids as molecular determinants that regulate

- coagulation. *Proc. Natl. Acad. Sci. U.S.A.* 2013;**110**:5875-5880.
20. Okada Y, Shimizu T, Maeno E, Tanabe S, Wang X, Takahashi N. Volume-sensitive chloride channels involved in apoptotic volume decrease and cell death. *J. Membr. Biol.* 2006;**209**:21-29.
21. Lang PA, Kaiser S, Myssina S, Wieder T, Lang F, Huber SM. Role of Ca^{2+} -activated K^+ channels in human erythrocyte apoptosis. *Am. J. Physiol.* 2003;**285**:C1553-1560.
22. Wolfs JL, Wielders SJ, Comfurius P, Lindhout T, Giddings JC, Zwaal RF, Bevers EM. Reversible inhibition of the platelet procoagulant response through manipulation of the Gardos channel. *Blood* 2006;**108**:2223-2238.
23. Suzuki J, Fujii T, Ishihara K, Kuba H, Nagata S. Calcium-dependent phospholipid scramblase activity of TMEM16 protein family members. *J. Biol. Chem.* 2013;**288**:13305-13316.
24. Skals M, Jensen UB, Ousingsawat J, Kunzelmann K, Leipziger J, Praetorius HA. Escherichia coli α -hemolysin triggers shrinkage of erythrocytes via $\text{K}_{\text{Ca}3.1}$ and TMEM16A channels with subsequent phosphatidylserine exposure. *J. Biol. Chem.* 2010;**285**:15557-15565.
25. Siljander P, Farndale RW, Feijge MA, Comfurius P, Kos S, Bevers EM, Heemskerk JW. Platelet adhesion enhances the glycoprotein VI-dependent procoagulant response: Involvement of p38 MAP kinase and calpain. *Arterioscler. Thromb. Vasc. Biol.* 2001;**21**:618-627.
26. Grgic I, Kaistha BP, Paschen S, Kaistha A, Busch C, Si H, Köhler K, Elsässer HP, Hoyer J, Köhler R. Disruption of the Gardos channel ($\text{K}_{\text{Ca}3.1}$) in mice causes subtle erythrocyte macrocytosis and progressive splenomegaly. *Pflügers Arch.* 2009;**458**:291-302.
27. Si H, Heyken WT, Wolffe SE, Tysiac M, Schubert R, Grgic I, Vilianovich L, Giebing G, Maier T, Gross V, Bader M, de Wit C, Hoyer J, Kohler R. Impaired endothelium-derived hyperpolarizing factor-mediated dilations and increased blood pressure in mice deficient of the intermediate-conductance Ca^{2+} -activated K^+ channel. *Circ. Res.* 2006;**99**:537-544.
28. Brahler S, Kaistha A, Schmidt VJ, Wolffe SE, Busch C, Kaistha BP, Kacik M, Hasenau AL, Grgic I, Si H, Bond CT, Adelman JP, Wulff H, de Wit C, Hoyer J, Kohler R. Genetic deficit of SK3 and IK1 channels disrupts the endothelium-derived hyperpolarizing factor vasodilator pathway and causes hypertension. *Circulation* 2009;**119**:2323-2332.
29. Ehlen HW, Chinenkova M, Moser M, Munter HM, Krause Y, Gross S, Brachvogel B, Wuelling M, Kornak U, Vortkamp A. Inactivation of anoctamin-6/Tmem16f, a regulator of phosphatidylserine scrambling in osteoblasts, leads to decreased mineral deposition in skeletal tissues. *J. Bone Miner. Res.* 2013;**28**:246-259.
30. Van Kruchten R, Cosemans JM, Heemskerk JW. Measurement of whole blood thrombus formation using parallel-plate flow chambers: a practical guide. *Platelets* 2012;**23**:229-242.
31. Van der Meijden PE, Feijge MA, Swieringa F, Gilio K, Nergiz-Unal R, Hamulyak K, Heemskerk JW. Key role of integrin $\alpha_{\text{IIb}}\beta_3$ signaling to Syk kinase in tissue factor-induced thrombin generation. *Cell. Mol. Life Sci.* 2012;**69**:3481-3492.
32. Kuijpers MJ, van der Meijden PE, Feijge MA, Mattheij NJ, May F, Govers-Riemslog J, Meijers JC, Heemskerk JW, Renné T, Cosemans JM. Factor Xlla regulates the pathological process of thrombus formation on ruptured plaques. *Arterioscler. Thromb. Vasc. Biol.* 2014;**34**:1674-1680.
33. Deppermann C, Cherpokova D, Nurden P, Schulz JN, Thielmann I, Kraft P, Vogtle T, Kleinschnitz C, Dutting S, Krohne G, Eming SA, Nurden AT, Eckes B, Stoll G, Stegner D, Nieswandt B. Gray platelet syndrome and defective thrombo-inflammation in Nbeal2-deficient mice. *J. Clin. Invest.* 2013, pii: 69210.
34. Holme S, Moroff G, Murphy S. A multi-laboratory evaluation of in vitro platelet assays: the tests for extent of shape change and response to hypotonic shock. *Transfusion* 1998;**38**:31-40.
35. De Witt SM, Swieringa F, Cavill R, Lamers MM, van Kruchten R, Mastenbroek T, Baaten C, Coort S, Pugh N, Schulz A, Scharer I, Jurk K, Zieger B, Clemetson KJ, Farndale RW, Heemskerk JW, Cosemans JM. Identification of platelet function defects by multi-parameter assessment of thrombus formation. *Nat. Commun.* 2014;**5**:4257.

36. Gilio K, Munnix IC, Mangin P, Cosemans JM, Feijge MA, van der Meijden PE, Olieslagers S, Chrzanowska-Wodnicka MB, Lillian R, Schoenwaelder S, Koyasu S, Sage SO, Jackson SP, Heemskerk JW. Non-redundant roles of phosphoinositide 3-kinase isoforms α and β in glycoprotein VI-induced platelet signaling and thrombus formation. *J. Biol. Chem.* 2009;**285**:33750-33762.
37. De Witt S, Swieringa F, Cosemans JM, Heemskerk JW. Thrombus formation on microspotted arrays of thrombogenic surfaces. *Nat. Protocol Exchange* 2014, 3309.
38. Kuijpers MJ, Schulte V, Bergmeier W, Lindhout T, Brakebusch C, Offermanns S, Fässler R, Heemskerk JW, Nieswandt B. Complementary roles of glycoprotein VI and $\alpha_2\beta_1$ integrin in collagen-induced thrombus formation in flowing whole blood ex vivo. *FASEB J.* 2003;**17**:685-687.
39. Burkhardt JM, Vaudel M, Gambaryan S, Radau S, Walter U, Martens LJ, Sickmann A, Zahedi RP. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood* 2012;**120**:e73-e82.
40. Wang M, Yang H, Zheng LY, Zhang Z, Tang YB, Wang GL, Du YH, Lv XF, Liu J, Zhou JG, Guan YY. Downregulation of TMEM16A calcium-activated chloride channel contributes to cerebrovascular remodeling during hypertension by promoting basilar smooth muscle cell proliferation. *Circulation* 2012;**125**:697-707.
41. Kunzelmann K, Nilius B, Owsianik G, Schreiber R, Ousingsawat J, Sirianant L, Wanitchakool P, Bevers EM, Heemskerk JW. Molecular functions of anoctamin 6 (TMEM16F): a chloride channel, cation channel, or phospholipid scramblase? *Pflügers Arch.* 2014;**466**:407-414.
42. Shimizu T, Ichara T, Sato K, Fujii T, Sakai H, Okada Y. TMEM16F is a component of a Ca^{2+} -activated Cl^- channel but not a volume-sensitive outwardly rectifying Cl^- channel. *Am. J. Physiol.* 2013;**304**:C748-759.
43. Harper MT, Camacho-Londono JE, Quick K, Camacho-Londono J, Flockerzi V, Philipp SE, Birnbaumer L, Freichei M, Poole AW. Transient receptor potential channels function as a coincidence signal mediating phosphatidylserine exposure. *Sci. Sign.* 2013;**6**:ra50.
44. Van Kruchten R, Braun A, Feijge MA, Kuijpers MJ, Rivera-Galdos R, Kraft P, Stoll G, Kleinschnitz G, Bevers EM, Nieswandt B, Heemskerk JW. Antithrombotic potential of blockers of store-operated calcium channels in platelets. *Arterioscler. Thromb. Vasc. Biol.* 2012;**32**:1717-1723.
45. Scudieri P, Caci E, Venturini A, Sondo E, Pianigiani G, Marchetti C, Ravazzolo R, Pagani F, Galletta LJ. Ion channel and lipid scramblase activity associated with expression of TMEM16F/ANO6 isoforms. *J. Physiol.* 2015;**593**:3829-3948.

Chapter 8

Altered protein expression, phosphorylation and cleavage in platelets from rare Scott syndrome patient revealed by advanced proteomics analysis

Mattheij NJ^{1*}, Solari FA^{2*}, Burkhardt J², Swieringa F¹, Collins PW³, Cosemans JM¹, Sickmann A², Heemskerk JW^{1*}, Zahedi RP^{2*}

¹Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands, ²Leibniz-Institut für Analytische Wissenschaften-ISAS-e.V., Dortmund, Germany, ³Arthur Bloom Haemophilia Centre, School of Medicine, Cardiff University, Cardiff, United Kingdom

*Equal contribution
Submitted

Abstract

Introduction: The Scott syndrome is a rare bleeding disorder characterised by mutations in the transmembrane protein, anoctamin-6. Platelets from Scott patients are impaired in Ca^{2+} -dependent phosphatidylserine exposure, membrane blebbing and microparticle formation. We hypothesized that also altered protein levels and post-translational protein modifications contribute to this complex phenotype.

Methods: Well-purified washed platelets from healthy control donors and a Scott patient were left unstimulated or were stimulated with thrombin. Platelets were also stimulated with the strong Ca^{2+} -elevating agonists, convulxin/thrombin or ionomycin, which in case of Scott platelets were greatly deficient in phosphatidylserine exposure and membrane blebbing. Samples were used for advanced quantitative (phospho)proteomics analysis and determination of neo-N-termini representing proteolytic cleavage sites.

Results: Quantitative proteomics analysis (2,225 unique proteins) indicated 125 proteins with decreased expression and 88 proteins with increased expression levels in Scott platelets compared to control platelets. Changed expression was most frequently observed in membrane receptors & channels, mitochondrial proteins, metabolic and signalling & adapter proteins, such in accordance with the relatively high numbers of proteins in these classes. In Scott platelets, anoctamin-6 could not be detected, whereas the channel protein aquaporin-1 was highly upregulated. Analysis of 1,566 unique phosphopeptides (corresponding to 716 proteins) indicated major differences in Scott and control platelets after stimulation with convulxin/thrombin or ionomycin. A limited number of Scott proteins were decreased in phosphorylation (*e.g.*, proteins linked to the cytoskeleton or regulating phosphatidylserine exposure); a much higher number of Scott proteins were increased in phosphorylation (*e.g.*, cytoskeleton-linked, membrane and signalling proteins). In total 1,613 neo-N-termini were identified in the Scott and control platelets, 180 of which were confirmed to be calpain-regulated (corresponding to 106 proteins). A distinct set of 23 neo-N-termini (corresponding to 23 proteins) was identified as caspase-regulated. In the Scott platelets, most calpain-produced neo-N-termini were down-regulated after convulxin/thrombin stimulation (in particular from cytoskeleton-linked and key signalling proteins), and upregulated after ionomycin stimulation (a variety of proteins).

Conclusion: Advanced proteomic profiling provides novel insight into the altered protein composition and post-translational protein machinery explaining the major Ca^{2+} - and cytoskeleton-dependent membrane alterations in Scott syndrome platelets.

Introduction

The Scott syndrome is a very rare, moderately mild bleeding disorder, identified in the clinic by a reduced prothrombin consumption of the blood serum. The syndrome is characterised by a congenital deficiency in calcium-dependent phospholipid scrambling.^{1,2} As a result of this defect, platelets and other blood cells are incapable to externalise the aminophospholipids, phosphatidylserine (PS) and phosphatidylethanolamine in response to strong, Ca^{2+} -mobilising agents.³⁻⁵ In platelets, the exposure of PS appears to be regulated by a high and persistent elevation in cytosolic Ca^{2+} or, alternatively, in a less Ca^{2+} -dependent way by apoptosis.^{6,7} Surface exposure of PS induces the binding of multiple coagulation factors to the platelet surface with as a result a potentially increased generation of thrombin.^{8,9} Defective PS exposure of Scott cells can hence explain the bleeding phenotype. Recently, a critical role was reported for the transmembrane protein anoctamin-6 (gene *ANO6*, alias *TMEM16F*), in the Ca^{2+} -dependent PS exposure of platelets.¹⁰ A role of this protein in the Scott syndrome was confirmed by the finding of dysfunctional mutations in the *ANO6* gene of two unrelated Scott patients.^{10,11} This anoctamin has also been identified as a Ca^{2+} -dependent ion channel with permeability to chloride

ions and cations.^{5,12} In one of the Scott patients - the only one healthy in the world and investigated in the current paper -, a different mutation is present in either of the alleles: a transition at the first nucleotide of intron 6 (IVS6 + 1G→A), disrupting the splice site consensus sequence of intron 6, and a single-nucleotide insertion in exon 11 (c.1219insT), predicting a frame shift at codon 411.¹¹ Both mutations are not considered to be compatible with normal translation.

Recent findings have indicated that the phenotype of Scott platelets is more complex than earlier anticipated. In the patient's platelets, agonist-induced PS exposure is greatly diminished but not completely absent, while apoptosis-induced PS exposure is also reduced.¹³ Furthermore, after Ca^{2+} elevation Scott platelets display: a reduced microparticle formation; a lack of membrane blebbing and formation of ballooning structures – responses that are strongly associated with PS exposure;¹⁴ diminished platelet-dependent fibrin formation;¹⁵ and alterations in phospholipid profiles implicated in membrane scrambling.¹⁶ We hypothesized that this complex phenotypical change is a consequence of substantial alterations in platelet signal transduction, which can be directly or indirectly related to a deficiency in anoctamin-6 expression.

To investigate the phenotype of Scott

Figure 1. Strategy and workflow for combined determination of platelet quantitative (phospho)proteomics.

Purified washed platelets from a healthy donor and the Scott patient remained resting or were stimulated by thrombin, convulxin/thrombin or ionomycin for 30 minutes, as described in the methods section. After lysis and digestion with trypsin, all the samples were subjected to individual labelling with 8-plex iTRAQ reagent (113, 114, 115, 116, 117, 118, 119 and 121). For assessment of the quantitative proteome and the phosphoproteome, all samples were pooled in a 1:1 ratio; 10% of the pooled sample was used for global proteome analysis and the remaining 90% for phosphoproteome analysis. 500 µg of the eight samples were labelled with one of the stable isotopic iTRAQ labels, *i.e.* iTRAQ 113, 114, 115, 116, 117, 118, 119 or 121. The labelled samples were multiplexed and fractionated in two different ways. Fractions were analysed by LC-MS on Orbitrap XL. To increase the coverage of the human platelet proteome, a two-step TiO₂ enrichment for phosphopeptides first with low and then with high specificity was conducted. Samples analysed by nano-LC-MS/MS on a Q-Exactive mass spectrometer.

platelets in more detail, we performed a quantitative analysis of the protein expression levels and of phosphorylation state in Scott platelets. We used highly sensitive quantitative mass spectrometric techniques, which have previously led to the identification and quantification of the majority of platelet proteins.¹⁷⁻²⁰ Furthermore, we determined the calpain cleavage pattern of activated Scott platelets following a novel analysis of neo-N-terminal protein cleavage sites. By taking advantage of recent technologies, all these post-translational modifications could be

assessed from small platelet samples and related to changes in platelet function.

Methods

Blood collection and platelet isolation

Blood was obtained from four healthy volunteers and a Scott syndrome patient^{11,13} after full informed consent (Helsinki declaration). Protocols were approved by the local Medical Ethics Committees. Blood samples were collected into 1/6 volume of acid-citrate glucose solution (ACD, 80 mM trisodium citrate, 52 mM citric acid and 180 mM glucose). Platelet-rich plasma (PRP) was obtained by

Assumed function class	Control #/class %	Quantitative Scott vs. control		Phospho sites Scott vs. control	
		decreased %	increased %	decreased %	increased %
1 Cytoskeleton actin-myosin	3.1	3.2 (4)	3.4 (3)	16.2 (6)	9.7 (60)
2 Cytoskeleton intermediate	0.3	0.8 (1)	0	0	0
3 Cytoskeleton microtubule	2.5	0.8 (1)	4.5 (4)	5.4 (2)	4.8 (30)
4 Cytoskeleton receptor-linked	1.6	0.8 (1)	3.4 (3)	13.5 (5)	9.0 (56)
5 Endosome proteins	1.0	0.8 (1)	0	0	0.6 (4)
6 ER & Golgi proteins	5.4	4.0 (5)	5.7 (5)	2.7 (1)	1.1 (7)
7 Glucose metabolism	1.1	0	2.3 (2)	0	0.5 (3)
8 Lysosome & peroxisome proteins	1.4	0.8 (1)	2.3 (2)	0	0.3 (2)
9 Membrane & protein trafficking	5.4	5.6 (7)	2.3 (2)	2.7 (1)	5.2 (32)
10 Membrane receptors & channels	8.1	11.2 (14)	6.8 (6)	2.7 (1)	14.2 (88)
11 Mitochondrial proteins	8.9	12.0 (15)	3.4 (3)	0	0.2 (1)
12 Other metabolism	10.9	15.3 (19)	20.5 (18)	8.1 (3)	1.9 (12)
13 Other nuclear proteins	3.1	1.6 (2)	4.5 (4)	0	3.1 (19)
14 Proteasome	5.7	4.8 (6)	0	5.4 (2)	2.3 (14)
15 Protein kinases & phosphatases	5.6	2.4 (3)	3.4 (3)	10.8 (4)	8.4 (52)
16 Protein processing	1.1	3.2 (4)	0	2.7 (1)	0.8 (5)
17 Secretory proteins	3.5	8.8 (11)	6.8 (6)	2.7 (1)	0.3 (2)
18 Signalling & adapter proteins	12.0	12.0 (15)	13.6 (12)	21.6 (8)	15.9 (99)
19 Small GTPases & regulators	5.1	1.6 (2)	6.8 (6)	2.7 (1)	11.4 (71)
20 Transcription & translation	6.4	2.4 (3)	6.8 (6)	0	4.2 (26)
21 Unknown	7.9	0	3.4 (3)	2.7 (1)	6.1 (38)
Total	100 (4190)	100 (125)	100 (88)	100 (37)	100 (621)

Table I. Overview of differences in the quantitative and phosphoproteome of Scott platelets compared to healthy control platelets. Unique proteins of the quantitative proteome and phosphoproteome were assigned to 21 assumed platelet function classes. Second column points to numbers of proteins per class (expressed as %) for healthy control platelets, according to the previously published proteome²⁰. For the quantitative proteome of Scott platelets, numbers of proteins are shown with decreased or increased expression. For the phosphoproteome of Scott platelets (combination of convulxin/thrombin and ionomycin stimulation), numbers of phosphopeptides are shown with decreased or increased phosphorylation.

centrifuging at 260 g for 15 minutes.²¹ After addition of 1/15 volume of ACD, platelets were pelleted by centrifugation at 870 g for 15 minutes. The platelets were resuspended in Hepes buffer pH 6.6 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂ and 0.1% glucose) by carefully excluding the bottom layer of red blood cells. After addition of ACD (1/15 volume) and apyrase (1 U/ml), platelets were centrifuged again at 2000 g for 5 minutes in an Eppendorf centrifuge, and resuspended in Hepes buffer pH 7.45 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 0.1% glucose), again by excluding the bottom layer of erythrocytes. Purity of the final platelet suspensions was checked with a Thrombocounter and from microscopic preparations. Contamination with red blood cells was <1:15,000, contamination with leukocytes was <1:20,000.

Stimulation of washed platelets

Purified, washed platelets (1.5 x 10⁸/ml) in Hepes buffer pH 7.45 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 0.1% glucose) containing 2 mM CaCl₂ were left untreated or were activated with thrombin (4 nM), convulxin/thrombin (100 ng/ml, 4 nM) or ionomycin (10 μM) for 30 minutes at 37°C under non-stirring conditions. Where indicated, Sub-samples were analysed by flow cytometry for PS exposure using FITC-labelled annexin A5,

as described before.¹³ In control experiments, washed platelets (1.5 x 10⁸/ml) were activated with ionomycin (10 μM) in the presence or absence of calpain inhibitor calpeptin (200 μM) for 30 minutes, or with the pro-apoptotic agent ABT-7373 (10 μM) in the presence or absence of caspase inhibitor QVD-OPh (20 μM) 60 minutes at at 37 °C. Chemicals and (ant)agonists were obtained from sources described before.²¹

For proteomics analysis, reactions were stopped by addition of 1 volume of lysis buffer (50 mM Tris, 1% SDS, 150 mM NaCl, 1 tablet PhosStop/10 ml, pH 7.8).²² Lysed samples were immediately frozen and stored at -80°C until usage.

Western blot analysis

Washed platelets (5 x 10⁸/ml) were left untreated or activated by convulxin (200 ng/ml) plus thrombin (8 nM) or ionomycin (20 μM) in the presence of 2 mM CaCl₂ for 30 minutes at 37 °C under non-stirring conditions. Samples of resting and activated platelets were lysed with ice-cold 4x lysis buffer (600 mM NaCl, 10 mM Tris, 4 mM EGTA, 4 mM EDTA, 4% NP40). Samples (10 μg proteins) were separated on 8% SDS-PAGE gels, and transferred to PVDF blotting membranes by semi-dry transfer.²¹ Membranes were immunoblotted with the following antibodies: Ab762 against the full length

β_3 -chain (1:10000), Ab754 against the intracellular amino acid 754 neo-N-terminus of the cleaved β_3 -chain (1:1000), or α -tubulin (1:1000). Incubation with secondary horse radish peroxidase-coupled antibody (1:1000) was overnight at 4 °C, and visualisation with an ECL system. Quantification was performed by densitometric analysis of stained blots.²¹

Sample digestion and preparation for quantitative (phospho)proteomics

Platelet proteomics analyses were performed, basically following procedures reported earlier.^{17,20,22} For each platelet sample (5×10^8 platelets), protein concentrations were determined using a bicinchoninic acid protein assay kit according to the manufacturer's protocol (Pierce, Thermo-Fisher). Cysteines were reduced by 30 minutes

incubation at 56 °C with 10 mM dithiothreitol. Free sulfhydryl groups were alkylated with 30 mM iodoacetamide for 30 minutes at room temperature. For filter-aided sample preparation, 150 μ g of protein per sample were loaded onto a 30 kDa molecular weight cut-off spin filter, as described elsewhere.^{23,24}

Proteins were digested in triethylammonium bicarbonate (TEAB) buffer pH 8.0, consisting of 50 mM TEAB, 200 mM guanidinium hydrochloride and 2 mM CaCl_2 , in the presence of trypsin (1:20 w/w, Sigma, T-1426) at 37 °C, as described before²². After incubation for 7 hours, digested peptides were collected by centrifugation at 13,800 g for 25 minutes. Filters were additionally washed with 50 μ l of 50 mM TEAB and 50 μ l of LC-MS grade water to increase

Figure 2. Differences in quantitative proteome of Scott and control platelets. In total 2,225 unique proteins were relatively quantified in platelet samples from the Scott patient and a healthy control subject, and were plotted as a function of the protein number per ratio class. Curve parameters: mean = 1.02, SD = 0.27. Ratios <0.48 (mean – 2 SD) and ratios >1.56 (mean + 2 SD) were considered as relevant differences. Of the 2,225 proteins, 213 proteins (9.5%) fell into these categories: 125 proteins with low expression and 88 proteins with high expression in Scott platelets. For pairs of healthy controls subjects ($n = 4$), similar analysis yielded ~40 proteins in either category < (mean – 2 SD) and > (mean + 2 SD).

Uniprot number	Gene name	Protein name	Copies/control platelet	Ratio Scott/control	Assumed function class
Top 15 decreased expression					
Q4KMQ2	ANO6	Anoctamin-6	3976	0.03	Membrane receptors & channels (10)
P18859	ATP5J	ATP synthase-coupling factor 6, mitochondrial	9996	0.09	Mitochondrial proteins (11)
P53602	MVD	Diphosphomevalonate decarboxylase	1256	0.12	Other metabolism (12)
P06702	S100A9	Protein S100-A9	1166	0.13	Signalling & adapter proteins (18)
Q5JRX3	PITRM1	Presequence protease, mitochondrial	1018	0.16	Mitochondrial proteins (11)
P28676	GCA	Grancalcin	1199	0.18	Secretory proteins (17)
P81605	DCD	Dermcidin	968	0.19	Secretory proteins (17)
P17655	CAPN2	Calpain-2 catalytic subunit	1425	0.19	Signalling & adapter proteins (18)
Q3SXM5	HSDL1	Inactive hydroxysteroid dehydrogenase-like protein 1	2370	0.19	Mitochondrial proteins (11)
P54886	ALDH18A1	Delta-1-pyrroline-5-carboxylate synthase	1475	0.21	Other metabolism (12)
P08311	CTSG	Cathepsin G	n.d.	0.24	Secretory proteins (17)
P22307	SCP2	Non-specific lipid-transfer protein	1727	0.24	Other metabolism (12)
Q8IZ07	ANKRD13A	Ankyrin repeat domain-containing protein 13A	1103	0.25	Unknown (21)
Q6NUK1	SLC25A24	Calcium-binding mitochondrial carrier protein SCaMC-1	2210	0.26	Mitochondrial proteins (11)
Q12792	TWF1	Twinfilin-1	1012	0.26	Cytoskeleton actin-myosin (1)
Top 15 increased expression					
P29972	AQP1	Aquaporin-1	n.d.	54.10	Membrane receptors & channels (10)
P18065	IGFBP2	Insulin-like growth factor-binding protein 2	975	8.78	Signalling & adapter proteins (18)
O14639	ABLM1	Actin-binding LIM protein 1	1120	7.64	Cytoskeleton receptor-linked (4)
Q96KC8	DNAJC1	DnaJ homolog subfamily C member 1	TiO ₂	7.38	ER & Golgi proteins (6)
P57088	TMEM33	Transmembrane protein 33	2497	6.23	Membrane receptors & channels (10)
O95671	ASMTL	N-acetylserotonin O-methyltransferase-like protein	1035	5.84	Other metabolism (12)
O60890	OPHN1	Oligophrenin-1	1527	5.21	Small GTPases & regulators (19)
Q92616	GCN1L1	Translational activator GCN1	808	5.02	Transcription & translation (20)
Q01415	GALK2	N-acetylgalactosamine kinase	1323	4.61	Other metabolism (12)
P68104	EEF1A1	Elongation factor 1-alpha 1	4521	4.05	Transcription & translation (20)
Q9P035	PTPLAD1	Very-long-chain 3-hydroxyacyl-ACP dehydratase 3	929	4.04	Other metabolism (12)
Q9UPM8	AP4E1	AP-4 complex subunit epsilon-1	632	3.98	ER & Golgi proteins (6)
Q12805	EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	1414	3.95	Secretory proteins (17)
Q96PD5	PGLYRP2	N-acetylmuramoyl-L-alanine amidase	n.d.	3.95	Secretory proteins (17)
P05388	RPLP0	60S acidic ribosomal protein P0	986	3.85	Transcription & translation (20)

Table II. Top 15 of decreased (top) and increased (bottom) expression levels of proteins in Scott platelets. Relative abundance is determined for 2,225 identified proteins in unstimulated platelets from the Scott patient and a healthy control subject (see Figure 2). Shown are the top 15 of proteins with highest decrease or increase in expression levels in the patient's platelets. Indicated are Uniprot numbers and common names of genes and proteins. Estimated copy numbers per platelet (healthy subject) are taken from the previously published proteome ²⁰. All proteins were assigned to 21 assumed platelet function classes, based on GeneOntology classifications. Abbreviations: n.d., not determined; TiO₂, TiO₂ enrichment protocol.

peptide yield. For quality control, the digestion performance was monitored as described before.^{17,20} Peptide concentrations were determined by NanoDrop (Thermo Scientific, Bremen, Germany) according to the manufacturer's protocol. For each digested sample, equal peptide amounts (~100 µg) were dried under vacuum. Pellets were reconstituted in iTRAQ 8-

plex solution buffer (AB Sciex, Dreieich, Germany) and labelled according to the manufacturer's protocol. The eight differently labelled activation states from the control subject and Scott patient were pooled in a 1:1 ratio and the volume was reduced under vacuum. Subsequently, 10% of the pooled sample was desalted by C₁₈ solid phase extraction (SPEC C₁₈ AR, 4 mg bed, Agilent Technologies), according to manufacturer's protocol²⁵. The pooled sample was dried under vacuum and reconstituted in 10 mM ammonium acetate pH 6.0 (solvent A).

Platelet global proteome analysis using iTRAQ labels

iTRAQ global proteome analysis was performed as a quality control to check for possible changes in relative protein abundance after platelet stimulation. This ensured that differences in phosphorylation pattern (up/down-regulated) are not due to changes in protein abundance. Therefore, 40 µg of the pooled peptide samples were separated by HPLC on a C₁₈ column (Agilent Zorbax; 1 mm inner diameter (ID) x 15 cm; 5 µm particle size; 300 Å pore size, Agilent Technologies). The U3000 HPLC system (Thermo Scientific) was used with a flow rate of 60 µl/minute and a binary gradient ranging from 3-60% solvent B (10 mM ammonium acetate, 84% acetonitrile, pH 6.0) in 75 minutes.

Twenty concatenated fractions were collected every minute. All fractions were individually analysed by nano LC-MS/MS, using an UltiMate 3000 nano-RSLC system online-coupled to a Q-Exactive mass spectrometer. Each fraction was loaded onto a trap column (Acclaim PepMap100 C₁₈ trap column; 100 µm x 2 cm) with 0.1% trifluoroacetic acid at flow rate of 20 µl/minute. This was followed by separation of peptides on the main column (PepMap100 C₁₈ main column; 75 µm x 50 cm), using a binary gradient ranging from 3-42% solvent B during 140 minutes at 60 °C and a flow rate of 250 nl/minute. In the Q-Exactive mass spectrometer, survey scans were acquired at resolution of 70,000 using an automatic gain control (AGC) target value of 3×10^6 . Subsequently, MS/MS spectra of the 15 most intense ions were acquired with (i) a resolution of 17,500, (ii) an isolation width of 2.0 *m/z*; (iii) a normalised collision energy of 35; (iv) an AGC target value of 1×10^6 ions; (v) a maximum injection time of 250 milliseconds; (vi) and dynamic exclusion of 12 seconds with underfill ratio of 10%. The first fixed mass was set to 105 *m/z*.

Global proteome data were interpreted as follows. Raw data were processed with the program Proteome Discoverer 1.4 (Thermo-Fisher Scientific); data were searched against the Uniprot human database (August 2012; 20,232 target

A

B

Figure 3. Quantitation of anoctamin-6 peptides in Scott platelets using parallel reaction monitoring (PRM). High-resolution quantification of tryptic anoctamin-6 fragments by PRM. (A) Representative data of the anoctamin-6 peptide VLSVDESIKPEQEFFTAPFEK (amino acids 167-188). Whereas the correct peptide at retention time of 31.2 minutes can only be detected in the sample from the control subject, the background peaks at this retention time have the same intensity for both samples. Owing to the high mass deviation of -35.2 ppm and the incorrect peak pattern, the residual signal at 31.03 minutes in the Scott sample (8x lower than in the control) cannot be assigned to this peptide. (B) Summary of the normalised abundance of four quantified anoctamin-6 peptides in Scott and control platelets, representing different parts of the 910 amino acids. Intensities of the control sample were set to 1.0 and compared to those of the patient sample. The minor residual signals in the Scott patient can be attributed to noise, because of deviation in mass and peak pattern.

Uniprot number	Gene name	Protein name	Phospho-site	Ratio Scott/control (phospho)			Assumed function class
				Thr	Cvx/Thr	Iono	
Top 15 decreased phospho-sites							
O95810	SDPR	Cavin-2 (PS-p68, phosphatidylserine-binding protein)	Ser114	0.62	0.27	0.12	Signalling & adapter proteins (18)
P05867	PLEK	Pleckstrin	Thr341	0.32	0.21	0.49	Cytoskeleton actin-myosin (1)
O75324	SNN	Stannin	Thr507	1.21	0.44	0.26	Signalling & adapter proteins (18)
P29350	PTPN6	Tyrosine-protein phosphatase non-receptor type 6	Ser82	1.01	0.47	0.26	Protein kinases & phosphatases (15)
Q05655	PRKCD	Protein kinase C delta type	Thr73	1.09	0.42	0.31	Protein kinases & phosphatases (15)
P54578	USP14	Ubiquitin carboxyl-terminal hydrolase 14	Ser109	0.77	0.41	0.33	Proteasome (14)
Q8NHG8	ZNRF2	E3 ubiquitin-protein ligase ZNRF2	Ser332	0.87	0.51	0.37	Proteasome (14)
Q99719	SEPT5	Septin-5 (Cell division control-related protein 1)	Ser698	0.29	0.69	0.23	Cytoskeleton receptor-linked (4)
Q9Y2Q0	ATP8A1	Phospholipid-transporting ATPase 1A (P4-ATPase flippase)	Ser49	0.46	0.54	0.40	Membrane receptors & channels (10)
Q86UX7	FERMT3	Fermitin family homolog 3 (Kindlin-3)	Thr311	0.92	0.53	0.45	Cytoskeleton receptor-linked (4)
Q9H4B7	TUBB1	Tubulin beta-1 chain	Tyr536	0.85	0.70	0.28	Cytoskeleton microtubule (3)
Q8N699	MYCT1	Myc target protein 1	Ser31	0.76	0.66	0.35	Signalling & adapter proteins (18)
Q13976	PRKG1	cGMP-dependent protein kinase 1	Ser1178	0.47	0.36	0.69	Protein kinases & phosphatases (15)
Q12982	BNIP2	BLC2	Ser114	0.31	0.19	0.47	Signalling & adapter proteins (18)
P61026	RAB10	Ras-related protein Rab-10	Ser1076	0.99	0.68	0.45	Small GTPases & regulators (19)
Uniprot number	Gene name	Protein name	Phospho-site	Ratio Scott/control (phospho)			Assumed function class
				Thr	Cvx/Thr	Iono	
Top 15 increased phospho-sites							
P78559	MAP1A	Microtubule-associated protein 1A	Ser391	6.23	6.54	8.02	Cytoskeleton microtubule (3)
Q9UDY2	TJP2	Tight junction protein ZO-2	Ser899	1.58	4.84	9.67	Membrane receptors & channels (10)
Q6VY07	PACS1	Phosphofurin acidic cluster sorting protein 1	Ser168	1.53	4.68	8.01	ER & Golgi proteins (6)
Q0ZGT2	NEXN	Nexlin	Thr144	1.71	3.39	9.16	Cytoskeleton actin-myosin (1)
O95819	MAP4K4	Mitogen-activated protein kinase kinase kinase 4	Thr144	1.48	4.03	7.88	Protein kinases & phosphatases (15)
Q8TF64	GIPC3	PDZ domain-containing protein GIPC3	Thr144	1.39	4.18	7.36	Unknown
Q9Y490	TLN1	Talin-1	Thr142	5.96	6.16	5.09	Cytoskeleton receptor-linked (4)
O60229	KALRN	Kalirin (Huntingtin-associated protein-interacting protein)	Thr517	1.34	3.98	6.32	Small GTPases & regulators (18)
Q96G03	PGM2	Phosphoglucomutase-2	Thr59	4.62	3.93	4.74	Glucose metabolism (7)
O14639	ABLIM1	Actin-binding LIM protein 1	Ser301	1.04	2.97	5.00	Cytoskeleton receptor-linked (4)
Q9P035	PTPLAD1	Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 3	Ser111	1.46	3.23	3.86	Other metabolism (12)
P42331	ARHGAP25	Rho GTPase-activating protein 25	Ser703	1.32	2.40	4.46	Small GTPases & regulators (18)
P78559	MAP1A	Microtubule-associated protein 1A	Ser86	1.42	2.31	4.11	Cytoskeleton microtubule (3)
Q8ND76	CCNY	Cyclin-Y (Cyclin box protein 1)	Ser138	0.91	2.07	4.22	Signalling & adapter proteins (18)
P27987	ITPKB	Inositol-trisphosphate 3-kinase B	Thr638	1.12	3.47	2.87	Signalling & adapter proteins (18)

Table III. Top 15 of decreased (top) and increased (bottom) phosphorylation patterns in proteins of Scott platelets. Ratios are determined for phosphorylation changes of 1,566 phosphopeptides from Scott and control platelets, stimulated with thrombin, convulxin/thrombin or ionomycin. Shown are the top 15 of highest decreases or increases in phosphorylation in Scott platelets (ranked for combination of convulxin/thrombin and ionomycin stimulation) compared to control platelets per agonist condition. Also indicated are Uniprot accessions and common names of genes and proteins. Proteins were assigned to 21 assumed platelet function classes, based on GeneOntology classifications. Colour code: red = decreased, green = increased in the patient's platelets.

sequences). Mascot and Sequest were used as search algorithms with the following settings: (i) trypsin as enzyme allowing two missed cleavages, (ii) iTRAQ 8-plex at N-termini and lysines and carbamidomethylation of Cys residues as

fixed modifications, (iii) oxidation of methionine as variable modification, (iv) mass tolerances for MS and MS/MS were set to 10 ppm and 0.02 Da, respectively. False discovery rate (FDR) estimation for the level of peptide spectrum matches

was performed using the peptide validator node with filtering for 1% FDR (high confidence filter) and the reporter ion quantifier node.

ratios for the 8 samples, an artificial 113/113 ratio was created and set to 1.0 per protein. For each protein, a median of all iTRAQ ratios (113/113, ..., 121/113) was calculated and the individual iTRAQ ratios were divided by this median to obtain relative abundance values. For normalisation of potential systematic errors, correction factors for each channel were calculated based on the median relative abundance over all proteins. Subsequently, ratios between the stimulated and basal conditions for the patient and control platelets were calculated. Proteins which had ratios larger than 2 x SD above or below the mean value were considered as regulated.

Label free global proteome analysis

To exclude isolation interference in ion-based quantifications, we verified the global proteome by an additional label-free quantification workflow. Therefore, 35 µg protein samples from unstimulated platelets were separately processed and digested, as mentioned above. After digestion control, samples were individually fractionated on a C₁₈ column (BioBasic-18; 0.5 mm ID x 15 cm; 5 µm particle size; 300 Å pore size). An UltiMate 3000 liquid chromatography

Unique proteins quantified if at least 2 unique peptides were considered. As Proteome Discoverer only provided 7

system (Thermo-Fisher Scientific) was used with a flow rate of 12.5 µl/minute and a binary gradient ranging from 3-60% solvent J (10 mM ammonium acetate, 84% acetonitrile, pH 6.0) in 75 minutes. Four concatenated fractions were collected. Each fraction was individually analysed by nano LC-MS/MS, using an UltiMate 3000 nano-RSLC system online-coupled with an Orbitrap Fusion mass spectrometer. Fractions were loaded onto a trap column (Acclaim PepMap100 C₁₈ trap column; 100 µm x 2 cm) with 0.1% trifluoroacetic acid at flow rate of 20 µl/minute, followed by separation on the main column (PepMap100 C₁₈ main column; 75 µm x 50 cm) using a binary gradient ranging from 3-42% solvent B during 100 minutes at 60 °C and a flow rate of 250 nl/minute. Survey scans were acquired at a resolution of 60,000 with target values of 4 x 10⁵ and maximum injection time of 50 milliseconds. Then, MS/MS spectra of the 20 most intense ions were acquired in the ion trap, using HCD with a collision energy of 35, a target value of 1 x 10⁴ ions, a maximum injection time of 50 milliseconds, and a dynamic exclusion of 12 seconds.

Data analysis and label free quantification were performed using the Progenesis LC-MS software version 4.1

from Nonlinear Dynamics (Newcastle upon Tyne, UK) in combination with the programme PeptideShaker 0.29.²⁵ Fractions were aligned pairwise and four peak lists were exported and searched with X!Tandem Jackhammer using searchGUI 1.18.4²⁶ as well as Mascot 2.4 (Matrix Science), and a concatenated target/decoy version of the human Uniprot database (August 2012; 20,232 target sequences). The following search parameters were used: (i) trypsin as protease with a maximum of two missed cleavages, (ii) carbamidomethylation of Cys as fixed, and (iii) oxidation of Met as

variable modification. MS and MS/MS tolerances were set to 10 ppm and 0.5 Da. Search results were combined using PeptideShaker 0.29.1 (<http://code.google.com/p/peptide-shaker/>) and filtered at an FDR of 1 % on the peptide spectrum match, peptide and protein levels. Results from paired fractions were reimported into Progenesis. Peptide sequences containing oxidized Met and pyro-Glu were omitted from further data analysis and only proteins with at least two unique peptides were considered for quantification. For all proteins,

Figure 4. Changes of phosphoproteome in Scott platelets subjected to strong activation. iTRAQ-labelled digests from control and Scott platelets were simultaneously analysed for phosphopeptide presence: unstimulated, or stimulated for 30 minutes with thrombin, convulxin/thrombin or ionomycin. Per quantified phosphopeptide, median ratios were calculated to determine relative changes in phosphorylation upon platelet activation with indicated agonist (activation ratios per subject). Activation ratios per subject were classified as changed when outside the range of mean $\pm$ 2 SD (mean = 0.97, SD = 0.18); hence, decreased (ratio $\leq$ 0.61), unchanged (ratio 0.61-1.33) or increased (ratio $\geq$ 1.33). Panels indicate changes in phosphopeptides for (un)stimulated Scott platelets; clockwise: green = decreased, grey = unchanged, red = increased. (A) Distribution of changes in phosphopeptide ratio of unstimulated platelets (ratio: Scott unstimulated/control unstimulated). (B-D) Distribution of changes in phosphopeptide ratios after platelet stimulation (ratio: effect agonist on Scott platelets/effect agonist on control platelets); stimulation with thrombin (B), convulxin/thrombin (C), or ionomycin (D).

Uniprot number	Gene name	Protein name	Cleavage site	AA P2	AA P1	AA P1'	Ratio Scott/control (cleavage)			Inhib.	Assumed function class
							Thr	Cxx/Thr	Iono		
Top 15 decreased N-terminal cleavage											
Q14432	PDE3A	cGMP-inhibited 3',5'-cyclic phosphodiesterase A	Asn596	Q	G	N	0.94	0.08	0.77	0%	Signalling & adapter proteins (18)
Q9UJU6	DBNL	Drebrin-like protein	Ala183	A	K	A	0.47	0.19	3.73	0%	Cytoskeleton actin-myosin (1)
Q9Y490	TLN1	Talin-1	Ala2504	I	A	A	0.93	0.22	1.73	0%	Cytoskeleton receptor-linked (4)
Q0ZGT2	NEXN	Nexlin (F-actin-binding protein)	Leu312	L	K	L	0.84	0.23	2.32	0%	Cytoskeleton actin-myosin (1)
Q15942	ZYX	Zyxin	Asp369	M	Q	D	0.92	0.23	1.26	0%	Cytoskeleton actin-myosin (1)
P53621	COPA	Coatomer subunit alpha	Ser413	K	R	S	0.73	0.23	0.06	1%	Membrane & protein trafficking (9)
O00429	DNM1L	Dynamin-1-like protein	Asp627	L	L	D	1.09	0.24	1.68	0%	Cytoskeleton microtubule (3)
Q7L591	DOK3	Docking protein 3	Gly412	L	H	G	1.11	0.25	1.09	0%	Signalling & adapter proteins (18)
O43294	TGFB1H	TGF beta-1-induced transcript 1 protein	Leu210	L	G	L	0.90	0.26	1.14	0%	Signalling & adapter proteins (18)
P50552	VASP	Vasodilator-stimulated phosphoprotein	Ser329	T	T	S	0.74	0.27	2.37	0%	Signalling & adapter proteins (18)
B2RUZ4	SMIM1	Small integral membrane protein 1	Ala25	L	G	A	0.86	0.27	1.74	0%	Cytoskeleton receptor-linked (4)
P49841	GSK3B	Glycogen synthase kinase-3 beta	Ala394	T	N	A	0.67	0.27	1.50	0%	Protein kinases & phosphatases (15)
Q95810	SDPR	Cavin-2 (PS-p68, phosphatidylserine-binding protein)	Tyr395	V	R	Y	0.48	0.30	1.52	2%	Signalling & adapter proteins (18)
Q15691	MAPRE1	Microtubule-associated protein RP/EB family member 1	Ala171	T	A	A	0.77	0.31	2.74	0%	Cytoskeleton microtubule (3)
O15117	FYB	FYN-binding protein	Gly93	R	F	G	0.77	0.32	2.74	1%	Signalling & adapter proteins (18)
Uniprot number	Gene name	Protein name	Cleavage site	AA P2	AA P1	AA P1'	Ratio Scott/control (cleavage)			Inhib.	Assumed function class
							Thr	Cxx/Thr	Iono		
Top 15 increased N-terminal cleavage											
P21333	FLNA	Filamin-A	Ser468	P	R	S	1.65	8.57	1.44	100%	Cytoskeleton receptor-linked (4)
A5A3E0	POTEF	POTE ankyrin domain family member F	Asp922	A	L	D	1.01	8.40	0.82	82%	Cytoskeleton actin-myosin (1)
P21333	FLNA	Filamin-A	Ser310	T	R	S	2.22	6.03	1.28	100%	Cytoskeleton receptor-linked (4)
P08514	ITGA2B	Integrin alpha-IIb	Gly367	P	R	G	2.38	3.72	2.00	100%	Membrane receptors & channels (10)
Q9UBC2	EPS15L1	Epidermal growth factor receptor substrate 15-like 1	Ser560	H	R	S	1.65	3.61	0.41	7%	Membrane & protein trafficking (9)
Q562R1	ACTBL2	Beta-actin-like protein 2	Val220	C	Y	V	1.03	3.45	0.82	46%	Cytoskeleton actin-myosin (1)
Q16643	DBN1	Drebrin	Ala635	W	D	A	3.41	2.83	2.32	65%	Cytoskeleton receptor-linked (4)
Q96U6	GMPPA	Mannose-1-phosphate guanyltransferase alpha	Ser413	S	R	S	2.76	2.81	1.26	100%	Other metabolism (12)
O14964	HGS	Hepatocyte growth factor-regulated tyr kinase substrate	Ser432	G	R	S	1.16	2.59	0.16	98%	Signalling & adapter proteins (18)
P07996	THBS1	Thrombospondin-1	Gly25	E	S	G	1.64	2.45	0.76	97%	Secretory proteins (17)
Q7LDG7	RASGRP2	RAS guanyl-releasing protein 2 (CalDAG-GEFI)	Ser487	L	R	S	2.23	2.38	1.11	91%	Small GTPases & regulators (19)
P35579	MYH9	Myosin-9	Ala625	Q	V	A	2.38	2.35	1.84	82%	Cytoskeleton actin-myosin (1)
P62942	FKBP1A	Peptidyl-prolyl cis-trans isomerase FKBP1A	Gly2	M	G	A	2.08	2.30	0.82	81%	Protein kinases & phosphatases (15)
Q95810	SDPR	Cavin-2 (PS-p68, phosphatidylserine-binding protein)	Tyr399	G	S	Y	3.96	2.29	2.56	1%	Signalling & adapter proteins (18)
P62937	PPIA	Peptidyl-prolyl cis-trans isomerase A	Val2	M	V	A	1.97	2.29	0.90	85%	Protein processing (16)

Table IV. Top 15 of decreased (top) and increased (bottom) neo-N-terminal cleavage sites in proteins of Scott platelets. Ratios are determined for the appearance of neo-N-termini of 1,613 peptides in Scott and control platelets, activated with thrombin, convulxin/thrombin or ionomycin. Shown are the top 15 of highest decreases or increases in neo-N-termini formed in Scott platelets (ranked for convulxin/thrombin), compared to control platelets per agonist condition. Ratios are calculated as: (effect of agonist in Scott platelets) / (effect of agonist in control platelets). Indicated are two amino acids (AA) P2 and P1 prior to the cleavage site, and the neo-N-terminal amino acid P1' at the cleavage site. Blue colour refers to amino acids with a predicted positive contribution to calpain-mediated cleavage. Colour code: red = decreased, green = increased in the patient's platelets. Column Inhib. refers to the confirmative experiment, showing residual cleavage in the presence of calpain inhibitor calpeptin in ionomycin-stimulated platelets from a control subject (0% = blue = no residual cleavage in the presence of calpeptin).

normalised abundances per sample were obtained, and ratios between Scott and control platelets were calculated.

Platelet phosphoproteome analysis

For phosphopeptide analysis of the pooled iTRAQ samples, a TiO₂-based phosphopeptide enrichment protocol was conducted, as described.²⁷ Enriched

phosphopeptides were fractionated with a U3000 HPLC-rapid separation liquid chromatography (Thermo-Fisher Scientific) system running in hydrophilic interaction liquid chromatography (HILIC) mode. Peptides were loaded on a self-packed HILIC column (Polar phase TSKgel Amide-80; 150 µm ID x 15 cm length; 5 µm particle size; 80 Å pore size, Tosoh

Bioscience, Germany) and separated using a binary gradient ranging from 5-40% solvent D (mobile phase solvent C: 98% acetonitrile, 0.1% trifluoroacetic acid; solvent D: 0.1% trifluoroacetic acid) within 40 minutes at a flow rate of 4 μ l/minute. Eluting peptides were collected in 9 fractions. The separation procedure was repeated as required.²²

LC-MS analysis of phosphopeptides

All HILIC fractions were analysed by nano-LC-MS/MS using an RSLC-nano system, online coupled to a LTQ-Orbitrap Velos mass spectrometer (both Thermo-Fisher Scientific). Peptides were pre-concentrated on a self-packed pre-column (Kinetex; 100 μ m ID x 2 cm length; 2.6 μ m particle size; 100 Å pore size) at a flow rate of 20 μ l/minute and further separated on a self-packed main column (Kinetex, 75 μ m ID x 30 cm length; 2.6 μ m particle size; 100 Å pore size) using a binary gradient (solvent E: 0.1% formic acid; solvent F: 84% acetonitrile, 0.1% formic acid) ranging from 3-45% solvent F during for 75 minutes at a flow rate of 250 nl/minute at 60 °C. In the LTQ-Orbitrap Velos MS higher collision-induced dissociation (HCD) fragmentation was used; survey scans were acquired with a resolution of 30,000 using the polysiloxane m/z 371.1012 as lock mass.²⁸ Subsequently, MS/MS spectra of the 5 most intense ions were acquired with (i) a resolution

of 7,500 (ii) an isolation width of 2.0 m/z ; (iii) a normalised collision energy of 50; (iv) an AGC target value of $1e^5$ ions; (v) a maximum injection time of 200 milliseconds; (vi) and a dynamic exclusion of 12 seconds. Reaction tubes containing 10% ammonium water were placed in front of the ion source to compensate for the iTRAQ-induced increase of peptide charge states.²⁹

Data analysis and confidence of determination of phosphopeptides

Raw data were processed with the program Proteome Discoverer 1.3 (Thermo-Fisher Scientific) using Sequest and Mascot as search algorithms. MS/MS spectra were searched against a human Uniprot database (July 2012; 20,232 target sequences) using the following search settings: (i) trypsin with a maximum of two missed cleavages; (ii) carbamidomethylation of Cys (+57.0214 Da) as well as iTRAQ 8-plex (+304.2053 Da) at peptide N-termini and Lys residues as fixed modifications; (iii) oxidation of Met (+15.9949 Da) as well as phosphorylation of Ser/Thr/Tyr (+79.9663 Da) as variable modifications; (iv) MS and MS/MS tolerances of 10 ppm and 0.02 Da, respectively. FDR estimation on the level of peptide spectrum matches (PSMs) was performed using the peptide validator node, and phosphorylation site localisation was determined using

phospho-RS.³⁰ The PSMs were filtered for 1% FDR (high confidence filter), and only phosphopeptides with phospho-RS probabilities $\geq 90\%$ were considered as confident. Raw data and Proteome Discoverer search results will be deposited in the ProteomeXchange repository.³¹ Obtained iTRAQ ratios were normalised based on the iTRAQ global proteome analysis in order to compensate for systematic errors. For determination of confident phosphorylation sites at the peptide and protein level a ready-to-use Excel macro provided by Mechtler lab (<http://ms.imp.ac.at/?goto=phosphors>) was used. Peptides were grouped based on their sequences, protein accessions and phosphorylation sites. Next, normalised abundance per channel was calculated, as mentioned above for the global proteome. Ratios between the Scott patient and control donor were calculated per condition. Cut-offs for potential regulation level were defined as for the iTRAQ global proteome.

Sample digestion and preparation for determination of neo N-terminal cleavage sites

Proteins were precipitated using ice-cold ethanol. Pellets from 8 selected samples were resuspended in 10 μ l 10% SDS under ultrasonication (3x for 10 seconds). These samples were then diluted to 1% SDS with 0.5 M TEAB containing 20% isopropanol (v/v). iTRAQ 8-plex reagents

in isopropanol (40 μ l) were added and allowed to react for 2 hours at 25 °C. Subsequently, all 8 samples were pooled and the reaction was quenched with 60 mM glycine (10 minutes at room temperature), followed by incubation with 130 mM hydroxylamine to reverse side reactions at hydroxyl groups (10 minutes at room temperature).

For proteolytic digestion, the multiplex sample was divided into two aliquots (160 μ g proteins each), which were subjected to ethanol precipitation. Samples were resuspended in 0.2 M guanidine HCl and 50 mM TEAB buffer and 2 mM CaCl₂ (final concentrations). Trypsin (Sigma, T1426) was added at enzyme/protein ratio of 1:20. After 14 hours trypsinisation at 37 °C, reactions were stopped with 1% trifluoroacetic acid, and the proteolytic digest was controlled using monolithic reversed phase chromatography, as described.²² Generated peptides were desalted using C₁₈ solid phase tips (SPEC C₁₈ AR, 4 mg bed, Agilent Technologies) as follows: activation with 100 μ l of 100% acetonitrile, followed by equilibration with 100 μ l of 0.1% trifluoroacetic acid. Bound peptides were washed two times with 0.1% of formic acid (v/v), and then eluted with 100 μ l of 70% acetonitrile with 0.1% formic acid (v/v). Samples were dried under vacuum and aliquots of 1 μ g were analysed with LC-MS to assess the labelling efficiency by calculating the

ratio between the modified and unmodified lysines at PSM level.

Charge-based fractional diagonal chromatography for determination of neo-N-terminal cleavage sites

The dried peptide pellets were resuspended in 52 μ l of SCX solvent G (10 mM KH_2PO_4 in 20% acetonitrile, pH 2.7). In the first dimension peptide samples (160 μ g) were separated at a flow rate of 80 μ l/minute using a U3000 HPLC system (Thermo-Fisher Scientific), a 150 x 1 mm polysulfoethyl A column (PolyLC; 5 μ m particle size; 200 Å pore size, Columbia), and a tertiary buffer system of strong cation exchange buffers, *i.e.* solvent G (see above), solvent H (10

mM KH_2PO_4 , 250 mM KCl in 20% acetonitrile, pH 2.7), and solvent I (10 mM KH_2PO_4 , 600 mM NaCl in 20% acetonitrile, pH 2.7). The following gradient steps were performed: 100% solvent G for 10 minutes; followed by a linear ramp from 0-15% solvent H in 9 minutes; after 9 minutes at 15%, solvent H was increased to 30% over 8 minutes; after 11 minutes at 30%, solvent H was increased to 100% in 5 minutes; after 5 minutes, solvent I was increased to 100% in 1 minute; after 5 minutes at 100% of solvent I, solvent G was increased to 100% in 1 minute; and the column was equilibrated for 20 minutes.

The five collected fractions (corresponding to charge states +1, +2,

Figure 5. Prediction model of calpain cleavage sites of platelet proteins. Analysis of in vitro digestion of a platelet lysate with calpain by LC-MS, identifying 2,224 unique peptides, corresponding to 375 proteins. Icelogo representation of P4-P4' positions (4 amino acids upstream to 4 amino acids downstream of cleavage site) to visualise consensus patterns. Large size characters indicate amino acids with a large positive or negative predictive contribution to calpain cleavage. In this model, calpain cleavage sites contained at least 2 positive amino acids at positions P2, P1 and P1'.

+3, +4 and >+4) were dried under vacuum and resuspended in 50 mM Na₂HPO₄ (pH 7.8). Free N-termini of internal peptides were blocked by trideutero to 100% in 5 minutes; after 5 minutes, solvent I was increased to 100% in 1 minute; after 5 minutes at 100% of solvent I, solvent G was increased to 100% in 1 minute; and the column was equilibrated for 20 minutes.

The five collected fractions (corresponding to charge states +1, +2, +3, +4 and >+4) were dried under vacuum and resuspended in 50 mM Na₂HPO₄ (pH 7.8). Free N-termini of internal peptides were blocked by trideutero (d3)-acetylation to induce a charge state shift. Therefore, trideutero N-hydroxysuccinimide acetate (d3-NHS acetate) was added to a final concentration of 20 mM and the sample was incubated for 1 hour at 37 °C, followed by a second addition step of 10 mM d3-NHS acetate. After 2 hours, reactions were quenched by 10 minutes treatment with 60 mM glycine, followed by 10 minutes treatment with 130 mM hydroxylamine (room temperature). Fractions were dried under vacuum and desalted using Poros Oligo R3 reversed-phase material (Applied Biosystems). Desalted fractions were solubilised in 52 µl buffer and were separately subjected to re-chromatography under the same conditions as above. Final fractions

enriched in N-terminal peptides were used for LC-MS analysis.

LC-MS analysis for determination of neo-N-terminal cleavage sites

Dried eluates were resuspended into 15 µl of 0.1% (v/v) trifluoroacetic acid, and each fraction was analysed by nano-LC-MS/MS using a Q-Exactive Plus mass spectrometer online-coupled to the nano-RSLC HPLC system (both Thermo-Fisher Scientific). LC separation was performed as follows: each fraction was loaded onto a C₁₈ reversed phase column (100 µm × 2 cm nano-trap) with 0.1% trifluoroacetic acid at a flow rate of 20 µl/minute for 10 minutes, followed by separation of the peptides on a C₁₈ reversed phase main column (75 µm x 50 cm) using a binary gradient from 3-42% solvent H at a flow rate of 0.25 µl/minute for 140 minutes at 60 °C.

Samples were measured in data-dependent acquisition mode acquiring full MS scans at a resolution of 70,000, and the 15 most abundant ions (top 15) were selected for MS/MS at a resolution of 17,500. Target values were set to 1 x 10⁶ for MS and 2 x 10⁵ for MS/MS scans. Maximum injection times were 120 and 200 milliseconds, respectively. A normalised collision energy of 30 was used, and the first fixed mass was set to 100 *m/z*. For strong cation exchange charge state fractions +3, +4 and +5, 10% ammonia solution was placed in front of

the ion source to compensate for increased charge states, as described ²⁸. For analysis of the +1 fraction, fragmentation of singly charged peptides was allowed, excluding *m/z* values of singly charged ions occurring in previous blank runs. Generated raw data were searched against the human Uniprot

database (December 2014; 13,414 target sequences). For peptide identification Proteome Discoverer software version 1.4 (Thermo-Fisher Scientific) was used with Mascot 2.4 (Matrix Science) as search engine, reporter ion quantifier and percolator nodes. Enzyme specificities were set to semi-Arg-C.

Figure 6. Class distribution of calpain-cleaved (A) and caspase-cleaved (B) proteins in platelets. Control platelets were stimulated with ionomycin (30 minutes) or ABT-737 (60 minutes) in the presence or absence of calpain inhibitor calpeptin or caspase inhibitor QVD-OPh (see Suppl. Table I). (A) Distribution of 106 identified calpain-cleaved proteins over platelet function classes (>3-fold increased cleavage with ionomycin and >50% reduction with calpeptin). (B) Distribution of 23 identified caspase-cleaved proteins over platelet function classes (>3-fold increased cleavage with ABT-737 and >50% reduction QVD-OPh).

To enable the quantification of two classes of N-terminal peptides, *i.e.* those with N-terminal iTRAQ label and those with endogenous N-acetylation, a two-step search strategy was performed. First, data were searched with iTRAQ-8plex (+304.2053 Da) as fixed modification at Lys and peptide N-termini; second, iTRAQ 8plex of Lys and N-terminal acetylation (+ 42.0105 Da) of N-termini were set as fixed modifications. In both cases, carbamidomethylation of Cys (+57.0214 Da) was selected as fixed and oxidation (+15.9949 Da) of methionine as variable modification. Mass tolerances were set to 10 ppm for MS and 0.02 Da for MS/MS. Identified peptides were filtered for high confidence corresponding to an FDR <1% at the PSM level, and a search engine rank of 1. The reporter ion quantifier node was adjusted according to the search settings.

Data analysis for determination of neo-N-terminal cleavage sites

Quantified modified peptides were grouped if they represented the same gene, sequence and modification state, to sum reporter intensities from each condition individually. Normalisation was done as described for the phosphoproteome data. Again, to scale the data per generated entry, each of the eight reporter intensities were divided by their median. The obtained scaled values were used to assess the normalised

abundance for each sample (condition). Ratios between samples were calculated, followed by a log2 transformation.

Assessment of consensus calpain cleavage motif. Lysates of unstimulated normal platelets were *in vitro* digested with calpain. Digestion was conducted for 30 minutes at 37 °C in 100 mM NaCl and 3 mM CaCl₂ (pH 7.5 with Tris) using filter-aided sample preparation; calpain (Calbiochem) was added in a protein/enzyme ratio of 2:1 (w/w). Control samples did not contain calpain. Monolithic HPLC confirmed the extensive generation of digested peptides, which was absent in the control sample. The calpain-digested sample was analysed with LC-MS, and data were processed as above, but with enzyme specificity set to 'none'. This resulted in the identification of 2,224 unique peptides at 1% FDR, corresponding to 375 identified proteins. Subsite nomenclature according to Schechter and Berger was used to assess the specificity of calpain cleavage.³² After defining the P4-P4' positions (*i.e.*, from 4 amino acids downstream to 4 amino acids upstream of the cleavage site), Icelogo software (<http://iomics.ugent.be/icelogoserver/main.html>)³³ was used to visualise potential consensus cleavage patterns. All unique peptides identified from the Scott patient N-terminal analysis were

then sorted according to their agreement with the established consensus motif.

Results and Discussion

Combined assessment of quantitative and phospho-proteome of Scott platelets

To assess the consequences of anoctamin 6 deficiency for platelet protein composition and phosphorylation, well-purified washed platelets (contamination with erythrocytes <1:15,000, with leukocytes <1:20,000) were obtained from healthy control donors and a Scott patient. The platelets were kept resting or were stimulated in CaCl₂-containing medium using with saturating, high concentrations of thrombin (8 nM), convulxin/thrombin (200 ng/ml, 8 nM) or ionomycin (20 µM) for a prolonged time of 30 minutes. For representative healthy control subjects, fractions with PS exposure of resting platelets or platelets stimulated for 30 minutes with thrombin, convulxin/thrombin or ionomycin were $0.5 \pm 0.2\%$, $9 \pm 2\%$, $51 \pm 3\%$ and $88 \pm 4\%$, respectively (means $\pm$ SEM, $n = 3$). For platelets from the Scott patient, corresponding fractions with PS exposure were $0.6 \pm 0.2\%$, $1.5 \pm 0.5\%$, $1.7 \pm 0.5\%$ and $2.0 \pm 0.6\%$, respectively (means $\pm$ SEM, $n = 3$). This confirmed that the patient's platelets were greatly impaired in agonist-induced PS exposure, such in agreement with earlier results with platelets from the same patient.¹³

Furthermore, the stimulated Scott platelets were unable to membrane blebbing and formation of balloon structures (data not shown). Samples of the resting and activated platelets (2×10^8 platelets) from patient and a control subject were then used for trypsin digestion and with 8-plex iTRAQ reagents (Figure 1). After combination of subsamples, 8-plex preparations were employed for quantifying relative protein expression levels, and after a TiO₂ enrichment procedure, for quantifying differences in phosphopeptide levels. Other samples were used for determination of neo-N-terminal residues originating from *in situ* protease activities during platelet activation.

Changes in quantitative proteome of Scott platelets

For platelets from the patient and the control subject, 2,225 unique proteins were quantified with at least 2 unique peptides in the label free analysis. This corresponds to approximately 55% of the estimated platelet proteome.²⁰ Estimated copy numbers per platelet ranged from 87,000 (filamin A) to <500 copies (detection threshold) for low abundant proteins. The relative abundances of proteins in control platelets were highly similar as described before.²⁰ For each individual protein, the ratio was calculated between values for Scott and control platelets. Plotting these

ratios as frequency distribution data resulted in a Gaussian distribution curve (Figure 2), with as mean 1.02 ± 0.27 (SD). Taking >95% confidence, we considered ratios with a deviance from mean larger or smaller than $2 \times \text{SD}$ as indicative for differential regulation. Based on these thresholds, a total of 9.6% proteins were potentially changed in expression in the Scott platelets, *i.e.* 125 proteins decreased and 88 proteins increased (Figure 2). When comparing pairs of healthy controls subjects ($n = 4$), a similar ratioed protein profile analysis gave ~40 proteins in either category (data not shown).

As described earlier and based on GeneOntology groupings,¹⁸ all proteins identified in the quantitative proteome were categorised into 21 assumed platelet function classes. As indicated in Table I, changes in expression were most frequently observed in proteins of the following classes: Membrane receptors & channels (class 10), Mitochondrial proteins (class 11), Other metabolism (class 12), and Signalling & adapter molecules (class 18). This is in accordance with the relatively high numbers of proteins in these classes.

The protein with the strongest down-regulation in Scott platelets was anoctamin 6 (gene *ANO6*, ratio <0.03). A targeted MS analysis using parallel reaction monitoring of four major peptide fragments of anoctamin 6 (*i.e.*

amino acids 97-114, 167-188, 191-199 and 649-661) indicated that the residual signal in the Scott samples could be attributed to noise, because of clear deviations in mass and peak pattern (Figure 3A-B). It was thus concluded that the level of anoctamin 6 in Scott platelets was below the detection limit, *i.e.* 50-100 times reduced in comparison to healthy controls.

The top 15 of most decreased protein levels in Scott platelets (Table II-top) further include mitochondrial proteins (genes *ATP5J*, *HSDL1*, *PITRM1*, *SLC25A24*) with different functions, as well as a number of proteases, *i.e.* the Ca^{2+} -dependent calpain-2 (*CAPN2*) and the secretory cathepsin G (*CTSG*). This top 15 of most increased protein levels in Scott platelets (Table II-bottom) surprisingly contains the membrane channel protein aquaporin-1 (*AQP1*), which was previously not identified in platelets;²⁰ proteins involved in transcription/translation (*EEF1A1*, *GCN1L1*, *RPLP0*); and further the Rho-GTPase-activating protein oligophrenin (*OPHN1*), a protein deficient in patients with rare oligophrenia which has previously been shown to be involved in glycoprotein-VI induced platelet activation.³⁴ Targeted re-analysis of the MS spectra confirmed the increased presence of *AQP1* peptides in the Scott sample (not shown).

A *All neo-N-termini*

B *Calpain-cleaved neo-N-termini*

Neo-N-termini: ■ decreased, ■ increased

C *Calpain-cleaved neo-N-termini*

Figure 7. Changes of protein cleavage pattern in Scott platelets subjected to strong activation. Samples of Scott and control platelets were left unstimulated (Unstim), or were stimulated for 30 minutes with thrombin (Thr), convulxin/thrombin (Cvx/Thr) or ionomycin (Iono). Median ratios were calculated per neo-N-terminal peptide to determine changes in protein cleavage upon platelet activation with indicated agonist, as for Figure 4 (ratios Scott/control). Ratio changes were classified as decreased (ratio ≤ 0.49), unchanged (ratio 0.50-1.99) or increased (ratio ≥ 2.00) in the Scott platelets. (A) Panels indicate changes in all 1,613 neo-N-terminal peptides per agonist condition for (un)stimulated Scott platelets; clockwise: green = decreased, grey = unchanged, red = increased. (B) Panels indicate changes in 110 neo-N-terminal peptides identified as calpain-cleaved (see Suppl. Table IA) per agonist condition. (C) Mean ratios Scott/control of 110 neo-N-terminal peptides identified as calpain-cleaved per agonist condition. * $P < 0.03$ vs. thrombin.

Changes in phosphorylation profile of activated Scott platelets

From the TiO₂-enriched samples, 1,566 phosphopeptides corresponding to 716 unique proteins could be relatively quantified in the eight samples from Scott and control platelets. In the absence of agonist, ratios for Scott/control platelets were decreased or increased in 5-9% of the phosphopeptides (Figure 4A). Long-term (30 minutes) stimulation with thrombin markedly enhanced the fraction of phosphopeptides with increased phosphorylation in the patient's platelets to 15% (Figure 4B), which fraction was further enhanced to 55% after stimulation with convulxin/thrombin and to 28% after stimulation with ionomycin (Figure 4C-D). When combining all phosphopeptide Scott/control ratios after stimulation with convulxin/thrombin and ionomycin - *i.e.* conditions where the patient's platelets are defective in PS exposure – much more proteins were increased in phosphorylation at one or more sites (*i.e.* 621) than decreased (*i.e.* 37), as indicated in Table I. Assignment tabling indicated that in majority Scott proteins of the following function classes were increased in phosphorylation: Cytoskeleton(-linked), Membrane receptors & channels, Protein kinases & phosphatases, Signalling & adaptor proteins, and Small GTPases and

regulators (Table 1). Zooming in to individual proteins revealed decreased phosphorylation (Table III-top) of key cytoskeletal proteins (genes *FERMT3*, *PLEK*, *SEPT5*, *TUBB1*), and proteins implicated in PS exposure, *i.e.* BLC2 (*BNIP2*), a phospholipid flippase (*ATP8A1*) and protein kinase Cδ (*PRKCD*)^{3,7,35}. The top 15 of highest increases in phosphorylation (Table III-bottom) include four cytoskeletal-(associated) proteins (genes *ABLIM1*, *MAP1A*, *NEXN*, *TNL1*), and the tight-junction protein ZO-2 (*TJP2*). These lists contain considerable overlap with the phosphoproteome changes reported for human platelets activated with thrombin or oxidised phospholipids.³⁶ Together, these data suggested that the altered phosphorylation pattern of Scott platelets upon strong stimulation in particular involves cytoskeletal (-linked) proteins and regulators of PS exposure.

Identification of platelet proteins cleaved by calpains or caspases

Platelet PS exposure is known to be accompanied by increased activity of cytosolic proteases. Thus, platelet stimulation with the strong Ca²⁺-elevating agents convulxin/thrombin or ionomycin leads to high activation of calpain isoforms,^{13,21} whereas platelet stimulation with the pro-apoptotic agent ABT-737 leads to caspase activation.⁷ As detailed in the methods section, we

developed a procedure for small scale assessment of neo-N-terminal peptides that are formed due to protein cleavage by active intra- or extra-cytosolic proteases. Given that the literature does not provide clear indications of the consensus sites in proteins for calpain- or caspase-induced cleavage, we followed two approaches to identify such substrates. In the first approach, lysates of control platelets were digested *in vitro* with purified calpain, after which the digested sample was analysed with LC-MS. This resulted in identification of 2,224 unique peptides corresponding to 375 proteins. Using the scheme created by Schechter and Berger,³¹ amino acids around the neo-N-termini were defined that were positively or negatively

predictive for cleavage by calpain (Figure 5). In the second approach, control platelets were stimulated with ionomycin or ABT-737 in the presence or absence of calpain inhibitor calpeptin or caspase inhibitor QVD-Oph.^{13,21} Analysis of the neo-N-termini by LC-MS revealed 227 neo-N-termini peptides with >3-fold increased levels in ionomycin-stimulated platelets, 180 of which were inhibited by calpeptin but not by QVD-Oph (Suppl. Table IA). These 180 calpain-cleaved neo-N-terminal peptides corresponded to 106 proteins, which almost completely overlapped with the proteins identified in the first approach (data not shown). Included in the list were proteins previously found to be cleaved in PS-exposing platelets, namely Src kinase

Figure 8. Reduced calpain-dependent cleavage of integrin β_3 chain in Scott platelets. Purified platelets from control subjects or Scott patient ($5 \times 10^8/\text{ml}$) were preincubated for 10 minutes with vehicle or one of the calpain inhibitors, calpeptin (200 μM) or MDL-28170 (200 μM). The platelets were unstimulated or were stimulated with convulxin/thrombin for 30 minutes. Western blots of samples were stained for full-length, intact integrin β_3 with Ab762 or cytosolic cleaved integrin β_3 with Ab754. Blots were reprobed with anti- α -tubulin Ab as protein loading control. Data are representative for 3 experiments. Lane 1, resting platelets; lanes 2-4, convulxin/thrombin stimulated platelets; lane 2, vehicle treatment; lane 3 calpeptin treatment; lane 4, MDL-28170 treatment.

(SRC) and talin-1 (*TLN1*).²¹ Function class analysis indicated that the majority of these proteins clustered into Cytoskeleton (-linked) classes, Membrane & protein trafficking, and Signalling & adapter proteins (Figure 6A). On the other hand, in apoptotic ABT-737-stimulated platelets, 45 neo-N-termini showed a >3-fold increase, 23 of which were inhibited by QVD-Oph but not calpeptin (Suppl. Table IB). The corresponding 23 proteins were mostly categorised as Cytoskeletal(-linked) and Signalling & adaptor proteins (Figure 6B). Together, these results point to patterns of non-overlapping cleavage sites by calpain and caspases of particularly cytoskeletal-associated and signalling proteins.

Changes in N-terminal proteome of activated Scott platelets

Unstimulated and stimulated platelets from the Scott patient and control subject were subsequently analysed for the presence of differential cleavage patterns. Abundance ratios of 1,613 quantified neo-N-terminal peptides (3 technical replicates) were assessed for the eight samples from Scott and control platelets (unstimulated; stimulated with thrombin, convulxin/thrombin or ionomycin). As indicated in Figure 7A, a limited number of cleaved peptides is increased or decreased (5-17%) in the Scott platelets when compared to the

control platelets, at all activation conditions. Interestingly, the top-15 of decreased neo-N-terminal peptides in the Scott patients (arranged according to ratios with the PS-exposing agonists, convulxin/thrombin and ionomycin) showed in the majority a cleavage pattern with two or more of our consensus amino acids for calpain cleavage and with a complete inhibitory effect of calpeptin, as established for control platelets (Table IV-top). These included cytoskeleton-linked proteins (genes *DBLN*, *DNM1L*, *MAPRE1*, *NEXN*, *SMIM1*, *TLN1* and *ZYX*) and key regulatory signalling proteins (*DOK3*, *FYB*, *GSK3B*, *PDE3A*, *VASP*). In contrast, the top-15 of *increased* expression of neo-N-terminal peptides in the Scott patients mostly concerned a variety of proteins with cleavage sites containing an arginine at position P₁ and a lack of inhibitory effect of calpeptin (Table IV-bottom). Hence, these cleavage sites are unlikely to be calpain-mediated. Collectively, this suggested that the activated Scott platelets are characterised by a decreased calpain-dependent formation of neo-N-termini. Interestingly, the majority of identified calpain cleavage sites differ from previous neo-N-termini lists from human platelets,³⁷ likely because these were established for stored, unstimulated platelets rather than from activated platelets, as in the present paper.

Further data analysis was then performed to confirm this hypothesis, by comparing only the neo-N-terminal peptides that were identified as calpain substrates (Suppl. Table IA). This resulted in a list of 110 peptides, which in case of unstimulated or thrombin-stimulated platelets were similar in expression for the Scott patient and control subject (Figure 7B). However, after convulxin/thrombin stimulation 58 neo-N-termini peptides were less present in the Scott platelets, and after ionomycin stimulation 38 peptides were more abundant in the Scott platelets. In agreement with this, the mean ratios between Scott and control platelets for all 110 peptides was lowered after convulxin/thrombin stimulation and raised after ionomycin stimulation (Figure 7C). Hence, these data pointed to a reduced calpain activity in the Scott platelets when stimulated with convulxin/thrombin, and an increased calpain activity after ionomycin stimulation. The latter difference is surprising, but can be explained by the protection of Scott platelets to PS exposure and phospholipid membrane dysregulation at the high intracellular Ca^{2+} concentration reached with ionomycin, which may prolong the calpain activity before the cells collapse. Further evidence for decreased calpain activity in convulxin/thrombin-stimulated Scott platelets came from

Western blotting of platelet samples.²¹

Using an antibody recognising the intact cytosolic N-terminal site of the integrin β_3 -chain (Ab762) and an antibody recognising a calpain-cleaved neo-N-terminal site in the β_3 -chain (Ab754), it appeared that Scott platelets showed a lower reduction in the Ab762 band and lower appearance of the Ab754 band (Figure 8). The staining with Ab754 was completely antagonised with two calpain antagonists, calpeptin and MDL-28170, thus demonstrating a calpain-mediated protein cleavage.

Conclusion

In this paper, we provide for the first time a full analysis of the protein composition, protein phosphorylation state and protein cleavage pattern after platelet stimulation with established strong Ca^{2+} -mobilising agonists (convulxin/thrombin and ionomycin). These agonists induce PS exposure and membrane blebbing in platelets from normal subjects, but not from the rare Scott patients. This advanced proteomic profiling provides novel insight into the altered protein composition and post-translational protein machinery explaining the major Ca^{2+} - and cytoskeleton-dependent membrane alterations in Scott syndrome platelets. Quantitative proteomics analysis reveals, in addition to a major reduction in anoctamin 6, up-regulation of the

channel protein aquaporin-1. Phosphopeptide analysis indicated major differences in Scott and control platelets, after stimulation with convulxin/thrombin or ionomycin, in proteins linked to the cytoskeleton,

regulating phosphatidylserine exposure, or implicated in signalling. Analysis of the neo-N-termini in Scott platelets revealed major changes in calpain-cleaved cytoskeleton-linked and key signalling proteins.

References

1. Toti F, Satta N, Fressinaud E, Meyer D, Freyssinet JM. Scott syndrome, characterized by impaired transmembrane migration of procoagulant phosphatidylserine and haemorrhagic complications, is an inherited disorder. *Blood*. 1996;**87**:1409-15.
2. Zwaal RF, Schroit AJ. Pathophysiological implications of membrane phospholipid asymmetry in blood cells. *Blood*. 1997;**89**:1121-32.
3. Bevers EM, Comfurius P, Dekkers DW, Harmsma M, Zwaal RF. Regulatory mechanisms of transmembrane phospholipid distributions and pathophysiological implications of transbilayer lipid scrambling. *Lupus*. 1998;**7** Suppl. 2:S126-31.
4. Thomas CP, Morgan LT, Maskrey BH, Murphy RC, Kuhn H, Hazen SL, Goodall AH, Hamali HA, Collins PW, O'Donnell VB. Phospholipid-esterified eicosanoids are generated in agonist-activated human platelets and enhance tissue factor-dependent thrombin generation. *J Biol Chem*. 2010;**285**:6891-903.
5. Kmit A, van Kruchten R, Ousingasawat J, Mattheij NJ, Senden-Gijsbers B, Heemskerk JW, Scheiber R, Bevers EM, Kunzelmann K. Calcium-activated and apoptotic phospholipid scrambling induced by Ano6 can occur independently of Ano6 ion currents. *Cell Death Disease*. 2013;**4**:e611.
6. Smeets EF, Heemskerk JW, Comfurius P, Bevers EM, Zwaal RF. Thapsigargin amplifies the platelet procoagulant response caused by thrombin. *Thromb Haemost*. 1993;**70**:1024-9.
7. Schoenwaelder SM, Yuan Y, Josefsson EC, White MJ, Tao Y, Mason KD, O'Reilly LA, Henley KJ, Ono A, Hsiao S, Willcox A, Roberts AW, Huang DCS, Salem HH, Kile BT, Jackson SP. Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function. *Blood*. 2009;**114**:663-6.
8. Cosemans JM, Schols SE, Stefanini L, de Witt S, Feijge MA, Hamulyak K, Deckmyn H, Bergmeier W, Heemskerk JW. Key role of glycoprotein Ib/V/IX and von Willebrand factor in platelet activation-dependent fibrin formation at low shear flow. *Blood*. 2011;**117**:651-60.
9. Heemskerk JW, Mattheij N, Cosemans JM. Platelet-based coagulation: different populations, different functions. *J Thromb Haemost*. 2013;**11**:2-11.
10. Suzuki J, Umeda M, Sims PJ, Nagata S. Calcium-dependent phospholipid scrambling by TMEM16F. *Nature*. 2010;**468**:834-8.
11. Castoldi E, Collins PW, Williamson PL, Bevers EM. Compound heterozygosity for 2 novel TMEM16F mutations in a patient with Scott syndrome. *Blood*. 2011;**117**:4399-400.
12. Kunzelmann K, Nilius B, Owsianik G, Schreiber R, Ousingasawat J, Sirianant L, Wanitchakool P, Bevers EM, Heemskerk JW. Molecular functions of anoctamin 6 (TMEM16F): a chloride channel, cation channel, or phospholipid scramblase? *Pflügers Arch*. 2014;**466**:407-14.
13. Mattheij NJ, Gilio K, van Kruchten R, Jobe SM, Wieschhaus AJ, Chishti AH, Collins P, Heemskerk JW, Cosemans JM. Dual mechanism of integrin $\alpha_{IIb}\beta_3$ closure in procoagulant platelets. *J Biol Chem*. 2013;**288**:13325-36.
14. Heemskerk JW, Kuijpers MJ, Munnix IC, Siljander PR. Platelet collagen receptors and coagulation. A characteristic platelet response as possible target for antithrombotic treatment. *Trends Cardiovasc Med*. 2005;**15**:86-92.
15. Wielders SJ, Broers J, ten Cate H, Collins PW, Bevers EM, Lindhout T. Absence of platelet-

- dependent fibrin formation in a patient with Scott syndrome. *Thromb Haemost.* 2009;**101**:76-82.
16. Clark SR, Thomas CP, Hammond VJ, Aldrovandi M, Wilkinson GW, Hart KW, Murphy RC, Collins PW, O'Donnell VB. Characterization of platelet aminophospholipid externalization reveals fatty acids as molecular determinants that regulate coagulation. *Proc Natl Acad Sci USA.* 2013;**110**:5875-80.
17. Burkhardt JM, Schumbrutski C, Wortelkamp S, Sickmann A, Zahedi RP. Systematic and quantitative comparison of digest efficiency and specificity reveals the impact of trypsin quality on MS-based proteomics. *J Proteomics.* 2012;**75**:1454-62.
18. Burkhardt JM, Gambaryan S, Watson SP, Jurk K, Walter U, Sickmann A, Heemskerk JW, Zahedi RP. What can proteomics tell us about platelets? *Circ Res.* 2014;**114**:1204-19.
19. Scholten A, Preisinger C, Corradini E, Bourgonje VJ, Hennrich ML, van Veen TA, Swaminathan PD, Joiner ML, Vos MA, Anderson ME, Heck AJ. Phosphoproteomics study based on in vivo inhibition reveals sites of calmodulin-dependent protein kinase II regulation in the heart. *J Am Heart Assoc.* 2013;**2**:e000318.
20. Burkhardt JM, Vaudel M, Gambaryan S, Radau S, Walter U, Martens L, Geiger J, Sickmann A, Zahedi RP. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood.* 2012;**120**:e73-82.
21. Mattheij NJ, Gilio K, van Kruchten R, Jobe SM, Wieschhaus AJ, Chishti AH, Collins P, Heemskerk JW, Cosemans JM. Dual mechanism of integrin $\alpha_{IIb}\beta_3$ closure in procoagulant platelets. *J Biol Chem.* 2013;**288**:13325-36.
22. Beck F, Geiger J, Gambaryan S, Veit J, Vaudel M, Nollau P, Kohlbacher O, Martens L, Walter U, Sickmann A, Zahedi RP. Time-resolved characterization of cAMP/PKA-dependent signaling reveals that platelet inhibition is a concerted process involving multiple signaling pathways. *Blood.* 2014;**123**:e1-e10.
23. Manza LL, Stamer SL, Ham AJ, Codreanu SG, Liebler DC. Sample preparation and digestion for proteomic analyses using spin filters. *Proteomics.* 2005;**5**:1742-5.
24. Wisniewski JR, Zougman A, Mann M. Combination of FASP and StageTip-based fractionation allows in-depth analysis of the hippocampal membrane proteome. *J Proteome Res.* 2009;**8**:5674-8.
25. Vaudel M, Burkhardt JM, Zahedi RP, Oveland E, Berven FS, Sickmann A, Martens L, Barsnes H. PeptideShaker enables reanalysis of MS-derived proteomics data sets. *Nat Biotechnol.* 2015;**33**:22-4.
26. Vaudel M, Barsnes H, Berven FS, Sickmann A, Martens L. SearchGUI: An open-source graphical user interface for simultaneous OMSSA and X!Tandem searches. *Proteomics.* 2015;**11**:996-9.
27. Engholm-Keller K, Birck P, Stirling J, Pociot F, Mandrup-Poulsen T, Larsen MR. TiSH: a robust and sensitive global phosphoproteomics strategy employing a combination of TiO₂, SIMAC, and HILIC. *J Proteomics.* 2012;**75**:5749-61.
28. Olsen JV, de Godoy LM, Li G, Macek B, Mortensen P, Pesch R, Makarov A, Lange O, Horning S, Mann M. Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. *Mol Cell Proteomics.* 2005;**4**:2010-21.
29. Thingholm TE, Palmisano G, Kjeldsen F, Larsen MR. Undesirable charge-enhancement of isobaric tagged phosphopeptides leads to reduced identification efficiency. *J Proteome Res.* 2010;**9**:4045-52.
30. Taus T, Kocher T, Pichler P, Paschke C, Schmidt A, Henrich C, Mechtler K. Universal and confident phosphorylation site localization using phosphoRS. *J Proteome Res.* 2011;**10**:5354-62.
31. Vizcaino JA, Cote RG, Csordas A, Dienes JA, Fabregat A, Foster JM, Griss J, Alpi E, Birim M, Contell J, O'Kelly G, Schoenegger A, Ovelheiro D, Perez-Riverol Y, Reisinger F, Rios D, Wang R, Hermjakob H. The PRoteomics IDentifications (PRIDE) database and associated tools: status in 2013. *Nucleic Acids Res.* 2013;**41**:D1063-9.
32. Schechter I, Berger A. On the size of the active site in proteases. I. Papain. *Biochem Biophys Res Commun.* 1967;**27**:157-62.
33. Maddelein D, Colaert N, Buchanan I, Hulstaert N, Gevaert K, Martens L. The iceLogo web server and

- SOAP service for determining protein consensus sequences. *Nucleic Acids Res.* 2015 (in press).
34. Bleijerveld OB, van Holten TC, Preisinger C, van der Smagt JJ, Farndale RW, Kleefstra T, Willemsen MH, Urbanus RT, de Groot PG, Heck AJ, Roest M, Scholten A. Targeted phosphotyrosine profiling of glycoprotein VI signaling implicates oligophrenin-1 in platelet filopodia formation. *Arterioscler Thromb Vasc Biol* 2013;**33**:1538-43.
 35. Gilio K, Harper MT, Cosemans JM, Konopatskaya O, Munnix IC, Prinzen L, Leitges M, Liu Q, Molkentin JD, Heemskerk JW, Poole AW. Functional divergence of platelet protein kinase C (PKC) isoforms in thrombus formation on collagen. *J Biol Chem.* 2010;**285**:23410-9.
 36. Zimman A, Titz B, Komisopoulou E, Biswas S, Graeber TG, Podrez EA. Phosphoproteomic analysis of platelets activated by pro-thrombotic oxidized phospholipids and thrombin. *Plos One.* 2014;**9**:e84488.
 37. Prudova A, Serrano K, Eckhard U, Fortelny N, Devine DV, Overall CM. TAILS N-terminomics of human platelets reveals pervasive metalloproteinase-dependent proteolytic processing in storage. *Blood.* 2014;**124**:e49-e60.

Supplemental Table IA

Uniprot number	Gene name	Protein name	Cleavage sites	Effect ionomycin (mean)			Function class
				vehicle	calpeptin	QVD-Oph	
ionomycin-induced neo-N-termini (ratio >3.0)							
P53396	ACLY	ATP-citrate synthase	A441	32.1	1%	120%	12
Q562R1	ACTBL2	Beta-actin-like protein 2	V220	3.1	46%	65%	1
P12814	ACTN1	Alpha-actinin-1	Y12, M13	20.8	1%	62%	1
P61160	ACTR2	Actin-related protein 2	S304	314.4	15%	90%	1
O43306	ADCY6	Adenylate cyclase type 6	V106	3.8	10%	70%	18
P54819	AK2	Adenylate kinase 2, mitochondrial	M2	4.3	81%	85%	11
Q4KMQ2	ANO6	Anoctamin-6	S38	13.4	0%	74%	10
Q8NEU8	APPL2	DCC-interacting protein 13-beta	D645	25.1	0%	64%	18
Q8N392	ARHGAP18	Rho GTPase-activating protein 18	E180	9.7	0%	76%	19
O43150	ASAP2	Arf-GAP with SH3, ANK and PH domains protein 2	K806	5.6	3%	91%	19
Q9NT62	ATG3	Ubiquitin-like-conjugating enzyme ATG3	A184	14.4	0%	85%	9
P06576	ATP5B	ATP synthase subunit beta, mitochondrial	T50	3.4	73%	82%	11
P21281	ATP6V1B2	V-type proton ATPase subunit B, brain isoform	A13	6.6	0%	91%	8
Q9UII2	ATPIF1	ATPase inhibitor, mitochondrial	G26	5.3	85%	91%	11
Q9UBW5	BIN2	Bridging integrator 2	K34, A412	64.8	0%	99%	4
Q05682	CALD1	Caldesmon	N30, T86, Q87, V88, Y160, Y161	24.8	1%	89%	1
P27797	CALR	Calreticulin	E18	3.7	98%	97%	6
Q01518	CAP1	Adenylyl cyclase-associated protein 1	V21	9.3	0%	89%	1
P04632	CAPNS1	Calpain small subunit 1	A68, E71, A74, Y87, A92	13.7	10%	91%	18
P78371	CDS2	T-complex protein 1 subunit beta	L6	33.5	0%	59%	6
O95674	CCT2	Phosphatidate cytidylyltransferase 2	A48	25.5	2%	71%	12
P30622	CLIP1	CAP-Gly domain-containing linker protein 1	S44	5.6	11%	102%	3
P09496	CLTA	Clathrin light chain A	A95	98.9	0%	98%	9
P10909	CLU	Clusterin	S131	3.6	84%	84%	17
Q8IY22	CMIP	C-Maf-inducing protein (c-Mip)	S439	6.3	8%	113%	18
Q99439	CNN2	Calponin-2	L14	21.5	3%	67%	1
Q6PJW8	CNST	Consortin	G18	37.2	0%	80%	10
P31146	CORO1A	Coronin-1A	A417, G423	17.8	2%	99%	1
Q9BR76	CORO1B	Coronin-1B	A428	13.3	0%	97%	18
P20674	COX5A	Cytochrome c oxidase subunit 5A, mitochondrial	S42	5.8	90%	90%	11
P10606	COX5B	Cytochrome c oxidase subunit 5B, mitochondrial	A32	3.8	99%	104%	11
P43155	CRA7	Carnitine O-acetyltransferase	K29	3.2	83%	93%	12
P46109	CRKL	Crk-like protein	A115	7.1	1%	72%	18
P10619	CTSA	Lysosomal protective protein	A29	3.4	87%	91%	8
Q14247	CTTN	Src substrate cortactin	S113, T337	6.0	16%	119%	1
Q16643	DBN1	Drebrin	L242	14.3	0%	78%	1
Q9UJU6	DBNL	Drebrin-like protein	A183	98.7	0%	88%	4
Q08495	DMTN	Dematin	A199	5.5	6%	97%	1
O00429	DNM1L	Dynamin-1-like protein	D627	37.6	0%	132%	3
O60496	DOK2	Docking protein 2	D358	30.3	1%	97%	18
Q7L591	DOK3	Docking protein 3	S209, G412, G413	9.1	3%	74%	18
P13639	EEF2	Elongation factor 2	V2	3.1	113%	107%	20
P55884	EIF3B	Eukaryotic translation initiation factor 3 subunit B	A97	4.5	1%	90%	20
P50402	EMD	Emerin	G106	7.1	106%	96%	1
Q9UBC2	EPS15L1	Epidermal growth factor receptor substrate 15-like 1	S560	5.6	7%	77%	6
Q9Y624	F11R	Junctional adhesion molecule A	S28	3.9	80%	86%	10
P00488	F13A1	Coagulation factor XIII A chain	S305	3.6	97%	95%	17
Q8N5J2	FAM63A	Protein FAM63A	A29	18.4	2%	53%	21
Q86UX7	FERMT3	Fermitin family homolog 3 (Kindlin-3)	T79, S199	4.5	81%	105%	4
Q86UX7	FERMT3	Fermitin family homolog 3 (Kindlin-3)	L334, G336, S337	122.3	0%	99%	4
P02671	FGA	Fibrinogen alpha chain	A20, D21, G538	7.3	76%	93%	17
Q9Y613	FHOD1	FH1/FH2 domain-containing protein 1	A2	3.9	75%	79%	4
P21333	FLNA	Filamin-A	A12, A13, G14, A15	61.5	0%	59%	4
P21333	FLNA	Filamin-A	A1026, G1526	15.3	0%	68%	4
P21333	FLNA	Filamin-A	S1520, Y1832	4.2	104%	99%	4
O15117	FYB	FYN-binding protein	G25, G93, G224, S256	18.5	1%	116%	18
Q14C86	GAPVD1	GTPase-activating protein and VPS9 domain protein 1	S746	25.4	0%	105%	19

P17174	GOT1	Aspartate aminotransferase, cytoplasmic	A2	4.5	67%	67%	12
P07359	GP1BA	Platelet glycoprotein Ib alpha chain	G574	23.2	0%	45%	10
P14770	GP9	Platelet glycoprotein IX	T17	4.3	128%	138%	10
P43304	GPD2	Glycerol-3-phosphate dehydrogenase, mitochondrial	A43	3.6	80%	89%	11
P62993	GRB2	Growth factor receptor-bound protein 2	D166	14.2	0%	69%	18
P49841	GSK3B	Glycogen synthase kinase-3 beta	A394	30.9	0%	90%	15
P06396	GSN	Gelsolin	G33, V53	3.9	108%	98%	1
P06396	GSN	Gelsolin	S412	4.2	12%	48%	1
P00390	GSR	Glutathione reductase, mitochondrial	Q48	26.9	0%	50%	11
P14317	HCLS1	Hematopoietic lineage cell-specific protein	V63, V308	13.3	6%	111%	18
P61978	HNRNPK	Heterogeneous nuclear ribonucleoprotein K	S366	14.4	0%	96%	20
P11021	HSPA5	78 kDa glucose-regulated protein (GRP-78)	A298	3.0	93%	98%	6
Q13418	ILK	Integrin-linked protein kinase	S324	3.6	118%	103%	15
Q13576	IQGAP2	Ras GTPase-activating-like protein IQGAP2	G15	12.3	0%	67%	19
P08514	ITGA2B	Integrin alpha-IIb	S432	3.5	103%	145%	10
P27987	ITPKB	Inositol-trisphosphate 3-kinase B	S352	16.7	6%	86%	12
Q8IVT5	KSR1	Kinase suppressor of Ras 1	A531	3.7	13%	81%	19
Q14847	LASP1	LIM and SH3 domain protein 1	K187, A191, S198	15.1	0%	78%	1
O43561	LAT	Linker for activation of T-cells family member 1	T174, A175	9.6	6%	122%	18
Q3ZCW2	LGALS1	Galectin-related protein	S24, S25, A29	13.2	1%	170%	21
P49257	LMAN1	Protein ERGIC-53	G316	6.9	1%	45%	6
Q93052	LPP	Lipoma-preferred partner	A302, N406	27.6	0%	88%	13
P50851	LRBA	Lipopolysaccharide-resp and beige-like anchor protein	V1591	25.4	0%	118%	18
Q32M24	LRRFP1	Leucine-rich repeat flightless-interacting protein 1	A710	64.7	0%	87%	20
Q9Y608	LRRFP2	Leucine-rich repeat flightless-interacting protein 2	A301, D352, A554, S560	35.1	1%	91%	18
Q9UNF1	MAGED2	Melanoma-associated antigen D2	S265	6.9	1%	76%	21
Q15691	MAPRE1	Microtubule protein RP/EB family member 1	A171	55.0	0%	110%	3
Q15555	MAPRE2	Microtubule protein RP/EB family member 2	S50, A171	35.3	1%	70%	3
Q9H3U5	MFSD1	Major facilitator superfamily domain protein 1	A13	6.5	0%	62%	10
Q13201	MMRN1	Multimerin-1 (EMILIN-4)	A296	3.4	18%	64%	17
Q9Y6C9	MTCH2	Mitochondrial carrier homolog 2	S56	3.2	81%	77%	18
Q96S97	MYADM	Myeloid-associated differentiation marker	T15	3.4	11%	114%	18
Q8N699	MYCT1	Myc target protein 1	S114	5.0	4%	79%	20
P35579	MYH9	Myosin-9	Q890, A891, L895, A897, I9	38.5	0%	88%	1
P55209	NAP1L1	Nucleosome assembly protein 1-like 1	M43, G58, E61, T62	9.4	90%	100%	13
O43639	NCK2	Cytoplasmic protein NCK2	Y271	21.0	0%	86%	18
Q0ZGT2	NEXN	Nexlin	L249, L312, G568, S569	38.1	8%	74%	1
P07237	P4HB	Protein disulfide-isomerase	D18	3.9	97%	97%	6
Q6VY07	PACS1	Phosphofurin acidic cluster sorting protein 1	S781	8.9	0%	141%	6
Q14432	PDE3A	cGMP-inhibited 3',5'-cyclic phosphodiesterase A	N596	23.6	0%	95%	18
O76074	PDE5A	cGMP-specific 3',5'-cyclic phosphodiesterase	Q15	62.8	0%	6%	18
P30101	PDI A3	Protein disulfide-isomerase A3	S25	4.0	100%	100%	6
P13667	PDI A4	Protein disulfide-isomerase A4	A24	4.1	87%	91%	6
Q96HC4	PDLIM5	PDZ and LIM domain protein 5	A392, Q395	5.2	10%	82%	18
P00491	PNP	Purine nucleoside phosphorylase	S59	4.4	80%	74%	11
Q9H2U2	PPA2	Inorganic pyrophosphatase 2, mitochondrial	A33	3.7	74%	80%	11
P02775	PPBP	Platelet basic protein	N44, G48	4.1	115%	108%	17
Q86WR7	PROSER2	Proline and serine-rich protein 2	A206, R369	6.2	6%	115%	21
Q14289	PTK2B	Protein-tyrosine kinase 2-beta (FAK)	A403	13.4	0%	88%	15
Q99952	PTPN18	Tyrosine-protein phosphatase non-receptor type 18	R398	4.6	15%	80%	15
P54727	RAD23B	UV excision repair protein RAD23 homolog B	A264	5.3	8%	92%	14
Q8TC12	RDH11	Retinol dehydrogenase 11	S229	4.0	70%	110%	12
Q96AA3	RFT1	Protein RFT1 homolog	G2	4.5	67%	70%	6
Q9H4X1	RGCC	Regulator of cell cycle RGCC	D70	3.1	25%	71%	18
Q9NS28	RGSI8	Regulator of G-protein signaling 18	S66	4.2	132%	107%	19
O14828	SCAMP3	Secretory carrier-associated membrane protein 3	A123	11.5	0%	69%	9
O95810	SDPR	Cavin-2 (PS-p68, Phosphatidylserine-binding protein)	T260, S398, Y399, A400	21.9	3%	94%	18
Q15019	SEPT2	Septin-2	A21	14.2	0%	60%	1
O75563	SKAP2	Src kinase-associated phosphoprotein 2 (Pyk2)	S271	3.1	20%	67%	18
O14745	SLC9A3R1	Na+/H+ exchange regulatory cofactor NHE-RF1	E121, A274, S280	30.0	0%	102%	18
Q14BN4	SLMAP	Sarcolemmal membrane-associated protein	G488	26.6	0%	74%	3
B2RUZ4	SMM1	Small integral membrane protein 1	A25	13.9	0%	103%	4

Protein expression, phosphorylation and cleavage in Scott platelets

O60749	SNX2	Sorting nexin-2	S119	14.2	1%	131%	9
P12931	SRC	Proto-oncogene tyrosine-protein kinase Src	G65, A79	30.3	0%	100%	18
Q9UEW8	STK39	STE20/SPS1-related proline-alanine-rich protein kinase	G436	41.8	1%	116%	18
Q9UMZ2	SYNRG	Synergisin gamma	G1119	23.2	0%	85%	9
Q96C24	SYTL4	Synaptotagmin-like protein 4	S346	8.3	1%	60%	9
P37802	TAGLN2	Transgelin-2	G5	3.4	37%	79%	17
Q4KMP7	TBC1D10B	TBC1 domain family member 10B (Rab27A-GAP-beta)	G312	3.5	10%	94%	19
Q8N9U0	TC2N	Tandem C2 domains nuclear protein	S194	105.5	0%	90%	13
Q92734	TFG	Protein TFG	L187	3.7	19%	88%	9
O43294	TGFB1H	TGF beta-1-induced transcript 1 protein	A163, L210	16.4	0%	113%	18
P07996	THBS1	Thrombospondin-1	N19, S405, G471	5.7	102%	102%	17
Q9UDY2	TJP2	Tight junction protein ZO-2	A129	5.7	0%	227%	18
Q9Y490	TLN1	Talin-1	Q163, S467, M468, Q473	16.5	12%	108%	4
Q9Y490	TLN1	Talin-1	A839, A1576, S1582	7.0	11%	69%	4
Q9Y490	TLN1	Talin-1	A2504	15.1	2%	150%	4
Q969X1	TMBIM1	Protein lifeguard 3	G68	26.6	0%	35%	8
Q8WWA1	TMEM40	Transmembrane protein 40	S8, S41, D119	29.4	5%	82%	10
Q9NYL9	TMOD3	Tropomodulin-3	T34, D178	31.4	0%	89%	1
P67936	TPM4	Tropomyosin alpha-4 chain	Q19	11.1	2%	50%	1
Q9Y3Q8	TSC22D4	TSC22 domain family protein 4	A188	31.7	0%	116%	20
Q99816	TSG101	Tumor susceptibility gene 101 protein	S202	3.5	19%	88%	9
P68366	TUBA4A	Tubulin alpha-4A chain	E433	19.1	0%	72%	3
Q9H4B7	TUBB1	Tubulin beta-1 chain	E26	20.3	2%	60%	3
Q9BZV1	UBXN6	UBX domain-containing protein 6	A89	3.5	16%	123%	14
P50552	VASP	Vasodilator-stimulated phosphoprotein	S128, A250, E265, E282	33.4	2%	82%	18
P50552	VASP	Vasodilator-stimulated phosphoprotein	S329, E330, T331	38.8	0%	89%	18
Q9BRP8	WIBG	Partner of Y14 and mago	Q123	4.7	0%	162%	20
O43516	WIPF1	WAS/WASL-interacting protein family member 1	S344, A394	24.4	4%	96%	1
Q15942	ZYX	Zyxin	V36, E111, Q243	5.0	32%	188%	4
Q15942	ZYX	Zyxin	Q368, D369, V378	25.1	0%	77%	4

Supplemental Table IB

Uniprot number	Gene name	Protein name	Cleavage sites	Effect ABT-737 (mean)			Assumed function c
				vehicle	QVD-Oph	calpeptin	
ABT-737-induced neo-N-termini (ratio >3.5)							
P12814	ACTN1	Alpha-actinin-1	S6	35.2	6%	115%	1
P54819	AK2	Adenylate kinase 2, mitochondrial	S4	4.8	81%	105%	11
Q8NEU8	APPL2	DCC-interacting protein 13-beta	D645	49.0	80%	102%	6
O43182	ARHGAP6	Rho GTPase-activating protein 6	S366	38.0	3%	113%	19
P06576	ATP5B	ATP synthase subunit beta, mitochondrial	A47	10.1	81%	96%	11
Q7Z460	CLASP1	CLIP-associating protein 1	G1219	8.7	12%	123%	3
P20674	COX5A	Cytochrome c oxidase subunit 5A, mitochondrial	S42	4.5	124%	114%	11
Q9UBC2	EPS15L1	Epidermal growth factor receptor substrate 15-like 1	S560	12.7	5%	92%	9
P00488	F13A1	Coagulation factor XIII A chain	Y305	3.5	90%	95%	17
Q86UX7	FERMT3	Fermitin family homolog 3 (Kindlin-3)	S345	82.8	3%	117%	4
Q86UX7	FERMT3	Fermitin family homolog 3 (Kindlin-3)	T79, S199, G336, S337	4.5	82%	97%	4
P02671	FGA	Fibrinogen alpha chain	A20, D21	7.2	94%	96%	17
Q9Y613	FHOD1	FH1/FH2 domain-containing protein 1	G10	55.8	3%	105%	1
P21333	FLNA	Filamin-A	G1337, G1505	53.1	8%	115%	4
P07359	GP1BA	Platelet glycoprotein Ib alpha chain	V481	12.8	15%	125%	10
P06396	GSN	Gelsolin	G33, V53	4.0	78%	95%	1
P06396	GSN	Gelsolin	G404	78.4	6%	101%	1
Q27J81	INF2	Inverted formin-2	A1052	33.8	37%	146%	1
P05106	ITGB3	Integrin beta-3	G27	7.2	111%	114%	10
O43561	LAT	Linker for activation of T-cells family member 1	S84	4.3	51%	145%	18
O75427	LRCH4	Leucine-rich repeat and calponin homology protein 4	S359	9.7	10%	112%	21
Q32MZ4	LRRFP1	Leucine-rich repeat flightless-interacting protein 1	A741	28.0	1%	148%	20
P35579	MYH9	Myosin-9	S1154	23.6	4%	117%	1
P55209	NAP1L1	Nucleosome assembly protein 1-like 1	G58	65.9	6%	106%	20
Q0ZGT2	NEXN	Nexilin	G568	90.4	4%	89%	1
P30101	PDIA3	Protein disulfide-isomerase A3	S25	3.8	108%	96%	6
P13667	PDIA4	Protein disulfide-isomerase A4	A24	4.4	107%	90%	6
O00151	PDLIM1	PDZ and LIM domain protein 1	S84	4.6	56%	98%	4
P47712	PLA2G4A	Cytosolic phospholipase A2	A523	35.2	5%	116%	18
Q9NS28	RGS18	Regulator of G-protein signaling 18 (RGS18)	S66	5.2	76%	98%	19
O75563	SKAP2	Src kinase-associated phosphoprotein 2 (Pyk2)	S271	54.3	1%	157%	18
Q9NRL3	STRN4	Striatin-4	G341	8.2	25%	172%	18
Q15833	STXBP2	Syntaxin-binding protein 2	A2	4.2	88%	93%	9
P07996	THBS1	Thrombospondin-1	N19, S405	6.4	115%	100%	17
Q9Y490	TLN1	Talin-1	E2007	4.3	93%	95%	4
Q9UKE5	TNIK	TRAF2 and NCK-interacting protein kinase	G853	5.6	15%	115%	15
Q5T4S7	UBR4	E3 ubiquitin-protein ligase UBR4	S2904	17.1	7%	108%	14
P45974	USP5	Ubiquitin carboxyl-terminal hydrolase 5	D768	38.0	2%	113%	14

Chapter 9

General discussion

In-depth analysis of the formation of procoagulant, phosphatidylserine-exposing platelets

As explained in **Chapter 1**, platelets become procoagulant due to calcium-or apoptosis-induced phospholipid scrambling and surface exposure of phosphatidylserine.¹⁻³ The exposure of phosphatidylserine, readily detected with the probe annexin A5, greatly stimulates the binding and activation of coagulation factors to produce factor Xa and thrombin.⁴⁻⁶ However, the overview **Chapter 2** indicates that the role of coagulation in thrombus formation and stabilisation is broader than earlier thought and also includes clot retraction. The combined ligands collagen and thrombin appears to be key triggers of the platelet procoagulant response, and evoke a potent Ca^{2+} signal via glycoprotein VI and the protease-activated receptors 1/4, respectively (**Chapter 3**). Another finding is the assistant role in phosphatidylserine exposure of the formation of a mitochondrial permeability transition pore (mPTP) and of the store-operated Ca^{2+} entry (SOCE) pathway, which pathways likely enforce the cytosolic Ca^{2+} mobilisation with Ca^{2+} from mitochondrial and extracellular origin, respectively.

In **Chapter 6**, it is described that platelet treatment with the pro-apoptotic agent ABT-737 results in the formation of two

distinct subpopulations of phosphatidylserine-exposing platelets, one with moderate and one with high annexin A5 binding. A relevant question arising is whether the moderate annexin A5-binding platelets are also limitedly active in supporting thrombin generation. However, this does not seem to be case, as the Jackson and Goodall groups have shown that thrombin generation curves obtained with ABT-737-stimulated platelets are similar to those obtained with collagen receptor peptide-stimulated platelets.^{7,8} Our *in vitro* experiments with Scott platelets – deficient in anoctamin-6 – showed a partly reduced ABT-737-induced formation in the patient's platelets of particularly the high annexin A5-binding fraction. On the other hand, the Scott platelets were unaffected in the ABT-737-induced and caspase-mediated phosphatidylserine exposure, thus excluding a major role of anoctamin-6 in the apoptosis pathway. Together, these data suggest the presence of a secondary Ca^{2+} -dependent mechanism of phosphatidylserine exposure, mediated by anoctamin-6 - and hence absent in Scott platelets - and responsible for the high annexin A5 binding fraction.

A previous finding from our laboratory is that the integrin $\alpha_{\text{IIb}}\beta_3$ becomes secondarily inactivated in platelets undergoing Ca^{2+} -dependent phosphatidylserine exposure.^{9,10} Based

on early reports this was explained by proteolytic degradation of the membrane cytoskeleton.¹¹ The studies described in **Chapter 4** have re-examined this in more detail, and provide molecular evidence that calpain cleaves several cytosolic proteins, not only the integrin β_3 -chain, but also signalling proteins operating downstream of integrin $\alpha_{IIb}\beta_3$ such as Src kinase and talin-1, in phosphatidylserine-exposing platelets stimulated via both the collagen and thrombin receptors. Control experiments indicated that platelet stimulation with thrombin alone did not induce integrin closure, protein cleavage nor phosphatidylserine exposure. These findings were confirmed in the neo-N-terminal proteomics analysis of **Chapter 8**, as further discussed below. Together, this suggests that in phosphatidylserine-exposing platelets both the integrin $\alpha_{IIb}\beta_3$ and integrin-dependent signalling pathways are abrogated due to intracellular cleavage of the concerning proteins.

A number of additional experiments in **Chapter 4** revealed the calpain isoform involved in this multiple protein cleavage. Intracellular cleavage of the integrin β_3 -chain, Src kinase and talin-1 in phosphatidylserine-exposing platelets could completely be prevented by calpeptin and MDL-28170, which are known to be specific inhibitors of calpain protease activity.^{12,13} On the other hand,

activated platelets from mice deficient in the calpain-1 isoform (*Capn1*^{-/-}) showed normal cleavage activity of these proteins. It is known that platelets contain both the calpain-1 and calpain-2 isoforms at appreciable levels, and that calpain-2 becomes active at higher cytosolic calcium concentrations in comparison to calpain-1.^{14,15} Hence, we concluded that especially the calpain-2 isoform is responsible for the proteolytic degradation of integrin-linked proteins. Unfortunately, this could not be confirmed in mice, since *Capn2*-deficient mice are embryonically lethal.¹⁶ For the future, it is an option to generate conditional and platelet-specific *Capn2*-deficient mice in order to confirm the proteolytic role of calpain-2 in phosphatidylserine-exposing platelets. Another finding of **Chapter 4** was that the general calpain inhibitors calpeptin and MDL-28170 abrogated the integrin β_3 -chain cleavage, but incompletely blocked the integrin closure. This points to the existence of a second mechanism of integrin closure in phosphatidylserine-exposing platelets that is not mediated by calpain. Markedly, in Scott platelets, integrin closure *was* completely inhibited by calpeptin or MDL-28170, suggesting that the second mechanism involves the channel activity of anoctamin-6. Considering that Scott syndrome platelets are essentially devoid of Ca^{2+} -dependent phospholipid scrambling and

membrane blebbing,¹⁷ it is not unlikely that the major membrane disturbances seen in phosphatidylserine-exposing platelets partially prevent the secondary closure of activated integrins.

Procoagulant platelets are known to undergo a dramatic morphological change by forming blebbing and ballooning structures, which processes are accompanied by degradation of the actin membrane skeleton.¹ The precise mechanisms underlying membrane blebbing and ballooning are still unclear, but the increased volume of phosphatidylserine-exposing platelets implicates fluid entry. Hypothetically, this can be either osmotically driven, *i.e.* following massive ion entry,¹ or due to the presence of water channels or aquaporins.¹⁸ In **Chapter 8**, we provide for a compensatory role of aquaporin-1 - which seems to be upregulated in Scott platelets showing limited blebbing. In **Chapter 7**, we observed that ionomycin-induced platelet ballooning was impaired in a hypertonic media, which is suggestive for at least a partial involvement of osmotic water entry. However, further research is needed to distinguish between the possibilities of ballooning driven by either ion or water channels. More evidence should come from currently performed inhibitor studies and experimentation with channel-deficient mice.

Phosphatidylserine-exposing platelets versus coated platelets

As indicated in **Chapter 1**, coated platelets have been identified in the literature in at least three distinct ways: (i) irreversible retention of factor V, fibrinogen and other proteins via transglutaminase-mediated cross-linking; (ii) high $\alpha_{IIb}\beta_3$ -independent, transglutaminase-dependent binding of fibrinogen; (iii) surface exposure of phosphatidylserine.^{19,20} The original hypothesis of the Dale group was that transglutaminases establish the formation of a protein coat around platelets by a serotonin-dependent cross-linking process.²⁰ In **Chapter 5**, we have taken this up and used a novel probe (Rhod-A14) to measure the activity of transglutaminases on the surface of activated platelets. Dual stimulation of human platelets via their collagen and thrombin receptors led to considerable incorporation of this probe in a way dependent on transglutaminase activity, as confirmed by various control experiments. The findings point to an incomplete overlap of the populations of phosphatidylserine-exposing and coated platelets (defined as platelets incorporating Rhod-A14). Furthermore, time courses indicated that the exposure of phosphatidylserine was faster than the incorporation of Rhod-A14. On the other hand, the Rhod-A14 binding (*i.e.*, formation of a protein coat) did not

prevent binding of labelled annexin A5 or factor Xa to the platelet surface. This suggests that, in at least part of the coated platelets, the procoagulant activity is maintained and, hence the protein coat must consist of a loose protein network. This in fact agrees with the literature, where coated platelets have been described as platelets capable to bind annexin A5 and factor V(a).^{21,22} Another relevant observation was that platelet stimulation with thrombin alone led to high fibrin(ogen) binding, but not to Rhod-A14 incorporation or phosphatidylserine exposure.

The precise role and origin of individual transglutaminases - tissue-type transglutaminase and coagulation factor XIII - was difficult to study in a human system, since factor XIII is present in both plasma and platelets, and platelets also contain tissue-type transglutaminase.^{21,23} For this reason, we used mice deficient in factor XIII-A, which is the factor XIII subunit containing the catalytic site. These animals survive normally and have normal platelet count.²⁴ Interestingly, incorporation of the Rhod-A14 probe was completely absent with the collagen/thrombin-activated knockout platelets, which suggested that mouse platelets have no or low expression of tissue-type transglutaminase. In contrast, fibrinogen binding and phosphatidylserine exposure were unaffected in the factor XIII-A-

deficient mice. We concluded that, in mouse, the formation of coated, transglutaminase-active platelets fully relies on the presence of factor XIII and that coated platelet formation is not required for platelet procoagulant activity. Subsequent flow perfusion studies, also using integrin inhibitors, indicated that murine factor XIII-A and integrin $\alpha_{IIb}\beta_3$ have a non-redundant role in the formation of fibrin fibres at the platelet surface under flow conditions. By implication, these results indicate that coated platelets provide a scaffold for the formation of fibrin in thrombus formation.

Together, the results of **Chapter 5** point a mechanism for coated platelet formation, as depicted in Figure 1: initial collagen/thrombin receptor-induced $\alpha_{IIb}\beta_3$ activation allows fibrin(ogen) binding, preceding the phosphatidylserine exposure. Subsequent activity of transglutaminases at the platelet surface leads to consolidation of the fibrin(ogen) at the platelet surface. This initial fibrin(ogen)-containing protein coat then acts as a scaffold for platelet-driven extension of fibrin fibres into the blood plasma (Figure 1). This scheme may illustrate the importance of coated platelet formation in thrombosis and haemostasis.

The Scott syndrome and anoctamin-6

Recently, a critical role was proposed for

anoctamin-6 (*ANO6* gene) in the regulation of phospholipid scrambling and phosphatidylserine exposure.²⁵ Indeed, dysfunctional mutations in the *ANO6* gene have been reported in two unrelated Scott patients in the USA and the United Kingdom²⁶. Furthermore, in a Scott patient from France,²⁷ also a mutation in the *ANO6* gene has also been traced (E. Freyssinet, personal communication). In **Chapters 6 and 7** we raised the question whether the anoctamin-6 channel can also be involved in other characteristic changes of procoagulant platelets, such as membrane blebbing/ballooning, microparticle formation, calpain-

dependent integrin cleavage and integrin closure. The data of **Chapter 6** indicated that the defect in phosphatidylserine exposure in the UK Scott patient was essentially confined to agonist/calcium-dependent phospholipid scrambling and did not extend to apoptosis-dependent phospholipid scrambling. The data of **Chapter 7** showed that in both human platelets (Scott) and mouse platelets with deficiency in anoctamin-6, the calcium/agonist-induced phosphatidylserine exposure was greatly diminished, but not completely abrogated. While some residual phosphatidylserine exposure was observed in platelets from the Scott

Figure 1. Scheme of formation of procoagulant and coated platelets. Resting platelets can develop into procoagulant, PS-exposing platelets without initial integrin activation when stimulated with the Ca^{2+} -ionophore ionomycin (lono). When stimulated via collagen and thrombin receptors (Cvx/Thr = convulxin/thrombin), procoagulant platelets show initial integrin activation, which is followed by integrin closure. Coated platelets are characterized by initial high fibrinogen binding, which crosslinks via transglutaminase activity (FXIIIa) to a coat of fibrin(ogen) at the platelets surface. Although coated platelets are covered with a fibrin coat, their ability to bind coagulation factors (FVa, FXa) is not affected.

patients and the *Ano6*-deficient mice, in either case the membrane blebbing and balloon formation was completely abolished (Table 1). Most likely, these 'swelling' responses are a consequence of ion influx via the membrane channel formed by anoctamin-6. Indeed, several groups have presented evidence that anoctamin-6 acts as a channel transmitting Ca^{2+} -dependent chloride and cation currents.²⁸⁻³⁰ The absence of cation/chloride influx could well explain the absence of membrane blebbing and ballooning in the patient's platelets. On the other hand, experiments in **Chapter 7** performed with sodium or chloride-free buffers did not show changes in the amount of ballooning, indicating that this

osmotic response is not driven by Na^+ or Cl^- influx alone. Whether anoctamin-6 is directly involved in membrane blebbing and ballooning, or whether it regulates the osmotic activity via other cation and anion channels is still to be established. The Scott syndrome has been described as a moderate bleeding disorder, with haemorrhagic episodes only after trauma or childbirth.³¹ Our data presented in **Chapter 7** point to limited hemostatic insufficiency in mice deficient in anoctamin-6, as mouse tail bleed times were moderately increased and platelet aggregation properties were unchanged. Furthermore, we measured non-zero, residual phosphatidylserine exposure of Scott and *Ano6*^{-/-} platelets

	Procoagulant	Apoptotic	Coated	Role for Ano6	Chapter/ref
Integrin activation	+	-	+	-	5
Integrin closure	+	-	+	+/-	4, 5
PS exposure	+	+	+	+	5, 6, 7
Ballooning	+	+	+	+	7, 8
Intracellular cleavage	+ (calpain)	+ (caspase)	+	-	4, 7, 8
Coagulation factor binding	+	+	+	+	5
High fibrinogen binding	-	-	+	-	5
Transglutaminase activity	-	?	+	?	5

Table 1. General characteristics of procoagulant, apoptotic and coated platelets. While there is a large overlap in properties between procoagulant and apoptotic platelets, a major difference between these two platelet populations is the inability of apoptotic platelets to display integrin activation and as a consequence lack of secondary integrin closure. Properties such as PS exposure, ballooning, intracellular protein cleavage and coagulation factor binding are displayed in both procoagulant and apoptotic platelets. Coated platelets have some additional characteristics in comparison to procoagulant platelets, such as initial high fibrinogen binding and surface-bound transglutaminase activity. The table furthermore highlights the role for the 'scramblase' protein anoctamin-6 in each of these platelet features. The chapters of this thesis describing the indicated characteristics of these platelet populations are shown in the last column.

during thrombus formation, which may offer an explanation for the limited severity of the bleeding phenotype. Western blots indicated greatly reduced expression of anoctamin-6 in platelets from the patient and the mice. Advanced proteomic profiling pointed to complete the absence of anoctamin-6 expression in the Scott cells, as shown in **Chapter 8**. Interestingly, in human and mouse blood samples we found evidence for the presence of alternative splice variants of the *ANO6* (*Ano6*) gene. These splice variants might be of functional importance during embryonic development and later life. However, our current data do not point to a significant role of these splice variants in the residual phosphatidylserine exposure or morphological changes in human Scott syndrome or mouse *Ano6*-deficient platelets.

As an overview of the present findings, Table 1 shows the role of anoctamin-6 in the formation of procoagulant, apoptotic and coated platelets. Overall, it appears that the procoagulant platelet response is highly dependent on anoctamin-6, while anoctamin-6 only plays a partial and minimal role in apoptotic and coated platelets.

Proteomics as a tool to understand the phenotypic properties of Scott platelets

In chapter 8 we use a novel tool to perform both quantitative proteomics,

phosphoproteomics and neo-N-terminal peptide identification in one single sample. The advantage of this tool is the ability to investigate the presence of the protein, the phosphorylation state of the protein and the presence of calpain specific cleavage sites at once. In this way the amount of activation is exactly equal between the phosphoproteome and calpain specific cleavage patterns. By using these methods we were able to identify thousands of proteins in healthy platelets as well as platelets from the Scott patient, and could determinate the amount of phosphorylation after specific activation and the amount of calpain-specific cleavage.

As described above, platelets from the Scott patient and anoctamin-6 deficient mice are greatly impaired in calcium-dependent phosphatidylserine exposure and membrane blebbing. **Chapter 8** describes that, in addition to anoctamin-6, the expression of several other proteins is changed in Scott platelets. In particular, Scott platelets displayed a marked up-regulation of the channel protein aquaporin-1, which was confirmed by peptide identification mapping. Aquaporin-1 expression in platelets has also been reported in recent proteomic analyses (Solari F et al. 2015 unpublished). Further research is needed to elucidate if in Scott platelets the water channel aquaporin-1 may have a compensatory role in

phosphatidylserine exposure and membrane blebbing.

Proteins which are further significantly decreased in protein levels in the Scott patients compared to control platelets, could be assigned to the functional classes mitochondrial proteins (genes *ATP5J*, *HSDL1*, *PITRM1*, *SLC25A24*) with different functions, as well as a number of proteases, *i.e.* the Ca^{2+} -dependent calpain-2 (*CAPN2*) and the secretory cathepsin G (*CTSG*). The decreased amount of the proteases present in the Scott patient could explain the decreased N-terminal cleavage we observed further on in Chapter 8. The top 15 of most increased protein levels in Scott platelets further includes proteins involved in transcription/translation (*EEF1A1*, *GCN1L1*, *RPLP0*); and the Rho-GTPase-activating protein oligophrenin (*OPHN1*). Previous literature pointed to an abnormal protein tyrosine phosphorylation pattern in Scott syndrome platelets.³² The phosphoproteome analysis of **Chapter 8** showed comparable phosphorylation profiles of Scott platelets and control platelets after thrombin stimulation. However, strong platelet activation with convulxin/thrombin or ionomycin greatly decreased the overlap in phosphorylation profile; much more proteins were increased in phosphorylation at one or more sites than decreased in Scott platelets in

comparison to the control platelets. Strikingly, only after these two specific conditions the patient's platelets are defective in phosphatidylserine exposure. The altered phosphorylation pattern of Scott platelets upon strong stimulation in particular involves cytoskeletal(-linked) proteins and regulators of phosphatidylserine exposure. This could explain in some part the deficiency of Scott platelets to induced the ballooning-structure and the lack of phosphatidylserine exposure.

It has been described that proteases of the calpain family - in platelets with high cytosolic Ca^{2+} concentration - and of the caspase family - in apoptotic platelets -, generated in activated platelets specific neo-N-termini.³³ We could identify 180 calpain-cleaved neo-N-terminal peptides after platelet activation by the Ca^{2+} -elevating agent ionomycin, which corresponded to 106 proteins. Specificity for calpain as the responsible protease was straightened by the fact that these terminal peptides could not be observed in the presence of the general calpain inhibitor calpeptin. Function class analysis indicated that the majority of these proteins clustered into the classes: Cytoskeleton (-linked), Membrane & protein trafficking, and Signalling & adapter proteins. In agreement with previously published data we observed cleavage of Src kinase (*SRC*) and talin-1 (*TLN1*) in phosphatidylserine-exposing

platelets (**Chapter 8**). Only a limited number of cleaved peptides is differential (increased + decreased 5-17%) between Scott platelets and control platelets. The top-15 decreased neo-N-terminal peptides in the Scott patient could be assigned to the functional classes: cytoskeleton-linked proteins (genes *DBLN*, *DNM1L*, *MAPRE1*, *NEXN*, *SMIM1*, *TLN1* and *ZYX*) and key regulatory signalling proteins (*DOK3*, *FYB*, *GSK3B*, *PDE3A*, *VASP*).

The observed decrease in calpain activity could be explained by the fact that Scott syndrome platelets appear to have decreased expression numbers of several proteases. But on the other hand,

decreased cleavage of proteins by calpain would make it possible that protein phosphorylation sites which are normally cleaved off in control platelets are still present in Scott platelets. In this way the increased phosphorylation pattern of multiple proteins in the Scott syndrome patients, could be explained by the decreased amount of protein cleavage. Hence, thanks to this novel combined proteomics analysis, we have additional clues on proteins which are differently regulated in Scott platelets and could conclude that a solely role for anoctamin-6 in the observed defects in Scott syndrome platelets can be excluded.

References

1. Heemskerk JW, Vuist WM, Feijge MA, Reutelingsperger CP, Lindhout T. Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine, and procoagulant activity of adherent platelets: evidence for regulation by protein tyrosine kinase-dependent Ca^{2+} responses. *Blood* 1997;**90**:2615-2625.
2. Schoenwaelder SM, Yuan Y, Josefsson EC, White MJ, Yao Y, Mason KD, O'Reilly LA, Henley KJ, Ono A, Hsiao S, Willcox A, Roberts AW, Huang DC, Salem HH, Kile BT, Jackson SP. Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function. *Blood* 2009;**114**:663-666.
3. Goodwin CA, Wheeler-Jones CP, Kakkar VV, Deadman JJ, Authi KS, Scully MF. Thrombin receptor activating peptide does not stimulate platelet procoagulant activity. *Biochem Biophys Res Commun* 1994;**202**:321-327.
4. Hemker HC, van Rijn JL, Rosing J, van Dieijen G, Bevers EM, Zwaal RF. Platelet membrane involvement in blood coagulation. *Blood Cells* 1983;**9**:303-317.
5. Thiagarajan P, Tait JF. Collagen-induced exposure of anionic phospholipid in platelets and platelet-derived microparticles. *J Biol Chem* 1991;**266**:24302-24307.
6. Bevers EM, Comfurius P, van Rijn JL, Hemker HC, Zwaal RF. Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. *Eur J Biochem* 1982;**122**:429-436.
7. Jackson SP, Schoenwaelder SM. Procoagulant platelets: are they necrotic? *Blood* 2010;**116**:2011-2018.
8. Vogler M, Hamali HA, Sun XM, Bampton ET, Dinsdale D, Snowden RT, Dyer MJ, Goodall AH, Cohen GM. BCL2/BCL-X(L) inhibition induces apoptosis, disrupts cellular calcium homeostasis, and prevents platelet activation. *Blood* 2011;**117**:7145-7154.
9. Munnix IC, Kuijpers MJ, Auger J, Thomassen CM, Panizzi P, van Zandvoort MA, Rosing J, Bock PE, Watson SP, Heemskerk JW. Segregation of platelet aggregatory and procoagulant microdomains in thrombus formation: regulation by transient integrin activation. *Arterioscler Thromb Vasc Biol* 2007; **27**:2484-2490.

10. Cosemans JM, Iserbyt BF, Deckmyn H, Heemskerk JW. Multiple ways to switch platelet integrins on and off. *J Thromb Haemost* 2008;**6**:1253-1261.
11. Fox JE. Regulation of platelet function by the cytoskeleton. *Adv Exp Med Biol* 1993;**344**:175-185.
12. Tsujinaka T, Kajiwaru Y, Kambayashi J, Sakon M, Higuchi N, Tanaka T, Mori T. Synthesis of a new cell penetrating calpain inhibitor (calpeptin). *Biochem Biophys Res Commun* 1988;**153**:1201-1208.
13. Mehdi S, Angelastro MR, Wiseman JS, Bey P. Inhibition of the proteolysis of rat erythrocyte membrane proteins by a synthetic inhibitor of calpain. *Biochem Biophys Res Commun* 1988;**157**:1117-1123.
14. Kraemer BF, Weyrich AS, Lindemann S. Protein degradation systems in platelets. *Thromb Haemost* 2013;**110**:920-924.
15. Randriamboavonjy V, Fleming I. All cut up! The consequences of calpain activation on platelet function. *Vascul Pharmacol* 2012;**56**:210-215.
16. Dutt P, Croall DE, Arthur JS, Veyra TD, Williams K, Elce JS, Greer PA. m-Calpain is required for preimplantation embryonic development in mice. *BMC Dev Biol* 2006;**6**:3.
17. Toti F, Satta N, Fressinaud E, Meyer D, Freyssinet JM. Scott syndrome, characterized by impaired transmembrane migration of procoagulant phosphatidylserine and hemorrhagic complications, is an inherited disorder. *Blood* 1996;**87**:1409-1415.
18. Suzuki M, Tanaka S. Structure and diverse functions of vertebrate aquaporins. *Seikagaku* 2014;**86**:41-53.
19. Dale GL. Coated-platelets: an emerging component of the procoagulant response. *J Thromb Haemost* 2005;**3**:2185-2192.
20. Dale GL, Friese P, Batar P, Hamilton SF, Reed GL, Jackson KW, Clemetson KJ, Alberio L. Stimulated platelets use serotonin to enhance their retention of procoagulant proteins on the cell surface. *Nature* 2002;**415**:175-179.
21. Alberio LJ, Clemetson KJ. All platelets are not equal: COAT platelets. *Curr Hematol Rep* 2004;**3**:338-343.
22. Colucci G, Stutz M, Rochat S, Conte T, Pavicic M, Reusser M, Giabbani E, Huynh A, Thurelmann C, Keller P, Alberio L. The effect of desmopressin on platelet function: a selective enhancement of procoagulant COAT platelets in patients with primary platelet function defects. *Blood* 2014;**123**:1905-1916.
23. Puszkin EG, Raghuraman V. Catalytic properties of a calmodulin-regulated transglutaminase from human platelet and chicken gizzard. *J Biol Chem* 1985;**260**:16012-16020.
24. Inbal A, Lubetsky A, Krapp T, Castel D, Shaish A, Dickneite G, Modis L, Muszbek L. Impaired wound healing in factor XIII deficient mice. *Thromb Haemost* 2005;**94**:432-437.
25. Suzuki J, Umeda M, Sims PJ, Nagata S. Calcium-dependent phospholipid scrambling by TMEM16F. *Nature* 2010;**468**:834-838.
26. Castoldi E, Collins PW, Williamson PL, Bevers EM. Compound heterozygosity for 2 novel TMEM16F mutations in a patient with Scott syndrome. *Blood* 2011;**117**:4399-4400.
27. Flores-Nascimento MC, Orsi FL, Yokoyama AP, Pereira FG, Lorand-Metze I, De Paula EV, Castro V, Annichino-Bizzacchi JM. Diagnosis of Scott syndrome in patient with bleeding disorder of unknown cause. *Blood Coagul Fibrinolysis* 2012;**23**:75-77.
28. Kunzelmann K, Nilius B, Owsianik G, Schreiber R, Ousingawatt J, Sirianant L, Wanitchakool P, Bevers EM, Heemskerk JW. Molecular functions of anoctamin 6 (TMEM16F): a chloride channel, cation channel, or phospholipid scramblase? *Pflugers Arch* 2014;**466**:407-414.
29. Martins JR, Faria D, Kongsuphol P, Reisch B, Schreiber R, Kunzelmann K. Anoctamin 6 is an essential component of the outwardly rectifying chloride channel. *Proc Natl Acad Sci U S A* 2011;**108**:18168-18172.
30. Tian Y, Schreiber R, Kunzelmann K. Anoctamins are a family of Ca²⁺-activated Cl⁻ channels. *J Cell Sci* 2012;**125**:4991-4998.
31. Satta N, Toti F, Fressinaud E, Meyer D, Freyssinet JM. Scott syndrome: an inherited defect of the procoagulant activity of platelets. *Platelets* 1997;**8**:117-124.
32. Dachary-Prigent J, Pasquet JM, Fressinaud E, Toti F, Freyssinet JM, Nurden AT. Aminophospholipid exposure, microvesiculation and abnormal protein tyrosine phosphorylation in the platelets of a patient with Scott syndrome: a study using physiologic agonists and local anaesthetics. *Br J Haematol* 1997;**99**:959-967.
33. Maddelein D, Colaert N, Buchanan I, Hulstaert N, Gevaert K, Martens L. The icLogo web server and SOAP service for determining protein consensus sequences. *Nucleic Acids Res* 2015;**43**:W543-6.

Summary

Platelets play important roles in thrombus formation. They adhere to the damaged vascular wall, form a platelet plug, and stimulate the coagulation process. It is considered that two types of platelets are formed in a growing thrombus: aggregating and procoagulant platelets. **Chapter 1** describes that these two platelet populations are discriminated by the rise in intracellular Ca^{2+} concentration. While a transient rise in intracellular calcium is sufficient for platelet integrin $\alpha_{\text{IIb}}\beta_3$ activation and secretion – two processes that are implicated in platelet aggregation –, a high, sustained rise in Ca^{2+} is required for the exposure of phosphatidyl serine (PS), which enables platelet to become procoagulant. Prolonged high intracellular Ca^{2+} concentrations activate the scrambling of membrane phospholipids, and as a consequence surface exposure of PS. This provides a surface for coagulation factor binding and ensuing thrombin generation. The underlying mechanisms and consequences of procoagulant platelet formation are investigated in this thesis. In **chapter 2**, background information is given on the multifaceted process of arterial thrombus formation and vascular occlusion. In this overview, we describe how the classical concept of arterial thrombus formation and vascular occlusion, driven by platelet activation and fibrin formation, can be extended

and fine-tuned. Key processes controlling the stability of a thrombus are: (i) initial platelet integrin activation controlling platelet aggregation, which can be reversible and result in shedding of platelet emboli; (ii) contact-dependent signaling, stabilizing the platelet aggregates; and (iii) thrombin and fibrin generation in plasma via the intrinsic and extrinsic coagulation pathways. The latter provide the thrombus with a fibrin network, but still allow shedding of platelet-fibrin emboli (microclots). A list of over 40 published studies with genetically modified mice points to key roles of platelet as well as plasma proteins in the control of thrombus stability after vascular injury.

Elaborating on the capability of procoagulant platelets in a thrombus to interact with (anti)coagulation factors and support blood coagulation, **chapter 3** provides an overview of the different ways how platelets act in a model of cell-based control of coagulation. Platelets with elevated Ca^{2+} and exposed PS stimulate thrombin and fibrin generation, whilst platelets with activated $\alpha_{\text{IIb}}\beta_3$ rather regulate clot retraction. Only few physiological agonists, particularly those stimulating the collagen receptor, glycoprotein VI (GPVI), are capable of inducing PS exposure by themselves. On the other hand, a larger number of agonists, including von Willebrand factor, fibrinogen, thrombin, ADP, antibodies

and thrombospondin, appears to enhance the process of (GPVI-mediated) PS exposure, by stimulating their respective receptors. In most cases the co-agonists act by increasing the Ca^{2+} signal towards a prolonged high Ca^{2+} rise. Mechanisms for Ca^{2+} elevation that contribute to PS exposure include: Ca^{2+} mobilization from the endoplasmic reticulum via inositol trisphosphate receptors; store-operated Ca^{2+} entry via STIM1 and Orai1 channels; and release of mitochondrial Ca^{2+} via mitochondrial permeability transition pore formation.

Chapter 4 aims to reveal how PS-exposing platelets disconnect from a thrombus by inactivation of their integrins. It was demonstrated that integrin $\alpha_{\text{IIb}}\beta_3$ inactivation in procoagulant platelets relies on a sustained high intracellular Ca^{2+} , stimulating intracellular cleavage of the integrin β_3 chain as well as talin, and Src kinase. Inhibition of calpain activity completely abolished protein cleavage, but only partly suppressed $\alpha_{\text{IIb}}\beta_3$ inactivation. Since the inactivation of $\alpha_{\text{IIb}}\beta_3$ was unchanged in platelets from mice deficient in calpain-1, we proposed a role of the calpain-2 isoform, with is known to become active at relatively high Ca^{2+} levels. Scott syndrome platelets displayed reduced $\alpha_{\text{IIb}}\beta_3$ inactivation with the remaining integrin activity fully dependent on calpain. In platelets from *Ppif*-deficient mice, lacking mitochondrial

permeability transition pore formation, we found that agonist-induced PS exposure and $\alpha_{\text{IIb}}\beta_3$ inactivation were reduced. Treatment of human platelets with cyclosporin A (targeting the same process) gave a similar phenotype. Together, these data point to a dual mechanism of $\alpha_{\text{IIb}}\beta_3$ inactivation via calpain(-2) cleavage of integrin-associated proteins and via anoctamin-6-dependent phospholipid scrambling with an assistant role of mitochondrial pore formation.

Coated platelets, formed by collagen and thrombin activation, have been characterized in different ways, such as by the formation of a protein coat of α -granular proteins, by exposure of procoagulant PS or by fibrinogen binding. Moreover, their functional role has remained unclear. In **chapter 5**, we used a novel transglutaminase probe, Rhod-A14, to identify subpopulations of platelets with a cross-linked protein coat, and compared these with other platelet activation characteristics, using a panel of platelet function assays. Platelet stimulation with convulxin/thrombin resulted in initial integrin $\alpha_{\text{IIb}}\beta_3$ activation, the appearance of a platelet population with high fibrinogen binding – independently of active integrins, but dependent on the presence of thrombin –, followed by PS exposure and binding of factors Va and Xa. Specifically, the transglutaminase-active platelets,

detected with Rhod-A14, formed as a subpopulation of the PS-exposing platelets. This staining pattern was confirmed in tissue-factor triggered platelet-rich plasma. Markedly, the high fibrin(ogen) and Rhod-A14 binding were antagonized by combined inhibition of transglutaminase activity and integrin $\alpha_{IIb}\beta_3$. In thrombi formed on collagen, Rhod-A14 again bound to a subpopulation of PS-exposing platelets via transglutaminase activity and later, under coagulant conditions, to fibrin fibers. Markedly, in thrombi from mice deficient in factor XIII, but not in wild-type thrombi, platelet-driven fibrin formation and Rhod-A14 binding were abolished by $\alpha_{IIb}\beta_3$ blockage. Together, this made us conclude that transglutaminase-active coated platelets, with initial $\alpha_{IIb}\beta_3$ activation and high fibrinogen binding, function in platelet-dependent fibrin fiber formation via factor XIII and $\alpha_{IIb}\beta_3$.

Scott syndrome, a bleeding disorder caused by defective phospholipid scrambling and PS exposure, has been associated with mutations in the gene for anoctamin-6 (*ANO6*, previously indicated as *TMEM16F*). In **chapter 6**, the processes of apoptosis- and agonist-induced PS exposure are studied in platelets from a Scott patient, lacking anoctamin-6. Apoptosis-dependent PS exposure was studied with the BH3-mimetic ABT-737. In control platelets,

ABT-737 gave two distinct fractions with moderate and high PS exposure. Markedly, the high PS-exposing fraction was substantially reduced in Scott platelets. From these and other observations, we concluded that, although anoctamin-6 is not critically involved in PS exposure upon apoptosis, it does increase PS exposure in a subset of the apoptotic platelets. High, but not moderate, PS exposure in platelets was suppressed by chelation of intracellular Ca^{2+} , whereas caspase inhibition completely abolished ABT-737-induced PS exposure in both Scott and control platelets. On the other hand, high PS exposure as induced by the high Ca^{2+} -mobilizing agonists convulxin/thrombin, fully relied on mitochondrial depolarization and was virtually absent in Scott platelets. Finally, PS exposure induced by collagen/thrombin was partly affected in Scott platelets, indicating that next to an anoctamin-6-dependent pathway, also another pathway to PS exposure exists. The residual PS exposure was insensitive to inhibition of caspases or mitochondrial depolarization. In sum, these results demonstrate that TMEM16F-dependent and -independent phospholipid scrambling can be induced in platelets upon apoptosis, aging, and activation.

In **chapter 7**, the role of anoctamin-6 in platelet responses was further studied using genetically modified mice deficient

in this protein. In addition, mice were used lacking other candidate proteins of phospholipid scramblase, *i.e.* anoctamin-1 and the Ca^{2+} -dependent K^+ channel Kca3.1 (Gardos channel). Anoctamin-6 deficient mice showed normal collagen-induced thrombus formation, but low residual PS exposure. Platelets from these mice did not display Ca^{2+} -dependent formation of ballooned structures, had reduced integrin β_3 -chain cleavage and a moderately affected apoptosis-dependent PS exposure. All these altered responses are observed in human Scott platelets as well. Markedly, deficiency in anoctamin-6 resulted in a reduced viability; and the surviving anoctamin-6-deficient mice had increased tail bleeding times. No platelet phenotype was observed in mice lacking the related ion-channel anoctamin-1, or mice lacking the Kca3.1 Gardos channels. In conclusion, mouse deficiency of Ano6 but not of other channels, affects viability and phenocopies the complex activation changes in hemostatically impaired human Scott platelets.

Considering that platelets from Scott patients have a complex phenotype – including lack of PS exposure, balloon formation and microparticle shedding –, we hypothesized in **chapter 8** that changes in several protein levels and post-translational protein modifications contribute to this phenotype. To investigate this, we compared platelets

from healthy control donors and a Scott patient by highly sensitive and advanced (phospho)proteomics analysis. Quantitative proteomics (2,225 unique proteins) indicated changed expression levels of about 200 proteins in the Scott platelets compared to control platelets. Alterations in expression were most frequently observed in proteins of the following function classes: membrane receptors & channels, mitochondrial proteins, metabolic and signaling & adapter proteins, such in accordance with the relatively high numbers of expressed proteins in these classes. In Scott platelets, anoctamin-6 could not be detected, whereas the channel protein aquaporin-1 was highly upregulated. Analysis of 1,566 unique phosphopeptides (corresponding to 716 proteins) indicated major phosphorylation differences between Scott and control platelets after stimulation with convulxin/thrombin or ionomycin. In Scott platelets, a limited number of proteins were decreased in phosphorylation (*e.g.*, proteins linked to the cytoskeleton or regulating PS exposure); a much higher number was increased in phosphorylation (*e.g.*, cytoskeleton-linked, membrane and signaling proteins). Furthermore, we identified a total of 1,613 neo-N-termini – in majority formed by intracellular cleavage of proteins –, with substantial differences between Scott and control

platelets. In total, we identified 180 neo-N-terminal peptides (corresponding to 106 proteins) that are generated due to calpain activity. In comparison, we identified a distinct set of 23 neo-N-termini (corresponding to 23 proteins) as caspase-regulated. In Scott platelets, most calpain-cleaved neo-N-termini were lower in level after convulxin/thrombin stimulation (in particular peptides from cytoskeleton-linked and key signaling proteins), and higher in level after ionomycin stimulation (a variety of proteins). In summary, we postulate that advanced

proteomic profiling provides novel insight into the altered protein composition and post-translational protein machinery explaining the major Ca^{2+} - and cytoskeleton-dependent membrane alterations in Scott syndrome platelets.

In **chapter 9**, the most important findings of this thesis are discussed in view of the current literature. It is argued that the mouse studies and the proteomic analyses employed have let to more insight into the complex mechanism underlying the procoagulant platelet response.

Samenvatting

Bloedplaatjes spelen een belangrijke rol in het proces van trombusvorming. Ze hechten aan een beschadigd bloedvat, vormen een bloedplaatjesprop, en stimuleren vervolgens het stollingsproces. In een aangroeiende trombus kunnen bloedplaatjes in twee vormen aanwezig zijn, namelijk als aggregerende cellen en als stollingsactieve (procoagulante) bloedplaatjes. In **hoofdstuk 1** van dit proefschrift is beschreven dat deze twee populaties van bloedplaatjes van elkaar onderscheiden kunnen worden door de stijging van hun intracellulaire vrije calciumconcentratie. Terwijl een kortstondige verhoging in vrij calcium voldoende is voor het activeren van integrine $\alpha_{IIb}\beta_3$ en de uitscheiding van actieve substanties – twee processen die betrokken zijn bij de plaatjesaggregatie –, is een continu verhoogd vrij calcium nodig voor het verschijnen van fosfatidylserine (PS) op het membraanoppervlakte, waardoor de stolling in gang gezet kan worden. Een blijvend verhoogd calcium zorgt voor de translocatie van PS van de binnenzijde naar de buitenzijde van het celmembraan. Hierna dient het geëxposeerde PS als bindingsplaats voor diverse stollingsfactoren, als gevolg waarvan via de stollingscascade trombine wordt gevormd. De signaleringspaden die in het bloedplaatje plaatsvinden voor deze procoagulante

activiteit, worden in dit proefschrift besproken.

In **hoofdstuk 2** wordt achtergrondinformatie gegeven over de processen die betrokken zijn bij vasculaire occlusie en een arteriële trombose, dat wil zeggen het afsluiten van een slagader door een bloedstolsel. In dit hoofdstuk wordt beschreven hoe het klassieke idee van occlusieve arteriële trombusvorming, aangestuurd door het activeren van bloedplaatjes en het vormen van een fibrinenetwerk, moet worden uitgebreid en verfijnd. Enkele belangrijke processen die de stabiliteit van een trombus beïnvloeden zijn: 1) de initiële activatie van integrines op bloedplaatjes, een proces dat omkeerbaar is waardoor bloedplaatjes van de trombus kunnen loslaten; 2) de intracellulaire signalering die nodig is voor stabiel contact tussen de bloedplaatjes, waardoor de aggregaten stabiliseren; en 3) de vorming van trombine en fibrine in het omliggende bloedplasma via de zogenoemde intrinsieke en extrinsieke stollingscascaden. Het resultaat is een trombus met een omringend fibrinenetwerk, waarvan echter nog steeds emboli kunnen loslaten die zowel bloedplaatjes als fibrine bevatten, de zogenaamde microclots. Een analyse van meer dan 40 gepubliceerde studies met genetisch gemodificeerde muizen laat zien dat eiwitten van zowel bloedplaatjes

als bloedplasma een belangrijke rol spelen bij het controleren van de stabiliteit van een trombus die gevormd wordt in de slagaders.

De stollingsactieve (procoagulante) bloedplaatjes in een trombus gaan een interactie aan met (anti)stollingsfactoren en daarmee de bloedstolling beïnvloeden. In **hoofdstuk 3** is een overzicht gegeven op welke verschillende manieren bloedplaatjes dit proces kunnen controleren. Terwijl bloedplaatjes met een verhoogd intracellulair calcium en geëxposeerd PS een sterk stimulerend effect hebben op de vorming van trombine en fibrine, zijn plaatjes met geactiveerd integrine $\alpha_{IIb}\beta_3$ actief in samentrekken van de trombus. Slechts enkele fysiologische agonisten, in het bijzonder activatoren van de collageenreceptor glycoproteïne VI (GPVI), zijn in staat om PS-expositie op te wekken. Aan de andere kant zijn er diverse plaatjesagonisten, zoals von Willebrand factor, fibrinogeen, trombine, ADP, antilichamen en trombospodine, die het proces van GPVI-gemedieerde PS-expositie kunnen versterken. In de meeste gevallen zorgt de co-stimulatie van GPVI met een van de andere receptoren voor een langdurige verhoging van het intracellulaire calcium. Mechanismen die betrokken zijn bij dit calciumsignaal en die dus bijdragen aan het exposeren van PS zijn: calcium-mobilisatie vanuit het endoplasmatisch

reticulum via inositoltrifosfaat-receptoren, extracellulaire calciuminstroom met behulp van de STIM1 en Orai1 eiwitten, en het vrijlaten van mitochondrieel calcium via de vorming van een permeabiliteits transitie-porie.

Het inactiveren van integrines op het membraan van PS-exposerende bloedplaatjes kan leiden tot het loslaten van plaatjes van een trombus. Het doel van **hoofdstuk 4** was om het mechanisme van integrine-inactivering te onthullen. Aangetoond wordt dat deze inactivering het gevolg is van een aanhoudend verhoogde intracellulaire calciumconcentratie en het intracellulair knippen van diverse signaleringseiwitten, zoals de integrine β_3 keten, taline en Src-kinase. Remmen van calpain (intracellulaire calciumproteases) resulteerde in een volledige onderdrukking van het knippen van bovenstaande eiwitten. Echter de inactivering van het integrine werd maar gedeeltelijk onderdrukt door dergelijke remmers. Verder bleek de inactivering van $\alpha_{IIb}\beta_3$ ongewijzigd in bloedplaatjes van muizen die deficient zijn in calpain-1, op basis waarvan wij concludeerden dat vooral de calpain-2 isovorm (werkzaam bij hogere calciumconcentraties) betrokken is bij het knippen en inactiveren van het integrine. Een interessante bevinding was dat bloedplaatjes van een Scott

patiënt een verminderde inactivering van $\alpha_{IIb}\beta_3$ lieten zien, waarbij de overgebleven inactivering volledig afhankelijk was van calpaïne-activiteit. Verder bleken ook de bloedplaatjes van Ppif-deficiënte muizen met een mitochondrieel defect verminderd in agonist-geïnduceerde PS-expositie alsmede in $\alpha_{IIb}\beta_3$ inactivering. Behandeling van mensen bloedplaatjes met cyclosporine A (gericht op eenzelfde mitochondrieel proces) gaf vergelijkbare effecten. Samen wijzen deze resultaten op een tweedelig mechanisme van inactivering van integrine $\alpha_{IIb}\beta_3$, namelijk een calpaïne(-2)-geïnduceerde knip van integrines en daarmee geassocieerde eiwitten, en de scrambling van membraanfosfolipiden met daarbij een rol van mitochondriële porievorming.

De zogenoemde gecoate bloedplaatjes worden gevormd na activering met zowel collageen als trombine. Deze populatie van bloedplaatjes wordt in de literatuur op verschillende manieren gekarakteriseerd, zoals door een mantel van α -granulaire eiwitten, de aanwezigheid van procoagulante activiteit met geëxposeerd PS, of door hoge binding van fibrinogeen aan de bloedplaatjes. Onduidelijk is ook de functionele rol van gecoate bloedplaatjes. In **hoofdstuk 5** hebben wij gebruik gemaakt van een nieuwe transglutaminase probe, Rhod-A14, om

de populatie van gecoate bloedplaatjes eenduidig te identificeren. Tevens beschrijft dit hoofdstuk experimenten om de functies van gecoate bloedplaatjes beter in kaart te brengen. Stimulering van bloedplaatjes met convulxine/trombine resulteert in een initiële activering van integrine $\alpha_{IIb}\beta_3$, en het verschijnen van een populatie met een hoge fibrinogeenbinding – welke niet afhankelijk is van integrines, maar wel van trombine. Vervolgens exposeren deze bloedplaatjes PS en binden ze de stollingsfactoren Va en Xa. Opmerkelijk is dat de transglutaminase-actieve bloedplaatjes slechts een subpopulatie vormen van de PS-exposerende bloedplaatjes. Soortgelijke resultaten zijn verkregen in bloedplaatjes-rijk plasma dat gestimuleerd is met weefselfactor. Een andere opmerkelijke bevinding is dat de hoge fibrinogeenbinding en de Rhod-A14 binding tegen kan worden gegaan, wanneer zowel de transglutaminase-activiteit als integrine $\alpha_{IIb}\beta_3$ geblokkeerd worden. Tijdens de trombusvorming aan collageen bindt de Rhod-A14 probe eerst aan PS-exposerende bloedplaatjes via transglutaminase-activiteit en in een later stadium aan de fibrinedraden die rond de trombus worden gevormd. In afwezigheid van stollingsfactor XIII (een transglutaminase) en gelijktijdig blokkeren van integrine $\alpha_{IIb}\beta_3$ blijken er geen fibrinedraden meer te ontstaan vanuit de bloedplaatjes in de trombus.

Concluderend kunnen we stellen dat de transglutaminase-actieve gecoatete bloedplaatjes, met initiële integrine $\alpha_{IIb}\beta_3$ activering en hoge fibrinogeen-binding, een rol spelen in de fibrinevorming via factor XIII en $\alpha_{IIb}\beta_3$.

Het Scott syndroom is een zeldzame bloedingsafwijking, welke geassocieerd is met een defect in de scrambling van de fosfolipiden en de expositie van PS. Patiënten met dit syndroom hebben mutaties in het gen anoctamine-6 (*ANO6*, voorheen aangeduid als *TMEM16F*). In **hoofdstuk 6** is de apoptose- en agonist-geïnduceerde PS-expositie bestudeerd in de bloedplaatjes van een Scott patiënt. Apoptose-geïnduceerde PS-expositie werd opgewekt met behulp van het medicijn ABT-737. In controle bloedplaatjes geeft ABT-737 twee verschillende fracties van PS-exponerende bloedplaatjes, namelijk met matige en hoge PS-expositie. In de Scott plaatjes zien we dat de fractie met hoge PS-expositie sterk verminderd is. Deze en andere observaties geven aan dat hoewel anoctamine-6 geen kritische rol speelt bij apoptose-geïnduceerde PS-expositie, het wel zorgt voor een toegenomen PS expositie in een subset van de bloedplaatjes. De fractie met hoge PS-expositie, maar niet die met matige PS-expositie, kan onderdrukt worden door wegvangen van het intracellulaire calcium. Remming van de caspase-activiteit leidt echter tot

volledige onderdrukking van de PS-expositie geïnduceerd door ABT-737 in de bloedplaatjes van patiënt en controles. De plaatjesagonisten convulxine en trombine veroorzaken hoge PS-expositie middels een proces, dat volledig afhankelijk is van mitochondriële depolarisatie. Deze PS-expositie is vrijwel afwezig in Scott bloedplaatjes. Anderzijds is de PS-expositie met collageen en trombine slechts gedeeltelijk afwezig in de Scott bloedplaatjes. Dit laatste geeft aan dat er naast de anoctamine-6 afhankelijke route, ook nog een andere route kan zorgen voor agonist-geïnduceerde PS-expositie. De residuele PS-expositie in bloedplaatjes van de Scott patiënt is niet gevoelig voor de remming van caspases of mitochondriële depolarisatie. Samengevat geven deze resultaten aan dat zowel anoctamine-6-afhankelijke als -onafhankelijke scrambling van fosfolipiden plaats vindt in bloedplaatjes die in apoptose gaan of middels hun collageen- en trombine-receptoren geactiveerd worden.

In **hoofdstuk 7** is de rol van anoctamine-6 bij de activering van bloedplaatjes verder onderzocht met behulp van genetisch gemodificeerde muizen, die dit eiwit niet tot expressie brengen. Daarnaast zijn muizen bestudeerd waarin andere kandidaateiwitten voor het fosfolipiden-scramblase ontbreken, namelijk anoctamine-1 of het calcium-afhankelijke kaliumkanaal Kca3.1

(Gardos-kanaal). Anoctamine-6 deficiënte muizen hadden een normale collageen-geïnduceerde trombusvorming (plaatjesaggregatie), maar een substantieel doch niet compleet defect in PS-expositie van de collageen-gehechte bloedplaatjes. Verder toonden bloedplaatjes van deze muizen geen calcium-afhankelijke ballonvorming, en waren ze aanmerkelijk verlaagd in het knippen van integrine β_3 . Ook lieten deze plaatjes een gedeeltelijk verminderde apoptose-afhankelijke PS-expositie zien. Al deze afwijkingen blijken ook te bestaan in de bloedplaatjes van een Scott patiënt. Uitschakelen van het anoctamine-6 gen in muizen leidde tot een duidelijk verminderde levensvatbaarheid; de enkele overlevende anoctamine-6 deficiënte muizen hadden een verhoogde staartbloedingstijd. Anderzijds konden wij geen afwijkend fenotype noteren in de bloedplaatjes van muizen, die anoctamine-1 of het Gardos-kanaal missen. Concluderend kan gesteld worden dat een defect in anoctamine-6 maar niet in de andere ionkanalen van invloed is op de levensvatbaarheid, en dat de bloedplaatjes van anoctamine-6 deficiënte muizen dezelfde kenmerken en defecten vertonen als aanwezig in bloedplaatjes van de Scott patiënt. Gezien het complexe fenotype van de Scott bloedplaatjes – defecten in PS-expositie, ballonvorming en

micropartikel-vorming –, postuleren wij in **hoofdstuk 8** dat er meerdere veranderingen moeten zijn in het eiwitpatroon van de bloedplaatjes van Scott patiënten. Om dit te onderzoeken hebben we de bloedplaatjes van gezonde controle-donoren vergeleken met die van een patiënt middels een zeer gevoelige en geavanceerde (fosfo)proteomics analyse. Kwantitatieve analyse van het proteoom van 2225 unieke eiwitten gaf aan dat minder dan 10% van de eiwitten veranderingen vertoonden in relatieve expressie. De veranderingen die er waren werden waargenomen in eiwitten van de volgende functieklassen: membraanreceptoren en kanalen, mitochondriële eiwitten, metabole eiwitten en signalering/adapter-eiwitten. Detailonderzoek toonde aan dat het eiwit anoctamine-6 niet te detecteren was in de Scott bloedplaatjes, terwijl het kanaaleiwit aquaporine-1 sterk verhoogd was ten opzichte van controle-bloedplaatjes. Analyse van het fosfoproteoom resulteerde in 1566 unieke fosfopeptiden (overeenkomend met 716 eiwitten). Er bleken duidelijke verschillen tussen bloedplaatjes van de patiënt en controle in de fosforyleringsstatus van diverse eiwitten, in het bijzonder na stimulatie met convulxine/trombine of ionomycine. In geactiveerde Scott bloedplaatjes vertoonde een beperkt aantal eiwitten

een verminderde fosforylering (bijvoorbeeld eiwitten van het cytoskelet). Anderzijds gaf een veel groter aantal eiwitten een verhoogde fosforylering wanneer vergeleken met controle-bloedplaatjes (eiwitten van het cytoskelet, celmembraan of signalering). In hetzelfde hoofdstuk identificeerden we in totaal 1613 neo-N-terminale peptiden – eiwitfragmenten gevormd door intracellulaire knip –, met ook daarin aanzienlijke verschillen tussen de Scott- en controle-bloedplaatjes. Van 180 neo-N-terminale peptiden (overeenkomend met 106 eiwitten) konden we vaststellen dat die gevormd worden als gevolg van calpaïne-activiteit. Daarnaast konden we van 23 neo-N-termini (23 eiwitten) bepalen, dat die geknipt worden door caspase-activiteit. In bloedplaatjes van de Scott patiënt maten we voornamelijk verlaging van de

neo-N-termini geproduceerd door calpaïne na stimulatie met convulxine/trombine (in het bijzonder van cytoskelet- of signaleringseiwitten) terwijl we een verhoging vonden na ionomycine-stimulatie. Samenvattend concluderen we dat geavanceerde proteomics profilering nieuwe inzichten verschaft in de veranderde eiwit-modificaties in Scott bloedplaatjes, die een verklaring kan geven van de calcium- en cytoskeleton-afhankelijke membraanveranderingen in deze bloedplaatjes.

In **hoofdstuk 9** worden de belangrijkste bevindingen van dit proefschrift besproken in het licht van de huidige literatuur. Beargumenteerd is hoe zowel de muizenstudies als de toegepaste proteomics technieken meer inzicht bieden in de complexiteit van de procoagulante bloedplaatjesrespons.

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Curriculum vitae

Nadine Mattheij werd geboren op 19 augustus 1987 te Sittard. In 1999 begon zij haar middelbare schoolopleiding aan het Trevianum College te Sittard, waar zij in 2005 haar VWO-diploma behaalde met als afstudeerprofiel Natuur & Gezondheid met Beeldende Vorming in het vrije deel. In 2005 begon zij de bacheloropleiding Moleculaire Levenswetenschappen aan de Universiteit Maastricht. Na het behalen van haar bachelor-grad in 2008, volgde zij aan dezelfde universiteit de masteropleiding Clinical Molecular Sciences. Haar afstudeerstage, naar de rol van endotheline-1 bij de vasoconstrictie, deed zij bij de vakgroep Farmacologie van de Universiteit Maastricht, waarna zij in 2010 haar master-diploma ontving. Op 1 augustus 2010 begon zij haar promotie-onderzoek binnen het Cardiovascular Research Institute Maastricht (CARIM) bij de vakgroep Biochemie van de Universiteit Maastricht. Onder leiding van Prof. Dr. J.W.M. Heemskerk en Dr. J.M.E.M. Cosemans voerde zij onderzoek uit in het domein trombose en hemostase, zoals beschreven in dit proefschrift. Als promovendus presenteerde zij haar onderzoek op internationale congressen in Maastricht, Bath (Groot-Brittannië), Amsterdam, Birmingham (Groot-Brittannië) en Le Bischoffshausen (Frankrijk). Een deel van dit onderzoek verrichtte zij in de laboratoria van Dr. S. Jobe in Atlanta (GA, USA), Prof. Dr. A. Chishti in Boston (MA, USA), Prof. Dr. B. Nieswandt in Würzburg (Duitsland), Prof. Dr. M. Torti in Pavia (Italië), en van Prof. Dr. K. Kunzelmann in Regensburg (Duitsland). Na werkzaam te zijn voor 7 maanden als Medical Advisory and Communication Specialist bij het farmaceutisch bedrijf Janssen Biologic te Leiden, startte ze in december 2015 als klinisch chemicus in opleiding aan het Zuyderland ziekenhuis te Sittard en Heerlen.

Nadine Mattheij was born on August 19 1987 in Sittard. In 1999 she started secondary school at the Trevianum in Sittard where she received her Atheneum degree in 2005 with the specialization Nature & Health with Visual Education in the free part. In 2005 she started her Bachelor in Molecular Life Sciences at Maastricht University. After earning her bachelor's degree in 2008, she studied at the same university the master Clinical Molecular LifeSciences. Her internship, investigating the role of endothelin-1 in vasoconstriction, was performed at the Department of Pharmacology at Maastricht University, after which she received her master's degree in 2010. On the 1st of August 2010 she started her PhD research at the Department of Biochemistry of the Cardiovascular Research Institute Maastricht (CARIM) at the University of Maastricht. Under direct supervision of Prof. Dr. J.W.M. Heemskerk and Dr. J.M.E.M. Cosemans she performed research in the field of thrombosis and hemostasis as described in this thesis. As a PhD student, she presented her research at international conferences in Maastricht, Bath (UK), Amsterdam, Birmingham (UK) and Le Bischenberg (France). Part of this research she conducted in the laboratories of Dr. S. Jobe in Atlanta (GA, USA), Prof. Dr. A. Chishti in Boston (MA, USA), Prof. Dr. B. Nieswandt in Würzburg (Germany), Prof. Dr. M. Torti in Pavia (Italy) and Prof. Dr. K. Kunzelmann in Regensburg (Germany). After working for seven months as a Medical Advisory and Communication Specialist at the pharmaceutical company Janssen Biologics in Leiden, she started in December 2015 as a clinical chemist in training at the Zuyderland hospital in Sittard and Heerlen.

Publications

Full papers

1. Meens MJ, **Mattheij NJ**, Nelissen J, Lemkens P, Compeer MG, Janssen BJ, De Mey JG. Calcitonin gene-related peptide terminates long-lasting vasopressor responses to endothelin 1 in vivo. *Hypertension*. 2011;58:99-106.
2. Meens MJ, **Mattheij NJ**, van Loenen PB, Spijkers LJ, Lemkens P, Nelissen J, Compeer MG, Alewijnse AE, De Mey. G-protein $\beta\gamma$ subunits in vasorelaxing and anti-endothelinergic effects of calcitonin gene-related peptide. *JG. Br J Pharmacol*. 2012;166:297-308.
3. Heemskerk JW, **Mattheij NJ**, Cosemans JM. Platelet-based coagulation: different populations, different functions. *J Thromb Haemost*. 2013;11:2-16.
4. Van Kruchten R, **Mattheij NJ**, Saunders C, Feijge MA, Swieringa F, Wolfs JL, Collins PW, Heemskerk JW, Bevers EM. Both TMEM16F-dependent and TMEM16F-independent pathways contribute to phosphatidylserine exposure in platelet apoptosis and platelet activation. *Blood*. 2013;121:1850-7.
5. **Mattheij NJ**, Gilio K, van Kruchten R, Jobe SM, Wieschhaus AJ, Chishti AH, Collins P, Heemskerk JW, Cosemans JM. Dual mechanism of integrin $\alpha_{IIb}\beta_3$ closure in procoagulant platelets. *J Biol Chem*. 2013;288:13325-36.
6. Kmit A, van Kruchten R, Ousingawatt J, **Mattheij NJ**, Senden-Gijsbers B, Heemskerk JW, Schreiber R, Bevers EM, Kunzelmann K. Calcium-activated and apoptotic phospholipid scrambling induced by Ano6 can occur independently of Ano6 ion currents. *Cell Death Dis*. 2013;4:e611.
7. Cosemans JM, Angelillo-Scherrer A, **Mattheij NJ**, Heemskerk JW. The effects of arterial flow on platelet activation, thrombus growth, and stabilization. *Cardiovasc Res*. 2013;99:342-52.
8. Kuijpers MJ, van der Meijden PE, Feijge MA, **Mattheij NJ**, May F, Govers-Riemslog J, Meijers, Renné T, Heemskerk JW, Cosemans JM. Factor XII regulates the pathological process of thrombus formation on ruptured plaques. *Arterioscler Thromb Vasc Biol*. 2014;34(8):1674-80.
9. Karshovska E, Zhao Z, Blanchet X, Schmitt MM, Bidzhekov K, Soehnlein O, von Hundelshausen P, **Mattheij NJ**, Cosemans JM, Megens RT, Koeppl T, Schober A, Hackeng TM, Weber C, Koenen RR. Hyperreactivity of junctional adhesion molecule A-deficient platelets accelerates atherosclerosis in hyperlipidemic mice. *Circ Res*. 2015;116(4):587-99.
10. Kuijpers MJ*, **Mattheij NJ***, Cipolla L, van Geffen JP, Lawrence T, Donners MM, Boon L, Lievens D, Torti M, Noels H, Gerdes N, Cosemans JM, Lutgens E,

- Heemskerk JW. Platelet CD40L modulates thrombus growth via phosphatidylinositol 3-Kinase β , and not via CD40 and I κ B Kinase α . *Arterioscler Thromb Vasc Biol.* 2015; 2015;35(6):1374-81.
11. Agbani EO, van den Bosch MT, Brown E, Williams CM, **Mattheij NJ**, Cosemans JM, Collins PW, Heemskerk JW, Hers I, Poole AW. Coordinated membrane ballooning and procoagulant spreading in human platelets. *Circulation.* 2015 Oct 13;132(15):1414-24.
 12. **Mattheij NJ**, Braun A, van Kruchten R, Pircher J, Castoldi E, Baaten C, Kuijpers M, Ehlen HW, Poole A, Vortkamp A, Schreiber R, Kunzelmann K, Nieswandt B, Cosemans JM, Heemskerk JW. The Scott syndrome protein anoctamin 6 regulates multiple responses in procoagulant platelets including membrane phospholipid scrambling. *FASEB J.* 2015 (Epub ahead of print)
 13. **Mattheij NJ**, Swieringa F, Lang-Berny M, May F, Suylen D, Nolte M, Hackeng T, McCarty O, van der Meijden PE, Heemskerk JW, Cosemans JM. Coated platelets: non-redundant roles of transglutaminases and integrin $\alpha_{IIb}\beta_3$ in platelet-fibrin formation distinct from phosphatidylserine exposure. *Resubmitted to Haematologica*
 14. **Mattheij NJ**, Solari F, Burkhart J, Swieringa F, Collins PW, Cosemans JM, Sickmann A, Heemskerk JW, Zahedi R. Altered phosphorylation profile of Scott syndrome platelets revealed by quantitative phosphoproteomics. *Submitted*
 15. Swieringa F*, Solari FA*, Feijge MA, **Mattheij NJ**, Beck F, Stumpel CT, Sickmann A, van der Meijden PE, Körver-Keularts IM, Zahedi RP*, Heemskerk JW*. Multiple changes in novel protein kinase A phosphorylation targets identified in platelets from patients with impaired G α signaling. *Submitted*
 16. Beck F, Geiger J, Gambaryan S, Loroach S, **Mattheij NJ**, Mindukshev I, Pötz O, Burkhart JM, Fufezan C, Heemskerk JW, Walter U, Zahedi RP, Sickmann A. Time-resolved phosphoproteomics points to novel central nodes in platelet activation and inhibition. *Submitted*

Abstracts

1. **Mattheij NJ**, Meens MJ, De Mey JG. G-protein $\beta\gamma$ subunits in vasorelaxing and anti-endothelinergic effects of calcitonin gene-related peptide. *Proc. TI Pharma spring meeting 2010*, Utrecht, The Netherlands.
2. Meens MJ, **Mattheij NJ**, van Essen H, Compeer MG, Janssen BJ, De Mey JG. Calcitonin gene-related peptide terminates endothelin-1-induced vasoconstriction

- and vasopressor responses. 12th Symposium on Vascular Neuroeffector Mechanisms, Odense, Denmark. *Acta Physiologica*, Suppl. July 2010.
3. **Mattheij NJ**, Meens MJ, De Mey JG. Role of G-protein subunits in arterial effects of CGRP. 12th Symposium on Vascular Neuroeffector Mechanisms, Odense, Denmark. *Acta Physiologica*, Suppl. July 2010.
 4. Van Kruchten R, Wolfs JL, Feijge MA, **Mattheij NJ**, Collins P, Heemskerk JW, Bevers EM. Role of TMEM16F in agonist-induced but not apoptosis-induced phosphatidylserine exposure in Scott platelets, XXIIIth Congress of the International Society on Thrombosis and Haemostasis (ISTH), Kyoto, Japan, 2011. *J Thromb Haemost*, Suppl. July 2011.
 5. **Mattheij NJ**, Gilio K, van Kruchten R, Feijge MA, Bevers EM, Jobe SM, Cosemans JM, Heemskerk JW. Regulation of integrin closure in procoagulant platelets. XXIIIth Congress of the International Society on Thrombosis and Haemostasis (ISTH), Kyoto, Japan, 2011. *J Thromb Haemost*, Suppl. July 2011.
 6. **Mattheij NJ**, Gilio K, Van Kruchten R, Shawn JM, Du X, Wieschhaus AJ, Chishti AH, Collins PW, Heemskerk JW, Cosemans JM. Regulation of integrin closure in procoagulant platelets. *Proc. British Society for Haemostasis & Thrombosis and the UK Platelet Annual Joint Scientific Meeting 2012*, Bath, United Kingdom.
 7. Van Kruchten R, **Mattheij NJ**, Saunders C, Feijge MA, Collins PW, Heemskerk JW, Bevers EM. Scott syndrome platelets demonstrate presence of TMEM16F-dependent and -independent phospholipid scrambling in both platelet apoptosis and platelet activation. *Proc. 1st EUPLAN Platelet Conference*, Maastricht, The Netherlands.
 8. **Mattheij NJ**, Gilio K, Van Kruchten R, Shawn JM, Wieschhaus AJ, Chishti AH, Collins PW, Heemskerk JW, Cosemans JM. Dual mechanism of integrin closure in procoagulant platelets. *Proc. 1st EUPLAN Platelet Conference*, Maastricht, The Netherlands.
 9. Kuijpers M, **Mattheij NJ**, Cipolla L, Cosemans JM, Lievens D, Donners M, Swieringa F, Mastenbroek T, Torti M, Lutgens E, Heemskerk JW. A role for CD40L signalling in platelet activation and thrombus formation that is independent of CD40. XXIVth Congress of ISTH, Amsterdam, The Netherlands. *J Thromb Haemost*, Suppl. June 2013.
 10. **Mattheij NJ**, Braun A, van Kruchten R, Cosemans JM, van der Meijden P, Baaten C, Ehlen H, Schreiber R, Vortkamp A, Collins P, Bevers EM, Ousingawatt J, Kunzelmann K, Nieswandt B, Heemskerk JW. The Scott syndrome protein anoctamin 6 (TMEM16F) regulates multiple cell death responses including

- membrane phospholipid scrambling in platelets. XXIVth Congress of ISTH, Amsterdam, The Netherlands. *J Thromb Haemost*, Suppl. June 2013
11. **Mattheij NJ**, Berny-Lang M, van de Vijver P, Hackeng T, McCarty O, Cosemans JM, Heemskerk JW. Functional characteristics of coated platelets. XXIVth congress of ISTH, Amsterdam, The Netherlands. *J Thromb Haemost*, Suppl. June 2013
 12. **Mattheij NJ**, Braun A, van Kruchten R, Cosemans JM, van der Meijden P, Baaten C, Ehlen H, Schreiber R, Vortkamp A, Collins P, Bevers EM, Ousingawati J, Kunzelmann K, Nieswandt B, Heemskerk JW. The Scott syndrome protein anoctamin 6 (TMEM16F) regulates multiple cell death responses including membrane phospholipid scrambling in platelets. *Proc. 15th UK Platelet Group Meeting*, Birmingham, United Kingdom
 13. **Mattheij NJ**, de Witt S, Mastenbroek T, Zieger B, Cosemans JM, Heemskerk JW. Human platelet STIM1 and ORAI1 are essential in store-operated calcium entry and thrombus formation under venous conditions. *Proc. 2nd EUPLAN Conference*, Le Bischoff, France.
 14. **Mattheij NJ**, Solari F, Burkhardt J, Swieringa F, Collins PW, Cosemans JM, Sickmann A, Zahedi R, Heemskerk JW. Altered phosphorylation profile of Scott syndrome platelets as revealed by quantitative phosphoproteomics. *Proc. 2nd EUPLAN Conference*, Le Bischoff, France.
 15. Solari F, **Mattheij NJ**, Burkhardt J, Swieringa F, Collins PW, Cosemans JM, Sickmann A, Heemskerk JW, Zahedi R. Calpain-dependent cleavage in highly activated platelets revealed by quantitative N-terminal ChFRADIC: alterations in Scott syndrome. *Proc. HUPO 13th Annual World Congress*, Madrid, Spain.
 16. **Mattheij NJ**, Solari F, Burkhardt J, Swieringa F, Collins PW, Cosemans JM, Sickmann A, Zahedi R, Heemskerk JW. Altered phosphorylation profile of Scott syndrome platelets as revealed by quantitative phosphoproteomics. *Proc. HUPO 13th Annual World Congress*, Madrid, Spain.

International oral presentations

1. **Mattheij NJ**, G-protein $\beta\gamma$ subunits in vasorelaxing and anti-endothelinergic effects of calcitonin gene-related peptide, 12th Symposium on Vascular Neuroeffector Mechanisms, Odense, Denmark (July 2010).
2. **Mattheij NJ**, Regulation of integrin closure in procoagulant platelets, 1st European Platelet Conference (EUPLAN), Maastricht, The Netherlands (September 2012).

3. **Mattheij NJ**, Regulation of integrin closure in procoagulant platelets, British Society on Haemostasis & Thrombosis and the UK platelet annual joint scientific Meeting 2012, Bath, United Kingdom (October 2012).
4. **Mattheij NJ**, The Scott syndrome protein anoctamin 6 (TMEM16F) regulates multiple cell death responses including membrane phospholipid scrambling in platelets, XXIVth Congress of the International Society on Thrombosis and Haemostasis, Amsterdam, The Netherlands (July 2013).
5. **Mattheij NJ**, Functional characteristics of coated platelets, XXIVth Congress of the International Society on Thrombosis and Haemostasis, Amsterdam, The Netherlands (July 2013).
6. **Mattheij NJ**, The Scott syndrome protein anoctamin 6 (TMEM16F) regulates multiple cell death responses including membrane phospholipid scrambling in platelets, 15th UK Platelet Group Meeting, Birmingham, United Kingdom (September 2013).
7. **Mattheij NJ**, Altered phosphorylation profile of Scott syndrome platelets as revealed by quantitative phosphoproteomics, 2nd EUPLAN Conference, Le Bischenberg, France (September 2014).

Appendix

Platelet CD40L Modulates Thrombus Growth Via Phosphatidylinositol 3-Kinase β , and Not Via CD40 and I κ B Kinase α

Kuijpers MJ^{1*}, Mattheij NJ^{1*}, Cipolla L², van Geffen JP¹, Lawrence
T³, Donners MM⁴, Boon L⁵, Lievens D⁶, Torti M², Noels
H⁷, Gerdes N⁶, Cosemans JM¹, Lutgens E^{5,8}, Heemskerk JW¹

¹Department of Biochemistry CARIM, Maastricht University, Maastricht, The Netherlands; ²Department of Biology and Biotechnology, University of Pavia, Pavia, Italy; ³Division of Biochemistry, Centre d'Immunologie de Marseille-Luminy, Aix-Marseille Université, Marseille; ⁴Department of Molecular Genetics, CARIM, Maastricht University, Maastricht, The Netherlands; ⁵Bioceros, Utrecht, The Netherlands; ⁶Institute for Cardiovascular Prevention, Ludwig-Maximilians-University, Munich, Germany; ⁷Institute for Molecular Cardiovascular Research (IMCAR), RWTH Aachen University, Aachen, Germany; ⁸Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

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Abstract

Objective - To investigate the roles and signaling pathways of CD40L and CD40 in platelet-platelet interactions and thrombus formation under conditions relevant for atherothrombosis. **Approach and Results** - Platelets from mice prone to atherosclerosis lacking CD40L (*Cd40lg^{-/-}Apoe^{-/-}*) showed diminished $\alpha_{IIb}\beta_3$ activation and α -granule secretion in response to glycoprotein VI stimulation, whereas these responses of CD40- deficient platelets (*Cd40^{-/-}Apoe^{-/-}*) were not decreased. Using blood from *Cd40lg^{-/-}Apoe^{-/-}* and *Cd40^{-/-}Apoe^{-/-}* mice, the glycoprotein VI-dependent formation of dense thrombi was impaired on atherosclerotic plaque material or on collagen, in comparison with *Apoe^{-/-}* blood. In all genotypes, addition of CD40L to the blood enhanced the growth of dense thrombi on plaques and collagen. Similarly, CD40L enhanced glycoprotein VI-induced platelet aggregation, even with platelets deficient in CD40. This potentiation was antagonized in *Pik3cb^{R/R}* platelets or by inhibiting phosphatidylinositol 3-kinase β (PI3K β). Addition of CD40L also enhanced collagen-induced Akt phosphorylation, which was again antagonized by absence or inhibition of PI3K β . Finally, platelets from *Chuk1^{A/A}Apoe^{-/-}* mice deficient in I κ B kinase α (IKK α), implicated in CD40 signaling to nuclear factor (NF) κ B, showed unchanged responses to CD40L in aggregation or thrombus formation. **Conclusions** - Under atherogenic conditions, CD40L enhances collagen-induced platelet-platelet interactions by supporting integrin $\alpha_{IIb}\beta_3$ activation, secretion and thrombus growth via PI3K β , but not via CD40 and IKK α /NF κ B. This role of CD40L exceeds the no more than modest role of CD40 in thrombus formation.

Introduction

Atherothrombosis is a life-threatening clinical condition that can result in myocardial infarction or stroke because of thrombus formation on rupture or erosion of an atherosclerotic plaque.¹ Both in vivo and in vitro studies have indicated that collagen is one of the main platelet-adhesive components of atherosclerotic plaques, causing platelet activation through the signaling collagen receptor, glycoprotein VI (GPVI).²⁻⁴ Collagen-dependent thrombus formation

is a complex and highly orchestrated process. Key steps are the adhesion of platelets to collagen, the activation of flowing platelets into aggregates via secreted autocrine mediators, such as ADP and thromboxane A₂; the activation of integrin $\alpha_{IIb}\beta_3$ via a phosphoinositide 3-kinase (PI3K) pathway to allow stable platelet-platelet interactions; and the formation of phosphatidylserine-exposing platelets, which support thrombin generation and fibrin formation.⁵ Platelets express substantial

levels of the membrane glycoprotein CD40 (TNFR-SF5), which is an ubiquitous member of the tumor necrosis factor receptor (TNFR) superfamily.^{6,7} The alleged counter-receptor is CD40L (TNF-SF5 and CD154), which belongs to the TNF superfamily and is present on human platelets at ≈ 1600 copies/platelet.⁸ In activated platelets, CD40L can be cleaved from the membrane by extracellular proteases, yielding a functional, extracellular peptide that is usually indicated as soluble CD40L.^{9,10} Several reports point to a key role of platelet CD40L in atherosclerosis and atherothrombosis. Patients with (re)current cardiovascular disease often show increased levels of circulating soluble CD40L, predominantly originating from platelets.^{11,12} In mouse models, deficiency in *Apoe* or *Ldlr* leads to elevation of plasma lipids and predisposes for atherosclerosis. In these atherogenic mice, we and others have reported that additional deficiency in CD40L (*Cd40lg*^{-/-}) or CD40 (*Cd40*^{-/-}) markedly impairs plaque development.^{13,14} Furthermore, we have shown that the injection of activated *Cd40lg*^{-/-} platelets to *Apoe*^{-/-} mice suppresses platelet-leukocyte aggregate formation, subsequent inflammation, and the platelet-dependent progression of atherosclerosis.¹⁵⁻¹⁷ The CD40L-CD40

axis may also play a role in the crosstalk of platelets themselves, and thus contribute to the stability of platelet thrombi. For instance, in mice deficiency in either CD40L or CD40 was found to protect against microvascular thrombosis when the animals were challenged by lipopolysaccharide.⁷ Other studies have suggested a mechanism, in which CD40L interacts with CD40-TRAF2 and stimulates the classical nuclear factor (NF) κ B pathway in platelets.^{18,19} In agreement with this postulation, pharmacological inhibition of NF κ B was shown to suppress platelet activation.²⁰ However, some reports indicate that the cleaved CD40L can interact with a different counter-receptor in both mouse and human platelets, namely the integrin β_3 chain.^{21,22} The binding of CD40L can support $\alpha_{IIb}\beta_3$ -dependent activation processes that have been implicated in thrombus stability. In this article, we aimed to unravel the roles and the underlying signaling pathways of CD40L and CD40 in platelet activation and thrombus formation under conditions relevant for atherothrombosis. For this purpose, we used blood from *Apoe*^{-/-} mice also deficient in CD40 or CD40L, prone to atherosclerosis, to investigate the activation pathways of platelets stimulated by collagen or plaque tissue via the GPVI receptor.

Figure 1. Agonist-induced integrin activation and secretion by *Apoe*^{-/-} platelets lacking CD40 or CD40L. Washed platelets from *Apoe*^{-/-}, *Cd40*^{-/-}*Apoe*^{-/-}, or *Cd40lg*^{-/-}*Apoe*^{-/-} mice were activated with the glycoprotein VI agonist convulxin (25–100 ng/mL), thrombin (0.25–2 nmol/L) or ADP (0.5–5 μmol/L). Using flow cytometry, activation of integrin α IIb β 3 was determined with PE-labeled JON/A monoclonal antibody (mAb; **A**), and P-selectin expression with FITC (fluorescein isothiocyanate)-labeled anti-CD62P mAb (**B**). Mean $\pm$ SEM (n=3–5), **P*<0.05.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

To compare the roles of platelet CD40 and CD40L under conditions relevant for atherosclerosis and atherothrombosis, we used *Apoe*^{-/-} mice with high plasma lipid levels and prone to atherosclerosis.²³ Platelets were isolated from *Apoe*^{-/-}, *Cd40*^{-/-}*Apoe*^{-/-}, and

Cd40lg^{-/-}*Apoe*^{-/-} mice, and stimulated with key receptor agonists, that is, convulxin activating the collagen receptor GPVI,²⁴ ADP as an agonist stimulating the P2Y₁/P2Y₁₂ receptors, and thrombin stimulating the PAR3/4 receptors.⁵ Platelet activation properties were compared by flow cytometry. Deficiency in CD40 led to increased integrin α IIb β 3 activation (measured with PE-JON/A monoclonal antibody) and

Figure 1. Agonist-induced integrin activation and secretion by *Apoe*^{-/-} platelets lacking CD40 or CD40L. Washed platelets from *Apoe*^{-/-}, *Cd40*^{-/-}*Apoe*^{-/-}, or *Cd40lg*^{-/-}*Apoe*^{-/-} mice were activated with the glycoprotein VI agonist convulxin (25–100 ng/mL), thrombin (0.25–2 nmol/L) or ADP (0.5–5 $\mu\text{mol/L}$). Using flow cytometry, activation of integrin $\alpha_{\text{IIb}}\beta_3$ was determined with PE-labeled JON/A monoclonal antibody (mAb; **A**), and P-selectin expression with FITC (fluorescein isothiocyanate)-labeled anti-CD62P mAb (**B**). Mean $\pm$ SEM (n=3–5), **P*<0.05.

increased α -granule secretion (measured with FITC (fluorescein isothiocyanate)-labeled anti-P-selectin monoclonal antibody), when the platelets were stimulated with a low or high dose of convulxin (Figure 1A and 1B). In marked contrast, deficiency in CD40L led to reduced $\alpha_{IIb}\beta_3$ activation and α -granule secretion after stimulation with convulxin. Responses triggered by thrombin or ADP were not changed in the CD40- or CD40L-deficient platelets. Thrombin, as a strong agonist, showed a rather steep dose-response curve with substantial integrin activation and P-selectin expression already at 0.25 nmol/L. At lower thrombin doses, these responses became highly variable (not shown). Stimulation with ADP resulted in high $\alpha_{IIb}\beta_3$ activation, but not in α -granule secretion, as previously demonstrated.²⁵ Taken together, these results suggest that, in *Apoe*^{-/-} mice, platelet CD40L and CD40 play nonidentical roles in GPVI-induced integrin activation and α -granule secretion.

Earlier experiments have demonstrated a primary role of GPVI in platelet adhesion and thrombus formation on plaque tissue or collagen at arterial flow conditions.^{2,3} We used this method of in vitro thrombus formation to investigate the roles of CD40 and CD40L in *Apoe*^{-/-} mice prone to develop atherosclerosis. Therefore, blood from *Apoe*^{-/-},

Cd40^{-/-}*Apoe*^{-/-}, or *Cd40lg*^{-/-}*Apoe*^{-/-} mice was perfused over murine plaque material at high wall shear rate, and the thrombi formed were analyzed by brightfield and fluorescence microscopy (Figure 2A). Image analysis gave for each genotype a similar deposition of platelets on plaque material (Figure 2B), albeit the overall size of platelet aggregates was smaller in the absence of CD40 or CD40L (Figure 2C). Whereas P-selectin expression was similar for all genotypes, phosphatidylserine exposure (indicating procoagulant activity) was significantly reduced for *Cd40*^{-/-}*Apoe*^{-/-} and *Cd40lg*^{-/-}*Apoe*^{-/-} platelets in comparison with *Apoe*^{-/-} platelets (Figure 2D and 2E). Blood perfusion over collagen gave comparable results: whereas platelet deposition was similar for each genotype, the build-up of large thrombi was consistently diminished in the absence of CD40L. This difference seemed most clearly from the area covered by multilayered aggregates, which was significantly lowered in the absence of CD40L, but not in the absence of CD40 (Figure 1A–1C in the online-only Data Supplement). Again, phosphatidylserine exposure was reduced in the absence of CD40 or CD40L, whereas P-selectin expression was unaltered (Figure 1D and 1E in the online-only Data Supplement). To determine the ability of cleaved CD40L to support thrombus formation, blood samples from *Apoe*^{-/-},

Figure 2. Increased thrombus formation on atherosclerotic plaque material by added CD40L in all genotypes. Blood from *Apoe*^{-/-}, *Cd40*^{-/-}*Apoe*^{-/-}, or *Cd40lg*^{-/-}*Apoe*^{-/-} mice was anticoagulated with PPACK (H-d-phenylalanyl-prolyl-arginyl chloromethyl ketone)/heparin, and perfused over murine plaque material at 1000 s⁻¹ for 4 minutes. Blood samples were preincubated with CD40L (1 $\mu\text{g}/\text{mL}$), where indicated. Thrombi were stained by postperfusion with FITC (fluorescein isothiocyanate)-labeled anti-P-selectin monoclonal antibody and AF647-annexin A5. **A**, Representative brightfield and fluorescence images (bar, 50 μm). **B**, Quantification of surface area coverage (%SAC) of platelet deposition. **C**, Quantification of integrated feature size from brightfield images. **P*<0.05 compared with no CD40L, #*P*<0.05 compared with *Apoe*^{-/-}. **D** and **E**, Quantification of P-selectin expression and phosphatidylserine exposure from fluorescence images. Mean $\pm$ SEM (*n*=3–5), **P*<0.05.

Cd40^{-/-}Apoe^{-/-}, or *Cd40lg^{-/-}Apoe^{-/-}* mice were preincubated with CD40L peptide before the perfusion. For all genotypes, this resulted in a substantial increase in thrombus formation on plaque material (Figure 2A) and on collagen (Figure 1A in the online-only Data Supplement). Quantification learned that in the presence of CD40L peptide larger platelet aggregates were formed on plaque material (Figure 2C), which were best detected as multilayered aggregates on collagen (Figure 1F in the online-only Data Supplement), regardless of the genotype, that is, also with platelets deficient in CD40 or CD40L. Expression of P-selectin and phosphatidylserine exposure were not affected by the CD40L preincubation (not shown). Further experiments using blood from *Apoe^{-/-}* mice indicated that preincubation of the

blood with a blocking antibody against CD40L caused a reduction in the formation of multilayered aggregates showing less $\alpha_{IIb}\beta_3$ activation, thus indicating a role of autologous CD40L in the thrombus growth (Figure II in the online-only Data Supplement).

Other authors have reported a stimulating effect of the cleaved CD40L on platelet aggregation in response to low doses of agonists.^{18,19} To investigate this in more detail, we examined this in *Apoe^{-/-}* mouse platelets also deficient in CD40L or CD40. Using platelets from *Apoe^{-/-}*, *Cd40^{-/-}Apoe^{-/-}*, or *Cd40lg^{-/-}Apoe^{-/-}* mice, a low collagen dose (0.5 $\mu\text{g/mL}$) did not result in appreciable aggregation nor shape change, whereas a high dose (5 $\mu\text{g/mL}$) induced maximal aggregation for all

Supplementary Figure II. Inhibition of CD40L modulates thrombus growth on collagen. Blood from *Apoe^{-/-}* mice was anticoagulated with PPACK/heparin, supplemented with fibrinogen-AF546 (10 $\mu\text{g/mL}$) and perfused over collagen at 1000 s⁻¹ for 4 minutes. Blood samples were pre-incubated with a blocking antibody against murine CD40L (20 $\mu\text{g/mL}$) or control. **A**, Quantification of surface area covered by multi-layered thrombi (%SAC as percentage of control). **B**, Quantification of fluorescence images of AF546-fibrinogen. Means $\pm$ SEM (n=3), **P*<0.05.

Figure 3. Enhancing role of CD40L in collagen-induced platelet aggregation and Akt phosphorylation. Washed platelets ($2.5 \times 10^8/\text{mL}$) from *Apoe*^{-/-}, *Cd40*^{-/-}*Apoe*^{-/-}, or *Cd40lg*^{-/-}*Apoe*^{-/-} mice were incubated with CD40L (1 $\mu\text{g}/\text{mL}$) for 30 minutes, and subsequently stimulated with the glycoprotein VI agonist, collagen (coll, 0.5–5 $\mu\text{g}/\text{mL}$). **A**, Representative aggregation traces. **B**, Maximal platelet aggregation (maximal change in light transmission). **C** and **D**, Platelet samples were taken from aggregation cuvettes after 10 minutes. Shown are representative western blots, and quantification of Akt phosphorylation at Ser473 and of total Akt. Lane 1: unstimulated platelets (unstim.), lane 2: collagen 5 $\mu\text{g}/\text{mL}$, lane 3: collagen 0.5 $\mu\text{g}/\text{mL}$, lane 4: collagen 0.5 $\mu\text{g}/\text{mL}$ +CD40L. Bars indicate relative phosphorylation of Akt (relative to total Akt), compared with high collagen (C), or compared with unstimulated platelets (D). Mean $\pm$ SEM ($n=3-4$), * $P<0.05$, # $P=0.06$.

genotypes (Figure 3A and 3B). Although exogenous CD40L alone was without effect, together with low collagen it caused near-maximal aggregation in all genotypes, including in platelets from the CD40-deficient mice. Taken together, these data point to a role of (cleaved) CD40L acting at least in part independently of CD40. In pilot experiments, we considered the possible involvement of another TNFR–TNF axis,

that is, of the LIGHT receptor (TNFR-SF14) and LIGHT (TNF-SF14), which were reported to be present in platelets.²⁶ However, a blocking antibody against this receptor was unable to inhibit the enhancement by CD40L of platelet responses (not shown).

Considering the major role of the PI3K/Akt signaling pathway in integrin activation,²⁷ we compared the ability of collagen to induce phosphorylation at

Ser473 of Akt in platelets from the CD40- and CD40L-deficient mice. The low dose of collagen (0.5 $\mu\text{g/mL}$) not causing platelet aggregation was also unable to stimulate Akt phosphorylation, whereas the high collagen dose (5 $\mu\text{g/mL}$) led to an increased phosphorylation by 4 to 6 times (Figure 3C). Markedly, this collagen-induced phosphorylation of Akt was diminished in *Cd40lg^{-/-}Apoe^{-/-}* platelets and tended to be lower in *Cd40^{-/-}Apoe^{-/-}*, when compared with *Apoe^{-/-}* platelets (Figure 3D). Addition of CD40L alone did not evoke Akt phosphorylation (not shown), but in combination with low collagen, it caused

a similar increase in phosphorylation in platelets from all genotypes (Figure 3C). Given that this phosphorylation is a prerequisite for integrin activation,^{28,29} these results suggest a key signaling role of PI3K- β in the stimulating effect of CD40L on collagen-induced platelet aggregation.

To further study the involvement of PI3K in CD40L-dependent platelet responses, we performed aggregation experiments with platelets from *Pik3cb^{R/R}* mice, expressing a catalytically inactive K805R mutant of PI3K- β , that is, an isoform that has previously been shown to control collagen-dependent signaling events.³⁰

Supplementary Figure III. Inhibition of PI3K- β abrogates CD40L enhancement of collagen-induced platelet aggregation. Washed platelets from wildtype mice ($2.5 \times 10^8/\text{mL}$) were pre-incubated with CD40L (1 $\mu\text{g/mL}$) and TGX-221 (1 μM), as indicated. Aggregation was induced with collagen (coll 0.5-5 $\mu\text{g/mL}$), and maximal amplitude of light transmission was measured. Means $\pm$ SEM ($n=4-10$), * $P<0.05$ compared to low collagen, ** $P<0.05$ compared to low collagen + CD40L.

Figure 4. Enhancing role of CD40L in collagen-induced platelet aggregation and Akt phosphorylation via PI3K- β . Washed platelets from *Pik3cb*^{R/R} or corresponding wildtype mice (*Pik3cb*^{WT/WT}) were activated with collagen (coll, 0.5–5 μ g/mL), as described in Figure 3. **A**, Representative aggregation traces. **B**, Quantification of platelet aggregation (maximal change in light transmission). Mean $\pm$ SEM (n=5–6). **C**, Representative western blots and quantification of Akt phosphorylation at Ser⁴⁷³ and of total Akt. Bars show relative phosphorylation of Akt (relative to total Akt), compared with high collagen (wild-type). Mean $\pm$ SEM (n=3), **P*<0.05 compared with wild-type.

When stimulated with high collagen, the aggregation of *Pik3cb*^{R/R} platelets was considerably reduced but not annulled (Figure 4A), which is in agreement with published results.³¹ With low collagen, exogenous CD40L failed to enhance the aggregation of *Pik3cb*^{R/R} platelets, in contrast to the large effect of CD40L seen with wildtype (*Pik3cb*^{WT/WT}) platelets (Figure 4A and 4B). Markedly, CD40L was also unable to stimulate Akt

phosphorylation in *Pik3cb*^{R/R} platelets (Figure 4C). Additional confirmation for a role of PI3K- β came from the observation that the PI3K- β -specific inhibitor, TGX-221, abolished the stimulating effect of CD40L on collagen-induced aggregation (Figure III in the online-only Data Supplement).

Perfusion experiments were subsequently performed with blood from *Pik3cb*^{R/R} and *Pik3cb*^{WT/WT} mice over a

collagen surface. In case of *Pik3cb^{R/R}* blood, the thrombi consisted of much smaller platelet aggregates, when compared with the wild-type (Figure IV in the online-only Data Supplement). For *Pik3cb^{R/R}* thrombi, P-selectin expression was unchanged, whereas phosphatidylserine exposure was reduced. Notably, addition of CD40L to blood samples increased the size of platelet aggregates for wild-type, but not

Pik3cb^{R/R} mice. Confirming experiments were performed in the presence of TGX-221. When TGX-221-treated *Apoe^{-/-}* blood, was flowed over collagen or plaque material, this resulted in thrombi with smaller platelet aggregates and reduced phosphatidylserine exposure (Figure V in the online-only Data Supplement). Largely based on pharmacological evidence, a role has been proposed for

Supplementary Figure IV. Enforcing role of CD40L in thrombus formation on collagen via PI3K-b. Blood from *Pik3cb^{R/R}* or corresponding wildtype mice (*Pik3cb^{WT/WT}*) was perfused over collagen at 1000 s^{-1} for 4 minutes. Pre-incubation with CD40L ($1\text{ }\mu\text{g/mL}$) was as indicated. **A**, Representative brightfield and fluorescence images (bar, $50\text{ }\mu\text{m}$). **B**, Quantification of integrated feature size. **C-D**, Quantification of P-selectin expression and phosphatidylserine exposure. Means $\pm$ SEM ($n=3-4$), * $P<0.05$ vs. *Pik3cb^{WT/WT}*.

NF κ B pathways in CD40L- and CD40-dependent platelet responses.¹⁹ To take this further, we first studied effects of the NF κ B inhibitor Ro-106-9920 (2.5–25 μ mol/L, targeting I- κ B α) and I κ B kinase (IKK) inhibitor VII (0.1–10 μ mol/L, targeting IKK β) on mouse platelet aggregation. Markedly, either compound caused a similar degree of inhibition on the aggregation responses induced by collagen alone or by collagen plus CD40L (data not shown). Because these compounds also suppressed thrombin- and collagen-induced calcium responses, we suspected off-target effects. For the I- κ B α inhibitor BAY 11-7082, an alternative mode of action in platelets has already been shown.³²

In nucleated cells, CD40 ligation is known to trigger several pathways leading to activation of NF κ B.³³ The noncanonical pathway proceeds via phosphorylation of the regulatory protein IKK α , which relieves the default inhibition of κ B kinase (p100) to induce signaling downstream of NF κ B.³⁴ To evaluate a contribution of this pathway in CD40L-mediated platelet activation, we used *Chuk1^{A/A}Apoe^{-/-}* mice, which are homozygous for an activation-resistant mutation of IKK α .³⁵ Markedly, in platelets from IKK α -deficient mice, the ability of CD40L to enhance aggregation was not affected (Figure 5A). Flow studies further indicated that the size of platelet aggregates on collagen was not

different for control *Apoe^{-/-}* and *Chuk1^{A/A}Apoe^{-/-}* blood (Figure 5B–5D). Surprisingly, phosphatidylserine exposure was increased in thrombi from *Chuk1^{A/A}Apoe^{-/-}* mice (Figure 5E). Together, these results indicate that the stimulatory effect of CD40L on thrombus growth is not mediated via the IKK α pathway to NF κ B.

Discussion

The glycoproteins CD40L and CD40 play major roles in autoimmune and inflammatory reactions mediated by T-cells, endothelial cells, and leukocytes, by stimulating signaling toward NF κ B activation.^{36,37} For the CD40L-CD40 dyad, involvement has been reported of the noncanonical and canonical NF κ B pathways.³³ Cross-talk of CD40L and CD40 has also been shown for platelet-leukocyte and platelet-endothelial cell interactions and, as such implicated in the progression of mouse atherosclerosis^{13,15,38} and neointima formation.¹⁶ Here, we investigated the importance of the CD40L-CD40 axis for establishment of platelet-platelet interactions using mice with an atherogenic *Apoe^{-/-}* background, that is, conditions where these platelet glycoproteins are considered to contribute to plaque progression. Our data indicate that CD40L and CD40 have, in part, different roles in platelet aggregation and thrombus growth.

Figure 5. Enhanced platelet aggregation and thrombus formation by CD40L are independent of IκB kinase α. **A**, Washed platelets from *Apoe*^{-/-} or *Chuk1*^{Δ/Δ}*Apoe*^{-/-} mice were activated with collagen (coll, 0.5–5 μg/mL), as described in Figure 3. Samples were preincubated with TGX-221 (1 μmol/L), where indicated. Data are quantification of platelet aggregation (maximal change in light transmission). Mean±SEM (n=3–6). **B**, Mouse blood anticoagulated with PPACK (H-d-phenylalanyl-prolyl-arginyl chloromethyl ketone)/heparin was perfused over collagen at 1000 s⁻¹ for 4 minutes. Thrombi were stained, as for Figure 2. Shown are representative brightfield and fluorescence images (bar, 50 μm). **C**, Quantification of percentage surface area coverage (%SAC) of platelets. **D** and **E**, Quantification of P-selectin expression and phosphatidylserine exposure from fluorescence images. Mean±SEM (n=6–7).

In studies of whole blood thrombus formation on plaque material or collagen, in which platelet adhesion and activation are known to be triggered by GPVI,^{2,39} we found that deficiency in CD40L or CD40 (not significant with collagen) impaired the formation of large, multilayered platelet aggregates. This reduced thrombus growth did not coincide with altered α -granule secretion (P-selectin expression), but was accompanied by reduced phosphatidylserine exposure, an event that also is mediated by the collagen receptor GPVI.²⁴ In accordance with these findings, others have reported that deficiency of CD40L alone (no *Apoe*^{-/-}) leads to reduced arterial thrombus formation in vivo.²¹ In earlier work, we have found that deficiency of CD40 alone causes a small, insignificant reduction in platelet deposition on collagen.¹⁶ Together, these results point to a moderate contribution of CD40L and CD40 in collagen-dependent thrombus growth.

Flow cytometric studies gave, in part, similar results. When triggered with the GPVI agonist convulxin, platelets from *Apoe*^{-/-} mice lacking CD40L showed a reduced integrin activation and secretion. However, platelets lacking CD40 responded by a higher integrin activation and higher P-selectin expression. The apparent different response of *Cd40*^{-/-}*Apoe*^{-/-} platelets, when compared

with the measurement of thrombus formation, can be explained by the fact that in flow cytometry all platelets become activated by the added GPVI agonist, whereas during thrombus formation only those platelets in direct contact with the collagen are activated via GPVI and the other platelets respond to paracrine agents.^{5,40} Hence, it seems that in thrombus-forming experiments the apparent gain-of-function of *Cd40*^{-/-}*Apoe*^{-/-} platelets is overruled by non-GPVI-mediated secondary processes.

The dissociation seen in flow cytometry between the phenotypes of CD40L or CD40 deficiency is one indication for, in part, dissimilar roles of platelets CD40L and CD40. Further confirmation for a role of CD40L independently of CD40 in platelet-platelet interactions comes from (1) the observed thrombus-reducing effect by blocking CD40L, and (2) the enhanced platelet aggregation and thrombus formation noticed by supplementation of CD40L even for *Cd40*^{-/-}*Apoe*^{-/-} platelets lacking CD40. Hence, we could not reproduce the finding from others that knockout of CD40 abolishes CD40L-dependent platelet aggregation.¹⁸ The reason for this difference remains unclear. Taken together, our data imply that, in the stimulating platelet aggregation and thrombus growth, CD40L acts at least, in part, independently of CD40.

Supplementary Figure V. Role of PI3K- β in thrombus formation on collagen or plaque material. Blood from *Apoe*^{-/-} mice was perfused over collagen or plaque material at 1000 s⁻¹ for 4 minutes. Blood samples were pre-incubated with TGX-221 (1 μM) or vehicle (DMSO). Thrombi were stained and images were analyzed as for Figure 2. **A, D**, Representative brightfield and fluorescence images (bar, 50 μm). **B, E**, Quantification of integrated feature size from brightfield images. **C, F**, Quantification of phosphatidylserine exposure (%SAC). Means $\pm$ SEM (n=3-5), **P*<0.05 vs. control.

Another novel finding is the role of PI3K- β (using deficient mice and inhibitor TGX-221) and its downstream target Akt (based on Ser473 phosphorylation) in the enhancing effect of CD40L on collagen-induced platelet aggregation and thrombus formation. This role of PI3K- β is in good agreement with the literature, demonstrating that this kinase is a major regulator of Akt phosphorylation and downstream integrin activation in

response to various platelet agonists.^{31,41-43} Other authors have suggested that CD40L in platelets signals via p38 mitogen-activated protein kinase,¹⁹ or via tyrosine phosphorylation of the integrin β_3 chain.²² These pathways may coexist and help to support the establishment of platelet-platelet interactions. Of interest, a comparable signaling pathway, involving PI3K and Akt, has been proposed for

endothelial cells, but in this case elicited by CD40 and leading to NF κ B activation.³⁷

The unchanged collagen-induced aggregation and thrombus formation with *Apoe*^{-/-} platelets deficient in IKK α activity (*Chuk1*^{A/A}), as well as the unchanged ability of CD40L to stimulate aggregation of these platelets, points to a no more than minor role of the noncanonical IKK α /NF κ B pathway - whether or not triggered via CD40 - in platelet-platelet interactions. In this respect, we like to note that evidence for a role of the NF κ B pathway in CD40-dependent platelet function is largely based on the use of pharmacological inhibitors.¹⁹ We suspect that at least some of these inhibitors have effects on platelet responses (eg, Ca²⁺ signaling), that are not mediated via NF κ B. However, several reports using IKK β knockout mice point to a regulatory role of the canonical NF κ B pathway in platelet secretion and glycoprotein shedding.^{44,45} Hence, we cannot rule out a role for this IKK β /NF κ B pathway in platelet activation responses evoked by CD40L independently of CD40.

Interestingly, in studying thrombus formation, we observed an increase in

phosphatidylserine exposure of *Chuk1*^{A/A}*Apoe*^{-/-} platelets, which we consider as further evidence that the IKK α /NF κ B pathway does not contribute to the role of CD40L. A mechanistic explanation for this unexpected finding is beyond the scope of this article but, not unlikely, the platelet proteome is altered by deficiency in one of the key enzymes of the NF κ B transcription factor pathway. Yet, the stimulating role of CD40L on platelet aggregation and thrombus formation seems to be retained in *Chuk1*^{A/A}*Apoe*^{-/-} platelets.

Reports to date indicate that the cleavage of CD40L from activated platelets proceeds relatively slow and involves the matrix metalloproteinases 2 and 9, as well as integrin $\alpha_{IIb}\beta_3$.^{9,10,46} Given these kinetics, our data suggest a role of both platelet-associated CD40L and cleaved CD40L in controlling thrombus growth. Taken together, we find that CD40L at least in part acting independently of CD40 is another of the (integrin-dependent) platelet-derived factors that contribute to the stable formation of large thrombi, especially under atherogenic conditions.

References

1. Ruggeri ZM. Platelets in atherothrombosis. *Nat Med.* 2002;**8**:1227-1234.
2. Cosemans JM, Kuijpers MJ, Lecut C, Loubele ST, Heeneman S, Jandrot-Perrus M, Heemskerk JW. Contribution of platelet glycoprotein VI to the thrombogenic effect of collagens in fibrous atherosclerotic lesions. *Atherosclerosis.* 2005;**181**:19-27.

3. Penz S, Reininger AJ, Brandl R, Goyal P, Rabie T, Bernlochner I, Rother E, Goetz C, Engelmann B, Smethurst PA, Ouwehand WH, Farndale R, Nieswandt B, Siess W. Human atheromatous plaques stimulate thrombus formation by activating platelet glycoprotein VI. *FASEB J*. 2005;**19**:898–909.
4. Kuijpers MJ, Gilio K, Reitsma S, Nergiz-Unal R, Prinzen L, Heeneman S, Lutgens E, van Zandvoort MA, Nieswandt B, Egbrink MG, Heemskerk JW. Complementary roles of platelets and coagulation in thrombus formation on plaques acutely ruptured by targeted ultrasound treatment: a novel intravital model. *J Thromb Haemost*. 2009;**7**:152–161.
5. Versteeg HH, Heemskerk JW, Levi M, Reitsma PH. New fundamentals in hemostasis. *Physiol Rev*. 2013;**93**:327–358.
6. Inwald DP, McDowall A, Peters MJ, Callard RE, Klein NJ. CD40 is constitutively expressed on platelets and provides a novel mechanism for platelet activation. *Circ Res*. 2003;**92**:1041–1048.
7. Gavins FN, Li G, Russell J, Perretti M, Granger DN. Microvascular thrombosis and CD40/CD40L signaling. *J Thromb Haemost*. 2011;**9**:574–581.
8. Burkhart JM, Vaudel M, Gambaryan S, Radau S, Walter U, Martens L, Geiger J, Sickmann A, Zahedi RP. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood*. 2012;**120**:e73–e82.
9. Choi WS, Jeon OH, Kim DS. CD40 ligand shedding is regulated by interaction between matrix metalloproteinase-2 and platelet integrin $\alpha(\text{IIb})\beta(3)$. *J Thromb Haemost*. 2010;**8**:1364–1371.
10. Rahman M, Zhang S, Chew M, Syk I, Jeppsson B, Thorlacius H. Platelet shedding of CD40L is regulated by matrix metalloproteinase-9 in abdominal sepsis. *J Thromb Haemost*. 2013;**11**:1385–1398.
11. Varo N, de Lemos JA, Libby P, Morrow DA, Murphy SA, Nuzzo R, Gibson CM, Cannon CP, Braunwald E, Schönbeck U. Soluble CD40L: risk prediction after acute coronary syndromes. *Circulation*. 2003;**108**:1049–1052.
12. Tousoulis D, Androulakis E, Papageorgiou N, Briasoulis A, Siasos G, Antoniadis C, Stefanadis C. From atherosclerosis to acute coronary syndromes: the role of soluble CD40 ligand. *Trends Cardiovasc Med*. 2010;**20**:153–164.
13. Mach F, Schönbeck U, Sukhova GK, Atkinson E, Libby P. Reduction of atherosclerosis in mice by inhibition of CD40 signalling. *Nature*. 1998;**394**:200–203.
14. Lutgens E, Lievens D, Beckers L, et al. Deficient CD40-TRAF6 signaling in leukocytes prevents atherosclerosis by skewing the immune response toward an antiinflammatory profile. *J Exp Med*. 2010;**207**:391–404.
15. Lutgens E, Gorelik L, Daemen MJ, de Muinck ED, Grewal IS, Kotliansky VE, Flavell RA. Requirement for CD154 in the progression of atherosclerosis. *Nat Med*. 1999;**5**:1313–1316.
16. Donners MM, Beckers L, Lievens D, et al. The CD40-TRAF6 axis is the key regulator of the CD40/CD40L system in neointima formation and arterial remodeling. *Blood*. 2008;**111**:4596–4604.
17. Lievens D, Zerneck A, Seijkens T, et al. Platelet CD40L mediates thrombotic and inflammatory processes in atherosclerosis. *Blood*. 2010;**116**:4317–4327.
18. Yacoub D, Hachem A, Théorêt JF, Gillis MA, Mourad W, Merhi Y. Enhanced levels of soluble CD40 ligand exacerbate platelet aggregation and thrombus formation through a CD40-dependent tumor necrosis factor receptor-associated factor-2/Rac1/p38 mitogen-activated protein kinase signaling pathway. *Arterioscler Thromb Vasc Biol*. 2010;**30**:2424–2433.
19. Hachem A, Yacoub D, Zaid Y, Mourad W, Merhi Y. Involvement of nuclear factor κB in platelet CD40 signaling. *Biochem Biophys Res Commun*. 2012;**425**:58–63.
20. Malaver E, Romaniuk MA, D'Atri LP, Pozner RG, Negrotto S, Benzadón R, Schattner M. NF- κB inhibitors impair platelet activation responses. *J Thromb Haemost*. 2009;**7**:1333–1343.
21. André P, Prasad KS, Denis CV, He M, Papalia JM, Hynes RO, Phillips DR, Wagner DD. CD40L stabilizes arterial thrombi by a $\beta(3)$ integrin-dependent mechanism. *Nat Med*. 2002;**8**:247–252.

22. Prasad KS, Andre P, He M, Bao M, Manganello J, Phillips DR. Soluble CD40 ligand induces beta3 integrin tyrosine phosphorylation and triggers platelet activation by outside-in signaling. *Proc Natl Acad Sci U S A*. 2003;**100**:12367–12371.
23. Nergiz-Unal R, Cosemans JM, Feijge MA, van der Meijden PE, Storey RF, van Giezen JJ, oude Egbrink MG, Heemskerk JW, Kuijpers MJ. Stabilizing role of platelet P2Y(12) receptors in shear-dependent thrombus formation on ruptured plaques. *PLoS One*. 2010;**5**:e10130.
24. Siljander P, Farndale RW, Feijge MA, Comfurius P, Kos S, Bevers EM, Heemskerk JW. Platelet adhesion enhances the glycoprotein VI-dependent procoagulant response: Involvement of p38 MAP kinase and calpain. *Arterioscler Thromb Vasc Biol*. 2001;**21**:618–627.
25. Kuijpers MJ, de Witt S, Nergiz-Unal R, van Kruchten R, Korporaal SJ, Verhamme P, Febbraio M, Tjwa M, Voshol PJ, Hoylaerts MF, Cosemans JM, Heemskerk JW. Supporting roles of platelet thrombospondin-1 and CD36 in thrombus formation on collagen. *Arterioscler Thromb Vasc Biol*. 2014;**34**:1187–1192.
26. Rowley JW, Oler AJ, Tolley ND, Hunter BN, Low EN, Nix DA, Yost CC, Zimmerman GA, Weyrich AS. Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. *Blood*. 2011;**118**:e101–e111.
27. Jackson SP, Schoenwaelder SM, Goncalves I, et al. PI 3-kinase p110beta: a new target for antithrombotic therapy. *Nat Med*. 2005;**11**:507–514.
28. Gibbins JM. Platelet adhesion signalling and the regulation of thrombus formation. *J Cell Sci*. 2004;**117**:3415–3425.
29. Gratacap MP, Guillermet-Guibert J, Martin V, Chicanne G, Tronchère H, Gaits-Iacovoni F, Payrastre B. Regulation and roles of PI3K β , a major actor in platelet signaling and functions. *Adv Enzyme Regul*. 2011;**51**:106–116.
30. Canobbio I, Stefanini L, Cipolla L, Ciraolo E, Gruppi C, Balduini C, Hirsch E, Torti M. Genetic evidence for a predominant role of PI3K β catalytic activity in ITAM- and integrin-mediated signaling in platelets. *Blood*. 2009;**114**:2193–2196.
31. Martin V, Guillermet-Guibert J, Chicanne G, Cabou C, Jandrot-Perrus M, Plantavid M, Vanhaesebroeck B, Payrastre B, Gratacap MP. Deletion of the p110beta isoform of phosphoinositide 3-kinase in platelets reveals its central role in Akt activation and thrombus formation in vitro and in vivo. *Blood*. 2010;**115**:2008–2013.
32. Lee HS, Kim SD, Lee WM, Endale M, Kamruzzaman SM, Oh WJ, Cho JY, Kim SK, Cho HJ, Park HJ, Rhee MH. A noble function of BAY 11-7082: Inhibition of platelet aggregation mediated by an elevated cAMP-induced VASP, and decreased ERK2/JNK1 phosphorylations. *Eur J Pharmacol*. 2010;**627**:85–91.
33. Oeckinghaus A, Hayden MS, Ghosh S. Crosstalk in NF- κ B signaling pathways. *Nat Immunol*. 2011;**12**:695–708.
34. Sun SC. Non-canonical NF- κ B signaling pathway. *Cell Res*. 2011;**21**:71–85.
35. Cao Y, Bonizzi G, Seagroves TN, Greten FR, Johnson R, Schmidt EV, Karin M. IKKalpha provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development. *Cell*. 2001;**107**:763–775.
36. Law CL, Grewal IS. Therapeutic interventions targeting CD40L (CD154) and CD40: the opportunities and challenges. In: Grewal IS, ed. *Therapeutics of the TNF Superfamily*. 2009;**647**:8–36.
37. Chakrabarti S, Blair P, Freedman JE. CD40-40L signaling in vascular inflammation. *J Biol Chem*. 2007;**282**:18307–18317.
38. Lievens D, Eijgelaar WJ, Biessen EA, Daemen MJ, Lutgens E. The multifunctionality of CD40L and its receptor CD40 in atherosclerosis. *Thromb Haemost*. 2009;**102**:206–214.
39. Nieswandt B, Brakebusch C, Bergmeier W, Schulte V, Bouvard D, Mokhtari-Nejad R, Lindhout T, Heemskerk JW, Zirngibl H, Fässler R. Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. *EMBO J*. 2001;**20**:2120–2130.
40. Kuijpers MJ, Schulte V, Bergmeier W, Lindhout T, Brakebusch C, Offermanns S, Fässler R, Heemskerk JW, Nieswandt B. Complementary roles of glycoprotein VI and alpha2beta1 integrin

in collagen-induced thrombus formation in flowing whole blood ex vivo. *FASEB J.* 2003;**17**:685–687.

41. Gilio K, Munnix IC, Mangin P, Cosemans JM, Feijge MA, van der Meijden PE, Olieslagers S, Chrzanowska-Wodnicka MB, Lillian R, Schoenwaelder S, Koyasu S, Sage SO, Jackson SP, Heemskerk JW. Nonredundant roles of phosphoinositide 3-kinase isoforms alpha and beta in glycoprotein VI-induced platelet signaling and thrombus formation. *J Biol Chem.* 2009;**284**:33750–33762.
42. Schoenwaelder SM, Ono A, Nesbitt WS, Lim J, Jarman K, Jackson SP. Phosphoinositide 3-kinase p110 beta regulates integrin alpha IIb beta 3 avidity and the cellular transmission of contractile forces. *J Biol Chem.* 2010;**285**:2886–2896.
43. Consonni A, Cipolla L, Guidetti G, Canobbio I, Ciraolo E, Hirsch E, Falasca M, Okigaki M, Balduini C, Torti M. Role and regulation of phosphatidylinositol 3-kinase β in platelet integrin $\alpha 2\beta 1$ signaling. *Blood.* 2012;**119**:847–856.
44. Karim ZA, Zhang J, Banerjee M, Chicka MC, Al Hawas R, Hamilton TR, Roche PA, Whiteheart SW. I κ B kinase phosphorylation of SNAP-23 controls platelet secretion. *Blood.* 2013;**121**:4567–4574.
45. Wei S, Wang H, Zhang G, Lu Y, An X, Ren S, Wang Y, Chen Y, White JG, Zhang C, Simon DI, Wu C, Li Z, Huo Y. Platelet I κ B kinase- β deficiency increases mouse arterial neointima formation via delayed glycoprotein Iba shedding. *Arterioscler Thromb Vasc Biol.* 2013;**33**:241–248.
46. Otterdal K, Pedersen TM, Solum NO. Release of soluble CD40 ligand after platelet activation: studies on the solubilization phase. *Thromb Res.* 2004;**114**:167–177.