Handbook of Experimental Pharmacology 210

Paolo Gresele
Gustav V.R. Born
Carlo Patrono
Clive P. Page *Editors*

Antiplatelet Agents

Springer

Handbook of Experimental Pharmacology

Volume 210

For further volumes:
http://www.springer.com/series/164

Paolo Gresele • Gustav V.R. Born • Carlo Patrono •
Clive P. Page
Editors

Antiplatelet Agents

Springer

Editors
Paolo Gresele
Department of Internal Medicine
University of Perugia
Perugia
Italy

Gustav V.R. Born
The William Harvey Research Institute
Charterhouse Square
London, EC1M6BQ
United Kingdom

Carlo Patrono
Istituto di Farmacologia
Università Cattolica del S. Cuore
Rome
Italy

Clive P. Page
Sackler Institute of Pulmonary Pharmacology
King's College London
London
United Kingdom

ISSN 0171-2004 ISSN 1865-0325 (electronic)
ISBN 978-3-642-29422-8 ISBN 978-3-642-29423-5 (eBook)
DOI 10.1007/978-3-642-29423-5
Springer Heidelberg New York Dordrecht London

Library of Congress Control Number: 2012945908

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Foreword
Platelets: Past, Present, and Future

This volume will be greatly welcomed by the biomedical community worldwide for bringing knowledge about platelets and their involvements up to date. Herewith are a few, I hope, relevant thoughts.

The Past: Platelets were discovered by Bizzozero in 1882. Knowledge about them increased slowly until the mid-twentieth century. I came to know about platelets from two, very different experiences: one came in late 1945 when, as a member of the Royal Army Medical Corps, I was one of the first foreign doctors in Hiroshima about 3 months after its destruction by the first atom bomb: innumerable local people were still dying from thrombocytopenic bleeding. The other was in Oxford in 1956 when, working on the storage of amines in cells, I remembered that platelets contained large amounts of 5-hydroxytryptamine and produced evidence for its ionic association with ATP.

Just about 50 years ago came the invention of platelet aggregometry. This method elucidated platelet function underlying haemostasis and thrombosis, initiated the discovery of aggregation inhibitors and permitted the discovery of the release reaction and of its inhibition by aspirin, which was the basis of the drug's 'second life' in the prevention and treatment of coronary thrombosis and stroke. Since then, of course, several aggregation inhibitors with different modes of action have come into effective therapy.

The present is dominated, as almost everywhere in biomedicine, by molecular biology. The origins of this are brilliantly recounted by Francis Crick himself in *What Mad Pursuit* and by Georgina Ferry in her excellent biography of Max Perutz. The molecular mechanisms underlying platelet function appear to be no less complex than those underlying the functions of proper, nucleated cells. So far, however, increasing knowledge of these mechanisms has had little effect in increasing therapeutic possibilities.

As to **the future**, the hope is that this may change and that further elucidation at the molecular level will provide new points for therapeutic attack. Meantime, other work is being done: on putative roles of platelets in inflammation, and in the metastatic spread of cancers.

But there is one deeply troubling thought: to the best of my knowledge, the reason why platelet deficiency is associated with microvascular bleeding is still unexplained. That question concerns the essential function of platelets, and it is extremely unsatisfactory that how platelets are connected with such bleeding remains unknown.

My own main interest now is to provide an **evolutionary explanation** for platelet aggregation by adenosine diphosphate (ADP). This was the first platelet-aggregating agent to be discovered and investigated. ADP induces haemostatic aggregation of platelets in man and other mammals but not of the functionally corresponding cells of other vertebrates including birds and reptiles, nor in those of other animal phyla. Different agents, viz. collagen and thromboxane A_2 (TXA_2), which activate platelets and analogous cells to prevent loss of blood and other body fluids, possess this function much earlier in evolution.

So why ADP, rather than any of the other extracellular transmitters, including Burnstock's ubiquitous extracellular ATP? The answer is suggested by comparing haemostasis in man and other mammals with the corresponding process in other animals. In mammals the blood pressure is high so that blood loss from injury is very rapid. Therefore, haemostasis must be very rapid, a requirement met by platelet aggregation: the activation time of human platelets at 37 °C is less than 100 ms; plasma clotting happens much more slowly. With ATP leaking from damaged cells, the dephosphorylating enzymes on cell surfaces ensure very rapid production of ADP. The further dephosphorylation to ineffective AMP is considerably slower, ensuring a temporary increase in local ADP concentration.

In vivo evidence indicates that ADP does indeed contribute essentially to haemostatic and thrombotic platelet aggregation. As other locally present agents, notably TXA_2, collagen, and thrombin are also effective, the addition of the ADP mechanism in mammals is presumably in the nature of a fail-safe system as employed by engineers. So, as always in science, it is best to end with a question: why not ADP also in birds?

Actually, I cannot do better than end with a quote from my late friend Gwyn Macfarlane, distinguished for discovering the cascade mechanism of blood coagulation: 'I live in the quiet of the country where one hears small things, and the other night I overheard two platelets talking as they drifted in some venous backwater.'

'You know', said one, 'I've got the strangest feeling that I've been here before.'

'What nonsense', said the other. 'Everyone knows that endothelium stretches to infinity. One doesn't go backwards—one just goes on.'

'Yes—until we stop.'

'One doesn't think about that—it's morbid.'

'I can't help it sometimes. One day endothelium will stop passing—everyone else will go on, but I shall be stuck there—dead. I say, I wonder what's on the other side?'

'What do you mean? On the other side of what?'

'On the other side of endothelium, of course.'

'It's a meaningless question. Endothelium encloses everything there is—it's the time-continuum. How can there be another side? It's a logical impossibility!'

'Well, I met a lymphocyte just now. He's certain he's been through the endothelium and has come back to another life on this side.'

'Lymphocytes! One doesn't pay any attention to them! All that mystical nonsense about re-incarnation. Next thing, they'll be saying we're all part of some Higher Organisation. . .!'

London, UK Gustav Born

Preface

Blood platelets attract growing interest among clinicians and basic scientists due to the increasing understanding of their role in diverse physiologic and pathologic phenomena and to the uninterrupted discovery of new functions.

The recent characterization of the protein biosynthetic activity of platelets, their complex RNA array, the genetic regulation of several of their functional activities, and their role in tissue repair and in inflammation have prompted a major reconsideration of the role of these cells. However, above all other aspects, it is the central role that platelets play in arterial thrombotic events which has been the object of great attention and of spectacular therapeutic advancements in the last few years.

It seemed thus timely to provide a comprehensive, detailed, and updated handbook for both clinicians and researchers on the basic and clinical aspects of drugs used to modulate platelet function and to review the wide range of anti-platelet drugs currently under development.

The book is divided into three parts, i.e. Pathophysiology, which provides all the basic knowledge on the structure and function of platelets and on their role in physiologic haemostasis and in atherothrombosis; Pharmacology, which discusses in detail the basic pharmacology of all the individual antiplatelet agents currently in use and all of the novel and future antiplatelet approaches; and Therapy, which examines systematically the current therapeutic indications for antiplatelet agents in the different clinical manifestations of ischaemic cardiovascular diseases.

Within these parts, 23 chapters cover extensively all the aspects of antiplatelet therapy, with a view on innovative aspects, like small interfering RNAs, signal transduction pathways as potential targets of antiplatelet therapy, or the pharmacologic modulation of the inflammatory activity of platelets.

A large group of leading experts in platelets and cardiovascular disorders, both clinicians and basic scientists, kindly accepted to contribute to this book thus providing the most updated and authoritative view on the state of the art in antiplatelet therapy. Particular care has been taken by the editors to ensure homogeneity and readability of the book and, to this end, each chapter reports a separate list of the main take-home messages and a table with the most significant

knowledge gaps on the subject. Simple figures and schematic diagrams help to provide an immediate and concise view on complex mechanisms and study results.

The book is intended to be a manual and a consultation textbook for all those who are interested in platelets, either for research purposes or for their daily clinical practice.

It is to be expected that blood platelets will continue to surprise us and that new therapeutic developments will take place in the next few years: this book intends to set the stage for the foreseeable future.

An insightful and entertaining foreword by Gustav V.R. Born, one of the fathers of the modern knowledge on platelets, opens the book. The enthusiastic contribution of 30 leading scientists, the editorial assistance of Springer Verlag, as well as the generous assistance of several co-workers at the Editor's institutions have made it possible to complete this ambitious project: we trust that it will be of help and interest to many readers worldwide.

The Editors

Contents

Part I
Pathophysiology

Platelets: Production, Morphology and Ultrastructure

Jonathan N. Thon and Joseph E. Italiano

Contents

J.N. Thon
Department of Medicine, Brigham and Women's Hospital, 1 Blackfan Circle, Karp 6, Boston, MA, USA

Harvard Medical School, Boston, MA, USA

J.E. Italiano (✉)
Department of Medicine, Brigham and Women's Hospital, 1 Blackfan Circle, Karp 6, Boston, MA, USA

Harvard Medical School, Boston, MA, USA

Department of Surgery, Vascular Biology Program, Children's Hospital, Boston, MA, USA
e-mail: jitaliano@rics.bwh.harvard.edu

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_1,

Abstract Platelets are anucleate, discoid cells, roughly 2–3 μm in diameter that function primarily as regulators of hemostasis, but also play secondary roles in angiogensis and innate immunity. Although human adults contain nearly one trillion platelets in circulation that are turned over every 8–10 days, our understanding of the mechanisms involved in platelet production is still incomplete. Platelets stem from large (30–100 μm) nucleated cells called megakaryocytes that reside primarily in the bone marrow. During maturation megakaryocytes extend long proplatelet elongations into sinusoidal blood vessels from which platelets ultimately release. During this process, platelets develop a number of distinguishable structural elements including: a delimited plasma membrane; invaginations of the surface membrane that form the open canalicular system (OCS); a closed-channel network of residual endoplasmic reticulum that form the dense tubular system (DTS); a spectrin-based membrane skeleton; an actin-based cytoskeletal network; a peripheral band of microtubules; and numerous organelles including α-granules, dense-granules, peroxisomes, lysosomes, and mitochondria. Proplatelet elongation and platelet production is an elaborate and complex process that defines the morphology and ultrastructure of circulating platelets, and is critical in understanding their increasingly numerous and varied biological functions.

Keywords Megakaryocyte maturation • Proplatelet production • Preplatelet interconversion • Platelet release • Morphology • Ultrastructure

1 Background

Human adults contain nearly one trillion blood platelets in circulation, each with an average lifespan of only 8–10 days. Among their primary functions, platelets serve as the "band-aids" of the bloodstream and respond to blood vessel injury by changing shape, secreting their granule contents, and aggregating to form a platelet clot. Platelets also play secondary roles in helping regulate angiogenesis and innate immunity. To maintain platelet counts of 150–400 $\times$ 10^9 platelets per liter of whole blood, roughly 100 billion new platelets must be produced daily from bone marrow megakaryocytes. Megakaryocytes are rare myeloid cells that constitute less than 0.1 % of the cellular population of the bone marrow, and their numbers can be modulated by factors like thrombopoietin (TPO), chemokines, or the localized expression of ligands for megakaryocyte surface receptors (Avecilla et al. 2004; Larson and Watson 2006a).

Megakaryocytes arise from pluripotent hematopoietic stem cells that are mostly restricted to the bone-proximal osteoblastic niche (Avecilla et al. 2004). These develop into burst- and colony-forming precursors, both of which express the CD34 antigen, and which continue to mature along an increasingly restricted lineage that culminates in the formation of precursors that develop into megakaryocytes (Fig. 1) (Ogawa 1993). Differentiation is predominantly driven by TPO signaling through the cMpl receptor, and is supported by additional growth factors such as interleukin-3 (IL-3), stem cell factor (SCF), IL-6, and IL-11 (Kaushansky 2006).

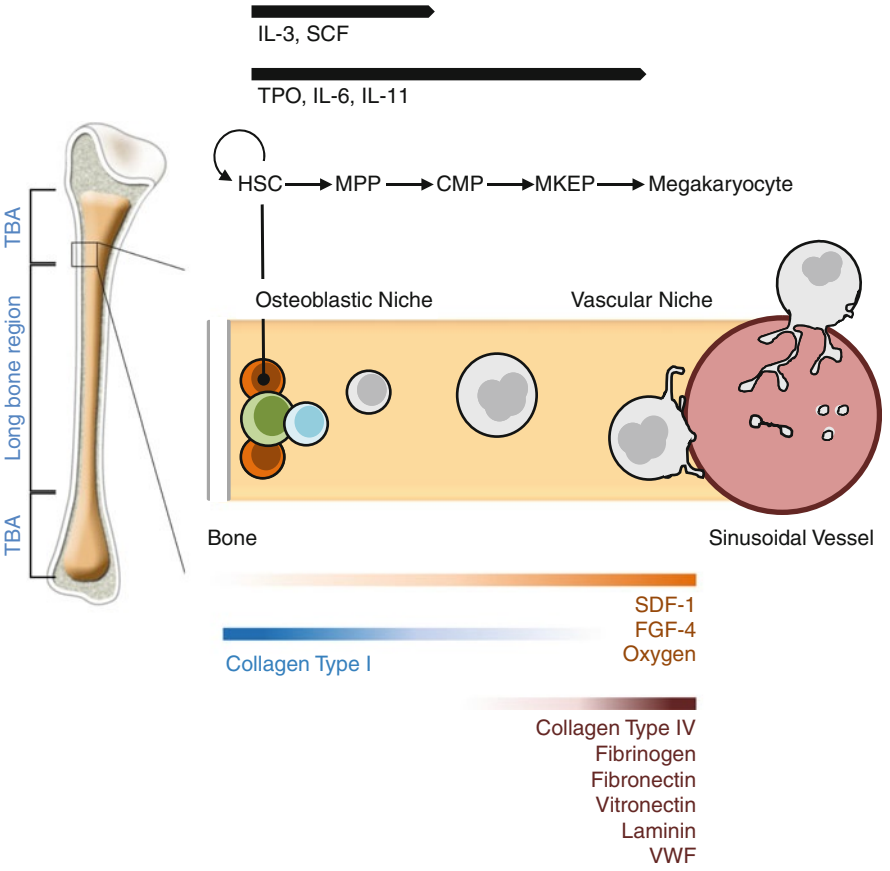


Fig. 1 Summary of megakaryocyte maturation and platelet production. Hematopoietic stem cells residing next to the endosteal bone surface produce progenitors that migrate to blood vessels at the center of the bone marrow cavity. Upon each division, a single daughter cell leaves the bone to proliferate and differentiate into various possible lineages. Differentiation down the megakaryocytic lineage is driven predominantly by TPO, and is supported by additional growth factors such as IL-3, SCF, IL-6, and IL-11. This process is assited by the extracellular matrix proteins type I and type IV collagen, fibrinogen, fibronectin, vitronectin, laminin, and VWF. Soluble factors such as SDF-1 and FGF-4, as well as the presence of intact sinusoids in the bone marrow help target megakaryocytes to the vascular niche during maturation. An elaborate intracellular program of nuclear amplification and protein production in maturing megakaryocytes precedes the mechanical extension of proplatelet elongations into the sinusoidal blood vessels of the bone marrow. Released proplatelets continue to mature in the vasculature and ultimately release individual platelets from their tips. *CMP* common myeloid progenitor, *HSC* hematopoietic stem cell, *IL* interleukin, *MKEP* megakaryocyte erythroid progenitor, *MPP* multipotent progenitor stem cell, *SCF* stem cell factor, *TBA* trabecular bone area, *TPO* thrombopoietin. Adapted from Yin and Li (2006)

Thrombopoietin is the principal regulator of thrombopoiesis and primate platelet counts can be reduced with an inhibitor against TPO without impairment of primary hemostasis (Kaushansky 2006; Tucker et al. 2010). These results suggest that suppressing platelet production without interfering with the hemostatic function

of platelets may offer a safe alternative to current therapies for prevention of stroke and heart attacks triggered by blood clotting (Tucker et al. 2010).

During maturation megakaryocytes undertake several rounds of chromosomal duplication without cell division (up to 64-fold) called endomitosis. This results in a single polylobulated nucleus as the megakaryocyte proceeds through G1, S, and G2 phases in succession, but fails to complete M phase, re-entering the G1 phase instead (Ravid et al. 2002). Megakaryocyte cytoplasmic volume increases proportionately with ploidy, which is used to direct protein and lipid synthesis in order to support platelet production (Bluteau et al. 2009). This increases the size of the megakaryocyte substantially (up to an approximate diameter of 100 μm) and fills it with platelet-specific organelles, granules, cytoskeletal proteins, and an extensive surface-connected internal membrane labyrinth called the invaginated membrane system. This specialized structure formerly called the demarcation membrane system (DMS) is continuous with the plasma membrane and is thought to constitute an extensive internal membrane reservoir that may facilitate platelet production.

As they mature, megakaryocytes migrate from the osteoblastic niche to a region proximal to the blood vessels of the bone marrow cavity, called the vascular niche. Such niches, while incompletely characterized, represent dynamic biological scaffolds that adapt to physiological requirements for rapid platelet production by regulating megakaryocyte maturation. The current model of platelet formation recognizes that upon reaching maturity bone marrow megakaryocytes will extend, through junctions in the lining of blood sinuses, long branching processes designated proplatelets. These dynamic intermediate structures are composed of tandem arrays of slender tubular projections with platelet-sized swellings at their ends. Released proplatelets continue to mature in the vasculature and ultimately release individual platelets from their tips (Italiano et al. 1999, 2007). A single megakaryocyte can give rise to 1,000–3,000 platelets (Stenberg and Levin 1989) before the residual nuclear material is eliminated by macrophage-mediated phagocytosis (Radley and Haller 1983). Indeed, mice lacking distinct hematopoietic transcription factors (such that they fail to produce proplatelets in culture) have severe thrombocytopenia, underscoring the correlation to platelet biogenesis in vivo (Lecine et al. 1998; Shivdasani et al. 1995; Shivdasani 2001). Because of the dramatic morphological changes that occur during thrombocytopoiesis, a better understanding of the processes that drive megakaryocyte maturation, proplatelet production, and platelet release is paramount.

2 Gaps in the Field

Our understanding of the molecular basis of platelet production has progressed substantially in recent years. But despite this progress, many questions remain: What mechanisms determine platelet size? How do specific genetic disorders result in unusually large or small platelets? What signals stimulate proplatelet production? Where does platelet biogenesis occur? Are there humoral factors that regulate the final stages of platelet production? And how do we grow functional platelets

in vitro on a large scale? Understanding these basic questions may lead to the translation of basic megakaryocyte and platelet biology into major clinical advances.

3 Mechanics of Proplatelet Elongation

Proplatelet production begins with the erosion of one pole of the megakaryocyte to generate large pseudopod-like structures that elongate, thin, and branch to yield slender tubular projections of uniform diameter (2–4 μm) (Fig. 2). This process is highly dependent upon a complex network of protein filaments that represent the molecular struts and girders of the cell. Tubulin and actin are both major components of this cytoskeletal network (Italiano et al. 1999). Megakaryocytes also contain a dense tubular network, and an expansive and interconnected membranous network of cisternae and tubules (the invaginated membrane system), which may represent a channeled system for granule release (Nakao and Angrist 1968). While the mechanism of membrane reorganization remains largely unknown, the invaginated membrane system is also thought to supply membrane during proplatelet formation and is largely supported by a spectrin-based membrane skeleton (Patel-Hett et al. 2011).

Microtubules and actin filaments are formed from thousands of molecules that assemble into linear polymers and play complementary roles in platelet production (Tablin et al. 1990). Proplatelet elongation, for example, is a microtubule-driven process (Tablin et al. 1990) which unfolds over a period of 4–10 h. Just prior to proplatelet formation, microtubules consolidate in a mass just beneath the cortical plasma membrane (Italiano et al. 1999). Proplatelet formation begins when these microtubules align into bundles and fill the cortex of the first blunt processes extended by megakaryocytes. Proplatelet shafts continue to become filled with thick bundles of (hundreds of) microtubules that undergo a thinning phase (to ~20 microtubules) and loop around within the proplatelet to reenter the shaft forming buds at the proplatelet tip (Hartwig and Italiano 2006). Proplatelet extension is due to the continuous polymerization of tubulin bundles at their free plus ends, and dynein-powered sliding of overlapping microtubules (Patel et al. 2005b). Mature megakaryocytes (like platelets) predominantly express the hematopoietic line-specific beta-1 tubulin isoform which plays a specialized role in platelet synthesis, structure, and function. The localization of beta-1 tubulin to the proplatelet marginal band is necessary for proplatelet production and beta-1 tubulin$^{-/-}$ mice exhibit thrombocytopenia resulting from a defect in generating proplatelets (Schwer et al. 2001).

Less is known about the role of actin in proplatelet production. Repeated actin-dependent bending and branching that bifurcates the proplatelet shaft is common, and serves to increase the number of free proplatelet ends from which platelets are thought to release (Hartwig and Italiano 2006). Branching occurs when a portion of the proplatelet shaft becomes bent, from which some of the microtubules within the loop separate from the bundle to form a new bulge in the shaft. Elongation/sliding of these microtubules then generates a new daughter proplatelet process.

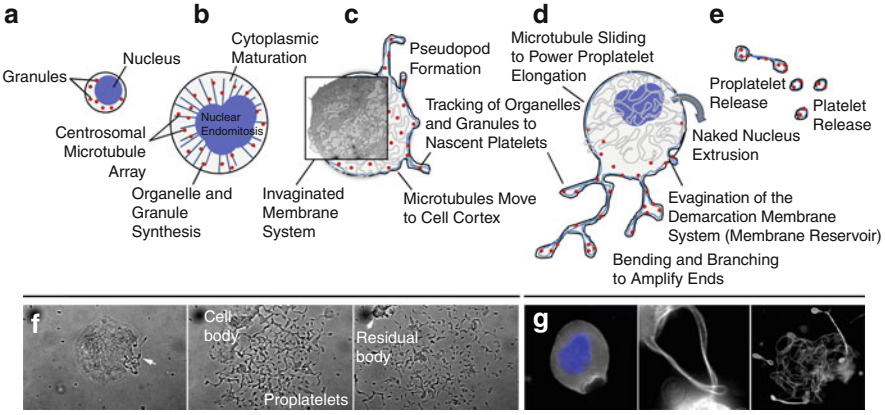


Fig. 2 Cytoskeletal mechanisms of proplatelet production and platelet release. A systematic series of events occurs as megakaryocytes transition from immature cells (**a**) to released platelets (**e**). (**b**) Immature megakaryocytes will undergo repeated cycles of nuclear endomitosis for the purpose of supporting organelle synthesis, and dramatic cytoplasmic maturation and expansion. (**c**) Prior to the onset of proplatelet formation, centrosomes disassemble and microtubules translocate to the cell cortex. A demarcation membrane system that is continuous with the surface membrane of the cell provides a reservoir of membrane for growth of the proplatelet processes. Thick bundles of microtubules fill the shafts and cortex of broad pseudopodia that are subsequently extended by the megakaryocyte. (**d**) Proplatelet production commences when the megakaryocyte cytoplasm starts to erode at one pole. Sliding of overlapping microtubules drives proplatelet elongation as organelles are tracked into proplatelet ends. Proplatelet formation continues to expand throughout the cell while bending and branching amplify existing proplatelet ends. Once the bulk of the megakaryocyte cytoplasm has been converted into proplatelets, the entire process ends in a rapid retraction that separates the released proplatelets from the residual cell body (nuclear extrusion). (**e**) The entire megakaryocyte cytoplasm is converted into a mass of proplatelets, which are released from the cell. As proplatelets elongate, their microtubule bundles twist bringing opposing bundles in contact, and allowing them to become zipped together in the proplatelet shaft. This forms loops at the ends of the proplatelets where granules and organelles become trapped. Sliding movements by microtubules in the shaft elongate released proplatelets further, and separate the ends from the shaft, mediating platelet release. (**f**) Differential interference contrast microscopy time-lapse sequence of a maturing megakaryocyte, showing the events that lead to elaboration of proplatelets in vitro. (**g**) Immunofluorescence microscopy sequence of the microtubule cytoskeleton and polylobulated nucleus of a proplatelet-producing megakaryocyte. Adapted from Hartwig and Italiano (2006) and Patel et al. (2005a)

In megakaryocytes, multivesicular bodies (MVBs) represent a developmental stage in α-granule and dense-granule maturation (Heijnen et al. 1998; Youssefian and Cramer 2000). Indeed, differences in intra-granular protein distribution of MVBs, α-granules, and dense-granules have been reported (van Nispen Tot Pannerden et al. 2010), and may reflect effective sorting and/or retention mechanisms in their formation. Microtubule bundles in the proplatelet shaft serve as tracks along which these granules move individually (Blair and Flaumenhaft 2009; Richardson et al. 2005). This process is sequential and bidirectional, culminating at the proplatelet tips where the cargo becomes trapped (Italiano et al. 2003). Defects of α-granule formation have been described in both humans

and mice, and often result from mutation or deletions in genes encoding vesicular trafficking proteins (Blair and Flaumenhaft 2009). Of these, SNAREs (soluble NSF attachment protein receptors) represent the core of the fusion machinery, and the distribution and association of vSNAREs (vesicular SNAREs, associated with granules) with tSNAREs (target SNAREs, associated with the plasma membrane) provides the basis for α-granule localization and secretion (Blair and Flaumenhaft 2009).

4 Regulation of Platelet Production

It is not surprising, given that megakaryocytes will migrate from the osteoblastic niche to the vascular niche, that the bone marrow stroma should contribute to megakaryocyte maturation (Avecilla et al. 2004; Larson and Watson 2006a). Indeed, the osteoblastic niche contains high levels of extracellular factors that prevent proplatelet formation (Fig. 1) (Avecilla et al. 2004). Adherence of megakaryocytes to PA6 stromal cells of the osteoblastic niche, for example, has been shown to keep megakaryocytes from producing proplatelets in vitro while promoting megakaryocyte growth and proliferation (Nagahisa et al. 1996). Likewise, soluble factors such as stromal derived factor-1 (SDF-1) and fibroblast growth factor-4 (FGF-4), as well as the presence of intact sinusoids in the bone marrow help target megakaryocytes to the vascular niche where they encounter extracellular factors that promote proplatelet production (Avecilla et al. 2004).

In addition to providing a suitable surface for attachment, extracellular matrices of the bone marrow can also regulate proplatelet production through specific matrix-receptor signaling (Larson and Watson 2006b). Type I collagen, the most abundant extracellular protein of the osteoblastic niche, completely suppresses proplatelet formation while promoting megakaryocyte spreading (Nilsson et al. 1998). This effect is thought to be regulated by the interaction of integrin $\alpha_2\beta_1$ with type I collagen, and the subsequent activation of the small GTPase Rho and the Rho-kinase ROCK (Chen and Shivdasani 2009), and could be overcome by the myosin IIA antagonist blebbistatin (Balduini et al. 2008). Phosphorylation of the regulatory light chain of myosin IIA by ROCK significantly attenuates the ATPase activity of myosin and suppresses proplatelet formation in both human and mouse megakaryocytes (Chen et al. 2007). Conversely, loss of myosin IIA function through dominant inhibitory mutations, targeted gene disruption, or manipulation of cultured megakaryocytes does not inhibit, and may indeed accelerate, proplatelet formation (Chen et al. 2007). As the megakaryocyte migrates toward the vascular niche, this attenuation is believed to disappear and proplatelet production is permitted to commence. Myosin IIA, the non-muscle myosin heavy chain product of the *MYH9* gene, has therefore been implicated in restraining proplatelet formation in developing megakaryocytes through this pathway until they are able to achieve terminal maturity in the bone marrow vascular niche (Chen et al. 2007). In the case of *MYH9*-related disorders, defective myosin IIA could promote premature proplatelet

formation in the osteoblastic niche through loss of attenuation, or result in defective proplatelet formation and reduced or abnormal platelet release in the vasculature (Chen and Shivdasani 2009). Indeed, patients with autosomal dominant inherited *MYH9*-related disorders (e.g., May-Hegglin anomaly) generally exhibit macrothrombocytopenia as well as variable degrees of hearing loss, nephritis, and cataracts (Chen and Shivdasani 2009).

Extracellular matrix proteins are a major component of the vascular niche as well, which is known to comprise type IV collagen, fibrinogen, fibronectin, vitronectin, laminin, and VWF (Larson and Watson 2006b). Type IV collagen, fibrinogen, and vitronectin have all been shown to promote proplatelet production, and antagonists directed against GPs Ibα, IIb, and IIbIIIa will inhibit this process when added to cultured megakaryocytes (Larson and Watson 2006a).

Src family kinases (SFKs) have also been demonstrated to play a critical role in integrin-induced megakaryocyte spreading, migration, and activation of PLCγ2 in primary bone marrow-derived megakaryocytes (Mazharian et al. 2011a, b). Megakaryocytes treated with an inhibitor of SFKs are unable to spread or migrate towards a gradient of SDF1α (Mazharian et al. 2011a, b). Interestingly, Dasatinib—a potent ATP-competitive inhibitor of multiple tyrosine kinases (such as SFKs) that is widely used for the treatment of Imatinib-resistant chronic myelogenous leukemia (CML)—was recently shown to cause thrombocytopenia in mice to a similar level observed in humans (Mazharian et al. 2011a, b). This effect was reversible and could be attributed to an impairment of megakaryocyte migration and proplatelet formation.

Despite these recent advances, it is still unclear how signaling through integrins by extracellular matrix proteins mediate terminal cytoskeletal changes that ultimately drive proplatelet production. Nevertheless, these represent attractive drug targets that can be exploited to regulate platelet levels without interfering with their hemostatic functions.

5 Terminal Stages of Platelet Release

Although the mechanics of proplatelet production have been studied, the terminal stages of platelet release remain poorly understood. This is partly due to significant limitations in the field, such as the asynchronous maturation of HSCs in culture, and our inability to synchronize proplatelet production. Megakaryocyte cultures always contain a complex mix of HSCs, immature megakaryocytes, proplatelet-producing megakaryocytes, released proplatelets and platelets which can range dramatically in size and shape (Behnke and Forer 1998; Italiano et al. 2007), and whose relative distribution changes during cell culture. As there are currently no methods available to isolate the multiple intermediate stages in platelet release, most studies to date have focused on the qualitative aspects of megakaryocyte maturation. Nevertheless, proplatelets have been identified both in vitro and in vivo (Leven 1987; Tablin et al. 1990), and proplatelet-producing megakaryocytes yield platelets that are

structurally and functionally similar to blood platelets (Choi et al. 1995; Italiano et al. 1999). Many important questions therefore remain concerning platelet formation, such as how and where are platelets actually released, what cytoskeletal forces are involved in this process, are platelets released in an intermediate "preplatelet" form, and what signals regulate each step of megakaryocyte maturation and platelet formation.

The repeated observation of proplatelets in the bone marrow, the correlation of proplatelet formation and platelet counts, and the elegant mechanisms of proplatelet packaging imply that proplatelets are essential intermediates in platelet production (Larson and Watson 2006a). Nevertheless, micrographs of proplatelet-producing megakaryocytes in situ or in vitro images of isolated megakaryocytes in culture have yielded only static snapshots and resulted in competing mechanistic models of platelet release. In vivo approaches, such as the use of live imaging with multiphoton intravital microscopy (Junt et al. 2007), have validated the proplatelet model of platelet production and suggest that platelets are formed upon the further fragmentation of cytoplasmic processes extended and released into the sinusoids of bone marrow by extravascularly located megakaryocytes (Behnke 1969; Choi et al. 1995; Radley and Scurfield 1980). It should be noted that trans-sinusoidal migration of whole megakaryocytes in these studies was rare, and the bone marrow sinusoidal diameter was observed to impose size constraints on released megakaryocyte fragments in vivo. Moreover, the presence of proplatelets in the peripheral blood of the CD41-EYFP$^{ki/+}$ mice implies that it is proplatelets, not megakaryocytes, that are directly responsible for individual platelet release (Junt et al. 2007). Indeed, almost all megakaryocyte fragments identified were 10–100 times as large as circulating platelets. As many of the shed megakaryocyte fragments were unbranched, and most exceeded platelet dimensions, this may be indicative of an intermediate stage in platelet development (Junt et al. 2007). Megakaryocytes routinely released heterogeneous particles with properties resembling immature proplatelets into bone marrow microvessels, suggesting proplatelet morphogenesis continues in peripheral blood to create individual platelets (Junt et al. 2007). This process is possibly assisted by intravascular shear forces in pulmonary arterioles and is consistent with observations that proplatelet counts are higher in pre-pulmonary vessels than in post-pulmonary vessels (Handagama et al. 1987), whereas platelet counts are higher in the latter (Howell and Donahue 1937).

The discovery of a new intermediate stage in platelet production (the preplatelet) and the demonstration that proplatelets are direct precursors to platelets in vitro/vivo confirms this new paradigm (Thon et al. 2011). The model of platelet production proposed by our lab predicts that megakaryocytes will release proplatelets into sinusoidal blood vessels where they undergo successive rounds of abscission along their midbody and at their ends to produce smaller barbell-shaped proplatelets and individual platelets (Fig. 2). This process is mediated by the formation of a cleavage furrow at the point of division and results in platelet release from the proplatelet ends at an increasing rate in time as more ends become available after each abscission event. Barbell-shaped proplatelets of ~30–50 μm-perimeter reversibly convert into preplatelets (anucleate discoid particles 2–10 μm across that have the

capacity to convert reversibly into elongated proplatelets by twisting microtubule-based forces). This process may represent a novel mechanism of microtubule reorganization and granule redistribution after each abscission event. During barbell proplatelet formation, dynamic and bidirectional assembly and reorganization of microtubule coils mediate platelet cytoskeleton remodeling as α- and dense-granules track to distal proplatelet tips. Platelets ultimately release from barbell-shaped proplatelet ends after a final abscission event at the proplatelet midsection. Interestingly, the release of platelets from the ends of proplatelets is potentiated by shear, and corresponds with earlier reports of higher platelet counts in post-pulmonary vessels.

6 Platelet Morphology and Ultrastructure

Platelets are small (2–3 μm), anucleate, disc-shaped cells of which two-thirds are present in the general circulation with the remaining third reversibly sequestered in the spleen. Platelets contain a number of distinguishable structural elements including: a delimited plasma membrane; invaginations of the surface membrane that form the open canalicular system (OCS); a closed-channel network of residual endoplasmic reticulum that form the dense tubular system (DTS); a spectrin-based membrane skeleton; an actin-based cytoskeletal network; a peripheral band of microtubules; and numerous organelles including α-granules, dense-granules, peroxisomes, lysosomes, and mitochondria (Fig. 3) (White 1968, 1972).

7 The Plasma Membrane

The plasma membrane is composed of a phospholipid bilayer in which cholesterol, glycolipids, and glycoproteins are embedded. Unlike erythrocytes which retain these molecules in the inner leaflet of their plasma membrane, platelets present these molecules on their surface, which may denote their multiple (and varied) functions in the body. Sodium- and calcium-adenosine triphosphate pumps in the plasma membrane control the intracellular ionic environment of the platelet, while the asymmetrical organization of phospholipids between the inner and outer leaflets regulates coagulation. Negatively charged phospholipids such as phosphatidylserine and phosphatidylinositol, for example, are predominantly expressed in the inner leaflet of the plasma membrane which maintains the platelet surface in a non-procoagulant state. Phospholipid asymmetry is maintained by ATP-dependent aminophospholipid translocases (flipases) that actively move negatively charged phospholipids from the outer to the inner leaflet and may also be supported by phospholipid interactions with cytoskeletal or cytoplasmic elements in the platelet. On platelet activation aminophospholipids become exposed on the platelet surface by ATP-dependent flopases and scramblases, triggering cell-surface-based coagulation reactions (Heemskerk et al. 2002).

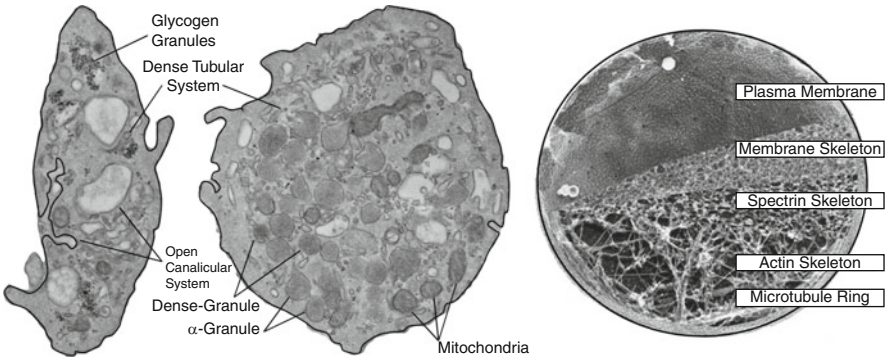


Fig. 3 Ultrastructural features observed in thin section electron micrographs of discoid platelets cut in the cross section and equitorial planes. Components include the open canalicular and dense tubular membrane systems, glycogen granules, α-granules, dense-granules, mitochondria, plasma membrane, membrane skeleton, spectrin skeleton, actin skeleton, and microtubule ring. Adapted from Hartwig et al. (1999)

The plasma membrane also contains dynamic, cholesterol- and sphingolipid-rich microdomains called lipid rafts that play important roles in signaling and intracellular trafficking in platelets. While notably devoid of caveolin, lipid rafts contain the protein markers flotillin (1 and 2) and stomatin, as well as the ganglioside GM_1. Other protein components of resting platelets include CD36, CD63, CD9, GP IIbIIIa, and GLUT-3. On platelet activation GP VI, FcγRIIa, GP Ib/IX/V, c-SRC, phosphatidic acid, and phosphoinositol 3-kinase products also partition into lipid rafts, which may mediate receptor clustering and regulate their activation. Indeed, the platelet membrane is densely packed with highly specific surface receptors that finely regulate signal-dependent platelet activation and may tailor α-granule release to coagulation, inflammation, atherosclerosis, antimicrobial host defense, angiogenesis, wound repair, or malignancy (Blair and Flaumenhaft 2009).

8 The Open Canalicular System

The surface-connected OCS comprises an elaborate series of plasma membrane-contiguous indentations and conduits that tunnel throughout the interior of the platelet (Behnke 1970). While its role in the cell remains somewhat controversial, the OCS is largely thought to serve three major functions; The first is as a mechanism for entry of external elements into the platelet, as well as a potential route for the release of granule contents to the platelet exterior. Indeed, upon platelet activation select α-granule subpopulations will either move to the center of the platelet or localize to the platelet periphery; denoting a possible mechanism for differential granule release (*see* Alpha-*Granules*). Secondly, the OCS may constitute an extensive internal membrane reservoir that may facilitate filopodia formation and spreading following platelet adhesion to an activating surface. This

process results in a dramatic increase in plasma membrane surface area compared to that of a resting platelet, and may be assisted by α- and dense-granules fusion to the plasma membrane upon granule release. Lastly, the OCS may function as a storage site for plasma membrane glycoproteins. GP Ib/IX, for example, becomes selectively sequestered to the OCS upon thrombin (and possibly plasmin) activation of the platelet (Michelson 1992). Conversely, membrane proteins of the OCS may become recruited to the platelet surface with activation; although α-granule fusion with the plasma membrane is likely a much larger contributor.

9 Dense Tubular System

First characterized histochemically by the presence of peroxidase activity, the DTS is a closed-channel network of residual endoplasmic reticulum primarily thought to sequester ionized calcium (Ca^{2+}) similar to the sarcotubules of the skeletal muscle (White 1972). The calcium binding protein calreticulin is likely responsible for calcium sequestration in the DTS, which is regulated by a number of downstream messenger molecules produced during platelet activation. Platelet activation with thrombin, for example, results in the phosphorylation of the Gα subunit of the G protein-coupled receptor PAR-1, which is responsible for activating the membrane-associated signal generating enzymes phospholipase C and phospholipase A_2 (Brass et al. 1993). Phospholipase C, in turn, generates the second messenger inositol 1,4,5 triphosphate (IP3), which binds IP3 type II receptors on the DTS membrane to stimulate Ca^{2+} release. The release of intracellular Ca^{2+} from the DTS results in GP IIbIIIa redistribution, cytoskeletal reorganization, and granule release; this makes regulation of intracellular Ca^{2+} stores in the DTS a useful target for anti-platelet drugs.

10 The Resting Platelet Cytoskeleton

The disc shape of the resting platelet is maintained by a well-defined and highly specialized cytoskeleton. This intricate system of molecular struts and girders preserves the shape and integrity of the platelet as it is exposed to high shear forces in the bloodstream. The three major cytoskeletal components of the resting platelet are (1) the spectrin-based membrane skeleton, (2) the actin cytoskeleton, and (3) the marginal microtubule coil.

11 The Spectrin-based Membrane Skeleton

The plasma membrane and OCS of the resting platelet are supported by an elaborate cytoskeletal network. The platelet is the only other cell besides the red blood cell whose membrane skeleton has been visualized at high resolution. Like the red

blood cell, the platelet membrane skeleton is also self-assembled of elongated spectrin strands that interconnect through actin filament binding, generating triangular pores. Platelets contain approximately 2,000 spectrin molecules (Hartwig and DeSisto 1991). This spectrin network laminates the cytoplasmic surface of both the OCS and plasma membrane systems. Although considerably less is understood about how the spectrin–actin network assembles and is attached to the plasma membrane in the platelet relative to the red blood cell, certain differences between the two skeletons have been defined. First, the spectrin strands composing the platelet membrane skeleton interconnect using the ends of long actin filaments instead of short actin oligomers (Hartwig and DeSisto 1991). These ends appear at the plasma membrane originating from filaments in the cytoplasm. Therefore, the spectrin lattice is assembled into a continuous network by its association with actin filaments. Second, tropomodulins are not present at sufficiently high levels to have a major role in the capping of the pointed ends of actin filaments; instead, experiments have demonstrated that a significant number (~2,000) of these ends are free in the resting platelet. Third, adducin is abundantly expressed and appears to cap most of the barbed ends of actin filaments in the resting cytoskeleton (Barkalow et al. 2003). Adducin is a key component of the membrane skeleton forming a triad complex with actin and spectrin. Capping of barbed filament ends by adducin also targets them to the spectrin-based membrane skeleton, as the affinity of spectrin for adducin–actin complexes is greater than for either adducing or actin alone.

12 The Actin Cytoskeleton

Actin, at a concentration of 0.5 mM, is the most abundant of all the platelet proteins with over two million molecules per platelet (Nachmias and Yoshida 1988). Similar to tubulin, actin is in a dynamic monomer–polymer equilibrium. Forty percent of the actin subunits assemble to form the 2,000–5,000 linear filaments present in the resting platelet (Hartwig and DeSisto 1991). The rest of the actin in the cytoplasm is maintained in storage as a 1-to-1 complex with β4-thymosin and is converted to filaments during platelet activation to drive cell spreading (Safer and Nachmias 1994). Evidence indicates that the filaments of the resting platelet are crosslinked at various points into a stiff cytoplasmic network, as platelets express high concentrations of actin crosslinking proteins including filamin and α-actinin (Rosenberg et al. 1981). Both filamin and α-actinin are homodimers in solution. Filamin subunits are elongated strands composed primarily of 24 repeats, each ~100 amino acids in length that are folded into IgG-like β-barrels. There are three filamin genes on chromosomes 3, 7, and X. Filamin A (on X) and filamin B (on 3) are expressed in platelets with filamin A present at greater than tenfold excess to filamin B. Filamin is recognized to be a prototypical scaffolding protein that attracts binding partners and positions them adjacent to the plasma membrane. Proteins bound by filamin members include the small GTPases, rho, rac, ralA, and cc42 with

ralA binding in a GTP-dependent manner; the exchange factors Trio and Toll; kinases such as PAK1; phosphatases; and transmembrane proteins. Critical to the structural organization of the resting platelet is the connection between filamin and the cytoplasmic tail of the GPIbα subunit of the GPIb–IX–V complex. Biochemical experiments have demonstrated that the majority of filamin (>90 %) in platelets is in complex with GPIbα (Kovacsovics and Hartwig 1996). This interaction has three consequences. First, it places filamin's self-association domain and corresponding partner proteins at the plasma membrane at the same time as presenting filamin's actin binding sites into the cytoplasm. Second, since a large fraction of filamin is bound to actin, it arranges the GPIb–IX–V complexes into linear rows on the surface of the platelet over the underlying filaments. Third, because the filamin linkages between actin filaments and the GPIb–IX–V complex pass through the pores of the spectrin lattice, it restrains the molecular movement of the spectrin strands in this lattice and holds the lattice in compression. The FLN–GPIbα connection is critical for the formation and release of discoid platelets by megakaryocytes as platelets lacking this connection are fragile and large and produced in low numbers. However, the role of the filamin–vWfR connection in platelet construction per se is not fully understood.

13 The Marginal Band of Microtubules

One of the most distinguishing features of the resting platelet is its marginal microtubule coil (White 1968). Alpha-beta tubulin dimers polymerize into microtubules under physiologic conditions, and in resting platelets, tubulin is equally separated between dimer and polymer fractions. The αβ tubulin subunits are in a dynamic equilibrium with microtubules such that reversible cycles of assembly–disassembly of microtubules are observed. Microtubules are long polymers, 24 nm in diameter, responsible for many types of cellular movements, such as the transport of organelles within the cell and the segregation of chromosomes during mitosis. The microtubule coil of the resting platelet, initially described in the late 1960s by James White, has been described as a single microtubule approximately 100 mm long and is coiled 8–12 times inside the periphery of the platelet (White 1968). The principal function of the microtubule coil is to maintain the disc shape of the resting platelet. Disassembly of platelet microtubules with drugs such as nocodazole, colhicine, or vincristine cause platelets to round and lose their discoid shape (White 1968). Chilling platelets to 4 °C also causes depolymerization of the microtubule coil and loss of the disc shape (White 1968). Furthermore, transgenic studies show that mice lacking the major hematopoietic-specific β-tubulin isoform (β1 tubulin) have platelets that lack the hallmark disc shape and contain defective marginal coils (Schwer et al. 2001). Genetic elimination of β1 tubulin in mice results in thrombocytopenia with mice having circulating platelet counts below 50 % of normal. Beta 1 tubulin-deficient platelets are spherical in shape and this appears to be due to defective marginal bands with reduced microtubule coilings. Whereas normal

platelets possess a marginal band consisting of 8–12 coils, β1 tubulin knockout platelets contain only 2–3 coils (Italiano et al. 2003; Schwer et al. 2001). A human β1 tubulin base pair substitution (AG → CC) resulting in a Q43P mutation has been identified, and demonstrated to cause both functional and structural platelet alterations (Freson et al. 2005). The Q43P β1-tubulin variant was found in 10.6 % of the general population and in 24.2 % of 33 unrelated patients with undefined congenital macrothrombocytopenia. Ultrastructural studies revealed large spherical platelets with a disrupted microtubule coil and structural alterations. Interestingly, platelets with the Q43P β1-tubulin variant showed a mild platelet dysfunction, with reduced adenosine triphosphate (ATP) release, thrombin-receptor-activating peptide (TRAP)-induced aggregation, and inhibited adhesion to collagen under flow. A more than doubled prevalence of the β1-tubulin variant was observed in healthy subjects not undergoing ischemic events, indicating it could confer an evolutionary advantage and protective cardiovascular role. The microtubules that make up the coil are decorated with proteins that modulate polymer stability (Kenney and Linck 1985). Kinesin and cytoplasmic dynein, the major microtubule motors, have been localized to platelets, but their roles in resting and activated platelets are not well understood.

14 Alpha-Granules

One of the most interesting characteristics of platelets is the large number of biologically active molecules that are stored in their granules. These molecules are poised to be precisely delivered at sites of vascular injury and function to recruit other cells in the blood. In resting platelets, granules are positioned close to the OCS membranes. During activation, the granules fuse and secrete into the OCS (Flaumenhaft 2003). Platelets have two major storage granules, α- and dense-granules. The most abundant are α-granules (~40–80 per platelet), which contain proteins essential for platelet adhesion during vascular repair (e.g., GPIIbIIIa, Fibrinogen, VWF). These granules are typically 200–500 nm in diameter and possess spherical shapes with a dark central core. They originate from the trans-Golgi network, where their characteristic dark nucleoid core becomes visible within the budding vesicles (Jones 1960). Alpha-granules obtain their molecular contents from both endogenous protein synthesis and by uptake and packaging of exogenous plasma proteins by receptor-mediated endocytosis and pinocytosis. Endogenously synthesized proteins such as β-thromboglobulin, platelet factor 4, and von Willebrand factor are detected in megakaryocytes before endocytosed proteins such as fibrinogen. In addition, synthesized proteins predominate in the Golgi complex, while endocytosed proteins are located in the peripheral regions of the cell. It has been established that uptake and delivery of fibrinogen to α-granules is mediated by the major membrane glycoprotein IIbIIIa (Coller et al. 1991). Numerous membrane proteins essential to platelet function are also packaged into α-granules, including GP IIbIIIa, P-selectin (CD62P), and CD36 (Blair and

Flaumenhaft 2009). Alpha-granules also have the bulk of cellular P-selectin in their membrane. Once inserted into the plasma membrane, P-selectin recruits neutrophils via the neutrophil counter receptor, the P-selectin glycoprotein ligand (PSGL1) (Diacovo et al. 1996). Alpha-granules also contain over 30 angiogenesis regulatory proteins, which allow them to function as mobile regulators of new blood vessel growth (Folkman et al. 2001). A body of experimental data suggests that specific classes of proteins may be differentially packaged into platelet α-granules, providing a mechanism for the differential release of proteins (Italiano et al. 2008).

Although there is very little known about the intracellular tracking of proteins in megakaryocytes and platelets, experiments using ultrathin cryosectioning and immunoelectron microscopy suggest that MVBs are an essential intermediate stage in the assembly of platelet α-granules (Heijnen et al. 1998). During development of megakaryocytes, these large (up to 0.5 mm) MVBs undergo a gradual transition from granules containing 30–70 nm internal vesicles to granules containing predominantly dense material. Studies examining the internalization kinetics of exogenous bovine serum albumin-gold particles and of fibrinogen, place the MVBs and α-granules sequentially in the endocytic pathway. MVBs contain the secretory proteins β-thromboglobulin and vWF, the platelet-specific membrane protein P-selectin, and the lysosomal membrane protein CD63, suggesting they are a precursor organelle for α-granules (Heijnen et al. 1998).

15 Dense-Granules

Dense-granules (or dense-bodies) are 250 nm in size, are identified in electron micrographs by virtue of their electron-dense cores, and function predominantly to recruit additional platelets to sites of vascular damage. Dense-granules house a variety of hemostatically active molecules that are secreted upon platelet activation, including catecholamines, serotonin, calcium, adenosine 5′-diphosphate (ADP), and adenosine 5′-triphosphate (ATP). ADP is a weak platelet agonist, triggering platelet shape change, granule release, and aggregation. Experiments have demonstrated that the transport of serotonin in platelet dense granules is essential for the process of liver regeneration (Lesurtel et al. 2006). Immunoelectron microscopy studies have also indicated that MVBs are an essential intermediate stage of dense-granule maturation and constitute a sorting compartment between α-granules and dense-granules (Youssefian and Cramer 2000).

16 Organelles

Platelets contain a relatively small number of mitochondria that are recognized in the electron microscope by their internal cisternae. Mitochondria provide an energy source for the platelet as it circulates in the blood for 7–10 days in humans.

Lysosomes and peroxisomes are also present in the cytoplasm of platelets. Lysosomes are also small organelles that contain a large assortment of degradative enzymes including cathepsin, β-galactosidase, aryl sulfatase, β-glucuronidase, and acid phosphatases. Lysosomes function primarily in the degradation of material ingested by phagocytosis or pinocytosis. Peroxisomes are small organelles that contain the enzyme catalase.

Knowledge Gaps

- What mechanisms determine platelet size?
- How do specific genetic disorders result in unusually large or small platelets?
- What signals stimulate proplatelet production?
- Where does platelet biogenesis occur?
- Are there humoral factors that regulate the final stages of platelet production?
- How do we grow functional platelets in vitro on a large scale?

Key Messages

- Approximately one trillion platelets circulate in an adult human
- 100 billion new platelets are produced daily from bone marrow megakaryocytes in order to maintain platelet counts of 150–400 $\times$ 10^9 platelets per liter of whole blood
- The primary role of platelets is to function as the "bandaids" of the blood
- Megakaryocytes are derived from hematopoietic stem cells in the bone marrow and migrate from the osteoblastic niche to the vascular niche during maturation
- Megakaryocytes undergo endomitosis and cytoplasmic maturation as they migrate
- Mature megakaryocytes remodel their cytoplasm into long cytoplasmic processes called proplatelets, which they extend into sinusoidal blood vessels
- Growth hormones and extracellular matrix proteins in the bone marrow direct hematopoietic stem cell differentiation, megakaryocyte maturation, and proplatelet production
- Platelet release occurs in the vasculature through barbell proplatelet and circular preplatelet intermediates
- Platelets contain an open canalicular system, a dense tubular system, organelles, and specialized secretory granules (α- and dense-granules)
- The disc shape of resting platelets is preserved by a cytoskeleton composed of spectrin microtubules, and actin filaments

Acknowledgments This work was supported in part by the National Institutes of Health grant Hl68130 (JEI). JEI is an American Society of Hematology Junior Faculty Scholar. JNT is an American Society of Hematology Scholar.

References

Avecilla ST, Hattori K, Heissig B, Tejada R, Liao F, Shido K, Jin DK, Dias S, Zhang F, Hartman TE, Hackett NR, Crystal RG, Witte L, Hicklin DJ, Bohlen P, Eaton D, Lyden D, de Sauvage F, Rafii S (2004) Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. Nat Med 10:64–71

Balduini A, Pallotta I, Malara A, Lova P, Pecci A, Viarengo G, Balduini CL, Torti M (2008) Adhesive receptors, extracellular proteins and myosin IIA orchestrate proplatelet formation by human megakaryocytes. J Thromb Haemost 6:1900–1907

Barkalow KL, Italiano JE Jr, Chou DE, Matsuoka Y, Bennett V, Hartwig JH (2003) Alpha-adducin dissociates from F-actin and spectrin during platelet activation. J Cell Biol 161:557–570

Behnke O (1969) An electron microscope study of the rat megakaryocyte. II. Some aspects of platelet release and microtubules. J Ultrastruct Res 26:111–129

Behnke O (1970) The morphology of blood platelet membrane systems. Ser Haematol 3:3–16

Behnke O, Forer A (1998) From megakaryocytes to platelets: platelet morphogenesis takes place in the bloodstream. Eur J Haematol Suppl 61:3–23

Blair P, Flaumenhaft R (2009) Platelet alpha-granules: basic biology and clinical correlates. Blood Rev 23:177–189

Bluteau D, Lordier L, Di Stefano A, Chang Y, Raslova H, Debili N, Vainchenker W (2009) Regulation of megakaryocyte maturation and platelet formation. J Thromb Haemost 7(Suppl 1):227–234

Brass LF, Hoxie JA, Manning DR (1993) Signaling through G proteins and G protein-coupled receptors during platelet activation. Thromb Haemost 70:217–223

Chen Z, Naveiras O, Balduini A, Mammoto A, Conti MA, Adelstein RS, Ingber D, Daley GQ, Shivdasani RA (2007) The May-Hegglin anomaly gene MYH9 is a negative regulator of platelet biogenesis modulated by the Rho-ROCK pathway. Blood 110:171–179

Chen Z, Shivdasani RA (2009) Regulation of platelet biogenesis: insights from the May-Hegglin anomaly and other MYH9-related disorders. J Thromb Haemost 7(Suppl 1):272–276

Choi ES, Nichol JL, Hokom MM, Hornkohl AC, Hunt P (1995) Platelets generated in vitro from proplatelet-displaying human megakaryocytes are functional. Blood 85:402–413

Coller BS, Seligsohn U, West SM, Scudder LE, Norton KJ (1991) Platelet fibrinogen and vitronectin in Glanzmann thrombasthenia: evidence consistent with specific roles for glycoprotein IIb/IIIA and alpha v beta 3 integrins in platelet protein trafficking. Blood 78:2603–2610

Diacovo TG, Roth SJ, Buccola JM, Bainton DF, Springer TA (1996) Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. Blood 88:146–157

Flaumenhaft R (2003) Molecular basis of platelet granule secretion. Arterioscler Thromb Vasc Biol 23:1152–1160

Folkman J, Browder T, Palmblad J (2001) Angiogenesis research: guidelines for translation to clinical application. Thromb Haemost 86:23–33

Freson K, De Vos R, Wittevrongel C, Thys C, Defoor J, Vanhees L, Vermylen J, Peerlinck K, Van Geet C (2005) The TUBB1 Q43P functional polymorphism reduces the risk of cardiovascular disease in men by modulating platelet function and structure. Blood 106:2356–2362

Handagama PJ, Feldman BF, Jain NC, Farver TB, Kono CS (1987) Circulating proplatelets: isolation and quantitation in healthy rats and in rats with induced acute blood loss. Am J Vet Res 48:962–965

Hartwig JH, DeSisto M (1991) The cytoskeleton of the resting human blood platelet: structure of the membrane skeleton and its attachment to actin filaments. J Cell Biol 112:407–425
Hartwig JH, Barkalow K, Azim A, Italiano J (1999) The elegant platelet: signals controlling actin assembly. Thromb Haemost 82:392–398
Hartwig JH, Italiano JE Jr (2006) Cytoskeletal mechanisms for platelet production. Blood Cells Mol Dis 36:99–103
Heemskerk JW, Bevers EM, Lindhout T (2002) Platelet activation and blood coagulation. Thromb Haemost 88:186–193
Heijnen HF, Debili N, Vainchencker W, Breton-Gorius J, Geuze HJ, Sixma JJ (1998) Multivesicular bodies are an intermediate stage in the formation of platelet alpha-granules. Blood 91:2313–2325
Howell WH, Donahue DD (1937) The production of blood platelets in the lungs. J Exp Med 65:177–203
Italiano JE Jr, Lecine P, Shivdasani RA, Hartwig JH (1999) Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. J Cell Biol 147:1299–1312
Italiano JE Jr, Bergmeier W, Tiwari S, Falet H, Hartwig JH, Hoffmeister KM, Andre P, Wagner DD, Shivdasani RA (2003) Mechanisms and implications of platelet discoid shape. Blood 101:4789–4796
Italiano JE Jr, Patel-Hett S, Hartwig JH (2007) Mechanics of proplatelet elaboration. J Thromb Haemost 5(Suppl 1):18–23
Italiano JE Jr, Richardson JL, Patel-Hett S, Battinelli E, Zaslavsky A, Short S, Ryeom S, Folkman J, Klement GL (2008) Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. Blood 111:1227–1233
Jones OP (1960) Origin of megakaryocyte granules from Golgi vesicles. Anat Rec 138:105–113
Junt T, Schulze H, Chen Z, Massberg S, Goerge T, Krueger A, Wagner DD, Graf T, Italiano JE Jr, Shivdasani RA, von Andrian UH (2007) Dynamic visualization of thrombopoiesis within bone marrow. Science 317:1767–1770
Kaushansky K (2006) Lineage-specific hematopoietic growth factors. N Engl J Med 354:2034–2045
Kenney DM, Linck RW (1985) The cytoskeleton of unstimulated blood platelets: structure and composition of the isolated marginal microtubular band. J Cell Sci 78:1–22
Kovacsovics TJ, Hartwig JH (1996) Thrombin-induced GPIb-IX centralization on the platelet surface requires actin assembly and myosin II activation. Blood 87:618–629
Larson MK, Watson SP (2006a) A product of their environment: do megakaryocytes rely on extracellular cues for proplatelet formation? Platelets 17:435–440
Larson MK, Watson SP (2006b) Regulation of proplatelet formation and platelet release by integrin alpha IIb beta3. Blood 108:1509–1514
Lecine P, Villeval J, Vyas P, Swencki B, Yuhui X, Shivdasani RA (1998) Mice lacking transcription factor NF-E2 provide in vivo validation of the proplatelet model of thrombocytopoiesis and show a platelet production defect that is intrinsic to megakaryocytes. Blood 92:1608–1616
Lesurtel M, Graf R, Aleil B, Walther DJ, Tian Y, Jochum W, Gachet C, Bader M, Clavien PA (2006) Platelet-derived serotonin mediates liver regeneration. Science 312:104–107
Leven RM (1987) Megakaryocyte motility and platelet formation. Scanning Microsc 1:1701–1709
Mazharian A, Ghevaert C, Zhang L, Massberg S, Watson SP (2011a) Dasatinib enhances megakaryocyte differentiation but inhibits platelet formation. Blood 117(19):5198–5206
Mazharian A, Thomas SG, Dhanjal TS, Buckley CD, Watson SP (2011b) Critical role of Src-Syk-PLC{gamma}2 signaling in megakaryocyte migration and thrombopoiesis. Blood 116:793–800
Michelson AD (1992) Thrombin-induced down-regulation of the platelet membrane glycoprotein Ib-IX complex. Semin Thromb Hemost 18:18–27
Nachmias VT, Yoshida K-I (1988) The cytoskeleton of the blood platelet: a dynamic structure. Adv Cell Biol 2:181–211
Nagahisa H, Nagata Y, Ohnuki T, Osada M, Nagasawa T, Abe T, Todokoro K (1996) Bone marrow stromal cells produce thrombopoietin and stimulate megakaryocyte growth and maturation but suppress proplatelet formation. Blood 87:1309–1316

Nakao K, Angrist AA (1968) Membrane surface specialization of blood platelet and megakaryocyte. Nature 217:960–961

Nilsson SK, Debatis ME, Dooner MS, Madri JA, Quesenberry PJ, Becker PS (1998) Immunofluorescence characterization of key extracellular matrix proteins in murine bone marrow in situ. J Histochem Cytochem 46:371–377

Ogawa M (1993) Differentiation and proliferation of hematopoietic stem cells. Blood 81:2844–2853

Patel-Hett S, Wang H, Begonja AJ, Thon JN, Alden EC, Wandersee NJ, An X, Mohandas N, Hartwig JH, Italiano JE Jr (2011) The spectrin-based membrane skeleton stabilizes mouse megakaryocyte membrane systems and is essential for proplatelet and platelet formation. Blood 118(6):1641–1652

Patel SR, Hartwig JH, Italiano JE Jr (2005a) The biogenesis of platelets from megakaryocyte proplatelets. J Clin Invest 115:3348–3354

Patel SR, Richardson JL, Schulze H, Kahle E, Galjart N, Drabek K, Shivdasani RA, Hartwig JH, Italiano JE Jr (2005b) Differential roles of microtubule assembly and sliding in proplatelet formation by megakaryocytes. Blood 106:4076–4085

Radley JM, Scurfield G (1980) The mechanism of platelet release. Blood 56:996–999

Radley JM, Haller CJ (1983) Fate of senescent megakaryocytes in the bone marrow. Br J Haematol 53:277–287

Ravid K, Lu J, Zimmet JM, Jones MR (2002) Roads to polyploidy: the megakaryocyte example. J Cell Physiol 190:7–20

Richardson JL, Shivdasani RA, Boers C, Hartwig JH, Italiano JE Jr (2005) Mechanisms of organelle transport and capture along proplatelets during platelet production. Blood 106:4066–4075

Rosenberg S, Stracher A, Lucas RC (1981) Isolation and characterization of actin and actin-binding protein from human platelets. J Cell Biol 91:201–211

Safer D, Nachmias VT (1994) Beta thymosins as actin binding peptides. Bioessays 16:590

Schwer HD, Lecine P, Tiwari S, Italiano JE Jr, Hartwig JH, Shivdasani RA (2001) A lineage-restricted and divergent beta-tubulin isoform is essential for the biogenesis, structure and function of blood platelets. Curr Biol 11:579–586

Shivdasani RA, Rosenblatt MF, Zucker-Franklin D, Jackson CW, Hunt P, Saris CJ, Orkin SH (1995) Transcription factor NF-E2 is required for platelet formation independent of the actions of thrombopoietin/MGDF in megakaryocyte development. Cell 81:695–704

Shivdasani RA (2001) Molecular and transcriptional regulation of megakaryocyte differentiation. Stem Cells 19:397–407

Stenberg PE, Levin J (1989) Mechanisms of platelet production. Blood Cells 15:23–47

Tablin F, Castro M, Leven RM (1990) Blood platelet formation in vitro. The role of the cytoskeleton in megakaryocyte fragmentation. J Cell Sci 97(Pt 1):59–70

Thon JN, Montalvo A, Patel-Hett S, Devine MT, Richardson JL, Ehrlicher A, Larson MK, Hoffmeister K, Hartwig JH, Italiano JE Jr (2011) Cytoskeletal mechanics of proplatelet maturation and platelet release. J Cell Biol 191:861–874

Tucker EI, Marzec UM, Berny MA, Hurst S, Bunting S, McCarty OJ, Gruber A, Hanson SR (2010) Safety and antithrombotic efficacy of moderate platelet count reduction by thrombopoietin inhibition in primates. Sci Transl Med 2:37ra45

van Nispen Tot Pannerden H, de Haas F, Geerts W, Posthuma G, van Dijk S, Heijnen HF (2010) The platelet interior revisited: electron tomography reveals tubular alpha granule subtypes. Blood 116(7):1147–1156

White JG (1968) Effects of colchicine and Vinca alkaloids on human platelets. I. Influence on platelet microtubules and contractile function. Am J Pathol 53:281–291

White JG (1972) Interaction of membrane systems in blood platelets. Am J Pathol 66:295–312

Yin T, Li L (2006) The stem cell niches in bone. J Clin Invest 116:1195–1201

Youssefian T, Cramer EM (2000) Megakaryocyte dense granule components are sorted in multivesicular bodies. Blood 95:4004–4007

Platelet Receptors

Alexandre Kauskot and Marc F. Hoylaerts

Contents

Abstract Well-understood functions for "traditional" platelet receptors are described, but "newer" receptors are equally discussed. Receptors are described biochemically (structure, ligand(s), protein partners, and function) and whenever possible, their clinical importance (mutations, polymorphisms, syndrome) are highlighted.

Keywords Platelet receptors • Adhesion • Aggregation • Amplification • Stabilization • Thrombosis • Bleeding

A. Kauskot
Center for Molecular and Vascular Biology, University of Leuven, Leuven, Belgium

M.F. Hoylaerts (✉)
Center for Molecular and Vascular Biology, University of Leuven, Leuven, Belgium

Center for Molecular and Vascular Biology, KU Leuven, Herestraat 49, 3000 Leuven, Belgium
e-mail: Marc.Hoylaerts@med.kuleuven.be

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_2,

1 Introduction

At sites of vascular injury, platelet receptor–ligand interactions are essential for the recruitment of circulating platelets to exposed subendothelial extracellular matrix components, as well as for the subsequent platelet activation and their aggregation. It is not surprising that those receptor–ligand interactions (Fig. 1), which control the initial capture of flowing platelets have attracted a lot of attention. Especially in smaller arteries and arterioles, where shear forces in blood are rather high, interactions between the platelet receptor glycoprotein membrane complex Ib (GPIb) and von Willebrand factor (VWF) are crucial. Synthesized by endothelial cells, VWF multimers are deposited in the subendothelial matrix; VWF is also recruited from the circulation onto collagen fibers, exposed to the blood in the ruptured vasculature (Savage et al. 1998). VWF tethers with GPIb, i.e. its fast off-rate does not allow stable platelet adhesion to the injured vessel wall. Or, platelet GPIb targets platelets to the vessel wall, where they roll over (sub)endothelium in the direction of the blood flow. Firm platelet adhesion implicates additional receptor–ligand couples (Table 1). Of prime importance are specific sequences on exposed collagen fibrils, recognized by the platelet collagen receptor GPVI, a process triggering potent platelet activation and complementing the minor activation brought about by the GPIb–VWF couple. These events switch on intracellular

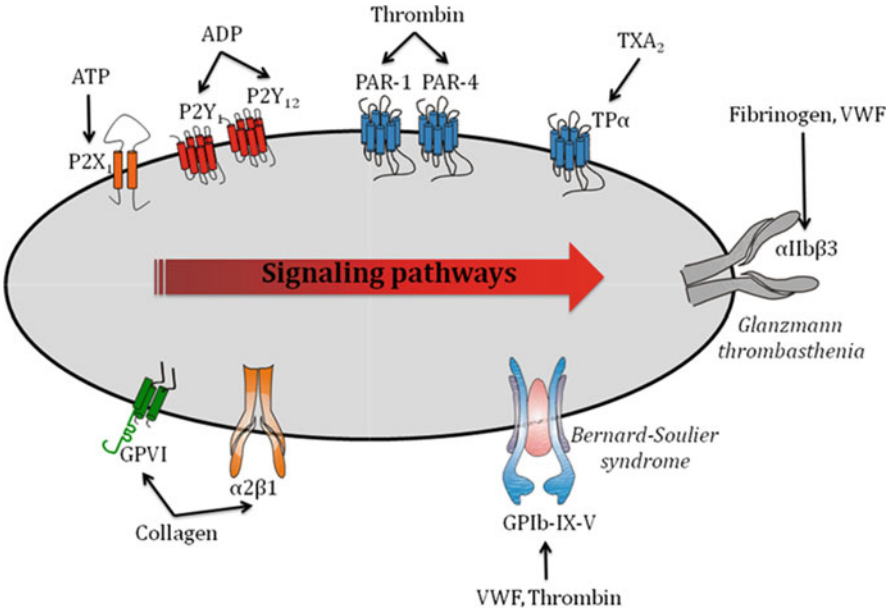


Fig. 1 Major platelet receptor–ligand interactions. Overview of well-known receptors on platelets and of their mode of activation driving to αIIbβ3 activation. Mutations in GPIb-IX-V and αIIbβ3 genes rise to Bernard–Soulier syndrome and Glanzmann thrombasthenia, respectively

Table 1 Platelet receptors in recruitment, adhesion, and aggregation

Receptors	Family	Ligands	Comments
Initiation of platelet recruitment			
GPIb-IX-V complex	Leucine-rich repeat family	VWF, thombin, FXI, FXII, P-selectin, HK, Mac-1, TSP-1	Bernard–Soulier syndrome
Platelet adhesion and aggregation			
GPVI	Ig superfamily	Collagen, laminin	
α2β1	Integrins	Collagen	
α5β1		Fibronectin	
α6β1		Laminin	
αvβ3		Vitronectin, fibrinogen, VWF, osteopontin	
αIIbβ3		Fibrinogen, fibrin, VWF, TSP-1, fibronectin, vitronectin	Glanzmann thrombasthenia
CD148	Tyrosine phosphatase receptor	unknown	Regulation of GPVI
CLEC-2	C-type lectin receptor	Podoplanin (platelets? CLEC-2?)	

signaling, amongst others leading to a conformational shift of membrane β-integrins on the platelet surface from a low to a high affinity state. This causes respectively firm platelet adhesion to collagen via $\alpha_2\beta_1$, in turn triggering platelet spreading, but also platelet aggregation via platelet $\alpha_{IIb}\beta_3$ interactions with fibrinogen, these interactions being essential in central blood vessels exposed to moderate shear stress (Varga-Szabo et al. 2008). To maintain hemostasis, this scheme requires additional fine-tuning via many more receptors, ranging from receptors recognized by soluble mediators, themselves released and produced by platelets, to receptors for thrombin, generated upon coagulation activation and receptor–co-receptor stabilizing pathways, all synergizing to control platelet activation and to arrest blood loss (Table 2).

Physiological platelet activation implies that platelets within the forming plug are sufficiently close to allow formation of direct and indirect bridges between adjacent platelets, allowing paracrine action by platelet-released molecules, favoring the transfer of information as in a neurological or immunological synapse. Together with fibrin formation on the activated platelets, this tight contact also restricts the diffusion of plasma factors, preventing premature fibrinolysis by plasmin over the growing thrombus. The late aggregate-stabilizing events prevent early disaggregation and/or embolization of the fresh platelet aggregate (Rivera et al. 2009) (Table 3).

Platelet receptors also mediate platelet interactions with activated/inflamed vascular endothelium and participate in pro-inflammatory leukocyte interactions with the vessel wall (Pitchford et al. 2003), but this is beyond the scope of this chapter. The currently available list of receptors with a specific role in platelet activation is probably incomplete. Novel proteomic and genomic strategies have hypothesized new candidate receptors, even when only based on the presence in

Table 2 Platelet receptors in the amplification phase

Receptors	Family	Ligands	Comments
$P2Y_1$	G protein-coupled receptors	ADP	
$P2Y_{12}$		ADP	
PAR1		Thrombin	High affinity
PAR4		Thrombin	Low affinity
TPα		Thromboxane	
PGE_2 receptor (EP3)		PGE_2	
PAF receptors		1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine	PAF: platelet-activating factor
Lysophosphatidic acid receptor		Lysophosphatidic acid	
Chemokine receptors		Chemokines	
V1a vasopressin receptor		Vasopressin	
A2a adenosine receptor		Adenosine	
β2 adrenergic receptor		Epinephrine	
Serotonin receptor		Serotonin (5-hydroxytryptamin)	
Dopamine receptor		Dopamine	
$P2X_1$	Ion channel	ATP	
c-mpl	Tyrosine kinase receptor	TPO	
Leptin receptor		Leptin	
Insulin receptor		Insulin	
PDGF receptor		PDGF	

such factors of a transmembrane and an extracellular domain. Consequently, some of these candidate receptors are orphans at present, without known ligand, even when murine knock-out models or/and genetic data (SNPs and mutations) suggest their involvement in (patho)physiological hemostasis (Fig. 2).

2 GPIb-IX-V in Initiation of Platelet Recruitment

Following arterial vascular injury, subendothelial collagen fibrils are disrupted and exposed to the circulation, alongside other subendothelial factors, including VWF, laminin, proteoglycans, fibronectin, vitronectin, etc. Whereas subendothelial VWF is believed to be bound to collagen VI (Hoylaerts et al. 1997), exposed collagen I and III fibers further recruit circulating VWF, providing a network of highly multimeric VWF strands, capable of tethering the platelet receptor glycoprotein Ibα (GPIbα), thus initiating platelet rolling on damaged (sub)endothelium (Wu et al. 2000).

Table 3 Platelet receptors in the stabilization phase and in the negative regulation of platelet activation

Receptors	Family	Ligands	Comments
Stabilization			
Ephr	Tyrosine kinase receptor	Ephrin	
Axl/Tyro3/Mer		Gas-6	
P-selectin	C-type lectin receptor family	PSGL-1, GPIb, TF	Soluble P-selectin: biomarker
TSSC6	Tetraspanins	?	
CD151		?	
CD36	Class B scavenger receptor	TSP1, oxLDL, VLDL, oxPL, collagen type V	Many functions
TLT-1	Ig superfamily	Fibrinogen?	TLT-1 soluble form correlated with DIC
PEAR1	Multiple EGF-like domain protein	?	Phosphorylated after platelet contact
Negative regulation			
VPAC1	G protein-coupled receptors	PACAP	
PECAM-1	Ig superfamily	PECAM-1, collagen, glycosaminoglycans	
G6b-B		?	
PGI_2 receptor (IP)	G protein-coupled receptors	PGI_2	Prostacyclin released from endothelial cells
PGD_2 receptor		PGD_2	
PGE_2 receptor (EP4)		PGE_2	

2.1 Biochemical Description

Four distinct transmembrane proteins, GPIbα (135 kDa), GPIbβ (26 kDa), GPIX (20 kDa), and GPV (82 kDa), assemble at the surface of bone marrow megakaryocytes to constitute a functional GPIb receptor. The four subunits are encoded by genes mapping to chromosomes 17p12 (*GPIBA*), 22q11.2 (*GPIBB*), 3q29(*GP5*), and 3q21 (*GP9*). These genes belong to the leucine-rich family of proteins and are almost exclusively expressed in platelets, with the exception of faint expression in certain endothelial cells (Wu et al. 1997). One complex of GPIb is formed by two chains of GPIbα, 2 GPIbβ, 2 GPIX et 1 GPV (2 :2 :2 :1). Assembly of the GPIb-IX-V complex depends critically on a molecular chaperone in the endoplasmic reticulum: gp96 (grp94, HSP90b1) (Staron et al. 2011). GPIbα, GPIbβ, and IX are present at 25,000 copies per platelet, GPV at 12,500 copies. GPIbα, GPIbβ, and GPIX are closely associated and are all required for efficient bioavailability of GPIb. Deficiency of a single subunit dramatically decreases the surface expression of the whole complex. GPV is more loosely associated and its absence does neither prevent the expression of GPIb, nor its interaction with VWF. It plays a role during thrombin binding to GPIbα (see below).

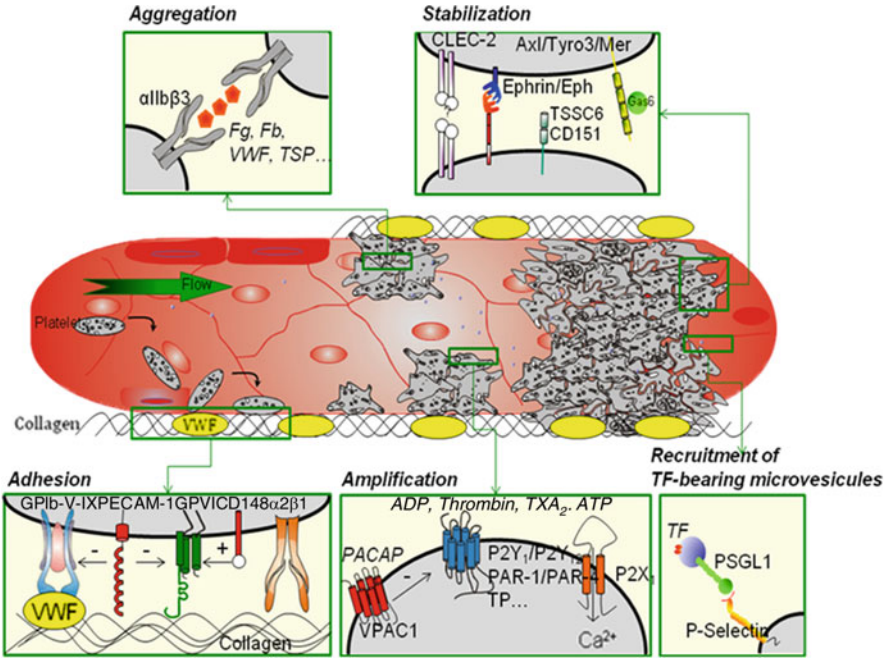


Fig. 2 Overview of platelet receptors involved in platelet activation. Vascular injury induces the exposure of the subendothelial extracellular matrix and leads to the deceleration of circulating platelets, enabling sustained contacts of platelet receptors with components of the ECM and leading to platelet activation. This platelet adhesion at the site of vessel damage is the first step in thrombus formation. It involves interactions of von Willebrand factor (VWF) with GPIb-IX-V and of collagen with GPVI and integrin α2β1. Platelet activation is associated with a change in platelet shape, granule secretion, thromboxane A_2 (TXA_2) synthesis, and intracellular signaling events leading to integrin αIIbβ3 activation. Its activation leads to a change in its conformation, enabling it to bind to ligands, such as fibrinogen and VWF. This promotes platelet–platelet interactions (platelet aggregation) and thrombus formation. Together with fibrin formation on the activated platelets, this tight contact prevents early disaggregation and/or embolization of the fresh platelet aggregate via various receptors and ligands

GPIbα contains 8 Leucine-Rich Repeat domains, one negatively charged region, containing 3 sulfated tyrosines, an O and N-glycosylated region, and a transmembrane and cytoplasmic domain. Platelet activation induces transient clearance of GPIb from the platelet surface, followed by a slow reappearance to normal levels, between 30 and 60 min. Translocation appears to be associated with the formation of GPIb clusters.

2.2 GPIb, an Omnivalent Receptor

The extracellular domain comprises the binding site for VWF, P-selectin, and Mac-1. In addition to its dynamic role in platelet recruitment onto VWF, the GPIb membrane complex functions as a receptor for coagulation factor XII

(Bradford et al. 2000), XI (Baglia et al. 2002), thrombin, the latter interaction studied in large detail (Adam et al. 2003; De Marco et al. 1994) and HK (High-molecular-weight kininogen) (Lanza 2006). Hence, GPIb is a receptor linking primary and secondary hemostasis. Thrombin binding to GPIbα appears to favor presentation of thrombin to its proper receptor, the Protease-Activated-Receptor 1 (see below). GPV may act as a negative modulator of thrombin-induced platelet activation, since cleavage of GPV by thrombin unmasks GPIb-IX and facilitates binding of thrombin to GPIbα (Ramakrishnan et al. 2001). GPIb interacts with vascular P-selectin, pointing to its function in inflammatory platelet pathways (Romo et al. 1999).

2.3 Bernard–Soulier Syndrome

Defects in three GPIb encoding genes give rise to a serious bleeding diathesis, accompanied by morphological platelet anomalies (giant platelets), collectively defined as the Bernard–Soulier Syndrome (BSS). BSS is a rare hereditary thrombocytopathy, first described in 1948 by Bernard and Soulier in a young male patient who had severe mucocutaneous bleeding, prolonged bleeding time with normal platelet count, and abnormally large platelets. Ultrastructural studies of affected platelets show a dilated open canalicular system, prominent dense tubular system, and vacuolization (Lanza 2006).

The BSS usually presents in newborn and during infancy, or early childhood as epistaxis, and/or gingival bleeding. Later bleeding complications will be associated with menstruation, trauma, surgery, or gastric ulcers. The BSS is rare with a reported prevalence of 1 in 1,000,000. It is an autosomal recessive disorder, linked with consanguinity (Lanza 2006). Patients have a prolonged bleeding time, thrombocytopenia, and larger platelets than the normal individual due to defective thrombopoiesis in GPIb defective megakaryocytes (Poujol et al. 2002).

Few mutations were reported that cause a gain of function in the GPIbα chain, leading to the so-called platelet-type GPIB (Pincus et al. 1991), showing a phenotype similar to that of certain subtypes of von Willebrand disease (Franchini et al. 2008).

3 Platelet Adhesion and Aggregation

3.1 Collagen Receptors

Collagens are the most abundant proteins in the subendothelial extracellular matrix (20–40% of total proteins in the aorta). In addition to providing mechanical strength to the blood vessel wall, collagen is essential in platelet adherence and platelet plug formation. Nine types of collagen reside in the vasculature, but only fibrillar collagen

type I, III, V, and VI and nonfibrillar collagen type IV and VIII are thrombogenic. Although platelets have various receptors for collagen, such as GPVI, α2β1, p65, p47, TIIICBP, GPIV, the integrin α2β1 and GPVI are considered major receptors for binding to collagen and activation of platelets (Nuyttens et al. 2011).

3.1.1 GPVI-FcRγ

Biochemical Description

Glycoprotein VI (GPVI) is a 63-kDa transmembrane protein consisting of two immunoglobulin (Ig)-like domains in the extracellular region connected to a highly glycosylated linker, a transmembrane domain, and a cytoplasmic tail. GPVI is a member of the Ig-like receptors within the leukocyte Ig-like receptor complex (LRC) on human chromosome 19q13.4. GPVI is expressed exclusively in platelets and megakaryocytes (around 3,700 copies per platelet), where it associates with the transmembrane adapter protein FcRγ. Surface expression of GPVI is dependent on FcRγ stabilization, through a salt bridge between the transmembrane GPVI domain residue Arg^{272} and FcRγ (Asp residues). The FcRγ is a covalent-linked homodimer with each chain containing one copy of an ITAM (Immunoreceptor Tyrosine-based Activation Motif) defined by the presence of two YxxL sequences separated by seven amino acids (Clemetson and Clemetson 2001). Phosphorylation of the ITAM motif by two Src kinases (Fyn and Lyn) associated with GPVI initiates platelet signaling, leading to potent platelet activation. GPVI is expressed on platelets as a mixture of monomers and dimers, with a stoichiometry of one GPVI to each FcRγ-chain covalent dimer. The affinity of collagen for monomeric GPVI is too low to mediate activation to physiological concentrations of collagen, but dimeric GPVI forms a unique conformation with higher affinity for collagen (Miura et al. 2002). $F(ab)_2$ fragments of antibodies to this structure induce platelet activation, while Fab fragments block activation by collagen. These results suggest a minimal signaling model in which activation is achieved through cross-linking of two GPVI dimers.

Moderate Bleeding in Patients

The role of GPVI deficiency for bleeding complications has remained uncertain for a long time. Early reports on modest bleeding complications could not be interpreted due to incomplete GPVI deficiencies reported (Arthur et al. 2007). Even, the study of various knock-out models did not clearly elucidate the role of GPVI in platelet function, with various mouse models showing different degrees of functional deficiency, depending on the model chosen (Konstantinides et al. 2006; Mangin et al. 2006; Massberg et al. 2003). The paradigm, that GPVI is a major collagen receptor on platelets, despite a moderate bleeding diathesis in men and mice, has recently been resolved by two different groups. They described

independent patients with a proven genetic GPVI defect. Collagen-induced platelet activation was absent, whereas GPVI quantification by flow cytometry evidenced incomplete antigen absence. With complete functional GPVI deficiency, only a mild bleeding phenotype was reported in both cases (Dumont et al. 2009; Hermans et al. 2009), suggesting that additional platelet–collagen interactions play an equally important role in platelet physiology, the next candidate being α2β1.

3.1.2 α2β1

Biochemical Description

The integrin α2β1 (or VLA2, CD49b/CD29, GPIa/IIa) is a collagen receptor composed of a 150-kDa α2 chain and a 130-kDa β1 chain. α2β1 is expressed at 1,730 ± 500 copies per platelet, with an expression profile ranging from 900 to 4,000 copies per platelets. α2 is the only platelet subunit to contain an I domain, a 200-residue inserted sequence. The crystal structure of this domain indicates that it comprises seven helices surrounding a core of five parallel β-strands, a short antiparallel β-strand, and a C-terminal helix (Clemetson and Clemetson 2001). The I-domain metal ion coordinating residues D151, T221, and D254 are all important for collagen binding to the integrin. The I-domain prefers Mg^{2+}/Mn^{2+} in this site. Several recognition sequences by α2β1 have been identified in collagen I and III: GFOGER, GLOGER, GASGER, GROGER, and GLOGEN, with different hierarchy and affinity in their recognition profile (Siljander et al. 2004). This interaction is dependent on Mg^{2+} and the GER sequence. α2β1 recognizes these sequences in the resting state, but platelet activation by classical agonists via intracellular signal transduction pathways will activate α2β1 via a structural rearrangement of the α2β1 domains (Cosemans et al. 2008), causing them to upregulate their affinity for their preferred ligand sequences, stereochemically positioned at regular positions on bundled collagen fibrils (Siljander et al. 2004).

Variable Membrane Expression and Function

Even when no strict deficiencies in α2β1 have been reported, hereditary variation in α2β1 receptor density is well known, at least in part due to several α2β1 allelic polymorphisms, such as the 873G/A and 807C/T polymorphisms (Moshfegh et al. 1999). The C807T and G873A polymorphisms are risk factors for thrombotic pathologies such as myocardial infarction. In some patients with inherited thrombocytopenia, a reduced α2β1 membrane density is coupled to prolonged bleeding times and severely reduced in vitro platelet adhesion to ligands for α2β1 (Santoro 1999). Yet, platelet aggregation tests in vitro manifest subnormal to normal platelet responses to collagen.

3.1.3 CD148 in Optimal Platelet Response to Collagen

General Description

The CD148 gene maps to chromosome 11p11.2, encoding a protein consisting of a large, glycosylated extracellular region containing eight fibronectin type III repeats, a single transmembrane domain, and a single cytoplasmic protein tyrosine phosphatase (PTP) domain. CD148 is the only receptor-like protein tyrosine phosphatase identified in platelets. CD148 is expressed in many cell types, including epithelial and endothelial cells, fibroblasts, and most hematopoietic cells. CD148 null mice exhibited a bleeding tendency and defective arterial thrombosis. CD148 plays a critical role in regulating GPVI/FcRγ chain expression. Indeed, CD148-deficient mouse platelets show reduced GPVI expression. Basal *Scr family kinases* (SFK) activity was found to be markedly reduced in CD148-deficient platelets, resulting in global hyporesponsiveness to agonists that signal through SFKs, including collagen and fibrinogen. CD148 maintains a pool of active SFKs in platelets by directly dephosphorylating the C-terminal inhibitory tyrosines of SFKs that are essential for platelet activation (Ellison et al. 2010; Senis et al. 2009).

CD148 in Disease

In view of the moderate bleeding complications in GPVI deficiency, it seems not surprising that CD148-related platelet dysfunction has not been reported. However, dysregulation of CD148 has been noted in other tissues: comparative analysis of tissue samples from normal gut and from patients with active Crohn's disease showed that leucocytes expressing CD148 are significantly upregulated in inflamed tissues. Furthermore, a marked loss of CD148 immunoreactivity was apparent in some carcinomas (Autschbach et al. 1999). Further work will need to define whether CD148 deficiencies affect GPVI-dependent platelet signaling reactions.

3.1.4 C-Type Lectin-Like Receptor 2: CLEC-2

Biochemical Description

The CLEC-2 gene maps to chromosome 12 and codes for a type II membrane protein C-type lectin receptor family with an extracellular carbohydrate-like recognition domain (CRD-like) that lacks the conserved amino acids for binding to sugars. It has a cytoplasmic tail of 31 amino acids that contains a single conserved YxxL sequence (known as a hem-immunoreceptor tyrosine-based activation motif, hem-ITAM). CLEC-2 is highly expressed on megakaryocytes and platelets, and at low level on peripheral blood mouse neutrophils. CLEC-2 was discovered via the snake venom rhodocytin, also known as aggretin and isolated from the venom of the Malayan

pit viper, *Calloselasma rhodostoma*. It was initially thought to mediate platelet activation through α2β1 and GPIbα based on the ability of high concentrations of antibodies to block activation. However, rhodocytin did not bind recombinant α2β1 and activates platelets deficient in integrin α2β1, GPIbα, and GPVI. Thus, rhodocytin appears to activate platelets through a novel receptor. Also, an antibody to CLEC-2 induces potent activation of human platelets, establishing CLEC-2 as a novel platelet activation receptor (Suzuki-Inoue et al. 2006). Furthermore, by using podoplanin—a ligand of CLEC-2 expressed on the surface of cells such as kidney podocytes, lung type I alveolar cells, and lymphatic endothelial cells but absent from vascular endothelial cells and platelets and an anti-CLEC-2 antibody, it was shown that Syk mediates phosphorylation of CLEC-2, with Src family kinases playing a critical role further downstream.

Recently, three studies claim, respectively deny a role for CLEC-2 in platelet activation at arteriolar shear rates. However, the underlying function is still elusive, as platelets do not express podoplanin, the only known endogenous ligand of CLEC-2. It is possible that CLEC-2 is involved in thrombus stabilization through homophilic interactions (Hughes et al. 2010; May et al. 2009; Suzuki-Inoue et al. 2010). Whether it plays a role in platelet plug formation, in contact with extravascular tissue, remains to be demonstrated.

3.2 Integrins

Integrins play an essential role in the cell metabolism of every cell, including platelets, the only difference being that the integrin ligands of platelets are partly extracellular matrix bound and insoluble, and partly soluble and present in the circulation. Correspondingly, heterodimeric receptors of the β1 and β3 integrin families mediate platelet adhesion and aggregation. In resting platelets, integrins are expressed in a low-affinity state but they shift to a high-affinity state and efficiently bind their ligands in response to cellular activation (Kasirer-Friede et al. 2007). The integrin receptor for collagen on platelets, α2β1 has been discussed in the previous paragraph, but platelets have several integrins at their disposal.

3.2.1 α5β1

General Description

α5β1 is the principal platelet receptor for matrix fibronectin through its RGD sequence. α5β1 supports resting platelet adhesion to fibronectin in static conditions. However, this interaction is unable to promote platelet tyrosine phosphorylation, calcium oscillation, or lamellipodia formation. The α5β1–fibronectin interaction is shear stress sensitive and quickly loses avidity at increasing shear stress. Thus, the

role of α5β1 may be limited to initiating the interaction of resting platelets with matrix fibronectin especially in injuries in the larger blood vessels, where shear forces are low, promoting the subsequent engagement of other integrins and other receptors to amplify platelet responses.

3.2.2 α6β1

General Description

α6β1 is the principal laminin receptor in platelets, not requiring platelet activation in order to bind laminin. Cations Mn^{2+}, Co^{2+}, and Mg^{2+} support adhesion while Ca^{2+}, Zn^{2+}, and Cu^{2+} do not. Binding of platelets to laminin through α6β1 does not induce platelet aggregation but platelets adherent to laminin trigger signaling pathways, inducing filopodia formation, with PI3K and cdc42 activities being higher than in platelets activated through αIIbβ3 (see below). Laminin has been known for many years to support adhesion of platelets through integrin α6β1, but its ability to activate GPVI was only recently discovered by demonstration of spreading of mouse platelets on a laminin surface and loss of activation in the absence of GPVI. The interaction between laminin and GPVI is dependent on the initial interaction with integrin α6β1, in contrast to collagen which initiates platelet activation through GPVI. This difference between the two matrix proteins may reflect the tenfold lower affinity of laminin for GPVI or the presence of a subpopulation of constitutively active α6β1. While the weak nature of the activation of GPVI by laminin argues against a significant role in the prevention of major bleeds, it is ideally suited to facilitate vessel repair after minor damage without the risk of forming occlusive thrombi (Ozaki et al. 2009).

3.2.3 αIIbβ3

Biochemical Description

αIIbβ3 is the most abundant surface-expressed integrin in platelets (40,000–80,000 copies per platelet), with an additional pool that can be recruited from internal membranes upon agonist-induced platelet activation. It is the major receptor on the platelet, also functionally (see below). The mature αIIb and β3 subunits are 148-kDa and 95-kDa proteins, respectively. αIIbβ3 binds several RGD containing ligands, including fibrinogen, fibrin, VWF, vitronectin, fibronectin, and thrombospondin. While ligand recognition typically occurs through an RGD tract, in the case of fibrinogen (the major platelet αIIbβ3 ligand), investigations of the αIIbβ3-mediated cell adhesion to immobilized fibrinogen have evidenced that the fibrinogen synergy gamma (400–411) sequence (dodecapeptide) by itself promotes cell attachment by initiating αIIbβ3 clustering and recruitment of intracellular proteins, while the RGD

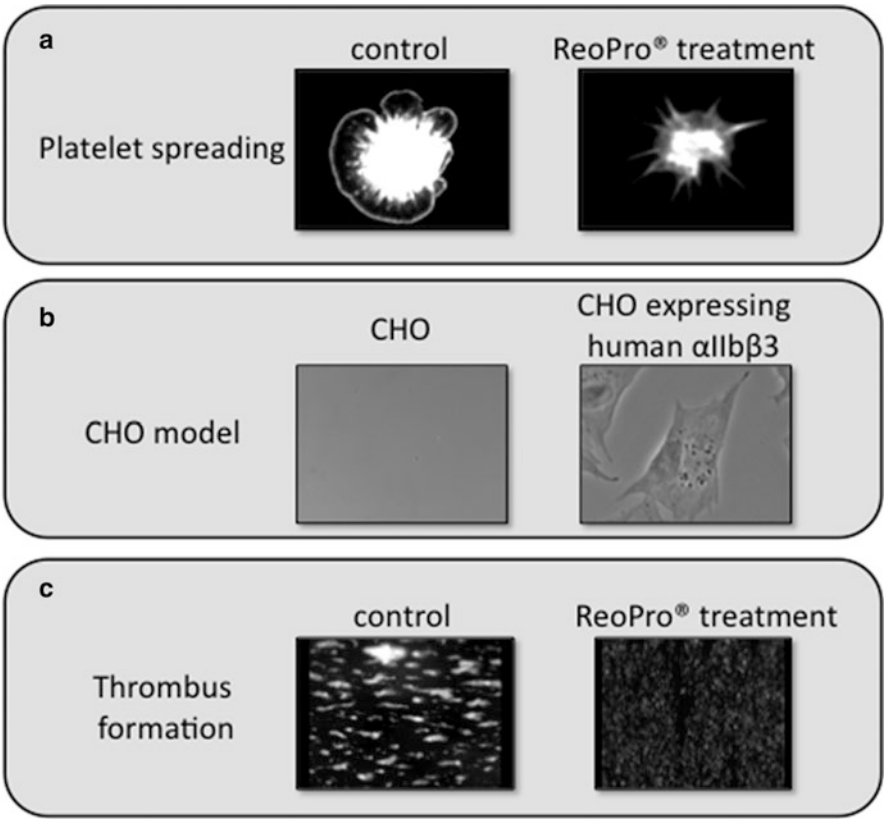


Fig. 3 Central role of αIIbβ3 in platelet activation. (**a**) Human platelets plated over a fibrinogen matrix during 25 min and upon additional incubation with (**a**, *left*) or without ReoPro® for 10 min, (**a**, *right*) (monoclonal antibody that blocks αIIbβ3). Treatment with ReoPro® impairs platelet spreading and induces the detachment of platelets from the fibrinogen matrix. (Platelets were stained with phalloidin–rhodamine for F-actin). (**b**) Chinese Hamster Ovary cells (CHO) without αIIbβ3 or transfected with the human integrin, spread over a fibrinogen matrix. Only cells expressing integrin show cell adhesion and spreading. (**c**) Whole blood was perfused over a collagen matrix at a shear rate of 1,500 s^{-1} during 4 min and thrombus formation monitored with fluorochrome (rhodamine). The treatment of blood with ReoPro® impairs platelet aggregation and thrombus formation, responsible for formation of a monolayer of platelets owing to GPIb and collagen receptors, interacting with collagen-bound VWF and specific collagen sequences, respectively

motif subsequently acts as a molecular switch on the β3 subunit to induce a conformational change necessary for full cell spreading (Salsmann et al. 2006).

This dominant integrin on the platelet surface mediates platelet aggregation through binding of plasma fibrinogen and serves as the principal receptor for platelet adhesion in vivo. The shift of integrin αIIbβ3 from a low- to a high-affinity state is considered the "final common pathway" of platelet activation. This shift is an absolute requirement for platelet αIIbβ3 to interact with fibrinogen (Fig. 3), itself a ligand with two receptor interaction sites, i.e., enabling interaction with separate platelets, constituting the basis of platelet aggregation.

Pathophysiology

The function of αIIbβ3 was first highlighted in the so-called Glanzmann thrombasthenia (GT) syndrome, a rare autosomal recessive bleeding syndrome affecting the megakaryocyte lineage and characterized by lack of platelet aggregation due to quantitative or qualitative defects in αIIbβ3. The sites of bleeding in GT are clearly defined: purpura, epistaxis, gingival hemorrhage, and menorrhagia are nearly constant features; gastrointestinal bleeding and hematuria are less common but can cause serious complications. GT platelets attach to subendothelium after injury, but platelet spreading on the exposed surface is defective. In addition to defective thrombus formation and clot retraction, other αIIbβ3 dependent processes are often absent. Although GT is a very rare disorder, occurring in ≈1:1,000,000 individuals worldwide, research efforts have led to the characterization of more than 170 distinct genetic abnormalities within αIIbβ3, resulting in quantitative and qualitative deficiencies of the receptor on the platelet surface. All mutations for αIIb and β3 are classified in the following database at http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu. Nonsense mutations and splice site mutations with frameshift are common. Certain mutations predominate in ethnic groups such as in Israel and in the French gypsy population. Recently, the estimated age of the French gypsy mutation founder was 300–400 years (Coller and Shattil 2008; Fiore et al. 2011; Kasirer-Friede et al. 2007; Nurden 2006; Nurden and Nurden 2008).

The current treatment options for GT are transfusion with normal human platelets or via therapeutic agents, limiting fibrinolyis (Amicar, tranexamic acid) or generating a procoagulant tendency (NovoSeven®). Unfortunately, frequently antibodies develop to transfused platelets, destroying them. In addition, the beneficial effect of expensive hemostatic agents is relatively short lived. Recently hematopoietic stem cell gene transfer was explored as a strategy to improve platelet function within a canine model for GT. Approximately 5,000 αIIbβ3 receptors formed on 10% of platelets. These modest levels allowed platelets to adhere to fibrinogen, form aggregates, and mediate retraction of a fibrin clot. These results fuel the hope that gene therapy could become a practical approach for treating inherited platelet defects, one day (Fang et al. 2011).

3.2.4 αvβ3

General Description

In general, the β3 gene is widespread, with αvβ3 being expressed in many cell types, including endothelial cells, osteoblasts, smooth muscle cells, and leukocytes, throughout the vascular bed. In platelets it is only present in a few hundred copies per platelet. There are notable differences between αvβ3 and αIIbβ3 in platelets. αvβ3 can bind several RGD-containing ligands, including osteopontin and adenovirus penton base, but vitronectin is the preferred ligand for αvβ3. High-affinity αvβ3 can be induced by agonists such as ADP and by direct integrin modulators

such as DTT and $MnCl_2$. Activated αvβ3 on platelets can bind osteopontin, present in atherosclerotic plaques and in the wall of injured but not normal arteries (Kasirer-Friede et al. 2007; Nurden 2006).

3.3 Receptors in Amplification Phase

Soluble platelet agonists play a critical role in platelet activation and thrombus formation. TXA_2 is synthesized by activated platelets ADP, ATP are released from damaged red blood cells and platelet dense granules, all serving to amplify ongoing platelet activation and recruit circulating platelets in developing platelet aggregates. These agonists activate platelets via G-protein-coupled receptors (GPCRs), a family of 7-transmembrane domain receptors that transmit signals through heterotrimeric G proteins and via the ion channel $P2X_1$. Early traces of thrombin generated during the initiation phase of coagulation by microvesicular tissue factor deposited on blebbing platelets (Varga-Szabo et al. 2008) will give rise to amplifying thrombin-induced platelet activation via protease-activated receptors (PARs), long before coagulation activation has come to completion. Thus, ADP will control platelet activation via two platelet receptors, i.e., $P2Y_1$ (Gq coupled) and $P2Y_{12}$ (Gi coupled), whereas ATP will induce rapid activation via Ca^{2+} influx through $P2X_1$. Thrombin bound onto GPIb will rapidly activate PAR1 in human platelets, whereas on murine platelets, thrombin can likewise be presented to PAR4 by PAR3. PAR4 requires higher thrombin concentrations and is not the prime thrombin receptor on human platelets.

Prostanoids are a family of bioactive lipid mediators that are formed by cyclooxygenase from arachidonic acid liberated from the cell membrane. They are involved in numerous physiological activities, including platelet aggregation, vasorelaxation and vasoconstriction, local inflammatory response, and leucocyte–endothelial cell adhesion. Amplification of platelet activation by TXA_2 synthesis and binding to the TXA_2/prostaglandin H2 receptor (TP) receptor are well known, because they constitute the aspirin-sensitive arm of platelet activation. By contrast, prostacyclin (PGI_2) and PGD_2 are known to inhibit platelet aggregation, whereas PGE_2 potentiates or inhibits platelet response in a dose-dependent manner.

3.3.1 $P2Y_1$

Biochemical Description

The $P2Y_1$ receptor is a 42-kDa protein that contains 373 amino acid residues and is widely distributed in many tissues including heart, blood vessels, smooth muscle cells, neural tissue, testis, prostate, ovary, and platelets. About 150 $P2Y_1$ receptor binding sites are expressed per platelet. Although present at the platelet surface, $P2Y_1$ is also abundantly represented in membranes of α-granules and elements of

the open canalicular system. The $P2Y_1$ receptor is absolutely required for ADP-induced platelet aggregation. Its pharmacological inhibition or genetic deficiency results in complete absence of platelet aggregation and shape change in response to ADP. ADP is a more potent agonist than ATP and their 2-methylthio derivatives are more potent than the parent compounds. UTP, UDP, CTP, and GTP are inactive. ATP is, in fact, a partial agonist at the $P2Y_1$ receptor and so, at low levels of receptor expression it acts as an antagonist. Overall, $P2Y_1$ accounts for about 20–30 % of the total ADP binding sites on the platelet surface (Gachet 2008).

Functional Variability

A common genetic variant at the $P2Y_1$ locus (dimorphism, 1622AG) is associated with platelet reactivity to ADP. This genotype effect partly explains the interindividual variation in platelet response to ADP, which may have clinical implications with regard to thrombotic risk.

3.3.2 $P2Y_{12}$

Biochemical Description

$P2Y_{12}$, which maps to chromosome 3q21-q25, is present in platelets, endothelial cells, glial cells, and smooth muscle cells. It contains 342 amino acid residues, including 4 extracellular Cys residues at positions 17, 97, 175, and 270: Cys 97 and Cys 175, which are linked by a disulphide bridge, are important for receptor expression; 2 potential N-linked glycosylation sites at the extracellular amino-terminus may modulate its activity.

$P2Y_{12}$ receptors exist predominantly as homo-oligomers situated in lipid rafts. On treatment with the active metabolite of clopidogrel (which covalently inhibits $P2Y_{12}$), the homo-oligomers are disrupted into nonfunctional dimers and monomers that are sequestered outside the lipid rafts.

ADP and some of its analogs, such as 2-methylthio-ADP and (*N*)-methanocarba-2-methylthio-ADP, stimulate $P2Y_{12}$, whereas adenosine triphosphate and its triphosphate analogs act as antagonists.

$P2Y_{12}$, the G_i-coupled platelet receptor for adenosine diphosphate (ADP), plays a central role in platelet function.

Patient Bleeding

Patients with congenital $P2Y_{12}$ defects display a mild to moderate bleeding diathesis, characterized by mucocutaneous bleedings and excessive postsurgical and posttraumatic blood loss. Defects of $P2Y_{12}$ should be suspected when ADP,

even at high concentrations (≥10 μM), is unable to induce full, irreversible platelet aggregation (Gachet 2008).

3.3.3 $P2X_1$

Description

Platelet dense granules also release ATP upon activation, reactive with an ion channel present on platelets, i.e. $P2X_1$. $P2X_1$ is a widely distributed ligand-gated ion channel, highly expressed in human megakaryocytes and platelets. ATP is the physiological agonist and ADP is an antagonist. Rapid desensitization of the $P2X_1$ receptor during platelet preparation made this receptor go unnoticed in vitro, for a long time. The $P2X_1$ gene maps to chromosome 17p13.2, encoding 399 amino acids, organized in two transmembrane domains (TM1 and TM2) separated by a large extracellular domain containing ten cysteine residues. Three molecules of ATP bind the extracellular domain of $P2X_1$, triggering conformational changes, resulting in the opening of a cationic pore, allowing rapid changes in the membrane permeability for monovalent and divalent cations, including Ca^{2+}, Na^{+}, and K^{+}. Activation of $P2X_1$ receptors triggers transient shape change, with platelets converted from a discoid to spherical shape. $P2X_1$ receptor activation furthermore amplifies platelet responses to low concentrations of other platelet agonists, with rapid kinetics. Yet, $P2X_1$ contributes equally to low and high levels of thromboxane A2 receptor activation. More significantly, $P2X_1$ receptor activation has been shown to be essential for enhanced platelet adhesion and thrombus formation under high shear rates (Oury et al. 2004). During various studies, $P2X_1$ has distinguished itself as a potential new drug target for antithrombotic therapy, especially for those conditions where mild long-term risk correction is required. $P2X_1$, indeed, seems a "safe" target, its inhibition causing mild effects on platelet function (Hu and Hoylaerts 2010).

3.3.4 Thromboxane Receptor

Biochemical Description

TXA_2 is generated from its precursor arachidonic acid through the cyclooxygenase pathway (Patrono et al. 2005). The TP receptor or TXA_2/prostaglandin (PG) H_2 receptor (57 kDa) is a membrane-bound seven transmembrane receptor, G-protein coupled and distributed widely in the cardiovascular system. Human TP receptors exist in two isoforms, TPα and TPβ, which differ in their C-terminal intracytoplasmic region. Both receptors are encoded by the same gene, but result from alternative splicing. Even though mRNA for both TPα and TPβ is found in platelets, only TPα is expressed in platelets, with TPβ being expressed in endothelial cells. TPα-mediated signal transduction occurs via several G proteins,

including Gq and $G_{12/13}$. TP receptors are also expressed in other cell types, relevant to atherothrombosis, such as smooth muscle cells, macrophages, and monocytes (Habib et al. 1999; Hirata et al. 1996).

Clinical Importance

In several patients, platelet dysfunction has been attributed to an abnormal platelet TXA_2 receptor. One mutation (Arg^{60}-Leu) in the first cytoplasmic loop of TPα was found in patients with a mild bleeding disorder, characterized in aggregation tests by altered responses to TXA_2, TXA_2 analogs, and other agonists (but not thrombin).

3.3.5 Prostaglandin E2 Receptors

Description

During inflammation, the synthesis of prostanoids in endothelial cells and smooth muscle cells is highly increased. The biosynthesis of PGE_2 is enhanced by inflammatory mediators in vascular smooth muscle cells and macrophages.

PGE_2 shows a biphasic, concentration-dependent effect on platelet aggregation. Although high concentrations inhibit platelet aggregation, lower concentrations enhance it. PGE_2 activates four G protein-coupled receptors EP1, EP2, EP3, and EP4. Each of these receptors has a distinct pharmacological signature and intracellular signal transduction. Stimulation of EP3 receptors results in elevation of free intracellular calcium levels, whereas stimulation of EP2 and EP4 receptors usually increases intracellular cAMP levels through activation of Gαs protein, resulting in a decrease of intracellular calcium levels. Human platelets contain mRNA for EP1 receptor, all of the EP3 splice variants, and EP4. However mRNA for EP2 receptors is lacking in platelets (Paul et al. 1998).

The proaggregatory effect of PGE_2 has been ascribed to the activation of the EP3 receptor, leading to inhibition of the increase in cAMP, increased mobilization of calcium, and elevated P-selectin expression in platelets. Mice lacking this receptor show an increased bleeding tendency and a decreased susceptibility to thromboembolism (Ma et al. 2001). It has also been demonstrated that PGE_2 produced by atherosclerotic plaques in mice can facilitate arterial thrombosis, acting via EP3 (Gross et al. 2007).

Defects in the EP3 receptor gene are not yet discovered in humans.

Recently, a selective EP4 agonist (ONO AE1-329) has been show to inhibit platelet aggregation, calcium mobilization, and upregulation of P-selectin. Furthermore EP4 activation enhances the inhibitory effect of aspirin. Conversely, two chemically distinct EP4 antagonists, GW627368x and ONO AE3-208, abrogated the inhibitory effect of ONO AE1-329 on platelet aggregation (Philipose et al. 2010). EP4 receptors might play an important role in the control of hemostasis by mediating the inhibitory effect of PGE_2, thereby balancing out the proaggregatory

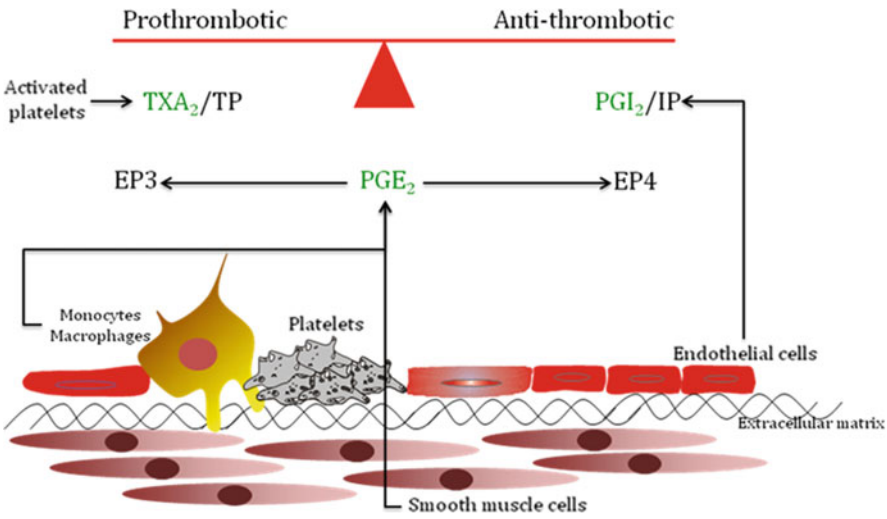


Fig. 4 The balance between thrombotic and antithrombotic effects of prostanoids. In response to vascular injury, PGI_2 produced by endothelial cells opposes the enhanced prothrombotic effect of TXA_2 produced by platelets. Smooth muscle cells, monocytes, and macrophages (accumulate in atherosclerotic plaques) release prostanoids such as PGE_2 during inflammation. PGE_2 shows a biphasic, dose-dependent effect on platelet aggregation

effect of EP3 receptors (Fig. 4). EP4 agonists might constitute a novel class of antithrombotic agents and be clinically useful in cases where aspirin or ADP antagonists are not warranted or are insufficient (Philipose et al. 2010).

3.3.6 Prostacyclin (PGI_2) Receptor

Prostaglandin I_2 (PGI_2) or prostacyclin is a derivative of arachidonic acid, released by vascular endothelial cells, serving as a potent vasodilator, inhibitor of platelet aggregation, and moderator of vascular smooth muscle cell proliferation–migration–differentiation (antiatherosclerotic) through a specific membrane-bound receptor, the prostacyclin receptor (IP receptor) (Fig. 4). The receptor belongs to the prostanoid family of G protein-coupled receptors and is widely distributed throughout the body with predominate cardiovascular expression on platelets and smooth muscle cells. The receptor has a molecular weight of 37–41 kDa (depending upon different states of glycosylation) and has been categorized as a Class A rhodopsin-like GPCR. Glycosylation of the extracellular domain is necessary for ligand binding, receptor activation, and membrane localization. In the cytoplasmic domain, a number of serine residues (S328 and S374) are thought to be phosphorylated by GPCR kinases or second-messenger-activated kinases (PKC and PKA) and play potential roles in either agonist-induced phosphorylation and/or kinase-mediated receptor desensitization (Stitham et al. 2007). Prostaglandins have two structural features, a

cyclopentane ring and side chains that are recognized by their receptor to stabilize ligand binding. The binding pocket of the receptor can accommodate the cyclopentane rings of PGI_2, PGE_1, and PGE_2. The IP is most commonly associated with coupling to the Gαs subunit of the heterotrimeric G-protein, which upon receptor activation stimulates membrane-bound adenylyl cyclase to catalyze the formation of the second messenger, cAMP. Animal studies using prostacyclin receptor knock-out mice have revealed increased propensities towards thrombosis, intimal hyperplasia, atherosclerosis, restenosis, as well as reperfusion injury.

Modification of IP and TP Function by Heterodimerization

IP and TP associate to form homo- and heterodimers. Interestingly, heterodimerization with the IP facilitated coupling of the TPα to cAMP generation (an IP-like cellular response) (Wilson et al. 2004) and rendered the TP sensitive to regulation by IP agonists (Wilson et al. 2007). Therefore, when IP and TP are present in the same cell, a common occurrence in cardiovascular tissues, cAMP can be generated via the PGI_2-IP/IP and TxA_2-IP/TP pathways. This signaling shift likely contributes to the limit placed by the IP on the deleterious cardiovascular effects of TP activation. One variant, IP^{R212C}, which occurs at low frequency (0.8 % in white and Asian cohorts), is associated with accelerated cardiovascular diseases. IP^{R212C} exerts a dominant action on the wild-type IP and TPα through dimerization. This likely contributes to accelerated cardiovascular disease in individuals carrying the variant allele (Ibrahim et al. 2010).

Clinical Importance

Two polymorphisms, which result in the amino acid mutations Val25Met and Arg21His were reported. The latter polymorphism exhibited a significant decrease in signaling upon activation of the IP receptor (Stitham et al. 2002). Other dysfunctional human prostacyclin receptor variants were described, some having defects in binding, activation, and/or protein stability/folding. Mutations (M113T, L104R, and R279C) in three highly conserved positions demonstrated severe misfolding resulting in impaired ligand binding and activation of cell surface receptors. In a cohort study, major coronary artery obstruction was significantly increased in the dysfunctional mutation group in comparison with the silent mutations (Stitham et al. 2011). Circulating platelet–leukocyte mixed conjugates and platelet microparticles are potential markers of inflammation in atherothrombotic disease. Epoprostenol (a synthetic salt of PGI_2 clinically used in pulmonary hypertension and transplantation as a potent inhibitor of platelet aggregation) inhibits human platelet–leukocyte conjugate and platelet microparticle formation in whole blood. Epoprostenol might reduce the inflammatory cell contribution to pulmonary hypertension and thrombosis (Tamburrelli et al. 2011).

3.3.7 Thrombin Receptors

Biochemical Description

As explained earlier, platelet activation by thrombin partially depends on GPIb-IX-V, but is primarily assured by two protease-activated receptors (PAR), i.e., PAR-1 and PAR-4. PARs consist of four members (PAR1-4), PAR-3 and PAR-4 being the mouse platelet receptors for thrombin. GPIb contains a high affinity binding site for thrombin within residues 268–287 at the N-terminal globular domain and can bind two separate thrombin molecules by interacting with both exosite I and II of thrombin. Binding of thrombin (immobilized, proteolytically inactive) to GPIb induces platelet adhesion and spreading and secretion; platelets from patients with the BSS which lack GPIb display impaired thrombin responsiveness (Adam et al. 2003). Or, GPIb is an enhancer of the thrombin response. PAR-1 and PAR-4 are activated by a unique irreversible proteolytic cleavage within the first extracellular loop exposing an N-terminus that serves as a tethered ligand. Short synthetic peptidomimetics of the N-terminus sequences, corresponding to the new N-terminus, upon cleavage by thrombin (SFLLR for PAR-1 and GYPGQV for PAR-4) can activate these receptors directly, reproducing most of the action of thrombin on platelets. This dual-receptor signaling model for thrombin implies that PAR-1 is the primary mediator, activating platelets at low concentration vs. PAR-4 as a back-up receptor, activated at higher thrombin concentrations. Qualitative differences in the dynamics of PAR-1 and PAR-4 activation might be relevant for sustained optimal platelet responses to thrombin. Thus, the PAR-4 mediated Ca^{2+} mobilization is slower and more prolonged than that of PAR-1 and it is switched off more slowly.

No patient has been identified with congenital deficiencies of PAR receptors. Pharmacological PAR-1 inhibitors are in development for the prevention of platelet-dependent thrombosis (Oestreich 2009).

3.4 Receptors in Stabilization Phase

Stabilization of platelet aggregates is a dynamic process requiring contact-dependent signaling and outside-in signaling through integrins, particularly αIIbβ3. Signals emanate from αIIbβ3 once ligand binding has occurred, thus triggering essential events for thrombus stabilization, such as cytoskeletal reorganization, enlarging platelet aggregates, development of a procoagulant surface, and clot retraction. These processes increase the local concentration of soluble platelet agonists inside the aggregate consisting of degranulating platelets. In addition to integrins, other actors perpetuate the platelet plug (Rivera et al. 2009).

3.4.1 Eph Kinase Receptors

Biochemical Description

Eph kinases are receptor with ligands, the ephrins, expressed on the surface of cells. Eph kinases are receptor tyrosine kinases with an extracellular ligand binding domain and an intracellular tyrosine kinase domain. The Eph B subfamily is distinguished from the Eph A subfamily by an insertion within the extracellular domain that helps to define the ligand preferences for the receptor. Interactions between Eph kinases and ephrins on adjacent cells play a central role in neuronal patterning and vasculogenesis. Human platelets express EphA4, EphB1, and ephrinB1. The former is constitutively associated with αIIbβ3 in both resting and activated platelets. Clustering of either EphA4 or ephrinB1 causes platelet adhesion to immobilized fibrinogen, but blockade of Eph/ephrin interactions hampers clot retraction by impairing β_3 phosphorylation. This results in inhibition of platelet aggregation at low agonist concentrations and results in smaller thrombi on collagen-coated surfaces under arterial flow conditions, causing premature disaggregation. These effects are partially due to the ability of ephrin B1 to activate Rap1, a Ras family member that supports integrin activation in platelets (Prevost et al. 2003, 2005).

3.4.2 Gas-6 and Its Receptors

Biochemical Description

Gas-6 (growth arrest-specific gene 6) is a vitamin K-dependent protein implicated in cell growth, adhesion, and migration, through its interactions with Tyro 3, Axl, and Mer tyrosine kinase receptors (TAM family). The TAM receptors are three related protein receptor tyrosine kinases that were first cloned in 1991 as orphan receptors, widely expressed in the vertebrate nervous system. The three receptors consist of two N-terminal immunoglobulin domains, followed by two fibronectin-III-like domains attached via a single-pass α-helical transmembrane domain to an intracellular tyrosine kinase domain. In common with other receptor tyrosine kinases, the functional receptors form both hetero- and homodimers. The structure of receptor–ligand complexes causes two N-terminal immunoglobulin domains to mediate binding to the ligand.

Mouse Gas-6 is found in plasma and in platelets' granules from which it is secreted upon activation. In contrast, in man Gas-6 is predominantly present in plasma. Mice deficient in Gas-6 or in one of its receptors show abnormal platelet responses to agonists and are protected against thrombosis, suggesting a major role of this axis in thrombus formation and vascular wall homeostasis. Recently, the role of Gas-6 in human platelet function has been clarified. Gas-6 reinforces αIIbβ3 outside-in signaling by activation of PI-3 K and Akt, and promotes β_3 phosphorylation and therefore clot retraction (Angelillo-Scherrer et al. 2005). These effects

constitute in fact, an enhancement and perpetuation of the thrombus-stabilizing role of ADP. The inhibition of Gas-6 signaling has been proposed as an attractive target for novel antithrombotic drugs (Cosemans et al. 2006, 2010).

3.4.3 Tetraspanin Superfamily

Tetraspanins, also called tetraspans or the transmembrane 4 superfamily (TM4SF), have four transmembrane domains, intracellular N and C-termini, and two extracellular domains (EC1, EC2), one short and one longer, typically 100 amino acid residue long loop. The key features are four or more cysteine residues in the EC2 domain, with two in a highly conserved "CCG" motif. Generally, tetraspanins are often thought to act as scaffolding proteins, anchoring multiple proteins to one area of the cell membrane.

CD151

Biochemical Description

The tetraspanin superfamily member CD151 (previously termed PETA-3/SFA-1) is broadly expressed in vascular, hematopoietic, and immune compartments, and is especially abundant in epithelia, endothelia, cardiac muscle, smooth muscle, megakaryocytes, platelets, and the immune system. CD151 is functionally linked to hemidesmosome formation, cancer metastasis, neurite outgrowth, vascular morphogenesis, cell migration, integrin trafficking, wound healing, immune responsiveness, and hemostasis. Recently, it has been found that CD151 appears to regulate fibrinogen-binding proteins, including integrin αIIbβ3. The absence of CD151 leads to smaller, unstable thrombi being formed in vivo (Orlowski et al. 2009).

TSSC6

General Description

Tumor-suppressing subchromosomal transferable fragment cDNA 6 (TSSC6), also previously known as Pan hematopoietic expression (Phemx), is a tetraspanin superfamily member. Its C-terminal cytoplasmic domain is relatively large (33 and 99 amino acids in mouse and human, respectively) compared to other tetraspanin superfamily members. TSSC6 is specifically expressed in hematopoietic organs and tissues where it may play a role in hematopoietic cell function. Absence of platelet TSSC6 affects the secondary stability of arterial thrombi in vivo upon vascular injury, by regulating integrin αIIbβ3 "outside-in" signaling events (Goschnick et al. 2006).

3.4.4 CD36

Biochemical Description

CD36, also known as scavenger receptor, GPIIIb, GPIV, GP88, FAT, SCARB3, or PASIV, is expressed on the surface of most cells, such as platelets, monocytes, endothelial cells, smooth muscle cells, and cardiomyocytes. The human gene is located on chromosome 7. The CD36 protein consists of a single peptide chain of 474 amino acids and has a molecular weight of 80–90 kDa. On platelets 10,000–25,000 molecules are present. CD36 has a "hairpin-like" configuration, containing two transmembrane domains, one near the N-terminus and the other near the C-terminus, separated by a large, glycosylated extracellular loop. Platelet ligands of CD36 identified include TSP1, oxidized phospholipids (oxPL), oxidized low-density lipoprotein (oxLDL), and long chain fatty acids. CD36 was first described as a collagen type I and III receptor on platelets. However, platelets from CD36-deficient patients respond normally to collagen stimulation. Even when CD36 binding to the nonfibrillar type V collagen is documented, today it is clear that CD36 is not a primary collagen receptor (Nergiz-Unal et al. 2011). Yet, platelet activation is modulated by CD36 interactions with various ligands. A new role for TSP-1 in promoting platelet aggregation through modulation of an inhibitory signaling pathway has been demonstrated. Through CD36, TSP-1 prevents cAMP/protein kinase A (PKA) signaling. Indeed, TSP-1 triggers CD36-dependent signals that reduce platelet sensitivity to PGE_1, diminishing its ability to inhibit platelet aggregation and arrest under conditions of flow (Roberts et al. 2010). On the other hand, oxLDL formed in the setting of hyperlipidemia and atherosclerosis can activate platelets in a CD36-dependent manner.

Clinical Relevance

The level of platelet CD36 surface expression is highly variable among individuals from a general population. This variability affects the functional response of platelets to oxLDL and is associated with inheritance of specific genotypic polymorphisms at the *CD36* locus in both Caucasians and African Americans (Ghosh et al. 2011).

3.4.5 TLT-1

General Description

Triggering Receptors Expressed on Myeloid cells (TREMs) are involved in the activation of various cell types of the innate immune system, including monocytes, macrophages, microglia, and neutrophils. The family is characterized by a single V-set immunoglobulin (Ig) domain, a short cytoplasmic tail, and a charged residue

in the transmembrane domain. TREM-like transcript-1 (TLT-1), a type 1 single Ig domain orphan receptor specific to platelet and megakaryocyte alpha-granules, relocates to the platelet surface upon platelet stimulation and its longer cytoplasmic tail carries a canonical ITIM (Immunoreceptor Tyrosine-based Inhibition Motif) capable of becoming phosphorylated and of binding Src homology-containing protein tyrosine phosphatase-1 (SHP-1), identifying TLT-1 as the only putative inhibitory member of the TREM cluster. The ability of anti-TLT-1 scFv to block aggregation of washed platelets suggested that TLT-1 facilitates thrombosis by interacting with a ligand or ligands on or in activated platelets. TLT-1 may act in concert with αIIbβ3 to facilitate fibrinogen/platelet interactions and/or higher order platelet aggregation (Giomarelli et al. 2007; Washington et al. 2009).

Patients

Septic patients, in contrast to healthy individuals, have substantial levels of soluble TLT-1 (sTLT-1) in their plasma that correlated with the presence of disseminated intravascular coagulation (DIC). The sTLT-1 directly promotes platelet aggregation in vitro at clinically relevant concentrations, which suggests that TLT-1 may be a novel, platelet-specific, secondary activation factor (see Sect. 4).

3.4.6 PEAR1

General Description

PEAR1 (Platelet Endothelial Aggregation Receptor-1) (also known as MEGF12 or JEDI) is a transmembrane protein of the multiple EGF-like domain protein family of 150 kDa. PEAR1 is mainly expressed in platelets, endothelial cells, and also in satellite glial cell precursors, where it is necessary for apoptotic neuron clearance in the embryonic dorsal root ganglia via an engulfment activity. PEAR1 is composed of an extracellular EMI domain (Emilin domain), 15 extracellular epidermal growth factor-like repeats (EGF-like repeats), and multiple cytoplasmic tyrosines and prolines. The intracellular domain structure contains 5 proline-rich domains and an NPXY motive, which may serve as a phosphotyrosine binding site. During platelet aggregation, PEAR1 is phosphorylated at Tyr-925 and Ser-953/1029, upon its oligomerization, in an αIIbβ3-dependent manner. PEAR1 phosphorylation is also observed, independently of αIIbβ3 during physical platelet approximation via centrifugation. Thus PEAR1 was hypothesized to be a platelet–platelet contact receptor (Nanda et al. 2005).

Functional Relevance

A possible function for PEAR1 was substantiated by several studies that have linked polymorphisms in PEAR1 with increased or decreased platelet responses to various

agonists. A PEAR1 promoter-region variant (rs2768759) was associated with increased aggregation in PRP, most strongly in response to epinephrine, in both pre- and postaspirin treatment conditions (Herrera-Galeano et al. 2008). Increased expression of PEAR1 might be an important cause of hyperactivity (Herrera-Galeano et al. 2008) and genetic variation within PEAR1, particularly rs41299597, seems to lead to an increased membrane expression of PEAR1 in activated platelets and elevated responsiveness to GPVI ligands (Jones et al. 2009a). A genome-wide meta-analyses linked the minor allele of the PEAR1 SNP (rs12566888) to a drop in aggregation response towards ADP and epinephrine in the European and African-ancestry sample (Johnson et al. 2010).

3.4.7 P-Selectin/PSGL-1 Couple

General Description

P-selectin is a cell adhesion molecule of the selectin family of 140 kDa. The primary ligand for P-selectin is PSGL-1 (P-selectin glycoprotein ligand-1), constitutively found on all leukocytes. The transient interactions between P-selectin and PSGL-1 allow leukocytes and activated platelets to roll along the venular endothelium. The formation of a fibrin network upon activation of the coagulation cascade is a critical event contributing to thrombus stability. Recent studies with a laser injury-induced thrombosis model in mice expressing a low level of tissue factor (TF) have shown that this fibrin formation depends on the monocyte-derived TF carried by microvesicules, with minimal contribution of vessel wall TF. These microvesicules are captured onto the thrombus through the interaction between P-selectin expressed on the surface of activated platelets and PSGL-1 present on microvesicules, hence delivering TF to the growing thrombus. Mice deficient in either PSGL-1 or P-selectin display thrombi with little TF and reduced thrombin generation, resulting in hampered thrombus size (Abdullah et al. 2009; Morel et al. 2008; Ramacciotti et al. 2009).

Cardiovascular Patients

Soluble P-selectin is present in the blood and circulates in man at 100 ng/ml. Elevated plasma sP-selectin is a major predictive factor of cardiovascular events related to platelet turnover and its activation and function. Increased expression of P-selectin is observed in coronary artery disease, acute myocardial infarction, stroke, and peripheral artery diseases.

3.5 Receptors in Negative Regulation of Platelet Activation

Arterial thrombus formation is a dynamic process, in which a state of surface passivation is preferable to limit thrombus growth. The essential roles of nitric

oxide (NO) and prostacyclin (PGI_2) in the negative regulation of platelets to prevent uncontrolled thrombosis have been well established. However, it is now recognized that various receptors with and without ITIM domain inhibit thrombus formation actively.

3.5.1 ITIM-Containing Receptors

ITIMs are defined by a consensus sequence (L/I/V/S)-X-Y-X-X-(L/V) and are commonly found in pairs separated by 15–30 amino acid residues. ITIM-containing receptors were originally identified by their ability to inhibit signaling by ITAM receptors. For example, PECAM-1 causes mild inhibition of platelet activation by the ITAM and ITAM-like receptors, GPVI and CLEC-2, and by the G protein-coupled receptor agonist, thrombin. The latter action is similar to that of G6b-B (see below), which upon cross-linking by a specific antibody inhibits activation of platelets by the GPVI-specific agonist, collagen-related peptide, and the G protein-coupled receptor agonist ADP.

3.5.2 PECAM-1

General Description

Platelet activation can be inhibited through the adhesion molecule PECAM-1 (or CD31). Like GPVI, PECAM-1 is a member of the Ig superfamily, with 6 extracellular Ig domains, transmembrane domain, and cytoplasmic tail. The cytoplasmic domain of PECAM-1 contains an ITIM, which becomes phosphorylated upon stimulation by homophilic interactions and/or clustering, facilitating the recruitment of tyrosine, serine/threonine, or possibly lipid phosphatases, and the consequent inhibition of kinase-dependent signaling. PECAM-1 plays a role in attenuating thrombus formation involving GPVI, GPIb, and thrombin-mediated platelet activation (Jones et al. 2009b).

3.5.3 G6b-B

Description

The list of novel plasma membrane proteins identified via proteomics includes the immunoglobulin superfamily member G6b of 241 amino acids (26-kDa), which undergoes extensive alternate splicing. G6b-B undergoes tyrosine phosphorylation and association with the SH2 domain-containing phosphatase, SHP-1, in stimulated platelets suggesting that it may play a novel role in limiting platelet activation. G6b-B is the only one of these variants to contain both a transmembrane region and two ITIM that support binding to the two SH2 domain-containing protein tyrosine

phosphatases, SHP1 and SHP2. The second ITIM in G6b-B is located around 20 amino acids downstream of the first ITIM and has a slightly different sequence mentioned earlier (TXYXXV) (Mori et al. 2008).

3.5.4 VPAC1/PACAP

Description and Patients

The pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide of the vasoactive intestinal peptide/secretin/glucagon superfamily. Studies in two related patients with a partial trisomy 18p revealed three copies of the PACAP gene and elevated PACAP concentrations in plasma. Patients suffer from severe mental retardation and have a bleeding tendency with mild thrombocytopenia. The PACAP receptor (vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide receptor 1 VPAC1) in platelets is coupled to adenylyl cyclase activation. Increased basal cAMP levels in patients' platelets have been found, providing a basis for the reduced platelet aggregation in these patients. Megakaryocyte-specific transgenic overexpression of PACAP in mice correspondingly increased PACAP release from platelets, reduced platelet activation, and prolonged the tail bleeding time. The therapeutic potential to manage arterial thrombosis or bleeding by administration of PACAP mimetics or inhibitors is being considered (Freson et al. 2004).

4 Proteolysis/Shedding of Platelet Receptors

Metalloproteinase-mediated ectodomain shedding of platelet receptors has emerged as a new mechanism for modulating platelet function. By regulating surface expression of GPVI and GPIbα, shedding not only irreversibly downregulates GPVI/GPIbα functions, but generates proteolytic fragments that might be unique biomarkers or modulators in plasma. Altered expression levels of GPIbα/GPVI are associated with both thrombotic propensity and platelet aging, suggesting an additional role in platelet clearance. Recently, the molecular GPVI shedding has been described to be dependent on FXa or/and other proteinases. Indeed, coagulation-induced GPVI shedding via FXa downregulates GPVI under procoagulant conditions. FXa inhibitors have an unexpected role in preventing GPVI downregulation. Furthermore, various platelet proteinases (at least ADAM10 and 17) regulate GPVI shedding (Bender et al. 2010).

Furthermore, the TLT-1 extracellular domain is shed from platelets during activation. Therefore, the possibility that sTLT-1 might be released under pathological conditions, resulting in changes in platelet function during those disease states has been advanced. Data from two independent cohorts confirmed the presence of increased levels of sTLT-1 in sepsis patients. The finding that patients who died

during sepsis had increasing levels of sTLT-1, whereas those who survived showed a decline in sTLT-1 during this same period, suggesting that monitoring of sTLT-1 levels could be an important prognostic indicator. Similarly, the correlation between sTLT-1 and D-dimers indicates association of sTLT-1 with the clinical manifestations of disseminated intravascular coagulation (DIC). These data substantiated the involvement of TLT-1 in the response to sepsis and indicate that sTLT-1 may provide a significant clinical tool for the diagnosis of disseminated intravascular coagulation associated with sepsis (Washington et al. 2009). Other proteins can be shed, like P-selectin, ICAM-1, and PECAM-1, the latter shed from human platelets during high shear stress. However, the concentrations released in plasma are insufficient to affect platelet activation; they may serve to monitor platelet activation and vascular injury in coronary artery disease. Indeed, a rapid increase of PECAM-1 has been reported after myocardial infarction (Soeki et al. 2003).

Recently, a clinical study showed that soluble P-selectin can establish the diagnosis of deep vein thrombosis (DVT) with a cutoff point of 90 ng/mL, when combined with a Wells score ≥ 2, with a positive predictive value of 100%. Also, soluble P-selectin can exclude the diagnosis of DVT with cutoff points below 60 ng/mL, when combined with a Wells score <2, with a negative predictive value of 96%. Based on their data, 32% of patients could potentially be diagnosed with DVT without the need of imaging exams (Ramacciotti et al. 2011). One of the conclusions of this study is that such biomarkers (like P-selectin) could establish or exclude the diagnosis for thrombosis in case where ultrasound imaging is not available. Also, in patients with the antiphospholipid syndrome, sP-selectin measurements had a good prognostic value in thrombosis prediction (Devreese et al. 2010).

5 Conclusion

Different types of receptors control platelet function at various levels, as highlighted earlier. Translation of receptor-delivered signals occurs via a series of intracellular signaling pathways, discussed in larger detail in other chapters. There is a wealth of platelet-specific and platelet-prone receptors (see "Key Messages"); yet we only have limited pharmacological tools to control platelet function in secondary thrombosis prevention or in bleeding (see "Knowledge Gaps"). The new functional insight in the so-called secondary platelet receptors should accelerate the development of additional pharmaceutical drugs to control platelet function.

Knowledge Gaps

- Some receptors are orphans i.e., without know ligand.
- Interplay between platelet receptors activation in hemostasis and its consequences in inflammation, immune system, wound healing, angiogenesis, antibacterial activity, etc.
- Limited clinical phenotypes after identification of the large number of functional polymorphisms and mutations in various receptors.
- Limited molecular understanding of the role in hemostasis and thrombosis of newly identified potential membrane receptors via genome-wide linkage association studies.
- Limited pharmacological translation of basal knowledge into new drugs.

Key Messages

- Receptors can be classified into categories, depending on their role in adhesion, aggregation, amplification, and stabilization.
- Hemostasis is only achieved when the majority of receptors act in concert. There is limited redundancy in receptor function.
- Polymorphisms in receptors or their expression add to large variability in platelet responses to various agonists.
- Newly discovered receptors may guide development of new antithrombotics.

Acknowledgments The CMVB is supported by the "programmafinanciering KULeuven" (PF/10/014). Financial support was given by KU Leuven project GOA09/13.

References

Abdullah NM, Kachman M, Walker A, Hawley AE, Wrobleski SK, Myers DD, Strahler JR, Andrews PC, Michailidis GC, Ramacciotti E, Henke PK, Wakefield TW (2009) Microparticle surface protein are associated with experimental venous thrombosis: a preliminary study. Clin Appl Thromb Hemost 15:201–208

Adam F, Bouton MC, Huisse MG, Jandrot-Perrus M (2003) Thrombin interaction with platelet membrane glycoprotein Ib alpha. Trends Mol Med 9:461–464

Angelillo-Scherrer A, Burnier L, Flores N, Savi P, DeMol M, Schaeffer P, Herbert JM, Lemke G, Goff SP, Matsushima GK, Earp HS, Vesin C, Hoylaerts MF, Plaisance S, Collen D, Conway EM, Wehrle-Haller B, Carmeliet P (2005) Role of Gas6 receptors in platelet signaling during thrombus stabilization and implications for antithrombotic therapy. J Clin Invest 115:237–246

Arthur JF, Dunkley S, Andrews RK (2007) Platelet glycoprotein VI-related clinical defects. Br J Haematol 139:363–372

Autschbach F, Palou E, Mechtersheimer G, Rohr C, Pirotto F, Gassler N, Otto HF, Schraven B, Gaya A (1999) Expression of the membrane protein tyrosine phosphatase CD148 in human tissues. Tissue Antigens 54:485–498

Baglia FA, Badellino KO, Li CQ, Lopez JA, Walsh PN (2002) Factor XI binding to the platelet glycoprotein Ib-IX-V complex promotes factor XI activation by thrombin. J Biol Chem 277:1662–1668

Bender M, Hofmann S, Stegner D, Chalaris A, Bosl M, Braun A, Scheller J, Rose-John S, Nieswandt B (2010) Differentially regulated GPVI ectodomain shedding by multiple platelet-expressed proteinases. Blood 116:3347–3355

Bradford HN, Pixley RA, Colman RW (2000) Human factor XII binding to the glycoprotein Ib-IX-V complex inhibits thrombin-induced platelet aggregation. J Biol Chem 275:22756–22763

Clemetson KJ, Clemetson JM (2001) Platelet collagen receptors. Thromb Haemost 86:189–197

Coller BS, Shattil SJ (2008) The GPIIb/IIIa (integrin alphaIIbbeta3) odyssey: a technology-driven saga of a receptor with twists, turns, and even a bend. Blood 112:3011–3025

Cosemans JM, Munnix IC, Wetzker R, Heller R, Jackson SP, Heemskerk JW (2006) Continuous signaling via PI3K isoforms beta and gamma is required for platelet ADP receptor function in dynamic thrombus stabilization. Blood 108:3045–3052

Cosemans JM, Iserbyt BF, Deckmyn H, Heemskerk JW (2008) Multiple ways to switch platelet integrins on and off. J Thromb Haemost 6:1253–1261

Cosemans JM, Van Kruchten R, Olieslagers S, Schurgers LJ, Verheyen FK, Munnix IC, Waltenberger J, Angelillo-Scherrer A, Hoylaerts MF, Carmeliet P, Heemskerk JW (2010) Potentiating role of Gas6 and Tyro3, Axl and Mer (TAM) receptors in human and murine platelet activation and thrombus stabilization. J Thromb Haemost 8:1797–1808

De Marco L, Mazzucato M, Masotti A, Ruggeri ZM (1994) Localization and characterization of an alpha-thrombin-binding site on platelet glycoprotein Ib alpha. J Biol Chem 269:6478–6484

Devreese K, Peerlinck K, Hoylaerts MF (2010) Thrombotic risk assessment in the antiphospholipid syndrome requires more than the quantification of lupus anticoagulants. Blood 115:870–878

Dumont B, Lasne D, Rothschild C, Bouabdelli M, Ollivier V, Oudin C, Ajzenberg N, Grandchamp B, Jandrot-Perrus M (2009) Absence of collagen-induced platelet activation caused by compound heterozygous GPVI mutations. Blood 114:1900–1903

Ellison S, Mori J, Barr AJ, Senis YA (2010) CD148 enhances platelet responsiveness to collagen by maintaining a pool of active Src family kinases. J Thromb Haemost 8:1575–1583

Fang J, Jensen ES, Boudreaux MK, Du LM, Hawkins TB, Koukouritaki SB, Cornetta K, Wilcox DA (2011) Platelet gene therapy improves hemostatic function for integrin {alpha}IIb{beta}3-deficient dogs. Proc Natl Acad Sci USA 108(23):9583–9588

Fiore M, Pillois X, Nurden P, Nurden AT, Austerlitz F (2011) Founder effect and estimation of the age of the French Gypsy mutation associated with Glanzmann thrombasthenia in Manouche families. Eur J Hum Genet 19(9):981–987

Franchini M, Montagnana M, Lippi G (2008) Clinical, laboratory and therapeutic aspects of platelet-type von Willebrand disease. Int J Lab Hematol 30:91–94

Freson K, Hashimoto H, Thys C, Wittevrongel C, Danloy S, Morita Y, Shintani N, Tomiyama Y, Vermylen J, Hoylaerts MF, Baba A, Van Geet C (2004) The pituitary adenylate cyclase-activating polypeptide is a physiological inhibitor of platelet activation. J Clin Invest 113:905–912

Gachet C (2008) P2 receptors, platelet function and pharmacological implications. Thromb Haemost 99:466–472

Ghosh A, Murugesan G, Chen K, Zhang L, Wang Q, Febbraio M, Anselmo RM, Marchant K, Barnard J, Silverstein RL (2011) Platelet CD36 surface expression levels affect functional responses to oxidized LDL and are associated with inheritance of specific genetic polymorphisms. Blood 117:6355–6366

Giomarelli B, Washington VA, Chisholm MM, Quigley L, McMahon JB, Mori T, McVicar DW (2007) Inhibition of thrombin-induced platelet aggregation using human single-chain Fv antibodies specific for TREM-like transcript-1. Thromb Haemost 97:955–963

Goschnick MW, Lau LM, Wee JL, Liu YS, Hogarth PM, Robb LM, Hickey MJ, Wright MD, Jackson DE (2006) Impaired "outside-in" integrin alphaIIbbeta3 signaling and thrombus stability in TSSC6-deficient mice. Blood 108:1911–1918

Gross S, Tilly P, Hentsch D, Vonesch JL, Fabre JE (2007) Vascular wall-produced prostaglandin E2 exacerbates arterial thrombosis and atherothrombosis through platelet EP3 receptors. J Exp Med 204:311–320

Habib A, FitzGerald GA, Maclouf J (1999) Phosphorylation of the thromboxane receptor alpha, the predominant isoform expressed in human platelets. J Biol Chem 274:2645–2651

Hermans C, Wittevrongel C, Thys C, Smethurst PA, Van Geet C, Freson K (2009) A compound heterozygous mutation in glycoprotein VI in a patient with a bleeding disorder. J Thromb Haemost 7:1356–1363

Herrera-Galeano JE, Becker DM, Wilson AF, Yanek LR, Bray P, Vaidya D, Faraday N, Becker LC (2008) A novel variant in the platelet endothelial aggregation receptor-1 gene is associated with increased platelet aggregability. Arterioscler Thromb Vasc Biol 28:1484–1490

Hirata T, Ushikubi F, Kakizuka A, Okuma M, Narumiya S (1996) Two thromboxane A2 receptor isoforms in human platelets. Opposite coupling to adenylyl cyclase with different sensitivity to Arg60 to Leu mutation. J Clin Invest 97:949–956

Hoylaerts MF, Yamamoto H, Nuyts K, Vreys I, Deckmyn H, Vermylen J (1997) von Willebrand factor binds to native collagen VI primarily via its A1 domain. Biochem J 324(Pt 1):185–191

Hu H, Hoylaerts MF (2010) The P2X1 ion channel in platelet function. Platelets 21:153–166

Hughes CE, Navarro-Nunez L, Finney BA, Mourao-Sa D, Pollitt AY, Watson SP (2010) CLEC-2 is not required for platelet aggregation at arteriolar shear. J Thromb Haemost 8:2328–2332

Ibrahim S, Tetruashvily M, Frey AJ, Wilson SJ, Stitham J, Hwa J, Smyth EM (2010) Dominant negative actions of human prostacyclin receptor variant through dimerization: implications for cardiovascular disease. Arterioscler Thromb Vasc Biol 30:1802–1809

Johnson AD, Yanek LR, Chen MH, Faraday N, Larson MG, Tofler G, Lin SJ, Kraja AT, Province MA, Yang Q, Becker DM, O'Donnell CJ, Becker LC (2010) Genome-wide meta-analyses identifies seven loci associated with platelet aggregation in response to agonists. Nat Genet 42:608–613

Jones CI, Bray S, Garner SF, Stephens J, de Bono B, Angenent WG, Bentley D, Burns P, Coffey A, Deloukas P, Earthrowl M, Farndale RW, Hoylaerts MF, Koch K, Rankin A, Rice CM, Rogers J, Samani NJ, Steward M, Walker A, Watkins NA, Akkerman JW, Dudbridge F, Goodall AH, Ouwehand WH (2009a) A functional genomics approach reveals novel quantitative trait loci associated with platelet signaling pathways. Blood 114:1405–1416

Jones CI, Garner SF, Moraes LA, Kaiser WJ, Rankin A, Ouwehand WH, Goodall AH, Gibbins JM (2009b) PECAM-1 expression and activity negatively regulate multiple platelet signaling pathways. FEBS Lett 583:3618–3624

Kasirer-Friede A, Kahn ML, Shattil SJ (2007) Platelet integrins and immunoreceptors. Immunol Rev 218:247–264

Konstantinides S, Ware J, Marchese P, Almus-Jacobs F, Loskutoff DJ, Ruggeri ZM (2006) Distinct antithrombotic consequences of platelet glycoprotein Ibalpha and VI deficiency in a mouse model of arterial thrombosis. J Thromb Haemost 4:2014–2021

Lanza F (2006) Bernard-Soulier syndrome (hemorrhagiparous thrombocytic dystrophy). Orphanet J Rare Dis 1:46

Ma H, Hara A, Xiao CY, Okada Y, Takahata O, Nakaya K, Sugimoto Y, Ichikawa A, Narumiya S, Ushikubi F (2001) Increased bleeding tendency and decreased susceptibility to thromboembolism in mice lacking the prostaglandin E receptor subtype EP(3). Circulation 104:1176–1180

Mangin P, Yap CL, Nonne C, Sturgeon SA, Goncalves I, Yuan Y, Schoenwaelder SM, Wright CE, Lanza F, Jackson SP (2006) Thrombin overcomes the thrombosis defect associated with platelet GPVI/FcRgamma deficiency. Blood 107:4346–4353

Massberg S, Gawaz M, Gruner S, Schulte V, Konrad I, Zohlnhofer D, Heinzmann U, Nieswandt B (2003) A crucial role of glycoprotein VI for platelet recruitment to the injured arterial wall in vivo. J Exp Med 197:41–49

May F, Hagedorn I, Pleines I, Bender M, Vogtle T, Eble J, Elvers M, Nieswandt B (2009) CLEC-2 is an essential platelet-activating receptor in hemostasis and thrombosis. Blood 114:3464–3472

Miura Y, Takahashi T, Jung SM, Moroi M (2002) Analysis of the interaction of platelet collagen receptor glycoprotein VI (GPVI) with collagen. A dimeric form of GPVI, but not the monomeric form, shows affinity to fibrous collagen. J Biol Chem 277:46197–46204

Morel O, Morel N, Freyssinet JM, Toti F (2008) Platelet microparticles and vascular cells interactions: a checkpoint between the haemostatic and thrombotic responses. Platelets 19:9–23

Mori J, Pearce AC, Spalton JC, Grygielska B, Eble JA, Tomlinson MG, Senis YA, Watson SP (2008) G6b-B inhibits constitutive and agonist-induced signaling by glycoprotein VI and CLEC-2. J Biol Chem 283:35419–35427

Moshfegh K, Wuillemin WA, Redondo M, Lammle B, Beer JH, Liechti-Gallati S, Meyer BJ (1999) Association of two silent polymorphisms of platelet glycoprotein Ia/IIa receptor with risk of myocardial infarction: a case-control study. Lancet 353:351–354

Nanda N, Bao M, Lin H, Clauser K, Komuves L, Quertermous T, Conley PB, Phillips DR, Hart MJ (2005) Platelet endothelial aggregation receptor 1 (PEAR1), a novel epidermal growth factor repeat-containing transmembrane receptor, participates in platelet contact-induced activation. J Biol Chem 280:24680–24689

Nergiz-Unal R, Rademakers T, Cosemans JM, Heemskerk JW (2011) CD36 as a multiple-ligand signaling receptor in atherothrombosis. Cardiovasc Hematol Agents Med Chem 9:42–55

Nurden AT (2006) Glanzmann thrombasthenia. Orphanet J Rare Dis 1:10

Nurden P, Nurden AT (2008) Congenital disorders associated with platelet dysfunctions. Thromb Haemost 99:253–263

Nuyttens BP, Thijs T, Deckmyn H, Broos K (2011) Platelet adhesion to collagen. Thromb Res 127 (Suppl 2):S26–S29

Oestreich J (2009) SCH-530348, a thrombin receptor (PAR-1) antagonist for the prevention and treatment of atherothrombosis. Curr Opin Investig Drugs 10:988–996

Orlowski E, Chand R, Yip J, Wong C, Goschnick MW, Wright MD, Ashman LK, Jackson DE (2009) A platelet tetraspanin superfamily member, CD151, is required for regulation of thrombus growth and stability in vivo. J Thromb Haemost 7:2074–2084

Oury C, Sticker E, Cornelissen H, De Vos R, Vermylen J, Hoylaerts MF (2004) ATP augments von Willebrand factor-dependent shear-induced platelet aggregation through Ca2+-calmodulin and myosin light chain kinase activation. J Biol Chem 279:26266–26273

Ozaki Y, Suzuki-Inoue K, Inoue O (2009) Novel interactions in platelet biology: CLEC-2/podoplanin and laminin/GPVI. J Thromb Haemost 7(Suppl 1):191–194

Patrono C, Garcia Rodriguez LA, Landolfi R, Baigent C (2005) Low-dose aspirin for the prevention of atherothrombosis. N Engl J Med 353:2373–2383

Paul BZ, Ashby B, Sheth SB (1998) Distribution of prostaglandin IP and EP receptor subtypes and isoforms in platelets and human umbilical artery smooth muscle cells. Br J Haematol 102:1204–1211

Philipose S, Konya V, Sreckovic I, Marsche G, Lippe IT, Peskar BA, Heinemann A, Schuligoi R (2010) The prostaglandin E2 receptor EP4 is expressed by human platelets and potently inhibits platelet aggregation and thrombus formation. Arterioscler Thromb Vasc Biol 30:2416–2423

Pincus MR, Dykes DC, Carty RP, Miller JL (1991) Conformational energy analysis of the substitution of Val for Gly 233 in a functional region of platelet GPIb alpha in platelet-type von Willebrand disease. Biochim Biophys Acta 1097:133–139

Pitchford SC, Yano H, Lever R, Riffo-Vasquez Y, Ciferri S, Rose MJ, Giannini S, Momi S, Spina D, O'Connor B, Gresele P, Page CP (2003) Platelets are essential for leukocyte recruitment in allergic inflammation. J Allergy Clin Immunol 112:109–118

Poujol C, Ware J, Nieswandt B, Nurden AT, Nurden P (2002) Absence of GPIbalpha is responsible for aberrant membrane development during megakaryocyte maturation: ultrastructural study using a transgenic model. Exp Hematol 30:352–360

Prevost N, Woulfe D, Tognolini M, Brass LF (2003) Contact-dependent signaling during the late events of platelet activation. J Thromb Haemost 1:1613–1627

Prevost N, Woulfe DS, Jiang H, Stalker TJ, Marchese P, Ruggeri ZM, Brass LF (2005) Eph kinases and ephrins support thrombus growth and stability by regulating integrin outside-in signaling in platelets. Proc Natl Acad Sci USA 102:9820–9825

Ramacciotti E, Hawley AE, Farris DM, Ballard NE, Wrobleski SK, Myers DD Jr, Henke PK, Wakefield TW (2009) Leukocyte- and platelet-derived microparticles correlate with thrombus weight and tissue factor activity in an experimental mouse model of venous thrombosis. Thromb Haemost 101:748–754

Ramacciotti E, Blackburn S, Hawley AE, Vandy F, Ballard-Lipka N, Stabler C, Baker N, Guire KE, Rectenwald JE, Henke PK, Myers DD Jr, Wakefield TW (2011) Evaluation of soluble P-Selectin as a marker for the diagnosis of deep venous thrombosis. Clin Appl Thromb Hemost 17(4):425–431

Ramakrishnan V, DeGuzman F, Bao M, Hall SW, Leung LL, Phillips DR (2001) A thrombin receptor function for platelet glycoprotein Ib-IX unmasked by cleavage of glycoprotein V. Proc Natl Acad Sci USA 98:1823–1828

Rivera J, Lozano ML, Navarro-Nunez L, Vicente V (2009) Platelet receptors and signaling in the dynamics of thrombus formation. Haematologica 94:700–711

Roberts W, Magwenzi S, Aburima A, Naseem KM (2010) Thrombospondin-1 induces platelet activation through CD36-dependent inhibition of the cAMP/protein kinase A signaling cascade. Blood 116:4297–4306

Romo GM, Dong JF, Schade AJ, Gardiner EE, Kansas GS, Li CQ, McIntire LV, Berndt MC, Lopez JA (1999) The glycoprotein Ib-IX-V complex is a platelet counterreceptor for P-selectin. J Exp Med 190:803–814

Salsmann A, Schaffner-Reckinger E, Kieffer N (2006) RGD, the Rho'd to cell spreading. Eur J Cell Biol 85:249–254

Santoro SA (1999) Platelet surface collagen receptor polymorphisms: variable receptor expression and thrombotic/hemorrhagic risk. Blood 93:3575–3577

Savage B, Almus-Jacobs F, Ruggeri ZM (1998) Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. Cell 94:657–666

Senis YA, Tomlinson MG, Ellison S, Mazharian A, Lim J, Zhao Y, Kornerup KN, Auger JM, Thomas SG, Dhanjal T, Kalia N, Zhu JW, Weiss A, Watson SP (2009) The tyrosine phosphatase CD148 is an essential positive regulator of platelet activation and thrombosis. Blood 113:4942–4954

Siljander PR, Hamaia S, Peachey AR, Slatter DA, Smethurst PA, Ouwehand WH, Knight CG, Farndale RW (2004) Integrin activation state determines selectivity for novel recognition sites in fibrillar collagens. J Biol Chem 279:47763–47772

Soeki T, Tamura Y, Shinohara H, Sakabe K, Onose Y, Fukuda N (2003) Increased soluble platelet/endothelial cell adhesion molecule-1 in the early stages of acute coronary syndromes. Int J Cardiol 90:261–268

Staron M, Wu S, Hong F, Stojanovic A, Du X, Bona R, Liu B, Li Z (2011) Heat-shock protein gp96/grp94 is an essential chaperone for the platelet glycoprotein Ib-IX-V complex. Blood 117:7136–7144

Stitham J, Stojanovic A, Hwa J (2002) Impaired receptor binding and activation associated with a human prostacyclin receptor polymorphism. J Biol Chem 277:15439–15444

Stitham J, Arehart EJ, Gleim SR, Douville KL, Hwa J (2007) Human prostacyclin receptor structure and function from naturally-occurring and synthetic mutations. Prostaglandins Other Lipid Mediat 82:95–108

Stitham J, Arehart E, Elderon L, Gleim SR, Douville K, Kasza Z, Fetalvero K, MacKenzie T, Robb J, Martin KA, Hwa J (2011) Comprehensive biochemical analysis of rare prostacyclin receptor

variants: study of association of signaling with coronary artery obstruction. J Biol Chem 286:7060–7069

Suzuki-Inoue K, Fuller GL, Garcia A, Eble JA, Pohlmann S, Inoue O, Gartner TK, Hughan SC, Pearce AC, Laing GD, Theakston RD, Schweighoffer E, Zitzmann N, Morita T, Tybulewicz VL, Ozaki Y, Watson SP (2006) A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. Blood 107:542–549

Suzuki-Inoue K, Inoue O, Ding G, Nishimura S, Hokamura K, Eto K, Kashiwagi H, Tomiyama Y, Yatomi Y, Umemura K, Shin Y, Hirashima M, Ozaki Y (2010) Essential in vivo roles of the C-type lectin receptor CLEC-2: embryonic/neonatal lethality of CLEC-2-deficient mice by blood/lymphatic misconnections and impaired thrombus formation of CLEC-2-deficient platelets. J Biol Chem 285:24494–24507

Tamburrelli C, Crescente M, Izzi B, Barisciano M, Donati MB, de Gaetano G, Cerletti C (2011) Epoprostenol inhibits human platelet-leukocyte mixed conjugate and platelet microparticle formation in whole blood. Thromb Res 128(5):446–451

Varga-Szabo D, Pleines I, Nieswandt B (2008) Cell adhesion mechanisms in platelets. Arterioscler Thromb Vasc Biol 28:403–412

Washington AV, Gibot S, Acevedo I, Gattis J, Quigley L, Feltz R, De La Mota A, Schubert RL, Gomez-Rodriguez J, Cheng J, Dutra A, Pak E, Chertov O, Rivera L, Morales J, Lubkowski J, Hunter R, Schwartzberg PL, McVicar DW (2009) TREM-like transcript-1 protects against inflammation-associated hemorrhage by facilitating platelet aggregation in mice and humans. J Clin Invest 119:1489–1501

Wilson SJ, Roche AM, Kostetskaia E, Smyth EM (2004) Dimerization of the human receptors for prostacyclin and thromboxane facilitates thromboxane receptor-mediated cAMP generation. J Biol Chem 279:53036–53047

Wilson SJ, Dowling JK, Zhao L, Carnish E, Smyth EM (2007) Regulation of thromboxane receptor trafficking through the prostacyclin receptor in vascular smooth muscle cells: role of receptor heterodimerization. Arterioscler Thromb Vasc Biol 27:290–296

Wu G, Essex DW, Meloni FJ, Takafuta T, Fujimura K, Konkle BA, Shapiro SS (1997) Human endothelial cells in culture and in vivo express on their surface all four components of the glycoprotein Ib/IX/V complex. Blood 90:2660–2669

Wu YP, Vink T, Schiphorst M, van Zanten GH, IJsseldijk MJ, de Groot PG, Sixma JJ (2000) 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 20:1661–1667

Platelet Signaling

Timothy J. Stalker, Debra K. Newman, Peisong Ma, Kenneth M. Wannemacher, and Lawrence F. Brass

Contents

T.J. Stalker • P. Ma • K.M. Wannemacher • L.F. Brass (✉)
Departments of Medicine and Pharmacology, University of Pennsylvania, Philadelphia, PA, USA
e-mail: brass@mail.med.upenn.edu

D.K. Newman
The BloodCenter of Wisconsin, Milwaukee, WI, USA

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_3,

Abstract This chapter summarizes current ideas about the intracellular signaling that drives platelet responses to vascular injury. After a brief overview of platelet activation intended to place the signaling pathways into context, the first section considers the early events of platelet activation leading up to integrin activation and platelet aggregation. The focus is on the G protein-mediated events utilized by agonists such as thrombin and ADP, and the tyrosine kinase-based signaling triggered by collagen. The second section considers the events that occur after integrin engagement, some of which are dependent on close physical contact between platelets. A third section addresses the regulatory events that help to avoid unprovoked or excessive platelet activation, after which the final section briefly considers individual variations in platelet reactivity and the role of platelet signaling in the innate immune response and embryonic development.

Keywords G proteins • G protein coupled receptors • Signal transduction • Thrombin • Collagen • ADP

1 Overview

The steps in stable platelet plug formation can be summarized in a three stage model as initiation, extension and stabilization, each of which is supported by signaling events within the platelet. *Initiation* occurs when moving platelets become tethered to and activated by collagen/von Willebrand factor (VWF) complexes within the injured vessel wall. This produces a platelet monolayer that supports the subsequent adhesion of activated platelets to each other. *Extension* occurs when additional platelets adhere to the initial monolayer and become activated. Thrombin, ADP and thromboxane A_2 (TxA_2) play an important role in this step, activating platelets via cell surface receptors coupled to heterotrimeric G proteins. Subsequent intracellular signaling activates integrin $\alpha_{IIb}\beta_3$ (glycoprotein (GP) IIb-IIIa in older literature) on the platelet surface, thereby enabling cohesion between platelets. *Stabilization* refers to the late events that help to consolidate the platelet plug and prevent premature disaggregation, in part by amplifying signaling within the platelet. Examples include outside-in signaling through integrins and contact-dependent signaling through receptors whose ligands are located on the surface of adjacent platelets. The net result is a hemostatic plug composed of activated platelets embedded within a cross-linked fibrin mesh, a structure stable enough to withstand the forces generated by flowing blood in the arterial circulation.

This three stage model arises from studies on platelets from individuals with monogenic disorders of platelet function and from mouse models in which genes of interest have been knocked out. However, recent observations suggest that the model is overly simplistic in presenting platelet accumulation after injury as a linear, unstoppable and nonreversible series of events. In fact, there is now ample evidence for spatial as well as temporal heterogeneity within a growing hemostatic plug (Yang et al. 2002; Reininger et al. 2006; Ruggeri et al. 2006; Nesbitt et al. 2009;

Bellido-Martin et al. 2011; Brass et al. 2011). This means that at any given time following injury there are fully activated platelets as well as minimally activated platelets, not all of which will inevitably become fully activated. Furthermore, with the passage of time, incorporated platelets draw closer together and many remain in stable contact with each other. This allows contact-dependent signaling to occur and produces a sheltered environment in which soluble molecules can accumulate. Thus, a more updated view of platelet activation needs to be less ordered than the three stage model, reflect differences in the extent of activation of individual platelets and incorporate the consequences of platelet:platelet interactions in a three dimensional space.

1.1 Molecular Events

Under steady state conditions, platelets circulate in an environment bordered largely by a continuous monolayer of endothelial cells. They move freely, but are quiescent. Once vascular injury has occurred, platelets are principally activated by locally exposed collagen, locally generated thrombin, platelet-derived thromboxane A_2 (TxA_2) and ADP that is either secreted from platelet dense granules or released from damaged cells. VWF serves as an essential accessory molecule. In the pre-injury state, VWF is found in plasma, within the vessel wall and in platelet α-granules. Additional VWF/collagen complexes form as collagen fibrils come into contact with plasma. Circulating erythrocytes facilitate adhesion to collagen by pushing platelets closer to the vessel wall, allowing GP Ibα on the platelet surface to be snared by the VWF A1 domain. Once captured, the drivers for platelet activation include the receptors for collagen (GP VI) and VWF (GP Ibα), thrombin (PAR1 and PAR4), ADP ($P2Y_1$ and $P2Y_{12}$) and thromboxane A_2 (TP) (Fig. 1).

In general terms, agonist-initiated platelet activation begins with the activation of one of the phospholipase C (PLC) isoforms expressed in platelets. By hydrolyzing membrane phosphatidylinositol-4,5-bisphosphate (PIP_2), PLC produces the second messenger inositol-1,4,5-trisphosphate (IP_3) needed to raise the cytosolic Ca^{2+} concentration. This leads to integrin activation via a pathway that currently includes a Ca^{2+}-dependent exchange factor (CalDAG-GEF), a switch (Rap1), an adaptor (RIAM), and proteins that interact directly with the integrin cytosolic domains (kindlin and talin) (Shattil et al. 2010). Which PLC isoform is activated depends on the agonist. Collagen activates PLCγ2 using a mechanism that depends on scaffold molecules and protein tyrosine kinases. Thrombin, ADP and TxA_2 activate PLCβ using G_q as an intermediary.

The rise in the cytosolic Ca^{2+} concentration that is triggered by most platelet agonists is essential for platelet activation. In resting platelets, the cytosolic free Ca^{2+} concentration is maintained at approximately 0.1 μM by limiting Ca^{2+} influx and pumping Ca^{2+} out of the cytosol either out across the plasma membrane or into the dense tubular system. In activated platelets, the Ca^{2+} concentration rises tenfold to >1 μM as Ca^{2+} pours back into the cytosol from two sources. The first

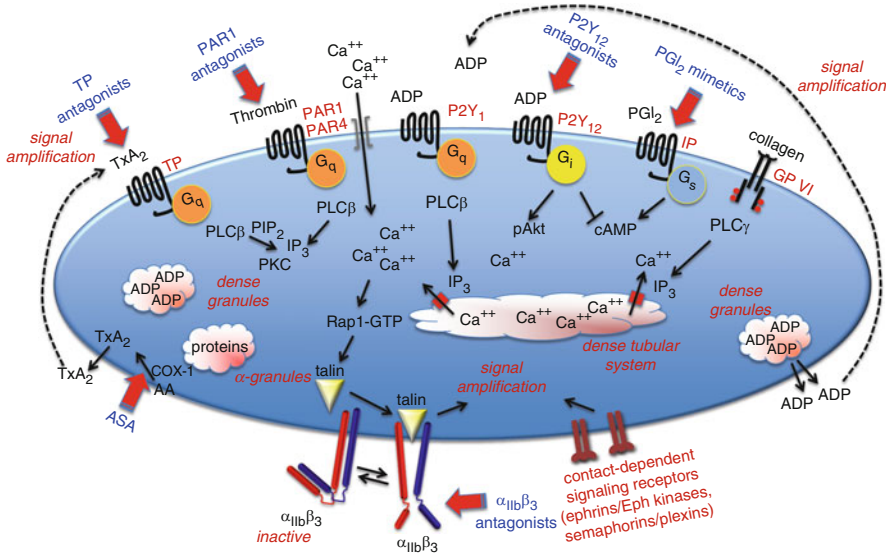


Fig. 1 An overview of some of the pathways that support platelet activation. Targets for antiplatelet agents that are currently in clinical use or in clinical trials are indicated in blue. PLC, phospholipase C; PKC, protein kinase C; IP_3, inositol-1,4,5-trisphosphate; TxA_2, thromboxane A_2; GP, glycoprotein; IP and TP, PGI_2 and TxA_2 receptors

is IP_3-mediated release of Ca^{2+} from the platelet dense tubular system (DTS). The second is Ca^{2+} influx across the platelet plasma membrane, an event triggered when depletion of the DTS Ca^{2+} pool produces a conformational change in STIM1, a protein located in the DTS membrane. This conformational change promotes the binding of STIM1 to Orai1 in the plasma membrane allowing Ca^{2+} entry (Varga-Szabo et al. 2011).

Ultimately, it is the binding of fibrinogen or another bivalent ligand to $\alpha_{IIb}\beta_3$ that enables platelets to stick to each other. Proteins that can substitute for fibrinogen include fibrin, VWF and fibronectin. Average expression levels of $\alpha_{IIb}\beta_3$ range from approximately 50,000 per cell on resting platelets to 80,000 on activated platelets. Mutations in $\alpha_{IIb}\beta_3$ that suppress its expression or function produce a bleeding disorder (Glanzmann's thrombasthenia) because platelets are unable to form stable aggregates. Antiplatelet agents such as Integrilin (eptifibatide) and ReoPro (abciximab) take advantage of this by blocking $\alpha_{IIb}\beta_3$.

2 The Early Events of Platelet Activation

Signaling within platelets begins with activation of receptors on the platelet surface by agonists such as collagen, thrombin, ADP, TxA_2 and epinephrine. With the exception of collagen, each of these works through one or more members of the G

protein coupled receptor superfamily. Some of the properties that are common to G protein coupled receptors (GPCRs) make them particularly well-suited for their tasks in platelets. Most bind their ligands with high affinity and each occupied receptor can activate multiple G proteins, amplifying the initial signal. In general, the GPCRs expressed on the platelet surface are present in low copy number, ranging from a few hundred (epinephrine receptors and $P2Y_1$ ADP receptors) to a few thousand (PAR1) copies per cell. Their duration of signaling is subject to receptor internalization, receptor desensitization and the accelerated inactivation of G proteins by members of the RGS (Regulators of G protein Signaling) family. This multiplicity of mechanisms means that platelet activation can be tightly regulated even at its earliest stages.

Although there is now growing evidence in cells other than platelets that GPCRs can signal by more than one mechanism, the events that have been described downstream of the GPCRs in platelets are mediated by heterotrimeric ($\alpha\beta\gamma$) G proteins. Human platelets express ten members of the G_s, G_i, G_q and G_{12} families. This includes at least one G_s, four G_i (G_{i1}, G_{i2}, G_{i3} and G_z), three G_q family members (G_q, G_{11} and G_{16}) and two G_{12} family members (G_{12} and G_{13}). As will be discussed below, much has been learned about the role of G proteins in platelets through studies on mice in which the genes encoding one or more forms of G_α have been disrupted.

2.1 Categorizing Critical Events

Faced with the profusion of signaling events underlying platelet activation, it can be helpful to divide them into categories. The first category begins with the activation of PLC, which cleaves membrane PIP_2 to produce IP_3 and diacylglycerol (DAG), the second messengers needed to raise the cytosolic Ca^{2+} concentration and activate some of the protein kinase C (PKC) isoforms found in platelets. As already noted, collagen activates PLCγ2 using adaptor molecules and tyrosine kinases; thrombin, ADP and TxA_2 activate PLCβ using G_q and, perhaps, G_i family members. The subsequent increase in cytosolic Ca^{2+} triggers downstream events including integrin activation and TxA_2 formation.

The second category of critical events involve monomeric G proteins in the Rho and Rac families, whose activation trigger the reorganization of the actin cytoskeleton that underlies filopodia and lamellopodia formation. Along with changes in the platelet's circumferential microtubular ring, filopodia and lamellopodia formation are the essence of platelet shape change. When platelets in suspension are activated by soluble agonists, shape change precedes platelet aggregation. Most platelet agonists can trigger shape change, the notable exception being epinephrine. The soluble agonists (thrombin, ADP and TxA_2) that trigger shape change typically act through receptors that are coupled to members of the G_q and G_{12} family.

The third category of critical events includes the suppression of cyclic adenosine monophosphate (cAMP) synthesis by adenylyl cyclase, provided the intracellular

cAMP concentration has been raised above baseline by the action of endothelium-derived PGI_2 and nitric oxide (NO). Inhibition of cAMP formation relieves a block on platelet signaling that otherwise serves to suppress inappropriate platelet activation. The agonists that inhibit cAMP formation in platelets do so by binding to receptors coupled to the G_i family members, especially G_{i2} (ADP and thrombin) and G_z (epinephrine). Suppression of adenylyl cyclase is clearly critical when cAMP levels are elevated. It is less clear that it is necessary under basal cAMP conditions.

The fourth category involves activation of the PI 3-kinase isoforms expressed in platelets, either by G_i family members (PI3Kγ) or by phosphotyrosine-dependent signaling pathways downstream of collagen receptors (PI3K$\alpha\beta\delta$). PI 3-kinases phosphorylate PI-4-P and PI-4,5-P_2 to produce PI-3,4-P_2 and PI-3,4,5-P_3. Among the best-described consequence of PI3K activation in platelets is the activation of the protein kinase, Akt. Knockout and inhibitor studies demonstrate that all three Akt isoforms are necessary for normal platelet activation (Chen et al. 2004; Woulfe et al. 2004; O'Brien et al. 2011). Knockout and inhibitor studies also show a G_i/PI3K-dependent mechanism for activating Rap1 (Woulfe et al. 2002; Yang et al. 2002) which, given the clinical utility of $P2Y_{12}$ antagonists as antiplatelet agents, seems to be a mechanism that is important for stabilizing platelet aggregates.

3 G proteins and Their Effectors in Platelets

Platelet agonists are not equally potent. Thrombin provides a robust stimulus for phosphoinositide hydrolysis and causes the largest and fastest increase in cytosolic Ca^{2+}. Collagen and ADP are more dependent on the synthesis and release of TxA_2 to achieve a maximal response. In the case of those whose receptors are GPCRs, potency is determined in part by which G proteins their receptors are coupled to, the number of receptor copies, the efficiency with which they activate the G proteins and the susceptibility of any given receptor/G protein pair to signal suppression.

3.1 G_q, Phosphoinositide Hydrolysis, Cytosolic Ca^{2+} and Integrin Activation

The critical role of G_q in platelets is reflected by the major defect in platelet activation observed in $G_{q\alpha}{}^{-/-}$ platelets (Offermanns et al. 1997). As already noted, agonists like thrombin, TxA_2 and (to a lesser extent) ADP whose receptors are coupled to G_q provide a strong stimulus for phosphoinositide hydrolysis in platelets by activating PLCβ (Fig. 2). Different isoforms of PLCβ can be activated by either G_α or $G_{\beta\gamma}$ (or both). PLCβ-activating α subunits are typically derived from

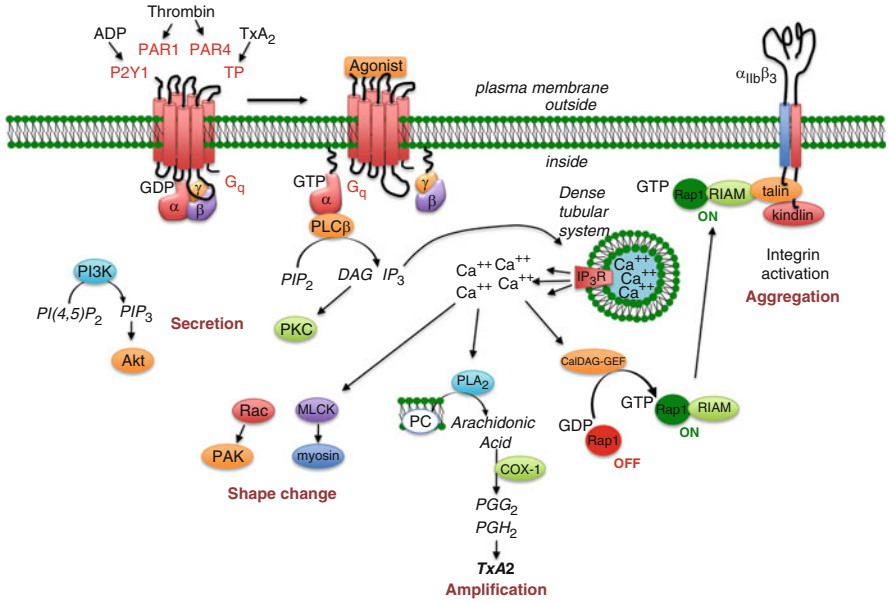


Fig. 2 G_q signaling in platelets. Agonists whose receptors are coupled to G_q are able to activate PLCβ via $G_{q\alpha}$. The potency with which the activation occurs varies with the agonist, with thrombin and TxA_2 providing a stronger stimulus for PLCβ-mediated phosphoinositide hydrolysis than ADP. Thrombin activates two G_q-coupled receptors on human platelets, PAR1 and PAR4, which differ somewhat in the kinetics of PLC activation. Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; COX-1, cyclooxygenase 1; DAG, diacylglycerol; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; IP3 R, IP_3 receptor; MLCK, myosin light chain kinase; PI3K, phosphatidylinositol 3-kinase; PAK, p21-activated kinase; PAR, protease-activated receptor; PG, prostaglandin; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; PLA_2, phospholipase A_2; PLCβ, phospholipase Cβ; TxA_2, thromboxane A_2

G_q in platelets, but in theory PLC can also be activated by $G_{\beta\gamma}$ derived from G_i family members. Whether this actually occurs in platelets remains to be demonstrated. In a recent study in which $G_{i2\alpha}$ was replaced with a gain of function mutant, there was an increase in G_{i2}-dependent activation of Akt, but no increase in the cytosolic Ca^{2+} response, which suggests that this mechanism is not a major contributor to PLC activation (Signarvic et al. 2010).

Once initiated, the rising Ca^{2+} concentration in activated platelets can trigger integrin activation via the CalDAG-GEF/Rap1/RIAM pathway (Lee et al. 2009; Shattil et al. 2010). This pathway accounts for the ability of Ca^{2+} ionophores to trigger platelet aggregation. Its biological relevance is reflected by the reduction of aggregation in platelets from Rap1 and CalDAG-GEF knockout mice (Crittenden et al. 2004; Chrzanowska-Wodnicka et al. 2005). That the CalDAG-GEF/Rap1/RIAM pathway is not the only way to accomplish integrin activation is reflected by the incomplete reduction of aggregation in $Rap1^{-/-}$ platelets and the ability of PKC

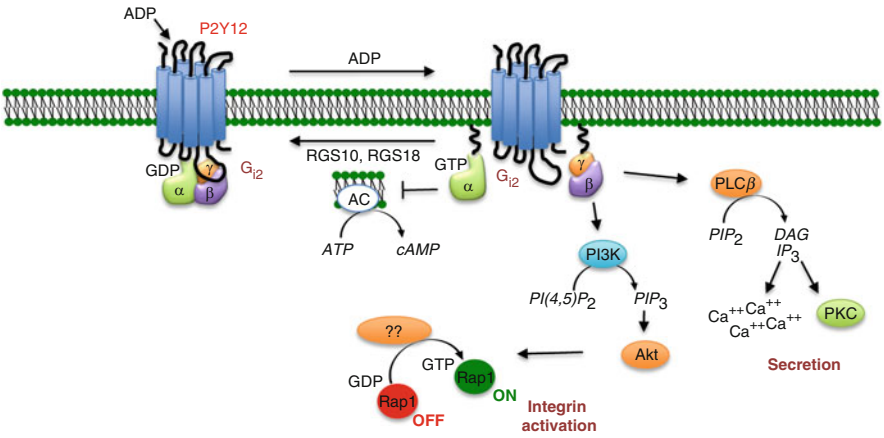


Fig. 3 G_i signaling in platelets. G_{i2} is the predominant G_i family member expressed in human platelets. In addition to inhibiting adenylyl cyclase (alleviating the repressive effects of cAMP), G_{i2} couples $P2Y_{12}$ ADP receptors to PI 3-kinase, Akt phosphorylation and Rap1B activation. Other effectors may exist as well. Abbreviations: AC, adenylyl cyclase; ADP, adenosine diphosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; PI3K, phosphatidylinositol 3-kinase; PLA_2, phospholipase A_2; PLCβ, phospholipase Cβ; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C

activators such as phorbol myristate acetate (PMA) to cause aggregation without causing an increase in cytosolic Ca^{2+} (Shattil and Brass 1987). PKC isoforms phosphorylate multiple cellular proteins on serine and threonine residues. Activation of PKC by PMA is sufficient to cause integrin activation, granule secretion and platelet aggregation in the absence of an increase in cytosolic Ca^{2+}. However, just as the ability of Ca^{2+} ionophores to activate platelets on their own does not fully account for platelet activation in vivo, neither does the response to PMA.

3.2 G_i Family Members, cAMP and PI 3-Kinase

Rising cAMP levels turn off signaling in platelets and an increase in cAMP synthesis is one of the mechanisms by which endothelial cells prevent inappropriate platelet activation. PGI_2 and NO released from endothelial cells cause $G_{s\alpha}$-mediated increases in adenylyl cyclase activity (PGI_2) and inhibit the hydrolysis of cAMP by phosphodiesterases (NO). When added to platelets in vitro, PGI_2 can cause a >10-fold increase in the platelet cAMP concentration, but even relatively small increases in cAMP levels (twofold or less) can impair thrombin responses (Keularts et al. 2000). Platelet agonists such as ADP and epinephrine inhibit PGI_2-stimulated cAMP synthesis by binding to receptors that are coupled to one or more G_i family members (Fig. 3). Disruption of the genes encoding $G_{i2\alpha}$ or $G_{z\alpha}$ causes an

increase in the basal cAMP concentration in mouse platelets (Yang et al. 2002). Conversely, loss of PGI_2 receptor (IP) expression causes a decrease in basal cAMP levels, enhances responses to agonists and predisposes mice to thrombosis in arterial injury models (Murata et al. 1997; Yang et al. 2002).

Although the G_i family members in platelets are most commonly associated with their role in the suppression of cAMP formation, this is not their only role. Neither the defect seen in platelets that are missing $G_{i\alpha}$ family members nor the response to ADP measured in the presence of $P2Y_{12}$ antagonists (Daniel et al. 1999) can be reversed solely by adding inhibitors of adenylyl cyclase. Other downstream effectors for G_i family members in platelets include PI 3-kinase, Src family members and Rap1B (Dorsam et al. 2002; Lova et al. 2002; Woulfe et al. 2002, 2004). The role of Rap1B in integrin activation was discussed in the context of G_q, Ca^{2+} and CalDAG-GEF. PI 3-kinases phosphorylate PI-4-P and PI-4,5-P_2 to produce PI-3,4-P_2 and PI-3,4,5-P_3. Human platelets express the α, β, γ and δ isoforms of PI 3-kinase, each of which is composed of a catalytic subunit and a regulatory subunit. The α, β and δ isoforms are activated by binding to phosphorylated tyrosine residues. The PI3Kγ isoform is activated by $G_{\beta\gamma}$ derived from G_i family members.

Much of what is known about the role of PI 3-kinase in platelets comes from studies with inhibitors such as wortmannin and LY294002, or from studies of gene-deleted mouse platelets (Jackson et al. 2004). Those studies have established that PI3K activation can occur downstream of both G_q and G_i family members and that effectors for PI3K in platelets include the serine/threonine kinase, Akt (Woulfe et al. 2004), and Rap1B (Jackson et al. 2005). Loss of the PI3Kγ isoform causes impaired platelet aggregation (Hirsch et al. 2001). Loss of PI3Kβ impairs Rap1B activation and thrombus formation in vivo, as do PI3Kβ-selective inhibitors (Jackson et al. 2005). Most of the Akt expressed in platelets appears to be the Akt2 isoform, but Akt1 and Akt3 (O'Brien et al. 2011) are present as well. All three appear to contribute to platelet activation, at least in mice. Deletion of the gene encoding Akt2 results in impaired thrombus formation and stability, and inhibits secretion (Woulfe et al. 2004). Loss of Akt1 inhibits platelet aggregation (Chen et al. 2004) and affects vascular integrity (Chen et al. 2005). Loss of Akt3 impairs aggregation and secretion (O'Brien et al. 2011).

3.3 G_q, G_{13} and the Reorganization of the Actin Cytoskeleton

At least two effector pathways are involved in the reorganization of the actin cytoskeleton that accompanies platelet activation: Ca^{2+}-dependent activation of myosin light chain kinase downstream of G_q family members and activation of Rho family members downstream of G_{13} (Fig. 4) (Klages et al. 1999; Offermanns 2001). Several proteins having both G_α-interacting domains and guanine nucleotide exchange factor (GEF) domains can link G_{12} family members to Rho family members, including p115RhoGEF (Fukuhara et al. 2001). With the exception of

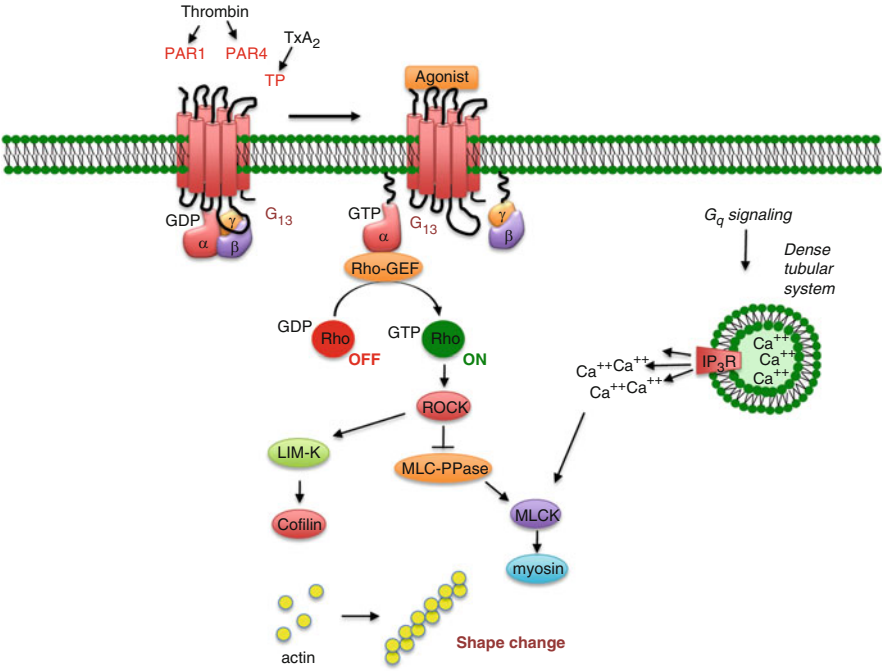


Fig. 4 $G_{12/13}$ signaling in platelets. Agonists whose receptors are coupled to the G_{12} family members expressed in platelets are able to trigger shape change, in part by Rho-dependent activation of kinases that include the Rho-activated kinase, p160 ROCK, and the downstream kinases, MLCK and LIM-K. Although G_{12} and G_{13} are both expressed, based on knockout studies, G_{13} is the dominant G_{12} family member in mouse platelets. Y27632 inhibits p160 ROCK. Abbreviations: GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; IP3 R, receptor for 1,4,5-IP_3; MLCK, myosin light chain kinase; PAR, protease-activated receptor; TxA_2, thromboxane A_2

ADP, shape change persists in platelets from mice that lack $G_{q\alpha}$ but is lost when $G_{13\alpha}$ expression is suppressed, alone or in combination with $G_{12\alpha}$ (Moers et al. 2003, 2004). These results indicate that for most platelet agonists, G_{13} signaling is essential for shape change and that the events underlying shape change are invoked by a combination of G_{13}- and G_q-dependent signals. A combination of inhibitor and genetic approaches suggest that G_{13}-dependent Rho activation leads to shape change via pathways that include the Rho-activated kinase (p160ROCK) and LIM-kinase (Klages et al. 1999; Wilde et al. 2000; Pandey et al. 2005). Activation of these kinases results in phosphorylation of myosin light chain kinase and cofilin, helping to regulate both actin filament formation and myosin. ADP, on the other hand, depends more heavily on G_q-dependent activation of PLC to produce shape change and is able to activate G_{13} only as a consequence of TxA_2 generation; hence ADP-induced shape change is lost when G_q signaling is suppressed.

3.4 Phospholipase A_2, Arachidonate, COX-1 and TxA_2 Production in Platelets

In addition to activating PLC, platelet agonists can activate phospholipase A_2 which cleaves membrane phospholipids, liberating arachidonate from the C2 position of the glycerol backbone (Fig. 2). Arachidonate can be transformed into a very large number of bioactive compounds, but TxA_2 is the key product in platelets. TxA_2 synthesis begins with the cyclooxygenase, COX-1, which forms PGG_2 and PGH_2 from arachidonate in a two step process that is inhibited by aspirin. PGH_2 is then metabolized to TxA_2 by thromboxane synthetase. Evidence suggests that phospholipase A_2 activation in platelets can occur in more than one way. It can clearly happen in response to an increase in cytosolic Ca^{2+} as the addition of a Ca^{2+} ionophore is sufficient to cause phospholipase A_2 activation. There is also evidence that MAPK pathway signaling activates phospholipase A_2, although not necessarily by direct phosphorylation of phospholipase A_2 (Börsch-Haubold et al. 1995, 1999). Once formed, TxA_2 can diffuse out of the platelet, activating nearby receptors in an autocrine or paracrine fashion before it is hydrolyzed to inactive TxB_2. Even though TxA_2 is a potent platelet activator, its half-life in aqueous solution is limited, which has implications for both its duration of action and its impact downstream from a growing thrombus. Studies on TxA_2-induced platelet activation are commonly performed with stable analogs such as U46619.

4 Signaling Responses by Selected Platelet Agonists

4.1 Platelet Activation by Collagen

Four distinct collagen receptors have been identified on human and mouse platelets. Two bind directly to collagen ($\alpha_2\beta_1$ and GP VI); the other two bind to collagen via VWF ($\alpha_{IIb}\beta_3$ and GP Ibα) (Fig. 5). Of these, GP VI is the most potent signaling receptor (Clemetson et al. 1999). The structure of the GP VI extracellular domain places it in the immunoglobulin superfamily. Its ability to generate signals rests on its constitutive association with the ITAM-containing Fc receptor γ-chain (Fc$R\gamma$). Loss of Fc$R\gamma$ affects collagen signaling in part because of loss of a necessary signaling element and in part because Fc$R\gamma$ is required for GP VI to reach the platelet surface. The $\alpha_2\beta_1$ integrin also appears to be necessary for an optimal interaction with collagen, supporting adhesion to collagen and acting as a source of integrin-dependent signaling after engagement (Keely and Parise 1996; Consonni et al. 2012). However, this appears to require an initial wave of signaling that activates $\alpha_2\beta_1$, much as the fibrinogen receptor, $\alpha_{IIb}\beta_3$ is activated by signaling within platelets. Human platelets with reduced expression of $\alpha_2\beta_1$ have impaired collagen responses, as do mouse platelets that lack β1 integrins when the ability of these platelets to bind to collagen is tested at high shear (Nieswandt et al. 2001; Kuijpers et al. 2003).

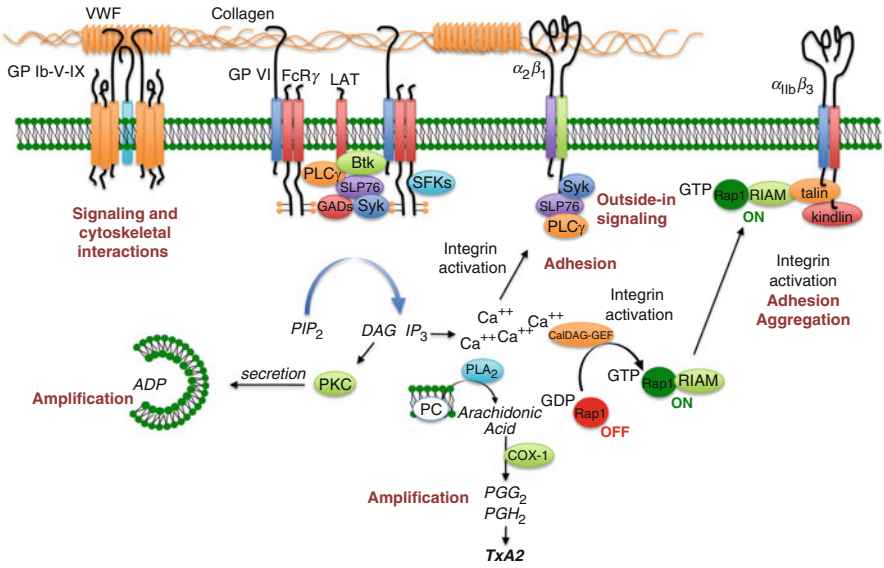


Fig. 5 Platelet activation by collagen. Platelets use several different molecular complexes to support platelet activation by collagen. These include (1) VWF-mediated binding of collagen to the GPIb-IX-V complex and integrin $\alpha_{IIb}\beta_3$, (2) a direct interaction between collagen and both the integrin $\alpha_2\beta_1$ and the GP VI/FcRγ-chain complex. Clustering of GP VI results in the phosphorylation of tyrosine residues in the FcRγ cytoplasmic domain, followed by the binding and activation of the tyrosine kinase, Syk. One consequence of Syk activation is the phosphorylation and activation of phospholipase Cγ, leading to phosphoinositide hydrolysis, secretion of ADP and the production and release of TxA_2

Signaling through GP VI can be studied in isolation with the snake venom protein, convulxin, or with synthetic "collagen-related" peptides (CRP), both of which bind to GP VI, but not to other collagen receptors. According to current models, collagen causes clustering of GP VI. Polymerization of soluble collagen and clustering of GP VI/FcRγ complexes contribute to the lag that is commonly observed when collagen is added to platelets in an aggregometer. This leads to the phosphorylation of FcRγ by Src family tyrosine kinases associated with a proline-rich domain in GP VI (Schmaier et al. 2009). Phosphorylation creates an ITAM motif that is recognized by the tandem SH2 domains of Syk. Association of Syk with the GP VI/FcRγ-chain complex activates Syk and leads to the phosphorylation and activation of $PLC\gamma_2$. Loss of Syk impairs collagen responses (Poole et al. 1997). $PLC\gamma_2$ hydrolyzes PIP_2, raising the cytosolic Ca^{2+} concentration and indirectly triggering Ca^{2+} influx across the platelet plasma membrane. The changes in the cytosolic Ca^{2+} concentration that occur when platelets adhere to collagen under flow can be visualized in real time (Nesbitt et al. 2003; Kulkarni et al. 2004).

4.2 Platelet Activation by ADP

ADP is stored in platelet dense granules and released upon platelet activation. It is also released from damaged cells at sites of vascular injury, serving as an autocrine and paracrine stimulus for recruiting additional platelets and stabilizing the hemostatic plug. Aggregation studies performed ex vivo show that all of the other platelet agonists are dependent to some extent on released ADP to elicit maximal platelet aggregation, although this dependence varies with the agonist and is dose-related. When added to platelets in vitro, ADP causes TxA_2 formation, protein phosphorylation, an increase in cytosolic Ca^{2+}, shape change, aggregation and secretion. It also inhibits cAMP formation. These responses are half-maximal at approximately 1 μM ADP. However, even at high concentrations, ADP is a comparatively weak activator of PLC, its utility as a platelet agonist resting more upon its ability to activate other pathways. Human and mouse platelets express two distinct GPCRs for ADP, denoted $P2Y_1$ and $P2Y_{12}$. $P2Y_1$ receptors couple to G_q. $P2Y_{12}$ receptors couple to G_i family members other than G_z. Optimal activation of platelets by ADP requires activation of both receptors. When $P2Y_1$ is blocked or deleted, ADP is still able to inhibit cAMP formation, but its ability to cause an increase in cytosolic Ca^{2+}, shape change and aggregation is greatly impaired, as it is in platelets from mice that lack $G_{q\alpha}$ (Offermanns et al. 1997). ${P2Y_1}^{-/-}$ mice have a minimal increase in bleeding time and show some resistance to thromboembolic mortality following injection of ADP, but no predisposition to spontaneous hemorrhage. Primary responses to platelet agonists other than ADP are unaffected and when combined with serotonin, which is a weak stimulus for PLC in platelets, ADP can still cause aggregation of ${P2Y_1}^{-/-}$ platelets. Taken together, these results show that platelet $P2Y_1$ receptors are coupled to $G_{q\alpha}$ and responsible for activation of PLC. $P2Y_1$ receptors can also activate Rac and the Rac effector, p21-activated kinase (PAK), but do not appear to be coupled to G_i family members.

As had been predicted by inhibitor studies and by the phenotype of a patient lacking functional $P2Y_{12}$, platelets from ${P2Y_{12}}^{-/-}$ mice do not aggregate normally in response to ADP (Foster 2001). ${P2Y_{12}}^{-/-}$ platelets retain $P2Y_1$-associated responses, including shape change and PLC activation, but lack the ability to inhibit cAMP formation in response to ADP. The G_i family member associated with $P2Y_{12}$ appears to be primarily G_{i2}, since platelets from ${G_{i2\alpha}}^{-/-}$ mice have an impaired response to ADP (Jantzen et al. 2001; Yang et al. 2002), while those lacking $G_{i3\alpha}$ or $G_{z\alpha}$ do not (Yang et al. 2000, 2002). Conversely, expression of a $G_{i2\alpha}$ variant that is resistant to the inhibitory effects of RGS proteins produces a gain of function in mouse platelets stimulated with ADP (Signarvic et al. 2010).

4.3 Platelet Activation by Thrombin

Platelet responses to thrombin are mediated by members of the protease-activated receptor (PAR) family of GPCRs. There are four members of this family, three of which (PAR1, PAR3 and PAR4) can be activated by thrombin. PAR1 and PAR4 are

expressed on human platelets; mouse platelets express PAR3 and PAR4. Receptor activation occurs when thrombin cleaves the extended N-terminus of each of these receptors, exposing a new N-terminus that serves as a tethered ligand (Vu et al. 1991). Synthetic peptides based on the sequence of the tethered ligand domain of PAR1 and PAR4 are able to activate the receptors, mimicking at least some of the actions of thrombin. While human PAR3 has been shown to signal in response to thrombin in transfected cells, PAR3 on mouse platelets appears to primarily serve to facilitate cleavage of PAR4 rather than to generate signals on its own (Nakanishi-Matsui et al. 2000).

Thrombin is able to activate platelets at concentrations as low as 0.1 nM. Although other platelet agonists can also cause phosphoinositide hydrolysis, none appear to be as efficiently coupled to phospholipase C as thrombin. Within seconds of the addition of thrombin, the cytosolic Ca^{2+} concentration increases tenfold, triggering downstream Ca^{2+}-dependent events, including the activation of phospholipase A_2. Thrombin also activates Rho, leading to rearrangement of the actin cytoskeleton and shape change, responses that are greatly reduced or absent in mouse platelets that lack $G_{13\alpha}$. Finally, thrombin is able to inhibit adenylyl cyclase activity in human platelets, either directly (via a G_i family member) or indirectly (via released ADP) (Barr et al. 1997; Kim et al. 2000).

4.4 Platelet Activation by Epinephrine

Compared to thrombin, epinephrine is a weak activator of human platelets. Nonetheless, there are reports of human families in which a mild bleeding disorder is associated with impaired epinephrine-induced aggregation and reduced numbers of catecholamine receptors. Epinephrine responses in platelets are mediated by α_{2A}-adrenergic receptors (Kaywin et al. 1978; Newman et al. 1978; Motulsky and Insel 1982). In both mice and humans, epinephrine is able to potentiate the effects of other agonists. Potentiation is usually attributed to the ability of epinephrine to inhibit cAMP formation, but there are clearly other effects as well. Epinephrine has no detectable direct effect on phospholipase C and does not cause shape change, although it can trigger phosphoinositide hydrolysis indirectly by stimulating TxA_2 formation. These results suggest that platelet α_{2A}-adrenergic receptors are coupled to G_i family members, but not G_q or G_{12} family members. Knockout studies show that epinephrine responses in mouse platelets are abolished when $G_{z\alpha}$ expression is abolished, while loss of $G_{i2\alpha}$ or $G_{i3\alpha}$ has no effect. G_z also appears to be responsible for the ability of epinephrine to activate Rap1B (Woulfe et al. 2002; Yang et al. 2002).

4.5 Platelet Activation by TxA_2

When added to platelets in vitro, stable thromboxane analogs such as U46619 cause shape change, aggregation, secretion, phosphoinositide hydrolysis, protein

phosphorylation and an increase in cytosolic Ca^{2+}, while having little if any direct effect on cAMP formation. Similar responses are seen when platelets are incubated with exogenous arachidonate (Gerrard and Carroll 1981). Once formed, TxA_2 can diffuse across the plasma membrane and activate other platelets (Fig. 1) (FitzGerald 1991). Like secreted ADP, release of TxA_2 amplifies the initial stimulus for platelet activation and helps to recruit additional platelets. This process is limited by the brief half-life of TxA_2 in solution, helping to confine the spread of platelet activation to the original area of injury. Loss of $G_{q\alpha}$ abolishes U46619-induced IP_3 formation and changes in cytosolic Ca^{2+}, but does not prevent shape change (Offermanns et al. 1997). Loss of $G_{13\alpha}$ abolishes TxA_2-induced shape change (Moers et al. 2003). In cells other than platelets, TPα and TPβ have been shown to couple to G_i family members (Gao et al. 2001), however, in platelets the inhibitory effects of U46619 on cAMP formation appear to be mediated by secreted ADP. These observations have previously been interpreted to mean that platelet TxA_2 receptors are coupled to G_q and $G_{12/13}$, but not to G_i family members. However, the gain of function recently observed in mouse platelets carrying an RGS protein resistant $G_{i2\alpha}$ variant suggests that this is still an open issue (Signarvic et al. 2010). $TP^{-/-}$ mice have a prolonged bleeding time. Their platelets are unable to aggregate in response to TxA_2 agonists and show delayed aggregation with collagen, presumably reflecting the role of TxA_2 in platelet responses to collagen (Thomas et al. 1998). The most compelling case for the contribution of TxA_2 signaling in human platelets comes from the successful use of aspirin as an antiplatelet agent. When added to platelets in vitro, aspirin abolishes TxA_2 generation (Fig. 1). It also blocks platelet activation by arachidonate and impairs responses to thrombin and ADP. The defect in thrombin responses appears as a shift in the dose/response curve, indicating that TxA_2 generation is supportive of platelet activation by thrombin, but not essential.

5 Some of the Later Events in Platelet Activation

As platelet activation in response to injury proceeds in vivo, previously mobile platelets come into increasingly stable contact with each other, eventually with sufficient stability and proximity that molecules on the surface of one platelet can interact directly with molecules on the surface of adjacent platelets. Although in theory this can occur anywhere within a growing hemostatic plug or thrombus, it is likely to occur most readily in the thrombus core where platelets appear to be closest together. Stable cohesive contacts between platelets require engagement of $\alpha_{IIb}\beta_3$ with one of its ligands, after which inward-directed (i.e., outside-in) signaling occurs through the integrin and through other molecules that can then engage with their counterparts *in trans*. Some of these are primarily signal-generating events that affect platelet activation and thrombus stability. Others serve primarily to help form contacts between platelets and create a protected space in which soluble molecules, including agonists, can accumulate.

5.1 Outside-In Signaling by Integrins

Activated $\alpha_{IIb}\beta_3$ bound to fibrinogen, fibrin or VWF provides the dominant cohesive strength that holds platelet aggregates together. It also contributes a further impetus for sustained platelet activation by serving as a scaffold for the assembly of signaling molecules (Prevost et al. 2007; Shattil 2009; Stegner and Nieswandt 2011). The term "outside-in signaling" refers to the effects of these molecules (Shattil and Newman 2004). Some of the protein–protein interactions that involve the cytoplasmic domains of $\alpha_{IIb}\beta_3$ help regulate integrin activation; others participate in outside-in signaling and clot retraction. Proteins that are capable of binding directly to the cytoplasmic domains of $\alpha_{IIb}\beta_3$ include β_3-endonexin, CIB1, talin, kindlin, myosin, Shc and the tyrosine kinases, Src, Fyn and Syk. Some interactions require the phosphorylation of tyrosine residues Y773 and Y785 in the β3 cytoplasmic domain by Src family members. Mutation of the corresponding tyrosine residues in mice produces platelets with impaired clot retraction and a tendency to re-bleed from tail bleeding time sites (Law et al. 1999a). Fibrinogen binding to the extracellular domain of activated $\alpha_{IIb}\beta_3$ stimulates an increase in the activity of Src family kinases and Syk (Law et al. 1999a, b). Studies of platelets from mice lacking these kinases suggest that these events are required for the initiation of outside-in signaling and for full platelet spreading, irreversible aggregation and clot retraction. There is also evidence that the ITAM-containing receptor, FcγRIIa, is the link between Src family kinase and Syk activation in human platelets activated by $\alpha_{IIb}\beta_3$ (Boylan et al. 2008).

5.2 Cell Surface Ligands and Receptors

The close proximity of one platelet to another can permit the direct binding of cell surface ligands to cell surface receptors on adjacent platelets. Several examples have been identified in platelets, including members of the ephrins and semaphorin families plus their respective receptors (Eph kinases and plexins). Ephrins are cell surface molecules attached by either a GPI anchor (ephrin A family members) or a single transmembrane domain (ephrin B family members). Eph kinases have a single transmembrane domain and a cytoplasmic domain that includes the catalytic domain and protein:protein interaction motifs. Human platelets express EphA4 and EphB1 plus their ligand, ephrinB1 (Prevost et al. 2002). Forced clustering of either EphA4 or ephrinB1 results in cytoskeletal changes leading to platelet spreading, as well as to increased adhesion to fibrinogen, Rap1B activation and granule secretion (Prevost et al. 2002, 2004). EphA4 associates with $\alpha_{IIb}\beta_3$ and Eph/ephrin interactions promote phosphorylation of the β_3 cytoplasmic domain. Conversely, blockade of Eph/ephrin interactions impairs clot retraction and causes platelet disaggregation at low agonist concentrations, resulting in impaired thrombus growth. It also inhibits platelet accumulation on collagen under flow (Prevost et al. 2002, 2005).

Semaphorin 4D (Sema4D, CD100) provides another example of a cell surface ligand involved in contact-dependent signaling in platelets. Like the ephrins, semaphorins are best known for their role in the developing nervous system, but they have also been implicated in organogenesis, vascularization and immune cell regulation. Semaphorins can either be secreted, bound to the plasma membrane via a transmembrane domain or held in place with a GPI anchor. Sema4D, which has a transmembrane domain, is expressed on the surface of both mouse and human platelets and is slowly shed from the surface of activated platelets by the metalloprotease, ADAM17 (Zhu et al. 2007). Human platelets express at least two receptors for sema4D: CD72 and a member of the plexin B family (Zhu et al. 2007). Mouse platelets express the plexin, but not CD72. Sema4D$^{-/-}$ mouse platelets have a defect in their responses to collagen and convulxin in vitro, and a reduced response to vascular injury in vivo (Zhu et al. 2007). Responses to thrombin, ADP and TxA_2 mimetics are normal. The collagen defect has been mapped to a failure to maximally activate Syk downstream of the collagen receptor, GP VI. Events in the pathway upstream of Syk occur normally in sema4D$^{-/-}$ platelets (Zhu et al. 2007; Wannemacher et al. 2010). Notably, these defects are observed only when platelets come in contact with each other and can be reversed by adding soluble recombinant sema4D. Thus, the evidence suggests that sema4D provides a contact-dependent boost in collagen-signaling. The platelets which are most likely to be activated via GP VI are those in the initial monolayer that accumulates on exposed collagen.

6 Regulators of Platelet Activation

The molecular mechanisms that drive platelet activation reflect an evolutionary compromise necessitated in part by the switch from nucleated thrombocytes to the more complex system of stationary megakaryocytes and circulating platelets found in mammals. This compromise can be thought of as establishing a threshold for platelet activation. If the threshold is too high, then platelets become useless for hemostasis. If too low, then the risk for unwarranted platelet activation rises. The set point normally found in humans and other mammals is established by balancing the intracellular signaling mechanisms that drive platelet activation forward in response to injury with regulatory mechanisms that either dampen those responses or prevent their initiation in the first place.

Regulatory events that impact platelet reactivity can be viewed as being extrinsic and intrinsic. A healthy endothelial monolayer provides a physical barrier that limits platelet activation. It also produces inhibitors of platelet activation including NO, prostacyclin (PGI_2) and the surface *ecto*-ADPase, CD39, which hydrolyzes plasma ADP that would otherwise sensitize platelets to activation by other agonists. In addition to these extrinsic regulators of platelet function, a number of intrinsic regulators of platelet activation have been identified. A few of them will be

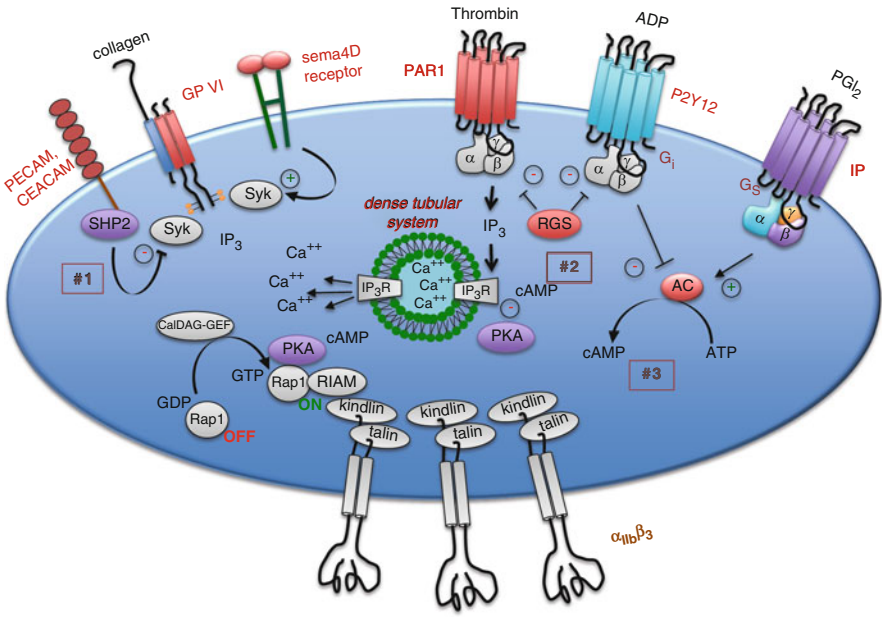


Fig. 6 Regulatory events that impact platelet activation. Although platelets are primed to respond rapidly to injury, a number of regulatory events have been described that can limit the rate and/or extent of the response. Examples shown in the figure include (1) PECAM and CEACAM, which reduce Syk activation downstream of the collagen receptor, GP VI, by recruiting the tyrosine phosphatase, SHP2, (2) RGS10 and RGS18 which shorten the duration of G protein-dependent signaling in platelets and (3) PGI_2, which stimulates cAMP formation in platelets and dampens platelet responsiveness via protein kinase A (PKA)

considered briefly here, including those that limit G protein-dependent signaling and those that impact platelet activation by collagen (Fig. 6).

6.1 Regulation of G Protein-Dependent Signaling

As already noted, most of the agonists which extend the platelet plug do so via G protein coupled receptors. The properties of these receptors make them particularly well-suited for this task. Because mechanisms exist that can limit the activation of G protein coupled receptors, platelet activation can be tightly regulated even at its earliest stages. Based largely on work in cells other than platelets, those regulatory mechanisms likely include receptor internalization, receptor phosphorylation, the binding of cytoplasmic molecules such as arrestin family members and the limits on signal duration imposed by RGS (Regulator of G protein Signaling) proteins. The role of RGS proteins in platelets is just beginning to be defined. In cells other than platelets, RGS proteins limit signaling intensity and duration by

accelerating the hydrolysis of GTP by activated G protein α subunits. At least 10 RGS proteins have been identified in platelets at the RNA level, but only RGS10 and RGS18 have been confirmed at the protein level. The evidence that RGS proteins are biologically relevant in platelets comes from studies on mice in which glycine 184 in the α subunit of G_{i2} has been replaced with serine, rendering it unable to interact with RGS proteins without impairing the ability of G_{i2} to interact with either receptors or downstream effectors. This substitution produces the predicted gain of platelet function in vitro and in vivo, even in the heterozygous state (Signarvic et al. 2010).

6.2 cAMP and Protein Kinase A

The best-known inhibitor of platelet activation is cAMP. As already noted, rising cAMP levels turn off signaling in platelets. Regulatory molecules released from endothelial cells cause $G_{s\alpha}$-mediated increases in adenylyl cyclase activity (PGI_2) and inhibit the hydrolysis of cAMP by phosphodiesterases (NO). Deletion of the genes encoding either $G_{i2\alpha}$ or $G_{z\alpha}$ causes an increase in the basal cAMP concentration in mouse platelets (Yang et al. 2002). cAMP phosphodiesterase inhibitors such as dipyridamole act as anti-platelet agents by raising cAMP levels. Conversely, loss of PGI_2 receptor (IP) expression in mice causes a decrease in basal cAMP levels, enhances responses to agonists, and predisposes mice to thrombosis in arterial injury models (Murata et al. 1997; Yang et al. 2002). Despite ample evidence that cAMP inhibits platelet activation, the mechanisms by which it does this are not fully defined. cAMP-dependent protein kinase A (PKA) is thought to be essential, but other mechanisms may be involved as well. Numerous substrates for the kinase have been described, including some G protein coupled receptors, IP_3 receptors, GP Ibβ, vasodilator-stimulated phosphoprotein (VASP) and Rap1, but it is still not clear whether a single substrate accounts for most of the effect or whether it is the accumulated effect of phosphorylation of many substrates.

6.3 Adhesion/Junction Receptors Contribute to Contact-Dependent Signaling

In addition to amplifying platelet activation, contact-dependent signaling can also help to limit thrombus growth and stability. Examples of this phenomenon include, PECAM-1, CEACAM1 and members of the CTX family of adhesion molecules. Knockouts of any of these in mice produce a gain of function, rather than a loss of function phenotype. Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a type-1 transmembrane protein with six extracellular domains, the most distal of which can form homophilic interactions in *trans* (Newman and Newman 2003).

The cytoplasmic domain contains two immunoreceptor tyrosine inhibitory motifs (ITIMs) that can bind the tyrosine phosphatase, SHP-2 (Jackson et al. 1997). PECAM1-deficient platelets exhibit enhanced responses to collagen in vitro and in vivo, consistent with a model in which PECAM-1 localizes SHP-2 to its substrates, including the GP VI signaling complex, thus providing a brake and preventing excessive platelet activation and thrombus growth (Patil et al. 2001; Falati et al. 2006; Moraes et al. 2010).

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is a second ITIM family member expressed on the platelet surface that can form homophilic and heterophilic interactions with other CEACAM superfamily members. In contrast to PECAM-1, the CEACAM1 ITIMs prefer SHP-1 over SHP-2, although either can become bound. The CEACAM1 knockout, like the PECAM-1 knockout produces a gain of function, showing increased platelet activation in vitro in response to collagen and increased thrombus formation in a $FeCl_3$ injury model (Wong et al. 2009). Thus, at least on the basis of the knockout results, CEACAM1 also appears to be a negative regulator of GP VI signaling.

7 Concluding Thoughts

This chapter represents our collective effort to summarize the efforts of a small, but passionate community of investigators who have worked to understand the molecular basis for platelet activation in vivo. In writing the chapter, we have focused on pathways, but have attempted to place those pathways in the context of observable events. The subject of platelet activation remains a work in progress and we have tried to incorporate perspectives that have been gained recently. We would like to close by pointing out some of the blank places on the platelet signaling map and by briefly reflecting on what might be coming next.

One of the biggest advances in the platelet field over the past 10 years has come from studies on how platelets are made. The process of proplatelet formation is described elsewhere in this volume. From the perspective of signal transduction and platelet response mechanisms, there are large unanswered questions about how a developing platelet at the tip of a proplatelet extension becomes equipped with the necessary molecular toolkit to not only drive a response to injury, but also to regulate that response to make it safe. Might some platelets be endowed with greater adhesive capabilities and be best at forming the initial monolayer on exposed collagen fibrils? Might others be particularly well suited for cohesive (platelet:platelet) interactions, either because of their complement of integrins or their density of receptors for soluble agonists such as ADP, thrombin and TxA_2? Heterogeneity among platelet populations within a single individual could easily extend to heterogeneity among individuals, perhaps contributing to risks for cardiovascular and cerebrovascular compromise (Bray 2007; Bray et al. 2007a, b; Watkins et al. 2009; Johnson et al. 2010; Kunicki and Nugent 2010; Musunuru et al. 2010). Finally, platelets contain a broad representation of megakaryocyte RNA

(Rowley et al. 2011), although not all of the proteins present in platelets are accompanied by the corresponding message (Cecchetti et al. 2011). Platelets also express the synthetic machinery needed to produce proteins (Weyrich et al. 1998; Denis et al. 2005; Schwertz et al. 2012). How do the changes that occur when platelets age in the circulation affect platelet function and how might the protein synthetic capability within the platelets help to withstand those changes or reconfigure the platelet during the hemostatic response?

A second source of recent energy in the platelet community has been the identification of new roles for platelets beyond those associated with the hemostatic response to injury. These include roles in embryonic development, innate immunity and oncogenesis. How do each of these roles draw on the molecular toolkit for platelet activation? Although the full answer to that question is not yet known, there appears to be only a partial overlap. Thus, for example, the separation of lymphatics from the vasculature depends in part on signaling pathways that are identical to those that drive platelet responses to collagen downstream of GP VI. However, instead of GP VI, the initiation of those signaling events involves a separate receptor, CLEC-2 (Hughes et al. 2010; Suzuki-Inoue et al. 2010). Similarly, platelet involvement in innate immunity appears to be driven in part by TLRs (Toll like receptors), molecules that have not appeared elsewhere in this chapter, but which are receiving increased attention for their role in platelets (Semple et al. 2011).

Knowledge Gaps

- Although many of the critical signaling pathways in platelets have been mapped, uncertainties remain about the complete mechanisms for activating $\alpha_{IIb}\beta_3$ and causing granule exocytosis.
- Gaps also remain in understanding how platelet activation is regulated so that an optimal response to local injury can be achieved without excessive accumulation of platelets.
- Platelets are generally treated as if they are all the same in each individual. However, it is not yet clear whether there are meaningful differences among platelets that optimize their ability to perform specific functions.
- Conversely, except in cases of well-defined monogenic disorders, it is not yet clear whether the differences observed in platelet aggregation among different individual donors necessarily have a definable molecular basis and a meaningful clinical impact.
- Finally, this chapter focuses on the hemostatic response to vascular injury. Uncertainty remains about the activation mechanisms that underlie pathological platelet activation. Some of them are undoubtedly the same as those employed in the hemostatic response. Others are proving not to be.

Key Messages

- Platelet activation during the response to vascular injury is a tightly coordinated process in which platelets are exposed to multiple activators and inhibitors, producing a response that is the net effect of all of these inputs.
- The most commonly encountered (and best understood) platelet agonists in vivo are thrombin, collagen, ADP, TxA_2 and epinephrine. With the exception of collagen, each of these agonists activates one or more G protein coupled receptor and signals through one or more classes of heterotrimeric G proteins. Many of these receptors have proved to prime targets for antiplatelet drugs, including the $P2Y_{12}$ and PAR1 antagonists as well as aspirin, which inhibits TxA_2 formation in platelets.
- Signal transduction during platelet activation is typically described one agonist and one pathway at a time, but it is in reality a signaling network in which different pathways can reinforce or oppose each other. Each pathway is also subject to the restraining effects of one or more inhibitors, including cAMP, RGS proteins and protein phosphatases, not all of which are fully understood.
- Once platelets are turned on, intracellular signaling that depends on a rise in cytosolic Ca^{2+} triggers a conformational change in the extracellular domain of $\alpha_{IIb}\beta_3$ (GP IIb-IIIa), exposing the fibrinogen (and fibrin) binding site on the integrin and allowing it mediate platelet aggregation.
- The development of integrin-mediated contacts between platelets makes possible a secondary wave of contact-dependent signaling that amplifies platelet accumulation and promotes formation of a stable thrombus.
- Platelet aggregation also promotes granule secretion and the release of molecules into the local surroundings that support the recruitment of additional platelets, but can also affect angiogenesis, the recruitment of leukocytes and wound healing.

References

Barr AJ, Brass LF, Manning DR (1997) Reconstitution of receptors and GTP-binding regulatory proteins (G proteins) in Sf9 cells: a direct evaluation of selectivity in receptor-G protein coupling. J Biol Chem 272:2223–2229

Bellido-Martin L, Chen V, Jasuja R, Furie B, Furie BC (2011) Imaging fibrin formation and platelet and endothelial cell activation in vivo. Thromb Haemost 105:776–782. doi:10.1160/TH10-12-0771

Börsch-Haubold AG, Kramer RM, Watson SP (1995) Cytosolic phospholipase A2 is phosphorylated in collagen- and thrombin-stimulated human platelets independent of protein kinase C and mitogen-activated protein kinase. J Biol Chem 270:25885–25892

Börsch-Haubold AG, Ghomashchi F, Pasquet S, Goedert M, Cohen P, Gelb MH, Watson SP (1999) Phosphorylation of cytosolic phospholipase A2 in platelets is mediated by multiple stress-activated protein kinase pathways. Eur J Biochem 265:195–203

Boylan B, Gao C, Rathore V, Gill JC, Newman DK, Newman PJ (2008) Identification of FcgammaRIIa as the ITAM-bearing receptor mediating alphaIIbbeta3 outside-in integrin signaling in human platelets. Blood 112:2780–2786. doi:10.1182/blood-2008-02-142125

Brass LF, Wannemacher KM, Ma P, Stalker TJ (2011) Regulating thrombus growth and stability to achieve an optimal response to injury. J Thromb Haemost 9(Suppl 1):66–75. doi:10.1111/j.1538-7836.2011.04364.x

Bray PF (2007) Platelet hyperreactivity: predictive and intrinsic properties. Hematol Oncol Clin North Am 21:633–645. doi:S0889-8588(07)00071-8 [pii] 10.1016/j.hoc.2007.06.002, v–vi

Bray PF, Howard TD, Vittinghoff E, Sane DC, Herrington DM (2007a) Effect of genetic variations in platelet glycoproteins Ibalpha and VI on the risk for coronary heart disease events in postmenopausal women taking hormone therapy. Blood 109:1862–1869. doi:blood-2006-03-013151 [pii] 10.1182/blood-2006-03-013151

Bray PF, Mathias RA, Faraday N et al (2007b) Heritability of platelet function in families with premature coronary artery disease. J Thromb Haemost 5:1617–1623. doi:JTH02618 [pii] 10.1111/j.1538-7836.2007.02618.x

Cecchetti L, Tolley ND, Michetti N, Bury L, Weyrich AS, Gresele P (2011) Megakaryocytes differentially sort mRNAs for matrix metalloproteinases and their inhibitors into platelets: a mechanism for regulating synthetic events. Blood 118:1903–1911. doi:10.1182/blood-2010-12-324517

Chen J, De S, Damron DS, Chen WS, Hay N, Byzova TV (2004) Impaired platelet responses to thrombin and collagen in AKT-1-deficient mice. Blood 104:1703–1710

Chen J, Somanath PR, Razorenova O, Chen WS, Hay N, Bornstein P, Byzova TV (2005) Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. Nat Med 11:1188–1196

Chrzanowska-Wodnicka M, Smyth SS, Schoenwaelder SM, Fischer TH, White GC 2nd (2005) Rap1b is required for normal platelet function and hemostasis in mice. J Clin Invest 115:680–687

Clemetson JM, Polgar J, Magnenat E, Wells TNC, Clemetson KJ (1999) The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcalphaR and the natural killer receptors. J Biol Chem 274:29019–29024

Consonni A, Cipolla L, Guidetti G et al (2012) Role and regulation of phosphatidylinositol 3-kinase beta in platelet integrin alpha2beta1 signaling. Blood 119:847–856. doi:10.1182/blood-2011-07-364992

Crittenden JR, Bergmeier W, Zhang Y et al (2004) CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation. Nat Med 10:982–986

Daniel JL, Dangelmaier C, Jin JG, Kim YB, Kunapuli SP (1999) Role of intracellular signaling events in ADP-induced platelet aggregation. Thromb Haemost 82:1322–1326

Denis MM, Tolley ND, Bunting M et al (2005) Escaping the nuclear confines: signal-dependent pre-mRNA splicing in anucleate platelets. Cell 122:379–391. doi:10.1016/j.cell.2005.06.015

Dorsam RT, Kim S, Jin JG, Kunapuli SP (2002) Coordinated signaling through both G12/13 and Gi pathways is sufficient to activate GPIIb/IIIa in human platelets. J Biol Chem 277:17948–17941

Falati S, Patil S, Gross PL et al (2006) Platelet PECAM-1 inhibits thrombus formation in vivo. Blood 107:535–541. doi:2005-04-1512 [pii] 10.1182/blood-2005-04-1512

FitzGerald GA (1991) Mechanisms of platelet activation: thromboxane A2 as an amplifying signal for other agonists. Am J Cardiol 68:11B–15B

Foster CJ (2001) Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine drugs using P2Yac-null mice. J Clin Invest 107:1591–1598

Fukuhara S, Chikumi H, Gutkind JS (2001) RGS-containing RhoGEFs: the missing link between transforming G proteins and Rho? Oncogene 20:1661–1668

Gao Y, Tang S, Zhou S, Ware JA (2001) The thromboxane A2 receptor activates mitogen-activated protein kinase via protein kinase C-dependent Gi coupling and Src-dependent phosphorylation of the epidermal growth factor receptor. J Pharmacol Exp Ther 296:426–433

Gerrard JM, Carroll RC (1981) Stimulation of protein phosphorylation by arachidonic acid and endoperoxide analog. Prostaglandins 22:81–94

Hirsch E, Bosco O, Tropel P et al (2001) Resistance to thromboembolism in PI3Kgamma-deficient mice. FASEB J 15:NIL307–NIL326

Hughes CE, Navarro-Nunez L, Finney BA, Mourao-Sa D, Pollitt AY, Watson SP (2010) CLEC-2 is not required for platelet aggregation at arteriolar shear. J Thromb Haemost 8:2328–2332. doi:10.1111/j.1538-7836.2010.04006.x

Jackson DE, Kupcho KR, Newman PJ (1997) Characterization of phosphotyrosine binding motifs in the cytoplasmic domain of platelet/endothelial cell adhesion molecule-1 (PECAM-1) that are required for the cellular association and activation of the protein-tyrosine phosphatase, SHP-2. J Biol Chem 272:24868–24875

Jackson SP, Yap CL, Anderson KE (2004) Phosphoinositide 3-kinases and the regulation of platelet function. Biochem Soc Trans 32:387–392

Jackson SP, Schoenwaelder SM, Goncalves I et al (2005) PI 3-kinase p110beta: a new target for antithrombotic therapy. Nat Med 11:507–514

Jantzen H-M, Milstone DS, Gousset L, Conley PB, Mortensen RM (2001) Impaired activation of murine platelets lacking Galphai2. J Clin Invest 108:477–483

Johnson AD, Yanek LR, Chen MH et al (2010) Genome-wide meta-analyses identifies seven loci associated with platelet aggregation in response to agonists. Nat Genet 42:608–613. doi:10.1038/ng.604

Kaywin P, McDonough M, Insel PA, Shattil SJ (1978) Platelet function in essential thrombocythemia: decreased epinephrine responsivenesss associated with a deficiency of platelet alpha-adrenergic receptors. N Engl J Med 299:505–509

Keely PJ, Parise LV (1996) The alpha2beta1 integrin is a necessary co-receptor for collagen-induced activation of Syk and the subsequent phosphorylation of phospholipase Cgamma2 in platelets. J Biol Chem 271:26668–26676

Keularts IMLW, Van Gorp RMA, Feijge MAH, Vuist WMJ, Heemskerk JWM (2000) alpha2A-adrenergic receptor stimulation potentiates calcium release in platelets by modulating cAMP levels. J Biol Chem 275:1763–1772

Kim S, Quinton TM, Cattaneo M, Kunapuli SP (2000) Evidence for diverse signal transduction pathways in thrombin receptor activating peptide (SFLLRN) and other agonist-induced fibrinogen receptor activation in human platelets. Blood 96:242a–240

Klages B, Brandt U, Simon MI, Schultz G, Offermanns S (1999) Activation of G12/G13 results in shape change and Rho/Rho-kinase-mediated myosin light chain phosphorylation in mouse platelets. J Cell Biol 144:745–754

Kuijpers MJ, Schulte V, Bergmeier W et al (2003) Complementary roles of glycoprotein VI and alpha2beta1 integrin in collagen-induced thrombus formation in flowing whole blood ex vivo. FASEB J 17:685–687

Kulkarni S, Nesbitt WS, Dopheide SM, Hughan SC, Harper IS, Jackson SP (2004) Techniques to examine platelet adhesive interactions under flow. Methods Mol Biol 272:165–186

Kunicki TJ, Nugent DJ (2010) The genetics of normal platelet reactivity. Blood 116:2627–2634. doi:10.1182/blood-2010-04-262048

Law DA, DeGuzman FR, Heiser P, Ministri-Madrid K, Killeen N, Phillips DR (1999a) Integrin cytoplasmic tyrosine motif is required for outside-in alphaIIbbeta3 signalling and platelet function. Nature 401:808–811

Law DA, Nannizzi-Alaimo L, Ministri K et al (1999b) Genetic and pharmacological analyses of Syk function in alphaIIbbeta3 signaling in platelets. Blood 93:2645–2652

Lee HS, Lim CJ, Puzon-McLaughlin W, Shattil SJ, Ginsberg MH (2009) RIAM activates integrins by linking talin to ras GTPase membrane-targeting sequences. J Biol Chem 284:5119–5127. doi:M807117200 [pii] 10.1074/jbc.M807117200

Lova P, Paganini S, Sinigaglia F, Balduini C, Torti M (2002) A Gi-dependent pathway is required for activation of the small GTPase Rap1B in human platelets. J Biol Chem 277:12009–12015
Moers A, Nieswandt B, Massberg S et al (2003) G13 is an essential mediator of platelet activation in hemostasis and thrombosis. Nat Med 9:1418–1422
Moers A, Wettschureck N, Gruner S, Nieswandt B, Offermanns S (2004) Unresponsiveness of platelets lacking both Galpha(q) and Galpha(13). Implications for collagen-induced platelet activation. J Biol Chem 279:45354–45359
Moraes LA, Barrett NE, Jones CI et al (2010) PECAM-1 regulates collagen-stimulated platelet function by modulating the association of PI3 Kinase with Gab1 and LAT. J Thromb Haemost 8:2530–2541. doi:JTH4025 [pii] 10.1111/j.1538-7836.2010.04025.x
Motulsky HJ, Insel PA (1982) [3 H]Dihydroergocryptine binding to alpha-adrenergic receptors of human platelets. A reassessment using the selective radioligands [3 H]prazosin, [3 H]yohimbine, and [3 H]rauwolscine. Biochem Pharmacol 31:2591–2597
Murata T, Ushikubi F, Matsuoka T et al (1997) Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. Nature 388:678–682
Musunuru K, Post WS, Herzog W et al (2010) Association of single nucleotide polymorphisms on chromosome 9p21.3 with platelet reactivity: a potential mechanism for increased vascular disease. Circ Cardiovasc Genet 3:445–453. doi:10.1161/CIRCGENETICS.109.923508
Nakanishi-Matsui M, Zheng YW, Sulciner DJ, Weiss EJ, Ludeman MJ, Coughlin SR (2000) PAR3 is a cofactor for PAR4 activation by thrombin. Nature 404:609–610
Nesbitt WS, Giuliano S, Kulkarni S, Dopheide SM, Harper IS, Jackson SP (2003) Intercellular calcium communication regulates platelet aggregation and thrombus growth. J Cell Biol 160:1151–1161
Nesbitt WS, Westein E, Tovar-Lopez FJ et al (2009) A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. Nat Med 15:665–673. doi:nm.1955 [pii] 10.1038/nm.1955
Newman PJ, Newman DK (2003) Signal transduction pathways mediated by PECAM-1: new roles for an old molecule in platelet and vascular cell biology. Arterioscler Thromb Vasc Biol 23:953–964
Newman KD, Williams LT, Bishopric NH, Lefkowitz RJ (1978) Identification of alpha-adrenergic receptors in human platelets by 3 H-dihydroergocryptine binding. J Clin Invest 61:395–402
Nieswandt B, Brakebusch C, Bergmeier W et al (2001) Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. EMBO J 20:2120–2130
O'Brien KA, Stojanovic-Terpo A, Hay N, Du X (2011) An important role for Akt3 in platelet activation and thrombosis. Blood 118:4215–4223. doi:10.1182/blood-2010-12-323204
Offermanns S (2001) In vivo functions of heterotrimeric G-proteins: studies in Galpha-deficient mice. Oncogene 20:1635–1642
Offermanns S, Toombs CF, Hu YH, Simon MI (1997) Defective platelet activation in Galphaq-deficient mice. Nature 389:183–186
Pandey D, Goyal P, Bamburg JR, Siess W (2005) Regulation of LIM-kinase 1 and cofilin in thrombin-stimulated platelets. Blood 107:575–583
Patil S, Newman DK, Newman PJ (2001) Platelet endothelial cell adhesion molecule-1 serves as an inhibitory receptor that modulates platelet responses to collagen. Blood 97:1727–1732
Poole A, Gibbins JM, Turner M et al (1997) The Fc receptor gamma-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. EMBO J 16:2333–2341
Prevost N, Woulfe D, Tanaka T, Brass LF (2002) Interactions between Eph kinases and ephrins provide a mechanism to support platelet aggregation once cell-to-cell contact has occurred. Proc Natl Acad Sci USA 99:9219–9224. doi:10.1073/pnas.142053899 142053899 [pii]
Prevost N, Woulfe DS, Tognolini M et al (2004) Signaling by ephrinB1 and Eph kinases in platelets promotes Rap1 activation, platelet adhesion, and aggregation via effector pathways that do not require phosphorylation of ephrinB1. Blood 103:1348–1355
Prevost N, Woulfe DS, Jiang H, Stalker TJ, Marchese P, Ruggeri ZM, Brass LF (2005) Eph kinases and ephrins support thrombus growth and stability by regulating integrin outside-in

signaling in platelets. Proc Natl Acad Sci USA 102:9820–9825. doi:0404065102 [pii] 10.1073/pnas.0404065102
Prevost N, Kato H, Bodin L, Shattil SJ (2007) Platelet integrin adhesive functions and signaling. Methods Enzymol 426:103–115. doi:S0076-6879(07)26006-9 [pii] 10.1016/S0076-6879(07)26006-9
Reininger AJ, Heijnen HF, Schumann H, Specht HM, Schramm W, Ruggeri ZM (2006) Mechanism of platelet adhesion to von Willebrand factor and microparticle formation under high shear stress. Blood 107:3537–3545. doi:2005-02-0618 [pii] 10.1182/blood-2005-02-0618
Rowley JW, Oler A, Tolley ND et al (2011) Genome wide RNA-seq analysis of human and mouse platelet transcriptomes. Blood. doi:10.1182/blood-2011-03-339705
Ruggeri ZM, Orje JN, Habermann R, Federici AB, Reininger AJ (2006) Activation-independent platelet adhesion and aggregation under elevated shear stress. Blood 108:1903–1910. doi:blood-2006-04-011551 [pii] 10.1182/blood-2006-04-011551
Schmaier AA, Zou Z, Kazlauskas A et al (2009) Molecular priming of Lyn by GPVI enables an immune receptor to adopt a hemostatic role. Proc Natl Acad Sci USA 106:21167–21172. doi:0906436106 [pii] 10.1073/pnas.0906436106
Schwertz H, Rowley JW, Tolley ND, Campbell RA, Weyrich AS (2012) Assessing protein synthesis by platelets. Methods Mol Biol 788:141–153. doi:10.1007/978-1-61779-307-3_11
Semple JW, Italiano JE Jr, Freedman J (2011) Platelets and the immune continuum. Nat Rev Immunol 11:264–274. doi:10.1038/nri2956
Shattil SJ (2009) The beta3 integrin cytoplasmic tail: protein scaffold and control freak. J Thromb Haemost 7(Suppl 1):210–213. doi:JTH3397 [pii] 10.1111/j.1538-7836.2009.03397.x
Shattil SJ, Brass LF (1987) Induction of the fibrinogen receptor on human platelets by intracellular mediators. J Biol Chem 262:992–1000
Shattil SJ, Newman PJ (2004) Integrins: dynamic scaffolds for adhesion and signaling in platelets. Blood 104:1606–1615
Shattil SJ, Kim C, Ginsberg MH (2010) The final steps of integrin activation: the end game. Nat Rev Mol Cell Biol 11:288–300. doi:nrm2871 [pii] 10.1038/nrm2871
Signarvic RS, Cierniewska A, Stalker TJ et al (2010) RGS/Gi2alpha interactions modulate platelet accumulation and thrombus formation at sites of vascular injury. Blood 116:6092–6100. doi:10.1182/blood-2010-05-283846
Stegner D, Nieswandt B (2011) Platelet receptor signaling in thrombus formation. J Mol Med 89:109–121. doi:10.1007/s00109-010-0691-5
Suzuki-Inoue K, Inoue O, Ding G et al (2010) Essential in vivo roles of the C-type lectin receptor CLEC-2: embryonic/neonatal lethality of CLEC-2-deficient mice by blood/lymphatic misconnections and impaired thrombus formation of CLEC-2-deficient platelets. J Biol Chem 285:24494–24507. doi:10.1074/jbc.M110.130575
Thomas DW, Mannon RB, Mannon PJ et al (1998) Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2. J Clin Invest 102:1994–2001
Varga-Szabo D, Braun A, Nieswandt B (2011) STIM and Orai in platelet function. Cell Calcium 50:270–278. doi:10.1016/j.ceca.2011.04.002
Vu T-KH, Hung DT, Wheaton VI, Coughlin SR (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 64:1057–1068
Wannemacher KM, Zhu L, Jiang H et al (2010) Diminished contact-dependent reinforcement of Syk activation underlies impaired thrombus growth in mice lacking Semaphorin 4D. Blood 116:5707–5715. doi:blood-2010-04-279943 [pii] 10.1182/blood-2010-04-279943
Watkins NA, Gusnanto A, de Bono B et al (2009) A HaemAtlas: characterizing gene expression in differentiated human blood cells. Blood 113:e1–9. doi:blood-2008-06-162958 [pii] 10.1182/blood-2008-06-162958
Weyrich AS, Dixon DA, Pabla R, Elstad MR, McIntyre TM, Prescott SM, Zimmerman GA (1998) Signal-dependent translation of a regulatory protein, Bcl-3, in activated human platelets. Proc Natl Acad Sci USA 95:5556–5561

Wilde JI, Retzer M, Siess W, Watson SP (2000) ADP-induced platelet shape change: an investigation of the signalling pathways involved and their dependence on the method of platelet preparation. Platelets 11:286–295

Wong C, Liu Y, Yip J et al (2009) CEACAM1 negatively regulates platelet-collagen interactions and thrombus growth in vitro and in vivo. Blood 113:1818–1828. doi:blood-2008-06-165043 [pii] 10.1182/blood-2008-06-165043

Woulfe D, Jiang H, Mortensen R, Yang J, Brass LF (2002) Activation of Rap1B by Gi family members in platelets. J Biol Chem 277:23382–23390

Woulfe D, Jiang H, Morgans A, Monks R, Birnbaum M, Brass LF (2004) Defects in secretion, aggregation, and thrombus formation in platelets from mice lacking Akt2. J Clin Invest 113:441–450

Yang J, Wu J, Kowalska MA et al (2000) Loss of signaling through the G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs. Proc Natl Acad Sci USA 97:9984–9989

Yang J, Wu J, Jiang H et al (2002) Signaling through Gi family members in platelets - Redundancy and specificity in the regulation of adenylyl cyclase and other effectors. J Biol Chem 277:46035–46042

Zhu L, Bergmeier W, Wu J et al (2007) Regulated surface expression and shedding support a dual role for semaphorin 4D in platelet responses to vascular injury. Proc Natl Acad Sci USA 104:1621–1626

Platelet Interaction with the Vessel Wall

Philip G. de Groot, Rolf T. Urbanus, and Mark Roest

Contents

Abstract Platelets have attracted a growing interest among basic scientists and clinicians, as they have been shown to play an important role in many physiological and pathophysiological conditions. Beyond hemostasis, platelets participate in wound healing, inflammation, infectious diseases, maintenance of the endothelial barrier function, angiogenesis, and tumor metastasis. Over the last 50 years enormous progress has been made in our understanding of the role of platelets in hemostasis. Platelets circulate in blood in a resting state, but they are able to react immediately upon a vessel wall injury by adhering to the exposed collagen, followed by platelet–platelet interaction to form a plug that effectively seals the injured vessel wall to prevent excessive blood loss. Comparable events will take place on a rupturing atherosclerotic plaque, which may result in a platelet-rich thrombus. This chapter will address the molecular basis of platelet adhesion and aggregation, the regulation of platelet function and the interaction of primary and secondary hemostasis.

P.G. de Groot (✉) • R.T. Urbanus • M. Roest
Department of Clinical Chemistry and Haematology (G03.550), University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands
e-mail: r.t.urbanus@umcutrecht.nl; m.roest@umcutrecht.nl

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_4,

Keywords Platelet adhesion • Platelet activation • Secondary hemostasis

1 Introduction

Blood platelets originate from megakaryocytes present in the bone marrow (Thiery and Bessis 1956). Every hour about eight billion platelets are released into the circulation, where they circulate for about 10 days before they are removed by the spleen. Platelets are in fact relatively simple cellular fragments (White 2002). With a volume of about 7–9 fL they are small and they lack a nucleus, a clear endoplasmatic reticulum, and Golgi apparatus. The platelet interior is filled with different organelles (Fig. 1). The dense bodies (δ-granules) are the storage pool of low molecular weight components such as adenosine diphosphate (ADP), serotonin, and Ca^{2+}. The α-granules are the most numerous organelles present in platelets and they contain a large variety of proteins, including adhesive proteins, clotting factors, protease inhibitors, growth factors, cytokines, and chemokines. The third organelles are the lysosomes that are full of hydrolytic enzymes. The content of all these organelles can be released upon platelet activation. Platelets also contain large intracellular membrane complexes: the surface-connected open canalicular system and the dense tubular system. The open canalicular system increases the total surface area of the platelets enormously and provides a route for different components to penetrate deeply into the cell. The dense tubular system can be distinguished from the open canalicular system by its amorphous content when visualized with an electron microscope. The dense tubular system originates from the rough endoplasmatic reticulum and is a storage site of intracellular calcium.

Platelets play a pivotal role in normal physiology. Their primary function is to maintain an undisturbed blood flow, as evidenced by the bleeding tendency observed in patients with quantitative and qualitative platelet disorders and the therapeutic efficacy of antiplatelet drugs for thrombotic complications. Besides their role in hemostasis, platelets participate in many other metabolic processes, including wound healing (Nurden 2011), inflammation (Semple et al. 2011), infectious diseases (Yeaman 2010), maintenance of the endothelial barrier function (Nachman and Rafii 2008), angiogenesis (Sabrkhany et al. 2011), and tumor metastasis (Gay and Felding-Habermann 2011). Blood platelets play a major role, or are involved in diseases responsible for the large majority of deaths and disabilities worldwide.

Platelets are an essential component of normal hemostasis. In 1885, Lubnitzky was the first to notice that, in flowing blood, platelets are primary responders in the formation of a hemostatic plug and that fibrin formation occurs as a secondary effect (Lubnitzky 1885). These findings were largely ignored during the next 70 years. It took until the 1960s until it became clear that a platelet response is the first step in the arrest of bleeding and that they are the key players of what now is called primary hemostasis (French et al. 1964). It became obvious that studies on the adhesion and aggregation of platelets were essential not only to understand the

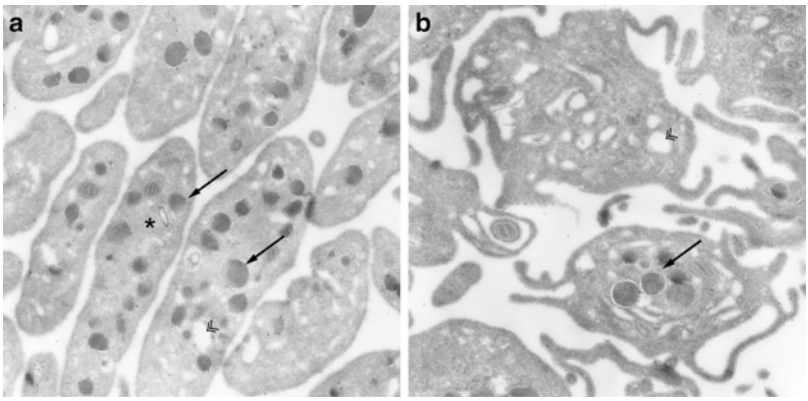


Fig. 1 The platelet interior. (**a**) Transmission electron micrograph (TEM) of resting platelets. Blood was collected in fixative, after which platelets were isolated. (**b**) TEM of thrombin-stimulated platelets. Washed platelets were stimulated with thrombin for 5 min prior to fixation. *Arrows* indicate α-granules, *asterisk* indicates a δ-granule, and the *double arrow* indicates the open canalicular system (OCS). Pictures courtesy of Dr. HFG Heijnen

pathophysiology of a bleeding tendency, but also for our understanding of the formation of arterial thrombi and thus for an optimal treatment of heart attacks and stroke. As a result, an explosive growth in publications on platelet function has been observed over the last 50 years and our knowledge on these little cells has increased concomitantly.

Platelets do not adhere to undamaged vessel walls. They circulate passively through the vascular tree, lined by an intact endothelium. Most platelets will never come into operation during their entire lifetime. Platelets circulate in our blood in a resting state until they are needed, but, if necessary, they can react immediately (Broos et al. 2011). They are circulating monitors of the integrity of the vessel wall and are designed to stop any leak. Platelets circulate in the blood close to the vessel wall on a continuous search for an injury. They have specific receptors on their surface that are able to recognize proteins that will be exposed to the blood after an injury has taken place. Platelets immediately respond when an injury is detected. They adhere, become activated, and start to interact with each other to form a platelet plug that covers the injury. The aggregated platelets also supply a surface to which coagulation factors can bind and fibrin can be formed to stabilize the plug. Platelet function is carefully regulated. Spontaneous or sustained platelet aggregation should be avoided at all times. An optimal platelet response is reached when blood loss is restrained without further damage caused by vascular occlusion. In normal physiology, the endothelium is critical in the control of the haemostatic response (van Hinsbergh 2011). This very efficient and strongly regulated process can be derailed when an atherosclerotic plaque is ruptured and a platelet thrombus is formed inside an intact blood vessel. Nonocclusive thrombi are far more frequently formed than occlusive thrombi (Davies et al. 1989). Mural thrombi are thought to be important contributors of the progression of an atherosclerotic plaque

(Ross and Glomset 1976), whereas an occlusive thrombus will result in the clinical manifestations of heart attack or stroke.

2 Platelet Adhesion

Platelet adhesion is the first step in the hemostatic response to a vascular injury. Platelet adhesion has several unique features. The adhesion of platelets occurs in flowing blood, which means that the adhesive process must occur rapidly and that the adhered platelet must withstand the exposed shear forces (Savage et al. 1996). This has lead to the evolution of adhesive receptors and ligands that are unique for platelet adhesion. Before the first interaction between a platelet and an injured vessel wall can take place, von Willebrand factor (vWF) present in plasma will adhere to the exposed collagens in the subendothelium (Fig. 2) (Sakariassen et al. 1979). The initial contact of the platelet can then take place via glycoprotein Ibα (GPIbα), one of the polypeptides of the platelet receptor complex GPIb–V–IX, with the immobilized vWF (Clemetson 2007). The interaction of vWF with GPIbα is transient and does not allow stable adhesion. This interaction initiates the tethering of platelets over the vessel wall. Platelets will roll over vWF in the direction of flow, driven by the shear forces exerted by the flowing blood (Savage et al. 1996). A continuous loss of GPIbα–vWF interactions at the downstream side of the platelet and the formation of new interactions at the upstream side mediate the rolling process. The rolling process slows down the platelets and enables interaction of platelets with vessel wall proteins via other receptors. The GPIbα–vWF interaction will also induce a weak intracellular signaling that will lead to integrin activation (Du 2007). The reduced velocity in combination with mild platelet activation allows subsequent interaction of additional receptors such as αIIbβ3 (glycoprotein IIb:IIIa; GPIIbIIIa), α2β1 and glycoprotein (GP)VI with vWF and collagen, respectively, resulting in firm adhesion (Savage et al. 1998). To further stabilize the interaction of platelets with subendothelium, platelets will spread (Fig. 3), thereby increasing the number of interactions with the surface. Spreading will help the platelets to withstand the shear forces exerted by the flowing blood. Platelet spreading is primarily mediated by the integrin αIIbβ3 (Weiss et al. 1991). Before stable adhesion and spreading is established, part of the platelets will detach and return to the circulation. The spread platelet provides a new surface for the next platelet to adhere to. The mechanism by which a circulating platelet attaches to already adhered platelets has many similarities to the adhesion of a platelet to the damaged vessel wall (Ruggeri 2000). The circulating platelet must attach to an adhered platelet when it is still subjected to shear forces. The adhered platelet will bind fibrinogen and vWF from the circulation via αIIbβ3 and GPIbα, respectively, creating an ideal surface for the next platelet to adhere.

Von Willebrand factor as adhesive surface is essential for optimal platelet adhesion at higher shear rates (Nieswandt et al. 2011). However, the subendothelium and connective tissue contain many other adhesive molecules, such as collagen types

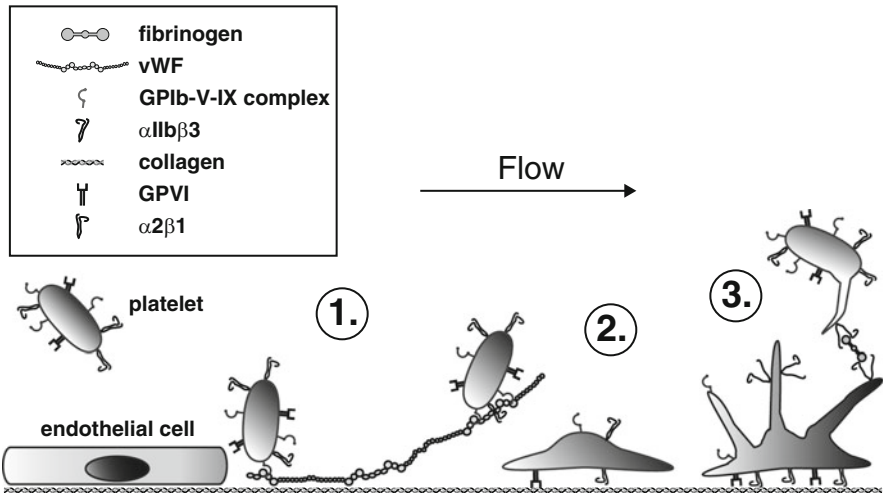


Fig. 2 Schematic overview of platelet adhesion. Platelets circulate in a resting state at high velocity. (1.) Upon vascular damage, the plasma protein Von Willebrand factor (vWF) will bind to the exposed subendothelial collagen via its A3 domain, after which it adopts its platelet-binding conformation. VWF then captures platelets from the circulation via the interaction of the vWF A1-domain with the GPIb–V–IX complex on the platelet. Since the interaction between vWF and GPIb–V–IX is transient, this will result in the slowing down and subsequent rolling of platelets over the injured vessel wall. (2.) The rolling platelet will then firmly adhere to the exposed collagen via the integrin α2β1. The interaction of GPVI with collagen will result in further platelet activation. (3.) Activated platelets will spread and undergo a shape change that dramatically increases their surface area. They will subsequently release the contents of the α- and δ-granules, which leads to the activation of the second layer of rolling platelets. Activated platelets express the integrin αIIbβ3 in its high-affinity fibrinogen-binding conformation, which will result in platelet aggregate formation

I, III, IV, V and VI, laminin, fibronectin and thrombospondin, for all of which platelets possess receptors (de Groot and Sixma 2002). All these adhesive proteins can support platelet adhesion at lower shear stresses. Which receptor(s) and extracellular protein(s) are involved in the different stages of adhesion depends on the vascular bed and nature of the injury. Local shear stress in combination with the composition of the exposed subendothelium, which depends on the site, type and severity of the injury, will probably determine the importance of the individual receptors. In the venous system, low shear rates allow the interaction of many different receptors with their ligands. In the arterial circulation, higher shear forces limit the participation of the majority of receptors and the major adhesive protein is thought to be vWF. VWF is present in the vessel wall, but its amount is often limited: the exposed collagen fibers present in the subendothelium rapidly bind plasma vWF (Lisman et al. 2006).

Platelets can adhere to laminin via α6β1 (Hindriks et al. 1992), to fibronectin via α5β1 (Beumer et al. 1995), to thrombospondin via GPIbα (Jurk et al. 2003), to fibrinogen via αIIbβ3 (Hantgan et al. 1992), and directly to collagen via α2β1 and GPVI (Pugh et al. 2010). The interaction of platelets with these different proteins

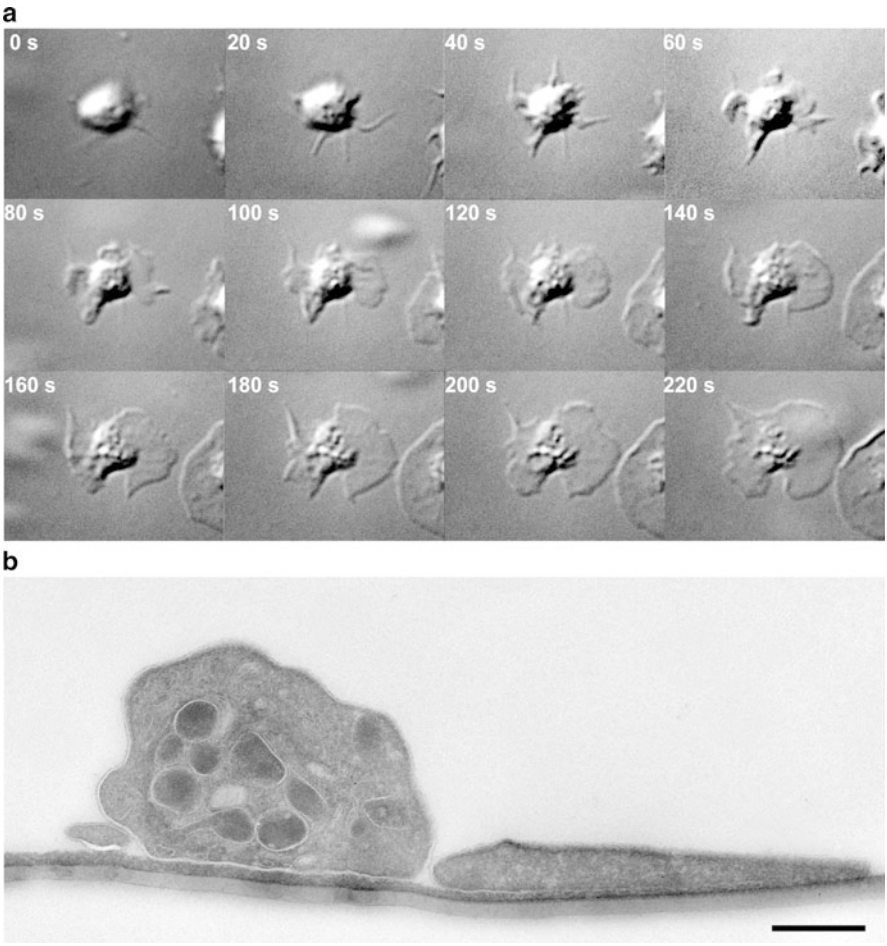


Fig. 3 Platelet spreading. (**a**) Washed platelets were perfused over a fibrinogen surface at a shear rate of 100 s^{-1}. Platelet spreading was visualized with differential interference microscopy. Snap shots were taken every 20 s. Pictures courtesy of Dr. V De Angelis. (**b**) TEM of surface-adhered platelets. Picture courtesy of Dr. HFG Heijnen

has been studied with isolated and purified proteins, but the contribution of the individual proteins in the much more complex subendothelium remains to be investigated. Some of these receptors have a very low surface density. However, there may be synergy of multiple substrate receptors. On the other hand, mutual interactions between proteins in the subendothelium might specifically shield off certain adhesive domains (Agbanyo et al. 1993). Studies with genetically modified mice have suggested significant roles for α5β1, α6β1, and αIIbβ3 in platelet adhesion (Gruner et al. 2003) indicating that platelet adhesion in vivo is a highly integrated process involving multiple interactions, none of which is essential by itself.

3 Von Willebrand Factor–GPIbα Interaction

It is apparent that vWF–GPIbα interactions are essential and unique for the adhesion of platelets at higher shear rates. VWF is present in plasma and subendothelium but there are additional sources of vWF in the Weibel-Palade bodies of endothelial cells and α-granules of platelets. The content of these organelles will be released when the hemostatic process is initiated and increase the local concentration. The plasma concentration of vWF is about 10 μg/mL, the concentration in platelets is about 280 ng/10^9 platelets (Sadler 2005). The normal range of plasma vWF is broad and strongly influenced by blood groups, with 25% lower levels in blood group O and high levels in individuals with AB loci (Sadler 2005). VWF is a large multimeric protein synthesized by endothelial cells and megakaryocytes. VWF present in the circulation cannot bind spontaneously to platelets, as the GPIbα binding site within vWF is cryptic. VWF must first bind to collagen via its A3-domain before it can interact with GPIbα via its A1-domain (Lankhof et al. 1996). The conformational changes that coincide with the binding to collagen and are necessary to expose the A1-domain are only partly understood. Other domains of vWF will probably shield the A1 domain (Ulrichts et al. 2006). Collagen is not the only binding site for vWF in the vessel wall, vWF can be immobilized via self-assembly (Savage et al. 2002). Circulating vWF can bind to vWF in the subendothelium via multiple domain interactions.

The overall shape of vWF in the circulation is dynamic. When vWF binds to collagen, shear stress will force vWF into an elongated conformation in the direction of flow, the ideal route for a platelet to roll on. This might explain why the hemostatic potency of vWF depends so strongly on its multimeric structure (Sixma et al. 1984). The length of the vWF molecules is accurately regulated. Individuals lacking the highest multimers of vWF, as in von Willebrand disease type 2a, have a severe bleeding tendency, while the presence of ultra-large multimers of vWF in the circulation will result in microangiopathy. VWF in the Weibel-Palade bodies of endothelial cells appears to be ultra-large. After secretion form the endothelial cells, the cleavage protease ADAMTS-13 will trim vWF to the length found in plasma (Dong 2005).

About 25,000 copies of the GPIb–V–IX complex are expressed on the surface of platelets. The complex consists of GPIbα, GPIbβ, GPV, and GPIX in a 2:4:1:2 ratio (Luo et al. 2007). The members of the complex belong to the leucine-rich repeat receptor family. GPIbα can bind many different ligands besides vWF, amongst others P-selectin, MAC-1, β2-glycoprotein I and the coagulation factors thrombin, VII, XI and XII (Andrews et al. 2003). The crystal structure of the complex between the extracellular part of GPIbα and the A1-domain of vWF showed GPIbα wrapped around one side of A1, providing two contact areas bridged by an area of solvated charge interaction (Huizinga et al. 2002). The bond between GPIbα and the A1-domain is termed a flex-bond, as it can exist in two states, a low-affinity and an extended, high-affinity state (Kim et al. 2010). One state is seen at low force; a second state begins to engage at a force of 10 pN and has greater force resistance

and an approximately 20-fold longer lifetime. Switches between the two states are induced by shear forces and determine the lifetime of an individual bond. This subtle interaction between shear stress and affinity of GPIbα for vWF allows the platelet to withstand a broad range of forces.

The essential role of vWF–GPIbα interaction is highlighted by the bleeding complications observed in patients with von Willebrand's disease and Bernard-Soulier Syndrome. Studies with mice lacking these proteins have confirmed the importance of this interaction (Denis et al. 1998; Ware et al. 2000). Furthermore, it has been shown that mice lacking these proteins are protected against thrombotic complications (Konstantinides et al. 2006). Deficiencies of ADAMTS-13 will result in thrombotic thrombocytopenic purpura, a syndrome characterized by the presence of ultra-large multimers of vWF, thrombocytopenia, hemolytic anemia, schistocytes, and organ failure (Chauhan et al. 2008).

4 Platelet Activation

The interaction between subendothelial collagen, vWF, and the GPIb–V–IX complex on platelets facilitates the initial capture of platelets from the circulation to the site of the injury. This interaction allows the direct binding of GPVI to collagen, which triggers immuno-receptor-based activation motif (ITAM)-regulated signaling pathways (Watson et al. 2010), leading to activation of adhered platelets (Fig. 4). Binding of GPVI to collagen furthermore triggers α2β1 inside-out activation (Chen et al. 2002). Activated α2β1 is important for firm adhesion of platelets to the collagen surface and further activation of the adhered platelets (Santoro 1986).

GPVI is a 62-kDa type I transmembrane receptor of the immunoglobulin superfamily of surface receptors, which is exclusively expressed in platelets and megakaryocytes. The signaling capacity of GPVI depends on its association with an Fc receptor (FcR) γ-chain homodimer. Each FcR γ-chain monomer contains a conserved ITAM-motif, which typically consists of two conserved YXXL motifs separated by 6–12 amino acids. Upon receptor cross-linking by the ligand, collagen, these two conserved ITAM tyrosine residues are phosphorylated by the Src family tyrosine kinases Fyn and Lyn. This phosphorylation then leads to recruitment and activation of the tyrosine kinase Syk, which regulates a complex downstream pathway that involves the adapter proteins LAT, Gads and SLP-76, the Tec family tyrosine kinases Btk and Tec, the GTP exchange factors Vav1 and Vav3, PI3-kinase isoforms and phospholipase C (PLC)γ2 [for detailed reviews, see (Watson et al. 2010; Jung and Moroi 2008)]. GPVI-deficient platelets aggregate normally on ADP and thrombin, but do not aggregate on collagen. GPVI-deficient patients have mildly prolonged bleeding times (Kato et al. 2003), which suggests that platelet activation by collagen can be by-passed by other activation processes. Inhibition of the GPVI pathway may reduce thrombosis risk (Nurden and Nurden 2011), which makes the GPVI-mediated signaling pathway an attractive target for novel antiplatelet therapy.

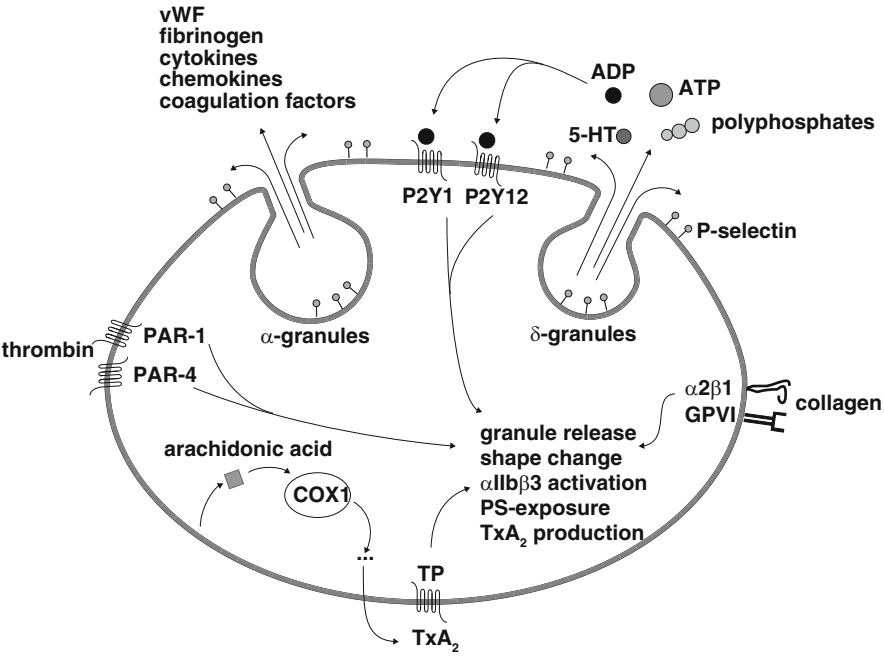


Fig. 4 Schematic overview of the major platelet activation pathways. Platelets can be activated by collagen via the integrin α2β1 and the platelet receptor GPVI, thrombin via the interaction with protease-activated receptor (PAR)-1 and -4 and ADP via the purinergic receptors P2Y1 and P2Y12. Interaction of each ligand with their respective receptors results in the transition of αIIbβ3 from a low- to a high-affinity conformation, platelet cytoskeletal rearrangement, the release of α- and δ-granule content and P-selectin expression. Dense granule ADP augments platelet activation. Upon platelet activation, arachidonic acid is liberated from the plasma membrane and converted to thromboxane A2 (TxA2) in a series of reactions that involves the enzyme cyclo-oxygenase (COX)-1. TxA2 furthers augments platelet activation

The integrin α2β1 requires an agonist-induced conformational change via inside-out signaling to bind to collagen. The underlying "inside-out" signaling events translate into talin binding to α2β1 (Nieswandt et al. 2009). Binding of α2β1 to collagen contributes to cellular activation both indirectly, by reinforcing GPVI–collagen interactions, and directly, by a series of intracellular signaling events, which are strikingly similar to GPVI-induced signaling and include Src, Syk, SLP-76, and PLCγ2. Thus, although structurally unrelated, the two major collagen receptors share important signaling molecules and act in a cooperative manner, reinforcing each other's activity. It is now generally accepted that they act synergistically in the process of α- and δ-granule release, activation of the integrins αIIbβ3 and α2β1, shape change and spreading, phosphatidylserine exposure, and matrix metalloproteinase activation.

Collagen-induced platelet activation triggers the formation of a baseline layer of aggregated platelets, but does not support additional layers at greater distance from

the subendothelium (Offermanns 2006). Second-wave mediators, such as ADP and thromboxane that are released from the baseline layer of activated platelets, are required for further thrombus growth. ADP is stored in the δ-granules of platelets. Upon platelet activation, ADP is rapidly released to cause auto-activation and to activate surrounding platelets. When ADP is secreted, it amplifies the response induced by other activators and it stabilizes platelet aggregates. Platelets contain two receptors for ADP: P2Y1 and P2Y12. Both receptors play a role in platelet aggregation and aggregate stabilization (Cattaneo 2011). Remarkably, although ADP stimulation of platelets has a strong effect on αIIbβ3 inside-out signaling, it has only minor impact on the release of α- and δ-granule content (Cattaneo et al. 2000).

P2Y1 is a G_q-coupled receptor that mediates the activation of PLCβ, which subsequently converts PIP2 into the second messengers DAG and IP3. IP3 opens calcium channels and DAG activates PKC and calDAG-GEFI (CD-GEFI). CD-GEFI contains binding sites for Ca^{2+} and diacylglycerol (DAG) and a guanine nucleotide exchange factor (GEF) domain catalyzing the activation of Rap1. Then the Rap1 effector molecule (RIAM) interacts with Talin-1 to unmask its integrin-binding site. The binding of Talin-1 to αIIbβ3 disrupts the salt bridges between the α and β subunits, which changes the integrin conformation from a low- to a high-affinity state. Kindlin-3 supports this process by binding to the β-tail. Individuals with mutations in Kindlin-3 show defects in αIIbβ3 activation and express a bleeding tendency.

P2Y12 is coupled to G_i-type G proteins, in particular G_{i2} (Offermanns 2006). Upon stimulation of the heterotrimeric receptor, the α-subunit is released from the βγ-complex. The βγ-complex is a second messenger that stimulates phosphoinositide 3-kinase (PI3K). The most important target protein of PI3K is Akt, which regulates platelet function by phosphorylating and inhibiting GSKβ. PI3K also activates the small GTPase Rap1b, which is a critical regulator of αIIbβ3 inside-out signaling. In addition, the liberated α-subunit of the G_{i2} protein inhibits adenylyl cyclase (AC) and thereby counteracts cyclic adenosine monophosphate (cAMP), which inhibits the release of Ca^{2+} from intracellular stores. This function is mainly important to overrule the inhibitory effects of prostacyclin (PGI_2) secreted by endothelial cells.

Patients with functional genetic modifications in P2Y12 show a variable bleeding diathesis, characterized by mucocutaneous bleedings, excessive postsurgical bleeding, and mild to moderate posttraumatic blood loss. These patients show impaired platelet reactivity to ADP even at high concentrations. P2Y12 is one of the most effective targets for platelet inhibition to prevent secondary cardiovascular disease events. Several different P2Y12 inhibitors have been widely used, including thienopyridines (ticlopidine, clopidogrel, and prasugrel), while direct P2Y12 inhibitors (tricagrelor) have entered the market (Storey 2011).

Analogous to ADP, thromboxane A2 can (auto-)activate platelets via a positive feedback loop during platelet activation. Thromboxane A2 is produced from arachidonic acid that is liberated from the plasma membrane by phospholipase A2, through conversion by cyclooxygenase-1 (COX1). Prevention of thromboxane

A2 formation in platelets by COX1 inhibitors, such as acetylsalicylic acid, belongs to the most commonly used medication in the prevention of secondary events of cardiovascular disease. Thromboxane A2 interacts with the thromboxane A2 receptor (TP) on platelets and triggers both G_q- and G_{13}-mediated signaling pathways (Stegner and Nieswandt 2011).

Another potent activator of platelets is thrombin, the final product of the coagulation system, which activates platelets via protease-activated receptor (PAR)-1 and PAR-4. The activation of PARs is a two-step process. First, the cryptic ligand is unmasked by proteolytic cleavage of the N-terminal domain of the receptor. Then, an intramolecular rearrangement allows the ligand and the receptor moieties to interact, which results in intracellular signaling. PAR-1 is activated at low thrombin concentrations, while PAR-4 mediates platelet activation at high thrombin concentrations (Leger et al. 2006). Both PAR-1 and PAR-4 couple to G_q, which mediates signaling through its interaction with PLCγ2 and the subsequent conversion of phosphatidylinositol bisphosphate (PIP2) into diacylglycerol and inositol-triphosphate (IP3). This opens calcium channels, and activates PKC and CD-GEFI, catalyzing the activation of Rap1 and Rap2 to regulate inside-out activation of αIIbβ3. In addition, PAR-1 and PAR-4 couple to G_{13} to activate guanidine nucleotide exchange factors (GEFs) for the small G-protein RhoA, which induces myosin light chain phosphorylation and myosin-dependent contraction. This pathway is of major importance for platelet shape change. Deficiency of G_{13} causes more dramatic effects for platelet adhesion than for integrin activation, aggregation or granule secretion. It has been suggested that PAR-1 and PAR-4 may also couple to G_i, although this interaction is still debatable.

5 Thrombus Stability

A growing thrombus typically consists of a dense inner core of fully activated platelets covered by a layer of loosely bound platelets. Platelet recruitment to this growing thrombus requires only minimal activation, since tethering platelets retain their discoid shape. Over time, some of these discoid platelets will become fully activated and release their granule content, whereas others will detach from the thrombus and embolize (Furie and Furie 2005; Nesbitt et al. 2009). The transition of αIIbβ3 from a low- to a high-affinity state that accompanies activation allows further platelet–platelet interactions to take place, a process dependent on the association of the integrin with its ligand and subsequent outside-in signaling. When close contacts between adjacent platelets are formed, several other surface molecules will interact with their counterparts and induce contact-dependent signaling, which regulates thrombus formation and stability.

One of the surface receptors reported to influence thrombus stability is CD40L (CD154), a receptor best known from its role in T-cell-mediated B-cell responses. Although platelets only express an estimated 600–1,000 copies of this receptor, it enhances the activation of αIIbβ3, the most abundant platelet receptor, by binding

to the extracellular portion of the β3 integrin via its KGD sequence (Andre et al. 2002). Interestingly, the absence of CD40L has no effect on hemostasis, but appears to protect against thrombosis in a murine thrombosis model. One of the most pronounced features in these mice is the increased embolization rate after thrombus induction, indicating thrombus instability. In humans, addition of soluble CD40L strongly augments thrombus formation under conditions of flow.

Activation of the integrin αIIbβ3 is also enhanced by the Eph kinases EphA4 and EphB1 and their ligand Ephrin B1. The formation of close contacts between adjacent platelets in a growing thrombus allows the interaction between the Eph kinases and Ephrin B1 to occur, which leads to intracellular signaling events. Interaction of the EphA4 or EphB1 kinases with Ephrin B1 results in the activation of Rap1, a small GTPase involved in αIIbβ3 engagement (Prevost et al. 2004). Moreover, EphA4 is constitutively associated with the β3 integrin, where it facilitates the phosphorylation of the β3 integrin cytoplasmic domain and its association with myosin (Prevost et al. 2005). Blockage of either the Eph kinases or Ephrin B1 results in decreased thrombus size and defects in clot retraction.

Semaphorin 4D is another platelet receptor involved in the regulation of stable thrombus formation. Engagement of semaphorin 4D by its ligands plexin-B1 and CD72, all of which are expressed by platelets, augment the response to collagen, but not to other stimuli. The effects of semaphorin 4D on the platelet response to collagen are self-limiting, as the protein is quickly shed from the membrane by the metalloprotease ADAM17. Similar to mice deficient in CD40L, mice deficient in semaphorin 4D show delayed arterial thrombus formation (Zhu et al. 2007). Interestingly, the function of another surface receptor involved in contact-dependent signaling, platelet and endothelial cell adhesion molecule (PECAM)-1, directly opposes the function of semaphorin 4D. PECAM-1 dimerization, either in trans or on the same platelet, specifically inhibits the collagen pathway of platelet activation, but has no influence on ADP or thrombin-induced activation (Newman and Newman 2003). Whereas binding of semaphorin 4D to CD72 results in the inactivation of SHP-1, which downregulates collagen signaling, PECAM-1 dimerization results in SHP-2 recruitment.

The vitamin K-dependent protein known as growth arrest-specific gene 6 (gas6) has also been implicated in the regulation of thrombus stability. Murine platelets have been shown to contain both gas6 and express its receptors Axl, Mer and Tyro3 on their surface, deficiency of which is associated with impaired arterial and venous thrombus formation (Angelillo-Scherrer et al. 2001). However, the presence of gas6 in human platelets remains controversial and the effects of gas6 on human platelets are questioned (Clauser et al. 2006). Nevertheless, there are reports that gas6 enhances the ADP-dependent activation of αIIbβ3 (Cosemans et al. 2010).

Most of the stimulatory regulators of contact-dependent signaling, i.e., CD40L, the Eph kinases and semaphorin 4D, are expressed in low levels on resting platelets, with increased surface expression upon platelet activation. Whether this additional pool of receptors originates from the open canalicular system or from α-granules is unclear. Another receptor involved in contact-dependent signaling that becomes surface-exposed upon platelet activation is endothelial cell-specific adhesion

molecule (ESAM). This receptor, a member of the CTX family of adhesion receptors, localizes to the junctions between adjacent platelets, where it negatively regulates stable platelet–platelet interactions. Similar to PECAM-1 knockouts, deficiency of ESAM is a gain-of-function mutation (Stalker et al. 2009), with ESAM-deficient mice exhibiting increased thrombus formation upon vascular challenge. The way in which ESAM regulates thrombus stability is currently unclear.

6 Platelets and the Endothelium

The vascular endothelium is the interface between blood and the subendothelial tissue and is critical for the regulation of the hemostatic response (van Hinsbergh 2011). Endothelial cells line the entire vasculature. Under normal conditions, circulating platelets do not adhere to intact endothelium. The endothelium releases substances that suppress unwanted platelet activation in an intact blood vessel. Damage of the endothelial cell layer and, to a lesser extent, endothelial cell dysfunction will promote a pro-thrombotic state.

The luminal surface of the endothelium is covered with a negatively charged glycocalyx. Resting platelets cannot bind to the surface of endothelial cells due to electrical repulsion by negatively charged heparan sulfates (Reitsma et al. 2011). This defense mechanism can be overcome by activated platelets. Binding of P-selectin or GPIbα on activated platelets to the protein PSGL-1 on the surface of the endothelium will result in rolling of platelets over the endothelium (Frenette et al. 1995). Platelet heparanase, which is released from activated platelets, can facilitate this interaction by degrading proteoglycans in the endothelial glycocalyx (Pries et al. 1997).

Endothelial cells contain prostacyclin synthase, an enzyme that converts endoperoxides into prostacyclin (Yuhki et al. 2010). Peroxides are synthesized from arachidonic acid by cyclo-oxygenases (COX) 1 and 2 in every cell, but the further processing of endoperoxides is cell type-specific. Prostacyclin binds to the prostanoid receptor IP, a seven transmembrane spanning G-protein-coupled receptor on the platelet membrane (Stitham et al. 2007). Binding of prostacyclin stimulates adenylyl cyclase activity. The resulting increase in platelet cyclic AMP leads to calcium re-uptake by the dense tubular system, thereby inhibiting stable platelet adhesion and platelet aggregation (Noe et al. 2010). Prostacyclin is one of the most potent inhibitors of platelet aggregation. Mice lacking the prostacyclin receptor IP show an increased thrombotic tendency, intimal hyperplasia, accelerated atherosclerosis, restenosis, and reperfusion injury (Yuhki et al. 2011). These observations have been supported in human population-based studies in which a significant increased coronary disease was observed in individuals with functional mutations in the prostacyclin receptor (Stitham et al. 2011). Furthermore, the world-wide withdrawal of selective COX-2 inhibitors due to an increased

incidence of myocardial infarction and thrombotic strokes in users points to the importance of COX-2-derived endothelial cell prostacyclin synthesis.

Nitric oxide (NO) is a second endothelial cell product that is involved in the maintenance of its antithrombotic properties (Palmer et al. 1987). Nitric oxide released from the endothelium diffuses through the platelet membrane and binds to the heme moiety of guanylyl cyclase. This will lead to increased levels of cyclic AMP and downregulation of platelet activation. The importance of NO as antithrombotic mediator was recently demonstrated in a mouse model of the antiphospholipid syndrome. The antiphospholipid syndrome is accompanied by recurrent thrombosis. Interaction of antiphospholipid antibodies with the LRP8 receptor on endothelial cells resulted in de-phosphorylation of NO synthase at its Ser1179 site, a reduced NO production and an increased tendency to thrombus formation (Ramesh et al. 2011). The antithrombotic effect of endothelial cells may be the consequence of a combined action of prostacyclin and nitric oxide. Synergistic antiaggregatory effects have been shown on platelets. Both prostacyclin and nitric oxide are not only involved in the prevention of platelet activation, they are also important regulators of the vascular tone.

In addition to the effects of prostacyclin and nitric oxide, endothelial cells can reduce platelet activation with a surface-bound ecto-ADPase (CD39) (Gayle et al. 1998). ADP released form platelets, which mediates an amplification loop for platelet activation, will be metabolized by CD39. The formed AMP will be further metabolized to adenosine, which inhibits platelet aggregation. CD39-deficient mice have an increased spontaneous thrombotic tendency, suggesting that CD39 plays an important role in the regulation of primary hemostasis. Besides these direct effects on platelet function, endothelial cells also influence platelet function indirectly via downregulation of thrombin formation via the expression of thrombomodulin (Esmon 2003), the synthesis of tissue factor pathway inhibitor (TFPI) (Broze et al. 1990), and the regulation of vWF mutimeric size via ADAMTS-13.

7 Interaction Between Primary and Secondary Hemostasis

The formation of a platelet plug to seal a defect in the vascular wall is only the first step in the prevention of blood loss. Without further consolidation, this platelet plug will dissolve within hours and merely delay bleeding. Platelet aggregates that are formed at a site of injury are therefore rapidly strengthened by the formation of an insoluble fibrin network. The importance of this process is illustrated by a test of platelet function, the bleeding time test, in individuals with reduced or deficient levels of coagulation factor (F)VIII (hemophilia A): despite initial staunching of bleeding from the skin lesion that is induced in this test due to the formation of a platelet plug, these individuals will re-bleed from the wound after several hours due to inadequate fibrin formation (Sixma and van den Berg 1984). Based on these and other observations, the process of platelet adhesion and plug formation is generally referred to as primary hemostasis, whereas the formation of a fibrin network that

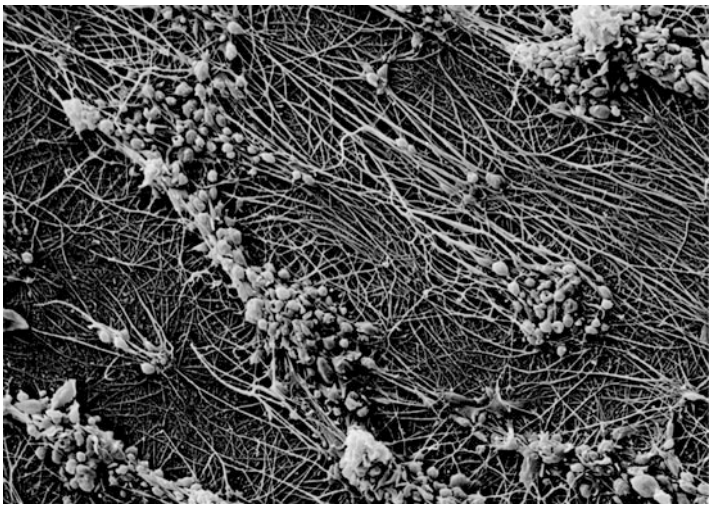


Fig. 5 Platelets and the coagulation system tightly interact during thrombus formation. Scanning electron micrograph of thrombi formed after perfusion of recalcified blood over endothelial cell matrix. Picture courtesy of Dr. HFG Heijnen

stabilizes this platelet aggregate is referred to as secondary hemostasis. We now know, however, that both primary hemostasis and secondary hemostasis tightly interact in the formation of a thrombus in vivo (Fig. 5).

Blood coagulation occurs when the soluble plasma protein fibrinogen is converted into insoluble fibrin fibers by the protease thrombin (Fig. 6). This enzyme is the final product of an intricate series of enzymatic reactions that starts with the exposure of blood to tissue factor (TF) (the extrinsic pathway of coagulation), or negatively charged surfaces such as glass (the intrinsic pathway of coagulation). We differentiate between these two pathways, because they largely depend on different enzymatic reactions. The intrinsic pathway of coagulation starts with activation of coagulation factor XII (FXII) on a negatively charged surface such as glass. This will lead to the activation of factor XI (FXI). Activated FXI (FXIa) will then activate factor IX, which forms a complex with its cofactor activated FVIII (FVIIIa) that is known as the intrinsic tenase complex and activates coagulation factor X (FX). The extrinsic pathway of coagulation starts with the exposure of TF to blood and the subsequent binding of circulating activated factor VII (FVIIa). This complex, known as the extrinsic tenase complex, can then activate FX. Both the intrinsic and the extrinsic pathway of coagulation culminate in the common pathway of coagulation, in which prothrombin is converted into thrombin by the prothrombinase complex, which consists of activated FX (FXa) and its cofactor activated factor V (FVa). Nevertheless, these differentiations largely apply to a diagnostic setting, because the initiation of coagulation by TF exposure is considered far more important than the activation of FXII by negatively charged surfaces in vivo. Moreover, the intrinsic and extrinsic pathway of coagulation partially

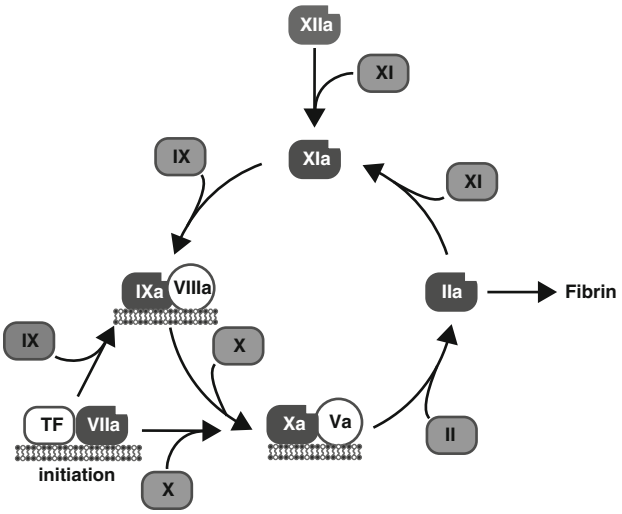


Fig. 6 The coagulation system. Coagulation is initiated by the exposure of flowing blood to extravascular TF, which rapidly binds circulating FVIIa. The TF–FVIIa complex activates FX, which—together with its cofactor FVa—converts prothrombin (FII) to thrombin (FIIa). Thrombin can then cleave fibrinogen into fibrin and coagulation ensues. Thrombin formation leads to more thrombin formation through a process called feedback activation, in which thrombin activates FXI. FXIa then activates FIX, which—together with its cofactor FVIIIa, (the intrinsic tenase complex) can then generate more FXa. Thrombin formation increases considerably through this process. When only small amounts of TF are exposed to blood, the TF–VIIa complex can also directly activate FIX. The intrinsic tenase complex then rapidly produces more FXa, which causes thrombin formation. FXII appears to be irrelevant for physiological hemostasis, but is reported to play a role in thrombosis

overlap, as the extrinsic tissue factor–factor VIIa complex can also activate FIX and the thrombin that is formed during the initial coagulation reaction causes feedback activation of FXI to generate more thrombin.

Platelets are considered crucial components of the coagulation reaction in vivo for several reasons. First of all, activated platelets are thought to provide the surface on which coagulation takes place. Most of the coagulation reactions take place on a surface containing the negatively charged phospholipid phosphatidylserine (PS). This greatly enhances the efficiency of the coagulation reaction, since it minimizes entropy, prevents the washout of activated coagulation factors by the flowing blood and ensures that coagulation only takes place at sites where it is needed. PS is normally present on the inner leaflet of the plasma membrane of every cell. PS exposure on the outer membrane is generally considered a marker for apoptosis and will result in rapid clearance. In platelets, a subpopulation expresses PS upon full activation, which is enough to support the coagulation reaction. The protein that mediates this PS exposure was recently identified as transmembrane protein 16F (TMEM16F) (Suzuki et al. 2010) in a patient with Scott Syndrome, a rare bleeding disorder characterized by defective PS exposure at sites of vascular injury.

A second reason why platelets are important regulators of coagulation is that platelet α-granules contain several coagulation proteins that are secreted upon activation, such as prothrombin and FV. Platelets contain approximately one-fifth of the entire FV pool in human circulation (Camire et al. 1998), which is both synthesized and taken up from blood plasma by the megakaryocyte. The importance of platelet FV for coagulation is also illustrated by the bleeding phenotype in severe FV-deficient individuals. Despite extremely low or undetectable levels of FV in the plasma of these individuals, the presence of minute amounts of FV in their platelets, which act as FV reservoirs, can ameliorate the severity of the bleeding phenotype associated with FV deficiency (Duckers et al. 2010).

Alpha-granules also contain several inhibitors of the coagulation reaction (Maynard et al. 2010), such as antithrombin and C1-inhibitor. Both of these molecules are serine protease inhibitors (SERPINS) and can inhibit several coagulation factors, amongst others FXIa, FIXa, FXa, and thrombin. Protein S is also reported to be in platelet α-granules. This protein acts as a cofactor for activated protein C, which degrades the cofactors FV and FVIII, thereby attenuating the coagulation reaction. Protein S also functions as a cofactor for tissue factor pathway inhibitor (TFPI) (Hackeng et al. 2006), an inhibitor of the extrinsic tenase complex, which has been reported to be present on the surface of activated platelets as well (Maroney et al. 2007).

The contact phase of the intrinsic pathway of coagulation, i.e. the activation of FXII by negatively charged surfaces, has been considered irrelevant for physiological hemostasis for a long time, because FXII deficiency is not associated with a bleeding phenotype. It has, however, gained renewed interest since the discovery that FXII plays a role in thrombus formation in a murine arterial thrombosis model (Renne et al. 2005). The mechanism via which FXII mediates this prothrombotic influence is currently under investigation. One of the first questions to address is how FXII is localized to the growing thrombus, as it lacks the ability to bind to negatively charged phospholipids to prevent being washed away by the flowing blood. One possibility is that it binds to platelet receptors. Indeed, FXII is reported to bind directly to platelets via the GPIbα subunit of the GPIb–V–IX complex (Bradford et al. 2000). Furthermore, it has been known for a long time that FXII can bind to negatively charged regions on exposed subendothelial collagen (Niewiarowski et al. 1964; Wilner et al. 1968). This brings us to the other issue that needs to be resolved: how does FXII get activated during thrombus formation? One possibility is the collagen, which has been shown to activate FXII (Wilner et al. 1968; Kawamoto and Kaibara 1990). Other potential activators of FXII in the setting of a growing thrombus are polyphosphates. Platelet δ-granules contain polyphosphates that are secreted upon activation (Ruiz et al. 2004). Recent experimental evidence suggests that polyphosphates can indeed activate FXII and mediate FXII-dependent thrombus formation (Muller et al. 2009). Moreover, polyphosphates have been shown to enhance both the activation of FV by thrombin and factor Xa activity, thereby influencing the propagation of coagulation (Smith et al. 2006). They have also been implicated as modulators of fibrin fiber thickness (Smith and Morrissey 2008). All of these processes have been attributed to different lengths of

polyphosphate polymers (Smith et al. 2010). Which of these effects of polyphosphates are most important in thrombus formation remains to be determined, although the polymer length of δ-granule polyphosphate seems to favor FV activation (Smith et al. 2010).

FXI has also been implicated as a major player in thrombosis, whereas its role in hemostasis seems to be of less importance. Deficiency of the molecule is associated with only a mild bleeding tendency, but appears to protect against excessive venous and arterial thrombus formation in in vivo models of thrombosis (Zhang et al. 2010; Wang et al. 2006; Rosen et al. 2002). Similar to FXII, FXI lacks phospholipid-binding capacity to support its localization to the growing thrombus in flowing blood. However, the enzyme can bind to two receptors on the platelet surface, GPIbα (Baglia et al. 2003) and LRP8 (White-Adams et al. 2009), which may retain FXIa on the platelet surface long enough for it to activate FIX. The role of FXI in thrombosis seems to be the amplification of thrombin formation via feedback activation on the growing platelet thrombus, but the exact mechanism of FXI-dependent thrombus formation is not fully understood. There are indications that the prothrombotic effect of FXI on thrombus formation is independent of both FXII and FVIIa (Tucker et al. 2009), which makes it hard to envision how FXI is activated.

Further strengthening of the thrombus by the fibrin network occurs when the clot contracts, a process driven by platelets and dependent on the interaction of the fibrinogen receptor αIIbβ3 with the actin cytoskeleton (Cohen et al. 1982). Interestingly, binding of αIIbβ3 to fibrin appears to involve a different epitope on the receptor than the epitope that binds fibrinogen, because some mutations that are associated with Glanzmann Thrombasthenia do not abolish the ability of platelets to mediate clot contraction, despite absent fibrinogen binding (Ward et al. 2000; Kiyoi et al. 2003).

8 Conclusions

A significant progress has been made over the last 50 years in our understanding of platelet function. We now have a detailed understanding of fundamental cellular processes and molecular interactions that determined platelet adhesion and activation. Thanks to structural biology, we have solved the structures of GPIbα and vWF and we start to understand the processes that underlie the flow-regulated interaction between these two unique proteins. We have unraveled the process of integrin activation at the atomic level. The next few years new information will be obtained from genomics, proteomics and system biology studies that will add new dimensions to our knowledge on platelet function in different parts of the vasculature, observations that may allow more rational drug design. As we are unable to identify the cause of a bleeding tendency in many patients at present, perhaps microarray-based gene expression analysis will allow a more in-depth analysis of bleeding disorders.

One of the major drawbacks in platelet research is the impossibility to genetically modify human platelets, making detailed studies on platelet function difficult. We completely rely on studies with genetically modified mice. We know that platelet function in mice can be different from platelet function in humans in many respects. The development of methods to produce platelets in significant amounts from cultured human megakaryocytes would significantly improve our possibilities to determine the importance of different signaling pathways for optimal platelet function.

Knowledge Gaps

- The differences in platelet responses in distinct parts of the vasculature.
- Regulation of secondary hemostasis by platelets.
- Approaches to modulate platelet function without risk of bleeding.
- Fine regulation of the release of granule contents.
- How do platelets participate in immunity and infections?

Key Messages

- The role of platelet in (primary) hemostasis.
- The regulation of platelet function.
- The roles of von Willebrand factor and glycoprotein Ibα in platelet adhesion.
- Signaling pathways involved in platelet activation.
- Factors determining the stability of a platelet thrombus.

References

Agbanyo FR, Sixma JJ, de Groot PG, Languino LR, Plow EF (1993) Thrombospondin-platelet interactions. Role of divalent cations, wall shear rate, and platelet membrane glycoproteins. J Clin Invest 92:288–296

Andre P, Prasad KS, Denis CV, He M, Papalia JM, Hynes RO, Phillips DR, Wagner DD (2002) CD40L stabilizes arterial thrombi by a beta3 integrin–dependent mechanism. Nat Med 8:247–252

Andrews RK, Gardiner EE, Shen Y, Whisstock JC, Berndt MC (2003) Glycoprotein Ib-IX-V. Int J Biochem Cell Biol 35:1170–1174

Angelillo-Scherrer A, de Frutos P, Aparicio C, Melis E, Savi P, Lupu F, Arnout J, Dewerchin M, Hoylaerts M, Herbert J, Collen D, Dahlback B, Carmeliet P (2001) Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. Nat Med 7:215–221

Baglia FA, Shrimpton CN, Lopez JA, Walsh PN (2003) The glycoprotein Ib-IX-V complex mediates localization of factor XI to lipid rafts on the platelet membrane. J Biol Chem 278:21744–21750

Beumer S, Heijnen HF, IJsseldijk MJ, Orlando E, de Groot PG, Sixma JJ (1995) Platelet adhesion to fibronectin in flow: the importance of von Willebrand factor and glycoprotein Ib. Blood 86:3452-3460

Bradford HN, Pixley RA, Colman RW (2000) Human factor XII binding to the glycoprotein Ib-IX-V complex inhibits thrombin-induced platelet aggregation. J Biol Chem 275: 22756–22763

Broos K, Feys HB, De Meyer SF, Vanhoorelbeke K, Deckmyn H (2011) Platelets at work in primary hemostasis. Blood Rev 25:155–167

Broze GJ Jr, Girard TJ, Novotny WF (1990) Regulation of coagulation by a multivalent Kunitz-type inhibitor. Biochemistry 29:7539–7546

Camire RM, Pollak ES, Kaushansky K, Tracy PB (1998) Secretable human platelet-derived factor V originates from the plasma pool. Blood 92:3035–3041

Cattaneo M (2011) Bleeding manifestations of congenital and drug-induced defects of the platelet P2Y12 receptor for adenosine diphosphate. Thromb Haemost 105(Suppl 1):S67–S74

Cattaneo M, Lecchi A, Lombardi R, Gachet C, Zighetti ML (2000) Platelets from a patient heterozygous for the defect of P2CYC receptors for ADP have a secretion defect despite normal thromboxane A2 production and normal granule stores: further evidence that some cases of platelet 'primary secretion defect' are heterozygous for a defect of P2CYC receptors. Arterioscler Thromb Vasc Biol 20:E101–E106

Chauhan AK, Walsh MT, Zhu G, Ginsburg D, Wagner DD, Motto DG (2008) The combined roles of ADAMTS13 and VWF in murine models of TTP, endotoxemia, and thrombosis. Blood 111:3452–3457

Chen H, Locke D, Liu Y, Liu C, Kahn ML (2002) The platelet receptor GPVI mediates both adhesion and signaling responses to collagen in a receptor density-dependent fashion. J Biol Chem 277:3011–3019

Clauser S, Bachelot-Lozat C, Fontana P, Gaussem P, Remones V, Aiach M, Borgel D (2006) Physiological plasma Gas6 levels do not influence platelet aggregation. Arterioscler Thromb Vasc Biol 26:e22

Clemetson KJ (2007) A short history of platelet glycoprotein Ib complex. Thromb Haemost 98:63–68

Cohen I, Gerrard JM, White JG (1982) Ultrastructure of clots during isometric contraction. J Cell Biol 93:775–787

Cosemans JM, Van Kruchten R, Olieslagers S, Schurgers LJ, Verheyen FK, Munnix IC, Waltenberger J, Angelillo-Scherrer A, Hoylaerts MF, Carmeliet P, Heemskerk JW (2010) Potentiating role of Gas6 and Tyro3, Axl and Mer (TAM) receptors in human and murine platelet activation and thrombus stabilization. J Thromb Haemost 8:1797–1808

Davies MJ, Bland JM, Hangartner JR, Angelini A, Thomas AC (1989) Factors influencing the presence or absence of acute coronary artery thrombi in sudden ischaemic death. Eur Heart J 10:203–208

de Groot PG, Sixma JJ (2002) Platelet adhesion. In: Gresele P, Page CP, Fuster V, Vermylen J (eds) Platelets in thrombotic and non thrombotic disorders: pathophysiology, pharmacology and therapeutics. Cambridge University Press, Cambridge

Denis C, Methia N, Frenette PS, Rayburn H, Ullman-Cullere M, Hynes RO, Wagner DD (1998) A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. Proc Natl Acad Sci USA 95:9524–9529

Dong JF (2005) Cleavage of ultra-large von Willebrand factor by ADAMTS-13 under flow conditions. J Thromb Haemost 3:1710–1716

Du X (2007) Signaling and regulation of the platelet glycoprotein Ib-IX-V complex. Curr Opin Hematol 14:262–269

Duckers C, Simioni P, Spiezia L, Radu C, Dabrilli P, Gavasso S, Rosing J, Castoldi E (2010) Residual platelet factor V ensures thrombin generation in patients with severe congenital factor V deficiency and mild bleeding symptoms. Blood 115:879–886

Esmon CT (2003) The protein C pathway. Chest 124:26S–32S

French JE, Macfarlane RG, Sanders AG (1964) The structure of haemostatic plugs and experimental thrombi in small arteries. Br J Exp Pathol 45:467–474

Frenette PS, Johnson RC, Hynes RO, Wagner DD (1995) Platelets roll on stimulated endothelium in vivo: an interaction mediated by endothelial P-selectin. Proc Natl Acad Sci USA 92:7450–7454

Furie B, Furie BC (2005) Thrombus formation in vivo. J Clin Invest 115:3355–3362

Gay LJ, Felding-Habermann B (2011) Contribution of platelets to tumour metastasis. Nat Rev Cancer 11:123–134

Gayle RB 3rd, Maliszewski CR, Gimpel SD, Schoenborn MA, Caspary RG, Richards C, Brasel K, Price V, Drosopoulos JH, Islam N, Alyonycheva TN, Broekman MJ, Marcus AJ (1998) Inhibition of platelet function by recombinant soluble ecto-ADPase/CD39. J Clin Invest 101:1851–1859

Gruner S, Prostredna M, Schulte V, Krieg T, Eckes B, Brakebusch C, Nieswandt B (2003) Multiple integrin-ligand interactions synergize in shear-resistant platelet adhesion at sites of arterial injury in vivo. Blood 102:4021–4027

Hackeng TM, Sere KM, Tans G, Rosing J (2006) Protein S stimulates inhibition of the tissue factor pathway by tissue factor pathway inhibitor. Proc Natl Acad Sci USA 103:3106–3111

Hantgan RR, Endenburg SC, Cavero I, Marguerie G, Uzan A, Sixma JJ, de Groot PG (1992) Inhibition of platelet adhesion to fibrin(ogen) in flowing whole blood by Arg-Gly-Asp and fibrinogen gamma-chain carboxy terminal peptides. Thromb Haemost 68:694–700

Hindriks G, Ijsseldijk MJ, Sonnenberg A, Sixma JJ, de Groot PG (1992) Platelet adhesion to laminin: role of Ca2+ and Mg2+ ions, shear rate, and platelet membrane glycoproteins. Blood 79:928–935

Huizinga EG, Tsuji S, Romijn RA, Schiphorst ME, de Groot PG, Sixma JJ, Gros P (2002) Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. Science 297:1176–1179

Jung SM, Moroi M (2008) Platelet glycoprotein VI. Adv Exp Med Biol 640:53–63

Jurk K, Clemetson KJ, de Groot PG, Brodde MF, Steiner M, Savion N, Varon D, Sixma JJ, Van Aken H, Kehrel BE (2003) Thrombospondin-1 mediates platelet adhesion at high shear via glycoprotein Ib (GPIb): an alternative/backup mechanism to von Willebrand factor. FASEB J 17:1490–1492

Kato K, Kanaji T, Russell S, Kunicki TJ, Furihata K, Kanaji S, Marchese P, Reininger A, Ruggeri ZM, Ware J (2003) The contribution of glycoprotein VI to stable platelet adhesion and thrombus formation illustrated by targeted gene deletion. Blood 102:1701–1707

Kawamoto Y, Kaibara M (1990) Procoagulant activity of collagen. Effect of difference in type and structure of collagen. Biochim Biophys Acta 1035:361–368

Kim J, Zhang CZ, Zhang X, Springer TA (2010) A mechanically stabilized receptor-ligand flex-bond important in the vasculature. Nature 466:992–995

Kiyoi T, Tomiyama Y, Honda S, Tadokoro S, Arai M, Kashiwagi H, Kosugi S, Kato H, Kurata Y, Matsuzawa Y (2003) A naturally occurring Tyr143His alpha IIb mutation abolishes alpha IIb beta 3 function for soluble ligands but retains its ability for mediating cell adhesion and clot retraction: comparison with other mutations causing ligand-binding defects. Blood 101:3485–3491

Konstantinides S, Ware J, Marchese P, Almus-Jacobs F, Loskutoff DJ, Ruggeri ZM (2006) Distinct antithrombotic consequences of platelet glycoprotein Ibalpha and VI deficiency in a mouse model of arterial thrombosis. J Thromb Haemost 4:2014–2021

Lankhof H, van Hoeij M, Schiphorst ME, Bracke M, Wu YP, Ijsseldijk MJ, Vink T, de Groot PG, Sixma JJ (1996) A3 domain is essential for interaction of von Willebrand factor with collagen type III. Thromb Haemost 75:950–958

Leger AJ, Covic L, Kuliopulos A (2006) Protease-activated receptors in cardiovascular diseases. Circulation 114:1070–1077

Lisman T, Raynal N, Groeneveld D, Maddox B, Peachey AR, Huizinga EG, de Groot PG, Farndale RW (2006) A single high-affinity binding site for von Willebrand factor in collagen III, identified using synthetic triple-helical peptides. Blood 108:3753–3756

Lubnitzky S (1885) Die Zusammensetzung des Thrombus in Arterienwunden in den ersten funf Tagen. Naunyn-Schmeidebergs Arch Pharmacol 19:185

Luo SZ, Mo X, Afshar-Kharghan V, Srinivasan S, Lopez JA, Li R (2007) Glycoprotein Ibalpha forms disulfide bonds with 2 glycoprotein Ibbeta subunits in the resting platelet. Blood 109:603–609

Maroney SA, Haberichter SL, Friese P, Collins ML, Ferrel JP, Dale GL, Mast AE (2007) Active tissue factor pathway inhibitor is expressed on the surface of coated platelets. Blood 109:1931–1937

Maynard DM, Heijnen HF, Gahl WA, Gunay-Aygun M (2010) The alpha-granule proteome: novel proteins in normal and ghost granules in gray platelet syndrome. J Thromb Haemost 8:1786–1796

Muller F, Mutch NJ, Schenk WA, Smith SA, Esterl L, Spronk HM, Schmidbauer S, Gahl WA, Morrissey JH, Renne T (2009) Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. Cell 139:1143–1156

Nachman RL, Rafii S (2008) Platelets, petechiae, and preservation of the vascular wall. N Engl J Med 359:1261–1270

Nesbitt WS, Westein E, Tovar-Lopez FJ, Tolouei E, Mitchell A, Fu J, Carberry J, Fouras A, Jackson SP (2009) A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. Nat Med 15:665–673

Newman PJ, Newman DK (2003) Signal transduction pathways mediated by PECAM-1: new roles for an old molecule in platelet and vascular cell biology. Arterioscler Thromb Vasc Biol 23:953–964

Nieswandt B, Varga-Szabo D, Elvers M (2009) Integrins in platelet activation. J Thromb Haemost 7(Suppl 1):206–209

Nieswandt B, Pleines I, Bender M (2011) Platelet adhesion and activation mechanisms in arterial thrombosis and ischaemic stroke. J Thromb Haemost 9(Suppl 1):92–104

Niewiarowski S, Bankowski E, Fiedoruk T (1964) Adsorption of Hageman factor (Factor XII) on collagen. Experientia 20:367–368

Noe L, Peeters K, Izzi B, Van Geet C, Freson K (2010) Regulators of platelet cAMP levels: clinical and therapeutic implications. Curr Med Chem 17:2897–2905

Nurden AT (2011) Platelets, inflammation and tissue regeneration. Thromb Haemost 105(Suppl 1):S13–S33

Nurden A, Nurden P (2011) Advances in our understanding of the molecular basis of disorders of platelet function. J Thromb Haemost 9(Suppl 1):76–91

Offermanns S (2006) Activation of platelet function through G protein-coupled receptors. Circ Res 99:1293–1304

Palmer RM, Ferrige AG, Moncada S (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327:524–526

Prevost N, Woulfe DS, Tognolini M, Tanaka T, Jian W, Fortna RR, Jiang H, Brass LF (2004) Signaling by ephrinB1 and Eph kinases in platelets promotes Rap1 activation, platelet adhesion, and aggregation via effector pathways that do not require phosphorylation of ephrinB1. Blood 103:1348–1355

Prevost N, Woulfe DS, Jiang H, Stalker TJ, Marchese P, Ruggeri ZM, Brass LF (2005) Eph kinases and ephrins support thrombus growth and stability by regulating integrin outside-in signaling in platelets. Proc Natl Acad Sci USA 102:9820–9825

Pries AR, Secomb TW, Jacobs H, Sperandio M, Osterloh K, Gaehtgens P (1997) Microvascular blood flow resistance: role of endothelial surface layer. Am J Physiol 273:H2272–H2279

Pugh N, Simpson AM, Smethurst PA, de Groot PG, Raynal N, Farndale RW (2010) Synergism between platelet collagen receptors defined using receptor-specific collagen-mimetic peptide substrata in flowing blood. Blood 115:5069–5079

Ramesh S, Morrell CN, Tarango C, Thomas GD, Yuhanna IS, Girardi G, Herz J, Urbanus RT, de Groot PG, Thorpe PE, Salmon JE, Shaul PW, Mineo C (2011) Antiphospholipid antibodies promote leukocyte-endothelial cell adhesion and thrombosis in mice by antagonizing eNOS via beta2GPI and apoER2. J Clin Invest 121:120–131

Reitsma S, Oude Egbrink MG, Heijnen VV, Megens RT, Engels W, Vink H, Slaaf DW, van Zandvoort MA (2011) Endothelial glycocalyx thickness and platelet-vessel wall interactions during atherogenesis. Thromb Haemost 106(5):939–46. doi:10.1160/TH11-02-0133

Renne T, Pozgajova M, Gruner S, Schuh K, Pauer HU, Burfeind P, Gailani D, Nieswandt B (2005) Defective thrombus formation in mice lacking coagulation factor XII. J Exp Med 202:271–281

Rosen ED, Gailani D, Castellino FJ (2002) FXI is essential for thrombus formation following FeCl3-induced injury of the carotid artery in the mouse. Thromb Haemost 87:774–776

Ross R, Glomset JA (1976) The pathogenesis of atherosclerosis (first of two parts). N Engl J Med 295:369–377

Ruggeri ZM (2000) Old concepts and new developments in the study of platelet aggregation. J Clin Invest 105:699–701

Ruiz FA, Lea CR, Oldfield E, Docampo R (2004) Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. J Biol Chem 279:44250–44257

Sabrkhany S, Griffioen AW, Oude Egbrink MG (2011) The role of blood platelets in tumor angiogenesis. Biochim Biophys Acta 1815:189–196

Sadler JE (2005) von Willebrand factor: two sides of a coin. J Thromb Haemost 3:1702–1709

Sakariassen KS, Bolhuis PA, Sixma JJ (1979) Human blood platelet adhesion to artery subendothelium is mediated by factor VIII-Von Willebrand factor bound to the subendothelium. Nature 279:636–638

Santoro SA (1986) Identification of a 160,000 dalton platelet membrane protein that mediates the initial divalent cation-dependent adhesion of platelets to collagen. Cell 46:913–920

Savage B, Saldivar E, Ruggeri ZM (1996) Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. Cell 84:289–297

Savage B, Almus-Jacobs F, Ruggeri ZM (1998) Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. Cell 94:657–666

Savage B, Sixma JJ, Ruggeri ZM (2002) Functional self-association of von Willebrand factor during platelet adhesion under flow. Proc Natl Acad Sci USA 99:425–430

Semple JW, Italiano JE Jr, Freedman J (2011) Platelets and the immune continuum. Nat Rev Immunol 11:264–274

Sixma JJ, van den Berg A (1984) The haemostatic plug in haemophilia A: a morphological study of haemostatic plug formation in bleeding time skin wounds of patients with severe haemophilia A. Br J Haematol 58:741–753

Sixma JJ, Sakariassen KS, Beeser-Visser NH, Ottenhof-Rovers M, Bolhuis PA (1984) Adhesion of platelets to human artery subendothelium: effect of factor VIII-von Willebrand factor of various multimeric composition. Blood 63:128–139

Smith SA, Morrissey JH (2008) Polyphosphate enhances fibrin clot structure. Blood 112:2810–2816

Smith SA, Mutch NJ, Baskar D, Rohloff P, Docampo R, Morrissey JH (2006) Polyphosphate modulates blood coagulation and fibrinolysis. Proc Natl Acad Sci USA 103:903–908

Smith SA, Choi SH, Davis-Harrison R, Huyck J, Boettcher J, Rienstra CM, Morrissey JH (2010) Polyphosphate exerts differential effects on blood clotting, depending on polymer size. Blood 116:4353–4359

Stalker TJ, Wu J, Morgans A, Traxler EA, Wang L, Chatterjee MS, Lee D, Quertermous T, Hall RA, Hammer DA, Diamond SL, Brass LF (2009) Endothelial cell specific adhesion molecule (ESAM) localizes to platelet-platelet contacts and regulates thrombus formation in vivo. J Thromb Haemost 7:1886–1896

Stegner D, Nieswandt B (2011) Platelet receptor signaling in thrombus formation. J Mol Med (Berl) 89:109–121

Stitham J, Arehart EJ, Gleim SR, Douville KL, Hwa J (2007) Human prostacyclin receptor structure and function from naturally-occurring and synthetic mutations. Prostaglandins Other Lipid Mediat 82:95–108

Stitham J, Arehart E, Elderon L, Gleim SR, Douville K, Kasza Z, Fetalvero K, MacKenzie T, Robb J, Martin KA, Hwa J (2011) Comprehensive biochemical analysis of rare prostacyclin receptor variants: study of association of signaling with coronary artery obstruction. J Biol Chem 286:7060–7069

Storey RF (2011) New P2Y inhibitors. Heart 97:1262–1267
Suzuki J, Umeda M, Sims PJ, Nagata S (2010) Calcium-dependent phospholipid scrambling by TMEM16F. Nature 468:834–838
Thiery JP, Bessis M (1956) Mechanism of platelet genesis; in vitro study by cinemicrophotography. Rev Hematol 11:162–174
Tucker EI, Marzec UM, White TC, Hurst S, Rugonyi S, McCarty OJ, Gailani D, Gruber A, Hanson SR (2009) Prevention of vascular graft occlusion and thrombus-associated thrombin generation by inhibition of factor XI. Blood 113:936–944
Ulrichts H, Udvardy M, Lenting PJ, Pareyn I, Vandeputte N, Vanhoorelbeke K, Deckmyn H (2006) Shielding of the A1 domain by the D'D3 domains of von Willebrand factor modulates its interaction with platelet glycoprotein Ib-IX-V. J Biol Chem 281:4699–4707
van Hinsbergh VW (2012) Endothelium-role in regulation of coagulation and inflammation. Semin Immunopathol 34(1):93–106. doi:10.1007/s00281-011-0285-5
Wang X, Smith PL, Hsu MY, Gailani D, Schumacher WA, Ogletree ML, Seiffert DA (2006) Effects of factor XI deficiency on ferric chloride-induced vena cava thrombosis in mice. J Thromb Haemost 4:1982–1988
Ward CM, Kestin AS, Newman PJ (2000) A Leu262Pro mutation in the integrin beta(3) subunit results in an alpha(IIb)-beta(3) complex that binds fibrin but not fibrinogen. Blood 96:161–169
Ware J, Russell S, Ruggeri ZM (2000) Generation and rescue of a murine model of platelet dysfunction: the Bernard-Soulier syndrome. Proc Natl Acad Sci USA 97:2803–2808
Watson SP, Herbert JM, Pollitt AY (2010) GPVI and CLEC-2 in hemostasis and vascular integrity. J Thromb Haemost 8:1456–1467
Weiss HJ, Turitto VT, Baumgartner HR (1991) Further evidence that glycoprotein IIb-IIIa mediates platelet spreading on subendothelium. Thromb Haemost 65:202–205
White JG (2002) Morphology and ultrastructure of platelets. In: Gresele P, Page CP, Fuster V, Vermylen J (eds) Platelets in thrombotic and non-thrombotic disorders: pathophysiology, pharmacology and therapeutics. Cambridge University Press, Cambridge
White-Adams TC, Berny MA, Tucker EI, Gertz JM, Gailani D, Urbanus RT, de Groot PG, Gruber A, McCarty OJ (2009) Identification of coagulation factor XI as a ligand for platelet apolipoprotein E receptor 2 (ApoER2). Arterioscler Thromb Vasc Biol 29:1602–1607
Wilner GD, Nossel HL, LeRoy EC (1968) Activation of Hageman factor by collagen. J Clin Invest 47:2608–2615
Yeaman MR (2010) Platelets in defense against bacterial pathogens. Cell Mol Life Sci 67:525–544
Yuhki K, Kashiwagi H, Kojima F, Kawabe J, Ushikubi F (2010) Roles of prostanoids in the pathogenesis of cardiovascular diseases. Int Angiol 29:19–27
Yuhki K, Kojima F, Kashiwagi H, Kawabe J, Fujino T, Narumiya S, Ushikubi F (2011) Roles of prostanoids in the pathogenesis of cardiovascular diseases: novel insights from knockout mouse studies. Pharmacol Ther 129:195–205
Zhang H, Lowenberg EC, Crosby JR, MacLeod AR, Zhao C, Gao D, Black C, Revenko AS, Meijers JC, Stroes ES, Levi M, Monia BP (2010) Inhibition of the intrinsic coagulation pathway factor XI by antisense oligonucleotides: a novel antithrombotic strategy with lowered bleeding risk. Blood 116:4684–4692
Zhu L, Bergmeier W, Wu J, Jiang H, Stalker TJ, Cieslak M, Fan R, Boumsell L, Kumanogoh A, Kikutani H, Tamagnone L, Wagner DD, Milla ME, Brass LF (2007) Regulated surface expression and shedding support a dual role for semaphorin 4D in platelet responses to vascular injury. Proc Natl Acad Sci USA 104:1621–1626

Platelets in Atherosclerosis and Thrombosis

Christian Schulz and Steffen Massberg

Contents

Abstract Rupture of an atherosclerotic plaque exposes a thrombogenic matrix, which instantly triggers platelet tethering and activation. We here delineate the sequence of events during arterial thrombus formation and dissect the specific role of the various platelet receptors in this process. We also discuss the interplay of platelets with circulating immune cells, which support arterial thrombosis by fibrin formation in a process that involves extracellular nucleosomes. In the second part of this chapter we describe the role of platelets in atherosclerotic lesion formation. Platelets adhere to the dysfunctional endothelium early during atherogenesis. They contain a large machinery of proinflammatory molecules, which can be released upon their activation. This prepares the ground for subsequent leukocyte recruitment and infiltration, and boosts the inflammatory process of the arterial wall. Together, platelets play a critical role in both acute and chronic processes of the

C. Schulz • S. Massberg (✉)
Deutsches Herzzentrum and I. Medizinische Klinik, Klinikum rechts der Isar, Technische Universitaet Muenchen, Munich, Germany
e-mail: chrisschulz@mytum.de; massberg@idi.harvard.edu

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_5,

vascular wall, which makes them an attractive target for pharmacological strategies to treat arterial thrombosis and, potentially, also atheroprogression.

Keywords Platelets • Plaque rupture • Adhesion • Thrombosis • Innate immune cells • Atherogenesis • Inflammation • Cytokines

Beyond their physiological implication in haemostasis, platelets are of major importance in the pathophysiology of various diseases. Studies over the last decades have revealed their critical role in thrombotic disorders (Furie and Furie 2008), cancer metastasis (Gasic et al. 1968) and inflammatory diseases, such as atherosclerosis (Massberg et al. 2002; Huo et al. 2003), acute lung injury (Zarbock et al. 2006a), autoimmune disease (Boilard et al. 2010), and sepsis (Klinger and Jelkmann 2002; Levi 2005). In this chapter, we address how platelets initiate and promote atherosclerotic lesion formation, and the mechanisms underlying arterial thrombosis following plaque rupture. Atherothrombosis manifests clinically as myocardial infarction, stroke, and peripheral occlusive diseases, which have major impact on mortality and morbidity worldwide. Understanding of these principals is, thus, a prerequisite for successful therapy and future studies.

1 Molecular Mechanisms of Platelet Accumulation Following Atherosclerotic Plaque Rupture

The primary trigger of arterial thrombosis is rupture of an atherosclerotic plaque. Under physiological conditions, platelets circulate within the vasculature without significant interactions with the vascular wall. The endothelium inhibits platelet activation and adhesion by releasing vasoactive substances such as nitric oxide (NO) and prostacyclin (PGI2) (Radomski et al. 1987). Disruption of endothelial integrity, however, leads to exposure of the thrombogenic subendothelial matrix, which consists mainly of collagen and proteoglycans, but also fibronectin and laminin as non-collagenic adhesion proteins. Fibrillar collagen is of major importance for platelet adhesion and aggregation at sites of vascular injury (Massberg et al. 2003; Schonberger et al. 2008; Kuijpers et al. 2007). In addition, endothelium-derived von Willebrand factor (VWF) binds to exposed subendothelial collagen and provides a thrombogenic matrix for arriving platelets.

Composition of the subendothelial matrix changes profoundly during the development of an atherosclerotic lesion. In the plaque numerous platelet-adhesive and activating molecules such as fibrin/fibrinogen, fibronectin, vitronectin, thrombospondin, oxidized LDL, lysophosphatidic acid and tissue factor (TF) accumulate (De Meyer et al. 1999; Dietrich et al. 2007; Siess et al. 1999; Mackman 2004). In addition, a wide spectrum of leucocyte subsets (Galkina and Ley 2007), lipids (Guyton and Klemp 1996), and cell debris (Lusis 2000) are found, which engage with platelets (Seizer et al. 2010; Langer et al. 2007). Collagen types I and III

represent 80%–90% of total collagenous protein in atherosclerotic lesions (Katsuda and Kaji 2003). While collagen type I heavily accumulates within the fibrous cap that overlies the lipid-rich core, collagen type III appears to be predominant at the plaque/thrombus interface in regions of plaque erosion (Fernandez-Ortiz et al. 1994; Schulz et al. 2008a). Interestingly, expression and arrangement of collagen subtypes not only determines plaque stability but also influences the type of adhesion receptors utilized by platelets during arterial thrombosis (Schulz et al. 2008a; Penz et al. 2005).

1.1 Tethering and Initial Activation of Platelets

Several sequential steps are required to allow platelet accumulation following rupture of an atherosclerotic plaque. Initially, platelets tether to the vessel wall; they subsequently become activated and, finally, adhere and aggregate to the site of injury (Fig. 1). The different steps within the interaction cascade of blood platelets at the vessel wall are mediated by a distinct set of adhesion molecules. Their role is closely linked to the prevailing shear stress. Because platelet adhesion to the ruptured atherosclerotic lesion occurs under the conditions of arterial shear stress, high-affinity interactions between platelet adhesion receptors and ligands within the subendothelial layer are required to promote tethering to the injured vessel wall. Notably, wall shear rates of up to 10,000 s^{-1} have been measured in the coronary vasculature occluded by 50%, and may even exceed 50,000 s^{-1} in severe stenosis (Strony et al. 1993; Mailhac et al. 1994). Platelet GPIb–V–IX is a critical receptor in establishing the first contact of platelets with a vascular lesion at high shear conditions by binding to immobilized VWF (Ruggeri 2007; Savage et al. 1996) (Fig. 1). Vessel wall VWF derives from endothelial cells, but VWF is also generated by megakaryocytes and contained in platelets. It is secreted from the endothelium towards the subendothelial matrix and into the vessel lumen, and contributes to circulating VWF. VWF multimers become immobilized to collagen fibrils via the A3 domain at the site of vascular injury, which allows the subsequent binding of platelets via GPIb–V–IX interacting with the VWF A1 domain (Ruggeri 2007). The interaction of GPIb–V–IX and VWF is characterized by a fast association rate that can tether platelets to the exposed subendothelium at high shear rates. It is supported by the multimeric nature of VWF, which provides a high local density of active A1 domain sites and forms multiple bonds. The size of VWF multimers is closely associated with thrombogenic functions, and is modulated by differential cleavage (Ruggeri 2004). Surprisingly, thrombus formation in $VWF^{-/-}$ mice is delayed but not absent (Dubois et al. 2007; Bergmeier et al. 2006). Thus, ligand(s) other then VWF seem to exist that alternatively mediate GPIbα-dependent platelet adhesion under arterial flow conditions. A likely candidate is thrombospondin-1, which is highly expressed in atherosclerotic lesions, and was recently shown to mediate platelet adhesion in vitro at shear rates of up to 4,000 s^{-1} (Jurk et al. 2003).

Another platelet membrane receptor, the collagen receptor glycoprotein VI (GPVI), is involved in platelet tethering following vascular injury (Fig. 1). Unlike GPIb–V–IX, GPVI binds directly to subendothelial collagen and mediates the activation of different platelet adhesion receptors required for subsequent stable arrest. Platelet collagen receptors are divided into those that directly interact with collagen, including GPVI, the integrin α2β1, and CD36, and those that interact indirectly through collagen-bound VWF, including GPIbα and the integrin αIIbβ3 (Clemetson and Clemetson 2001). Platelet GPVI has been identified as the major direct platelet collagen receptor (Massberg et al. 2003; Moroi et al. 1996, 1989a; Kuijpers et al. 2003). It represents a 60- to 65-kDa type I transmembrane glycoprotein that belongs to the immunoglobulin superfamily (Jandrot-Perrus et al. 2000). In humans, GPVI forms a complex with the Fc receptor γ-chain on the cell surface (Gibbins et al. 1997; Zheng et al. 2001). Ligand binding to GPVI triggers tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif of the Fc receptor γ-chain, which initiates downstream signalling and platelet activation. This is a critical step that is mandatory for subsequent platelet adhesion. Importantly, stimulation of GPVI induces platelet activation that leads to secretion and inside-out signalling of the integrins α2β1 and αIIbβ3. This stabilizes platelet interaction with the arterial wall and mediates platelet aggregation (Cabeza et al. 2004; Nieswandt and Watson 2003). Platelets deficient in GPVI show loss of collagen-induced adhesion and aggregation in vitro (Moroi et al. 1989a; Sugiyama et al. 1987). Likewise, functional blocking with anti-GPVI monoclonal antibodies attenuates ex vivo platelet aggregation in response to collagen and collagen-related peptides, which mimic the collagen triple helix (Sugiyama et al. 1993; Schulte et al. 2001). GPVI is a major determinant of arterial thrombus formation in vivo, and platelet tethering at sites of arterial injury, as well as firm adhesion, is almost abolished in the absence of functional GPVI (Massberg et al. 2003).

GPIb–V–IX and VWF are both critical for the first contact of platelets with the vascular lesion. This interaction is insufficient to provide stable bonds, which are required for firm platelet arrest at physiological shear rates. However, binding via GPIbα also induces a shift in the activation status of platelet integrins from low to high affinity, which is important for platelet aggregate formation (Ruggeri 2007). Likewise, GPVI induces inside-out signalling following collagen binding (Jung and Moroi 1998; Nieswandt et al. 2001). Platelets lacking GPVI cannot activate integrins and fail to adhere to and aggregate on collagen under flow conditions in vitro (Nieswandt et al. 2001). Of note, at very high shear stress above 10,000 s^{-1}, as seen in a stenotic vessel, platelet aggregation does not require integrin activation and is solely dependent on GPIbα (Ruggeri 2007; Bergmeier et al. 2006; Reininger et al. 2006).

1.2 Firm Adhesion and Aggregation of Platelets

Integrins are heterodimeric transmembrane receptors composed of α and β subunits. Platelets contain the β1 integrins α2β1 (collagen receptor), α5β1

(fibronectin receptor), and α6β1 (laminin receptor), and the β3 integrins αIIbβ3 (fibrinogen receptor) and αvβ3 (vitronectin receptor) (Bennett 2005). However, among the two β3 integrins expressed by platelets, only αIIbβ3 is present with a high copy number of approximately 80,000 per platelet (Wagner et al. 1996). It binds to ligands containing an RGD sequence that includes fibrin/fibrinogen (main ligand), fibronectin, thrombospondin, vitronectin, and VWF. Upon activation, ligand-binding sites in the region of the integrin complex shift to a high-affinity state. This is critical for stable platelet arrest (Jung and Moroi 1998; Nieswandt et al. 2001; Kasirer-Friede et al. 2002). The binding of αIIbβ3 is strongly dependent on Ca^{2+} and leads to formation of platelet aggregates by the bridging of adjacent platelets. Importantly, the initial binding of fibrinogen to αIIbβ3 is a reversible process that is followed by an irreversible stabilization of the fibrinogen linkage to the αIIbβ3 complex. Different ligands bind to β3 integrins depending on the prevailing shear conditions and VWF appears most important at high shear rates (Ruggeri 2007). In contrast to αIIbβ3, the integrin αvβ3 is only present in low numbers on platelets (few hundred copies per platelet). Although αvβ3 has been shown to mediate platelet adhesion to osteopontin and vitronectin in vitro (Bennett et al. 1997), its role for arterial thrombosis remains elusive (Bennett 2005).

β1 integrins mediate platelet adhesion to the matrix proteins collagen, fibronectin and laminin. Following platelet tethering and activation, they act in concert with αIIbβ3 integrins, to promote the stable arrest of platelets. α2β1 facilitates the function of GPVI in the platelet activation process that leads to thrombus formation. However, α2β1 integrin is not indispensable for arterial thrombosis. Indeed, GPVI-induced platelet aggregation remains present even in the functional absence of this integrin (Kuijpers et al. 2003), and α2β1-deficient animals do not present with bleeding abnormalities (Holtkotter et al. 2002).

1.3 Amplification of Platelet Activation

After initiation of platelet adhesion and aggregation, platelets form and release various molecules that not only reinforce platelet thrombus formation but can also contribute to coagulation or control of blood flow (see also chapter on primary haemostasis). ADP and thromboxane (Tx)A2 are released from platelets into the extracellular space during thrombus formation. They activate surrounding platelets through G-protein-coupled receptors by paracrine and autocrine mechanisms and trigger inside-out signalling to integrins (Offermanns 2006). In addition, locally produced pro-thrombotic factors, such as thrombin, critically contribute to platelet activation and aggregation. By these means, circulating, yet non-activated platelets become recruited into the lesion site where they aggregate with adhering platelets and contribute to thrombus growth. Furthermore, TxA2 has vasoconstricting activity and thus supports thrombus formation by reducing blood flow velocity.

Whereas the latter mediators induce strong platelet activation and aggregation, not all platelets necessarily undergo rapid shape change during thrombus formation.

In fact, platelets can associate with the growing thrombus even in the absence of intracellular calcium, an essential mediator of platelet activation (Furie and Furie 2007). A recent study using high-resolution imaging revealed that discoid non-activated platelets, which form filamentous membrane tethers in a shear-dependent manner, translocate over firmly-adherent platelets in vivo (Nesbitt et al. 2009). Importantly, these discoid platelets can accumulate in high numbers at the distal site of the thrombus where lower shear rates prevail. Here, they remain available and can become activated and recruited into the growing thrombus. In addition to platelet-activating molecules secreted by platelets themselves, the constituents of atheromatous plaque, for example chemokines such as stromal cell-derived factor (SDF)-1α (CXCL12), (Abi-Younes et al. 2000) can be released into the lumen of the blood vessel to promote platelet activation.

Of the different platelet-activating molecules, GPVI has emerged as a highly attractive target for specific antiplatelet therapy in arterial thrombosis. As summarized above, GPVI is the major platelet collagen receptor, and genetic ablation or its pharmacological inhibition can diminish arterial thrombus formation. Importantly, absence of GPVI results in a relatively mild bleeding phenotype in humans (Moroi et al. 1989b; Dumont et al. 2009), as compared to other inherited disorders (Salles et al. 2008). While GPVI-deficient patients lack collagen-induced aggregation and adhesion, they show only mild bleeding time prolongation and normal platelet aggregation (Moroi et al. 1989a). Clinical studies targeting GPVI have therefore been initiated (Ungerer et al. 2011). Another pharmacological strategy recently introduced has been by harnessing antibodies against platelet GPIIIa. These single-chain fragments bind to and dissolve platelet thrombi without affecting integrin function (Zhang et al. 2010). Such antibodies could have beneficial effects through αIIbβ3 inhibition without haemorrhagic complications (Nieswandt et al. 2011).

1.4 Crosstalk Between Platelets, Innate Immunity and Coagulation

After activation, platelets release a set of potent chemokines and inflammatory mediators (also see Fig. 4 below). Through the release of chemokines, but also by the expression of adhesion receptors such as P-selectin, platelets recruit leucocytes, particularly monocytes and neutrophils, into growing thrombi (Fig. 1). Various ligand–receptor pairs have been identified that can mediate platelet-leucocyte-aggregate formation. Firm binding of monocytes and polymorphonuclear neutrophils to platelets has been attributed to the interaction of P-selectin with P-selectin glycoprotein ligand-1 (PSGL-1); binding of the β2 integrin CD11b (MAC-1) to platelet GPIbα; and to a fibrinogen bridged binding mechanism of CD11b (and also cellular adhesion molecules, i.e. ICAM-1) to platelet αIIbβ3 (von Hundelshausen et al. 2009; Zarbock et al. 2006b, 2007; Rinder et al. 1991; Romo

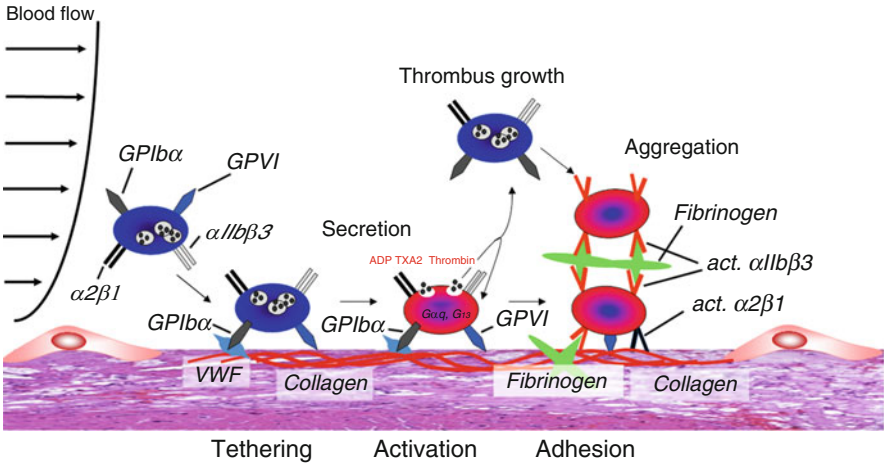


Fig. 1 Mechanisms of platelet recruitment following plaque rupture

et al. 1999). In vitro, blocking P-selectin on platelets or its leucocyte-expressed ligand PSGL-1 inhibits about half of the firm adhesion of monocytes to surface-bound platelets under arterial flow conditions (Kuijper 1998). This supports the current paradigm that, in addition to P-selectin–PSGL-1 interactions, additional (multiple) adhesion receptors are involved in firm adhesion of leucocytes to surface-bound platelets. More recently, other molecules involved in leucocyte adhesion to platelets have also been described. These include the junctional adhesion molecule (JAM)-A (F11R, CD321) and EMMPRIN (CD147). JAM-A is a ligand of the β2 integrin CD11a/CD18 (LFA-1) (Ostermann et al. 2002), while leucocyte EMMPRIN binds to the platelet GPVI receptor (Schulz et al. 2011). Interestingly, both JAM-A and EMMPRIN are expressed by platelets and leucocytes. This may lead to homotypical cell interactions depending on the prevailing shear stress (Ostermann et al. 2002; Schulz et al. 2011). However, homotypical binding of platelets and leucocytes is less likely to occur at high shear stress (Schulz et al. 2011).

Innate immune cells are essential for thrombus growth and thrombus stabilization (Fig. 2). They co-operate with platelets to trigger fibrin formation. Several leucocyte subsets, particularly monocytes, but also microparticles and platelets, express tissue factor (TF) (Mackman 2004). TF initiates the blood coagulation cascade and drives the formation of thrombin and fibrin—a step that is indispensable to thrombus stabilization and its growth. TF-dependent fibrin production is supported by the release of thiol isomerase enzymes, namely protein disulfide isomerase (PDI), and also ERp57, from activated platelets (Reinhardt et al. 2008; Schulz et al. 2010; Holbrook et al. 2010). They trigger conversion of TF from the functionally inactive to the active form (Reinhardt et al. 2008; Schulz et al. 2010). This process, referred to as TF decryption, represents an initial step in coagulation activation. Although other sources of both tissue factors (e.g. microparticles, monocytes) and thiol isomerases (e.g. endothelium) exist that have an important

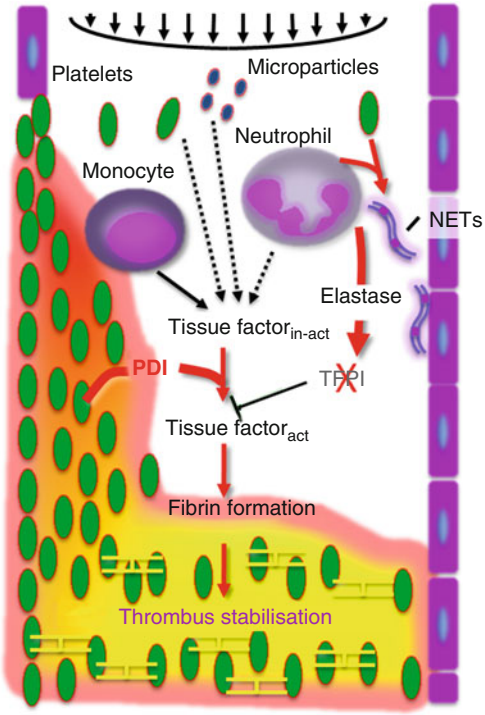


Fig. 2 Crosstalk between thrombosis and inflammation

role for fibrin generation and thrombus formation in vivo (Zwicker et al. 2011; Jasuja et al. 2010), we have recently shown that platelet-derived PDI supports TF-dependent blood coagulation at sites of vascular injury in vivo (Reinhardt et al. 2008). In addition, neutrophils recruited in a platelet-dependent manner release serine proteases including elastase, which degrade tissue factor pathway inhibitor (TFPI), a highly potent endogenous antagonist to TF (Massberg et al. 2010). This further fosters local fibrin formation (Fig. 2).

Finally, platelets support the release of nuclear extracellular traps (NETs) by neutrophils within thrombi. NETs are meshworks of DNA fibres that comprise histones and antimicrobial proteins that act together to provide prothrombotic scaffolds that support fibrin formation in vitro (Fuchs et al. 2010) and in large arteries in vivo (Massberg et al. 2010) (Fig. 2).

In conclusion, platelets, inflammation and coagulation appear important processes for thrombus formation following plaque rupture. Platelets not only initiate thrombus formation, but also orchestrate the subsequent leucocyte accumulation during coagulation. Indeed, a growing body of evidence now suggests that targeting platelets, and specifically platelet adhesion, could be an effective strategy for the treatment of atherothrombosis. In concordance with findings from experimental models (Kuijpers et al. 2009; Reininger et al. 2010), large-scale clinical trials

have demonstrated superiority of antiplatelet treatments over conventional anticoagulation therapy following coronary artery intervention (Schomig et al. 1996; Bertrand et al. 1998; Leon et al. 1998; Urban et al. 1998).

2 Platelets in Atherosclerotic Lesion Development

Increasing evidence now suggests that platelets, as well as being cellular mediators of thrombotic events that occur late in atherosclerosis, also play a role very early in the disease process.

2.1 Platelet Adhesion to the Dysfunctional Endothelium

In the early stages of atherosclerotic lesion formation, inflammation of the arterial wall impairs endothelial function and initiates recruitment of platelets and leucocytes (Lusis 2000; Ross 1999) (Fig. 3). Notably, platelets can adhere firmly to the inflamed endothelium in the absence of endothelial disruption (Frenette et al. 1995; Massberg et al. 1998). This interaction involves platelet αIIbβ3 and GPIbα, as well as endothelial ICAM-1 and αvβ3 integrin interactions (Gawaz et al. 1997; Bombeli et al. 1998; Meyer dos Santos et al. 2011; Schulz et al. 2007; Massberg et al. 2002, 2005). Fibrinogen, fibronectin, and VWF function as bridging molecules for αIIbβ3-mediated adhesion (Bombeli et al. 1998). In addition, a wide range of adhesion and platelet-activating molecules are expressed on the endothelial surface including: selectins (P-, E-selectin), cellular adhesion molecules (ICAM, VCAM, PECAM), and chemokines (Wagner and Frenette 2008). These support the recruitment of circulating platelets. P-selectin, for example, plays a role in transient platelet adhesion to the endothelium (Frenette et al. 1995). PECAM, in contrast, can support firm adhesion (Rosenblum et al. 1996) while endothelium-bound Fractalkine (CX3CL1) has been shown to activate adherent platelets in vitro (Meyer dos Santos et al. 2011; Schulz et al. 2007). Proinflammatory mediators are expressed on dysfunctional endothelium at early stages of atherogenesis (Cybulsky and Gimbrone 1991; Cottone et al. 2007; Szmitko et al. 2003; Zibara et al. 2000). In parallel, local production of endothelium-derived platelet inhibitors, such as NO and PGI2, is impaired (Busse et al. 1993). This further enhances platelet activation and accumulation at the arterial wall.

It is now widely accepted that platelet recruitment and secretion are critical events that not only lead to the clinical manifestation of atherothrombosis, but also initiate and maintain the chronic-inflammatory processes that leads to the formation of an atherosclerotic lesion. Application of intravital microscopy in mouse models of atherosclerosis shows that platelet adhesion occurs even before the first appearance of atherosclerotic lesions (Massberg et al. 2002). Platelet recruitment also clearly precedes firm adhesion of leucocytes (Massberg et al. 2002). When adhered to endothelial surfaces, platelets support leucocyte adhesion by surface expression

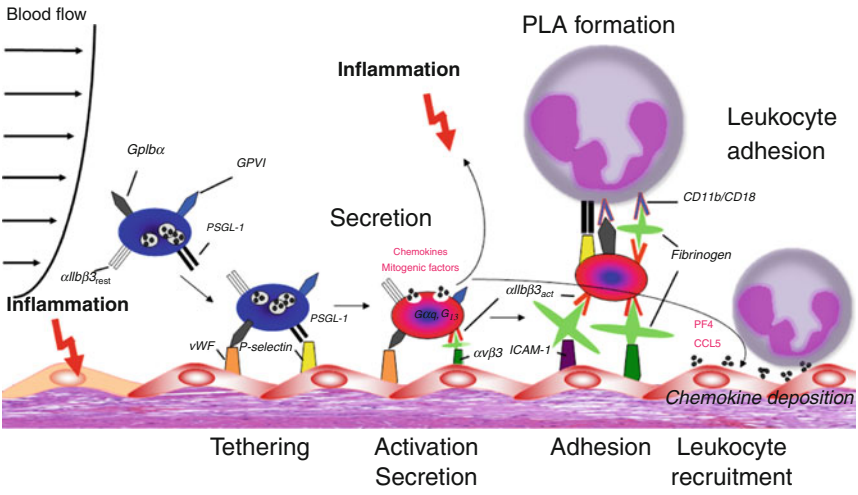


Fig. 3 Mechanisms of platelet recruitment to dysfunctional endothelial cells

of adhesion molecules such as P-selectin (Kuijper 1998; Schulz et al. 2007; Zwaginga et al. 1999) (Fig. 3).

2.2 Proinflammatory Machinery of Platelets

Platelets contain a machinery of bioactive mediators, inflammatory molecules and growth factors, which can drive or support inflammatory processes in the arterial wall (Fig. 4). Activation of adherent platelets leads to liberation of their cellular content, which drives the inflammatory process in the arterial wall in various ways:

1. Platelets present and deliver chemokines to the luminal surface of the vessel wall, which triggers arrest of leucocytes (Huo et al. 2003; von Hundelshausen et al. 2001). Platelet-derived inflammatory mediators were also found in atherosclerotic plaque (von Hundelshausen et al. 2001; Schober et al. 2002; Pitsilos et al. 2003).
2. Interaction with platelets triggers an inflammatory response of the endothelium that results in the surface expression of adhesion molecules and secretion of chemokines (Gawaz et al. 1998, 2000, 2002; Henn et al. 1998). In this manner, platelets modulate the chemotactic and adhesive properties of the vascular wall. This can subsequently lead to leucocyte recruitment, cell proliferation and the migration of inflammatory cells into plaque tissue.
3. Platelet-derived activating factors, most prominently ADP and TxA2 but also chemokines (Massberg et al. 2006), are secreted and stimulate surrounding platelets in a paracrine (autocrine) manner. This fosters platelet aggregate formation and contributes to the release of stored cytokines and mitogenic factors.

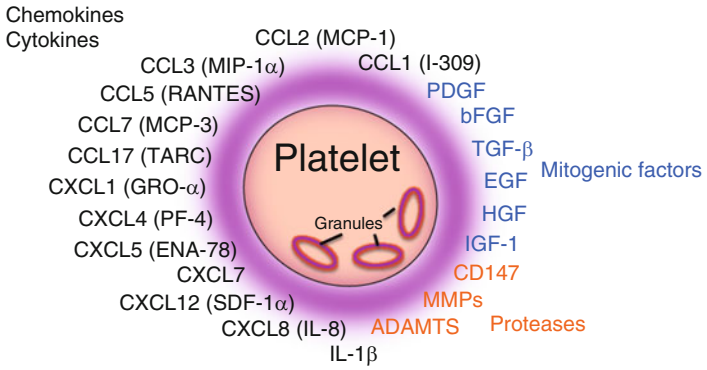


Fig. 4 Platelet-released proinflammatory molecules

Platelets are capable of producing inflammatory molecules, for example, interleukin (IL)-1β (Lindemann et al. 2001). The discovery of platelet protein synthesis, however, came as a surprise because platelets are anucleate cells that lack genomic DNA. Instead, megakaryocytes and platelets contain mRNA and the machinery for pre-mRNA splicing (Denis et al. 2005). Platelets can rapidly translate mRNA into proteins within minutes (Schulz et al. 2010; Lindemann et al. 2001). Upon stimulation, platelets can release more than 300 proteins, some of which, eg. CD154 (see below), appear to be involved in processes other than haemostasis (Coppinger et al. 2007; Maynard et al. 2007).

Alpha granules contain various membrane proteins (e.g. P-selectin), glycoproteins (e.g. fibrinogen, VWF), coagulation factors (e.g. protein S, factor V) and mitogenic factors [e.g. platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β]. Dense granules store platelet activating factors (e.g. ADP, serotonin) and a huge diversity of chemokines (e.g. CXCL7, CCL5 (RANTES), CXCL4 (platelet factor (PF)4), CCL3 and others) (Smyth et al. 2009; Semple et al. 2011). Interestingly, platelets may differentially store and release their cellular content (Italiano et al. 2008; Sehgal and Storrie 2007). Pro- and anti-angiogenic factors have been found to be separately packed in distinct granule populations; and treatment with specific agonists results in their differential release from platelets (Italiano et al. 2008; Chatterjee 2011). In a similar manner, major proteins such as VWF and fibrinogen are stored in different types of α-granules (Sehgal and Storrie 2007). It is tempting to speculate that proteins are separated in platelets by their functional (e.g. proinflammatory vs. haemostatic; pro- vs. anti-angiogenic) role. These may be released depending on the type of vascular injury (Chatterjee 2011). However, this concept was only recently challenged by the systematic quantification of 15 α-granule proteins with angiogenic functions. As there was no coclustering, the findings from this study supported a notion that there is random delivery of proteins to individual platelet granules (Kamykowski et al. 2011). It remains unclear how proteins, especially those with potentially antagonistic functions, are stored and released from platelets. Undoubtedly, inhibition of platelet secretion represents an

attractive target to diminish atherogenesis because of the high inflammatory capacity of the platelet releasate (Fig. 4). However, none of the present anti-atherosclerotic strategies specifically inhibits this process.

CD154 is among the various proinflammatory mediators expressed by activated platelets, which plays an important role in atherosclerosis. CD154 interacts with CD40, which is constitutively expressed on numerous cells including endothelial cells, macrophages, smooth muscle cells, as well as platelets. During platelet–endothelial cell contact, the interaction between CD154 and CD40 triggers the expression of chemokines (e.g. CCL2) and adhesion receptors (ICAM-1, VCAM-1) on the surface of endothelial cells. This promotes recruitment of leucocytes (Andre et al. 2002; Li et al. 2008). CD154–CD40 interactions also induce tissue factor expression on endothelium and macrophages, but down-regulate thrombomodulin expression, which increases local prothrombotic activity (Miller et al. 1998; Slupsky et al. 1998). Platelets are the main source of soluble CD154 (Heeschen et al. 2003), and circulating CD154 is significantly elevated in atherosclerosis (Heeschen et al. 2003). Surface expression and release of CD40L from platelets is induced by cross-linking of αIIbβ3 upon platelet adhesion and activation (Henn et al. 1998; May et al. 2002; Furman et al. 2004). Importantly, platelet-derived inflammatory mediators also effect maturation of antigen-presenting cells (Semple et al. 2011; Kissel et al. 2006). Specifically CD154 was shown to induce B- and T-cell differentiation (Semple et al. 2011) and plays a role in T- and B-cell homoeostasis, which likely impacts on atherogenesis. Indeed, inhibition or absence of CD154 attenuates lesion formation in atherosclerosis-prone mice (Heeschen et al. 2003) and it was recently shown that platelet-derived CD154 is a major trigger of atherosclerotic lesion formation (Lievens et al. 2010).

Apart from regulating inflammatory functions, ligation of CD40 on vascular cells, including smooth muscle cells and endothelial cells, coordinates the expression or secretion of substantial components of the proteolytic system in the subendothelial space. These include plasminogen activator receptors and matrix-degrading metalloproteinases (MMPs), which are endopeptidases that modify the extracellular matrix (Galis et al. 1995). Interestingly, activity of MMPs can be regulated by various platelet-derived inflammatory mediators including IL-1β and TGF-β (Siwik et al. 2000; Feinberg et al. 2000). By these means platelets may contribute to plaque destabilization (Galis et al. 1995). While platelets induce MMP secretion by other cells, they also host MMPs, specifically MMP2 and EMMPRIN, which are released upon activation (Schulz et al. 2011; Sawicki et al. 1998; Gresele et al. 2011). Platelet–monocyte interactions via EMMPRIN can stimulate nuclear factor kappaβ-driven inflammatory pathways in monocytes, such as MMP and cytokine induction (Schmidt et al. 2008). MMP2 can also modulate the shedding of CD154 exposed on the surfaces of activated platelets (Choi et al. 2010). Together, αIIbβ3-mediated platelet–endothelium adhesion, regulating platelet CD154 surface expression and secretion of cytokines may induce matrix degradation by MMPs. This might significantly contribute to both the initiation of atherosclerotic lesion formation and plaque rupture (Andre et al. 2002; Galis et al. 1995; Gresele et al. 2011).

Like CD154, platelets present and secrete various chemokines, which can be delivered to the luminal surface of the vessel wall. CCL5 and CXCL4 are platelet-derived chemokines that accumulate on inflamed endothelium in vivo (Fig. 3). They induce adhesion of monocytes and lymphocytes and can drive atherogenensis (Huo et al. 2003; von Hundelshausen et al. 2001). CXCL4 also effects neutrophil adhesion (Kasper et al. 2004). Interestingly, concerted action of CCL5 and CXCL4 is more effective than each single chemokine in mediating monocyte adhesion under flow (von Hundelshausen et al. 2005). They associate in platelets to form heteromers. Specific blockade of CCL5 and CXCL4 significantly attenuates atherosclerotic lesion formation in mice (Koenen et al. 2009). Thus, platelet-derived chemokines might present a potential target for the treatment and prevention of atherosclerosis.

Various other chemokines, e.g. CXCL1 and CCL3, have been found in platelets. In mouse models of atherosclerosis, global absence of CXCL1 diminishes atherosclerotic lesion formation (Boisvert et al. 2006). Bone-marrow transplantation of atherosclerosis-prone mice demonstrates that CXCL1 expressed in the vascular wall seems more important than blood-borne CXCL1 (Boisvert et al. 2006). Likewise, CXCL7 is highly abundant in platelets, and is detected in the vascular wall following carotid artery injury in mice (Hristov et al. 2007). The importance of this chemokine in atherogenesis, however, remains elusive and the role of this and other chemokines stored within platelet granules requires further investigation.

In summary, interaction of platelets with endothelial cells triggers the secretion of chemokines and the expression of adhesion molecules that promotes the adhesion of leucocytes. Adhesion of platelets to the endothelial surface generates signals for recruitment and extravasation of monocytes during atherosclerotic plaque formation, a process that seems important for atherogenesis. Indeed, platelet adhesion to the endothelium of atherosclerosis-prone mice coincided with gene expression of inflammatory mediators that precedes atherosclerotic plaque invasion by leucocytes (Massberg et al. 2002). Moreover, transfusion of pre-activated platelets promotes plaque formation (Huo et al. 2003). In contrast, prolonged blockade of platelet adhesion profoundly reduces leucocyte accumulation in the arterial intima and attenuates atherosclerotic lesion formation in atherosclerosis-prone vascular beds (Massberg et al. 2002, 2005; Schulz et al. 2008a).

Thus, platelet–vessel wall interaction at lesion-prone sites significantly contributes to the initiation and maintenance of the inflammatory process of atherosclerosis. Inhibition of platelet adhesion has, therefore, become a potential target to diminish atheroprogression. Inhibitors of platelet activation such as acetylsalicylic acid and clopidogrel have been successfully applied for the treatment of atherothrombosis in man. This treatment regime was insufficient to reduce atherosclerotic lesion development in mouse models when applied in doses comparable to humans (Schulz et al. 2008b). However, genetic ablation or specific pharmacological blockade of platelet receptors that mediate adhesion to inflamed endothelium (αIIbβ3) and subendothelial matrix proteins (GPVI) reduce atherosclerotic lesion formation

(Schulz et al. 2008a; Massberg et al. 2005). Simple intraperitoneal application of a soluble GPVI dimer, which effectively blocks the GPVI receptor in humans and mice in vivo (Ungerer et al. 2011; Massberg et al. 2004), reduces aortic arch lesion formation by 35% in $ApoE^{-/-}$ mice (Schulz et al. 2008a). Thus GPVI-mediated platelet adhesion, most likely to collagenous structures exposed in microerosions of the atherosclerotic arterial wall, appears to be involved in atheroprogression. Similarly, absence of αIIb reduces platelet recruitment to the carotid artery, and protects $ApoE^{-/-}$ mice from atherosclerotic plaque formation, supporting a critical role of platelets and platelet αIIb integrin in this process (Massberg et al. 2005). In contrast, $\beta3^{-/-}$ animals displayed accelerated atherosclerosis (Weng et al. 2003). As described above, the β3 integrin subfamily consists of two integrins, αIIbβ3 and αvβ3. αIIbβ3 is expressed exclusively by megakaryocytes and platelets and mediates platelet adhesion, aggregation, and activation. Instead, αvβ3 integrin is present on a variety of vascular cells, including monocytes, fibroblasts, endothelial cells, and smooth muscle cells, where it modulates multiple cellular functions, including proliferation, migration, and inflammation (Weng et al. 2003; Reynolds et al. 2002). Hence, β3-null mutants lack both αIIbβ3 and αvβ3, resulting in enhanced atherosclerotic lesion formation. This is in line with observations in humans with Glanzmann thrombasthenia. These patients, which lack both platelet fibrinogen and vitronectin receptors are not protected from atherosclerosis (Shpilberg et al. 2002). Thus, distinct platelet receptors are involved in the adhesion process to inflamed endothelium of the arterial wall, mediate secretion and ultimately contribute to atherosclerotic lesion formation.

3 Conclusion

Platelet adhesion and aggregation following the rupture of atherosclerotic plaques is increasingly thought as a significant cause of acute vascular events. Platelets adhere to the exposed subendothelial matrix in a multistep process, which involves the concerted action of GPVI, GPIbα, and the integrins α2β1 and αIIbβ3. The contribution of these receptors to platelet adhesion is dependent on the prevailing shear stress and composition of the plaque matrix and arterial wall. Locally secreted molecules such as tissue factor strongly contribute to platelet activation and fibrin generation. This process is influenced by platelets, which secrete thiol isomerase enzymes that control tissue factor activity. Thus, platelets play a central role in the process of arterial thrombosis. Novel imaging techniques that identified local fibrin formation or display a high-resolution picture of non-activated adherent platelets help to decipher the cascade of events during arterial thrombosis.

It is now evident that platelets cross-link the processes of haemostasis and inflammation, although their role in innate immune response is yet to be completely understood. As described in the second part of this chapter, platelets initiate and promote atherosclerotic lesion formation. Adhesion of platelets to the vascular endothelium at lesion-prone sites early in the process of atherogenesis leads to

atheroma formation through the release of cytokines and mitogenic factors. Proinflammatory molecules released from activated platelets (e.g. CD154) modulate differentiation of antigen-presenting cells. Thereby platelets alter homoeostasis and recruitment of innate immune cells into atherosclerotic plaque. Most surprisingly, platelets are also capable of protein synthesis to fill their treasure box of inflammatory molecules. Novel drugs that prevent the initial interaction of platelets with the arterial wall, or inhibit the process of platelet secretion, are therefore likely to provide an efficacious therapy for patients with a high atherosclerotic risk profile.

Knowledge Gaps

- The mechanisms underlying thrombus formation are nowadays more clearly understood. However, the ultimate antiplatelet agent is still missing. Novel antiplatelet strategies, which are highly efficient and well manageable at the same time, are warranted.
- Platelets trigger and modulate atherogenesis. However, important mechanisms are yet to be resolved, eg. how platelets interact with innate immune cells, such as dendritic cells and granulocytes, and how platelets support their recruitment to vascular lesions. Vice versa, platelets may be taken up and cleared by local macrophages.
- We will need to more precisely understand platelet protein synthesis and the signals, which trigger the release of cytokines and growth factors.
- It remains yet to be proven whether blocking platelet secretion represents an efficient strategy to inhibit inflammatory processes of the vascular wall.
- It would be interesting if the pathophysiological mechanisms underlying the role of platelets in atherosclerosis could be attributed to other diseases, e.g. cancer metastasis and autoimmune processes.

Key Messages

- Platelet adhesion to the arterial wall is a multistep process, which predominantly involves GPIb–V–IX (tethering), collagen receptors (firm adhesion) and integrins (aggregation). The type of receptor used by platelets is dependent on the prevailing shear stress.
- Activated platelets release molecules that reinforce platelet thrombus formation and contribute to blood coagulation.
- Platelets support accumulation of innate immune cells at the site of vascular injury, and initiate the release of nuclear extracellular traps by neutrophils. Innate immune cells produce tissue factor, which is critical for thrombus stabilization.

(continued)

- Platelets contain numerous bioactive mediators, inflammatory molecules and growth factors, which support inflammatory processes in the arterial wall.
- Platelets play a critical role in atherosclerosis. Platelet adhesion to the arterial wall occurs early during atherogenesis and before the first appearance of macroscopic vascular lesions. Platelet accumulation precedes and also supports firm adhesion of leucocytes at the inflamed endothelium.
- Inhibition of platelet secretion and platelet interactions with the arterial wall represent potential strategies to limit atheroprogression.

References

Abi-Younes S, Sauty A, Mach F, Sukhova GK, Libby P, Luster AD (2000) The stromal cell-derived factor-1 chemokine is a potent platelet agonist highly expressed in atherosclerotic plaques. Circ Res 86:131–138

Andre P, Nannizzi-Alaimo L, Prasad SK, Phillips DR (2002) Platelet-derived CD40L: the switch-hitting player of cardiovascular disease. Circulation 106:896–899

Bennett JS (2005) Structure and function of the platelet integrin alphaIIbbeta3. J Clin Invest 115:3363–3369

Bennett JS, Chan C, Vilaire G, Mousa SA, DeGrado WF (1997) Agonist-activated alphavbeta3 on platelets and lymphocytes binds to the matrix protein osteopontin. J Biol Chem 272:8137–8140

Bergmeier W, Piffath CL, Goerge T et al (2006) The role of platelet adhesion receptor GPIbalpha far exceeds that of its main ligand, von Willebrand factor, in arterial thrombosis. Proc Natl Acad Sci USA 103:16900–16905

Bertrand ME, Legrand V, Boland J et al (1998) Randomized multicenter comparison of conventional anticoagulation versus antiplatelet therapy in unplanned and elective coronary stenting. The full anticoagulation versus aspirin and ticlopidine (fantastic) study. Circulation 98:1597–1603

Boilard E, Nigrovic PA, Larabee K et al (2010) Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. Science 327:580–583

Boisvert WA, Rose DM, Johnson KA et al (2006) Up-regulated expression of the CXCR2 ligand KC/GRO-alpha in atherosclerotic lesions plays a central role in macrophage accumulation and lesion progression. Am J Pathol 168:1385–1395

Bombeli T, Schwartz BR, Harlan JM (1998) Adhesion of activated platelets to endothelial cells: evidence for a GPIIbIIIa-dependent bridging mechanism and novel roles for endothelial intercellular adhesion molecule 1 (ICAM-1), alphavbeta3 integrin, and GPIbalpha. J Exp Med 187:329–339

Busse R, Fleming I, Hecker M (1993) Signal transduction in endothelium-dependent vasodilatation. Eur Heart J 14(Suppl I):2–9

Cabeza N, Li Z, Schulz C et al (2004) Surface expression of collagen receptor Fc receptor-gamma/glycoprotein VI is enhanced on platelets in type 2 diabetes and mediates release of CD40 ligand and activation of endothelial cells. Diabetes 53:2117–2121

Chatterjee M Blood. 2011 Apr 7;117(14):3907–11. PMID: 21330475

Choi WS, Jeon OH, Kim DS (2010) CD40 ligand shedding is regulated by interaction between matrix metalloproteinase-2 and platelet integrin alpha(IIb)beta(3). J Thromb Haemost 8:1364–1371

Clemetson KJ, Clemetson JM (2001) Platelet collagen receptors. Thromb Haemost 86:189–197
Coppinger JA, O'Connor R, Wynne K et al (2007) Moderation of the platelet releasate response by aspirin. Blood 109:4786–4792
Cottone S, Mule G, Nardi E et al (2007) Microalbuminuria and early endothelial activation in essential hypertension. J Hum Hypertens 21:167–172
Cybulsky MI, Gimbrone MA Jr (1991) Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. Science 251:788–791
De Meyer GR, Hoylaerts MF, Kockx MM, Yamamoto H, Herman AG, Bult H (1999) Intimal deposition of functional von Willebrand factor in atherogenesis. Arterioscler Thromb Vasc Biol 19:2524–2534
Denis MM, Tolley ND, Bunting M et al (2005) Escaping the nuclear confines: signal-dependent pre-mRNA splicing in anucleate platelets. Cell 122:379–391
Dietrich T, Perlitz C, Licha K et al (2007) ED-B fibronectin (ED-B) can be targeted using a novel single chain antibody conjugate and is associated with macrophage accumulation in atherosclerotic lesions. Basic Res Cardiol 102:298–307
Dubois C, Panicot-Dubois L, Gainor JF, Furie BC, Furie B (2007) Thrombin-initiated platelet activation in vivo is vWF independent during thrombus formation in a laser injury model. J Clin Invest 117:953–960
Dumont B, Lasne D, Rothschild C et al (2009) Absence of collagen-induced platelet activation caused by compound heterozygous GPVI mutations. Blood 114:1900–1903
Feinberg MW, Jain MK, Werner F et al (2000) Transforming growth factor-beta 1 inhibits cytokine-mediated induction of human metalloelastase in macrophages. J Biol Chem 275:25766–25773
Fernandez-Ortiz A, Badimon JJ, Falk E et al (1994) Characterization of the relative thrombogenicity of atherosclerotic plaque components: implications for consequences of plaque rupture. J Am Coll Cardiol 23:1562–1569
Frenette PS, Johnson RC, Hynes RO, Wagner DD (1995) Platelets roll on stimulated endothelium in vivo: an interaction mediated by endothelial P-selectin. Proc Natl Acad Sci USA 92:7450–7454
Fuchs TA, Brill A, Duerschmied D et al (2010) Extracellular DNA traps promote thrombosis. Proc Natl Acad Sci USA 107:15880–15885
Furie B, Furie BC (2007) In vivo thrombus formation. J Thromb Haemost 5(Suppl 1):12–17
Furie B, Furie BC (2008) Mechanisms of thrombus formation. N Engl J Med 359:938–949
Furman MI, Krueger LA, Linden MD, Barnard MR, Frelinger AL 3rd, Michelson AD (2004) Release of soluble CD40L from platelets is regulated by glycoprotein IIb/IIIa and actin polymerization. J Am Coll Cardiol 43:2319–2325
Galis ZS, Muszynski M, Sukhova GK, Simon-Morrissey E, Libby P (1995) Enhanced expression of vascular matrix metalloproteinases induced in vitro by cytokines and in regions of human atherosclerotic lesions. Ann N Y Acad Sci 748:501–507
Galkina E, Ley K (2007) Leukocyte influx in atherosclerosis. Curr Drug Targets 8:1239–1248
Gasic GJ, Gasic TB, Stewart CC (1968) Antimetastatic effects associated with platelet reduction. Proc Natl Acad Sci USA 61:46–52
Gawaz M, Neumann FJ, Dickfeld T et al (1997) Vitronectin receptor (alpha(v)beta3) mediates platelet adhesion to the luminal aspect of endothelial cells: implications for reperfusion in acute myocardial infarction. Circulation 96:1809–1818
Gawaz M, Neumann FJ, Dickfeld T et al (1998) Activated platelets induce monocyte chemotactic protein-1 secretion and surface expression of intercellular adhesion molecule-1 on endothelial cells. Circulation 98:1164–1171
Gawaz M, Brand K, Dickfeld T et al (2000) Platelets induce alterations of chemotactic and adhesive properties of endothelial cells mediated through an interleukin-1-dependent mechanism. Implications for atherogenesis. Atherosclerosis 148:75–85

Gawaz M, Page S, Massberg S et al (2002) Transient platelet interaction induces MCP-1 production by endothelial cells via I kappa B kinase complex activation. Thromb Haemost 88:307–314

Gibbins JM, Okuma M, Farndale R, Barnes M, Watson SP (1997) Glycoprotein VI is the collagen receptor in platelets which underlies tyrosine phosphorylation of the Fc receptor gamma-chain. FEBS Lett 413:255–259

Gresele P, Falcinelli E, Loffredo F et al (2011) Platelets release matrix metalloproteinase-2 in the coronary circulation of patients with acute coronary syndromes: possible role in sustained platelet activation. Eur Heart J 32:316–325

Guyton JR, Klemp KF (1996) Development of the lipid-rich core in human atherosclerosis. Arterioscler Thromb Vasc Biol 16:4–11

Heeschen C, Dimmeler S, Hamm CW et al (2003) Soluble CD40 ligand in acute coronary syndromes. N Engl J Med 348:1104–1111

Henn V, Slupsky JR, Grafe M et al (1998) CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature 391:591–594

Holbrook LM, Watkins NA, Simmonds AD, Jones CI, Ouwehand WH, Gibbins JM (2010) Platelets release novel thiol isomerase enzymes which are recruited to the cell surface following activation. Br J Haematol 148:627–637

Holtkotter O, Nieswandt B, Smyth N et al (2002) Integrin alpha 2-deficient mice develop normally, are fertile, but display partially defective platelet interaction with collagen. J Biol Chem 277:10789–10794

Hristov M, Zernecke A, Bidzhekov K et al (2007) Importance of CXC chemokine receptor 2 in the homing of human peripheral blood endothelial progenitor cells to sites of arterial injury. Circ Res 100:590–597

Huo Y, Schober A, Forlow SB et al (2003) Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. Nat Med 9:61–67

Italiano JE Jr, Richardson JL, Patel-Hett S et al (2008) Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. Blood 111:1227–1233

Jandrot-Perrus M, Busfield S, Lagrue AH et al (2000) Cloning, characterization, and functional studies of human and mouse glycoprotein VI: a platelet-specific collagen receptor from the immunoglobulin superfamily. Blood 96:1798–1807

Jasuja R, Furie B, Furie BC (2010) Endothelium-derived but not platelet-derived protein disulfide isomerase is required for thrombus formation in vivo. Blood 116:4665–4674

Jung SM, Moroi M (1998) Platelets interact with soluble and insoluble collagens through characteristically different reactions. J Biol Chem 273:14827–14837

Jurk K, Clemetson KJ, de Groot PG et al (2003) Thrombospondin-1 mediates platelet adhesion at high shear via glycoprotein Ib (GPIb): an alternative/backup mechanism to von Willebrand factor. FASEB J 17:1490–1492

Kamykowski J, Carlton P, Sehgal S, Storrie B (2011) Quantitative immunofluorescence mapping reveals little functional coclustering of proteins within platelet {alpha}-granules. Blood 118:1370–1373

Kasirer-Friede A, Ware J, Leng L, Marchese P, Ruggeri ZM, Shattil SJ (2002) Lateral clustering of platelet GP Ib-IX complexes leads to up-regulation of the adhesive function of integrin alpha IIbbeta 3. J Biol Chem 277:11949–11956

Kasper B, Brandt E, Bulfone-Paus S, Petersen F (2004) Platelet factor 4 (PF-4)-induced neutrophil adhesion is controlled by src-kinases, whereas PF-4-mediated exocytosis requires the additional activation of p38 MAP kinase and phosphatidylinositol 3-kinase. Blood 103:1602–1610

Katsuda S, Kaji T (2003) Atherosclerosis and extracellular matrix. J Atheroscler Thromb 10:267–274

Kissel K, Berber S, Nockher A, Santoso S, Bein G, Hackstein H (2006) Human platelets target dendritic cell differentiation and production of proinflammatory cytokines. Transfusion 46:818–827

Klinger MH, Jelkmann W (2002) Role of blood platelets in infection and inflammation. J Interferon Cytokine Res 22:913–922

Koenen RR, von Hundelshausen P, Nesmelova IV et al (2009) Disrupting functional interactions between platelet chemokines inhibits atherosclerosis in hyperlipidemic mice. Nat Med 15:97–103

Kuijper PH, Gallardo Torres HI, Houben LA, Lammers JW, Zwaginga JJ, Koenderman L (1998) P-selectin and MAC-1 mediate monocyte rolling and adhesion to ECM-bound platelets under flow conditions. J Leukoc Biol 64:467–73

Kuijpers MJ, Schulte V, Bergmeier W et al (2003) Complementary roles of glycoprotein VI and alpha2beta1 integrin in collagen-induced thrombus formation in flowing whole blood ex vivo. FASEB J 17:685–687

Kuijpers MJ, Pozgajova M, Cosemans JM et al (2007) Role of murine integrin alpha2beta1 in thrombus stabilization and embolization: contribution of thromboxane A2. Thromb Haemost 98:1072–1080

Kuijpers MJ, Gilio K, Reitsma S et al (2009) Complementary roles of platelets and coagulation in thrombus formation on plaques acutely ruptured by targeted ultrasound treatment: a novel intravital model. J Thromb Haemost 7:152–161

Langer HF, Daub K, Braun G et al (2007) Platelets recruit human dendritic cells via Mac-1/JAM-C interaction and modulate dendritic cell function in vitro. Arterioscler Thromb Vasc Biol 27:1463–1470

Leon MB, Baim DS, Popma JJ et al (1998) A clinical trial comparing three antithrombotic-drug regimens after coronary-artery stenting. Stent Anticoagulation Restenosis Study Investigators. N Engl J Med 339:1665–1671

Levi M (2005) Platelets in sepsis. Hematology 10(Suppl 1):129–131

Li G, Sanders JM, Bevard MH et al (2008) CD40 ligand promotes Mac-1 expression, leukocyte recruitment, and neointima formation after vascular injury. Am J Pathol 172:1141–1152

Lievens D, Zernecke A, Seijkens T et al (2010) Platelet CD40L mediates thrombotic and inflammatory processes in atherosclerosis. Blood 116:4317–4327

Lindemann S, Tolley ND, Dixon DA et al (2001) Activated platelets mediate inflammatory signaling by regulated interleukin 1beta synthesis. J Cell Biol 154:485–490

Lusis AJ (2000) Atherosclerosis. Nature 407:233–241

Mackman N (2004) Role of tissue factor in hemostasis, thrombosis, and vascular development. Arterioscler Thromb Vasc Biol 24:1015–1022

Mailhac A, Badimon JJ, Fallon JT et al (1994) Effect of an eccentric severe stenosis on fibrin (ogen) deposition on severely damaged vessel wall in arterial thrombosis. Relative contribution of fibrin(ogen) and platelets. Circulation 90:988–996

Massberg S, Enders G, Leiderer R et al (1998) Platelet-endothelial cell interactions during ischemia/reperfusion: the role of P-selectin. Blood 92:507–515

Massberg S, Brand K, Gruner S et al (2002) A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation. J Exp Med 196:887–896

Massberg S, Gawaz M, Gruner S et al (2003) A crucial role of glycoprotein VI for platelet recruitment to the injured arterial wall in vivo. J Exp Med 197:41–49

Massberg S, Konrad I, Bultmann A et al (2004) Soluble glycoprotein VI dimer inhibits platelet adhesion and aggregation to the injured vessel wall in vivo. FASEB J 18:397–399

Massberg S, Schurzinger K, Lorenz M et al (2005) Platelet adhesion via glycoprotein IIb integrin is critical for atheroprogression and focal cerebral ischemia: an in vivo study in mice lacking glycoprotein IIb. Circulation 112:1180–1188

Massberg S, Konrad I, Schurzinger K et al (2006) Platelets secrete stromal cell-derived factor 1alpha and recruit bone marrow-derived progenitor cells to arterial thrombi in vivo. J Exp Med 203:1221–1233

Massberg S, Grahl L, von Bruehl ML et al (2010) Reciprocal coupling of coagulation and innate immunity via neutrophil serine proteases. Nat Med 16:887–896

May AE, Kalsch T, Massberg S, Herouy Y, Schmidt R, Gawaz M (2002) Engagement of glycoprotein IIb/IIIa (alpha(IIb)beta3) on platelets upregulates CD40L and triggers CD40L-dependent matrix degradation by endothelial cells. Circulation 106:2111–2117

Maynard DM, Heijnen HF, Horne MK, White JG, Gahl WA (2007) Proteomic analysis of platelet alpha-granules using mass spectrometry. J Thromb Haemost 5:1945–1955

Meyer dos Santos S, Klinkhardt U, Scholich K et al (2011) The CX3C chemokine fractalkine mediates platelet adhesion via the von Willebrand receptor glycoprotein Ib. Blood 117:4999–5008

Miller DL, Yaron R, Yellin MJ (1998) CD40L-CD40 interactions regulate endothelial cell surface tissue factor and thrombomodulin expression. J Leukoc Biol 63:373–379

Moroi M, Jung SM, Okuma M, Shinmyozu K (1989a) A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. J Clin Invest 84:1440–1445

Moroi M, Jung SM, Okuma M, Shinmyozu K (1989b) A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. J Clin Invest 84:1440–1445

Moroi M, Jung SM, Shinmyozu K, Tomiyama Y, Ordinas A, Diaz-Ricart M (1996) Analysis of platelet adhesion to a collagen-coated surface under flow conditions: the involvement of glycoprotein VI in the platelet adhesion. Blood 88:2081–2092

Nesbitt WS, Westein E, Tovar-Lopez FJ et al (2009) A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. Nat Med 15:665–673

Nieswandt B, Watson SP (2003) Platelet-collagen interaction: is GPVI the central receptor? Blood 102:449–461

Nieswandt B, Brakebusch C, Bergmeier W et al (2001) Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. EMBO J 20:2120–2130

Nieswandt B, Pleines I, Bender M (2011) Platelet adhesion and activation mechanisms in arterial thrombosis and ischaemic stroke. J Thromb Haemost 9(Suppl 1):92–104

Offermanns S (2006) Activation of platelet function through G protein-coupled receptors. Circ Res 99:1293–1304

Ostermann G, Weber KS, Zernecke A, Schroder A, Weber C (2002) JAM-1 is a ligand of the beta (2) integrin LFA-1 involved in transendothelial migration of leukocytes. Nat Immunol 3:151–158

Penz S, Reininger AJ, Brandl R et al (2005) Human atheromatous plaques stimulate thrombus formation by activating platelet glycoprotein VI. FASEB J 19:898–909

Pitsilos S, Hunt J, Mohler ER et al (2003) Platelet factor 4 localization in carotid atherosclerotic plaques: correlation with clinical parameters. Thromb Haemost 90:1112–1120

Radomski MW, Palmer RM, Moncada S (1987) The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. Br J Pharmacol 92:639–646

Reinhardt C, von Bruhl ML, Manukyan D et al (2008) Protein disulfide isomerase acts as an injury response signal that enhances fibrin generation via tissue factor activation. J Clin Invest 118:1110–1122

Reininger AJ, Heijnen HF, Schumann H, Specht HM, Schramm W, Ruggeri ZM (2006) Mechanism of platelet adhesion to von Willebrand factor and microparticle formation under high shear stress. Blood 107:3537–3545

Reininger AJ, Bernlochner I, Penz SM et al (2010) A 2-step mechanism of arterial thrombus formation induced by human atherosclerotic plaques. J Am Coll Cardiol 55:1147–1158

Reynolds LE, Wyder L, Lively JC et al (2002) Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. Nat Med 8:27–34

Rinder HM, Bonan JL, Rinder CS, Ault KA, Smith BR (1991) Activated and unactivated platelet adhesion to monocytes and neutrophils. Blood 78:1760–1769

Romo GM, Dong JF, Schade AJ et al (1999) The glycoprotein Ib-IX-V complex is a platelet counterreceptor for P-selectin. J Exp Med 190:803–814

Rosenblum WI, Nelson GH, Wormley B, Werner P, Wang J, Shih CC (1996) Role of platelet-endothelial cell adhesion molecule (PECAM) in platelet adhesion/aggregation over injured but not denuded endothelium in vivo and ex vivo. Stroke 27:709–711

Ross R (1999) Atherosclerosis–an inflammatory disease. N Engl J Med 340:115–126

Ruggeri ZM (2004) Platelet and von Willebrand factor interactions at the vessel wall. Hämostaseologie 24:1–11

Ruggeri ZM (2007) The role of von Willebrand factor in thrombus formation. Thromb Res 120 (Suppl 1):S5–S9

Salles II, Feys HB, Iserbyt BF, De Meyer SF, Vanhoorelbeke K, Deckmyn H (2008) Inherited traits affecting platelet function. Blood Rev 22:155–172

Savage B, Saldivar E, Ruggeri ZM (1996) Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. Cell 84:289–297

Sawicki G, Sanders EJ, Salas E, Wozniak M, Rodrigo J, Radomski MW (1998) Localization and translocation of MMP-2 during aggregation of human platelets. Thromb Haemost 80:836–839

Schmidt R, Bultmann A, Fischel S et al (2008) Extracellular matrix metalloproteinase inducer (CD147) is a novel receptor on platelets, activates platelets, and augments nuclear factor kappaB-dependent inflammation in monocytes. Circ Res 102:302–309

Schober A, Manka D, von Hundelshausen P et al (2002) Deposition of platelet RANTES triggering monocyte recruitment requires P-selectin and is involved in neointima formation after arterial injury. Circulation 106:1523–1529

Schomig A, Neumann FJ, Kastrati A et al (1996) A randomized comparison of antiplatelet and anticoagulant therapy after the placement of coronary-artery stents. N Engl J Med 334:1084–1089

Schonberger T, Siegel-Axel D, Bussl R et al (2008) The immunoadhesin glycoprotein VI-Fc regulates arterial remodelling after mechanical injury in ApoE-/- mice. Cardiovasc Res 80:131–137

Schulte V, Snell D, Bergmeier W, Zirngibl H, Watson SP, Nieswandt B (2001) Evidence for two distinct epitopes within collagen for activation of murine platelets. J Biol Chem 276:364–368

Schulz C, Schafer A, Stolla M et al (2007) Chemokine fractalkine mediates leukocyte recruitment to inflammatory endothelial cells in flowing whole blood: a critical role for P-selectin expressed on activated platelets. Circulation 116:764–773

Schulz C, Penz S, Hoffmann C et al (2008a) Platelet GPVI binds to collagenous structures in the core region of human atheromatous plaque and is critical for atheroprogression in vivo. Basic Res Cardiol 103:356–367

Schulz C, Konrad I, Sauer S et al (2008b) Effect of chronic treatment with acetylsalicylic acid and clopidogrel on atheroprogression and atherothrombosis in ApoE-deficient mice in vivo. Thromb Haemost 99:190–195

Schulz C, Leuschen NV, Frohlich T et al (2010) Identification of novel downstream targets of platelet glycoprotein VI activation by differential proteome analysis: implications for thrombus formation. Blood 115:4102–4110

Schulz C, von Bruhl ML, Barocke V et al (2011) EMMPRIN (CD147/basigin) mediates platelet-monocyte interactions in vivo and augments monocyte recruitment to the vascular wall. J Thromb Haemost 9:1007–1019

Sehgal S, Storrie B (2007) Evidence that differential packaging of the major platelet granule proteins von Willebrand factor and fibrinogen can support their differential release. J Thromb Haemost 5:2009–2016

Seizer P, Schiemann S, Merz T et al (2010) CD36 and macrophage scavenger receptor a modulate foam cell formation via inhibition of lipid-laden platelet phagocytosis. Semin Thromb Hemost 36:157–162

Semple JW, Italiano JE Jr, Freedman J (2011) Platelets and the immune continuum. Nat Rev Immunol 11:264–274

Shpilberg O, Rabi I, Schiller K et al (2002) Patients with Glanzmann thrombasthenia lacking platelet glycoprotein alpha(IIb)beta(3) (GPIIb/IIIa) and alpha(v)beta(3) receptors are not protected from atherosclerosis. Circulation 105:1044–1048

Siess W, Zangl KJ, Essler M et al (1999) Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. Proc Natl Acad Sci USA 96:6931–6936

Siwik DA, Chang DL, Colucci WS (2000) Interleukin-1beta and tumor necrosis factor-alpha decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts in vitro. Circ Res 86:1259–1265

Slupsky JR, Kalbas M, Willuweit A, Henn V, Kroczek RA, Muller-Berghaus G (1998) Activated platelets induce tissue factor expression on human umbilical vein endothelial cells by ligation of CD40. Thromb Haemost 80:1008–1014

Smyth SS, McEver RP, Weyrich AS et al (2009) Platelet functions beyond hemostasis. J Thromb Haemost 7:1759–1766

Strony J, Beaudoin A, Brands D, Adelman B (1993) Analysis of shear stress and hemodynamic factors in a model of coronary artery stenosis and thrombosis. Am J Physiol 265:H1787–H1796

Sugiyama T, Okuma M, Ushikubi F, Sensaki S, Kanaji K, Uchino H (1987) A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia. Blood 69:1712–1720

Sugiyama T, Ishibashi T, Okuma M (1993) Functional role of the antigen recognized by an antiplatelet antibody specific for a putative collagen receptor in platelet-collagen interaction. Int J Hematol 58:99–104

Szmitko PE, Wang CH, Weisel RD, de Almeida JR, Anderson TJ, Verma S (2003) New markers of inflammation and endothelial cell activation: Part I. Circulation 108:1917–1923

Ungerer M, Rosport K, Bultmann A et al (2011) Novel antiplatelet drug revacept (Dimeric Glycoprotein VI-Fc) specifically and efficiently inhibited collagen-induced platelet aggregation without affecting general hemostasis in humans. Circulation 123:1891–1899

Urban P, Macaya C, Rupprecht HJ et al (1998) Randomized evaluation of anticoagulation versus antiplatelet therapy after coronary stent implantation in high-risk patients: the multicenter aspirin and ticlopidine trial after intracoronary stenting (MATTIS). Circulation 98:2126–2132

von Hundelshausen P, Weber KS, Huo Y et al (2001) RANTES deposition by platelets triggers monocyte arrest on inflamed and atherosclerotic endothelium. Circulation 103:1772–1777

von Hundelshausen P, Koenen RR, Sack M et al (2005) Heterophilic interactions of platelet factor 4 and RANTES promote monocyte arrest on endothelium. Blood 105:924–930

von Hundelshausen P, Koenen RR, Weber C (2009) Platelet-mediated enhancement of leukocyte adhesion. Microcirculation 16:84–96

Wagner DD, Frenette PS (2008) The vessel wall and its interactions. Blood 111:5271–5281

Wagner CL et al (1996) Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets. Blood 88(3):907–914

Weng S, Zemany L, Standley KN et al (2003) Beta3 integrin deficiency promotes atherosclerosis and pulmonary inflammation in high-fat-fed, hyperlipidemic mice. Proc Natl Acad Sci USA 100:6730–6735

Zarbock A, Singbartl K, Ley K (2006a) Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation. J Clin Invest 116:3211–3219

Zarbock A, Singbartl K, Ley K (2006b) Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation. J Clin Invest 116:3211–3219

Zarbock A, Polanowska-Grabowska RK, Ley K (2007) Platelet-neutrophil-interactions: linking hemostasis and inflammation. Blood Rev 21:99–111

Zhang W, Li YS, Nardi MA et al (2010) Dissolution of arterial platelet thrombi in vivo with a bifunctional platelet GPIIIa49-66 ligand which specifically targets the platelet thrombus. Blood 116:2336–2344

Zheng YM, Liu C, Chen H, Locke D, Ryan JC, Kahn ML (2001) Expression of the platelet receptor GPVI confers signaling via the Fc receptor gamma -chain in response to the snake venom convulxin but not to collagen. J Biol Chem 276:12999–13006

Zibara K, Chignier E, Covacho C et al (2000) Modulation of expression of endothelial intercellular adhesion molecule-1, platelet-endothelial cell adhesion molecule-1, and vascular cell adhesion molecule-1 in aortic arch lesions of apolipoprotein E-deficient compared with wild-type mice. Arterioscler Thromb Vasc Biol 20:2288–2296

Zwaginga JJ, Torres HI, Lammers J, Sixma JJ, Koenderman L, Kuijper PH (1999) Minimal platelet deposition and activation in models of injured vessel wall ensure optimal neutrophil adhesion under flow conditions. Arterioscler Thromb Vasc Biol 19:1549–1554

Zwicker JI, Trenor CC 3rd, Furie BC, Furie B (2011) Tissue factor-bearing microparticles and thrombus formation. Arterioscler Thromb Vasc Biol 31:728–733

Part II
Pharmacology

Aspirin and Other COX-1 Inhibitors

Carlo Patrono and Bianca Rocca

Contents

Abstract Currently available antiplatelet drugs interfere with the process of platelet activation and aggregation by selectively blocking key enzymes involved in the synthesis of platelet agonists, or membrane receptors mediating activation signals. Pharmacological interference with critical molecular pathways of platelet activation and aggregation may reduce the risk of atherothrombotic complications through mechanisms that are also responsible for an increased risk of bleeding. Acetylsalicylic acid (aspirin) represents a prototypic antiplatelet agent. The aim of this chapter is to integrate our current understanding of the molecular mechanism of action of aspirin with the results of clinical trials and epidemiological studies

C. Patrono (✉) • B. Rocca
Department of Pharmacology, Catholic University School of Medicine, Largo F. Vito, 1, 00168 Rome, Italy
e-mail: carlo.patrono@rm.unicatt.it; b.rocca@tiscali.it

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_6,

assessing its efficacy and safety. Moreover, the antiplatelet properties of reversible inhibitors of the same drug target will also be reviewed.

Keywords Aspirin • Atherothrombosis • Cyclooxygenase inhibitors • Gastrointestinal bleeding • Interindividual variability

1 Introduction

Currently available antiplatelet drugs interfere with the process of platelet activation and aggregation by selectively blocking key enzymes involved in the synthesis of platelet agonists, or membrane receptors mediating activation signals. Pharmacological interference with critical molecular pathways of platelet activation and aggregation may reduce the risk of atherothrombotic complications through mechanisms that are also responsible for an increased risk of bleeding (Patrono et al. 2008; Patrono and Rocca 2010). Acetylsalicylic acid (aspirin) represents a prototypic antiplatelet agent. The historical aspects of the development of low-dose aspirin as an antithrombotic drug have been reviewed elsewhere (Born and Patrono 2006). The aim of this chapter is to integrate our current understanding of the molecular mechanism of action of aspirin with the results of clinical trials and epidemiological studies assessing its efficacy and safety. Moreover, the antiplatelet properties of reversible inhibitors of the same drug target will also be reviewed.

2 Mechanism of Action

The best characterized mechanism of action of aspirin reflects its capacity to permanently inactivate the cyclooxygenase (COX) activity of prostaglandin H-synthase (PGHS)-1 and -2 (also referred to as COX-1 and COX-2) (Roth and Majerus 1975; Roth et al. 1975; Smith et al. 1996). These isozymes catalyze the first committed step in prostanoid biosynthesis, i.e., the conversion of arachidonic acid (AA) to PGH_2 (Fig. 1). PGH_2 is the immediate precursor of PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane $(TX)A_2$. COX-1 and COX-2 are homodimers of a ~ 72 kDa monomeric unit. Each dimer has three independent folding units: an epidermal growth factor-like domain, a membrane-binding domain, and an enzymatic domain (Smith et al. 1996). Within the enzymatic domain, there is a peroxidase catalytic site and a separate, but adjacent, site for COX activity at the apex of a narrow, hydrophobic channel.

The molecular mechanism of permanent inactivation of COX activity by aspirin is through blockade of the COX channel as a consequence of acetylation of a strategically located serine residue (Ser529 in the human COX-1, Ser516 in the human COX-2), that prevents access of the substrate to the catalytic site of the enzyme (Loll et al. 1995). The hydrophobic environment of the COX channel

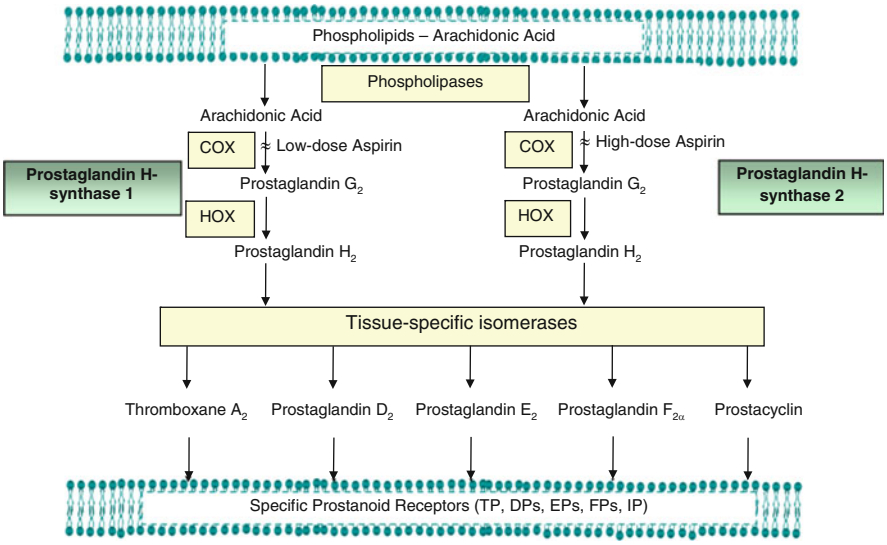


Fig. 1 Arachidonic acid metabolism and mechanism of action of aspirin. Arachidonic acid, a 20-carbon fatty acid containing four double bonds, is liberated from the sn2 position in membrane phospholipids by several forms of phospholipase, which are activated by diverse stimuli. Arachidonic acid is converted by cytosolic prostaglandin H synthases, which have both cyclooxygenase (COX) and hydroperoxidase (HOX) activity, to the unstable intermediate prostaglandin H_2. The synthases are colloquially termed cyclooxygenases and exist in two forms, COX-1 and COX-2. Low-dose aspirin selectively inhibits platelet COX-1 in vivo, and high-dose aspirin inhibits both COX-1 and -2. Prostaglandin H_2 is converted by tissue-specific isomerases to multiple prostanoids. These bioactive lipids activate specific cell membrane receptors of the superfamily of G-protein-coupled receptors. *DP* prostaglandin D_2 receptor, *EP* prostaglandin E_2 receptor, *FP* prostaglandin $F_{2\alpha}$ receptor, *IP* prostacyclin receptor, *TP* thromboxane receptor

stabilizes the modified serine side chain against hydrolysis. Inhibition of COX-1-dependent platelet function can be achieved with low doses of aspirin given once daily. In contrast, inhibition of COX-2-dependent pathophysiological processes (e.g., hyperalgesia and inflammation) requires larger doses of aspirin (probably because acetylation is determined by the oxidative state of the enzyme and is inhibited in cells with high peroxide tone) (Bala et al. 2008) and a much shorter dosing interval (because nucleated cells rapidly resynthesize the enzyme).

Human platelets and vascular endothelial cells process PGH_2 to produce primarily TXA_2 and PGI_2, respectively (Smith et al. 1996). TXA_2 induces platelet aggregation and vasoconstriction, while PGI_2 inhibits platelet aggregation and induces vasodilation (Smith et al. 1996). While TXA_2 is largely a COX-1-derived product (mostly from platelets) and thus highly sensitive to aspirin inhibition, vascular PGI_2 can derive from both COX-1 and to a greater extent, even under physiological conditions, from COX-2 (McAdam et al. 1999). COX-1-dependent PGI_2 production occurs transiently in response to agonist stimulation, e.g., bradykinin (Clarke et al. 1991), and is sensitive to aspirin inhibition. COX-2-mediated PGI_2 production occurs long

term, in response to laminar shear stress (Topper et al. 1996), and is largely unaffected by conventional antiplatelet doses of aspirin. This may explain the substantial residual PGI_2 biosynthesis in vivo at daily doses of aspirin in the range of 30–100 mg (FitzGerald et al. 1983), despite transient suppression of COX-1-dependent PGI_2 release (Clarke et al. 1991). It is not convincingly established that more profound suppression of PGI_2 formation by higher doses of aspirin is sufficient to initiate or predispose to thrombosis. However, two distinct lines of evidence suggest that PGI_2 is an important antithrombotic autacoid. The first is the observation that mice lacking the PGI_2 receptor had increased susceptibility to experimental thrombosis (Murata et al. 1997). The second is the characterization of the enhanced risk of myocardial infarction associated with COX-2 inhibitors (Kearney et al. 2006), that also supports the concept of PGI_2 acting as an important mechanism of thromboresistance in the setting of inadequate inhibition of platelet TXA_2 biosynthesis (Grosser et al. 2006).

3 Pharmacokinetics and Pharmacodynamics

Aspirin is rapidly absorbed in the stomach and upper intestine (Grosser et al. 2010). Peak plasma levels occur 30–40 min after aspirin ingestion, and inhibition of TXA_2-dependent platelet function is evident by 1 h. In contrast, it can take up to 3–4 h to reach peak plasma levels after administration of enteric-coated aspirin. The oral bioavailability of regular aspirin tablets is approximately 40–50% over a wide range of doses (Pedersen and FitzGerald 1984). A lower bioavailability has been reported for some enteric-coated tablets (Cox et al. 2006) and sustained-release, microencapsulated preparations (Pedersen and FitzGerald 1984). Poor absorption from the higher pH environment of the small intestine and lower bioavailability of some enteric-coated preparations may result in inadequate platelet inhibition, particularly in heavier subjects (Cox et al. 2006). A controlled-release formulation with negligible systemic bioavailability has been developed in an attempt to achieve selective inhibition of platelet TXA_2 production without suppressing systemic PGI_2 synthesis (Clarke et al. 1991). This was used successfully in the Thrombosis Prevention Trial (see below), but it remains unknown whether there is any advantage to the controlled-release formulation vis-à-vis plain aspirin.

The plasma concentration of aspirin decays with a half-life of 15–20 min in the human circulation (Grosser et al. 2010). Despite its rapid clearance, the inhibitory effect of aspirin lasts for the life span of the platelet (Burch et al. 1979) because aspirin irreversibly inactivates COX-1 (Roth and Majerus 1975; Roth et al. 1975). Aspirin also acetylates the enzyme in megakaryocytes before new platelets are released into the circulation (Demers et al. 1980). The mean life span of human platelets is approximately 8–10 days. Therefore, about 10–12% of circulating platelets are replaced every 24 h. Low-dose aspirin has at least two distinct drug targets contributing to its sustained antiplatelet effect: (1) acetylation of platelet COX-1 which occurs presystemically, i.e., in the portal blood (Pedersen and FitzGerald 1984), and is cumulative upon repeated daily dosing (Patrignani et al. 1982);

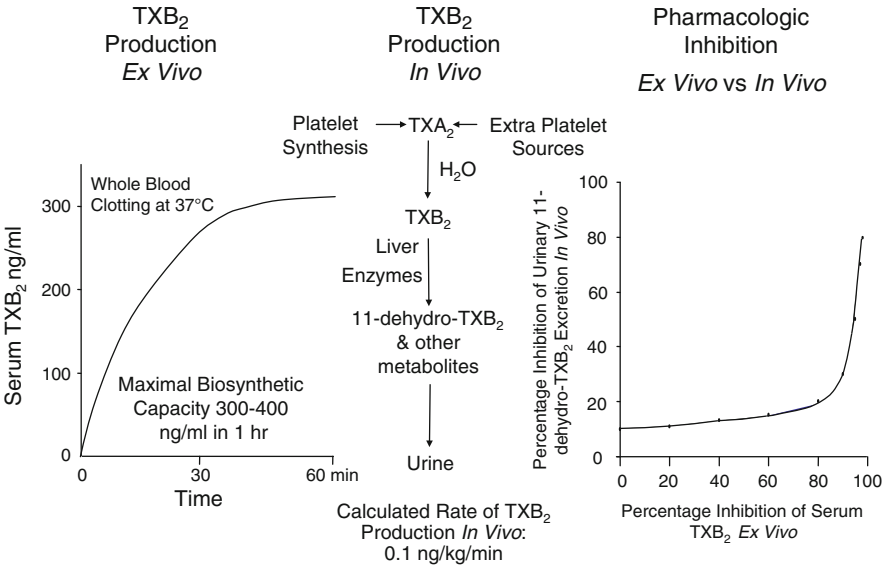


Fig. 2 Maximal capacity of human platelets to synthesize thromboxane B_2 (TXB_2), rate of TXB_2 production in healthy subjects, and relationship between the inhibition of platelet cyclooxygenase activity and TXB_2 biosynthesis in vivo. The *left panel* depicts the level of TXB_2 production stimulated by endogenous thrombin during whole blood clotting at 37 °C. The *center panel* shows the metabolic fate of thromboxane A_2 (TXA_2) in vivo and the calculated rate of its production in healthy subjects on the basis of TXB_2 infusions and measurement of its major urinary metabolite. The *right panel* depicts the nonlinear relationship between inhibition of serum TXB_2 measured ex vivo and the reduction in the excretion of thromboxane metabolite measured in vivo

this represents the major determinant of the virtually complete suppression of platelet TXA_2 production; and (2) acetylation of megakaryocyte COX-1 and COX-2 (Rocca et al. 2002) which is dependent on systemic bioavailability of the drug and contributes to the long-lasting duration of TXA_2 suppression, because newly released platelets express acetylated COX isozymes derived from the bone marrow progenitors during a substantial fraction of the 24-h dosing interval. Abnormal megakaryopoiesis as may occur in essential thrombocythemia and other disease states, as well as reduced systemic bioavailability of aspirin (as may occur in obesity), may limit the duration of platelet COX-1 suppression and possibly require a shorter dosing interval (Dragani et al. 2010; Pascale et al. 2012). Upon aspirin withdrawal, the recovery of TXA_2 biosynthesis in vivo is somewhat faster than predicted by the rate of platelet turnover (FitzGerald et al. 1983), possibly because of the nonlinear relationship between inhibition of platelet COX-1 activity and inhibition of TXA_2 biosynthesis in vivo (Fig. 2) (Reilly and FitzGerald 1987; Santilli et al. 2009). Because full suppression of TXA_2-dependent platelet function requires >97% inhibition of COX-1 activity (Santilli et al. 2009), even a modest recovery of this activity—as can be detected 2–3 days after aspirin withdrawal—can sustain a full aggregatory response.

4 Antithrombotic Effects of Aspirin

A number of issues related to the antithrombotic effect of aspirin have been debated during the past 20 years. These include the following: (1) the optimal dose of aspirin in order to maximize clinical efficacy and minimize gastrointestinal (GI) toxicity; (2) the suggestion that part of the antithrombotic effect of aspirin is unrelated to inhibition of platelet TXA_2; and (3) the possibility that some patients may be "resistant" to its antiplatelet effect (Patrono et al. 2008).

4.1 The Optimal Dose of Aspirin

Aspirin inhibits platelet COX-1 activity in a dose-dependent fashion with a ceiling effect at 100 mg given as a single dose, or at 30 mg given daily through cumulative inactivation of the drug target (Patrignani et al. 1982; Patrono et al. 1985). Placebo-controlled randomized trials have shown that aspirin is an effective antithrombotic agent when used long term in doses ranging between 50 and 100 mg once daily, and there is a suggestion that it is effective in doses as low as 30 mg once daily (Patrono et al. 2005; Antithrombotic Trialists Collaboration 2002). Aspirin in a dose of 75 mg once daily was shown to be effective in reducing the risk of acute myocardial infarction or death in patients with unstable angina and chronic stable angina, as well as in reducing stroke or death in patients with transient cerebral ischemia, and the risk of postoperative stroke after carotid endarterectomy (reviewed in Patrono et al. 2005, 2008). In the European Stroke Prevention Study (ESPS)-2, aspirin 25 mg twice daily was effective in reducing the risks of stroke and of the composite outcome stroke or death in patients with prior stroke or transient ischemic attack (TIA) (Diener et al. 1996). Moreover, in the European Collaboration on Low-dose Aspirin in Polycythemia vera (ECLAP) trial, aspirin 100 mg daily was effective in preventing thrombotic complications in patients with polycythemia vera, despite a higher than normal platelet count (Landolfi et al. 2004). However, the same antiplatelet regimen was not proven effective in preventing the major vascular complications of patients with diabetes mellitus (Ogawa et al. 2008; Belch et al. 2008) and patients with asymptomatic atherosclerosis (as detected by a low ankle/brachial index) (Fowkes et al. 2010), raising the possibility that the accelerated platelet turnover associated with these vascular disorders may limit the duration of the antiplatelet effect of low-dose aspirin given once daily (Patrono and Rocca 2010). The lowest effective daily dose of aspirin for various vascular disorders is shown in Table 1.

The clinical effects of different doses of aspirin have been compared directly in a relatively small number of randomized trials. In the United Kingdom-TIA (UK-TIA) study, no difference in efficacy was found between 300 and 1,200 mg daily (Farrel et al. 1991). In a study of 3,131 patients after a TIA or minor ischemic stroke, aspirin in a dose of 30 mg was compared with a dose of 283 mg once daily, and the hazard ratio for the group receiving the lower dose was 0.91 (95% CI, 0.76–1.09) (The Dutch

Table 1 Vascular disorders for which aspirin has been shown to be effective and lowest effective dose

Disorder	Lowest effective daily dose (mg)
Transient ischemic attack and ischemic stroke[a]	50
Pregnancy-induced hypertension	60
Men at high cardiovascular risk	75
Essential hypertension	75
Chronic stable angina	75
Unstable angina[a]	75
Severe carotid artery stenosis[a]	75
Polycythemia vera	100
Acute myocardial infarction	160
Acute ischemic stroke[a]	160
Atrial fibrillation	325

[a]Higher doses have been tested in other trials and not found to confer any greater risk reduction

TIA Trial Study Group 1991). The ASA and Carotid Endarterectomy (ACE) trial reported that the risk of stroke, myocardial infarction, or death within 3 months of carotid endarterectomy was significantly lower for patients taking 81 mg or 325 mg aspirin than for those taking 650 mg or 1,300 mg daily (6.2% vs. 8.4%; $p = 0.03$) (Taylor et al. 1999). Moreover, in the CURRENT-OASIS 7 trial of 25,086 patients with acute coronary syndromes, the 30-day primary outcome of cardiovascular death, myocardial infarction, or stroke occurred in 4.2% of patients assigned to a higher dose of aspirin (300–325 mg daily) as compared with 4.4% of those assigned to a lower dose (75–100 mg daily) (hazard ratio, 0.97; 95% CI, 0.86–1.09; $P = 0.61$) (Current-Oasis 7 Investigators 2010). Thus, there is no convincing evidence from randomized trials that have compared different doses of aspirin that higher doses are more effective than lower doses in reducing the risk of serious vascular events. These clinical findings are consistent with saturability of platelet COX-1 inactivation at daily doses as low as 30 mg (Patrignani et al. 1982).

There is evidence, however, that a daily dose of 300 mg produces fewer GI side effects than 1,200 mg (Farrel et al. 1991). There is also some evidence that a daily dose of 30 mg produces fewer side effects than 300 mg (The Dutch TIA Trial Study Group 1991). The Clopidogrel in Unstable angina to prevent Recurrent Events (CURE) Investigators have retrospectively investigated the relationship between the aspirin dose (the CURE protocol recommended 75–325 mg daily) and risk of major bleeding (Peters et al. 2003). This study was a randomized comparison of clopidogrel with placebo on a background of aspirin therapy. Patients with acute coronary syndromes receiving aspirin $\leq$100 mg daily had the lowest rate of major or life-threatening bleeding complications both in the placebo and clopidogrel arms of the trial. Bleeding risks increased with increasing aspirin dose, with or without clopidogrel (Peters et al. 2003).

In summary, the saturability of the antiplatelet effect of aspirin at low doses, the lack of a dose–response relationship in clinical studies evaluating its antithrombotic effects, and the dose dependence of its side-effects (see below) all support the use of

as low a dose of aspirin as has been found to be effective in the treatment of various vascular disorders (Table 1). Use of the lowest effective dose of aspirin (50–100 mg daily for long-term treatment) is currently the most appropriate strategy to maximize its efficacy and minimize its toxicity (Patrono et al. 2005).

4.2 *Effects of Aspirin Not Related to TXA_2 Suppression*

Aspirin has been reported to have effects on hemostasis and thrombosis that are unrelated to its ability to inactivate platelet COX-1. These include dose-dependent inhibition of platelet function, enhancement of fibrinolysis, and suppression of plasma coagulation (reviewed in Patrono et al. 2008).

In contrast to the well-characterized and saturable inhibition of COX-1 by aspirin, the putative mechanisms underlying the nonprostaglandin effects of aspirin on hemostasis and thrombosis are dose-dependent and less clearly defined, i.e., no alternative drug target has been characterized. Moreover, all of the available evidence suggesting dose-dependent effects of aspirin is indirect and inconsistent with the failure to show a dose effect in individual randomized clinical trials (Farrel et al. 1991; The Dutch TIA Trial Study Group 1991; Taylor et al. 1999; Current-Oasis 7 Investigators 2010) and in the ATT overview analysis (Antithrombotic Trialists Collaboration 2002). This failure to show a dose effect is consistent with the saturability of the aspirin effect on platelet COX-1 (Patrignani et al. 1982). For example, in studies with purified COX-1 and with isolated platelets, nanomolar concentrations of aspirin will completely block PG synthesis within 20 min after exposure (Roth and Majerus 1975; Roth et al. 1975). Higher concentrations and longer exposures will not alter aspirin's inhibitory effect on PG synthesis because of its saturability. Exactly the same feature (maximal effect at low doses, absence of dose effect) is apparent in clinical trials with aspirin as an antithrombotic agent (Patrono et al. 2005). Thus, the consistency of dose requirements and saturability of the effects of aspirin in acetylating the platelet enzyme (Burch et al. 1979), inhibiting TXA_2 production (Patrignani et al. 1982; Patrono et al. 1985), and preventing atherothrombotic complications (Patrono et al. 2005; Antithrombotic Trialists Collaboration 2002) constitute the best evidence that aspirin prevents thrombosis through inhibition of TXA_2 production. It is likely, therefore, that any of the potential effects of aspirin on other determinants of arterial thrombosis are much less important than the inhibition of platelet COX-1 activity (Patrono et al. 2008).

4.3 *Aspirin "Resistance"*

The term "aspirin resistance" has been used to describe a number of different phenomena, including the inability of aspirin to: (1) protect individuals from

thrombotic complications, (2) cause a prolongation of the bleeding time, (3) reduce TXA_2 production, or (4) produce a typical effect on in vitro tests of platelet function (Patrono 2003). The fact that some patients may experience recurrent vascular events despite aspirin therapy should be properly referred to as "treatment failure" rather than aspirin resistance. Treatment failure is a common phenomenon occurring with all drugs (e.g., lipid-lowering or antihypertensive drugs). Given the multifactorial nature of atherothrombosis, it is not surprising that only a fraction (usually one-quarter to one-third) of all vascular complications can be prevented by any single preventive strategy (Davì and Patrono 2007).

It has been reported that a variable proportion (up to one-fourth) of aspirin-treated patients with cerebrovascular, coronary or peripheral arterial disease only achieve partial inhibition of platelet aggregation at initial testing, and some (up to one-third) appear to develop "resistance" to aspirin over time, even with increasing doses (reviewed in Patrono et al. 2008). However, the majority of these studies shared the following major limitations: (1) biochemical or witnessed verification of patient's adherence to the prescribed therapy was absent; (2) there was only a single measurement of any given test; (3) the intra- and inter-subject variability of the assay over time was usually not reported; (4) the criteria to define the normal versus the "aspirin resistant" range and the assay conditions differed among studies; (5) the daily doses of aspirin were heterogeneous, ranging from 75 to 1,300 mg; and (6) none of these studies was properly controlled (reviewed in Patrono et al. 2008).

Lack of biochemical assessment of compliance is a major issue in studies investigating aspirin "unresponsiveness." Interestingly, a study in 190 patients with a history of myocardial infarction compared arachidonate-induced platelet aggregation in patients while receiving their usual aspirin therapy, after 7 days of withdrawal and 24 h after a single witnessed intake of aspirin 325 mg (Schwartz et al. 2005). While 9% of patients who declared having taken their usual therapy failed to show inhibition of platelet aggregation, this percentage dropped to $<1\%$ (1 patient out of 190) after a witnessed dose (Schwartz et al. 2005). Furthermore, this single patient admitted NSAID intake, 12 h before testing. Similar results were reported in the study by Lev et al. where, after a witnessed dose of 325 mg of aspirin, the mean of AA-induced platelet aggregation became $<20\%$ (the established limit to define "resistance") in formerly resistant patients (Lev et al. 2006). Therefore, questionnaires cannot be a reliable tool to assess the compliance to any given treatment, including aspirin, and studies not relying on salicylate measurements or serum TXB_2 have a major, intrinsic bias seriously hampering the interpretation of results. Furthermore, the few studies directly comparing different functional assays failed to find any significant agreement between tests, suggesting that aspirin nonresponsiveness may be highly test-specific (Santilli et al. 2009).

Currently available antiplatelet drugs are characterized by individual pharmacokinetic (PK) features that may explain the delivery of variable amounts of the active moiety of the drug to its site(s) of action in different individuals, and therefore provide a PK basis for interindividual variability in pharmacological response (Rocca and Patrono 2005). Inadequate bioavailability of intact acetylsalicylic acid or thiol-containing active metabolites of thienopyridines, because of galenic

or genetic reasons, may be sufficient to explain a reduced antiplatelet effect in some individuals without the need for claiming "resistance" of the drug target (Rocca and Patrono 2005).

Moreover, there seems to be relatively limited room for pharmacodynamic (PD) variability in response to aspirin or thienopyridines, with the notable exception of a PD interaction reducing the antiplatelet effect of aspirin (Rocca and Patrono 2005). Concomitant treatment with some readily available over-the-counter (OTC) NSAIDs, such as ibuprofen, may limit the antiplatelet effects of low-dose aspirin and contribute, together with nonadherence, to many reports of so-called aspirin "resistance" (Catella-Lawson et al. 2001). This is due to competition for a common docking site within the COX-1 channel (arginine-120), to which aspirin has to anchor in order to selectively acetylate Ser-529 (Loll et al. 1995). This interaction has been reported also between naproxen and low-dose aspirin (Capone et al. 2005; Anzellotti et al. 2011), but does not occur with rofecoxib (Catella-Lawson et al. 2001), celecoxib (Renda et al. 2006), or diclofenac (Catella-Lawson et al. 2001), i.e., drugs endowed with moderate to high COX-2 selectivity (FitzGerald and Patrono 2001). Although the clinical consequences of this interaction between aspirin and some traditional NSAIDs are uncertain, its occurrence in elderly patients with concomitant cardiovascular and osteoarthritic diseases may account for the occasional finding of less-than-complete suppression of platelet COX-1 activity in this setting.

Based on a PD analysis of the variability in response to aspirin and $P2Y_{12}$ blockers, there seems to be no solid grounds for the practice of phenotyping patients as being "resistant" or "nonresponders" to these drugs based on a single measurement of platelet function, performed at a variable time point after dosing, and using a largely arbitrary response threshold (Rocca and Patrono 2005). Moreover, unless the primary mechanism underlying a repeated finding of less-than-expected inhibition of platelet function at a standardized time point (e.g., 24 h after a witnessed administration) is characterized in the individual patient, changing his/her antiplatelet therapy will represent a purely empirical exercise.

The information from randomized clinical trials that we have briefly reviewed above should provide an estimate of the pharmacologically plausible difference in clinical event rates between patients who are fully responsive to antiplatelet therapy and those who are poor responders. Given the effect size of aspirin monotherapy (i.e., a 20–30% relative risk reduction in high-risk patients), one would reasonably expect that the relative risk of poor responders to aspirin may range between 1.3 and 1.5 (under a worst case scenario of no protective effects of the drug) as compared to good responders, assuming comparability of patients' characteristics and adherence to prescribed therapy. Krasopoulos et al. (2008) have reported a systematic review and meta-analysis of 20 studies that related a baseline diagnosis of aspirin "resistance" (based on a variety of biochemical and functional tests) to the risk of experiencing some sort of vascular complication during follow-up of patients with cardiovascular disease. The sample size of these studies ranged between 28 and 326 patients. Approximately one-quarter of these 2,930 patients were phenotyped as being aspirin "resistant" at baseline. The risk of experiencing any cardiovascular event was, on average, fourfold (odds ratio, 3.85; 95% CI, 3.08–4.80) higher in

"resistant" as compared to aspirin-"sensitive" patients, with odds ratios of individual studies ranging between 0.87 and 142 (Krasopoulos et al. 2008).

If these numbers were real, then aspirin "resistance" would represent the strongest predictor of cardiovascular morbidity, inasmuch as the typical odds ratio for the association of traditional risk factors with cardiovascular outcomes rarely is higher than 2.0 (Antithrombotic Trialists' (ATT) Collaboration 2009). Alternatively, one should seek other explanations for these unexpected findings. These include: (1) misclassification of patients as being "resistant" on "nonresponders" based on a single determination of platelet function using an arbitrary response threshold; (2) co-segregation of genetic variants influencing PK/PD variability with genetic traits adversely affecting the natural history of atherothrombosis; and (3) variable medication adherence in the two subgroups. It should be emphasized that compliance in these studies was typically ascertained by verbal interviews or questionnaires that largely underestimate the true rate of medication adherence. Nonadherence to medications has been documented to occur in over 60% of patients with cardiovascular disease (Baroletti and Dell'Orfano 2010). Self-reported medication adherence in these patients is <40% for the combination of aspirin, β-blocker and statin and falls to 21% when it is based on more than two consecutive follow-up surveys over 6 ± 12 months (Baroletti and Dell'Orfano 2010). Several studies have shown that both primary and secondary nonadherence lead to increased risk of cardiovascular events and mortality (Baroletti and Dell'Orfano 2010).

In a single center, prospective study of 700 consecutive aspirin-treated patients presenting for coronary angiographic evaluation, residual platelet COX-1 activity (as reflected by serum TXB_2), and COX-1-independent platelet function measured by PFA-100 collagen-ADP closure time, but not COX-1-dependent functional assays (e.g., AA-stimulated platelet markers), correlated with subsequent major cardiovascular events (Frelinger et al. 2009). Based on these findings the authors concluded that multiple mechanisms, including but not confined to inadequate inhibition of platelet COX-1, are responsible for poor clinical outcomes in aspirin-treated patients, and therefore the term "aspirin resistance" is inappropriate (Frelinger et al. 2009). The results of this study (Frelinger et al. 2009) based on serum TXB_2 determinations are consistent with the results of the two earlier studies (Eikelboom et al. 2002, 2008), in which Eikelboom et al. measured baseline urinary 11-dehydro-TXB_2 excretion in a subgroup of aspirin-treated participants in the HOPE (Eikelboom et al. 2002) and CHARISMA (Eikelboom et al. 2008) trials, and described an association of increasing risk for the primary end-point with increasing quartiles of TXB_2 metabolite excretion (odds ratio, 1.8 and 1.7 in each study, respectively, for the highest quartile relative to the lowest quartile). The effect size of this association is somewhat smaller and perhaps more realistic than in other studies based on functional measurements. Neither Frelinger's study (Frelinger et al. 2009) nor Eikelboom's studies (Eikelboom et al. 2002, 2008) addressed rigorously the issue of medication adherence, although the former excluded from follow-up two patients who had serum TXB_2 in the range of aspirin-free healthy controls. Of course, some of the included patients may well

have been irregularly noncompliant and displayed serum TXB_2 levels outside the control range but still in the nonfunctional range of platelet COX-1 inhibition (i.e., <97%) (Fig. 2), depending on the time elapsed since the last aspirin intake. Moreover, these studies—by virtue of their design—did not provide insight into the mechanism(s) underlying incomplete inhibition of platelet COX-1 activity (Frelinger et al. 2009) or "aspirin-resistant" TXA_2 biosynthesis (Eikelboom et al. 2002, 2008), nor did they assess reproducibility of these abnormal biochemical phenotypes.

Therefore, the term "resistance" is uninformative of the mechanism(s) contributing to the interindividual variability in response to aspirin or clopidogrel, and is potentially misleading. Thus, it implies that drug response can be measured with a standardized method that has direct bearing to clinical efficacy and, depending on its results, may lead to a change in antiplatelet therapy. In fact, the relationship of the various functional indexes of platelet capacity that can be measured ex vivo to the actual occurrence of platelet activation and inhibition in vivo is far from being established (Davì and Patrono 2007). Therefore, we (Rocca and Patrono 2005) and others (Frelinger et al. 2009; Cattaneo 2010) have suggested that the term "resistance" should be abandoned in order to advance our understanding of the distinct determinants of the interindividual variability in response to aspirin or $P2Y_{12}$ blockers.

If measurements of platelet biochemistry and/or function during antiplatelet treatment are intended to provide complementary prognostic information to better define the cardiovascular risk profile of the patient, then we need a paradigm shift along the following lines: (1) standardizing the methods to be routinely employed; (2) obtaining two to three repeated measurements from each subject to assess consistency of results; (3) drawing blood samples at a fixed time interval (e.g., 24 h for aspirin and thienopyridines) after dosing; (4) describing the results as being in the low, intermediate, or high range of response in the relevant patient population; (5) relating these variable functional responses to the occurrence of cardiovascular and bleeding outcomes in adequately sized observational studies.

If the same measurements are intended to provide guidance for tailoring antiplatelet therapy in the individual patient, then we need randomized clinical trials assessing the clinical effectiveness of testing versus nontesting in patients with inadequate response to standard regimens of antiplatelet therapy (Patrono et al. 2011). Several ongoing trials are evaluating whether adjustment of antiplatelet therapy by use of functional thresholds defined to identify high versus low on-treatment platelet reactivity with aspirin or clopidogrel can improve efficacy while maintaining safety versus standard therapy. It should be acknowledged that both the size (442–2,783 patients) and duration of follow-up (6–12 months) of these trials may be largely inadequate to detect relatively small differences between the two randomized strategies (Patrono et al. 2011). A general strategy of using higher doses of aspirin, clopidogrel or both in the early phase of acute coronary syndromes was not proven successful by the CURRENT-OASIS 7 trial (2010).

5 Clinical Efficacy of Low-Dose Aspirin

5.1 Prevention of Atherothrombosis in Different Clinical Settings

The efficacy and safety of aspirin are documented from analysis of approximately 70 randomized clinical trials that included over 115,000 patients at variable risk of thrombotic complications of atherosclerosis (Antithrombotic Trialists Collaboration 2002).

Aspirin has been tested in patients representing the whole spectrum of atherothrombosis, from apparently healthy, low-risk individuals to patients presenting with an acute myocardial infarction or an acute ischemic stroke; similarly, trials have extended for as short as a few weeks' duration or as long as 10 years (Patrono et al. 2005; Antithrombotic Trialists Collaboration 2002). Although aspirin has been shown consistently to be effective in preventing fatal and/or nonfatal vascular events in these trials, both the size of the proportional effects and the absolute benefits of antiplatelet therapy are somewhat heterogeneous in different clinical settings.

In the Second International Study of Infarct Survival (ISIS-2) (1988), a single tablet of aspirin, 162.5 mg, started within 24 h of the onset of symptoms of a suspected myocardial infarction and continued daily for 5 weeks produced highly significant reductions in the risk of vascular mortality (by 23%), nonfatal reinfarction (by 49%), and nonfatal stroke (by 46%). There was no increase in hemorrhagic stroke or GI bleeding in the aspirin-treated patients and only a small increase in minor bleeding (ISIS-2 Collaborative Group 1988). Treatment of 1,000 patients with suspected acute myocardial infarction with aspirin for 5 weeks will result in approximately 40 patients in whom a major vascular event is prevented (Antithrombotic Trialists Collaboration 2002), with a proportional odds reduction of 30%.

Two separate trials with a similar protocol, the International Stroke Trial (IST) (1997) and the Chinese Acute Stroke Trial (CAST) (1997), tested the efficacy and safety of early aspirin use in acute ischemic stroke. Approximately 40,000 patients were randomized within 48 h of the onset of symptoms to 2–4 weeks of daily aspirin therapy (300 mg and 160 mg, respectively) or placebo. An overview of the results of both trials suggests an absolute benefit of nine fewer deaths or nonfatal strokes per 1,000 patients in the first month of aspirin therapy (Antithrombotic Trialists Collaboration 2002). The proportional odds reduction in fatal or nonfatal vascular events is only 10% in this setting. Although the background risk of hemorrhagic stroke was threefold higher in CAST than in IST, the absolute increase in this risk associated with early use of aspirin was similar in the two studies (excess 2 per 1,000 patients) (International Stroke Trial Collaborative Group 1997; CAST (Chinese Acute Stroke Trial) Collaborative Group 1997). The broad clinical implications of these findings are discussed in elsewhere. In terms of their research implications, these results are consistent with biochemical evidence of episodic platelet activation during the first 48 h after the onset of symptoms of an acute

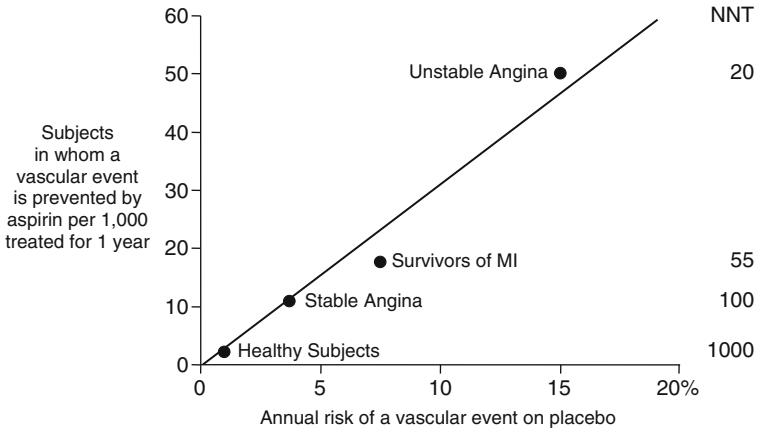


Fig. 3 The absolute risk of vascular complications is the major determinant of the absolute benefit of antiplatelet prophylaxis. Data are plotted from placebo-controlled aspirin trials in different clinical settings. For each category of patients, the abscissa denotes the absolute risk of experiencing a major vascular event as recorded in the placebo arm of the trial(s). The absolute benefit of antiplatelet treatment is reported on the ordinate as the number of subjects in whom an important vascular event (nonfatal myocardial infarction, nonfatal stroke, or vascular death) is actually prevented by treating 1,000 subjects with aspirin for 1 year. Numbers needed to treat (NNT) to prevent one event in each clinical setting are also displayed on the right hand side of the figure

ischemic stroke and with suppression of in vivo TXA_2 biosynthesis in patients receiving low-dose aspirin in this setting (Van Kooten et al. 1994).

Long-term aspirin therapy confers conclusive net benefit on the risk of subsequent myocardial infarction, stroke, or vascular death among subjects with high risk of vascular complications. These include patients with chronic stable angina, patients with prior myocardial infarction, patients with unstable angina, and patients with TIA or minor stroke, as well as other high-risk categories (Patrono et al. 2005, 2008; Antithrombotic Trialists Collaboration 2002). The proportional effects of long-term aspirin therapy on vascular events in these different clinical settings appear rather homogeneous, ranging between 20% and 25% odds reduction based on an overview of all randomized trials (Antithrombotic Trialists Collaboration 2002). However, individual trial data show substantial heterogeneity, ranging from no statistically significant benefits in patients with peripheral vascular disease to approximately 50% risk reduction in patients with unstable angina (Antithrombotic Trialists Collaboration 2002). Although other factors may play a role, we interpret these findings as reflecting the variable importance of TXA_2 as a mechanism amplifying the hemostatic response to plaque destabilization in different clinical settings (Patrono et al. 2008). In terms of absolute benefit, these protective effects of aspirin translate into avoidance of a major vascular event in 50 per 1,000 patients with unstable angina treated for 6 months and in 36 per 1,000 patients with prior myocardial infarction, stroke, or TIA treated for approximately 30 months (Fig. 3) (Antithrombotic Trialists Collaboration 2002).

For patients with different manifestations of ischemic heart or brain disease, a widespread consensus exists in defining a rather narrow range of recommended daily doses, i.e., 75–160 mg, for the prevention of myocardial infarction, stroke, or vascular death (Becker et al. 2008; Albers et al. 2008). This is supported by separate trial data in patients randomized to treatment with low-dose aspirin or placebo as well as by an overview of all antiplatelet trials showing no obvious dose dependence, from indirect comparisons, for the protective effects of aspirin (Antithrombotic Trialists Collaboration 2002). There is no convincing evidence that the dose requirement for the antithrombotic effect of aspirin varies in different clinical settings.

Among most high-risk patient groups, the expected number avoiding a serious vascular event by using aspirin substantially exceeds the number experiencing a major bleed. However, it is unclear whether aspirin might benefit people who, whilst apparently healthy, are at intermediate risk of serious vascular events. The Antithrombotic Trialists' (ATT) Collaboration has addressed this question in an individual participant data meta-analysis of all large randomized trials of the primary prevention of vascular events (Antithrombotic Trialists' (ATT) Collaboration 2009). In the six trials among 95,000 low-risk individuals, with mean follow-up 6.9 years, aspirin allocation yielded a 12% relative risk reduction in serious vascular events, from an annual rate of 0.57% to 0.51% (Antithrombotic Trialists' (ATT) Collaboration 2009). This effect was mainly due to a reduction in nonfatal myocardial infarction, from 0.23% to 0.18% per year. The net effect on stroke was not significant, reflecting a small reduction in presumed ischemic stroke and counterbalancing effects on hemorrhagic stroke and other stroke (Antithrombotic Trialists' (ATT) Collaboration 2009). There was no significant reduction in vascular mortality. Aspirin allocation increased GI (or other extracranial) bleeds from 0.07% to 0.1% per year (Antithrombotic Trialists' (ATT) Collaboration 2009). The risks of serious vascular events and of major extracranial bleeds were predicted by the same independent risk factors (age, male gender, diabetes mellitus, current smoking, blood pressure, and body mass index), so individuals at higher risk of vascular complications also had a high risk of bleeding (Antithrombotic Trialists' (ATT) Collaboration 2009).

While for secondary prevention the net benefits of adding aspirin to other preventive measures would substantially exceed the bleeding hazards, irrespective of age and gender, in people without preexisting vascular disease the benefits of adding long-term aspirin to other, safer, forms of primary prevention (e.g., statins and antihypertensive drugs) would be of similar magnitude as the hazards (Antithrombotic Trialists' (ATT) Collaboration 2009). Hence, the currently available trial results do not seem to justify general guidelines advocating the routine use of aspirin in all apparently healthy individuals above a moderate level of coronary risk, unless additional long-term benefits of aspirin therapy (Rothwell et al. 2010, 2011) become firmly established. This emphasizes the need for trials of low-dose aspirin among specific groups at increased risk of vascular complications, such as those aged over 70 years and people with diabetes mellitus (Patrono and Rocca 2010).

5.2 Atrial Fibrillation

Moderate-dose warfarin alone (international normalized ratio [INR], 2.0–3.0) is very effective in reducing the risk of stroke in patients with nonvalvular atrial fibrillation (Singer et al. 2008). The efficacy of aspirin in doses between 75 and 325 mg has been compared with warfarin and placebo in three randomized trials of patients with nonvalvular atrial fibrillation (Singer et al. 2008). Pooled analysis of the three studies shows a relative risk reduction in favor of aspirin over placebo of about 25% (range, 14–44%) (Singer et al. 2008). On pooled analysis, warfarin was significantly more effective than aspirin, with a 47% relative risk reduction (range, 28–61%, $p < 0.01$) (Singer et al. 2008). Thus, aspirin appears to be effective in preventing stroke in patients with atrial fibrillation but is substantially less effective than warfarin. The optimal dose of aspirin for patients with atrial fibrillation is uncertain. The largest effect of aspirin was seen in the SPAF1 trial, which used a daily dose of 325 mg (Stroke Prevention in Atrial Fibrillation Study 1991). However, extrapolating from trials of low-dose aspirin in other clinical indications, Singer et al. (2008) suggested that the best balance of efficacy and safety is achieved at 75–100 mg daily.

5.3 Deep Venous Thrombosis

The Pulmonary Embolism Prevention (PEP) trial (2000) has established that aspirin is effective in preventing venous thromboembolism after surgery for hip fracture. This was a double-blind multicenter study of 13,356 patients undergoing surgery for hip fracture and of an additional 4088 patients undergoing elective hip or knee arthroplasty. Patients were assigned aspirin 160 mg or placebo once daily for 5 weeks, with the first dose starting before surgery. Other forms of prophylaxis were allowed and either heparin or low molecular weight heparin was used in about 40% of the patients. Among the 13,356 patients with hip fracture, aspirin produced a 36% reduction in symptomatic deep venous thrombosis (DVT) or PE (absolute risk reduction 0.9%; $p = 0.0003$). A similar relative risk reduction in patients who were assigned aspirin was observed in patients who also received heparin (Pulmonary Embolism Prevention (PEP) Trial Collaborative Group 2000). This study therefore clearly shows that aspirin reduces the incidence of fatal PE and symptomatic nonfatal DVT or PE in patients with hip fracture. The results of the PEP trial are consistent with the meta-analysis performed by the Antiplatelet Trialists' Collaboration (1994), and supercede the findings in most of the previous trials. However, because more effective methods of thromboprophylaxis are readily available, the American College of Chest Physicians (ACCP) Evidence-Based Clinical Practice Guidelines recommend against the use of aspirin alone as thromboprophylaxis against venous thromboembolism for any patient group (Geerts et al. 2008).

5.4 Placental Insufficiency

The pathogenesis of preeclampsia and fetal growth retardation is related to reduced placental blood flow, which is believed to be caused by constriction and/or thrombosis of small placental arteries (Bates et al. 2008). The initial reports that low-dose aspirin therapy reduces the risk of severe low birth weight among newborns, and the risk of cesarean section in mothers with pregnancy-induced hypertension, led to the widespread use of prophylactic aspirin to prevent preeclampsia. Subsequently, several larger trials reported no beneficial effects of aspirin (Bates et al. 2008).

A systematic review (Duley et al. 2001) of data from 39 trials in over 30,000 women showed that antiplatelet therapy (mostly aspirin 60 mg daily) is associated with a 15% decrease in the risk of preeclampsia. This effect was consistent, regardless of risk status (moderate or high risk), dose of aspirin or gestation at trial entry. There was some evidence that there may be greater benefits for women given >75 mg aspirin, although the numbers of women in the subgroup were small and so there is potential for random error. There was also an 8% reduction in the risk of preterm birth and a 14% reduction in the risk of fetal or neonatal death for women allocated antiplatelet therapy (Duley et al. 2001). Remaining questions are whether particular subgroups of high-risk women might have greater benefit and whether earlier treatment (i.e., before 12 weeks) or aspirin doses >75 mg would have additional benefits without an increase in adverse effects (Duley et al. 2001). The potential involvement of extraplatelet sources of vasoactive eicosanoids expressing COX-2 in response to a local growth-promoting milieu might contribute, at least in part, to the limited efficacy of low-dose aspirin therapy in this setting.

6 Adverse Effects of Aspirin

Aspirin does not cause a generalized bleeding abnormality unless it is given to patients with an underlying hemostatic defect, such as hemophilia, uremia, or that induced by anticoagulant therapy. Aspirin-induced impairment of primary hemostasis cannot be separated from its antithrombotic effect and is similar at all doses equal to or greater than 75 mg daily (Patrono et al. 2005).

The balance between preventing vascular occlusion and causing excess bleeding with aspirin depends critically on the absolute thrombotic vs. hemorrhagic risk of the patient. Thus, in individuals at low risk for vascular occlusion (e.g., $\leq 1\%$ per year), a very small absolute benefit is offset by exposure of a large number of healthy subjects to undue bleeding complications. In contrast, in patients at high risk of cardiovascular or cerebrovascular complications (e.g., >3% per year), the absolute benefit of aspirin prophylaxis clearly outweighs the harm (Table 2). For example, the absolute excess of major bleeds (i.e., those requiring transfusion) in acute myocardial infarction is approximately 1/100th the absolute number of major vascular events avoided by aspirin therapy (Antithrombotic Trialists Collaboration 2002).

Table 2 Benefit and harm of antiplatelet prophylaxis with aspirin in different settings

Clinical setting	Benefit[a] Number of patients in whom a major vascular event is avoided per 1,000/year	Harm[b] Number of patients in whom a major GI bleeding event is caused per 1,000/year
Men at low to high cardiovascular risk	1–2	1–2
Essential hypertension	1–2	1–2
Chronic stable angina	10	1–2
Prior myocardial infarction	20	1–2
Unstable angina	50	1–2

[a]Benefits are calculated from randomized trial data reviewed in this chapter and depicted in Fig. 3
[b]Excess of upper GI bleedings are estimated from a background rate of 1–2 events per 1,000 per year in the general population of nonusers and a relative risk of 2.0 associated with aspirin prophylaxis. Such an estimate assumes comparability of other risk factors for upper GI bleeding, such as age and concomitant use of NSAIDs, and may actually underestimate the absolute risk in an elderly population exposed to "primary" prevention

The overall risk of major extracranial and intracranial hemorrhage associated with antiplatelet drugs is difficult to assess in individual trials because their incidence is low, i.e., <1% per year, making detection of even a 50–60% relative risk increase unrealistic in most trials of a few thousand patients.

Aspirin-induced GI toxicity, as detected in randomized clinical trials, appears to be dose related in the range of 30–1,300 mg daily (Patrono et al. 2008). This, along with studies of the relationship of efficacy to dose, is based largely on indirect comparisons of different trials and on a limited number of randomized, direct comparisons of different aspirin doses, as reviewed above. Such a dose–response relationship is thought to reflect at least two COX-1-dependent components, dose-dependent inhibition of COX-1 in the GI mucosa and dose-independent (within the range of examined doses) inhibition of COX-1 in platelets (Patrono et al. 2005, 2008). Thus, it is not surprising that the antithrombotic effect of aspirin can be dissociated, at least in part, from its most common side effect. However, even when administered at low doses, aspirin can cause serious GI bleeding, as reported in studies using 30–50 mg daily (Diener et al. 1996; The Dutch TIA Trial Study Group 1991). Because of the underlying prevalence of gastric mucosal erosions related to concurrent use of other NSAIDs and/or *Helicobacter pylori* infection in the general population, it should be expected that any antiplatelet dose of aspirin will cause more bleeding from preexisting lesions than a placebo. Consistent with this mechanistic interpretation, the relative risk of hospitalization due to upper GI bleeding associated with low-dose aspirin therapy (75–300 mg daily) was comparable to that of clopidogrel, i.e., 1.8 (95% CI, 1.6–2.0) vs. 1.7 (95% CI, 1.2–2.2), respectively, in a large cohort study with nested case–control analysis (García Rodríguez et al. 2011).

In the overview of the Antithrombotic Trialists' Collaboration, information was available on 787 major extracranial hemorrhages in 60 trials recording at least one such hemorrhage (Antithrombotic Trialists Collaboration 2002). These were generally defined as hemorrhages that were fatal or required transfusion; among them,

159 (20%) caused death. Overall, the proportional increase in risk of a major extracranial bleed with antiplatelet therapy was about one-half (odds ratio 1.6; 95% CI, 1.4–1.8), with no significant difference between the proportional increases observed in each of the five high-risk categories of patients. After allowing for noncompliance in the trials, these estimates are compatible with the twofold excess observed in case–control studies (García Rodríguez et al. 2001, 2011).

Several epidemiological studies have found a dose–response relationship between aspirin prescription and upper GI complications, as reviewed by García Rodríguez et al. (2001).

The widely held belief that enteric-coated and buffered varieties of aspirin are less likely to occasion major upper GI bleeding than plain tablets was tested in data from a multicenter case–control study (Kelly et al. 1996). The relative risks of upper GI bleeding for plain, enteric-coated, and buffered aspirin at average daily doses of $\leq$325 mg was 2.6, 2.7, and 3.1, respectively. At doses >325 mg, the relative risk was 5.8 for plain and 7.0 for buffered aspirin; there were insufficient data to evaluate enteric-coated aspirin at this dose level (Kelly et al. 1996). Similar conclusions were reached by a case–control study using data from the UK General Practice Research Database (De Abajo and García Rodríguez 2001).

Suppressing acid secretion is known to reduce the risk of ulcers associated with regular use of NSAIDs. In high-risk patients (history of previous ulcer bleeding) taking low-dose aspirin for 6 months, omeprazole and *H. pylori* eradication were associated with similar rates of recurrent bleeding (0.9% vs. 1.9%) (Chan et al. 2001), although clinically important differences between the two preventive strategies could not be excluded owing to the small sample size ($n = 250$).

Two relatively small studies (Chan et al. 2005; Lai et al. 2006) have challenged current guidelines that recommend clopidogrel for patients who have major GI contraindications to aspirin, principally recent significant bleeding from a peptic ulcer or gastritis. Both studies enrolled patients who developed ulcer bleeding after the use of low-dose aspirin. In the study of Chan et al. (2005), after healing of ulcers and eradication of *H. pylori*, if present, 320 patients were randomly assigned to receive either 75 mg of clopidogrel daily or 80 mg of aspirin daily plus 20 mg of esomeprazole twice daily for 12 months. The cumulative incidence of recurrent bleeding was 8.6% (95% CI, 4.1–13.1%) among patients who received clopidogrel and 0.7% (95% CI, 0–2.0%) among those who received aspirin plus esomeprazole ($P = 0.001$) (Chan et al. 2005). In the study of Lai et al. (2006), 170 patients with prior ulcer bleeding were randomly assigned to treatment with clopidogrel 75 mg daily or aspirin 100 mg daily and esomeprazole 20 mg daily for 1 year. The cumulative incidence of recurrent ulcer complications was 13.6% and 0%, respectively (95% CI for the difference, 6.3–20.9%, $P = 0.0019$) (Lai et al. 2006). The consistent findings of two independent studies suggest that the combination of esomeprazole and low-dose aspirin is superior to clopidogrel in preventing recurrent ulcer bleeding in patients with a history of aspirin-related ulcer bleeding.

Substantially less information is available concerning the risk of intracranial hemorrhage associated with aspirin use. In the Nurses' Health Study cohort of approximately 79,000 women aged 34–59 years, infrequent use of aspirin (1–6

tablets per week) was associated with reduced risk of ischemic stroke, while high frequency of use (15 or more aspirin per week) was associated with increased risk of subarachnoid hemorrhage, particularly among older or hypertensive women (Iso et al. 1999). In the overview of the ATT Collaboration (2002), the overall absolute excess of intracranial hemorrhage due to aspirin therapy was less than 1 per 1,000 patients per year in high-risk trials, with somewhat higher risks in patients with cerebrovascular disease. As noted earlier, the meta-analysis of primary prevention trials suggests that aspirin was associated with 5 additional hemorrhagic strokes per 1,000 among moderate-risk participants (risk of coronary event >1% per year) over 5 years (i.e., ~1/1,000 per year), but substantially less than this among low-risk participants (Antithrombotic Trialists' (ATT) Collaboration 2009).

Low-dose aspirin therapy has not been reported to affect renal function or blood pressure control, consistently with its lack of effect on renal prostaglandins that derive primarily from constitutively expressed COX-2 in the human kidney (FitzGerald and Patrono 2001). Moreover, aspirin 75 mg daily did not affect blood pressure nor the need for antihypertensive therapy in intensively treated hypertensive patients (Hansson et al. 1998). The suggestion that the use of aspirin and other antiplatelet agents is associated with reduced benefit from enalapril in patients with left ventricular systolic dysfunction is not supported by the results of a large meta-analysis of myocardial infarction trials (Latini et al. 2000). Similarly, no negative interaction occurs between ACE inhibition and the cardiovascular benefits of low-dose aspirin in intensively treated hypertensive patients (Zanchetti et al. 2002). The ACE Inhibitors Collaborative Group has carried out a systematic overview of data for 22,060 patients from six long-term randomized trials of ACE inhibitors to assess whether aspirin altered the effects of ACE inhibitor therapy on major clinical outcomes (Teo et al. 2002). Even though results from these analyses cannot rule out the possibility of some sort of interaction, they show unequivocally that even if aspirin is given, the addition of ACE inhibitor therapy produced substantial additional benefit in all major vascular outcomes. Therefore, in the absence of clear contraindications, concomitant use of aspirin and ACE inhibitors should be considered in all patients at high risk of major vascular events (Teo et al. 2002).

7 Reversible COX Inhibitors

In the absence of definitive randomized studies, traditional NSAIDs have long been thought to pose no cardiovascular hazard or to be somewhat cardioprotective. Because of their reversible mechanism of action in inhibiting platelet COX-1 and of their short half-lives, most traditional NSAIDs inhibit TXA_2-dependent platelet activation only transiently and incompletely in the vast majority of users (Grosser et al. 2010). A notable exception is provided by naproxen which, when administered regularly at 500 mg twice daily, has been shown to inhibit TXA_2 biosynthesis in vivo to the same extent as low-dose aspirin (Capone et al. 2004), due to its relative COX-1 selectivity and longer half-life than other commonly used

NSAIDs. Consistently with differential PK/PD, naproxen has been shown to produce no cardiovascular hazard, in contrast to other traditional NSAIDs (notably, diclofenac and ibuprofen) that display the same cardiotoxic phenotype associated with coxibs (Kearney et al. 2006).

The only reversible COX inhibitors that have been tested in randomized clinical trials for their antithrombotic efficacy are sulfinpyrazone, indobufen, flurbiprofen, and triflusal (reviewed in Patrono et al. 2008). Sulfinpyrazone is a uricosuric agent structurally related to the anti-inflammatory agent phenylbutazone. When used at the highest approved dosage of 200 mg qid, the drug inhibits platelet COX activity by approximately 60%, after conversion from an inactive sulfoxide to an active sulfide metabolite (Pedersen and FitzGerald 1985). The conflicting or negative results obtained in randomized clinical trials of sulfinpyrazone in patients with myocardial infarction or unstable angina (Patrono et al. 2008) are not surprising in light of the drug being a weak COX inhibitor with no other established antiplatelet mechanism of action (Pedersen and FitzGerald 1985).

In contrast, indobufen is a very potent inhibitor of platelet COX-1 activity and has comparable biochemical, functional, and clinical effects to those of a standard dose of aspirin. Thus, at therapeutic plasma levels achieved after oral dosing of 200 mg bid, indobufen inhibits serum TXB_2 by >95% throughout the dosing interval and reduces urinary thromboxane metabolite excretion to an extent quite comparable to aspirin (Patrono et al. 2008). The finding that indobufen is as effective as aspirin in preventing coronary graft occlusion (Patrono et al. 2008) is mechanistically consistent with the concept of platelet COX-1 inhibition largely accounting for the antithrombotic effect of aspirin, as discussed above. Indobufen has also been investigated in a small placebo-controlled study of patients with heart disease at increased embolic risk and compared with warfarin and ticlopidine in patients with nonrheumatic atrial fibrillation and patients with recent reversible cerebral ischemia, respectively (reviewed in Patrono et al. 2008). However, none of these studies in over 4,000 patients clearly established an advantage of indobufen vs. standard treatments, although the 95% CIs for these comparisons are wide.

Flurbiprofen has been evaluated in a single placebo-controlled, randomized trial of 461 patients with acute myocardial infarction (Brochier 1993). The 6-month reinfarction rate was significantly lower in the flurbiprofen group (3%) than in the placebo group (10.5%) with extremely low mortality rate (1.1%) in both groups. The small sample size of the study limits interpretation of these findings.

Triflusal, a salicylic acid derivative, reversibly inhibits platelet COX activity after conversion to a long-lived metabolite, 2-hydroxy-4-trifluoromethyl-benzoic acid (Ramis et al. 1990). While the half-life of the parent compound is only about 30 min, that of the deacetylated metabolite approximates 2 days. Although triflusal is claimed to have negligible effects on vascular PGI_2 production, this is likely to reflect the experimental conditions used for the assessment of PGI_2 production ex vivo (Patrono et al. 2008). The limited sample size of head-to-head comparisons of triflusal vs. aspirin in patients randomized within 24 h of acute myocardial infarction (Cruz-Fernandez et al. 2000) and in patients with cerebrovascular disease (Matías-Guiu et al. 2003) precludes unequivocal interpretation of the similar rates of major vascular events in the two treatment groups.

Knowledge Gaps

- The benefit/risk profile of aspirin in the primary prevention of atherothrombosis in people at intermediate cardiovascular risk (i.e., between 1.5% and 3.0% per annum) because of diabetes mellitus, advanced age or a cluster of risk factors is still uncertain.
- The factors influencing the rate of renewal of platelet COX-1 and the adequacy of a 24-h dosing interval are still insufficiently characterized.
- The potential chemopreventive effect of aspirin at antiplatelet doses has still to be completely clarified.

Key Messages

- The consistency of dose requirements and saturability of the effects of aspirin in acetylating platelet COX-1, inhibiting TXA_2 production, and preventing atherothrombotic complications constitute the best evidence that aspirin prevents arterial thrombosis through permanent inactivation of platelet COX-1.
- The saturability of the antiplatelet effect of aspirin at low doses, the lack of a dose–response relationship in clinical studies evaluating its antithrombotic effects, and the dose-dependence of its side-effects all support the use of as low a dose of aspirin as has been found to be effective in the treatment and prevention of atherothrombosis in different clinical settings.
- Use of the lowest effective dose of aspirin (50–100 mg daily for long-term treatment, with a single loading dose of 160–200 mg for acute ischemic syndromes) is currently the most appropriate strategy to maximize its efficacy and minimize its toxicity.
- The term "resistance" is uninformative of the mechanism(s) contributing to the interindividual variability in response to low-dose aspirin. There seems to be no solid grounds for the practice of phenotyping patients as being "resistant" or "non-responder" to aspirin based on a single measurement of platelet function, performed at a variable time point after dosing, and using a largely arbitrary response threshold.
- Unless the primary mechanism (e.g., noncompliance, interaction with other NSAIDs, or accelerated renewal of the drug target) underlying a repeated finding of inadequate platelet COX-1 suppression at 24 h after a witnessed aspirin administration is characterized in the individual patient, changing his/her antiplatelet therapy is not justified.

(continued)

Acknowledgments The authors' studies were supported by a grant from the European Commission (EICOSANOX Integrated Project 005033). The expert editorial assistance of Daniela Basilico is gratefully acknowledged.

References

Albers GW, Amarenco P, Easton JD, Sacco RL, Teal P (2008) Antithrombotic and thrombolytic therapy for ischemic stroke: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest 133:630S–669S

Antiplatelet Trialists' Collaboration (1994) Collaborative overview of randomized trials of antiplatelet therapy: III. Reduction in venous thrombosis and pulmonary embolism by antiplatelet prophylaxis among surgical and medical patients. BMJ 308:235–243

Antithrombotic Trialists Collaboration (2002) Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high-risk patients. BMJ 324:71–86

Antithrombotic Trialists' (ATT) Collaboration (2009) Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. Lancet 373:1849–1860

Anzellotti P, Capone ML, Jeyam A, Tacconelli S, Bruno A, Tontodonati P, Di Francesco L, Grossi L, Renda G, Merciaro G, Di Gregorio P, Price TS, Garcia Rodriguez LA, Patrignani P (2011) Low-dose naproxen interferes with the antiplatelet effects of aspirin in healthy subjects: recommendations to minimize the functional consequences. Arthritis Rheum 63:850–859

Bala M, Chin CN, Logan AT, Amin T, Marnett LJ, Boutaud O, Oates JA (2008) Acetylation of prostaglandin H2 synthases by aspirin is regulated by the redox state of the enzymes. Biochem Pharmacol 75:1472–1481

Baroletti S, Dell'Orfano H (2010) Medication adherence in cardiovascular disease. Circulation 121:1455–1458

Bates SM, Greer IA, Pabinger I, Sofaer S, Hirsh J (2008) Venous thromboembolism, thrombophilia, antithrombotic therapy, and pregnancy: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest 133:844S–866S

Becker RC, Meade TW, Berger PB, Ezekowitz M, O'Connor CM, Vorchheimer DA, Guyatt GH, Mark DB, Harrington RA, American College of Chest Physicians (2008) The primary and secondary prevention of coronary artery disease: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest 133:776S–814S

Belch J, MacCuish A, Campbell I, Cobbe S, Taylor R, Prescott R, Lee R, Bancroft J, MacEwan S, Shepherd J, Macfarlane P, Morris A, Jung R, Kelly C, Connacher A, Peden N, Jamieson A, Matthews D, Leese G, McKnight J, O'Brien I, Semple C, Petrie J, Gordon D, Pringle S, MacWalter R (2008) The prevention of progression of arterial disease and diabetes (POPADAD) trial: factorial randomised placebo controlled trial of aspirin and antioxidants in patients with diabetes and asymptomatic peripheral arterial disease. BMJ 337:1030–1036

Born GVR, Patrono C (2006) Antiplatelets drugs. Br J Pharmacol 147:S241–S251

Brochier ML (1993) Evaluation of flurbiprofen for prevention of reinfarction and reocclusion after successful thrombolysis or angioplasty in acute myocardial infarction. The Flurbiprofen French Trial. Eur Heart J 14:951–957

Burch JW, Stanford PW, Majerus PW (1979) Inhibition of platelet prostaglandin synthetase by oral aspirin. J Clin Invest 61:314–319

Capone ML, Tacconelli S, Sciulli MG, Grana M, Ricciotti E, Minuz P, Di Gregorio P, Merciaro G, Patrono C, Patrignani P (2004) Clinical pharmacology of platelet, monocyte and vascular cyclooxygenase inhibition by naproxen and low-dose aspirin in healthy subjects. Circulation 109:1468–1471

Capone ML, Sciulli MG, Tacconelli S, Grana M, Ricciotti E, Renda G, Di Gregorio P, Merciaro G, Patrignani P (2005) Pharmacodynamic interaction of naproxen with low-dose aspirin in healthy subjects. J Am Coll Cardiol 45:1295–1301

CAST (Chinese Acute Stroke Trial) Collaborative Group (1997) CAST: randomised placebo-controlled trial of early aspirin use in 20,000 patients with acute ischemic stroke. Lancet 349:1641–1649

Catella-Lawson F, Reilly MP, Kapoor SC, Cucchiara AJ, DeMarco S, Tournier B, Vyas SN, FitzGerald GA (2001) Cyclooxygenase inhibitors and the antiplatelet effects of aspirin. N Engl J Med 345:1809–1817

Cattaneo M (2010) New $P2Y_{12}$ inhibitors. Circulation 121:171–179

Chan FK, Chung SCS, Suen BY, Lee YT, Leung WK, Leung VK, Wu JC, Lau JY, Hui Y, Lai MS, Chan HL, Sung JJ (2001) Preventing recurrent upper gastrointestinal bleeding in patients with elicobacter pylori infection who are taking low-dose aspirin or naproxen. N Engl J Med 344:967–973

Chan FK, Ching JY, Hung LC, Wong VW, Leung VK, Kung NN, Hui AJ, Wu JC, Leung WK, Lee VW, Lee KK, Lee YT, Lau JY, To KF, Chan HL, Chung SC, Sung JJ (2005) Clopidogrel versus aspirin and esomeprazole to prevent recurrent ulcer bleeding. N Engl J Med 352:238–244

Clarke RJ, Mayo G, Price P, FitzGerald GA (1991) Suppression of thromboxane A_2 but not systemic prostacyclin by controlled-release aspirin. N Engl J Med 325:1137–1141

Cox D, Maree AO, Dooley M, Conroy R, Byrne MF, Fitzgerald DJ (2006) Effect of enteric coating on antiplatelet activity of low-dose aspirin in healthy volunteers. Stroke 37:2153–2158

Cruz-Fernandez JM, Lopez-Bescos L, García-Dorado D, López García-Aranda V, Cabadés A, Martín-Jadraque L, Velasco JA, Castro-Beiras A, Torres F, Marfil F, Navarro E (2000) Randomized comparative trial of triflusal and aspirin following acute myocardial infarction. Eur Heart J 21:457–465

Current-Oasis 7 Investigators (2010) Dose comparisons of clopidogrel and aspirin in acute coronary syndromes. N Engl J Med 363:930–942

Davì G, Patrono C (2007) Platelet activation and atherothrombosis. N Engl J Med 357:2482–2494

De Abajo FJ, García Rodríguez LA (2001) Risk of upper gastrointestinal bleeding and perforation associated with low-dose aspirin as plain and enteric-coated formulations. BMC Clin Pharmacol 1:1

Demers LM, Budin R, Shaikh B (1980) The effects of aspirin on megakaryocyte prostaglandin production. Proc Soc Exp Biol Med 163:24–29

Diener HC, Cunha L, Forbes C, Sivenius J, Smets P, Lowenthal A (1996) European Stroke Prevention Study: II. Dipyridamole and acetylsalicylic acid in the secondary prevention of stroke. J Neurol Sci 143:1–13

Dragani A, Pascale S, Recchiuti A, Mattoscio D, Lattanzio S, Petrucci G, Mucci L, Ferrante E, Habib A, Ranelletti FO, Ciabattoni G, Davì G, Patrono C, Rocca B (2010) The contribution of cyclooxygenase-1 and -2 to persistent thromboxane biosynthesis in aspirin-insensitive essential thrombocythemia: implications for antiplatelet therapy. Blood 115:1054–1061

Duley L, Henderson-Smart D, Knight M, King J (2001) Antiplatelet drugs for prevention of pre-eclampsia and its consequences: systematic review. BMJ 322:329–333

Eikelboom JW, Hirsh J, Weitz JI, Johnston M, Yi Q, Yusuf S (2002) Aspirin resistant thromboxane biosynthesis and the risk of myocardial infarction, stroke, or cardiovascular death in patients at high risk for cardiovascular events. Circulation 105:1650–1655

Eikelboom JW, Hankey GJ, Thom J, Bhatt DL, Steg PG, Montalescot G, Johnston SC, Steinhubl SR, Mak KH, Easton JD, Hamm C, Hu T, Fox KA, Topol EJ, Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management and Avoidance (CHARISMA) Investigators (2008) Incomplete inhibition of thromboxane biosynthesis by acetylsalicylic acid: determinants and effect on cardiovascular risk. Circulation 118:1705–1712

Farrel B, Godwin J, Richards S, Warlow C (1991) The United Kingdom transient ischaemic attack (UK-TIA) aspirin trial: final results. J Neurol Neurosurg Psychiatry 54:1044–1054

FitzGerald GA, Patrono C (2001) The coxibs, selective inhibitors of cyclooxygenase-2. N Engl J Med 345:433–442

FitzGerald GA, Oates JA, Hawiger J, Maas RL, Roberts LJ 2nd, Lawson JA, Brash AR (1983) Endogenous biosynthesis of prostacyclin and thromboxane and platelet function during chronic administration of aspirin in man. J Clin Invest 71:676–688

Fowkes FG, Price JF, Stewart MC, Butcher I, Leng GC, Pell AC, Sandercock PA, Fox KA, Lowe GD, Murray GD, Aspirin for Asymptomatic Atherosclerosis Trialists (2010) Aspirin for prevention of cardiovascular events in a general population screened for a low ankle brachial index: a randomized controlled trial. JAMA 303:841–848

Frelinger AL III, Li Y, Linden MD, Barnard MR, Fox ML, Christie DJ, Furman MI, Michelson AD (2009) Association of cyclooxygenase-1-dependent and -independent platelet function assays with adverse clinical outcomes in aspirin-treated patients presenting for cardiac catheterization. Circulation 120:2586–2596

García Rodríguez LA, Hernández-Díaz S, de Abajo FJ (2001) Association between aspirin and upper gastrointestinal complications: systematic review of epidemiologic studies. Br J Clin Pharmacol 52:563–571

García Rodríguez LA, Lin KJ, Hernández-Díaz S, Johansson S (2011) Risk of upper gastrointestinal bleeding with low-dose acetylsalicylic acid alone and in combination with clopidogrel and other medications. Circulation 123:1108–1115

Geerts WH, Bergqvist D, Pineo GF, Heit JA, Samama CM, Lassen MR, Colwell CW (2008) Prevention of venous thromboembolism: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest 133:381S–453S

Grosser T, Fries S, FitzGerald GA (2006) Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. J Clin Invest 116:4–15

Grosser T, Smyth E, FitzGerald GA (2010) Anti-inflammatory, antipyretic, and analgesic agents; pharmacotherapy of gout. In: Goodman & Gilman's the pharmacological basis of therapeutics, 12th edn. McGraw Hill, New York

Hansson L, Zanchetti A, Carruthers SG, Dahlöf B, Elmfeldt D, Julius S, Ménard J, Rahn KH, Wedel H, Westerling S (1998) Effects of intensive blood-pressure lowering and low-dose aspirin in patients with hypertension: principal results of the Hypertension Optimal Treatment (HOT) randomised trial. Lancet 351:1755–1762

International Stroke Trial Collaborative Group (1997) The International Stroke Trial (IST): a randomised trial of aspirin, subcutaneous heparin, both, or neither among 19,435 patients with acute ischemic stroke. Lancet 349:1569–1581

ISIS-2 Collaborative Group (1988) Randomised trial of intravenous streptokinase, oral aspirin, both or neither among 17,187 cases of suspected acute myocardial infarction: ISIS-2. Lancet 2:349–360

Iso H, Hennekens CH, Stampfer MJ, Rexrode KM, Colditz GA, Speizer FE, Willett WC, Manson JE (1999) Prospective study of aspirin use and risk of stroke in women. Stroke 30:1764–1771

Kearney PM, Baigent C, Godwin J, Halls H, Emberson JR, Patrono C (2006) Do selective cyclooxygenase-2 inhibitors and traditional non-steroidal anti-inflammatory drugs increase the risk of atherothrombosis? Meta-analysis of randomised trials. BMJ 332:1302–1308

Kelly JP, Kaufman DW, Jurgelon JM, Sheehan J, Koff RS, Shapiro S (1996) Risk of aspirin-associated major upper-gastrointestinal bleeding with enteric-coated or buffered product. Lancet 348:1413–1416

Krasopoulos G, Brister SJ, Beattie WS, Elliot RF, Buchanan MR (2008) Aspirin "resistance" and risk of cardiovascular morbidity: systematic review and meta-analysis. BMJ 336:195–198

Lai KC, Chu KM, Hui WM, Wong BC, Hung WK, Loo CK, Hu WH, Chan AO, Kwok KF, Fung TT, Wong J, Lam SK (2006) Esomeprazole with aspirin versus clopidogrel for prevention of recurrent gastrointestinal ulcer complications. Clin Gastroenterol Hepatol 4:860–865

Landolfi R, Marchioli R, Kutti J, Gisslinger H, Tognoni G, Patrono C, Barbui T, for the European Collaboration on Low-Dose Aspirin in Polycythemia Vera Investigators (2004) Efficacy and safety of low-dose aspirin in polycythemia vera. N Engl J Med 350:114–124

Latini R, Tognoni G, Maggioni AP, Baigent C, Braunwald E, Chen ZM, Collins R, Flather M, Franzosi MG, Kjekshus J, Køber L, Liu LS, Peto R, Pfeffer M, Pizzetti F, Santoro E, Sleight P, Swedberg K,

Tavazzi L, Wang W, Yusuf S (2000) Clinical effects of early ACE inhibitor treatment for acute myocardial infarction are similar in the presence and absence of aspirin: meta-analysis of individual data from nearly 96,000 randomized patients. J Am Coll Cardiol 35:1801–1807

Lev EI, Patel RT, Maresh KJ, Guthikonda S, Granada J, DeLao T, Bray PF, Kleiman NS (2006) Aspirin and clopidogrel drug response in patients undergoing percutaneous coronary intervention: the role of dual drug resistance. J Am Coll Cardiol 47:27–33

Loll PJ, Picot D, Garavito RM (1995) The structural basis of aspirin activity inferred from the crystal structure of inactivated prostaglandin H_2 synthase. Nat Struct Biol 2:637–643

Matías-Guiu J, Ferro JM, Alvarez-Sabín J, Torres F, Jiménez MD, Lago A, Melo T (2003) Comparison of triflusal and aspirin for prevention of vascular events in patients after cerebral infarction. The TACIP Study: a randomized, double-blind, multicenter trial. Stroke 34:840–848

McAdam BF, Catella-Lawson F, Mardini IA, Kapoor S, Lawson JA, FitzGerald GA (1999) Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. Proc Natl Acad Sci USA 96:272–277

Murata T, Ushikubi F, Matsuoka T, Hirata M, Yamasaki A, Sugimoto Y, Ichikawa A, Aze Y, Tanaka T, Yoshida N, Ueno A, Oh-ishi S, Narumiya S (1997) Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. Nature 388:678–682

Ogawa H, Nakayama M, Morimoto T, Uemura S, Kanauchi M, Doi N, Jinnouchi H, Sugiyama S, Saito Y, for the JPAD Trial Investigators (2008) Low-dose aspirin for primary prevention of atherosclerotic events in patients with type 2 diabetes. A randomized controlled trial. JAMA 300:2134–2141

Pascale S, Petrucci G, Dragani A, Habib A, Zaccardi F, Pocaterra D, Ragazzoni E, Rolandi G, Rocca B, Patrono C (2012) Thromboxane biosynthesis in aspirin-treated essential thrombocythemia is explained by abnormal megakaryopoiesis and accelerated renewal of the drug target: evidence from biochemical measurements and inhibitor trials. Blood 119:3595–3603

Patrignani P, Filabozzi P, Patrono C (1982) Selective cumulative inhibition of platelet thromboxane production by low-dose aspirin in healthy subjects. J Clin Invest 69:1366–1372

Patrono C (2003) Aspirin resistance: definition, mechanisms and clinical read-outs. J Thromb Haemost 1:1710–1713

Patrono C, Rocca B (2010) The future of antiplatelet therapy in cardiovascular disease. Annu Rev Med 61:49–61

Patrono C, Ciabattoni G, Patrignani P, Pugliese F, Filabozzi P, Catella F, Davì G, Forni L (1985) Clinical pharmacology of platelet cyclooxygenase inhibition. Circulation 72:1177–1184

Patrono C, García Rodríguez LA, Landolfi R, Baigent C (2005) Low-dose aspirin for the prevention of atherothrombosis. N Engl J Med 353:2373–2383

Patrono C, Baigent C, Hirsh J, Roth G (2008) Antiplatelet drugs: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest 133:199S–233S

Patrono C, Andreotti F, Arnesen H, Badimon L, Baigent C, Collet J-P, De Caterina R, Gulba D, Huber K, Husted S, Kristensen SD, Morais J, Neumann F-J, Rasmussen LH, Siegbahn A, Steg P-G, Storey RF, Van de Werf F, Verheugt F (2011) Antiplatelet agents for the treatment and prevention of atherothrombosis. Eur Heart J 32(23):2922–2932

Pedersen AK, FitzGerald GA (1984) Dose-related kinetics of aspirin: presystemic acetylation of platelet cyclo-oxygenase. N Engl J Med 311:1206–1211

Pedersen AK, FitzGerald GA (1985) Cyclooxygenase inhibition, platelet function and drug metabolite formation during chronic administration of sulfinpyrazone in man. Clin Pharmacol Ther 37:36–42

Peters RJG, Mehta SR, Fox KAA, Zhao F, Lewis BS, Kopecky SL, Diaz R, Commerford PJ, Valentin V, Yusuf S (2003) Effects of aspirin dose when used alone or in combination with clopidogrel in patients with acute coronary syndromes. Observations from the Clopidogrel in Unstable angina to prevent Recurrent Events (CURE) study. Circulation 108:1682–1687

Pulmonary Embolism Prevention (PEP) Trial Collaborative Group (2000) Prevention of pulmonary embolism and deep vein thrombosis with low dose aspirin: Pulmonary Embolism Prevention (PEP) trial. Lancet 355:1295–1302

Ramis J, Torrent J, Mis R, Conte L, Barbanoj MJ, Jané J, Forn J (1990) Pharmacokinetics of triflusal after single and repeated doses in man. Int J Clin Pharmacol Ther Toxicol 28:344–349

Reilly IAG, FitzGerald GA (1987) Inhibition of thromboxane formation *in vivo* and *ex vivo*: implications for therapy with platelet inhibitory drugs. Blood 69:180–186

Renda G, Tacconelli S, Capone ML, Sacchetta D, Santarelli F, Sciulli MG, Zimarino M, Grana M, D'Amelio E, Zurro M, Price TS, Patrono C, De Caterina R, Patrignani P (2006) Celecoxib, ibuprofen, and the antiplatelet effect of aspirin in patients with osteoarthritis and ischemic heart disease. Clin Pharmacol Ther 80:264–274

Rocca B, Patrono C (2005) Determinants of the interindividual variability in response to antiplatelet drugs. J Thromb Haemost 8:1597–1602

Rocca B, Secchiero P, Ciabattoni G, Ranelletti FO, Catani L, Guidotti L, Melloni E, Maggiano N, Zauli G, Patrono C (2002) Cyclooxygenase-2 expression is induced during human megakaryopoiesis and characterizes newly formed platelets. Proc Natl Acad Sci USA 99:7634–7639

Roth GJ, Majerus PW (1975) The mechanism of the effect of aspirin on human platelets: I. Acetylation of a particulate fraction protein. J Clin Invest 56:624–632

Roth GJ, Stanford N, Majerus PW (1975) Acetylation of prostaglandin synthase by aspirin. Proc Natl Acad Sci USA 72:3073–3077

Rothwell PM, Wilson M, Elwin CE, Norrving B, Algra A, Warlow CP, Meade TW (2010) Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials. Lancet 376:1741–1750

Rothwell PM, Fowkes FGR, Belch JFF, Ogawa H, Warlow CP, Meade TW (2011) Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patients data from randomised trials. Lancet 377:31–41

Santilli F, Rocca B, De Cristofaro R, Lattanzio S, Pietrangelo L, Habib A, Pettinella C, Recchiuti A, Ferrante E, Ciabattoni G, Davì G, Patrono C (2009) Platelet cyclooxygenase inhibition by low-dose aspirin is not reflected consistently by platelet function assays. J Am Coll Cardiol 53:667–677

Schwartz KA, Schwartz DE, Ghosheh K, Reeves MJ, Barber K, DeFranco A (2005) Compliance as a critical consideration in patients who appear to be resistant to aspirin after healing of myocardial infarction. Am J Cardiol 95:973–975

Singer DE, Albers GW, Dalen JE, Fang MC, Go AS, Halperin JL, Lip GY, Manning WJ (2008) Antithrombotic therapy in atrial fibrillation: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest 133:546S–592S

Smith WL, Garavito RM, DeWitt DL (1996) Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J Biol Chem 271:33157–33160

Stroke Prevention in Atrial Fibrillation Study (1991) Final results. Circulation 84:527–539

Taylor DW, Barnett HJM, Haynes RB, Ferguson GG, Sackett DL, Thorpe KE, Simard D, Silver FL, Hachinski V, Clagett GP, Barnes R, Spence JD (1999) Low-dose and high-dose acetylsalicylic acid for patients undergoing carotid endarterectomy: a randomised controlled trial. ASA and Carotid Endarterectomy (ACE) Trial Collaborators. ASA and Carotid Endarterectomy (ACE) Trial Collaborators. Lancet 353:2179–2184

Teo KK, Yusuf S, Pfeffer M, Torp-Pedersen C, Kober L, Hall A, Pogue J, Latini R, Collins R, ACE Inhibitors Collaborative Group (2002) Effects of long-term treatment with angiotensin-converting-enzyme inhibitors in the presence or absence of aspirin: a systematic review. Lancet 360:1037–1043

The Dutch TIA Trial Study Group (1991) A comparison of two doses of aspirin (30 mg vs 283 mg a day) in patients after a transient ischemic attack or minor ischemic stroke. N Engl J Med 325:1261–1266

Topper JN, Cai J, Falb D, Gimbrone MA Jr (1996) Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. Proc Natl Acad Sci USA 93:10417–10422

Van Kooten F, Ciabattoni G, Patrono C, Schmitz PIM, Van Gijn J, Koudstaal PJ (1994) Evidence for episodic platelet activation in acute ischemic stroke. Stroke 25:278–281

Zanchetti A, Hansson L, Leonetti G, Rahn KH, Ruilope L, Warnold I, Wedel H (2002) Low-dose aspirin does not interefere with the blood pressure-lowering effects of antihypertensive therapy. J Hypertens 20:1015–1022

Thienopyridines and Other ADP-Receptor Antagonists

Isabell Bernlochner and Dirk Sibbing

Contents

I. Bernlochner • D. Sibbing (✉)
Deutsches Herzzentrum and 1. Medizinische Klinik rechts der Isar, Lazarettstrasse 36, 80636 München, Germany
e-mail: dirk@sibbing.net

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_7,

Abstract Platelet P2Y12 receptor inhibition plays a pivotal role in preventing thrombotic vascular events in patients with ACS and in patients undergoing percutaneous coronary intervention (PCI). Among the P2Y12 receptor inhibitors, the group of thienopyridines include ticlopidine, clopidogrel and prasugrel, all of which are orally administered prodrugs leading to irreversible P2Y12 receptor inhibition. Non-thienopyridine derivatives including ticagrelor, cangrelor and elinogrel do not require metabolic activation and lead to a reversible P2Y12 receptor inhibition in contrast to thienopyridines. The extend of platelet inhibition is subject to the administered antiplatelet agent and influenced by individual genetic and clinical factors. Insufficient platelet inhibition, termed high platelet reactivity (HPR) is associated with an increased risk for ischemic events after PCI whereas exceeding platelet inhibition results in an increased bleeding risk. Pharmacologic properties and clinical outcome data differ substantially between the existing P2Y12 receptor inhibitors. Whether individualized antiplatelet treatment incorporating different P2Y12 receptor inhibitors improves patients' clinical outcomes warrants further investigation.

Keywords Platelet reactivity • Platelet function testing • P2Y12 receptor inhibition • Thienopyridines • Non-thienopyridines

1 Introduction

Activation and aggregation of platelets at sites of vascular injury or in the vicinity of implanted stent struts is pivotal in the development of thrombotic events during and after acute coronary syndromes (ACSs) or percutaneous coronary interventions (PCIs). Adenosine diphosphate (ADP), released from dense granules of platelets, is a key mediator in this setting and the ADP P2Y12 receptor pathway plays a central role in activating glycoprotein (GP) IIb/IIIa receptors (Dorsam and Kunapuli 2004; Lange and Hillis 2004; Gachet 2006).

Binding of the glycoprotein (GP) IIb/IIIa receptor with its primary ligand fibrinogen and cross-linking of platelets is considered as "the final common pathway" of platelet aggregation leading to stable platelet-rich thrombus formation and possible subsequent vessel occlusion. A dual antiplatelet treatment regimen with platelet P2Y12 receptor antagonists in addition to treatment with the cyclooxygenase inhibitor aspirin has become the cornerstone of treatment in ACS patients and in patients undergoing coronary stent placement to prevent occlusive thrombotic vascular events (Smith et al. 2006; Hamm et al. 2011). Among the P2Y12 receptor antagonists, thienopyridines and non-thienopyridine derivatives exist with different pharmacokinetic and pharmacodynamic properties. The group of thienopyridines

includes ticlopidine, clopidogrel and prasugrel. These agents are orally administered prodrugs, which irreversibly inhibit the prothrombotic effects of ADP on the platelet P2Y12 receptor after hepatic conversion to their active metabolites. The first generation thienopyridine ticlopidine has been replaced by the second generation thienopyridine clopidogrel, due to its superior safety profile (Berger et al. 1999; Muller et al. 2000; Quinn and Fitzgerald 1999; Moussa et al. 1999). In combination with aspirin, clopidogrel is currently the most commonly used P2Y12 receptor inhibitor in ACS patients and after PCI (Anderson et al. 2007; Bassand et al. 2007). Prasugrel is a third generation thienopyridine that has been developed to overcome major shortcomings of clopidogrel, namely its large interindividual response variability (Serebruany et al. 2005), its delayed onset of action (Muller et al. 2001) and the phenomenon of a high proportion (~20–30 %) of patients with insufficient inhibition of platelet aggregation (high platelet reactivity, HPR) (Bonello et al. 2010).

Novel P2Y12 inhibitory drugs include the non-thienopyridine derivatives ticagrelor, cangrelor and elinogrel. Those drugs have in common that they are direct-acting P2Y12 receptor inhibitors that lead—in contrast to thienopyridines—to a reversible P2Y12 receptor inhibition. While ticagrelor has received regular approval in Europe and USA in 2011 based on the results of the PLATelet Inhibition and Patient Outcomes (PLATO) trial (Wallentin et al. 2009), cangrelor and elinogrel are still under phase II and III investigation (Bhatt et al. 2009; Harrington et al. 2009; Leonardi et al. 2010).

In this book chapter, mechanistic data as well as data from clinical trials investigating these P2Y12 receptor antagonists will be summarized with a focus on pharmacokinetic and pharmacodynamic properties of these agents.

2 Thienopyridine Derivatives

Thienopyridines are prodrugs that are rapidly absorbed in the intestine after oral administration. For an overview of in vivo bioactivation, see Table 1 and Fig. 1. To generate their active metabolites, which irreversibly bind and inhibit the ADP P2Y12 receptor for the lifespan of the platelet, they require in vivo bioactivation via the cytochrome P450 (CYP P450) system, mainly located in the liver. The affinity of the different thienopyridines to the different hepatic CYP isoenzymes is different and in part explains their pharmacodynamic efficacy and their propensity to interference by other drugs. Disturbances in intestinal absorption, bioactivation and P2Y12 receptor interaction influences the efficacy of thienopyridine therapy. Three generations of thienopyridines exist, namely the 1st generation thienopyridine ticlopidine, the 2nd generation thienopyridine clopidogrel and 3rd generation thienopyridine prasugrel. These drugs exhibit different pharmacokinetic and pharmacodynamic properties leading to characteristic safety and efficacy profiles of the individual agents, which shall be summarized as follows:

Table 1 Pharmacological properties of P2Y12 receptor inhibitors

P2Y12 inhibitor	Dose	Intake	Metabolism	Mode of inhibition	Time to platelet inhibition	Completed phase III clinical studies
Thienopyridines						
Ticlopidine (1st generation)	250 mg twice daily (Picard-Fraire 1983)	Oral	Prodrug CYP P450 dependent (multi-step)	Irreversible	~3–5 days	STARS (Leon et al. 1998) ISAR (Schomig et al. 1996)
Clopidogrel (2nd generation)	300–600 mg LD 75 mg MD	Oral	Prodrug CYP P450 dependent (2 steps)	Irreversible	~2–4 h	CAPRIE (Committee CS 1996) CURE (Yusuf et al. 2001) CREDO (Steinhubl et al. 2002)
Prasugrel (3rd generation)	60 mg LD 5–10 mg MD	Oral	Prodrug CYP P450 dependent (single step)	Irreversible	~1–2 h	TRITON-TIMI 38 (Wiviott et al. 2007b)
Non-thienopyridines						
Ticagrelor	180 mg LD 90 mg MD twice daily	Oral	Direct-acting No biotransformation	Reversible	~1–2 h	PLATO (Wallentin et al. 2009)
Cangrelor	30 μg/kg bolus, 4 μg/kg/min infusion	i.v.	Direct-acting No biotransformation	Reversible	~15–30 min	CHAMPION-PCI CHAMPION-PLATFORM (Bhatt et al. 2009; Harrington et al. 2009)
Elinogrel	Oral: 50–150 mg Intravenous: 80 or 120 mg bolus twice daily	Oral or i.v.	Direct-acting No biotransformation	Reversible	Not well characterized dependent on oral or intravenous	Outstanding

LD loading dose, *MD* maintenance dose, *i.v.* intravenous

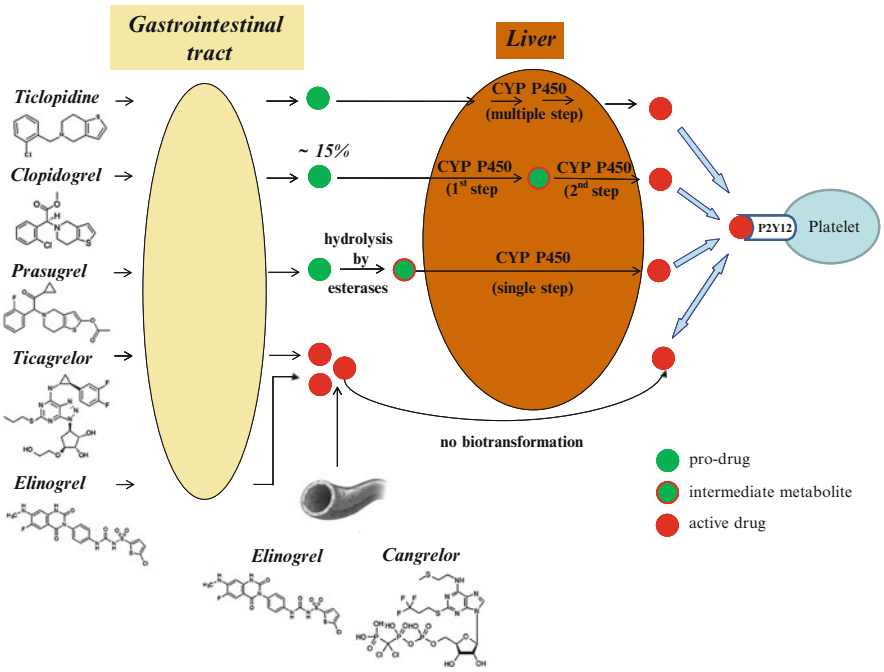


Fig. 1 Metabolism of P2Y12 inhibitory antiplatelet drugs. Clopidogrel, prasugrel and ticagrelor require intestinal absorption, cangrelor is applied intravenous (i.v.), elinogrel exists in both oral and intravenous formulation. Ticlopidine, clopidogrel and prasugrel are pro drugs that require hepatic cytochrome P450 (CYP)-dependent metabolism steps to generate their active compounds. Ticagrelor, cangrelor and elinogrel are direct-acting drugs and do not require biotransformation. CYP P450 = cytochrome P450

3 Ticlopidine—First Generation Thienopyridine

3.1 Pharmacological Data

Ticlopidine requires bioactivation via different hepatic CYP P450 pathways, of which CYP3A4, CYP2B6 and also CYP2C19 pathways have been identified yet (Farid et al. 2010; Dalvie and O'Connell 2004) (see Fig. 1). Although several ticlopidine metabolites were identified in humans, the active metabolite of ticlopidine remains unknown (Farid et al. 2010). Maximum plasma concentrations of a ticlopidine standard dose (250 mg twice daily) are reached 1–3 h after administration (Wallentin 2009) and maximal inhibition of ADP-induced platelet aggregation is observed after 3–5 days with continued dosing (Farid et al. 2010). Platelet function recovery occurs 3–4 days after discontinuation of ticlopidine maintenance dose (Wallentin 2009).

3.2 Clinical Data

Ticlopidine was the first thienopyridine that received regular FDA approval in 1991 to reduce ischemic events in CAD patients. The introduction of a dual antiplatelet inhibitory therapy through the combination of aspirin and ticlopidine after coronary stent placement had shown to reduce the risk of ischemic complications as compared to aspirin alone or as compared to aspirin–anticoagulant therapy (Schomig et al. 1996; Leon et al. 1998). The Intracoronary Stenting and Antithrombotic Regimen (ISAR) study showed a significant reduction in major cardiac events with aspirin–ticlopidine therapy compared with aspirin–anticoagulant therapy (Schomig et al. 1996). In the Stent Anticoagulation Restenosis (STARS) study, patients were randomized to receive either aspirin alone, aspirin plus coumadin, or aspirin plus ticlopidine for 4 weeks after coronary stent placement (Leon et al. 1998). The study demonstrated a significant superiority of combined aspirin–ticlopidine therapy over aspirin–anticoagulant therapy or aspirin mono therapy for the reduction of the combined primary ischemic endpoint of death, myocardial infarction, stent thrombosis or target vessel revascularization (Leon et al. 1998).

Questions raised by these trials were the timing of ticlopidine administration and the benefit of pretreatment of antiplatelet therapy before PCI due to delayed antiplatelet potential observed with ticlopidine therapy (Quinn and Fitzgerald 1999). Through analyses of the data of the "Evaluation of Platelet IIb/IIIa Inhibitor for Stenting" (EPISTENT) trial, Steinhubl and colleagues found that ticlopidine pretreatment before PCI was associated with a significant decrease in the incidence of the composite endpoint of death, myocardial infarction or target vessel revascularization at 1 year for patients who had not received additional therapy with the platelet IIb/IIIa inhibitor abciximab (Steinhubl et al. 2001a).

Despite its proven clinical benefit, ticlopidine therapy is associated with a wide range of toxic side effects like exanthemas and gastrointestinal problems, but also fatal complications such as severe neutropenia, thrombotic thrombocytopenic purpura and aplastic anemia (Quinn and Fitzgerald 1999; Elias et al. 1993). Because of these toxic side effects and due to ticlopidine's slow onset of action it seemed mandatory at that time to develop drugs with better safety and efficacy profiles.

4 Clopidogrel: Second Generation Thienopyridine

4.1 Pharmacologic Data

The orally administered second generation thienopyridine clopidogrel is absorbed through adenosine triphosphate binding cassette (ABC) efflux transporters located in the apical membrane of the intestinal mucosa (Taubert et al. 2006) (see Fig. 1). Esterases inactivate the majority of the absorbed prodrug and produce an inactive carboxylic acid derivative of clopidogrel. Only about 15 % of the absorbed prodrug is further metabolized to its active thiol metabolite in two sequential steps by

different hepatic CYP isoenzymes (CYP 3A4/5, CYP 2C19, CYP 1A2, CYP 2C9 and CYP 2B6). It has been shown that the polymorphically expressed isoenzyme CYP2C19 plays a dominant role in this process, probably because it affects both metabolic steps required for clopidogrel's active metabolite generation (Kazui et al. 2010). Clopidogrel's active thiol metabolite irreversibly inhibits the active site of the P2Y12 receptor via disulfide bridge binding.

After oral intake of clopidogrel, maximum plasma levels of clopidogrel's active metabolite is generated after 1–2 h in a dose-dependent manner with highest concentrations of clopidogrel's active metabolite and highest levels of platelet inhibition after administration of a single high loading dose of 600 mg (after 2–3 h) (Muller et al. 2001; Wallentin 2009; von Beckerath et al. 2005; Price et al. 2006; Montalescot et al. 2006). Several studies compared the pharmacological effects of 300, 600 and 900 mg loading doses of clopidogrel to achieve rapid and sufficient platelet inhibition (Muller et al. 2001; von Beckerath et al. 2005; Montalescot et al. 2006). The Intracoronary Stenting and Antithrombotic Regimen: Choose Between 3 High Oral Doses for Immediate Clopidogrel Effect (ISAR CHOICE) trial revealed that loading with 600 mg resulted in higher plasma concentrations of clopidogrel's active metabolite compared to loading with 300 mg and no further increase of clopidogrel active metabolite with administration of 900 mg clopidogrel loading dose probably due to saturable absorption, metabolism, or both (von Beckerath et al. 2005). After discontinuation of clopidogrel treatment, platelet aggregation values return to baseline aggregation measurements between 3 and 5 days (Price et al. 2011a).

4.2 Clinical Data

Clopidogrel was approved by the US Food and Drug Administration (FDA) in 1997 to reduce the incidence of ischemic events in patients with atherosclerotic vascular disease following the results of the CAPRIE (Clopidogrel vs. Aspirin in Patients at Risk of Ischemic Events) trial (Committee CS 1996) that assessed 19,185 patients with atherosclerotic vascular disease at risk of ischemic events and found that long-term administration of clopidogrel as more effective than aspirin in reducing the combined risk of ischemic stroke, myocardial infarction, or vascular death (Committee CS 1996). The CURE study assessed 12,562 patients with non-ST-elevation ACS who were randomly assigned to treatment with clopidogrel or placebo in addition to aspirin for 3–6 months and found that clopidogrel pre-treatment followed by long-term therapy was beneficial in reducing major cardiovascular events, compared with placebo (Yusuf et al. 2001). Since then a number of trials were conducted to better characterize clopidogrel's pharmacological properties and to identify possible advantages and disadvantages of the drug. In relation to clopidogrel's advantages, it turned out that clopidogrel—in comparison to ticlopidine—had a markedly better overall safety profile with considerable less toxic side effects (Bertrand et al. 2000). As a result, clopidogrel has replaced ticlopidine almost completely in clinical practice nowadays and has become standard of care in addition to aspirin for patients

undergoing PCI with stenting (Smith et al. 2006). Limitations of clopidogrel therapy are discussed in the following section:

5 Clopidogrel Response Variability and the Phenomenon of HPR

Not more than 2 years after the official approval of clopidogrel by the FDA, Geiger et al. reported the first case of clopidogrel treatment failure in the literature in 1999 (Geiger et al. 1999). In 2003, Gurbel et al. were among the first to describe the phenomenon of an interindividual variability in the platelet response to a 300 mg loading dose of clopidogrel in patients undergoing elective coronary stenting (Gurbel et al. 2003). Further on, Serebruany et al. reported in a larger cohort of 544 patients on high interindividual platelet aggregation variability after clopidogrel administration (Serebruany et al. 2005). Extensive variability in clopidogrel responsiveness following administration of even a high loading dose of 600 mg of clopidogrel was observed in CAD patients undergoing coronary stenting (Sibbing et al. 2010a). Figure 2 demonstrates a wide dispersion of platelet aggregation measurements induced by ADP after loading with clopidogrel (600 mg). In accord with clopidogrel response variability, a relevant proportion of clopidogrel-treated patients (about 20–30 %) show ineffective platelet inhibition by clopidogrel, termed clopidogrel low-response or high platelet reactivity (HPR) which is associated with a high risk for ischemic complications (Bonello et al. 2010) (for an overview of studies see Table 2). On the other side, enhanced clopidogrel response can be associated with bleeding complications (Sibbing et al. 2010b; Cuisset et al. 2009a; Mokhtar et al. 2010). To address the pharmacodynamic limitations of clopidogrel, the Clopidogrel Optimal Loading Dose Usage to Reduce Recurrent Events/Optimal Antiplatelet Strategy for Interventions (CURRENT/OASIS 7) trial assessed the efficacy and safety of intensified aspirin and clopidogrel treatment regimens in ACS patients (Mehta et al. 2010a, b). The study showed that an increased clopdiogrel therapy (600 mg clopidogrel loading dose followed by 150 mg/d for 7 days) had no influence on the outcome of the overall study population but significantly reduced the incidence of ischemic events as compared to standard clopidogrel treatment (300 mg clopidogrel loading dose followed by 75 mg/d) without increasing bleeding events in ACS patients undergoing PCI (Mehta et al. 2010a, b).

Several genetic and non-genetic factors that may influence clopidogrel metabolism and may therefore contribute to HPR are summarized in Table 3. Interferences with clopidogrel bioactivation may occur during intestinal absorption, hepatic bioactivation or at the inhibitory site of the platelet P2Y12 receptor.

6 Non-genetic Factors for Clopidogrel Response Variability

Apart from patient non-compliance (Serebruany et al. 2009), several co-morbidities have been found to influence antiplatelet efficacy of clopidogrel (Table 3). High body mass index, elevated inflammatory biomarkers and diabetes mellitus have

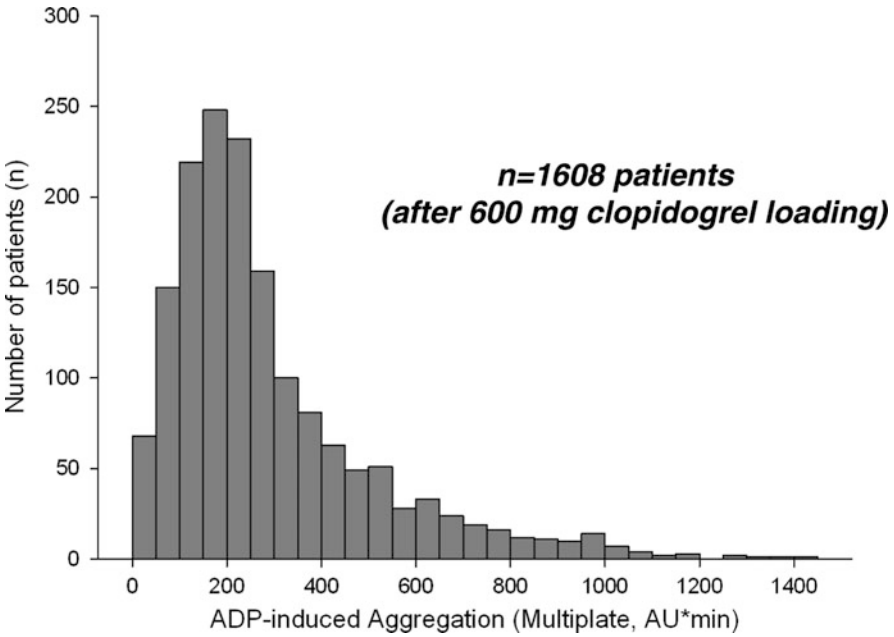


Fig. 2 Variability in clopidogrel responsiveness. The distribution of platelet function measurements in a study cohort ($n = 1{,}608$) (Sibbing et al. 2009b) of PCI-treated patients following clopidogrel loading (600 mg). Values obtained with the Multiplate assay are shown as ADP-induced aggregation units (AU) × min. *ADP*, adenosine diphosphate

been associated with higher platelet aggregation values on clopidogrel therapy (Bernlochner et al. 2010; Geisler et al. 2007; Sibbing et al. 2006, 2007). Specifically in acute clinical settings like ACS or myocardial infarction complicated by cardiogenic shock, clopidogrel shows little antiplatelet action (Matetzky et al. 2004; Geisler et al. 2008; Osmancik et al. 2009) presumably due to both impaired enteric absorption of the prodrug and reduced drug metabolism and bioactivation in the acute setting.

Important co-administered drugs are presented in Table 3. All of them are—like clopidogrel—substrates of the hepatic CYP P450 system and therefore affect clopidogrel's active metabolite generation. Concerning proton pump inhibitors (PPIs), pharmacodynamic data demonstrated an extenuated clopidogrel efficacy in patients who received PPIs which counted especially for omeprazole but not for pantoprazole (Siller-Matula et al. 2009; Gilard et al. 2008; Sibbing et al. 2009a; Cuisset et al. 2009b). However, clinical data are conflicting concerning the association between PPI use and clinical outcome in patients given clopidogrel (Bhatt et al. 2010; O'Donoghue et al. 2009; Ho et al. 2009; Juurlink et al. 2009). If PPI prescription is needed, specific PPIs, for instance pantoprazole, that have shown to have less pharmacodynamic interactions with clopidogrel should be preferred in these patients (Sibbing and Kastrati 2009).

Table 2 Studies relating clopidogrel low responsiveness with higher ischemic risk

Study	*N*	Device	Setting	Outcomes
Barragan et al. (2003)	46	VASP	PCI (all comers)	ST
Blindt et al. (2007)	99	VASP	PCI (high risk)	ST (6 months)
Bonello et al. (2007)	144	VASP	PCI (all comers)	MACE (6 months)
Breet et al. (2010)	1,069	LTA VerifyNow Plateletworks IMPACT-R PFA-100	Elective PCI	MACE (1 year)
Buonamici et al. (2007)	804	LTA	PCI with DES implantation	ST (6 months)
Cuisset et al.(2009a)	598	LTA	PCI (ACS)	ST (30 days)
Cuisset et al. (2006)	106	LTA	PCI (ACS)	MACE (30 days)
Eshtehardi et al. (2010)	219	MEA	PCI	MACE (30 days)
Frere et al. (2007)	195	VASP LTA	PCI (ACS)	MACE (30 days)
Geisler et al. (2010)	1,019	LTA	PCI (all comers)	ST (3 months)
Geisler et al. (2006)	379	LTA	PCI (all comers)	MACE (3 months)
Gori et al. (2008)	746	LTA	PCI with DES implantation	ST (6 months)
Gurbel et al. (2008)	297	LTA	Elective PCI	MACE (2 years)
Gurbel et al. (2005a)	192	LTA	Elective PCI	MACE (6 months)
Gurbel et al. (2005b)	120	LTA	Elective PCI	ST (over 1.5 years)
Hochholzer et al.(2006)	802	LTA	Elective PCI	MACE (30 days)
Marcucci et al. (2009)	683	VerifyNow	PCI in ACS patients	MACE (1 year)
Matetzky et al. (2004)	60	LTA	PCI in STEMI patients	MACE (6 months)
Migliorini et al. (2009)	215	VerifyNow	PCI with stenting	Cardiac mortality (3 years)
Patti et al. (2008)	160	VerifyNow	PCI (all comers)	MACE (30 days)
Price et al. (2008)	380	VerifyNow	PCI with DES implantation	MACE (6 months)
Sibbing et al. (2009b)	1,608	MEA	PCI with DES implantations (elective and ACS)	Definite ST (30 days)
Siller-Matula et al. (2010)	416	MEA	PCI (all comers)	ST (6 months)
Trenk et al. (2008)	797	LTA	Elective PCI	MACE (1 year)
Wang et al. (2009)	386	LTA	Elective PCI	MACE (1 year)

ACS acute coronary syndrome, *DES* drug eluting stent, *LTA* light transmission aggregometry, *MACE* major adverse cardiovascular event, *MEA* multiple electrode aggregometry, *PCI* percutaneous coronary intervention, *ST* stent thrombosis

Initial concerns about the potential of statins to reduce the antiplatelet efficacy of clopidogrel (Lau et al. 2003; Neubauer et al. 2003) were not confirmed by cumulative data from large clinical studies that did not find any clinically significant interaction between statins and clinical outcomes (Gorchakova et al. 2004; Serebruany et al. 2004; Saw et al. 2003, 2007).

Calcium channel blockers (CCBs) decrease clopidogrel response with a consecutive higher risk for ischemic cardiovascular events (Gremmel et al. 2010; Harmsze et al. 2010; Siller-Matula et al. 2008); this was not confirmed in a large cohort of CAD patients with coronary stenting and clopidogrel treatment (Sarafoff et al. 2011).

Table 3 Variables that influence clopidogrel responsiveness

Non-genetic factors	Genetic factors
Non-compliance	CYP2C19 gene variants:
Clinical factors and co-morbidities:	CYP2C19*2 loss of function
Body mass index	CYP2C19*17 gain of function
Diabetes mellitus	CYP2C19*3,*4,*5,*6,*7,*8 loss of function
Renal insufficiency	CYP3A4 gene variants
Systemic inflammation	CYP3A5 gene variants
Acute coronary syndromes	MDR1 gene variants
Cardiogenic shock	ITGB3 gene variants
Ejection fraction	P2Y12 gene variants
Age	PON-1 gene variants
Gender	
Smoking	
Co-medication:	
Proton pump inhibitors	
Calcium-channel blockers	
Coumarin derivatives	
Statins	

CYP cytochrome P, *MDR-1* multidrug resistance protein 1, *PON-1* paraoxonase-1

Phenprocoumon therapy has been demonstrated to significantly attenuate the antiplatelet effect of clopidogrel in a larger observational trial of 1,223 CAD patients after coronary stent placement under dual maintenance antiplatelet therapy with aspirin and clopidogrel (Sibbing et al. 2010c). Patients treated with phenprocoumon in addition to aspirin and clopidogrel exhibited significantly higher ADP-induced platelet aggregation values which might be explained through the fact that both drugs are metabolized through CYP2C9 and CYP3A4 pathways (Sibbing et al. 2010c; Beinema et al. 2008; Ufer et al. 2004).

7 Genetic Factors for Clopidogrel Response Variability

Inherited single nucleotide polymorphisms (SNPs) of genes encoding for enzymes and proteins that are involved in absorption and metabolism of clopidogrel have been reported to account for 5.2 % (Hochholzer et al. 2010) up to 12 % (Shuldiner et al. 2009) of clopidogrel response variability. Selected gene variants that have been reported to be associated with clopidogrel response variability are summarized in Table 3. Clopidogrel's active metabolite formation depends on numerous hepatic CYP isoenzymes, namely CYP3A4/5, CYP2C19, CYP1A2, CYP2C9 and CYP2B6, of which the polymorphically expressed isoenzyme CYP2C19 plays a dominant role because it affects both metabolic steps of clopidogrel bioactivation (Kazui et al. 2010; Lau et al. 2004).

Among SNPs within the CYP2C19 gene with functional impact on the CYP2C19 enzyme activity, the loss-of-function CYP2C19*2 is a frequent allelic variant that results in a complete loss of the CYP2C19 enzyme activity. CYP

2C19*2 has an allelic frequency of about 15–30 % (Mega et al. 2010a; Steinhubl 2010) with the majority of people being heterocygous carriers and a homocygous prevalence of CYP2C19*2 of only 2 % (Mega et al. 2010a). Several studies have demonstrated that the presence of the CYP2C19*2 allelic variant is associated with attenuated response to clopidogrel with a consecutive increased risk for ischemic events including stent thrombosis (Shuldiner et al. 2009; Collet et al. 2009; Giusti et al. 2007; Hulot et al. 2010; Mega et al. 2009; Sibbing et al. 2010d; Simon et al. 2009; Trenk et al. 2008; Wallentin et al. 2010). A large meta-analysis including more than 9,000 PCI-treated CAD patients further confirmed existing data and also found a significant association between both homozygous and heterozygous *2 allele carriage on adverse cardiovascular events, particularly ST (Mega et al. 2010a).

Other, less frequent CYP2C19 loss of function alleles (CYP 2C19 *3,*4,*5,*6, *7 or *8) have also been associated with an increased risk of ischemic events in CAD patients on clopidogrel therapy as well (Shuldiner et al. 2009; Collet et al. 2009; Mega et al. 2009; Wallentin et al. 2010; Hulot et al. 2006; Giusti et al. 2009).

In contrast to CYP2C19*2 loss-of-function allelic variant, the CYP2C19*17 genetic variant is associated with an increased enzyme function of CYP2C19 resulting in an ultrarapid hepatic drug metabolism (Sim et al. 2006). In a large study population of 1,542 clopidogrel-treated patients, we found a significant association between CYP2C19*17 carrier status and lower ADP-induced platelet aggregation values in a gene-dose-dependent manner and consequently in an increased bleeding risk in *17 carriers (Sibbing et al. 2010d, e). These results were confirmed by a genetic substudy of the PLATO trial (Wallentin et al. 2010) and a recently published prospective study conducted by Campo et al. (2011) where CYP2C19*17 carriage in clopidogrel-treated patients was also associated with higher rates of major bleeding complications. In contrast, Paré et al. did not find an increased bleeding risk in patients with either ACS or atrial fibrillation carrying the *17 allele but also did not find any influence of *2 on patients' outcomes (Pare et al. 2010) which might be explained through the fact that the rate of coronary stent placement was very low (~15 %) in this study population.

Conflicting data exists concerning the influence of a common SNP (ABCB1 3435C → T) within the ABCB1 gene that encodes for the intestinal multidrug resistance protein 1 (MDR 1) which is involved in the intestinal absorption of clopidogrel. It has been previously reported that CAD patients under clopidogrel therapy with this genetic variant suffer from a higher ischemic event rate in comparison to patients with wild-type genotype (Mega et al. 2010a, b; Simon et al. 2009). In contrast, a genetic substudy of the PLATO trial found no significant influence of polymorphisms of ABCB1 on patients' outcomes (Wallentin et al. 2010).

Recently, it was suggested that paraoxonase-1 (PON1) Q192R polymorphism in the paraoxonase gene encoding for the paraoxonase-1 (PON-1) enzyme may be the key player in clopidogrel active metabolite formation and to be associated with increased platelet reactivity as well as higher ischemic event rates in comparison to those with wild-type genotype (Bouman et al. 2011). In addition, Bouman et al. questioned the previously indicated important role of CYP2C19*2 for clopidogrel efficacy (Bouman et al. 2011). Results of this study needed external confirmation

and were challenged by other clinical studies that did not find any significant impact of PON1 on either clopidogrel responsiveness or clinical outcomes of CAD patients after coronary stent placement (Trenk et al. 2011; Sibbing et al. 2011; Lewis et al. 2011; Fontana et al. 2011).

8 HPR and Clinical Outcome

The definition for an inadequate antiplatelet efficacy of clopidogrel differs across studies and is dependent on the platelet function device used to measure ADP-induced platelet aggregation. A consensus on the definition of HPR, according to the most commonly used methods of platelet function testing, has been reached recently (Bonello et al. 2010). HPR occurs in a significant proportion of patients and has been linked with adverse cardiovascular events in numerous studies. Relevant studies are summarized in Table 2. Steinhubl and co-workers first blew the whistle in 2001 by describing an association between increased platelet reactivity and worse clinical outcome of PCI-treated patients (Steinhubl et al. 2001b). However, their focus was on GP IIb/IIIa inhibitors and not on P2Y12 receptor inhibition. In the following years, numerous studies employing different methods of platelet function testing confirmed a relationship between HPR and clinical outcome in the setting of clopidogrel treatment (Hochholzer et al. 2006; Buonamici et al. 2007; Barragan et al. 2003; Gurbel et al. 2005a). In patients with STEMI undergoing PCI, Matetzky et al. were among the first to report that a low response to clopidogrel treatment was associated with higher rates of ischemic events (Matetzky et al. 2004). Hochholzer et al. performed the first dedicated Impact of Extent of Clopidogrel Induced Platelet Inhibition During Elective Stent Implantation on Clinical Event Rate (EXCELSIOR) study that prospectively enrolled clopidogrel-treated patients to test for an association of platelet aggregation measurements and 30-day clinical outcome (Hochholzer et al. 2006). The authors reported on significantly higher rates of adverse cardiovascular events in patients with platelet aggregation values greater than the median. With a focus on ST, the RECLOSE (Low Responsiveness to Clopidogrel and Sirolimus- or Paclitaxel-Eluting Stent Thrombosis) trial assessed platelet aggregation after a clopidogrel loading of 600 mg and found a higher incidence for the combined endpoint of definite or probable ST according to ARC criteria in clopidogrel non-responders compared with responders (Buonamici et al. 2007). In 2008, Price and co-workers were among the first who used a point-of-care platelet function method, namely VerifyNowP2Y12, to describe the association between HPR and clinical outcome in stable CAD patents after coronary stent placement (Price et al. 2008). In accordance with the other studies, HPR was also associated with significantly higher rates of adverse cardiac events. In 2009, we demonstrated a significant association between clopidogrel low response and the occurrence of definite ST in a large cohort of 1,608 enrolled patients with CAD after PCI using the Multiplate analyzer as another point-of-care platelet function testing method (Sibbing et al. 2009b). Our study lends support to the hypothesis of a potential threshold effect of

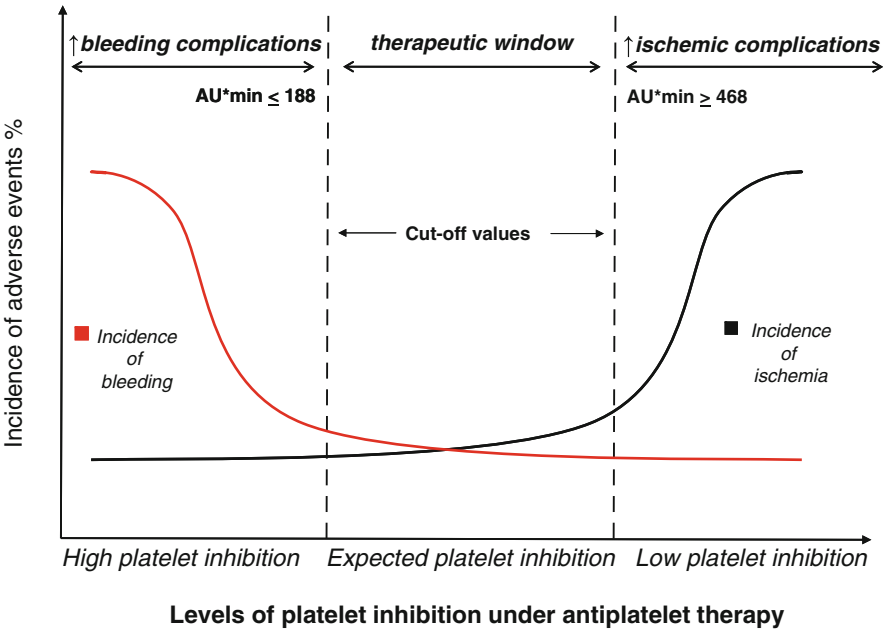


Fig. 3 Expected therapeutic window of P2Y12 receptor inhibition. Based on P2Y12 receptor reactivity patients can be categorized in three different groups: patients with high platelet inhibition and a consecutive higher bleeding risk, patients with expected platelet inhibition and patients with low platelet inhibition and a consecutive higher risk for ischemic complications. ROC-based cut-off values according to multiple electrode aggregometry for bleeding events ($\leq$188 AU $\times$ min) and ischemic events ($\geq$468 AU $\times$ min) are based on prior studies in clopidogrel-treated patients after PCI (Sibbing et al. 2009b, 2010b). *AU* aggregation units

HPR, since adverse events accumulated in the highest quintile of platelet reactivity instead of increasing gradually across quintiles.

9 Therapeutic Window for Clopidogrel Therapy and Value of Personalized Antiplatelet Therapy

Accompanied by the large interindividual response variability observed with clopidogrel therapy, ischemic events occur in patients with HPR, whereas high levels of platelet inhibition are associated with increased bleeding rates (Sibbing et al. 2010b; Cuisset et al. 2009a; Mokhtar et al. 2010). Platelet function testing is becoming increasingly established in clinical routine and it can be assumed that monitoring of platelet aggregation in patients under antiplatelet therapy will become an important component of routine cardiac care. Similar to the established therapeutic window based on international normalized ratio (INR) measurements to guide coumarin-derivative therapy, establishment of such a therapeutic window as outlined in Fig. 3 is conceptually conceivable to guide antiplatelet therapy in order

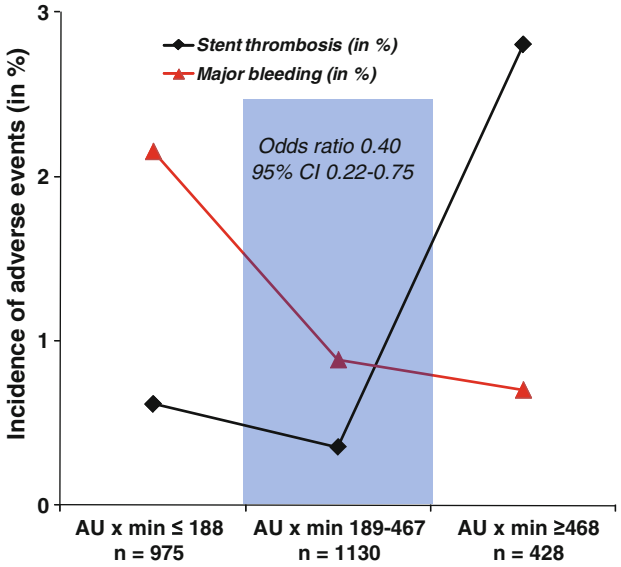


Fig. 4 Levels of P2Y12 receptor inhibition and adverse events. Incidence of adverse events (30 day definite or probable ST, *black line*; in-hospital major bleeding events, *red line*) after coronary stent placement. Patients ($n = 2{,}533$) are stratified into groups of normal responders (AUC 189 to 467), low responders (AUC $\geq$ 468) and enhanced responders (AUC $\leq$ 188). Patients with normal platelet reactivity showed the lowest risk for adverse events (OR 0.40; $p = 0.003$) as compared to the remaining patients. *ST* stent thrombosis, *AU* aggregation units, *OR* odds ratio. Figure modified from Sibbing et al. (2010f)

to improve patient care. Indeed, we recently found initial evidence for the existence of a therapeutic window for P2Y12 receptor inhibition after clopidogrel loading assessed with the Multiplate Analyzer (Sibbing et al. 2010f). As demonstrated in Fig. 4, patients with "normal platelet reactivity" defined according to ROC-based-cut-off values (Sibbing et al. 2009b, 2010b) showed the lowest risk of ST or bleeding as compared to the remaining patients at 30 days after coronary stent placement. Recently, our findings were confirmed in a smaller study by Campo et al., who prospectively evaluated HPR in clopidogrel-treated patients after PCI and defined a therapeutic window according to VerifyNow platelet function measurements (Campo et al. 2011). In line with these findings, focus was lately set on tailoring antiplatelet treatment to overcome HPR and initial findings reported promising clinical approaches (Bonello et al. 2008, 2009). However, results from recently published large scale randomized trials including GRAVITAS (Price et al. 2011b) and TRIGGER-PCI (Trenk 2011) failed to show a benefit of an intensified clopidogrel treatment (Price et al. 2011b) or switching from clopidogrel to more potent antiplatelet therapy with the newer thienopyridine prasugrel (Trenk 2011) in CAD patients treated with PCI. Possible reasons for the negative study results were attributed to the used platelet function assays and the chosen HPR cut-off values but predominantly to the fact that mainly stable patients with low event rates were

included in both studies leaving little room to further improve patients' outcomes. By now, the potential benefit of tailoring antiplatelet therapy based on platelet function testing is not known and it might be speculated that diagnosis of HPR and subsequent intensifying of P2Y12 receptor inhibition might play a dominant role in high-risk ACS patients who exhibit a higher prothrombotic state (Davi and Patrono 2007). Future studies are necessary to evaluate the value of platelet function based tailored antiplatelet treatment strategies in these high-risk patients with focus on the clinical efficacy and safety of more potent new antiplatelet agents.

10 Prasugrel: Third Generation Thienopyridine

10.1 Pharmacologic Data

Like clopidogrel, prasugrel is also an orally administered prodrug, which is converted to its intermediate metabolite by intestinal enzymes (Fig. 1). In contrast to clopidogrel, only one hepatic CYP P450 metabolism step is necessary for the conversion in its active form (Farid et al. 2010; Rehmel et al. 2006). Therefore, genetic variants of hepatic CYP enzymes (e.g., CYP2C19) do not seem to have any significant influence on the pharmacokinetics and pharmacodynamics of prasugrel (Brandt et al. 2007a; Varenhorst et al. 2009). Peak concentrations of prasugrel's active metabolite are measured about 30 min after oral intake and its concentration is dose-proportional between 5 and 60 mg (Wiviott et al. 2010). As compared to clopidogrel, prasugrel leads to a more effective and more rapid platelet inhibition with a therapeutic level of inhibition observed within about 1–2 h after oral intake of a 60 mg loading dose (Brandt et al. 2007b; Jakubowski et al. 2007; Jernberg et al. 2006; Wallentin et al. 2008) due to a more rapid generation of a higher level of prasugrel's active metabolite (Payne et al. 2007). Like all thienopyridines, prasugrel leads to an irreversible inhibition of the P2Y12 receptor lasting for the entire lifespan of the platelet. After discontinuation of prasugrel treatment, platelet aggregation values return to baseline within 7–9 days (Price et al. 2006, 2011a; Jakubowski et al. 2007).

In the phase II safety Joint Utilization of Medications to Block Platelets Optimally (JUMBO)-TIMI 26 study of prasugrel compared to clopidogrel, 904 patients undergoing elective or urgent PCI were randomized to either clopidogrel (300 mg loading/75 mg maintenance dose) or three different prasugrel dosing strategies (40 mg loading/7.5 mg maintenance dose; 60 mg loading/10 mg maintenance dose or 60 mg loading/15 mg maintenance dose) and followed up for 30 days (Wiviott et al. 2005). In this study, the primary safety endpoint (TIMI major and minor bleeding) did not differ significantly among treatment groups (prasugrel vs. clopidogrel) and—although the study was not powered for efficacy endpoints—there were numerically lower incidences of the primary efficacy composite endpoint (30-day major adverse cardiac events) and of the secondary endpoints myocardial infarction, recurrent ischemia and clinical target vessel thrombosis.

Results of this trial were the basis for large phase III efficacy and safety trials (Wiviott et al. 2005). Results of the PRINCIPLE-TIMI 44 study revealed that—even when compared to high clopidogrel treatment doses (600 mg loading/150 mg maintenance dose)—prasugrel resulted in a greater antiplatelet effect already 30 min after loading, as well as after 6 h and remained consistent under maintenance therapy in patients with stable CAD and planned PCI (Wiviott et al. 2007a). Despite these pharmacodynamic advantages, the phenomenon of HPR may also occur in prasugrel-treated patients. Recently, a prospective multicenter study that measured vasodilator-stimulated phosphoprotein (VASP) index in ACS patients ($n = 300$) after coronary stent placement was published and reported a considerable high rate of patients with HPR after prasugrel loading (60 mg) (Bonello et al. 2011). HPR was observed in 76 patients (25.2 %) which was associated with a significantly higher incidence of adverse cardiovascular events. Additional confirmatory studies are required to further clarify the association between prasugrel therapy and HPR.

10.2 Clinical Data

The third generation thienopyridine prasugrel was approved by the FDA in 2009 based on the results of the phase III, randomized, double blind TRITON-TIMI 38 trial that evaluated 13,608 patients with ACSs undergoing PCI (Wiviott et al. 2007b). This study demonstrated a higher efficacy of prasugrel with a significant reduction in the incidence of the primary endpoint (cardiovascular death, myocardial infarction, stroke) and a reduction of stent thrombosis in comparison to clopidogrel (Wiviott et al. 2007b). In accord with the greater platelet inhibition through prasugrel, TRITON-TIMI 38 showed a significant increase in TIMI major (2.4 vs. 1.8, $P = 0.03$) and life-threatening bleeding (1.4 % vs. 0.9 %, $P = 0.01$). Besides, patients who underwent coronary artery bypass graft (CABG) surgery suffered from significantly higher rates of bleeding complications when treated with prasugrel as compared to clopidogrel (13.4 % vs. 3.2 %, hazard ratio 4.73, 95 % CI 1.90–111.82, $P < 0.001$) (Wiviott et al. 2007b). Pharmacokinetic analyses revealed that prasugrel's active metabolite exposure was higher in patients weighing <60 kg and in patients >75 years of age (Wrishko et al. 2009). Reflecting the clinical outcome data of TRITON-TIMI 38, it is recommended to reduce prasugrel maintenance dose to 5 mg per day in patients >75 years and in patients weighing less than 60 kg after loading dose administration of 60 mg.

Results of a substudy of TRITON-TIMI 38 revealed that especially patients with diabetes mellitus seem to profit from prasugrel therapy with a significant greater reduction in ischemic events without an increase in major bleeding complications in these patients. These results might be explained through an increased platelet reactivity in diabetes patients (Wiviott et al. 2008). Similar results were found in

patients with ST-segment elevation myocardial infarction (STEMI) who also had a greater clinical benefit of prasugrel as compared to clopidogrel without an increase in bleeding complications (Montalescot et al. 2009). Especially for those patients, an intensified platelet inhibition through prasugrel is of particular benefit since the majority of patients with STEMI clopidogrel does not achieve sufficient platelet inhibition with standard clopidogrel treatment (Osmancik et al. 2009).

However, prasugrel—like clopidogrel—causes irreversible platelet inhibition with a slow offset of antiplatelet efficacy that lasts 7–9 days (Price et al. 2011a). Therefore, to reduce bleeding complications especially in patients who require urgent surgery, for instance CABG surgery, reversible P2Y12 receptor antagonists with a rapid on- and offset of antiplatelet action might be preferable.

11 Non-thienopyridine Derivatives

Novel, non-thienopyridine derivatives are direct binding P2Y12 receptor agonists that have two potential benefits in comparison to thienopyridines: first, they are active drugs which do not require metabolic activation; second, the binding to the P2Y12 receptor is completely reversible with a faster recovery of the platelet function within hours or days after cessation of the drug intake (Cattaneo 2010). The cyclopentyl-triazolo-pyrimidine ticagrelor is an orally administered P2Y12 ADP receptor antagonist, cangrelor is administered intravenously and belongs to a family of ATP analogs and elinogrel exists in both, intravenous and oral formulations and is a competitive P2Y12 ADP antagonist that competes with ADP at the ADP binding site of the P2Y12 receptor (Wallentin 2009).

12 Ticagrelor

12.1 Pharmacologic Data

The cyclopentyl-triazolo-pyrimidine Ticagrelor (AZD6140) is the first reversible oral P2Y12 receptor inhibitor. After intestinal absorption, ticagrelor—as a direct-acting drug—reversibly binds to the P2Y12 receptor with no need of previous metabolic activation (Fig. 1). After rapid absorption, ticagrelor undergoes enzymatic degradation to at least one active metabolite with similar pharmacologic properties (Wallentin 2009). In contrast to thienopyridines, which block the ADP binding site of the P2Y12 receptor directly, ticagrelor inhibits the binding of ADP to the receptor in a non-competitive manner suggesting the existence of an independent receptor binding site for cyclopentyl-triazolo-pyrimidines (van Giezen et al. 2009). Ticagrelor achieves a better and more consistent platelet inhibition

with faster onset and offset of action as compared to clopidogrel (Gurbel et al. 2009). Maximum plasma concentration and maximum platelet inhibition is reached at about 2 h after dosing and maintains for about 12 h with plasma concentrations starting to decline around 12 h after cessation of ticagrelor therapy (Husted et al. 2006; Teng and Butler 2010; Storey et al. 2010a).

The ONSET/OFFSET study (Randomized Double-Blind Assessment of the ONSET and OFFSET of the Antiplatelet Effects of Ticagrelor Versus Clopidogrel in Patients With Stable Coronary Artery Disease) demonstrated that inhibition of platelet aggregation with ticagrelor (180 mg loading dose, 90 mg twice daily maintenance dose) drops more rapidly as compared to clopidogrel 600 mg loading dose, 75 mg/day maintenance dose) with normal platelet reactivity attained about 5 days after ticagrelor cessation (Gurbel et al. 2009). A platelet function substudy of the PLATO study compared the antiplatelet effects of clopidogrel (300–600 mg loading dose, 75 mg/day maintenance dose) and ticagrelor (180 mg loading dose, 90 mg twice daily maintenance dose) assessed with light transmittance aggregometry, VerifyNow P2Y12 and VASP phosphorylation assays in patients with ACSs (Storey et al. 2010a). In this study, ticagrelor achieved greater suppression of platelet reactivity compared with clopidogrel after loading dose administration and during maintenance therapy. HPR occurred more frequently in the clopidogrel group as compared to the ticagrelor group (Storey et al. 2010a). In addition, the RESPOND study demonstrated that ticagrelor was sufficient to overcome clopidogrel low response assessed with three different platelet function methods in 98 patients with stable CAD (Gurbel et al. 2010a).

Besides its antiplatelet effects, ticagrelor has also shown to inhibit adenosine uptake in human erythrocytes in vitro (Björkman and van Giezen 2007) and therefore augmented adenosine-induced coronary blood flow and reactive hyperemia in animal models in a dose-dependent manner (Björkman and van Giezen 2007; van Giezen et al. 2011) which could have additional benefits in ACS patients (van Giezen et al. 2011).

12.2 Clinical Data

Ticagrelor received regulatory approval in Europe and in the US for the reduction of thrombotic events in patients with ACSs in 2011. In the PLATO study, ticagrelor significantly reduced the primary efficacy endpoint (death from vascular causes, myocardial infarction or stroke) at 1 year (9.8 % of patients in ticagrelor group vs. 11.7 patients in clopidogrel group, hazard ratio 0.84, 95 % CI 0.77–0.92, $P < 0.001$) (Wallentin et al. 2009) and the superiority of ticagrelor over clopidogrel was observed irrespective of CYP2C19 polymorphisms (Wallentin et al. 2010). According to safety endpoints, no differences in overall bleeding complications were observed between the two treatment groups. Despite the more effective platelet inhibition with ticagrelor, there was no increased bleeding risk in patients undergoing CABG probably due to ticagrelor's advantage of a reversible

P2Y12 receptor inhibition, which makes this drug attractive in the scenario of planned CABG surgery. Nonetheless, there were small but significant increases in the risk of non-CABG related and non-procedure related spontaneous bleedings (including fatal intracranial bleeding and fatal bleeding of other types) with ticagrelor (Wallentin et al. 2009). Notably, PLATO was the first study to demonstrate a reduction of overall mortality with a new P2Y12 receptor inhibitor in comparison to clopidogrel although the study was not powered to detect differences in the mortality rate (Schomig 2009). Several explanations for the mortality benefits with ticagrelor beyond its superior antiplatelet effects have been posited including its potential to modulate endogenous adenosine concentration with subsequent favourable vascular effects (Wallentin et al. 2011). Notably, subgroup analysis of the PLATO trial revealed that ticagrelor did not show a benefit over clopidogrel in North America with a significant treatment-by-region interaction ($P = 0.045$) and a non-significant trend of better outcome with clopidogrel in North America (Mahaffey et al. 2011; Gaglia and Waksman 2011). Besides the fact that this interaction could be a play of chance, subgroup analyses revealed that a higher median aspirin maintenance dose administered in the US patients was associated with a less treatment effect of ticagrelor (Mahaffey et al. 2011) resulting in a FDA panel recommendation to consider a low (75–100 mg) aspirin maintenance dose regarding ticagrelor treatment (Gaglia and Waksman 2011). Several attempts for a biological explanation for a less efficacy of ticagrelor in the presence of high aspirin doses have been made (Mahaffey et al. 2011) but further studies are required to provide insights into possible interactions with ticagrelor and aspirin.

In contrast to thienopyridines, ticagrelor therapy is associated with new, in fact not life-threatening side effects including dose-dependent dyspnea (Wallentin et al. 2009; Storey et al. 2010b; Cannon et al. 2007), apparently without influence on cardiac or pulmonary function, bradyarrhythmia including ventricular pauses >2.5 s and an increase in serum creatinine and uric acid which altogether led to higher rates of study drug discontinuation as compared to clopidogrel in PLATO (Wallentin et al. 2009). Together with the drug's side effects and its requirement for twice daily dosing due to its shorter half-life, patient compliance is a notable issue in case of ticagrelor therapy.

Like thienopyridines, ticagrelor can only be administered orally which is a potential shortcoming in cases when patients are unable to swallow for example in the setting of an acute cardiogenic shock. In these cases, drugs with the ability of an intravenous administration might be preferable.

13 Cangrelor

13.1 Pharmacologic Data

Cangrelor (AR-C69931MX) is a short-acting adenosine triphosphate (ATP) analogue, which must be administered intravenously and leads to a reversible P2Y12

receptor blockade (Greenbaum et al. 2006) (Fig. 1). Like ticagrelor, cangrelor is a direct-acting drug which does not require metabolic activation. Cangrelor stands out through its very rapid onset of action and the very short time required to reach maximal and steady state platelet inhibition within 15–30 min upon continuous infusion (Jacobsson et al. 2002; Storey et al. 2001a, b; Ferreiro et al. 2009) (Table 1). Rapid degradation through dephosphorylation leads to a very short plasma half-life of only a few minutes resulting in a recovery of normal platelet function within less than 1 h (Storey et al. 2001a, b). These pharmacologic properties might suggest an advantageous role for cangrelor in patients who need rapid platelet inhibition but cannot intake oral drugs and patients who require urgent surgery.

Notably, a competitive pharmacodynamic effect between cangrelor and thienopyridines has been observed at the platelet P2Y12 receptor with a reduced binding capacity of clopidogrel's or prasugrel's active metabolites in the presence of cangrelor resulting in a diminished thienopyridine mediated platelet inhibition (Dovlatova et al. 2008; Steinhubl et al. 2008). Therefore, thienopyridines cannot achieve sufficient platelet inhibition until cangrelor is released (Storey 2011) which is an important issue since the majority of patients with cangrelor treatment would require a switching to oral antiplatelet treatment with clopidogrel or prasugrel in the course of the therapy.

13.2 Clinical Data

Two phase III clinical trials, CHAMPION-PCI (Cangrelor vs. Standard Therapy to Achieve Optimal Management of Platelet Inhibition) and CHAMPION-PLATFORM (Cangrelor vs. Placebo to Achieve Optimal Management of Platelet Inhibition) failed to demonstrate a superior efficacy of cangrelor (30 μg/kg bolus followed by 4 μg/kg/min infusion) to reduce ischemic peri-procedural events in comparison or in addition to clopidogrel (600 mg loading dose) in patients undergoing PCI although significantly higher levels of platelet inhibition were achieved with cangrelor (Bhatt et al. 2009; Harrington et al. 2009). Several reasons for these unexpected findings were discussed including endpoint selection and trial design (Harrington et al. 2009). In both studies, patients in the cangrelor group received clopidogrel dosing at the discontinuation of the cangrelor-infusion which might not have been enough time to avoid pharmacological interaction between the study drugs on the one hand and might have created a time window with insufficient platelet inhibition or even increased platelet reactivity on the other hand (Steinhubl et al. 2008; Storey 2011).

There was a trend toward increased bleeding rates (mainly minor bleedings) in patients who received cangrelor in CHAMPION-PCI (Harrington et al. 2009). In CHAMPION-PLATFORM, according to two of the three different bleeding definitions which were used, no difference in the rates of major bleeding complications were seen, especially not among elderly patients or patients of prior stroke

history, who were at increased bleeding risk already (Bhatt et al. 2009). Concerning side effect, cangrelor—like ticagrelor—treatment was associated with significant higher rates of mild dyspnea in both studies (Bhatt et al. 2009; Harrington et al. 2009).

The phase II randomized double-blind BRIDGE (maintenance of platelet inhiBition with cangRelor after dIscontinuation of thienopyriDines in patients undergoing surGEry; NCT 00767507) study is currently recruiting patients receiving cangrelor infusion vs. placebo before CABG surgery to test if cangrelor has an acceptable safety profile without excessive peri-operative bleeding complications.

14 Elinogrel

Elinogrel (PRT060128) is another direct reversible inhibitor of the platelet P2Y12 receptor. Unlike cangrelor, it can be administered both orally and intravenously with a plasma half-life of about 8–12 h (Ueno et al. 2010) (Fig. 1). The oral administration of elinogrel (60 mg) to previously stented stable CAD patients with HPR resulted in an additional platelet inhibitory effect within 4 h and a reduction in the proportion of patients with HPR independent of the CYP2C19 status (Gurbel et al. 2010b). Platelet function recovery has been demonstrated to occur within 24 h after elinogrel administration (Oestreich 2010).

The small pilot phase II ERASE-MI (The Early Rapid Reversal of Platelet Thrombosis With Intravenous PRT060128 Before PCI to Optimize Reperfusion in Acute MI; NCT00546260) trial examined the safety and tolerability of escalating doses (10, 20, 40, and 60 mg) of elinogrel administered as single intravenous bolus in addition to clopidogrel 600 mg loading dose and a further 300 mg dose of clopidogrel 4 h after PCI in patients with STEMI before PCI (Berger et al. 2009). Within this population, bleeding events were equal and no differences in serious adverse events were observed among the different treatment groups (Berger et al. 2009). The recently completed phase II INNOVATE-PCI (INtraveNous and Oral administration of elinogrel, a selective and reversible P2Y12 receptor inhibitor versus clopidogrel to eVAluate Tolerability and Efficacy in non-urgent Percutaneous Coronary Interventions patients) study (NCT00751231) (Leonardi et al. 2010) evaluated both intravenous and oral elinogrel and investigated the safety and efficacy of elinogrel in comparison to clopidogrel in patients undergoing elective PCI. Patients were randomized to 80 mg or 120 mg of intravenously administered elinogrel before PCI followed by 50 mg, 100 mg or 150 mg orally elinogrel or a 300 or 600 mg loading dose of clopidogrel before PCI followed by 75 mg daily doses for up to 120 days (Leonardi et al. 2010). Results of this study were reported at the European Society of Cardiology (ESC) congress 2010 in Stockholm and revealed that elinogrel was associated with a more rapid and more potent platelet inhibition as compared to clopidogrel which was sustained after switching to the oral dosing. No significant differences of TIMI major or minor bleedings were observed between the treatment groups. The study was underpowered for ischemic endpoints. Larger phase III clinical trials are needed to further assess elinogrel efficacy and safety.

15 Advantages and Disadvantages of Irreversible versus Reversible P2Y12 Inhibition

The thienopyridines clopidogrel and prasugrel cause an irreversible P2Y12 receptor inhibition lasting for the entire lifespan of a platelet (7–10 days) which is advantageous in the chronic phase after coronary stent placement for sufficient long term platelet inhibition for up to 1 year as it is required in ACS patients and/or after drug eluting stent placement (Hamm et al. 2011). However, irreversible platelet inhibition is a disadvantage especially in patients who require urgent surgery, because a markedly higher rate of bleeding complications is associated with antiplatelet therapy (Cuisset et al. 2009a; Mokhtar et al. 2010; Chen et al. 2004). Furthermore, thienopyridines are orally administered drugs that require bioactivation resulting in a delayed onset of platelet inhibition (2–4 h for clopidogrel and 1–2 h for prasugrel, see Table 1) which might cause insufficient platelet inhibition especially in the acute setting of MI.

Reversible P2Y12 receptor inhibitors are direct-acting drugs that do not require bioactivation and therefore offer the great advantage of a rapid onset and offset of antiplatelet action which is especially advantageous in acute settings when immediate platelet inhibition is required as well as in situations when urgent surgery is necessary to reduce bleeding complications. The advantage of intravenously administered drugs like cangrelor and elinogrel emerges in acute settings when patients are mechanically ventilated and unable to swallow. Due to the short half-life of reversible antiplatelet agents they require continuous or repeat dosing during the day which makes patient compliance a more notable issue for these drugs.

16 Conclusion

Inhibition of the ADP P2Y12 receptor in addition to aspirin has shown to significantly improve the clinical outcome of patients in different clinical settings. The thienopyridine clopidogrel has successfully displaced ticlopidine due to an improved pharmacologic profile and a reduced spectrum of side effects and is according to current international guidelines standard-of-care therapy for P2Y12 receptor inhibition. Despite its proven clinical benefits, clopidogrel is associated with a high variability in platelet response leading to an insufficient platelet inhibition in up to 30 % of patients which is associated with a significantly higher incidence of ischemic cardiovascular events in patients after coronary stent placement.

As a result, more potent antiplatelet drugs have been developed recently. The new thienopyridine prasugrel and the non-thienopyridine ticagrelor have shown to decrease the rate of ischemic events in comparison to clopidogrel after percutaneous coronary intervention with a particularly favourable benefit risk ratio for prasugrel in high-risk populations such as patients with ST-segment elevation

myocardial infarction, diabetics or patients without sufficient response to clopidogrel. Reversible antiplatelet action is achieved with the non-thienopyridin ticagrelor and might deliver a great advantage especially for patients who need urgent surgery to minimize bleeding complications. Other new P2Y12 receptor inhibitors with reversible P2Y12 receptor inhibition include cangrelor and elinogrel which are currently under phase II and phase III investigation.

However, recent findings concerning the existence of a therapeutic window of P2Y12 receptor inhibition demonstrate that not all patients profit from an intensified antiplatelet therapy and too intensive platelet inhibition is associated with an increased bleeding risk. Therefore, the decision to switch from clopidogrel to a more potent P2Y12 receptor inhibitor has to be carefully evaluated for each patient. Individual genetic and clinical risk factors influencing platelet response should be incorporated in combination with measurements from validated platelet function assays to individualize antiplatelet therapy.

Knowledge Gaps

- Optimal use of drugs in acute vs. chronic settings
- Genetic factors affecting prasugrel efficacy
- Genetic factors affecting ticagrelor efficacy
- Establishment of therapeutic window of P2Y12 inhibition
- Value of personalized P2Y12 receptor inhibition based on platelet function testing in acute vs. chronic settings

Key Messages

- P2Y12 receptor antagonists in addition to aspirin have become the cornerstone of treatment after percutaneous coronary intervention to prevent occlusive thrombotic vascular events
- Thienopyridines require metabolic activation by the hepatic CYP P450 system and lead to an irreversible inhibition of platelet aggregation to a different extend depending on the substance
- The non-thienopyridine ticagrelor is the first approved reversible P2Y12 receptor inhibitor that has demonstrated to significantly reduce ischemic events without increasing bleeding complications
- The non-thienopyridine cangrelor is administrated intravenously and has failed to demonstrate superior efficacy to reduce ischemic events in comparison or in addition to clopidogrel
- The non-thienopyridine elinogrel is administered both, orally and intravenously, and has demonstrated good safety and tolerability in dose escalating studies and no increased bleeding complications as compared to clopidogrel has been demonstrated yet

(continued)

- Platelet function monitoring is a promising tool to guide tailored antiplatelet therapy
- A therapeutic window of antiplatelet therapeutic efficacy—based on established platelet function testing methods—may be used to guide medical antiplatelet P2Y12 receptor inhibition

References

Anderson JL, Adams CD, Antman EM et al (2007) ACC/AHA 2007 guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non ST-Elevation Myocardial Infarction): developed in collaboration with the American College of Emergency Physicians, the Society for Cardiovascular Angiography and Interventions, and the Society of Thoracic Surgeons: endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation and the Society for Academic Emergency Medicine. Circulation 116:e148–e304

Barragan P, Bouvier JL, Roquebert PO et al (2003) Resistance to thienopyridines: clinical detection of coronary stent thrombosis by monitoring of vasodilator-stimulated phosphoprotein phosphorylation. Catheter Cardiovasc Interv 59:295–302

Bassand JP, Hamm CW, Ardissino D et al (2007) Guidelines for the diagnosis and treatment of non-ST-segment elevation acute coronary syndromes. Eur Heart J 28:1598–1660

Beinema M, Brouwers JR, Schalekamp T et al (2008) Pharmacogenetic differences between warfarin, acenocoumarol and phenprocoumon. Thromb Haemost 100:1052–1057

Berger PB, Bell MR, Rihal CS et al (1999) Clopidogrel versus ticlopidine after intracoronary stent placement. J Am Coll Cardiol 34:1891–1894

Berger JS, Roe MT, Gibson CM et al (2009) Safety and feasibility of adjunctive antiplatelet therapy with intravenous elinogrel, a direct-acting and reversible P2Y12 ADP-receptor antagonist, before primary percutaneous intervention in patients with ST-elevation myocardial infarction: the Early Rapid ReversAl of platelet thromboSis with intravenous Elinogrel before PCI to optimize reperfusion in acute Myocardial Infarction (ERASE MI) pilot trial. Am Heart J 158(998–1004):e1001

Bernlochner I, Steinhubl S, Braun S et al (2010) Association between inflammatory biomarkers and platelet aggregation in patients under chronic clopidogrel treatment. Thromb Haemost 104:1193–1200

Bertrand ME, Rupprecht HJ, Urban P et al (2000) Double-blind study of the safety of clopidogrel with and without a loading dose in combination with aspirin compared with ticlopidine in combination with aspirin after coronary stenting: the clopidogrel aspirin stent international cooperative study (CLASSICS). Circulation 102:624–629

Bhatt DL, Lincoff AM, Gibson CM et al (2009) Intravenous platelet blockade with cangrelor during PCI. N Engl J Med 361:2330–2341

Bhatt DL, Cryer BL, Contant CF et al (2010) Clopidogrel with or without omeprazole in coronary artery disease. N Engl J Med 363:1909–1917

Björkman JA KI, van Giezen JJJ. (2007) AZD6140 inhibits adenosine uptake into erythrocytes and enhances coronary blood flow after local ischemia or intracoronary adenosine infusion. Circulation 116:II_28 (Abstract 245)

Blindt R, Stellbrink K, de Taeye A et al (2007) The significance of vasodilator-stimulated phosphoprotein for risk stratification of stent thrombosis. Thromb Haemost 98:1329–1334

Bonello L, Paganelli F, Arpin-Bornet M et al (2007) Vasodilator-stimulated phosphoprotein phosphorylation analysis prior to percutaneous coronary intervention for exclusion of postprocedural major adverse cardiovascular events. J Thromb Haemost 5:1630–1636

Bonello L, Camoin-Jau L, Arques S et al (2008) Adjusted clopidogrel loading doses according to vasodilator-stimulated phosphoprotein phosphorylation index decrease rate of major adverse cardiovascular events in patients with clopidogrel resistance: a multicenter randomized prospective study. J Am Coll Cardiol 51:1404–1411

Bonello L, Camoin-Jau L, Armero S et al (2009) Tailored clopidogrel loading dose according to platelet reactivity monitoring to prevent acute and subacute stent thrombosis. Am J Cardiol 103:5–10

Bonello L, Tantry US, Marcucci R et al (2010) Consensus and future directions on the definition of high on-treatment platelet reactivity to adenosine diphosphate. J Am Coll Cardiol 56:919–933

Bonello L, Pansieri M, Mancini J et al (2011) High on-treatment platelet reactivity after prasugrel loading dose and cardiovascular events after percutaneous coronary intervention in acute coronary syndromes. J Am Coll Cardiol 58:467–473

Bouman HJ, Schomig E, van Werkum JW et al (2011) Paraoxonase-1 is a major determinant of clopidogrel efficacy. Nat Med 17:110–116

Brandt JT, Close SL, Iturria SJ et al (2007a) Common polymorphisms of CYP2C19 and CYP2C9 affect the pharmacokinetic and pharmacodynamic response to clopidogrel but not prasugrel. J Thromb Haemost 5:2429–2436

Brandt JT, Payne CD, Wiviott SD et al (2007b) A comparison of prasugrel and clopidogrel loading doses on platelet function: magnitude of platelet inhibition is related to active metabolite formation. Am Heart J 153(1):66.e9–66.e16

Breet NJ, van Werkum JW, Bouman HJ et al (2010) Comparison of platelet function tests in predicting clinical outcome in patients undergoing coronary stent implantation. JAMA 303:754–762

Buonamici P, Marcucci R, Migliorini A et al (2007) Impact of platelet reactivity after clopidogrel administration on drug-eluting stent thrombosis. J Am Coll Cardiol 49:2312–2317

Campo G, Parrinello G, Ferraresi P et al (2011) Prospective evaluation of on-clopidogrel platelet reactivity over time in patients treated with percutaneous coronary intervention relationship with gene polymorphisms and clinical outcome. J Am Coll Cardiol 57:2474–2483

Cannon CP, Husted S, Harrington RA et al (2007) Safety, tolerability, and initial efficacy of AZD6140, the first reversible oral adenosine diphosphate receptor antagonist, compared with clopidogrel, in patients with non-ST-segment elevation acute coronary syndrome: primary results of the DISPERSE-2 trial. J Am Coll Cardiol 50:1844–1851

Cattaneo M (2010) New P2Y(12) inhibitors. Circulation 121:171–179

Chen L, Bracey AW, Radovancevic R et al (2004) Clopidogrel and bleeding in patients undergoing elective coronary artery bypass grafting. J Thorac Cardiovasc Surg 128:425–431

Collet JP, Hulot JS, Pena A et al (2009) Cytochrome P450 2C19 polymorphism in young patients treated with clopidogrel after myocardial infarction: a cohort study. Lancet 373:309–317

Committee CS (1996) A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. Lancet 348:1329–1339

Cuisset T, Frere C, Quilici J et al (2006) High post-treatment platelet reactivity identified low-responders to dual antiplatelet therapy at increased risk of recurrent cardiovascular events after stenting for acute coronary syndrome. J Thromb Haemost 4:542–549

Cuisset T, Cayla G, Frere C et al (2009a) Predictive value of post-treatment platelet reactivity for occurrence of post-discharge bleeding after non-ST elevation acute coronary syndrome. Shifting from antiplatelet resistance to bleeding risk assessment? EuroIntervention 5:325–329

Cuisset T, Frere C, Quilici J et al (2009b) Comparison of omeprazole and pantoprazole influence on a high 150-mg clopidogrel maintenance dose the PACA (Proton Pump Inhibitors And Clopidogrel Association) prospective randomized study. J Am Coll Cardiol 54:1149–1153

Dalvie DK, O'Connell TN (2004) Characterization of novel dihydrothienopyridinium and thienopyridinium metabolites of ticlopidine in vitro: role of peroxidases, cytochromes p450, and monoamine oxidases. Drug Metab Dispos 32:49–57

Davi G, Patrono C (2007) Platelet activation and atherothrombosis. N Engl J Med 357:2482–2494

Dorsam RT, Kunapuli SP (2004) Central role of the P2Y12 receptor in platelet activation. J Clin Invest 113:340–345

Dovlatova NL, Jakubowski JA, Sugidachi A et al (2008) The reversible P2Y antagonist cangrelor influences the ability of the active metabolites of clopidogrel and prasugrel to produce irreversible inhibition of platelet function. J Thromb Haemost 6:1153–1159

Elias M, Reichman N, Flatau E (1993) Bone marrow aplasia associated with ticlopidine therapy. Am J Hematol 44:289–290

Eshtehardi P, Windecker S, Cook S et al (2010) Dual low response to acetylsalicylic acid and clopidogrel is associated with myonecrosis and stent thrombosis after coronary stent implantation. Am Heart J 159:891–898 e891

Farid NA, Kurihara A, Wrighton SA (2010) Metabolism and disposition of the thienopyridine antiplatelet drugs ticlopidine, clopidogrel, and prasugrel in humans. J Clin Pharmacol 50:126–142

Ferreiro JL, Ueno M, Angiolillo DJ (2009) Cangrelor: a review on its mechanism of action and clinical development. Expert Rev Cardiovasc Ther 7:1195–1201

Fontana P, James R, Barazer I et al (2011) Relationship between paraoxonase-1 activity, its Q192R genetic variant and clopidogrel responsiveness in the ADRIE study. J Thromb Haemost 9:1664–1666

Frere C, Cuisset T, Quilici J et al (2007) ADP-induced platelet aggregation and platelet reactivity index VASP are good predictive markers for clinical outcomes in non-ST elevation acute coronary syndrome. Thromb Haemost 98:838–843

Gachet C (2006) Regulation of platelet functions by P2 receptors. Annu Rev Pharmacol Toxicol 46:277–300

Gaglia MA Jr, Waksman R (2011) Overview of the 2010 Food and Drug Administration Cardiovascular and Renal Drugs Advisory Committee meeting regarding ticagrelor. Circulation 123:451–456

Geiger J, Brich J, Honig-Liedl P et al (1999) Specific impairment of human platelet P2Y(AC) ADP receptor-mediated signaling by the antiplatelet drug clopidogrel. Arterioscler Thromb Vasc Biol 19:2007–2011

Geisler T, Langer H, Wydymus M et al (2006) Low response to clopidogrel is associated with cardiovascular outcome after coronary stent implantation. Eur Heart J 27:2420–2425

Geisler T, Anders N, Paterok M et al (2007) Platelet response to clopidogrel is attenuated in diabetic patients undergoing coronary stent implantation. Diabetes Care 30:372–374

Geisler T, Grass D, Bigalke B et al (2008) The Residual Platelet Aggregation after Deployment of Intracoronary Stent (PREDICT) score. J Thromb Haemost 6:54–61

Geisler T, Zurn C, Simonenko R et al (2010) Early but not late stent thrombosis is influenced by residual platelet aggregation in patients undergoing coronary interventions. Eur Heart J 31:59–66

Gilard M, Arnaud B, Cornily JC et al (2008) Influence of omeprazole on the antiplatelet action of clopidogrel associated with aspirin: the randomized, double-blind OCLA (Omeprazole CLopidogrel Aspirin) study. J Am Coll Cardiol 51:256–260

Giusti B, Gori AM, Marcucci R et al (2007) Cytochrome P450 2C19 loss-of-function polymorphism, but not CYP3A4 IVS10 + 12G/A and P2Y12 T744C polymorphisms, is associated with response variability to dual antiplatelet treatment in high-risk vascular patients. Pharmacogenet Genomics 17:1057–1064

Giusti B, Gori AM, Marcucci R et al (2009) Relation of cytochrome P450 2C19 loss-of-function polymorphism to occurrence of drug-eluting coronary stent thrombosis. Am J Cardiol 103:806–811

Gorchakova O, von Beckerath N, Gawaz M et al (2004) Antiplatelet effects of a 600 mg loading dose of clopidogrel are not attenuated in patients receiving atorvastatin or simvastatin for at least 4 weeks prior to coronary artery stenting. Eur Heart J 25:1898–1902

Gori AM, Marcucci R, Migliorini A et al (2008) Incidence and clinical impact of dual nonresponsiveness to aspirin and clopidogrel in patients with drug-eluting stents. J Am Coll Cardiol 52:734–739

Greenbaum AB, Grines CL, Bittl JA et al (2006) Initial experience with an intravenous P2Y12 platelet receptor antagonist in patients undergoing percutaneous coronary intervention: results from a 2-part, phase II, multicenter, randomized, placebo- and active-controlled trial. Am Heart J 151:689 e681–689 e610

Gremmel T, Steiner S, Seidinger D et al (2010) Calcium-channel blockers decrease clopidogrel-mediated platelet inhibition. Heart 96:186–189

Gurbel PA, Bliden KP, Hiatt BL et al (2003) Clopidogrel for coronary stenting: response variability, drug resistance, and the effect of pretreatment platelet reactivity. Circulation 107:2908–2913

Gurbel PA, Bliden KP, Guyer K et al (2005a) Platelet reactivity in patients and recurrent events post-stenting: results of the PREPARE POST-STENTING Study. J Am Coll Cardiol 46:1820–1826

Gurbel PA, Bliden KP, Samara W et al (2005b) Clopidogrel effect on platelet reactivity in patients with stent thrombosis: results of the CREST Study. J Am Coll Cardiol 46:1827–1832

Gurbel PA, Antonino MJ, Bliden KP et al (2008) Platelet reactivity to adenosine diphosphate and long-term ischemic event occurrence following percutaneous coronary intervention: a potential antiplatelet therapeutic target. Platelets 19:595–604

Gurbel PA, Bliden KP, Butler K et al (2009) Randomized double-blind assessment of the ONSET and OFFSET of the antiplatelet effects of ticagrelor versus clopidogrel in patients with stable coronary artery disease: the ONSET/OFFSET study. Circulation 120:2577–2585

Gurbel PA, Bliden KP, Butler K et al (2010a) Response to ticagrelor in clopidogrel nonresponders and responders and effect of switching therapies: the RESPOND study. Circulation 121:1188–1199

Gurbel PA, Bliden KP, Antonino MJ et al (2010b) The effect of elinogrel on high platelet reactivity during dual antiplatelet therapy and the relation to CYP2C19*2 genotype: first experience in patients. J Thromb Haemost 8:43–53

Hamm CW, Bassand JP, Agewall S et al (2011) ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: The Task Force for the management of acute coronary syndromes (ACS) in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC). Eur Heart J 32:2999–3054

Harmsze AM, Robijns K, van Werkum JW et al (2010) The use of amlodipine, but not of P-glycoprotein inhibiting calcium channel blockers is associated with clopidogrel poor-response. Thromb Haemost 103:920–925

Harrington RA, Stone GW, McNulty S et al (2009) Platelet inhibition with cangrelor in patients undergoing PCI. N Engl J Med 361:2318–2329

Ho PM, Maddox TM, Wang L et al (2009) Risk of adverse outcomes associated with concomitant use of clopidogrel and proton pump inhibitors following acute coronary syndrome. JAMA 301:937–944

Hochholzer W, Trenk D, Bestehorn HP et al (2006) Impact of the degree of peri-interventional platelet inhibition after loading with clopidogrel on early clinical outcome of elective coronary stent placement. J Am Coll Cardiol 48:1742–1750

Hochholzer W, Trenk D, Fromm MF et al (2010) Impact of cytochrome P450 2C19 loss-of-function polymorphism and of major demographic characteristics on residual platelet function after loading and maintenance treatment with clopidogrel in patients undergoing elective coronary stent placement. J Am Coll Cardiol 55:2427–2434

Hulot JS, Bura A, Villard E et al (2006) Cytochrome P450 2C19 loss-of-function polymorphism is a major determinant of clopidogrel responsiveness in healthy subjects. Blood 108:2244–2247

Hulot JS, Collet JP, Silvain J et al (2010) Cardiovascular risk in clopidogrel-treated patients according to cytochrome P450 2C19*2 loss-of-function allele or proton pump inhibitor coadministration: a systematic meta-analysis. J Am Coll Cardiol 56:134–143

Husted S, Emanuelsson H, Heptinstall S et al (2006) Pharmacodynamics, pharmacokinetics, and safety of the oral reversible P2Y12 antagonist AZD6140 with aspirin in patients with atherosclerosis: a double-blind comparison to clopidogrel with aspirin. Eur Heart J 27:1038–1047

Jacobsson F, Swahn E, Wallentin L et al (2002) Safety profile and tolerability of intravenous AR-C69931MX, a new antiplatelet drug, in unstable angina pectoris and non-Q-wave myocardial infarction. Clin Ther 24:752–765

Jakubowski JA, Matsushima N, Asai F et al (2007) A multiple dose study of prasugrel (CS-747), a novel thienopyridine P2Y12 inhibitor, compared with clopidogrel in healthy humans. Br J Clin Pharmacol 63:421–430

Jernberg T, Payne CD, Winters KJ et al (2006) Prasugrel achieves greater inhibition of platelet aggregation and a lower rate of non-responders compared with clopidogrel in aspirin-treated patients with stable coronary artery disease. Eur Heart J 27:1166–1173

Juurlink DN, Gomes T, Ko DT et al (2009) A population-based study of the drug interaction between proton pump inhibitors and clopidogrel. CMAJ 180:713–718

Kazui M, Nishiya Y, Ishizuka T et al (2010) Identification of the human cytochrome P450 enzymes involved in the two oxidative steps in the bioactivation of clopidogrel to its pharmacologically active metabolite. Drug Metab Dispos 38:92–99

Lange RA, Hillis LD (2004) Antiplatelet therapy for ischemic heart disease. N Engl J Med 350:277–280

Lau WC, Waskell LA, Watkins PB et al (2003) Atorvastatin reduces the ability of clopidogrel to inhibit platelet aggregation: a new drug-drug interaction. Circulation 107:32–37

Lau WC, Gurbel PA, Watkins PB et al (2004) Contribution of hepatic cytochrome P450 3A4 metabolic activity to the phenomenon of clopidogrel resistance. Circulation 109:166–171

Leon MB, Baim DS, Popma JJ et al (1998) A clinical trial comparing three antithrombotic-drug regimens after coronary-artery stenting. Stent Anticoagulation Restenosis Study Investigators. N Engl J Med 339:1665–1671

Leonardi S, Rao SV, Harrington RA et al (2010) Rationale and design of the randomized, double-blind trial testing INtraveNous and Oral administration of elinogrel, a selective and reversible P2Y(12)-receptor inhibitor, versus clopidogrel to eVAluate Tolerability and Efficacy in non-urgent Percutaneous Coronary Interventions patients (INNOVATE-PCI). Am Heart J 160:65–72

Lewis JP, Fisch AS, Ryan K et al (2011) Paraoxonase 1 (PON1) gene variants are not associated with clopidogrel response. Clin Pharmacol Ther 90(4):568–74

Mahaffey KW, Wojdyla DM, Carroll K et al (2011) Ticagrelor compared with clopidogrel by geographic region in the Platelet Inhibition and Patient Outcomes (PLATO) trial. Circulation 124:544–554

Marcucci R, Gori AM, Paniccia R et al (2009) Cardiovascular death and nonfatal myocardial infarction in acute coronary syndrome patients receiving coronary stenting are predicted by residual platelet reactivity to ADP detected by a point-of-care assay: a 12-month follow-up. Circulation 119:237–242

Matetzky S, Shenkman B, Guetta V et al (2004) Clopidogrel resistance is associated with increased risk of recurrent atherothrombotic events in patients with acute myocardial infarction. Circulation 109:3171–3175

Mega JL, Close SL, Wiviott SD et al (2009) Cytochrome p-450 polymorphisms and response to clopidogrel. N Engl J Med 360:354–362

Mega JL, Simon T, Collet JP et al (2010a) Reduced-function CYP2C19 genotype and risk of adverse clinical outcomes among patients treated with clopidogrel predominantly for PCI: a meta-analysis. JAMA 304:1821–1830

Mega JL, Close SL, Wiviott SD et al (2010b) Genetic variants in ABCB1 and CYP2C19 and cardiovascular outcomes after treatment with clopidogrel and prasugrel in the TRITON-TIMI 38 trial: a pharmacogenetic analysis. Lancet 376:1312–1319

Mehta SR, Tanguay JF, Eikelboom JW et al (2010a) Double-dose versus standard-dose clopidogrel and high-dose versus low-dose aspirin in individuals undergoing percutaneous

coronary intervention for acute coronary syndromes (CURRENT-OASIS 7): a randomised factorial trial. Lancet 376:1233–1243

Mehta SR, Bassand JP, Chrolavicius S et al (2010b) Dose comparisons of clopidogrel and aspirin in acute coronary syndromes. N Engl J Med 363:930–942

Migliorini A, Valenti R, Marcucci R et al (2009) High residual platelet reactivity after clopidogrel loading and long-term clinical outcome after drug-eluting stenting for unprotected left main coronary disease. Circulation 120:2214–2221

Mokhtar OA, Lemesle G, Armero S et al (2010) Relationship between platelet reactivity inhibition and non-CABG related major bleeding in patients undergoing percutaneous coronary intervention. Thromb Res 126:e147–e149

Montalescot G, Sideris G, Meuleman C et al (2006) A randomized comparison of high clopidogrel loading doses in patients with non-ST-segment elevation acute coronary syndromes: the ALBION (Assessment of the Best Loading Dose of Clopidogrel to Blunt Platelet Activation, Inflammation and Ongoing Necrosis) trial. J Am Coll Cardiol 48:931–938

Montalescot G, Wiviott SD, Braunwald E et al (2009) Prasugrel compared with clopidogrel in patients undergoing percutaneous coronary intervention for ST-elevation myocardial infarction (TRITON-TIMI 38): double-blind, randomised controlled trial. Lancet 373:723–731

Moussa I, Oetgen M, Roubin G et al (1999) Effectiveness of clopidogrel and aspirin versus ticlopidine and aspirin in preventing stent thrombosis after coronary stent implantation. Circulation 99:2364–2366

Muller C, Buttner HJ, Petersen J et al (2000) A randomized comparison of clopidogrel and aspirin versus ticlopidine and aspirin after the placement of coronary-artery stents. Circulation 101:590–593

Muller I, Seyfarth M, Rudiger S et al (2001) Effect of a high loading dose of clopidogrel on platelet function in patients undergoing coronary stent placement. Heart 85:92–93

Neubauer H, Gunesdogan B, Hanefeld C et al (2003) Lipophilic statins interfere with the inhibitory effects of clopidogrel on platelet function–a flow cytometry study. Eur Heart J 24:1744–1749

O'Donoghue ML, Braunwald E, Antman EM et al (2009) Pharmacodynamic effect and clinical efficacy of clopidogrel and prasugrel with or without a proton-pump inhibitor: an analysis of two randomised trials. Lancet 374:989–997

Oestreich JH (2010) Elinogrel, a reversible P2Y12 receptor antagonist for the treatment of acute coronary syndrome and prevention of secondary thrombotic events. Curr Opin Investig Drugs 11:340–348

Osmancik P, Jirmar R, Hulikova K et al (2009) A comparison of the VASP index between patients with hemodynamically complicated and uncomplicated acute myocardial infarction. Catheter Cardiovasc Interv 75:158–166

Pare G, Mehta SR, Yusuf S et al (2010) Effects of CYP2C19 genotype on outcomes of clopidogrel treatment. N Engl J Med 363:1704–1714

Patti G, Nusca A, Mangiacapra F et al (2008) Point-of-care measurement of clopidogrel responsiveness predicts clinical outcome in patients undergoing percutaneous coronary intervention results of the ARMYDA-PRO (Antiplatelet therapy for Reduction of MYocardial Damage during Angioplasty-Platelet Reactivity Predicts Outcome) study. J Am Coll Cardiol 52:1128–1133

Payne CD, Li YG, Small DS et al (2007) Increased active metabolite formation explains the greater platelet inhibition with prasugrel compared to high-dose clopidogrel. J Cardiovasc Pharmacol 50:555–562

Picard-Fraire C (1983) Ticlopidine hydrochloride: relationship between dose, kinetics, plasma concentration and effect on platelet function. Thromb Res Suppl 4:119–128

Price MJ, Coleman JL, Steinhubl SR et al (2006) Onset and offset of platelet inhibition after high-dose clopidogrel loading and standard daily therapy measured by a point-of-care assay in healthy volunteers. Am J Cardiol 98:681–684

Price MJ, Endemann S, Gollapudi RR et al (2008) Prognostic significance of post-clopidogrel platelet reactivity assessed by a point-of-care assay on thrombotic events after drug-eluting stent implantation. Eur Heart J 29:992–1000

Price MJ, Logan DK, Walder JS et al (2011a) Recovery of platelet function following discontinuation of prasugrel or clopidogrel maintenance dosing in aspirin-treated subjects with stable coronary disease: the recovery trial. J Am Coll Cardiol 57:E1636

Price MJ, Berger PB, Teirstein PS et al (2011b) Standard- vs high-dose clopidogrel based on platelet function testing after percutaneous coronary intervention: the GRAVITAS randomized trial. JAMA 305:1097–1105

Quinn MJ, Fitzgerald DJ (1999) Ticlopidine and clopidogrel. Circulation 100:1667–1672

Rehmel JL, Eckstein JA, Farid NA et al (2006) Interactions of two major metabolites of prasugrel, a thienopyridine antiplatelet agent, with the cytochromes P450. Drug Metab Dispos 34:600–607

Sarafoff N, Neumann L, Morath T et al (2011) Lack of impact of calcium-channel blockers on the pharmacodynamic effect and the clinical efficacy of clopidogrel after drug-eluting stenting. Am Heart J 161:605–610

Saw J, Steinhubl SR, Berger PB et al (2003) Lack of adverse clopidogrel-atorvastatin clinical interaction from secondary analysis of a randomized, placebo-controlled clopidogrel trial. Circulation 108:921–924

Saw J, Brennan DM, Steinhubl SR et al (2007) Lack of evidence of a clopidogrel-statin interaction in the CHARISMA trial. J Am Coll Cardiol 50:291–295

Schomig A (2009) Ticagrelor–is there need for a new player in the antiplatelet-therapy field? N Engl J Med 361:1108–1111

Schomig A, Neumann FJ, Kastrati A et al (1996) A randomized comparison of antiplatelet and anticoagulant therapy after the placement of coronary-artery stents. N Engl J Med 334:1084–1089

Serebruany VL, Midei MG, Malinin AI et al (2004) Absence of interaction between atorvastatin or other statins and clopidogrel: results from the interaction study. Arch Intern Med 164:2051–2057

Serebruany VL, Steinhubl SR, Berger PB et al (2005) Variability in platelet responsiveness to clopidogrel among 544 individuals. J Am Coll Cardiol 45:246–251

Serebruany V, Cherala G, Williams C et al (2009) Association of platelet responsiveness with clopidogrel metabolism: role of compliance in the assessment of “resistance”. Am Heart J 158:925–932

Shuldiner AR, O’Connell JR, Bliden KP et al (2009) Association of cytochrome P450 2C19 genotype with the antiplatelet effect and clinical efficacy of clopidogrel therapy. JAMA 302:849–857

Sibbing D, Kastrati A (2009) Risk of combining PPIs with thienopyridines: fact or fiction? Lancet 374:952–954

Sibbing D, von Beckerath O, Schomig A et al (2006) Diabetes mellitus and platelet function after administration of aspirin and a single 600 mg dose of clopidogrel. J Thromb Haemost 4:2566–2568

Sibbing D, von Beckerath O, Schomig A et al (2007) Impact of body mass index on platelet aggregation after administration of a high loading dose of 600 mg of clopidogrel before percutaneous coronary intervention. Am J Cardiol 100:203–205

Sibbing D, Morath T, Stegherr J et al (2009a) Impact of proton pump inhibitors on the antiplatelet effects of clopidogrel. Thromb Haemost 101:714–719

Sibbing D, Braun S, Morath T et al (2009b) Platelet reactivity after clopidogrel treatment assessed with point-of-care analysis and early drug-eluting stent thrombosis. J Am Coll Cardiol 53:849–856

Sibbing D, Morath T, Braun S et al (2010a) Clopidogrel response status assessed with Multiplate point-of-care analysis and the incidence and timing of stent thrombosis over six months following coronary stenting. Thromb Haemost 103:151–159

Sibbing D, Schulz S, Braun S et al (2010b) Antiplatelet effects of clopidogrel and bleeding in patients undergoing coronary stent placement. J Thromb Haemost 8:250–256
Sibbing D, von Beckerath N, Morath T et al (2010c) Oral anticoagulation with coumarin derivatives and antiplatelet effects of clopidogrel. Eur Heart J 31:1205–1211
Sibbing D, Gebhard D, Koch W et al (2010d) Isolated and interactive impact of common CYP2C19 genetic variants on the antiplatelet effect of chronic clopidogrel therapy. J Thromb Haemost 8:1685–1693
Sibbing D, Koch W, Gebhard D et al (2010e) Cytochrome 2C19*17 allelic variant, platelet aggregation, bleeding events, and stent thrombosis in clopidogrel-treated patients with coronary stent placement. Circulation 121:512–518
Sibbing D, Steinhubl SR, Schulz S et al (2010f) Platelet aggregation and its association with stent thrombosis and bleeding in clopidogrel-treated patients: initial evidence of a therapeutic window. J Am Coll Cardiol 56:317–318
Sibbing D, Koch W, Massberg S et al (2011) No association of paraoxonase-1 Q192R genotypes with platelet response to clopidogrel and risk of stent thrombosis after coronary stenting. Eur Heart J 32:1605–1613
Siller-Matula JM, Lang I, Christ G et al (2008) Calcium-channel blockers reduce the antiplatelet effect of clopidogrel. J Am Coll Cardiol 52:1557–1563
Siller-Matula J, Spiel A, Lang I et al (2009) Effects of pantoprazole and esomeprazole on platelet inhibition by clopidogrel. Am Heart J 157:148.e141–148.e145
Siller-Matula JM, Christ G, Lang IM et al (2010) Multiple electrode aggregometry predicts stent thrombosis better than the vasodilator-stimulated phosphoprotein phosphorylation assay. J Thromb Haemost 8:351–359
Sim SC, Risinger C, Dahl ML et al (2006) A common novel CYP2C19 gene variant causes ultrarapid drug metabolism relevant for the drug response to proton pump inhibitors and antidepressants. Clin Pharmacol Ther 79:103–113
Simon T, Verstuyft C, Mary-Krause M et al (2009) Genetic determinants of response to clopidogrel and cardiovascular events. N Engl J Med 360:363–375
Smith SC Jr, Feldman TE, Hirshfeld JW Jr et al (2006) ACC/AHA/SCAI 2005 guideline update for percutaneous coronary intervention: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (ACC/AHA/SCAI Writing Committee to Update 2001 Guidelines for Percutaneous Coronary Intervention). Circulation 113:e166–e286
Steinhubl SR (2010) Genotyping, clopidogrel metabolism, and the search for the therapeutic window of thienopyridines. Circulation 121:481–483
Steinhubl SR, Ellis SG, Wolski K et al (2001a) Ticlopidine pretreatment before coronary stenting is associated with sustained decrease in adverse cardiac events: data from the Evaluation of Platelet IIb/IIIa Inhibitor for Stenting (EPISTENT) Trial. Circulation 103:1403–1409
Steinhubl SR, Talley JD, Braden GA et al (2001b) Point-of-care measured platelet inhibition correlates with a reduced risk of an adverse cardiac event after percutaneous coronary intervention: results of the GOLD (AU-Assessing Ultegra) multicenter study. Circulation 103:2572–2578
Steinhubl SR, Berger PB, Mann JT 3rd et al (2002) Early and sustained dual oral antiplatelet therapy following percutaneous coronary intervention: a randomized controlled trial. JAMA 288:2411–2420
Steinhubl SR, Oh JJ, Oestreich JH et al (2008) Transitioning patients from cangrelor to clopidogrel: pharmacodynamic evidence of a competitive effect. Thromb Res 121:527–534
Storey RF (2011) Pharmacology and clinical trials of reversibly-binding P2Y12 inhibitors. Thromb Haemost 105(Suppl 1):S75–81
Storey RF, Newby LJ, Heptinstall S (2001a) Effects of P2Y(1) and P2Y(12) receptor antagonists on platelet aggregation induced by different agonists in human whole blood. Platelets 12:443–447

Storey RF, Oldroyd KG, Wilcox RG (2001b) Open multicentre study of the P2T receptor antagonist AR-C69931MX assessing safety, tolerability and activity in patients with acute coronary syndromes. Thromb Haemost 85:401–407

Storey RF, Angiolillo DJ, Patil SB et al (2010a) Inhibitory effects of ticagrelor compared with clopidogrel on platelet function in patients with acute coronary syndromes: the PLATO (PLATelet inhibition and patient Outcomes) PLATELET substudy. J Am Coll Cardiol 56:1456–1462

Storey RF, Bliden KP, Patil SB et al (2010b) Incidence of dyspnea and assessment of cardiac and pulmonary function in patients with stable coronary artery disease receiving ticagrelor, clopidogrel, or placebo in the ONSET/OFFSET study. J Am Coll Cardiol 56:185–193

Taubert D, von Beckerath N, Grimberg G et al (2006) Impact of P-glycoprotein on clopidogrel absorption. Clin Pharmacol Ther 80:486–501

Teng R, Butler K (2010) Pharmacokinetics, pharmacodynamics, tolerability and safety of single ascending doses of ticagrelor, a reversibly binding oral P2Y(12) receptor antagonist, in healthy subjects. Eur J Clin Pharmacol 66:487–496

Trenk D. (2011) TRIGGER-PCI: a prospective, randomized trial of prasugrel vs. clopidogrel in clopidogrel-hyporesponsive patients with stable ischemic heart disease undergoing percutaneous coronary intervention. Transcatheter Cardiovascular Therapeutics 2011, San Francisco, 9 Nov 2011

Trenk D, Hochholzer W, Fromm MF et al (2008) Cytochrome P450 2C19 681G > A polymorphism and high on-clopidogrel platelet reactivity associated with adverse 1-year clinical outcome of elective percutaneous coronary intervention with drug-eluting or bare-metal stents. J Am Coll Cardiol 51:1925–1934

Trenk D, Hochholzer W, Fromm MF et al (2011) Paraoxonase-1 Q192R polymorphism and antiplatelet effects of clopidogrel in patients undergoing elective coronary stent placement. Circ Cardiovasc Genet 4:429–436

Ueno M, Rao SV, Angiolillo DJ (2010) Elinogrel: pharmacological principles, preclinical and early phase clinical testing. Future Cardiol 6:445–453

Ufer M, Svensson JO, Krausz KW et al (2004) Identification of cytochromes P450 2C9 and 3A4 as the major catalysts of phenprocoumon hydroxylation in vitro. Eur J Clin Pharmacol 60:173–182

van Giezen JJ, Nilsson L, Berntsson P et al (2009) Ticagrelor binds to human P2Y(12) independently from ADP but antagonizes ADP-induced receptor signaling and platelet aggregation. J Thromb Haemost 7:1556–1565

van Giezen JJ, Sidaway J, Glaves P et al. (2012) Ticagrelor inhibits adenosine uptake in vitro and enhances adenosine-mediated hyperemia responses in a canine model. J Cardiovasc Pharmacol Ther

Varenhorst C, James S, Erlinge D et al (2009) Genetic variation of CYP2C19 affects both pharmacokinetic and pharmacodynamic responses to clopidogrel but not prasugrel in aspirin-treated patients with coronary artery disease. Eur Heart J 30:1744–1752

von Beckerath N, Taubert D, Pogatsa-Murray G et al (2005) Absorption, metabolization, and antiplatelet effects of 300-, 600-, and 900-mg loading doses of clopidogrel: results of the ISAR-CHOICE (Intracoronary Stenting and Antithrombotic Regimen: Choose Between 3 High Oral Doses for Immediate Clopidogrel Effect) Trial. Circulation 112:2946–2950

Wallentin L (2009) P2Y(12) inhibitors: differences in properties and mechanisms of action and potential consequences for clinical use. Eur Heart J 30:1964–1977

Wallentin L, Varenhorst C, James S et al (2008) Prasugrel achieves greater and faster P2Y12receptor-mediated platelet inhibition than clopidogrel due to more efficient generation of its active metabolite in aspirin-treated patients with coronary artery disease. Eur Heart J 29:21–30

Wallentin L, Becker RC, Budaj A et al (2009) Ticagrelor versus clopidogrel in patients with acute coronary syndromes. N Engl J Med 361:1045–1057

Wallentin L, James S, Storey RF et al (2010) Effect of CYP2C19 and ABCB1 single nucleotide polymorphisms on outcomes of treatment with ticagrelor versus clopidogrel for acute coronary syndromes: a genetic substudy of the PLATO trial. Lancet 376:1320–1328

Wallentin L, Becker RC, James SK et al (2011) The PLATO trial reveals further opportunities to improve outcomes in patients with acute coronary syndrome. Editorial on Serebruany. "Viewpoint: Paradoxical excess mortality in the PLATO trial should be independently verified" (Thromb Haemost 2011; 105.5). Thromb Haemost 105:760–762

Wang ZJ, Zhou YJ, Liu YY et al (2009) Impact of clopidogrel resistance on thrombotic events after percutaneous coronary intervention with drug-eluting stent. Thromb Res 124:46–51

Wiviott SD, Antman EM, Winters KJ et al (2005) Randomized comparison of prasugrel (CS-747, LY640315), a novel thienopyridine P2Y12 antagonist, with clopidogrel in percutaneous coronary intervention: results of the Joint Utilization of Medications to Block Platelets Optimally (JUMBO)-TIMI 26 trial. Circulation 111:3366–3373

Wiviott SD, Trenk D, Frelinger AL et al (2007a) Prasugrel compared with high loading- and maintenance-dose clopidogrel in patients with planned percutaneous coronary intervention: the Prasugrel in Comparison to Clopidogrel for Inhibition of Platelet Activation and Aggregation-Thrombolysis in Myocardial Infarction 44 trial. Circulation 116:2923–2932

Wiviott SD, Braunwald E, McCabe CH et al (2007b) Prasugrel versus clopidogrel in patients with acute coronary syndromes. N Engl J Med 357:2001–2015

Wiviott SD, Braunwald E, Angiolillo DJ et al (2008) Greater clinical benefit of more intensive oral antiplatelet therapy with prasugrel in patients with diabetes mellitus in the trial to assess improvement in therapeutic outcomes by optimizing platelet inhibition with prasugrel-Thrombolysis in Myocardial Infarction 38. Circulation 118:1626–1636

Wiviott SD, Antman EM, Braunwald E (2010) Prasugrel. Circulation 122:394–403

Wrishko RE, Ernest CS 2nd, Small DS et al (2009) Population pharmacokinetic analyses to evaluate the influence of intrinsic and extrinsic factors on exposure of prasugrel active metabolite in TRITON-TIMI 38. J Clin Pharmacol 49(8):984–98

Yusuf S, Zhao F, Mehta SR et al (2001) Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med 345:494–502

Glycoprotein IIb/IIIa Antagonists

Karen M. Hook and Joel S. Bennett

Contents

Abstract Mortality from ischemic cardiac disease in adults has been dramatically reduced by the development of novel therapies for inhibiting platelet function. Circulating platelets are maintained in a resting state and are activated at sites of vascular injury by exquisitely controlled mechanisms, thereby maintaining vascular integrity without causing intravascular thrombosis. As it became clear that platelets

K.M. Hook
The Carole and Ray Neag Comprehensive Cancer Center, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT USA
e-mail: khook@uchc.edu

J.S. Bennett (✉)
Division of Hematology/Oncology, Perelman School of Medicine, University of Pennsylvania, 914 BRB II/III, 421 Curie Blvd., Philadelphia, PA 19104, USA
e-mail: bennetts@mail.med.upenn.edu

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_8,

play a central role in arterial thrombosis, the processes of platelet activation, adhesion, and aggregation became logical targets for the development of antithrombotic agents.

Keywords Glycoprotein IIb/IIIa • Abciximab • Eptifibatide • Tirofiban

1 Introduction

Mortality from ischemic cardiac disease in adults has been dramatically reduced by the development of novel therapies for inhibiting platelet function. Circulating platelets are maintained in a resting state and are activated at sites of vascular injury by exquisitely controlled mechanisms, thereby maintaining vascular integrity without causing intravascular thrombosis. As it became clear that platelets play a central role in arterial thrombosis, the processes of platelet activation, adhesion, and aggregation became logical targets for the development of antithrombotic agents. Because acute arterial occlusion results from platelet thrombi that form on ruptured atherosclerotic plaques and after the iatrogenic arterial wall injury that occurs during percutaneous coronary interventions (PCI), therapeutic strategies to constrain platelet responses in each of these situations have led to improved clinical outcomes for acute myocardial infarction (AMI) and fewer restenotic events following PCI.

The discovery in the late 1970s that the platelet glycoprotein (GP) IIb/IIIa is a platelet receptor for fibrinogen and that fibrinogen binding to GPIIb/IIIa is directly responsible for platelet aggregation (Fig. 1a) (Yin et al. 2007) provided the impetus for the elucidation of the structure and function of GPIIb/IIIa, also known as the integrin αIIbβ3 (Bennett et al. 1982). The inhibition of ligand binding to GPIIb/IIIa prevents platelet aggregation (Fig. 1b) (Springer et al. 2008) and pharmacologic inhibition of fibrinogen binding to GPIIb/IIIa is particularly potent because it is the final mediator of platelet aggregation.

There are currently three GPIIb/IIIa inhibitors approved by the United States Food and Drug Administration (FDA): abciximab, eptifibatide, and tirofiban. Their role in the management of patients with acute coronary syndromes (ACS), with or without PCI, continues to evolve as newer antithrombotic and antiplatelet agents are introduced. Nevertheless, their efficacy and safety has been demonstrated in large, randomized, controlled clinical trials and they are utilized worldwide.

The unique pharmacodynamic profiles of each of the three available agents allow for choices in therapy that can be tailored to the clinical situation. Appropriate dosing is mandatory for sufficient platelet inhibition, effective bedside use, and conservation of desired clinical outcomes. This chapter will review the pharmacology of the available GPIIb/IIIa inhibitors.

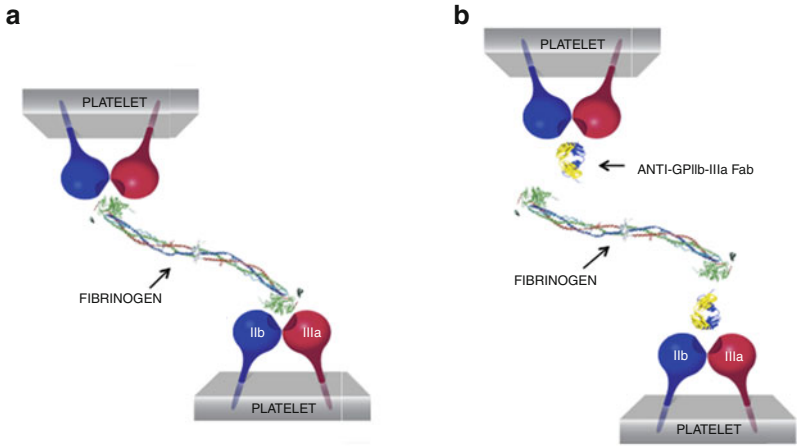


Fig. 1 Mechanism of GPIIb/IIIa-mediated platelet aggregation. (**a**) Platelet agonists initiate intracellular signals that convert the inactive GPIIb-IIIa on resting platelets to its active conformation, as illustrated in the figure. This exposes a binding site for macromolecular ligands such as fibrinogen on the extracellular domain of GPIIb/IIIa. In the presence of calcium ions, fibrinogen molecules bind to active GPIIb-IIIa molecules on adjacent platelets, crosslinking the platelets into aggregates. (**b**) By binding to the extracellular domain ligand binding site, GPIIb/IIIa antagonists, such as the monoclonal antibody Fab fragment depicted in the figure, prevent ligand binding to GPIIb/IIIa, thereby preventing platelet aggregation. Modified from Yin et al. (2007) Science 315:187 and from Springer et al. (2008) J Cell Biol 182:791

2 GPIIb/IIIa Structure

GPIIb/IIIa, a member of the integrin family of adhesion receptors, is a calcium-dependent heterodimer composed of two type I transmembrane glycoproteins, GPIIb (αIIb) and GPIIIa (β3) (Xiao et al. 2004). Both GPIIb and GPIIIa contain large extracellular domains, single transmembrane domains, and short cytosolic tails that associate with cytoskeletal and signaling proteins. The latter enables GPIIb/IIIa to transmit signals bidirectionally, either into the platelet cytosol following ligand binding ("outside-in" signaling) or from the cytosol to the GPIIb/IIIa extracellular domain ("inside-out" signaling) (Bennett et al. 1982).

Primary hemostasis, the formation of a hemostatic platelet plug in response to disrupted vascular endothelium, occurs when fibrinogen and/or von Willebrand factor bound to GPIIb/IIIa cross-links adjacent platelets into aggregates. Platelet aggregation is an active, metabolic process for which specific physiologic conditions must be met. Only agonist-stimulated platelets express the high-affinity GPIIb/IIIa conformation that is able to bind soluble macromolecular ligands (Bennett and Vilaire 1979). In addition, ligand binding to GPIIb/IIIa, as well as platelet aggregation, requires the presence of divalent cations (most often a calcium concentration greater than 100 nMol/L) and a temperature in the physiologic range (Shattil et al. 1985).

Congenital GPIIb/IIIa deficiency or dysfunction results in the autosomal recessive bleeding disorder Glanzmann thrombasthenia (GT). Patients with GT present with mucocutaneous bleeding, often severe, during infancy. The inability of GT platelets to aggregate in response to physiologic agonists such as adenosine diphosphate (ADP), epinephrine, collagen, or thrombin is diagnostic and is due to the inability of GT platelets to bind sufficient amounts of fibrinogen to aggregate.

GPIIb and GPIIIa are encoded by separate genes located on the long arm of human chromosome 17 (Bray et al. 1986). Nevertheless, GPIIb and GPIIIa biosynthesis is not coordinated. Thus, assembly of GPIIb/IIIa heterodimers occurs in the endoplasmic reticulum from nascent GPIIb and GPIIIa monomers. Correctly folded GPIIb/IIIa is then transported to the Golgi complex where posttranslational processing is completed and only correctly processed GPIIb/IIIa is expressed on the platelet surface (Duperray et al. 1989).

GPIIb contains 1,008 amino acids and is composed of a heavy chain with a molecular mass of 116 kDa that is disulfide linked to a light chain with a molecular mass of 25 kDa (Shattil et al. 1985). GPIIb is translated as pro-GPIIb from a single mRNA that encodes both the heavy and light chains and later undergoes proteolytic cleavage by a furin-like enzyme in the Golgi complex. GPIIIa is a single chain protein containing 762 amino acids, 56 cysteine residues, and 28 disulfide bonds. The disulfide bonds are concentrated in three regions of the extracellular portion of the molecule: a cysteine-rich, protease-resistant N-terminus, a protease-sensitive central region, and a disulfide-rich, protease-resistant core (Nurden 2007). The extracellular domain of GPIIb folds into a β propeller configuration, whereas the fold of the GPIIIa extracellular domain is complex, consisting of a series of nested domains, one of which (the I-like domain) resembles the A domains of von Willebrand factor and the I-domains appended to a number of integrin α subunits. The GPIIb β propeller domain and the GPIIIa I-like domain associate to form a nodular head containing the GPIIb/IIIa ligand binding site. The GPIIb β propeller and the GPIIIa I-like domains also contain divalent cation binding sites; the GPIIb sites are likely involved in GPIIb folding and the GPIIIa sites make important contributions to ligand binding. The ligand-binding site on the resting (inactive) conformation of GPIIb/IIIa is not exposed and only becomes available for ligand binding following platelet stimulation.

There are approximately 80,000 copies of GPIIb/IIIa per platelet, making it the most densely expressed platelet protein (Wagner et al. 1996). Based on the estimated platelet surface area, GPIIb/IIIa molecules are spaced less than 200 Å apart (Coller 1997). Additional GPIIb/IIIa is present in the membranes of platelet α-granules and can be translocated to the platelet surface after platelet activation.

3 Ligand Binding to GPIIb/IIIa

Crystal structures for the extracellular domains of GPIIb/IIIa and the related integrin αvβ3 suggest that these domains are bent over on themselves when the integrins are inactive and are extended when the integrins are active. Thus, it has

been proposed, but not universally accepted, that the conformational changes accompanying GPIIb/IIIa extension are responsible for ligand-binding site exposure (O'Toole et al. 1990).

In the crystal structure of the GPIIb/IIIa extracellular domain, ligands bind to a "specificity determining" loop in the β3 I-like domain and to a "cap" composed of four loops on the upper surface of the GPIIb β propeller domain. Ligand binding to GPIIb/IIIa requires divalent cations and platelet aggregation does not occur in the presence of the divalent cation chelator EDTA. Of the eight divalent cation-binding sites identified in the crystal structure of the αvβ3 extracellular domain, only the three located in the β3 (GPIIIa) I-like domain participate in ligand binding and the cation at one site, the β3 MIDAS (metal ion-dependent adhesion site), is in direct contact with ligand.

Fibrinogen is the major GPIIb/IIIa ligand and is composed of pairs of Aα, Bβ, and γ chains folded into three nodular domains. Peptides corresponding to either the carboxyl terminal 10–15 amino acids of the γ chain located at each end of the molecule or to two RGD motifs located in the α chain inhibit fibrinogen binding to GPIIb/IIIa, but only the γ chain sequence is required for the binding of intact fibrinogen to GPIIb/IIIa. Thus, the ability of RGD-based peptides and peptidomimetics inhibit GPIIb/IIIa function seems paradoxical, but recent crystal structures of RGD and γ-chain peptides bound to GPIIb–IIIa reveal that the RGD and γ chain binding sites on GPIIb/IIIa overlap.

4 Development of Glycoprotein IIb/IIIa Inhibitors

The commonly used antiplatelet agents aspirin and clopidogrel prevent platelet aggregation indirectly by inhibiting signal transduction pathways leading to GPIIb/IIIa activation. However, because ligand binding to GPIIb/IIIa represents the limiting interaction for platelet aggregation, three drugs specifically targeting this interaction were developed and are currently in clinical use. They include abciximab (ReoPro®, Eli Lily & Company, Indianapolis, IN, USA) (ReoPro (Abciximab) Prescribing Information 2005), eptifibatide (Integrilin®, Schering-Plough, Kenilworth, NJ, USA) (Integrilin (Eptifibatide) Prescribing Information 2009), and tirofiban (Aggrastat®, Medicure, Somerset, NJ, USA) (Aggrastat (Tirofiban) Prescribing Information 2008). The drugs were approved for use with aspirin and heparin for the treatment of ischemic heart disease based on the results of large clinical trials. Their entrance into the therapeutic arsenal against heart disease was timely, as they provided a way to improve reperfusion time following ischemia and minimize coronary restenosis due to iatrogenic vascular injury following PCI. Their clinical efficacy is considered equivalent, but differences exist with regard to cost, pharmacokinetic profiles, dosing schedules, and risk of adverse events (Koutouzis et al. 2010). Table 1 lists the comparative features of the drugs.

The efficacy of GPIIb/IIIa inhibitors correlates directly with the extent of platelet inhibition (Hobbach and Schuster 2003). Eighty percent occupancy of the

Table 1 Currently available GPIIb/IIIa antagonists

	Abciximab	Eptifibatide	Tirofiban
Structure	Humanized murine monoclonal antibody, Fab fragment	Cyclic heptapeptide containing an KGD sequence	Nonpeptide small molecule, rationally designed after the RGD sequence
FDA approval year	1994	1998	1998
2009 ACC/AHA Recommendation (Kushner et al. 2009)	Class IIa (Level of Evidence A) for use at the time of primary PCI in STEMI Class IIb (Level of evidence B) for use prior to primary PCI in STEMI	Class IIa (Level of Evidence B) for use at the time of primary PCI in STEMI Class IIb (Level of evidence B) for use prior to primary PCI in STEMI	Class IIa (Level of Evidence B) for use at the time of primary PCI in STEMI Class IIb (Level of evidence B) for use prior to primary PCI in STEMI
2011 European Society of Cardiology Guidelines for use in NSTEMI (Hamm et al. 2011)	Class I (Level of Evidence B) for use with dual antiplatelet therapy for high-risk PCI if the bleeding risk is low	Class I (Level of Evidence B) for use with dual antiplatelet therapy for high-risk PCI if the bleeding risk is low Class IIa (Level of Evidence C) for use with aspirin (but not P2Y12 antagonists) for high-risk patients undergoing angiography Class IIb (Level of Evidence C) for use with dual antiplatelet therapy for high-risk angiography if ongoing ischemia and low bleeding risk	Class I (Level of Evidence B) for use with dual antiplatelet therapy for high-risk PCI if the bleeding risk is low Class IIa (Level of Evidence C) for use with aspirin (but not P2Y12 antagonists) for high-risk patients undergoing angiography Class IIb (Level of Evidence C) for use with dual antiplatelet therapy for high-risk angiography if ongoing ischemia and low bleeding risk
Suggested bolus dose	0.25 mg/kg (The EPIC Investigators 1994)	180 μg/kg (The PURSUIT Trial Investigators 1998) For PCI, a second 180 mcg/kg bolus at 10 min (The ESPRIT Investigators 2000)	0.4 μg/kg/min over 30 min (The Platelet Receptor Inhibition in Ischemic Syndrome Management in Patients Limited By Unstable Signs and Symptoms (PRISM-PLUS) Study Investigators 1998)

Suggested infusion rate	0.125 μg/kg/min for 12–24 h (Koutouzis et al. 2010)	2.0 μg/kg/min for 72–96 h (Kushner et al. 2009)	0.1 μg/kg/min for 48–96 h (The EPIC Investigators 1994)
Dosing in the presence of renal insufficiency (Aggrastat (Tirofiban) Prescribing Information 2008)	No adjustment needed	Reduce dose by 50 % if CrCl is ≤50 mL/min Contraindicated in hemodialysis	Reduce dose by 50 % if CrCl is ≤30 mL/min
Time to platelet function recovery (h)	72	3–4	3–4
Antidote	Donor platelet transfusion (Koutouzis et al. 2010)	None	None
Dissociation constant (nM)	5	120	15

GPIIb/IIIa present on the platelet surface is required to reduce the in vitro platelet aggregation response to ADP to 20% of normal. This level of GPIIb/IIIa inhibition was required in a canine model of acute coronary injury with high-grade stenosis (Gold et al. 1988) to prevent coronary thrombosis while also limiting vessel reocclusion. It is generally accepted that receptor blockade of 50% or less is ineffective for clinical use.

Unlike the monitoring strategies used with warfarin, there is no means for calculating therapeutic drug levels of GPIIb/IIIa inhibitors or for titrating to a desired dose range. It is possible that inter-patient variability causes dose-level disparities for the same administered amount. For example, in the GOLD study (Steinhubl et al. 2001), a point-of-care device (Ultegra Rapid Platelet Function Assay, Accumetrics, Inc) was used to measure the extent of GPIIb/IIIa inhibition at 10 min, 1 h, 8 h, and 24 h after the administration of one of the three available GPIIb/IIIa antagonists to 485 patients undergoing PCI (Steinhubl 2000). When measured at 10 min, patients who achieved 95% platelet inhibition had a major adverse cardiac event (MACE) rate of 6.4%, whereas patients who did not achieve this level of inhibition had a MACE rate of 14.4%. Patients in whom GPIIb/IIIa inhibition was $<70\%$ at 8 h had a significantly greater incidence of MACE than patients with inhibition $\geq 70\%$ (25% vs. 8.1%). There was no correlation between bleeding events and measurements of platelet inhibition for any of the time intervals. However, despite the initial promise of results such as these, routine monitoring of platelet inhibition during the administration of GPIIb/IIIa antagonists is not currently recommended standard of care. In vitro measurements of platelet inhibition are fraught with inconsistencies, depending on the anticoagulant used in the assay. For example, when citrate is used, calcium chelation reduces the amount of free calcium available for GPIIb/IIIa-ligand engagement (Marciniak et al. 2001). As a result, platelet aggregation is diminished and the antiplatelet efficacy of GPIIb/IIIa antagonists overestimated, resulting in the administration of doses too low to be effective (The IMPACT II Investigators 1997).

5 Abciximab

Abciximab, the first GPIIb/IIIa antagonist to be developed, was approved by the FDA in December 1994 for the prevention of ischemic complications of angioplasty. Later, it was approved for PCI with stents and as medical therapy for unstable angina. Abciximab is a 48 kDa chimeric human/murine monoclonal antibody Fab fragment. The Fc portion of the antibody was removed from the final structure to minimize the immunogenicity of the murine portion of the molecule. Allergic or hypersensitivity reactions during or after the infusion are exceedingly uncommon. However, drug-related thrombocytopenia occurs in approximately 2% of patients given the drug for the first time and in a higher percentage of patients given the drug again (Curtis et al. 2002). Acute thrombocytopenia following initial exposure to abciximab appears to be due to naturally

occurring antibodies against the murine portion of molecule. When the occurrence of thrombocytopenia is delayed for 5–10 days, it is thought to be due to new antibodies that bind to abciximab-coated platelets that can persist in the circulation for up to 2 weeks after a single dose (Aster 2005).

Abciximab binds to platelets with high affinity, but it is not bound irreversibly. Most administered abciximab is bound to GPIIb/IIIa with a clinically insignificant amount free in the circulation. Unbound abciximab is degraded by proteolysis within 30 min. Ten minutes after a bolus dose, 50% of abciximab is bound to platelets. Abciximab can redistribute among platelets and among endothelial cells and leukocytes where it is bound to the vitronectin receptor $\alpha v\beta 3$. When measured at 8 days, 29% of platelet GPIIb/IIIa is occupied by abciximab and at 15 days, abciximab is still detectable on 13% of platelets (Casserly and Topol 2002).

The abciximab-binding site on GPIIb/IIIa appears to be located in the specificity determining loop of the GPIIIa I-like domain, close to its MIDAS domain, and adjacent to the RGD-binding site. Abciximab likely inhibits ligand binding to GPIIb/IIIa by steric hindrance. It is unique among the three available GPIIb/IIIa inhibitors in that it also binds to $\alpha v\beta 3$, as well as the integrin $\alpha_M\beta_2$ expressed on leukocytes. It has been postulated that abciximab binding to $\alpha_M\beta_2$ diminishes leukocyte infiltration and inflammation in areas of myocardial ischemia.

The first substantial clinical trial of abciximab in humans was the EPIC trial reported in 1994 (Koutouzis et al. 2010) in which abciximab as added to heparin and aspirin in high-risk patients undergoing PCI. The goal was to improve the rates of postangioplasty ischemic complications, estimated to occur in 10–20% of patients. Patients were randomized to receive an abciximab bolus alone (0.25 mg/kg), the same bolus plus an abciximab infusion (10 μg/min) (Tcheng et al. 1994), or placebo with a primary end point of a combination of death, myocardial infarction (MI), or revascularization at 30 days. The primary end point was significantly decreased in the group receiving the bolus plus infusion (8.3%) compared to placebo (12.8%), whereas patients in the bolus only arm had a rate of 30-day ischemic events of 11.5%. This study defined bolus plus infusion as the required administration schedule for abciximab.

In EPIC, heparin was administered at a fixed dose and bleeding occurred in 14% of patients in the bolus plus infusion arm, in 11% of patients receiving the bolus only, and in 7% of patients receiving placebo. Moreover, patients with the lowest body weight had the highest risk of bleeding. Thus, in the subsequent EPILOG (The EPILOG Investigators 1997) study, heparin administration was weight based and there was no statistically significant difference in major bleeding rates, with an absolute difference in the two study arms of only 2%.

Currently, PCI is accompanied by coronary artery stenting. Stent thrombosis occurs in approximately 1–3% of patients, typically occurring 7–10 days after stent placement, possibly due to iatrogenic vessel wall injury, mural thrombus formation, side-branch closure, and distal embolization. The EPISTENT study addressed whether impairing GPIIb/IIIa function with abciximab improved the safety and benefit of PCI with stenting. Patients were randomized to stenting plus placebo, stenting plus an abciximab bolus and 12 h infusion, or balloon angioplasty plus

abciximab. All patients were also given aspirin and the stented patients ticlopidine. The lowest 30-day rates of ischemic events (death, MI, or need for urgent revascularization) occurred in 5.3% of patients who received abciximab and a stent, compared to 6.9% of patients receiving abciximab with balloon angioplasty (6.9%) and 10.8% of patients receiving placebo and a stent. Differences in rates of bleeding among the three treatment arms were not statistically significant.

In the ADMIRAL trial (Montalescot et al. 2001), abciximab was again superior to placebo in patients receiving stents with no difference in major bleeding. The GUSTO-IV trial (2001) then addressed whether adding abciximab to heparin and aspirin was beneficial in patients not scheduled for early coronary revascularization. In contrast to the CAPTURE trial (1997) where an abciximab infusion, given to patients with refractory unstable angina for 18–24 h before PCI and continued for 1 h after the procedure, significantly reduced the primary end point of death, MI, or need for urgent revascularization at 30 days, no benefit was observed in GUSTO-IV for either 24 or 48 h infusions compared to placebo. The difference between GUSTO-IV and CAPTURE was that patients in the latter trial underwent PCI. This suggests that abciximab is most effective in situations where procedure-related vascular damage is likely to provide a strong stimulus for platelet-mediated thrombosis.

6 Small Molecule GP IIb/IIIa Antagonists

The synthesis of rationally designed small molecule GPIIb/IIIa antagonists was based on the observations that peptides containing the sequence Arg-Gly-Asp (RGD) (Bennett et al. 1988), as well as RGD-containing disintegrins (Gould et al. 1990) in snake venoms, inhibit ligand binding to GPIIb/IIIa on agonist-stimulated platelets and inhibit platelet aggregation as well.

7 Eptifibatide

Eptifibatide is a synthetic cyclic heptapeptide based on the Lys-Gly-Asp (KGD) motif of the snake venom disintegrin barbourin. It binds exclusively to GPIIb/IIIa with a dissociation constant of 120 nM. When infused into humans, eptifibatide rapidly and reversibly inhibits platelet aggregation with only modest prolongation of the bleeding time. Clearance is primarily renal (98%) and eptifibatide must be dose reduced in patients with renal failure and not given to patients receiving dialysis. It was approved by the FDA in 1998.

Eptifibatide binding to GPIIb/IIIa is enhanced at low calcium concentrations, such as those present in citrate-anticoagulated blood. Thus, preliminary calculations (Harrington et al. 1995) of the eptifibatide dose needed to inhibit GPIIb/IIIa function by 80%, based on in vitro measurements made in the presence of

citrate, overestimated its efficacy. When an early phase II study (Tcheng et al. 1995) suggested there was increased bleeding in patients receiving eptifibatide, the dose was reduced further. Accordingly, IMPACTII (The PURSUIT Trial Investigators 1998), the first major clinical trial of eptifibatide, did not improve the composite end point for post-PCI ischemic events. However, post hoc analyses suggested that patients were under-dosed, as the regimen of a 135 μg/kg bolus followed by either a 0.5 or 0.75 μg/kg/min infusion produced only 50% GP IIb/IIIa blockade (Phillips et al. 1997).

In the dose exploration phase of the PRIDE clinical trial, the protease inhibitor PPACK (D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone) was used instead of citrate as the in vitro anticoagulant (Tcheng et al. 2001). It was then found that of three different dosing regimens, a double bolus of 180 μg/kg was required to achieve >80% of inhibition of platelet aggregation. One hour after a single bolus, only 50% of patients had sufficient platelet inhibition, whereas at a higher bolus dose of 250 μg/kg, bleeding times were unacceptably prolonged. The desired infusion rate is 2.0 μg/kg/min.

In the next major clinical trial, PURSUIT (Kushner et al. 2009), patients with unstable angina or non-Q-wave MI were randomized to receive placebo or an 180 μg/kg eptifibatide bolus followed by a continuous eptifibatide infusion of either 1.3 or 2.0 μg/kg/min for 72 h or until hospital discharge, whichever came first. If PCI was performed at 72 h, the infusion was extended for an additional 24 h. The difference in the composite end point (death or nonfatal MI at 30 days) between patients receiving placebo versus eptifibatide was statistically significant (15.7% vs. 14.2%) and was reached after 4 days of therapy. Bleeding events were higher for eptifibatide and these patients required slightly more transfused blood. Thus, the PURSUIT trial demonstrated that eptifibatide, at doses that effectively impaired GPIIb/IIIa function, was of moderate, but significant, benefit to patients with acute coronary syndromes.

In the ESPRIT trial (Kushner et al. 2009), an eptifibatide dosing regimen consisting of two 180 μg/kg boluses 10 min apart followed by a 2.0 μg/kg infusion was studied in patients undergoing nonurgent PCI with stent placement. After 12 months of follow up, there was a statistically significant difference in the composite end point of death or MI in favor of the eptifibatide arm (8.0%) compared to the placebo arm (12.4%). These results suggested that long-term outcomes for nonurgent stent placement could be improved by GPIIb/IIIa blockade.

8 Tirofiban

Tirofiban is an RGD-based peptidomimetic analog of tyrosine that specifically binds to GPIIb/IIIa on resting platelets with a dissociation constant of 15 nM and inhibits the platelet aggregation in platelet-rich plasma with IC_{50}s ranging from 31 to 66 nM depending on the platelet agonist. Tirofiban was produced by the

sequential modification of a lead compound identified during a directed search for compounds with amino and carboxylate functionalities separated by distances of 10–20 Å and that inhibited ADP-stimulated platelet aggregation. The advantages of tirofiban include a short onset of action and a rapid return of platelet function when an infusion is stopped. In healthy subjects, its half-life in plasma ranges from 1 to 2 h and renal excretion accounts for 40% of its plasma clearance.

In the RESTORE trial (1997), a 10 μg/kg bolus of tirofiban followed by a 0.15 μg/kg/min infusion (Kereiakes et al. 1996) appeared to reduce ischemic risk at 2 days and 7 days in patients undergoing PCI. However, at 30 days, there was no change in the incidence of ischemic outcomes compared to patients receiving aspirin and heparin alone.

The effect of tirofiban on patients with unstable angina or non-ST-segment elevation myocardial infarction (NSTEMI) was then studied in the PRISM (The Platelet Receptor Inhibition in Ischemic Syndrome Management) (PRISM Investigators 1998) and PRISM-PLUS trials (Wagner et al. 1996). In PRISM, aspirin-treated patients were randomized to receive either heparin or tirofiban (0.6 μg/kg/min for 30 min followed by 0.15 μg/kg/min for 47.5 h). At 48 h, there was a significant 32% decrease in the composite end point of death, refractory ischemia, MI, but at 7 and 30 days, the decrease was no longer significant. In PRISM-PLUS, patients with MI were given aspirin and randomly assigned to receive either tirofiban at the same dose given in PRISM, heparin, or two-thirds the tirofiban dose given in PRISM plus heparin for a minimum of 48 h. At day 7 day, patients receiving tirofiban had significantly decreased mortality (1.1% for patients receiving tirofiban vs. 4.6% for patients receiving heparin) and the beneficial effect persisted at 6 months. There was no difference in major bleeding. This study suggested that GPIIb/IIIa antagonists, in addition to be useful for patients undergoing PCI, could also be beneficial for patients being treated medically for unstable angina and NSTEMI.

The TARGET study (Topol et al. 2001) then directly compared tirofiban and abciximab, although the lower tirofiban dosing regimen used in RESTORE was used in TARGET. Under these conditions, tirofiban failed to meet its end point of noninferiority to abciximab, but it is likely that the dose of tirofiban was insufficient (Schneider et al. 2002).

In the ON-TIME 2 study (Van't Hof et al. 2008), patients with ST-segment elevation myocardial infarction (STEMI) who were candidates for PCI were given aspirin, heparin, clopidogrel, and either placebo or tirofiban as a 5 μg/kg bolus in the ambulance or treating center at a median time of 55 min before angiography. Patients receiving tirofiban had less ST-segment elevation immediately and at 1 h and a lower rate of death, MI, or revascularization at 30 days. This study found that prehospital administration of higher dose tirofiban hastened ST-segment recovery and improved clinical outcome after PCI, suggesting that additional antiplatelet therapy besides clopidogrel was needed for patients with STEMI undergoing PCI.

9 Oral GP IIb/IIIa Inhibitors

With the success of parenteral GPIIb/IIIa antagonists for acute use, oral formulations were developed to create a maintenance phase of platelet inhibition. Anticipated advantages would include ease of administration and lack of laboratory monitoring. By 2001, three agents (xemilofiban, orbofiban, and sibrafiban) had progressed to phase III clinical trials in humans. Each was an RGD-containing pro-drug that was converted metabolically to its active form. Surprisingly these agents revealed increased bleeding, thrombocytopenia, and mortality.

A meta-analysis of four randomized, placebo-controlled trials—EXCITE, (xemilofiban); OPUS TIMI (orbofiban); and SYMPHONY and 2nd SYMPHONY (sibrafiban)—totaling 33,326 patients (Chew et al. 2001), found a 31% increase in mortality. Further, the odds ratio for major bleeding was significantly increased to 1.74 (95% CI 1.52–2.00). On the basis of these disappointing results, further development of oral antagonists was discontinued.

A leading hypothesis to explain the clinical failure of the oral agents is that when they bind to GPIIb/IIIa, they cause it to assume its active conformation, thereby facilitating, rather than hindering, thrombus formation (Cox 2004). Consistent with this hypothesis, when thrombocytopenia occurred, it was attributed to immune destruction due to the exposure of neoepitopes by antagonist binding to GPIIb/IIIa. On the other hand, the increased mortality associated with the use of the oral antagonists was not due to an increased number of ischemic events. Thus, it is also possible that off-target effects or inter-patient variation led to inconsistent peak plasma levels with a net result of chronic under-dosing.

10 Observations from Clinical Trials

Early clinical trials demonstrated the benefit of GPIIb/IIIa antagonists in reducing post-PCI thrombotic complications. Later studies found that GPIIb/IIIa antagonists were also beneficial in high-risk UA (Hamm et al. 1999) and NSTEMI patients who did not undergo PCI (Boersma et al. 2002), arguing for clinical benefit beyond preventing procedural complications.

However, it is not intuitively obvious why clinical outcomes measured months later were improved for patients who receive the GPIIb/IIIa antagonist infusion for a matter of hours. One possibility is that the timing of GPIIb/IIIa antagonists plays a role in outcomes. For example, when GPIIb/IIIa antagonists are given “upstream” or prior to a planned PCI, the objective is prevention of an acute thrombotic process, whereas “downstream” administration allows for selective use in high-risk patients during or after PCI. However, a meta-analysis (De Luca et al. 2001) of studies of GPIIb/IIIa antagonists found no change in 30-day mortality (2.0% vs. 2.0%) or MI (7.0% vs. 7.6%,) for upstream versus downstream administration. On the other hand, there was a significantly greater incidence of major bleeding for patients who received upstream GPIIb/IIIa antagonists (1.8% vs. 1.3%).

11 Risk of Bleeding with GP IIb/IIIa Antagonists

An updated Cochrane Database analysis from 2010 of 62,417 patients indicated that GPIIb/IIIa antagonists reduce the risk of death or MI in patients with acute coronary syndromes at 30 days (OR 0.65, 95% CI 0.60–0.72) and 6 months (OR 0.70, 95% CI 0.61–0.81). The trade-off is an increased risk of severe bleeding (OR 1.38, 95% CI 1.20–1.59). Bleeding is the most common noncardiac adverse event related to PCI, more so than MI (Feit et al. 2007). The prevalence of bleeding end points compared to placebo reported in representative trials of abciximab, eptifibatide, and tirofiban is given in Table 2.

Abbreviated infusion times appear to decrease the risk of bleeding (Kirtane et al. 2006). For example, in a single-center, nonrandomized, observational study (Kini et al. 2008), bolus-only abciximab or eptifibatide resulted in no change in major cardiac adverse events, a lower cost, a shorter hospital stay, and less bleeding events. The rates of overall bleeding for patients given bolus-only vs. standard administration were 4.9% vs. 7% ($P < 0.05$). Further, for patients deemed low risk (i.e., patients without acute MI, obvious filling defect on angiography, or high thrombus burden), a brief infusion of eptifibatide (less than 2 h) was noninferior to an 18-h infusion in preventing ischemic outcomes measured at 30 days and major bleeding was less frequent in the group receiving the shorter infusion (1.0% vs. 4.2%) (Fung et al. 2009).

In studies where women were found to have a higher bleeding risk, they were found to have been given an inappropriately high dose of GPIIb/IIIa antagonist 25% of the time, compared to men who were given excessive doses 4.4% of the time (Alexander et al. 2006). Overall, patients given inadvertent excessive doses were more likely to be female, over the age of 75, have renal insufficiency, low body weight, diabetes, and heart failure. As expected, patients given an excess dose have a higher risk of major bleeding. Major sites of bleeding associated with the administration of GPIIb/IIIa antagonists are listed in Table 3.

12 Procedural Factors Affecting Bleeding Risk

Several variables, listed in Table 4, can affect bleeding risk in patients given GPIIb/IIIa antagonists. Baseline characteristics associated with an increased bleeding risk during PCI are age >55, female gender, renal insufficiency, anemia, administration of low molecular weight heparin within 48 h of procedure, and severe circulatory impairment at presentation (Nikolsky et al. 2007; Mehta et al. 2009).

Vascular closure devices (Applegate et al. 2002) improve local hemostasis and minimize major bleeding (Sanborn et al. 2010) from femoral access sites. Using the radial artery approach decreases bleeding by 58% compared to the femoral approach and appears to be more cost-effective, even when a femoral closure device is used (Mehta et al. 2009; Moscucci et al. 2003). Women develop more

Table 2 A comparison of bleeding end points[a] in selected major clinical trials of the GPIIb/IIIa antagonists

Trial	GPIIb/IIIa antagonist	Bleeding in treated patients (%)	Bleeding in placebo controls (%)	*P*-value
EPILOG (Casserly and Topol 2002)	Abciximab	3.5	3.1	0.7
PURSUIT (Hamm et al. 2011)	Eptifibatide	10.6	9.1	0.02
PRISM-PLUS (The PURSUIT Trial Investigators 1998)	Tirofiban	4.0	3.0	0.34

[a]Bleeding end points: intracranial/intraocular bleeding; access site bleeding requiring intervention; ≥5 cm diameter hematoma; hemoglobin drop (≥4 g/dL with no source or ≥3 g/dL with source); reoperation for bleeding; or blood product transfusion

Table 3 Bleeding associated with the administration of GPIIb/IIIa antagonists (Cox 2004; De Luca et al. 2001; Husted 2008; Winchester et al. 2011a; Labinaz et al. 2007; Latour-Perez 2001)

	Incidence (%)
Groin hematoma	60–80
Gastrointestinal bleeding	15
Retroperitoneal hemorrhage	5–10
Intracranial hemorrhage	0.09
Major bleeding (defined in Table 2)	1.5–4

Table 4 Risk factors for GPIIb/IIIa antagonist-associated bleeding during PCI (Kini et al. 2008; Fung et al. 2009; Alexander et al. 2006; Winchester et al. 2011a; Labinaz et al. 2007; Moscucci et al. 2003; Tcheng 2000; Choussat et al. 2000; De Carlo et al. 2009)

Baseline patient characteristics
Age >65
Female gender
Diabetes
Hypertension
Renal insufficiency with CrCl < 60 ml/min
Anemia: <13 g/dL in men; <12 g/dL in women
Findings at the time of ACS diagnosis
No prior PCI
ST segment deviation >1 mm
Cardiac biomarker elevation
Cardiac shock
Need for intra-aortic balloon pump
Choice of antiplatelet or antithrombotic agent
GPI + heparin
GPI + bivalirudin
Procedural factors
Extended duration of procedure
Late sheath removal
Femoral vs. radial access site
No arterial seal device

local hematomas during radial access compared to men receiving the same therapy (OR 4.40, 95% CI 2.49–7.81, $p < 0.001$) (Tizon-Marcos et al. 2009), but this finding was unrelated to major bleeding, need for surgical intervention, or change in the primary outcome of ischemic complications.

Age, as a continuous variable, correlated with higher bleeding risk with GPIIb/IIIa antagonists (Lopes et al. 2009). On the other hand, in a nonrandomized retrospective analysis (Germing et al. 2010), for patients over age 80 undergoing PCI for ACS, there was no difference in incidence of femoral hematoma, femoral pseudoaneurysm, or need for blood transfusion depending on whether or not the patient received a GPIIb/IIIa antagonist during their procedure. Among the 439 patients in that study, there were no major hemorrhagic events.

A creatinine clearance of 70 mL/min or less did not appear to increase the risk for major (OR 1.18, 95% CI 0.99–1.39) or minor (OR 1.01, 95% CI 0.83–1.23) bleeding in patients given abciximab (Best et al. 2003). Both eptifibatide and tirofiban require dose reduction in renal failure (see Table 1) and eptifibatide is contraindicated in dialysis patients (Tsai et al. 2009).

13 Use of GP IIb/IIIa Antagonists with Newer Agents

PCI was introduced in the United States in 1994 and the CADILLAC trial (Stone et al. 2002) established that stents were superior to balloon angioplasty alone for patients with acute MI. The use of abciximab, combined with stent deployment in this study, resulted in the lowest rates of postprocedure revascularization (15.7% vs. 5.2%).

However, the initial studies of GPIIb/IIIa antagonists that led to their approval by the FDA and their routine use during PCI were performed before the era of double antiplatelet drug therapy and bivalirudin. Clopidogrel was approved by the FDA in 1997 and was studied as an upstream intervention, prior to PCI with or without abciximab in the ISAR-REACT study (Kastrati et al. 2004). Clopidogrel improved 30-day ischemia outcomes and the addition of abciximab did not change this result. Thus, clopidogrel loading alone prior to PCI has become the standard of care.

The pharmacokinetics of clopidogrel require a 2–6 h window between administration and peak effect, depending on dose. In the ISAR-REACT2 study (Kastrati et al. 2006), the addition of abciximab to 600 mg of clopidogrel benefitted the highest risk NSTEMI patients with elevated serum troponin levels and in the CLEAR PLATELETS study (Gurbel et al. 2005) addition of eptifibatide to clopidogrel for elective PCI resulted in the highest rates of in vitro platelet inhibition measured by flow cytometry and aggregometry.

For patients deemed "poor responders" to clopidogrel, the addition of tirofiban improves their 30-day major cardiac event rate (3.8% vs. 10.7%) (Valgimigli et al. 2009). Prasugrel, approved by the FDA in 2009 (Effient (prasugrel) Prescribing Information 2010), outperformed clopidogrel in patients with ACS, irrespective of the use of GPIIb/IIIa antagonists in TRITON-TIMI trial (O'Donoghue et al. 2009).

Although GPIIb/IIIa antagonists and prasugrel have a rapid onset of action, there was no increased risk of bleeding when the two agents were given together. Prasugrel is indicated in patients with poor response to clopidogrel, but clopidogrel remains the first-line therapy for all other patients, due to its superior bleeding and safety profile.

In 2011, a meta-analysis (Winchester et al. 2011b) of trials using all three GP IIb/IIIa antagonists in elective PCI with stents and clopidogrel found that the addition of GPIIb/IIIa antagonists reduced the incidence of MI (5.1% vs. 8.3%), but did not change overall mortality (0.3% vs. 0.5%). Furthermore, there was no increased risk of major bleeding when GPIIb/IIIa antagonists were added to the standard regimen (1.2% vs. 0.9%), but there was more minor bleeding (3.0% vs. 1.7%).

Bivalirudin, a direct thrombin inhibitor, was approved by the FDA in 2000 (Angiomax (bivalirudin) Prescribing Information 2005). Early evidence suggested that bivalirudin may provide superior outcomes in reducing post-PCI ischemic events when used alone, compared to either the thienopyridines or the GPIIb/IIIa antagonists (Saw et al. 2004). Compared to GPIIb/IIIa antagonists, bivalirudin administration to high-risk patients with STEMI undergoing PCI may increase the rate of acute stent thrombosis (Stone et al. 2008), but decreases overall adverse events at 30 days and decreases the incidence of bleeding.

14 Guidelines for the Use of GP IIb/IIIa Antagonists

Based on an analysis of studies such as those discussed earlier, the Task Force for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation (NSTEMI) of the European Society of Cardiology (ESC) published guidelines (Koutouzis et al. 2010) in 2011 for the use of GPIIb/IIIa antagonists (Table 1). Because GPIIb/IIIa antagonists do not appear to reduce the incidence of death or MI in medically managed patients with NSTEMI, exhibit their major benefit when given during PCI, and are associated with an increase in major bleeding complications, the ESC recommended against routine upstream administration of these drugs. However, upstream administration of tirofiban or eptifibatide, that is prior to angiography, can be considered, in addition to aspirin and a $P2Y_{12}$ inhibitor, when there is ongoing ischemia and the bleeding risk is low or when patients have not been preloaded with a $P2Y_{12}$ inhibitor. Further, administration of GPIIb/IIIa antagonists should be considered during PCI in patients already receiving aspirin and a $P2Y_{12}$ inhibitor when troponin is elevated or there is visible thrombus.

A subsequent systematic overview of published clinical trials of the upstream use of small molecule GPIIb/IIIa inhibitors in patients of with NSTEMI reached similar conclusions (De Luca et al. 2001). Although upstream use was associated with a trend toward fewer ischemic events, this modest benefit was associated with an increased risk of bleeding. Thus, the periprocedural use of these drugs in

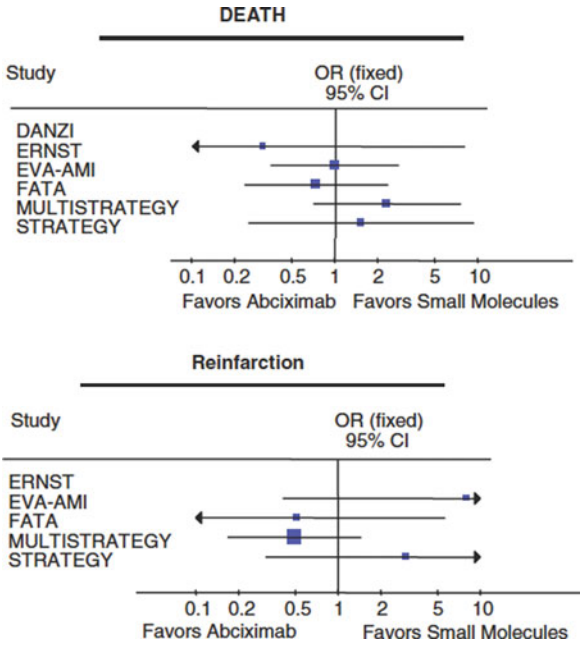


Fig. 2 Comparison of abciximab and small molecule GPIIb/IIIa antagonists with regard to death and reinfarction at 30 days. The size of the data markers is proportional to the statistical weight of each trial. Modified from De Luca et al. (2009) J Am Coll Cardiol 53:1668 with permission

high-risk patients undergoing PCI likely balances the benefits and risks of these drugs in this group of patients.

With regard to the use of GPIIb/IIIa antagonists for the treatment of ST-segment elevation myocardial infarction (STEMI), guidelines for the management of such patients were published by the ESC in 2008 (Van de Werf et al. 2008) and guidelines from the American College of Cardiology/American Heart Association for the treatment of STEMI with PCI were updated in 2009 (Aggrastat (Tirofiban) Prescribing Information 2008). Both sets of guidelines indicate that in the era of double antiplatelet therapy (i.e., aspirin and a $P2Y_{12}$ inhibitor), the additional benefit of adding a GPIIb/IIIa antagonist is uncertain, although they may play a role when the thrombus burden is large or when patients have not received a thienopyridine.

Recent meta-analyses of clinical trials comparing with the efficacy of abciximab with either of the two small molecule GPIIb/IIIa antagonists in patients with STEMI who underwent PCI revealed that when the drugs were given at optimal doses, there were no differences between them with regard to angiographic, electrocardiographic, or clinical outcome drugs or in bleeding complications (Fig. 2) (De Luca et al. 2009; Gurm et al. 2009). Thus, based on our current state of knowledge, the choice of antagonist should be based on availability, cost, and the unique pharmacology of each agent.

15 Future Indications for GP IIb/IIIa Antagonists

Besides their use in PCI for ACS or UA/NSTEMI, the uses of GPIIb/IIIa antagonists are also being studied for peripheral arterial disease, carotid artery disease, and for other sites of arterial thrombosis. However, initial studies of GPIIb/IIIa antagonists in stroke have not demonstrated improved outcomes.

There are reports (Wohrle et al. 2003; Thiele et al. 2008) suggesting that an intracoronary bolus is superior to intravenous bolus administration. Additionally, a novel balloon catheter system (Prati et al. 2010) is being studied to improve local drug delivery to the coronary thrombosis.

Newer GPIIb/IIIa antagonists are being developed to improve upon the side effects attributed to the currently available agents. Antagonist binding to GPIIb/IIIa without inducing conformational changes might minimize the creation of immunogenic neoepitopes and prevent the development of immune-mediated thrombocytopenia (Zhu et al. 2012). Further, different binding sites either GPIIb or GPIIIa might compete with ligand binding without causing partial agonist effects and subsequent platelet aggregation. Finally, point-of-care testing to monitor the degree of platelet inhibition is being pursued to improve safety and bleeding risks.

Knowledge Gaps

- The role of GP IIb/IIIa antagonists as a treatment option for acute coronary syndromes continues to evolve, with the advent of novel antiplatelet and anticoagulant therapies.
- Improvements in the bleeding risks of GP IIb/IIIa inhibitors will require further research into ultra-short acting preparations or the development of antidotes.
- Oral GP IIb/IIIa antagonists are currently not approved for clinical use due to poor safety profile.
- The utility of point-of-care testing to monitor the antiplatelet effect of GP IIb/IIIa antagonism in vivo must be refined before it can be recommended for clinical use.
- GP IIb/IIIa antagonists may be useful for treatment of arterial thrombosis in sites other than the coronary vessels.

Key Messages

- GP IIb/IIIa blockade presents a uniquely effective means of platelet inhibition due to its physiologic function as the final common mediator of platelet aggregation.

(continued)

- The three currently available GP IIb/IIIa antagonists are: abciximab, eptifibatide, and tirofiban.
- Efficacy among the three available agents, in terms of reducing morbidity and mortality of acute coronary syndromes, is considered equivalent.
- Differences in pharmacokinetics, metabolism, adverse events, and cost allow the selection of a particular GP IIb/IIIa antagonist to be tailored to the clinical situation.
- The major side effects of GP IIb/IIIa antagonists are bleeding and thrombocytopenia.

References

Aggrastat (Tirofiban) Prescribing Information (2008) Medicure, Somerset, NJ. Available at http://www.medicure.com/aggrastat/Aggrastat_PI_HiRes.pdf. Accessed 13 Feb 2011

Alexander KP, Chen AY, Newby LK, for the CRUSADE investigators et al (2006) Sex differences in major bleeding with glycoprotein IIb/IIIa inhibitors: results from the CRUSADE (Can Rapid risk stratification of Unstable angina patients Suppress ADverse outcomes with Early implementation of the ACC/AHA guidelines) initiative. Circulation 114:1380–1387

Angiomax (bivalirudin) Prescribing Information (2005) The Medicines Company, Parsippany, NJ. Accessed 13 Feb 2011

Applegate RJ, Grabarczyk MA, Little WC et al (2002) Vascular closure devices in patients treated with anticoagulation and IIb/IIIa receptor inhibitors during percutaneous revascularization. J Am Coll Cardiol 40:78–83

Aster RH (2005) Immune thrombocytopenia caused by glycoprotein IIb/IIIa inhibitors. Chest 127: S35–S95

Bennett JS, Vilaire G (1979) Exposure of platelet fibrinogen receptors by ADP and epinephrine. J Clin Invest 64:1393–1401

Bennett JS, Vilaire G, Cines DB (1982) Identification of the fibrinogen receptor on human platelets by photoaffinity labeling. J Biol Chem 257:8049–8054

Bennett JS, Shattil S, Power J, Gartner T (1988) Interaction of fibrinogen with its platelet receptor: differential effects of alpha and gamma chain fibrinogen peptides of the glycoprotein IIb-IIIa complex. J Biol Chem 263:12948–12953

Best PJ, Lennon R, Gersh BJ et al (2003) Safety of abciximab in patients with chronic renal insufficiency who are undergoing percutaneous coronary interventions. Am Heart J 146:345–350

Boersma E, Harrington RA, Moliterno DJ et al (2002) Platelet glycoprotein IIb/IIIa inhibitors in acute coronary syndromes: a meta-analysis of all major randomised clinical trials. Lancet 359:189–198 (correction in Lancet 2002;359:2120)

Bray PF, Rosa JP, Lingappa VR et al (1986) Biogenesis of the platelet receptor for fibrinogen: evidence for separate precursors for glycoproteins IIb and IIIa. Proc Natl Acad Sci USA 83:1480–1484

Casserly I, Topol E (2002) Glycoprotein IIb/IIIa –antagonists – from bench to practice. Cell Mol Life Sci 59:1–23

Chew DP, Bhatt DL, Sapp S, Topol EJ (2001) Increased mortality with oral platelet glycoprotein IIb/IIIa antagonists: a meta-analysis of phase III multicenter randomized trials. Circulation 103:201–206

Choussat R, Black A, Bossi I, Fajadet J, Marco J (2000) Vascular complications and clinical outcome after coronary angioplasty with platelet IIb/IIIa receptor blockade. Comparison of transradial vs transfemoral arterial access. Eur Heart J 21:662–667

Coller BS (1997) Platelet GPIIb/IIIa antagonists: the first anti-integrin receptor therapeutics. J Clin Invest 99:1467–1471

Cox D (2004) Oral GPIIb/IIIa antagonists: what went wrong? Curr Pharm Des 10:1587–1596

Curtis BR, Swyers J, Divgi A, McFarland JG, Aster RH (2002) Thrombocytopenia after second exposure to abciximab is caused by antibodies that recognize abciximab-coated platelets. Blood 99:2054–2059

De Carlo M, Borelli G, Gistri R et al (2009) Effectiveness of the transradial approach to reduce bleedings in patients undergoing urgent coronary angioplasty with GPIIb/IIIa inhibitors for acute coronary syndromes. Catheter Cardiovasc Interv 74:408–415

De Luca G, Navarese EP, Cassetti E, Verdoia M, Suryapranata H (2001) Meta-analysis of randomized trials of glycoprotein IIb/IIIa inhibitors in high-risk acute coronary syndromes patients undergoing invasive surgery. Am J Cardiol 107:198–203

De Luca G, Ucci G, Casetti E, Marino P (2009) Benefits from small molecule administration as compared with abciximab among patients with ST-segment elevation myocardial infarction treated with primary angioplasty. A meta-analysis. J Am Coll Cardiol 53:1668–1673

Duperray A, Troesch A, Berthier R et al (1989) Biosynthesis and assembly of platelet GP IIb/IIIa in human megakaryocytes: evidence that assembly between pro-GPIIb and GPIIIa is a prerequisite for expression of the complex on the cell surface. Blood 74:1603–1611

Effient (prasugrel) Prescribing Information (2010) Eli Lily & Company, Indianapolis, IN. Available at http://www.pi.lilly.com/us/effient.pdf. Accessed 13 Feb 11

Feit F, Voeltz MD, Attubato MJ et al (2007) Predictors and impact of major hemorrhage on mortality following percutaneous coronary intervention from the REPLACE-2 trial. Am J Cardiol 100:1364–1369

Fung AY, Saw J, Starovoytov A et al (2009) Abbreviated infusion of eptifibatide after successful coronary intervention: the BRIEF PCI (Brief Infusion of Eptifibatide Following Percutaneous Coronary Intervention) randomized trial. J Am Coll Cardiol 53:837–845

Germing A, Bojara W, Lawo T et al (2010) Bleeding complications associated with glycoprotein IIb/IIIa inhibitors in patients 80 years of age and older undergoing percutaneous coronary intervention. Exp Clin Cardiol 15:e57–e60

Gold HK, Coller BS, Yasuda T et al (1988) Rapid and sustained coronary artery recanalization with combined bolus injection of recombinant tissue-plasminogen activator and monoclonal antiplatelet GP IIb/IIIa antibody in a canine preparation. Circulation 77:670–677

Gould RJ, Polokoff MA, Friedman PA et al (1990) Disintegrins: a family of integrin inhibitory proteins from viper venoms. Proc Soc Exp Biol Med 195:168–171

Gurbel PA, Bliden KP, Zaman KA et al (2005) Clopidogrel loading with eptifibatide to arrest the reactivity of platelets: result of the Clopidogrel Loading with Epitifibatide to Arrest the Reactivity of Platelets (CLEAR PLATELETS) study. Circulation 111:1153–1159

Gurm HS, Tamhane U, Meier P, Grossman M, Chetcuti S, Bates ER (2009) A comparison of abciximab and small molecule GP IIb/IIIa inhibitors in patients undergoing primary percutaneous coronary intervention. A meta-analysis of contemporary randomized controlled trials. Circ Cardiovasc Interv 21:230–236

Hamm CW, Heeschen C, Goldmann B, for CAPTURE investigators et al (1999) Benefit of abciximab in patients with refractory unstable angina in relation to serum troponin T levels. New Engl J Med 340:1623–1629

Hamm CJ, Bassand J-P, Agewall S et al (2011) ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation. Eur Heart J. doi:10.1093/eurheartj/ehr236. http://eurheartj.oxfordjournals.org/content/early/2011/09/21/eurheartj.ehr236.extract. Accessed 2 Nov 11

Harrington RA, Keliman NS, Kottke-Marchant K et al (1995) Immediate and reversible platelet inhibition after intravenous administration of a peptide glycoprotein IIb/IIIa inhibitor during percutaneous coronary intervention. Am J Cardiol 76:1222–1227

Hobbach H-P, Schuster P (2003) Pharmacodynamic and clinical trials of glycoprotein IIb/IIIa inhibitors and potential relationship of results to dosing. Z Kardiol 92:213–218

Husted S (2008) Benefits and risks with antiplatelet therapy: how great a problem is bleeding? Eur Heart J 10(Suppl I):I19–I24

Integrilin (Eptifibatide) Prescribing Information (2009) Schering-Plough, Kenilworth, NJ. Available at http://www.spfiles.com/piintegrilin.pdf. Accessed 13 Feb 2011

Kastrati A, Mhelilli J, Schuhlen H, for the Intracoronary Stenting and Antithrombotic Regimen-Rapid Early Action for Coronary Treatment Study Investigators et al (2004) A clinical trial of abciximab in elective percutaneous coronary interventions after pretreatment with clopidogrel. New Engl J Med 350:232–238

Kastrati A, Mehilli J, Neumann FJ, for the ISAR-REACT-2 Trial Investigators et al (2006) Abciximab in patients with acute coronary syndromes undergoing percutaneous coronary intervention after clopidogrel pretreatment: the ISAR-REACT-2 randomized trial. JAMA 295:1531–1538

Kereiakes DJ, Kleiman NS, Ambrose J et al (1996) Randomized double-blind, placebo-controlled dose ranging study of tirofiban (MK-383) platelet IIb/IIIa blockade in high risk patients undergoing coronary angioplasty. J Am Coll Cardiol 27:536–542

Kini AS, Chen VHT, Krishnan P et al (2008) Bolus-only versus bolus + infusion of glycoprotein IIb/IIIa inhibitors during percutaneous coronary intervention. Am Heart J 156:513–519

Kirtane AJ, Piazza G, Murphy SA, for the TIMI Study Group et al (2006) Correlates of bleeding events among moderate-to high-risk patients undergoing percutaneous coronary intervention and treated with eptifibatide: observations from the PROTECT-TIMI-30 trial. J Am Coll Cardiol 47:2374–2379

Koutouzis M, Lagerqvist B, Oldgren J et al (2010) Long-term results following switch from abciximab to eptifibatide during percutaneous coronary intervention. Clin Cardiol 33:686–692

Kushner FG, Hand M, Smith SC Jr et al (2009) 2009 focused updates: ACC/AHA guidelines for the management of patients with ST-elevation myocardial infarction (updating the 2004 guideline and 2007 focused update) and ACC/AHA/SCAI guidelines on percutaneous coronary intervention (updating the 2005 guideline and 2007 focused update): a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. J Am Coll Cardiol 54:2205–2241

Labinaz M, Ho C, Banerjee S, Martin J, Chen S, Mensinkai S (2007) Meta analysis of clinical efficacy and bleeding risk with intravenous glycoprotein IIb/IIIa antagonists for percutaneous coronary intervention. Can J Cardiol 23:963–970

Latour-Perez J (2001) Risks and benefits of Glycoprotein IIb/IIIa antagonists in acute coronary syndrome. Ann Pharmacother 35:472–479

Lopes RD, Alexander KP, Manoukian SV et al (2009) Advanced age, antithrombotic strategy, and bleeding in non-ST-segment elevation acute coronary syndrome: results from the ACUITY (Acute Catheterization and Urgent Intervention Triage Strategy) trial. J Am Coll Cardiol 53:1021–1030

Marciniak SJ, Jordan R, Mascelli M (2001) Effect of CA+2 chelation on the platelet inhibitory activity of the GP IIb/IIIa antagonists abciximab, eptifibatide, and tirofiban. Thromb Haemost 85:539–543

Mehta SK, Frutkin AD, Lindsey JB, on behalf of the National Cardiovascular Data Registry (2009) Bleeding in patients undergoing percutaneous coronary intervention: the development of a clinical risk algorithm from the National Cardiovascular Data Registry. Circ Cardiovasc Interv 2:222–229

Montalescot G, Barragan P, Wittenberg O et al (2001) Platelet glycoprotein IIb/IIIa inhibition with coronary stenting for acute myocardial infarction. New Engl J Med 344:1895–1903

Moscucci M, Fox KAA, Cannon CP, for the GRACE Investigators et al (2003) Predictors of major bleeding in acute coronary syndromes: the Global Registry of Acute Coronary Events. Eur Heart J 24:1815–1823

Nikolsky E, Mehran R, Dangas G et al (2007) Development and validation of a prognostic risk score for major bleeding in patients undergoing percutaneous coronary intervention via the femoral approach. Eur Heart J 28:1936–1945

Nurden AT (2007) Platelet membrane glycoproteins: a look back into the past and a view to the future. Thromb Haemost 98:49–54

O'Donoghue M, Antman EM, Braunwald E, for the TIMI Study Group et al (2009) The efficacy and safety of prasugrel with and without a glycoprotein IIb/IIIa inhibitor in patients with acute coronary syndromes undergoing percutaneous intervention: a TRITON-TIMI 38 (Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition With Prasugrel-Thrombolysis in Myocardial Infarction 38) analysis. J Am Coll Cardiol 54:678–685

O'Toole TE, Loftus JC, Du XP et al (1990) Affinity modulation of the αIIbβ3 integrin (platelet GP IIb-IIIa) is an intrinsic property of the receptor. Cell Regul 1:883–893

Phillips DR, Teng W, Arfsten A et al (1997) Effect of Ca+2 on GP IIb-IIIa interactions with integrilin: enhanced GP IIb-IIIa binding and inhibition of platelet aggregation by reductions in the concentration of ionized calcium in plasma anticoagulated with citrate. Circulation 96:1488–1494

Prati F, Capodanno D, Pawlowski T et al (2010) Local delivery versus intracoronary infusion of abciximab in patients with acute coronary syndromes. JACC Cardiovasc Interv 3:928–934

ReoPro (Abciximab) Prescribing Information (2005) Eli Lily & Company, Indianapolis, IN. Available at http://www.centocorothobiotech.com/cobi/assets/reopro.pdf. Accessed 13 Feb 2011

Sanborn TA, Ebrahimi R, Manoukian SV et al (2010) Impact of femoral vascular closure devices and antithrombotic therapy on access site bleeding in acute coronary syndromes: the Acute Catheterization and Urgent Intervention Triage Strategy (ACUITY) trial. Circ Cardiovasc Interv 3:57–62

Saw J, Lincoff AM, DeSmet W, for REPLACE-2 Investigators et al (2004) Lack of clopidogrel pretreatment effect on the relative efficacy of bivalirudin with provisional glycoprotein IIb/IIIa blockade compared to heparin with routine glycoprotein IIb/IIIa blockade: a REPLACE-2 substudy. J Am Coll Cardiol 44:1194–1199

Schneider DJ, Hermann HC, Lakkis N et al (2002) Enhanced early inhibition of platelet aggregation with an increased bolus of tirofiban. Am J Cardiol 90:1421–1423

Shattil SJ, Brass LF, Bennett JS, Pandhi P (1985) Biochemical and functional consequences of dissociation of the platelet membrane glycoprotein IIb-IIIa complex. Blood 66:92–98

Springer TA, Zhu J, Xiao T (2008) Structural basis for distinctive recognition of fibrinogen gammaC peptide by the platelet integrin alphaIIbbeta3. J Cell Biol 182:791–800

Steinhubl SR (2000) Assessing the optimal level of platelet inhibition with GP IIb/IIIa inhibitors in patients undergoing coronary intervention: rationale and design of the GOLD study. J Thromb Thrombolysis 9:199–205

Steinhubl SR, Talley DJ, Braden GA et al (2001) Point-of-care measured platelet inhibition correlates with a reduced risk of an adverse cardiac event after percutaneous intervention. Results of the GOLD (AU-Assessing Ultegra) Multicenter Study. Circulation 103:2572–2578

Stone GW, Crines CL, Cox DA et al (2002) Comparison of angioplasty with stenting, without abciximab, in acute myocardial infarction. New Engl J Med 346:957–966

Stone GW, Witzenbichler B, Guagliumi G et al (2008) Bivalirudin during primary PCI in acute myocardial infarction. New Engl J Med 358:2218–2230

Tcheng JE (2000) Clinical challenges of platelet glycoprotein IIb/IIIa receptor inhibitor therapy: bleeding, reversal, thrombocytopenia, and retreatment. Am Heart J 139:538–545

Tcheng JE, Ellis SG, George BS et al (1994) Pharmacodynamics of chimeric glycoprotein IIb/IIIa integrin antiplatelet antibody Fab 7E3 in high-risk coronary angioplasty. Circulation 90:1757–1764

Tcheng JE, Harrington RA, Kottke-Marchant K et al (1995) Multicenter, randomized, double-blind placebo-controlled trial of the platelet integrin glycoprotein IIb/IIIa blocker Integrelin in elective coronary intervention. Circulation 91:2151–2157

Tcheng JE, Talley JD, O'Shea JC et al (2001) Clinical pharmacology of higher dose eptifibatide in percutaneous coronary intervention (the PRIDE study). Am J Cardiol 88:1097–1102

The CAPTURE Trial Investigators (1997) Randomised placebo-controlled trial of abciximab before and during coronary intervention in refractory unstable angina: the CAPTURE Study. Lancet 349:1429–1435

The EPIC Investigators (1994) Use of a monoclonal antibody directed against the glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty. New Engl J Med 330:956–961

The EPILOG Investigators (1997) Platelet glycoprotein IIb/IIIa receptor blockade and low-dose heparin during percutaneous coronary revascularization. New Engl J Med 336:1689–1696

The ESPRIT Investigators (2000) Novel dosing regimen of eptifibatide in planned coronary stent implantation (ESPRIT): a randomised, placebo controlled trial. Lancet 356:2037–2044

The GUSTO IV-ACS Investigators (2001) Effect of glycoprotein IIb/IIIa receptor blocker abciximab on outcome in patients with acute coronary syndromes with early coronary revascularisation: the GUSTO IV-ACS randomised trial. Lancet 357:1915–1924

The IMPACT II Investigators (1997) Randomised placebo-controlled trial of effect of eptifibatide on complications of percutaneous coronary intervention: IMPACT II. Lancet 349:1422–1428

The Platelet Receptor Inhibition in Ischemic Syndrome Management (PRISM) Study Investigators (1998) A comparison of aspirin plus tirofiban with aspirin plus heparin for unstable angina. New Engl J Med 338:1498–1505

The Platelet Receptor Inhibition in Ischemic Syndrome Management in Patients Limited By Unstable Signs and Symptoms (PRISM-PLUS) Study Investigators (1998) Inhibition of the platelet glycoprotein IIb/IIIa receptor with tirofiban in unstable angina and non-Q-wave myocardial infarction. New Engl J Med 338:1488–1497

The PURSUIT Trial Investigators (1998) Inhibition of platelet glycoprotein IIb/IIIa with eptifibatide in patients with acute coronary syndromes. Platelet glycoprotein IIb/IIIa in unstable angina: receptor suppression using Integrilin therapy. New Engl J Med 339:436–443

The RESTORE Investigators (1997) Effects of platelet glycoprotein IIb/IIIa blockade with tirofiban on adverse cardiac events in patients with unstable angina or acute myocardial infarction undergoing coronary angioplasty. Randomized Efficacy Study of Tirofiban for Outcomes and REstenosis. Circulation 96:1445–1453

Thiele H, Schindler K, Friedenberger J et al (2008) Intracoronary compared with intravenous bolus abciximab application in patients with ST-elevation myocardial infarction undergoing primary percutaneous coronary intervention. Circulation 118:49–57

Tizon-Marcos H, Bertrand OF, Rodes-Cabau J et al (2009) Impact of female gender and transradial coronary stenting with maximal antiplatelet therapy on bleeding and ischemic outcomes. Am Heart J 157:740–745

Topol EJ, Moliterno DJ, Hermann HC, for the TARGET Investigators et al (2001) Comparison of two platelet glycoprotein IIb/IIIa inhibitors, tirofiban and abciximab, for the prevention of ischaemic events with percutaneous coronary revascularisation. New Engl J Med 344:1888–1894

Tsai TT, Maddox TM, Roe MT, for the National Cardiovascular Data Registry et al (2009) Contraindicated medication use in dialysis patients undergoing percutaneous coronary intervention. JAMA 302:2458–2464

Valgimigli M, Campo G, de Cesare N et al (2009) Intensifying platelet inhibition with tirofiban in poor responders to aspirin, clopidogrel, or both agents undergoing elective coronary intervention. Circulation 119:3215–3222

Van de Werf F, Bax J, Betriu A, The task force on the management of ST-segment elevation acute myocardial infarction of the European Society of Cardiology et al (2008) Management of acute myocardial infarction in patients presenting with persistent ST-segment elevation. Eur Heart J 39:2909–2945

Van't Hof AW, Ten Berg J, Heestermans T et al (2008) Prehospital initiation of tirofiban in patients with ST-elevation myocardial infarction undergoing primary angioplasty (ON-TIME 2): a multicentre, double-blind, randomised controlled trial. Lancet 372:537–546

Wagner CL, Mascelli MA, Neblock DS et al (1996) Analysis of GP IIb/IIIa receptor number by quantification of 7E3 binding to human platelets. Blood 88:907–914

Winchester DE, Wen X, Brearley WD, Park KE, Anderson RD, Bavry AA (2011a) Efficacy and safety of glycoprotein IIb/IIIa inhibitors during elective coronary revascularization: a meta-analysis of randomized trials performed in the era of stents and thienopyridines. J Am Coll Cardiol 57:1190–1199

Winchester DE, Wen X, Brearley WD et al (2011b) Efficacy and safety of glycoprotein IIb/IIIa inhibitors during elective coronary revascularization: a meta-analysis of randomized trials performed in the era of stents and thienopyridines. J Am Coll Cardiol 57:1190–1199

Wohrle J, Grebe OC, Nusser T et al (2003) Reduction of major adverse cardiac events with intracoronary compared with intravenous bolus application of abciximab in patients with acute myocardial infarction or unstable angina undergoing coronary angioplasty. Circulation 107:1840–1843

Xiao T, Takagi J, Coller BS, Wang J, Springer TA (2004) Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. Nature 432:59–67

Yin H, Slusky JS, Berger BW et al (2007) Computational design of peptides that target transmembrane helices. Science 315:1817–1822

Zhu J, Choi WS, McCoy JG et al (2012) Struture-guided design of a high-affinity platelet integrin alpha 2b beta 3 receptor antagonist that disrupts Mg2+ binding to the MIDAS. Sci Transl Med 125:125ra32

Targeting Phosphodiesterases in Anti-platelet Therapy

Matthew T. Rondina and Andrew S. Weyrich

Contents

Abstract There are two primary modes of platelet inhibition: blockade of membrane receptors or neutralization of intracellular pathways. Both means of inhibition have proven benefits in the prevention and resolution of atherothrombotic events. With regard to intracellular inhibition, phosphodiesterases (PDEs) are fundamental for platelet function. Platelets possess several PDEs (PDE2, PDE3 and PDE5) that catalyze the hydrolysis of cyclic adenosine 3′-5′-monophosphate (cAMP) and cyclic guanosine 3′-5′-monophosphate (cGMP), thereby limiting the levels of intracellular nucleotides. PDE inhibitors, such as cilostazol and

M.T. Rondina
The Molecular Medicine Program and Department of Internal Medicine, University of Utah, Salt Lake City, UT 84112, USA

A.S. Weyrich (✉)
The Molecular Medicine Program and Department of Internal Medicine, University of Utah, Salt Lake City, UT 84112, USA

University of Utah, Eccles Institute of Human Genetics, Building 533, Room 4220, 15 North 2030 East, Salt Lake City, UT 84112, USA
e-mail: andy.weyrich@u2m2.utah.edu

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_9,

dipyridamole, dampen platelet function by increasing cAMP and cGMP levels. This review focuses on the roles of PDE inhibitors in modulating platelet function, with particular attention paid to drugs that have anti-platelet clinical indications.

Keywords Phosphodiesterase • Platelet • Thrombosis • Anti-Platelet Agents • Cilostazol • Dipyridamole

1 Introduction

Two prominent phases of atherothrombosis exist: a prolonged luminal narrowing and an acute thrombotic stage. Inhibition of platelet function has demonstrated benefits in both stages, especially in the prevention and treatment of acute thrombosis. There are several different classes of pharmaceutical agents with demonstrated efficacy for the prevention and treatment of atherothrombosis. For example, some anti-platelet agents target surface receptors that play a key role in platelet aggregation (e.g., the ADP receptor or integrin $\alpha_{IIb}\beta_3$). Other anti-platelet agents function by suppressing intracellular signaling pathways to either block the production of pro-aggregating factors or to increase the activity of platelet inhibitors. Aspirin, for example, reduces platelet aggregation by neutralizing cycloxygenase-1 (COX-1) and preventing thromboxane A_2 synthesis. Phosphodiesterase (PDE) inhibitors attenuate platelet activity by increasing cAMP and/or cGMP. Elevation of cAMP and cGMP levels subsequently dampens cytoskeletal rearrangement in platelets, activation of integrin $\alpha_{IIb}\beta_3$, and platelet secretion.

In this chapter, we will review the effects of PDE inhibitors on platelet function. In doing so, we will discuss the expression and function of PDEs in platelets focusing on anti-PDE drugs that are used clinically. We conclude with a brief summary of additional functions of PDE inhibitors that may have pleiotropic effects for the prevention and treatment of cardiovascular disease.

2 Platelet Phosphodiesterases

cAMP and cGMP are critical intracellular second messengers that modulate platelet functions (Haslam et al. 1999; Colman et al. 2004; Gresele et al. 2011). Arguably, interest in cyclic nucleotides began over 40 years ago when prostaglandin E_1 (PGE_1) was shown to inhibit platelet responses through cAMP-dependent mechanisms (Haslam et al. 1978, 1999). The critical role of cGMP in regulating platelet function was subsequently elucidated when nitrovasodilators, such as nitroprusside, were shown to inhibit platelet aggregation and, in parallel, increase cGMP levels (Haslam et al. 1978, 1999). It is now known that platelets have the ability to synthesize cGMP in response to nitric oxide released by nitrovasodilators.

Because of their key roles in regulating cellular signaling and function, it is critical for cells to limit the formation and activity of cyclic nucleotides. PDEs are essential in this process because they catalyze the hydrolysis of cAMP and cGMP to

Table 1 Phosphodiesterases (PDEs) in platelets

Family	Substrate	Clinically approved inhibitors
PDE2	cGMP = cAMP	None
PDE3	cAMP > cGMP	Cilostazol, milrinone, anagrelide
PDE5	cGMP	Dipyridamole, sildenafil, vardenafil, tadalafil

inactive 5′-AMP and 5′-GMP, respectively (Francis et al. 2011). In mammalian tissues, 11 families of PDEs (PDE1-11) have been described. The molecular mechanisms of the physiologic functions of these PDEs have been discussed in detail recently and the reader is referred to here for a more comprehensive review on the subject (Bender et al. 2006). In the soluble fraction of platelet extracts, three distinct PDEs have been isolated: PDE2, PDE3, and PDE5 (Hidaka et al. 1976). PDE2 and PDE3 hydrolyze cAMP and cGMP while PDE5 prefers cGMP as a substrate (Table 1). Together, these three isozymes account for the majority (more than 90 %) of platelet PDE activity.

2.1 Phosphodiesterase 2

PDE2, a dual substrate enzyme that hydrolyzes cAMP and cGMP equally well, contains two GAF domains (GAF-A and GAF-B) critical for normal physiologic functions (Fig. 1) (Haslam et al. 1999). GAFs are found in a variety of proteins, but the acronym is derived from the names of the first three classes of proteins recognized to contain this domain: cGMP-binding PDEs, *Anabaena* Adenylal cyclases, and *Escherichia coli* FhlA (Zoraghi et al. 2004; Aravind et al. 1997). The GAF domain structure has several functions in PDEs, including cGMP binding and dimerization of PDE monomers. The GAF-A domain mediates dimerization of PDE2 while the GAF-B domain binds cGMP (Zoraghi et al. 2004). Upon binding of cGMP to GAF-B, PDE2 is stimulated, resulting in a conformational change in the protein and an increase in enzyme activity in platelets (Haslam et al. 1999; Bender et al. 2006; Zoraghi et al. 2004). Thus, elevated concentrations of cGMP stimulate PDE2 (Bender et al. 2006).

The highest levels of PDE2 are observed in the brain, but PDE2 activity is also found in cardiac muscle, endothelial cells, and platelets. Purification of the enzyme from platelets demonstrates that PDE2 hydrolyzes cAMP and cGMP at similar rates (Grant et al. 1990). There are three known splice variants of PDE2: PDE2A1, PDE2A2, and PDE2A3 (Beavo et al. 1995). Nevertheless, there are no demonstrated differences in the kinetic behavior of the splice variants and all are involved in subcellular targeting (Yang et al. 1994). In platelets, these splice variants have not been well characterized and some investigators have suggested that the PDE2 isoform in platelets is unlikely to be PDE2A2—which is soluble—as the PDE2 isozyme in platelets localizes to the cell membrane (Haslam et al. 1999; Yang et al. 1994; Russwurm et al. 2009). However, this premise may deserve

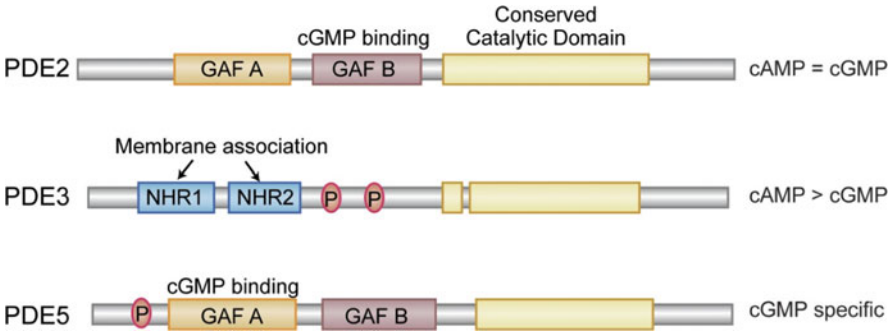


Fig. 1 The domain organization of platelet PDEs

reconsideration given the recent data from our group which used next-generation RNA-sequencing to show that PDE2A2 is the major isoform of PDE2 in human platelets (Rowley et al. 2011).

No known diseases are associated with PDE2 dysfunction and as deletion of PDE2A in mice is embryonically lethal (Stephenson et al. 2009), in vivo studies to characterize PDE2-driven platelet responses have been limited by this technical obstacle. Investigators have thus largely relied on pharmacological blockade of PDE2 to determine the molecular pathways by which PDE2 regulates platelet functions. The PDE2 inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) potentiates the inhibitory effects of nitroprusside on thrombin-induced aggregation (Dickinson et al. 1997), but this may be explained by concomitant neutralization of adenosine deaminase (Gresele et al. 2011). A new series of natural PDE2 inhibitors (purified from safrole obtained from *Ocotea pretiosa*) have recently been shown to block arachidonic acid and collagen-induced aggregation (Brito et al. 2010; Lima et al. 1999). Like EHNA, the effect of these inhibitors is enhanced in the presence of sodium nitroprusside (Brito et al. 2010).

PDE2 inhibitors are primarily used as research tools and have been studied far more extensively in other cells. There are no registered PDE2 inhibitors for clinical use (Table 1). Novel inhibitors that are more selective for PDE2, however, have recently been developed (i.e., 9-(6-phenyl-2-oxohex-3-yl)-2-(3,4-dimethoxybenzyl)-purin-6one [PDP]) but not yet tested on platelets (Gresele et al. 2011; Diebold et al. 2009).

2.2 Phosphodiesterase 3

Like PDE2, isoforms of PDE3 hydrolyze cAMP and cGMP with a preference for cAMP (Table 1). One unique feature of PDE3 is that hydrolysis of cAMP is blocked by cGMP, leading to the alternative name of PDE3: the cGMP-inhibited PDE (Bender et al. 2006). In fact, there is evidence that cGMP exerts most of its anti-platelet effects by acting as a competitive inhibitor of PDE3A (Maurice et al. 1990).

PDE3 contains two regions [NH_2-terminal hydrophobic regions (NHR1 and NHR 2)] that provide for membrane association (Francis et al. 2011) (Fig. 1). Two PDE3 genes have been identified: PDE3A and PDE3B (Bender et al. 2006). Both PDE3A and PDE3B have been found in vascular smooth muscle cells (Palmer et al. 2000) while PDE3B is unique to adipocytes, β cells, macrophages, and T-lymphocytes (Shakur et al. 2001). PDE3A is distinct to oocytes and platelets (Shakur et al. 2001). Three variants of PDE3A have been identified (PDE3A1/2/3) (Choi et al. 2001; Wechsler et al. 2002). PDE3A1 encodes the full-length PDE3A that possesses both the NHR1 and NHR2 domains and the catalytic domain (Fig. 1) (Omori et al. 2007). PDE3A2 encodes a protein containing the NHR2 and catalytic domain while placental PDE3A3 encodes a protein that has the catalytic domain only (Wechsler et al. 2002; Kasuya et al. 1995). Platelets primarily express the transcript for PDE3A1 (Rowley et al. 2011).

PDE3A, which was originally purified from outdated banked platelets, has a very low K_m for cAMP (Colman et al. 2004; Grant et al. 1984). Levels of PDE3A in human platelets treated with PGE_1 and PGI_2 increase and parallel the increase seen in cAMP levels (Colman et al. 2004). Several groups have shown that increases in PDE3A activity are mediated by protein kinase A and C (Grant et al. 1988; Macphee et al. 1988; Hunter et al. 2009).

Unlike PDE2, gene disruption of PDE3 in mice is not embryonic lethal. However, PDE3A deficient female mice are infertile as their oocytes contain higher levels of cAMP and fail to undergo spontaneous maturation (Masciarelli et al. 2004). Subtype-selective knockout mice studies demonstrate that PDE3A is the primary PDE3 responsible for regulating platelet function (Sun et al. 2007). Compared to wild-type, littermate controls, resting cAMP levels in platelets are twice as high in PDE3A knockout mice and platelets from PDE3A, but not PDE3B, knockout mice fail to respond to PDE3 inhibitors (Sun et al. 2007). The functional significance of PDE3A has also been demonstrated in studies showing that knockout mice are protected against collagen/epinephrine-induced pulmonary thrombosis and death (Sun et al. 2007).

PDE3A inhibitors reduce platelet aggregation in response to most agonists (Tani et al. 1992; Muggli et al. 1985). Thus, they are attractive targets for anti-platelet therapy in human diseases (Colman et al. 2004; Gresele et al. 2011). Three specific PDE3A inhibitors are currently approved for clinical use and others are in development (Gresele et al. 2011) (Table 1). A thorough discussion of the pharmacological features and clinical trial data with these agents is beyond the scope of this chapter but key data are briefly summarized in the following paragraph.

Milrinone, which increases intraplatelet cAMP levels and thus inhibits platelet aggregation in whole blood and platelet-rich plasma, is currently used clinically for the treatment of congestive heart failure (Gresele et al. 2011; Colucci et al. 1991). Anagrelide is a potent inhibitor of platelet aggregation and a platelet-reducing agent used to treat thrombocythemia (e.g., secondary to myeloproliferative disorders) (Seiler et al. 1987; Silverstein et al. 1988). Cilastazol, which is described in more detail below, is approved for the treatment of intermittent claudication (Faxon et al. 2004).

2.3 Phosphodiesterase 5

Historically, PDE5 was described as the cGMP-specific or cGMP-binding PDE (Bender et al. 2006). The structural basis for PDE5 to selectively hydrolyze cGMP was solved when it was determined that PDE5 has two GAF domains and, that in contrast to PDE2, cGMP binds the GAF-A domain (Fig. 1). Cyclic nucleotide binding to the GAF-A domain is over 100-fold more selective for cGMP over cAMP (Zoraghi et al. 2005). Binding of cGMP to the GAF-A domain significantly increases enzymatic activity (~10-fold) (Bender et al. 2006) and binding is stabilized by phosphorylation events (Bender et al. 2006; Francis et al. 2002).

PDE5 was first found in platelets and then in vascular smooth muscle and endothelial cells, with high expression levels subsequently reported in the corpus cavernosum and lung (Lin et al. 2006). PDE5 has three major isoforms (PDE5A1/2/3). However, these isoforms differ only in the initial portion of exon 1 and there are no obvious functional differences in PDE5A1, PDE5A2, and PDE5A3 (Kass et al. 2007). Nevertheless, the variants may allow for differential control of PDE5A gene expression in various cells (Bender et al. 2006). Next-gen RNA-sequencing suggests that all three PDE5A isoforms are expressed by platelets (Rowley et al. 2011).

Although gene targeted mice lacking PDE5A have not been studied, PDE5A is well recognized to regulate vascular smooth contraction through regulation of cGMP (Bender et al. 2006). In addition, PDE5 is an important regulator of platelet function. Inhibition of PDE5 increases platelet cGMP levels and amplifies the ability of nitric oxide to inhibit platelet aggregation and secretion (Ito et al. 1996; Dunkern et al. 2005). As alluded to above, part of the effect of PDE5 occurs through cGMP-mediated inhibition of PDE3 (Bender et al. 2006).

PDE5A inhibitors used in the clinic to treat erectile dysfunction include sildenafil (Viagra), vardenafil (Levirate), and tadalafil (Cialis). Similar to other PDE inhibitors, sildenafil potentiates the effect of nitroprusside on platelets (Wallis et al. 1999). Sildenafil also inhibits collagen-induced aggregation, ADP-stimulated activation of $\alpha_{IIb}\beta_3$, and prolongs bleeding time in healthy volunteers after acute administration (Berkels et al. 2001; Halcox et al. 2002). Vardenafil reduces calcium influx in thrombin-stimulated platelets while tadalafil reduces the expression of surface P-selectin (De Bon et al. 2010).

Another PDE5A inhibitor, dipyridamole, is used in combination with aspirin for the prevention of secondary stroke. In addition to inhibiting PDE5, dipyridamole blocks the reuptake of adenosine by red blood cells and inhibits other PDEs (Ahn et al. 1989). The anti-thrombotic properties of dipyridamole are described in more detail below.

3 PDE Inhibitors as Anti-platelet Agents

Although there are several preclinical and registered PDE inhibitors, cilostazol and dipyridamole have more established anti-thrombotic actions. As briefly mentioned above, cilostazol and dipyridamole inhibit platelet PDEs (Fig. 2). Their effect on platelet function and value in the clinic is described below.

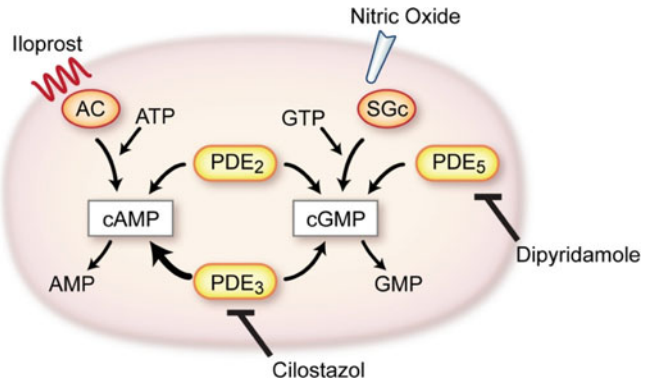


Fig. 2 Schematic representation of the three isoforms of PDEs in platelets. The anti-platelet effects of cilostazol and dipyridamole are discussed in detail

3.1 Cilostazol

Cilostazol is a potent anti-platelet agent registered in the United States since 1999 and in many other countries, including Japan since 1988, for the treatment of intermittent claudication in patients with peripheral vascular disease (usual dose 100 mg twice daily; BID) (Okuda et al. 1993). Intermittent claudication, a pain or ache in the lower extremity muscle groups that occurs with walking and resolved with rest, is a debilitating condition that may severely restrict a person's ability to ambulate and is associated with arterial occlusive disease (Criqui et al. 1985). Cilostazol has also been tested in randomized controlled trials (RCTs) for the secondary prevention of stroke (Shinohara et al. 2010), cardiovascular disease (Han et al. 2009; Suh et al. 2011), and post-stent stenosis (Ge et al. 2005; Yamagami et al. 2012; Jennings et al. 2010). Although differences in study design and patient characteristics exist, many of the results were encouraging, particularly in trials compared to placebo. Nevertheless, as cilostazol is primarily registered for the treatment of claudication and other uses may be off-label or investigational, the remainder of this discussion will focus on the evidence supporting the use of cilostazol in patients with intermittent claudication.

The mechanism by which cilostazol improves claudication is not entirely understood but likely involves one or more processes. As discussed briefly above, cilostazol prevents primary and secondary platelet aggregation by selectively and specifically inhibiting PDE3 in platelets ($IC_{50} = 0.2$ μm) (Schror et al. 2002). Cilostazol also inhibits adenosine uptake in cells leading to increased adenosine levels, which consequently increases intracellular cAMP levels (Schror et al. 2002). Cilostazol has also been shown to prevent platelet activation through shear stress during exercise, which may be particularly critical at foci of arterial bifurcation in patients with peripheral artery disease (Dobesh et al. 2009). Additionally, cilostazol inhibits platelet surface P-selectin expression, thromboxane B_2 production, platelet

factor 4 (PF4) release (Schror et al. 2002; Kariyazono et al. 2001; Igawa et al. 1990), and may also cause increases in high-density lipoprotein and a decrease in triglyceride levels (Thompson et al. 2002). Cilostazol does not prevent synthesis of the vasodilator prostacyclin (Dobesh et al. 2009).

Cilostazol is quickly absorbed when taken orally (time-to-peak plasma concentration 2.4 h) with a half-life of approximately 10 h. Metabolism occurs primarily through the CYP3A5 and CYP2C19 pathways (two isozymes of the cytochrome P450 system) and <1 % of orally administered drug is excreted in the urine without being metabolized (Schror et al. 2002; Akiyama et al. 1985). The metabolism of cilostazol is significantly affected by polymorphisms in the CYP3A5 and CYP2C19 pathways, which are common and result in an estimated coefficient of variation of 40–60 % (Yoo et al. 2010; Bramer et al. 1999). As a result, the dose of cilostazol should be reduced in patients who are taking CYP3A5 or CYP2C19 inhibitors concomitantly.

The clinical efficacy of cilostazol has been studied in multiple, prospective clinical trials. Although study designs differed, most patients were stable (e.g., patients with limb-threatening ischemia, ulcerations, and gangrene were excluded) with an ability to walk at least 30–60 m. In a published meta-analysis of 8 RCTs, cilostazol was superior to placebo in improving walking distances (50 % improvement for maximum distance and 67 % for pain-free distance) (Thompson et al. 2002). In a head-to-head prospective trial of cilostazol versus pentoxifylline (a methylxanthine derivative also used for the treatment of claudication), cilostazol treatment significantly increased walking distances (Dawson et al. 2000). Initial improvements in walking distances with cilostazol are often apparent within 2–4 weeks of treatment initiation (Dawson et al. 2000) but patients may see continued progress for up to 6 months.

Despite its potent antiplatelet effects, cilostazol does not eliminate the need for aspirin or clopidogrel in patients with intermittent claudication. In prospective, clinical trials studying patients on aspirin and cilostazol versus aspirin plus placebo (doses 75–325 mg/day), no significant increases in bleeding events were seen (Thompson et al. 2002). Similarly, the combination of clopidogrel and aspirin has not been demonstrated to significantly increase bleeding events in postmarketing studies, although more data are needed (Hiatt et al. 2005).

In clinical trials, the most common reported side effects of cilostazol include headache (34 %), loose stools (15 %), and diarrhea (19 %). Headache was the most common reason for drug discontinuation and appears to be dose dependent (1.3 % for 50 mg BID and 3.7 % for 100 mg BID) (Pratt et al. 2001). Cilostazol is contraindicated in heart failure as these patients are often on other PDE3 inhibitors (e.g., milrinone) which also increase cAMP levels. Nevertheless, clinical trial data have not demonstrated an increased risk of cardiovascular mortality in patients with heart failure.

3.2 Dipyridamole

Dipyridamole, initially used as a coronary vasodilator, has several anti-platelet effects that have recently been reviewed (Gresele et al. 2011; Eisert 2007).

As shown in Fig. 2, one of these includes inhibition of PDE5 that in turn increases cGMP levels (Eisert 2007; Aktas et al. 2003). Dipyridamole also stimulates prostacyclin production and blocks RBC-mediated uptake of the vasodilator adenosine (Klabunde et al. 1983; Neri Serneri et al. 1981). Adenosine-reuptake blockade leads to inhibition of platelet aggregation in whole blood (Gresele et al. 1983, 1986). By scavenging free radicals that inactivate cyclooxygenase, dipyridamole has key antioxidant properties that potentiate the inhibition of platelet activation and thrombin generation (Chakrabarti et al. 2008). In addition to affecting platelet function, dipyridamole has proven anti-thrombotic properties. Dipyridamole inhibits the activation of platelets as they contact extracellular matrix (Eldor et al. 1986). It also reduces thrombus formation and fibrinogen accumulation at the surface of damaged arteries (van Ryn et al. 2003; Venkatesh et al. 2010).

Oral ingestion of dipyridamole requires low pH (~4) for absorption, which needs to be taken into consideration in order to achieve relevant plasma levels (Eisert 2007). Dipyridamole is indicated as an adjunct to coumarin anti-coagulants in the prevention of post-operative thromboembolic complications associated with cardiac valve replacement (Stein et al. 1998; Chesebro et al. 1985). Dipyridamole is also given in combination with low-dose aspirin to reduce the rate of recurrent strokes (Eisert 2007). In several prospective trials and in meta-analysis data, patients randomized to dipyridamole had significantly greater reductions in the risk of stroke (Diener et al. 1996; Halkes et al. 2006; Verro et al. 2008). This efficacy is reflected in the current guidelines from the American College of Chest Physicians recommending that patients with stroke or TIA should be prescribed dual therapy with extended-release dipyridamole (200 mg BID) plus aspirin over aspirin alone (Adams et al. 2008). A recent large clinical trial showed that the rates of recurrent stroke were similar in patients receiving aspirin and dipyridamole combination therapy or clopidogrel (Sacco et al. 2008). Like cilostazol, the most common side effect of dipyridamole is headache at the onset of therapy (Theis et al. 1999).

4 Summary

PDEs are appealing targets for anti-platelet therapy. Indeed, drugs that target PDEs are in development and cilostazol and dipyridamole have established anti-platelet effects that are mediated, in part, via inhibition of PDEs in platelets. Because PDEs are expressed by a variety of cells, it is not surprising that PDE inhibitors may have other benefits in the treatment of cardiovascular disease beyond their direct effect on platelets. As an example, dipyridamole blocks de novo synthesis of monocyte chemotactic protein-1 (MCP-1) and matrix metalloproteinase-9 (MMP-9) by platelet-leukocyte aggregates (Franks et al. 2010; Weyrich et al. 2005, 2006). Similarly, cilostazol inhibits the expression of MCP-1 in endothelial cells by mechanisms that involve increased cAMP (Nishio et al. 1997). Both cilostazol and dipyridamole inhibit inflammatory responses mediated by NF-κB (Weyrich

et al. 2005; Wang et al. 2008; Jung et al. 2010). Although not rigorously tested, it is possible that the combinatorial anti-platelet and anti-inflammatory properties of cilostazol and dipyridamole could intervene in atherosclerosis disease progression. At a minimum, a deeper understanding of PDE inhibition in platelets and other cells is warranted as we search for effective agents in the treatment of acute and chronic cardiovascular disease.

Knowledge Gaps

- A deeper understanding of the role of PDEs in human platelets
- The development of a therapeutic strategy that specifically inhibits PDEs in platelets but not other cells
- The potential clinical benefit of combining lipid-lowering drugs with cilostazol or dipyridamole to prevent atherosclerosis disease progression

Key Messages

- PDE inhibitors, such as cilostazol and dipyridamole, inhibit platelet function by increasing cyclic adenosine 3′-5′-monophosphate (cAMP) and cyclic guanosine 3′-5′-monophosphate (cGMP) levels and are effective clinically.
- Cilostazol inhibits phosphodiesterase (PDE) 3 and has demonstrated clinical efficacy in reducing symptoms of claudication.
- Dipyridamole (a PDE5 inhibitor), given in combination with low-dose aspirin, reduces the rates of recurrent stroke and is recommended in patients with a history of stroke or transient ischemic attack (TIA).

References

Adams RJ, Albers G, Alberts MJ et al (2008) Update to the AHA/ASA recommendations for the prevention of stroke in patients with stroke and transient ischemic attack. Stroke 39:1647–1652

Ahn HS, Crim W, Romano M et al (1989) Effects of selective inhibitors on cyclic nucleotide phosphodiesterases of rabbit aorta. Biochem Pharmacol 38:3331–3339

Akiyama H, Kudo S, Shimizu T et al (1985) The metabolism of a new antithrombotic and vasodilating agent, cilostazol, in rat, dog and man. Arzneimittelforschung 35:1133–1140

Aktas B, Utz A, Hoenig-Liedl P et al (2003) Dipyridamole enhances NO/cGMP-mediated vasodilator-stimulated phosphoprotein phosphorylation and signaling in human platelets: in vitro and in vivo/ex vivo studies. Stroke 34:764–769

Aravind L, Ponting CP et al (1997) The GAF domain: an evolutionary link between diverse phototransducing proteins. Trends Biochem Sci 22:458–459

Beavo JA et al (1995) Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. Physiol Rev 75:725–748

Bender AT, Beavo JA et al (2006) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. Pharmacol Rev 58:488–520

Berkels R, Klotz T, Sticht G et al (2001) Modulation of human platelet aggregation by the phosphodiesterase type 5 inhibitor sildenafil. J Cardiovasc Pharmacol 37:413–421

Bramer SL, Forbes WP, Mallikaarjun S et al (1999) Cilostazol pharmacokinetics after single and multiple oral doses in healthy males and patients with intermittent claudication resulting from peripheral arterial disease. Clin Pharmacokinet 37(Suppl 2):1–11

Brito FC, Kummerle AE, Lugnier C et al (2010) Novel thienylacylhydrazone derivatives inhibit platelet aggregation through cyclic nucleotides modulation and thromboxane A2 synthesis inhibition. Eur J Pharmacol 638:5–12

Chakrabarti S, Freedman JE et al (2008) Dipyridamole, cerebrovascular disease, and the vasculature. Vascul Pharmacol 48:143–149

Chesebro JH, Steele PM, Fuster V et al (1985) Platelet-inhibitor therapy in cardiovascular disease. Effective defense against thromboembolism. Postgrad Med 78(48–50):57–71

Choi YH, Ekholm D, Krall J et al (2001) Identification of a novel isoform of the cyclic-nucleotide phosphodiesterase PDE3A expressed in vascular smooth-muscle myocytes. Biochem J 353:41–50

Colman RW et al (2004) Platelet cyclic adenosine monophosphate phosphodiesterases: targets for regulating platelet-related thrombosis. Semin Thromb Hemost 30:451–460

Colucci WS et al (1991) Cardiovascular effects of milrinone. Am Heart J 121:1945–1947

Criqui MH, Fronek A, Barrett-Connor E et al (1985) The prevalence of peripheral arterial disease in a defined population. Circulation 71:510–515

Dawson DL, Cutler BS, Hiatt WR et al (2000) A comparison of cilostazol and pentoxifylline for treating intermittent claudication. Am J Med 109:523–530

De Bon E, Bonanni G, Saggiorato G et al (2010) Effects of tadalafil on platelets and endothelium in patients with erectile dysfunction and cardiovascular risk factors: a pilot study. Angiology 61:602–606

Dickinson NT, Jang EK, Haslam RJ et al (1997) Activation of cGMP-stimulated phosphodiesterase by nitroprusside limits cAMP accumulation in human platelets: effects on platelet aggregation. Biochem J 323(Pt 2):371–377

Diebold I, Djordjevic T, Petry A et al (2009) Phosphodiesterase 2 mediates redox-sensitive endothelial cell proliferation and angiogenesis by thrombin via Rac1 and NADPH oxidase 2. Circ Res 104:1169–1177

Diener HC, Cunha L, Forbes C et al (1996) European Stroke Prevention Study. 2. Dipyridamole and acetylsalicylic acid in the secondary prevention of stroke. J Neurol Sci 143:1–13

Dobesh PP, Stacy ZA, Persson EL et al (2009) Pharmacologic therapy for intermittent claudication. Pharmacotherapy 29:526–553

Dunkern TR, Hatzelmann A et al (2005) The effect of Sildenafil on human platelet secretory function is controlled by a complex interplay between phosphodiesterases 2, 3 and 5. Cell Signal 17:331–339

Eisert WG (2007) Dipyridamole. In: Michelson AD (ed) Platelets. Academic, San Diego, CA, pp 1165–1179

Eldor A, Vlodavsky I, Fuks Z et al (1986) Different effects of aspirin, dipyridamole and UD-CG 115 on platelet activation in a model of vascular injury: studies with extracellular matrix covered with endothelial cells. Thromb Haemost 56:333–339

Faxon DP, Creager MA, Smith SC Jr et al (2004) Atherosclerotic Vascular Disease Conference: Executive summary: Atherosclerotic Vascular Disease Conference proceeding for healthcare professionals from a special writing group of the American Heart Association. Circulation 109:2595–2604

Francis SH, Bessay EP, Kotera J et al (2002) Phosphorylation of isolated human phosphodiesterase-5 regulatory domain induces an apparent conformational change and increases cGMP binding affinity. J Biol Chem 277:47581–47587

Francis SH, Blount MA, Corbin JD et al (2011) Mammalian cyclic nucleotide phosphodiesterases: molecular mechanisms and physiological functions. Physiol Rev 91:651–690

Franks ZG, Campbell RA, Weyrich AS et al (2010) Platelet-leukocyte interactions link inflammatory and thromboembolic events in ischemic stroke. Ann N Y Acad Sci 1207:11–17

Ge J, Han Y, Jiang H et al (2005) RACTS: a prospective randomized antiplatelet trial of cilostazol versus ticlopidine in patients undergoing coronary stenting: long-term clinical and angiographic outcome. J Cardiovasc Pharmacol 46:162–166

Grant PG, Colman RW et al (1984) Purification and characterization of a human platelet cyclic nucleotide phosphodiesterase. Biochemistry 23:1801–1807

Grant PG, Mannarino AF, Colman RW et al (1988) cAMP-mediated phosphorylation of the low-Km cAMP phosphodiesterase markedly stimulates its catalytic activity. Proc Natl Acad Sci USA 85:9071–9075

Grant PG, Mannarino AF, Colman RW et al (1990) Purification and characterization of a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from the cytosol of human platelets. Thromb Res 59:105–119

Gresele P, Zoja C, Deckmyn H et al (1983) Dipyridamole inhibits platelet aggregation in whole blood. Thromb Haemost 50:852–856

Gresele P, Arnout J, Deckmyn H et al (1986) Mechanism of the antiplatelet action of dipyridamole in whole blood: modulation of adenosine concentration and activity. Thromb Haemost 55:12–18

Gresele P, Momi S, Falcinelli E et al (2011) Anti-platelet therapy: phosphodiesterase inhibitors. Br J Clin Pharmacol 72(4):634–646

Halcox JP, Nour KR, Zalos G et al (2002) The effect of sildenafil on human vascular function, platelet activation, and myocardial ischemia. J Am Coll Cardiol 40:1232–1240

Halkes PH, van Gijn J, Kappelle LJ et al (2006) Aspirin plus dipyridamole versus aspirin alone after cerebral ischaemia of arterial origin (ESPRIT): randomised controlled trial. Lancet 367:1665–1673

Han Y, Li Y, Wang S et al (2009) Cilostazol in addition to aspirin and clopidogrel improves long-term outcomes after percutaneous coronary intervention in patients with acute coronary syndromes: a randomized, controlled study. Am Heart J 157:733–739

Haslam RJ, Davidson MM, Davies T et al (1978) Regulation of blood platelet function by cyclic nucleotides. Adv Cyclic Nucleotide Res 9:533–552

Haslam RJ, Dickinson NT, Jang EK et al (1999) Cyclic nucleotides and phosphodiesterases in platelets. Thromb Haemost 82:412–423

Hiatt WR et al (2005) The US experience with cilostazol in treating intermittent claudication. Atheroscler Suppl 6:21–31

Hidaka H, Asano T et al (1976) Human blood platelet 3′: 5′-cyclic nucleotide phosphodiesterase. Isolation of low-Km and high-Km phosphodiesterase. Biochim Biophys Acta 429:485–497

Hunter RW, Mackintosh C, Hers I et al (2009) Protein kinase C-mediated phosphorylation and activation of PDE3A regulate cAMP levels in human platelets. J Biol Chem 284:12339–12348

Igawa T, Tani T, Chijiwa T et al (1990) Potentiation of anti-platelet aggregating activity of cilostazol with vascular endothelial cells. Thromb Res 57:617–623

Ito M, Nishikawa M, Fujioka M et al (1996) Characterization of the isoenzymes of cyclic nucleotide phosphodiesterase in human platelets and the effects of E4021. Cell Signal 8:575–581

Jennings DL, Kalus JS et al (2010) Addition of cilostazol to aspirin and a thienopyridine for prevention of restenosis after coronary artery stenting: a meta-analysis. J Clin Pharmacol 50:415–421

Jung WK, Lee DY, Park C et al (2010) Cilostazol is anti-inflammatory in BV2 microglial cells by inactivating nuclear factor-kappaB and inhibiting mitogen-activated protein kinases. Br J Pharmacol 159:1274–1285

Kariyazono H, Nakamura K, Shinkawa T et al (2001) Inhibition of platelet aggregation and the release of P-selectin from platelets by cilostazol. Thromb Res 101:445–453
Kass DA, Champion HC, Beavo JA et al (2007) Phosphodiesterase type 5: expanding roles in cardiovascular regulation. Circ Res 101:1084–1095
Kasuya J, Goko H, Fujita-Yamaguchi Y et al (1995) Multiple transcripts for the human cardiac form of the cGMP-inhibited cAMP phosphodiesterase. J Biol Chem 270:14305–14312
Klabunde RE et al (1983) Dipyridamole inhibition of adenosine metabolism in human blood. Eur J Pharmacol 93:21–26
Lima LM, Ormelli CB, Brito FF et al (1999) Synthesis and antiplatelet evaluation of novel aryl-sulfonamide derivatives, from natural safrole. Pharm Acta Helv 73:281–292
Lin CS, Lin G, Xin ZC et al (2006) Expression, distribution and regulation of phosphodiesterase 5. Curr Pharm Des 12:3439–3457
Macphee CH, Reifsnyder DH, Moore TA et al (1988) Phosphorylation results in activation of a cAMP phosphodiesterase in human platelets. J Biol Chem 263:10353–10358
Masciarelli S, Horner K, Liu C et al (2004) Cyclic nucleotide phosphodiesterase 3A-deficient mice as a model of female infertility. J Clin Invest 114:196–205
Maurice DH, Haslam RJ et al (1990) Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP. Mol Pharmacol 37:671–681
Muggli R, Tschopp TB, Mittelholzer E et al (1985) 7-Bromo-1,5-dihydro-3,6-dimethylimidazo [2,1-b]quinazolin-2(3H)- one (Ro 15-2041), a potent antithrombotic agent that selectively inhibits platelet cyclic AMP-phosphodiesterase. J Pharmacol Exp Ther 235:212–219
Neri Serneri GG, Masotti G, Poggesi L et al (1981) Enhanced prostacyclin production by dipyridamole in man. Eur J Clin Pharmacol 21:9–15
Nishio Y, Kashiwagi A, Takahara N et al (1997) Cilostazol, a cAMP phosphodiesterase inhibitor, attenuates the production of monocyte chemoattractant protein-1 in response to tumor necrosis factor-alpha in vascular endothelial cells. Horm Metab Res 29:491–495
Okuda YKY, Yamashita K et al (1993) Cilostazol. Cardiovascu Drug Rev 11:451–465
Omori K, Kotera J et al (2007) Overview of PDEs and their regulation. Circ Res 100:309–327
Palmer D, Maurice DH et al (2000) Dual expression and differential regulation of phosphodiesterase 3A and phosphodiesterase 3B in human vascular smooth muscle: implications for phosphodiesterase 3 inhibition in human cardiovascular tissues. Mol Pharmacol 58:247–252
Pratt CM et al (2001) Analysis of the cilostazol safety database. Am J Cardiol 87:28D–33D
Rowley JW, Oler AJ, Tolley ND et al (2011) Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. Blood 118:e101–e111
Russwurm C, Zoidl G, Koesling D et al (2009) Dual acylation of PDE2A splice variant 3: targeting to synaptic membranes. J Biol Chem 284:25782–25790
Sacco RL, Diener HC, Yusuf S et al (2008) Aspirin and extended-release dipyridamole versus clopidogrel for recurrent stroke. N Engl J Med 359:1238–1251
Schror K et al (2002) The pharmacology of cilostazol. Diabetes Obes Metab 4(Suppl 2):S14–S19
Seiler S, Arnold AJ, Grove RI et al (1987) Effects of anagrelide on platelet cAMP levels, cAMP-dependent protein kinase and thrombin-induced Ca++ fluxes. J Pharmacol Exp Ther 243:767–774
Shakur Y, Holst LS, Landstrom TR et al (2001) Regulation and function of the cyclic nucleotide phosphodiesterase (PDE3) gene family. Prog Nucleic Acid Res Mol Biol 66:241–277
Shinohara Y, Katayama Y, Uchiyama S et al (2010) Cilostazol for prevention of secondary stroke (CSPS 2): an aspirin-controlled, double-blind, randomised non-inferiority trial. Lancet Neurol 9:959–968
Silverstein MN, Petitt RM, Solberg LA Jr et al (1988) Anagrelide: a new drug for treating thrombocytosis. N Engl J Med 318:1292–1294
Stein PD, Alpert JS, Dalen JE et al (1998) Antithrombotic therapy in patients with mechanical and biological prosthetic heart valves. Chest 114:602S–610S

Stephenson DT, Coskran TM, Wilhelms MB et al (2009) Immunohistochemical localization of phosphodiesterase 2A in multiple mammalian species. J Histochem Cytochem 57:933–949

Suh JW, Lee SP, Park KW et al (2011) Multicenter randomized trial evaluating the efficacy of cilostazol on ischemic vascular complications after drug-eluting stent implantation for coronary heart disease: results of the CILON-T (influence of CILostazol-based triple antiplatelet therapy ON ischemic complication after drug-eluting stenT implantation) trial. J Am Coll Cardiol 57:280–289

Sun B, Li H, Shakur Y et al (2007) Role of phosphodiesterase type 3A and 3B in regulating platelet and cardiac function using subtype-selective knockout mice. Cell Signal 19:1765–1771

Tani T, Sakurai K, Kimura Y et al (1992) Pharmacological manipulation of tissue cyclic AMP by inhibitors. Effects of phosphodiesterase inhibitors on the functions of platelets and vascular endothelial cells. Adv Second Messenger Phosphoprotein Res 25:215–227

Theis JG, Deichsel G, Marshall S et al (1999) Rapid development of tolerance to dipyridamole-associated headaches. Br J Clin Pharmacol 48:750–755

Thompson PD, Zimet R, Forbes WP et al (2002) Meta-analysis of results from eight randomized, placebo-controlled trials on the effect of cilostazol on patients with intermittent claudication. Am J Cardiol 90:1314–1319

van Ryn J, Lorenz M, Merk H et al (2003) Accumulation of radiolabelled platelets and fibrin on the carotid artery of rabbits after angioplasty: effects of heparin and dipyridamole. Thromb Haemost 90:1179–1186

Venkatesh PK, Pattillo CB, Branch B et al (2010) Dipyridamole enhances ischaemia-induced arteriogenesis through an endocrine nitrite/nitric oxide-dependent pathway. Cardiovasc Res 85:661–670

Verro P, Gorelick PB, Nguyen D et al (2008) Aspirin plus dipyridamole versus aspirin for prevention of vascular events after stroke or TIA: a meta-analysis. Stroke 39:1358–1363

Wallis RM, Corbin JD, Francis SH et al (1999) Tissue distribution of phosphodiesterase families and the effects of sildenafil on tissue cyclic nucleotides, platelet function, and the contractile responses of trabeculae carneae and aortic rings in vitro. Am J Cardiol 83:3C–12C

Wang F, Li M, Cheng L et al (2008) Intervention with cilostazol attenuates renal inflammation in streptozotocin-induced diabetic rats. Life Sci 83:828–835

Wechsler J, Choi YH, Krall J et al (2002) Isoforms of cyclic nucleotide phosphodiesterase PDE3A in cardiac myocytes. J Biol Chem 277:38072–38078

Weyrich AS, Denis MM, Kuhlmann-Eyre JR et al (2005) Dipyridamole selectively inhibits inflammatory gene expression in platelet-monocyte aggregates. Circulation 111:633–642

Weyrich AS, Kraiss LW, Prescott SM et al (2006) New roles for an old drug: inhibition of gene expression by dipyridamole in platelet-leukocyte aggregates. Trends Cardiovasc Med 16:75–80

Yamagami H, Sakai N, Matsumaru Y et al (2012) Periprocedural cilostazol treatment and restenosis after carotid artery stenting: The Retrospective Study of In-Stent Restenosis after Carotid Artery Stenting (ReSISteR-CAS). J Stroke Cerebrovasc Dis 21(3):193–199

Yang Q, Paskind M, Bolger G et al (1994) A novel cyclic GMP stimulated phosphodiesterase from rat brain. Biochem Biophys Res Commun 205:1850–1858

Yoo HD, Cho HY, Lee YB et al (2010) Population pharmacokinetic analysis of cilostazol in healthy subjects with genetic polymorphisms of CYP3A5, CYP2C19 and ABCB1. Br J Clin Pharmacol 69:27–37

Zoraghi R, Corbin JD, Francis SH et al (2004) Properties and functions of GAF domains in cyclic nucleotide phosphodiesterases and other proteins. Mol Pharmacol 65:267–278

Zoraghi R, Bessay EP, Corbin JD et al (2005) Structural and functional features in human PDE5A1 regulatory domain that provide for allosteric cGMP binding, dimerization, and regulation. J Biol Chem 280:12051–12063

PAR-1 Inhibitors: A Novel Class of Antiplatelet Agents for the Treatment of Patients with Atherothrombosis

Sergio Leonardi and Richard C. Becker

Contents

Abstract Stroke and myocardial infarction are leading causes of death and disability worldwide. Typically, these events are triggered by the rupture or erosion of "vulnerable" atherosclerotic plaque, a phenomenon termed atherothrombosis.

Three platelet activation pathways are presumed to be particularly important in the genesis of atherothrombosis and are triggered by 1) cyclo-oxygenase (COX)-1 mediated thromboxane A2 (TXA2) synthesis and activation via the TXA2 receptor, 2)

S. Leonardi
Duke Clinical Research Institute, Duke University Medical Center, 2400 Pratt Street, Durham, NC 27710, USA

Department of Cardiology, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
e-mail: sergio.leonardi@duke.edu

R.C. Becker (✉)
Duke Clinical Research Institute, Duke University Medical Center, 2400 Pratt Street, Durham, NC 27710, USA

Division of Cardiology, Duke University School of Medicine, Durham, NC, USA

Division of Hematology, Duke University School of Medicine, Durham, NC, USA
e-mail: richard.becker@duke.edu

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_10,

adenosine diphosphate (ADP) via the P2Y12 receptor, and 3) thrombin via the protease activated receptor (PAR)-1.

Despite the efficacy of aspirin and of a growing family of P2Y12 receptor antagonists on the first 2 pathways, major cardiovascular events continue to occur in patients with coronary and cerebrovascular disease, suggesting that thrombin-mediated platelet activation may contribute to these adverse events.

Recently, a novel class of antiplatelet agents able to inhibit thrombin-mediated platelet activation has been developed, PAR-1 inhibitors. In this chapter, we will discuss the rationale underlying the development of this novel class of agents focus on the two drugs in the most advanced stages of development: vorapaxar (SCH530348) and atopaxar (E5555).

Keywords Atherothrombosis • Acute coronary syndromes • Antiplatelet agents • PAR-1 inhibitors

1 Introduction

Stroke and myocardial infarction are leading causes of death and disability worldwide. Typically, these events are triggered by the rupture or erosion of a predisposed, "vulnerable" atherosclerotic plaque complicated by the development of a superimposed thrombus. This phenomenon, termed acute atherothrombosis, is primarily mediated by the activation of platelets. Also, activated platelets in combination with proliferative cellular phenomena may contribute to chronic atherothrombotic disease, such as restenosis after stent implantation or obstructive coronary artery disease (CAD).

Of the several pathways involved in platelet activation, three are presumed to be particularly important and triggered by (1) cyclo-oxygenase (COX)-1-mediated thromboxane A_2 (TXA_2) synthesis and activation via the TXA_2 receptor, (2) adenosine diphosphate (ADP) via the $P2Y_{12}$ receptor, and (3) thrombin via the protease-activated receptor (PAR)-1, with thrombin being the most potent of these agonists (Davì and Patrono 2007). Despite the efficacy of aspirin and of a growing family of $P2Y_{12}$ receptor antagonists on the first two pathways, major cardiovascular events continue to occur in patients with coronary and cerebrovascular disease, suggesting that thrombin-mediated platelet activation may contribute to these adverse events.

Recently, a novel class of antiplatelet agents able to inhibit thrombin-mediated platelet activation has been developed, PAR-1 inhibitors. These compounds, also called thrombin receptor antagonists, inhibit the cellular actions of thrombin via a selective antagonism of PAR-1. In this chapter, we will discuss the rationale underlying the development of this novel class of agents and focus on the two drugs in the most advanced stages of development: vorapaxar (SCH530348) and atopaxar (E5555).

2 Rationale for Developing PAR-1 Inhibitors

The armamentarium of antiplatelet agents available to physicians for the management of patients with atherothrombosis is increasing rapidly. After the introduction of aspirin many decades ago, an irreversible inhibitor of cyclooxygenase-1 (COX-1) that produces a permanent defect in TXA_2-mediated platelet activation and recruitment (Patrono et al. 2005), active research has been performed on the inhibition of the platelet $P2Y_{12}$ receptor.

This has led to the development of thienopyridines, a class of orally active prodrugs whose active metabolite inhibits irreversibly the $P2Y_{12}$ receptor. After ticlopidine, the first-in-class compound limited by a suboptimal safety profile, clopidogrel was developed. This second generation, better-tolerated thienopyridine has been used extensively in patients with acute and chronic atherothrombosis and has proved to be particularly useful in patients undergoing stent implantation (The Clopidogrel in Unstable Angina to Prevent Recurrent Events Trial Investigators 2001). After clopidogrel, the inhibition of the $P2Y_{12}$ receptor has been further refined with prasugrel, a third generation thienopyridine with a faster and more predictable generation of the active metabolite than clopidogrel (Wallentin et al. 2008) and ticagrelor, a member of a different class of reversible $P2Y_{12}$ inhibitors referred to as cyclopentyl-triazolo-pyrimidines. Both these compounds proved to have superior efficacy than clopidogrel (Wallentin et al. 2009; Wiviott et al. 2007) but at the price of an increased risk of bleeding, especially with prasugrel. Intravenous $P2Y_{12}$ inhibitors [cangrelor (Harrington et al. 2009; Bhatt et al. 2009) and elinogrel (Leonardi et al. 2010)] are also in advanced development. Considering the abundance of antiplatelet drugs, one may question whether an additional class of antiplatelet agents is needed. We believe that there are several potential reasons.

First, despite the availability of increasingly potent $P2Y_{12}$ inhibitors, ischemic events are not uncommon in patients with atherothrombosis. Up to 10.2% of patients experience a major cardiovascular ischemic event within 1 year even with the latest generation drugs (Wallentin et al. 2009; Wiviott et al. 2007). In addition, the improved efficacy profile of $P2Y_{12}$ antagonists has been accompanied by a parallel increase in the risk of bleeding. In the Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel–Thrombolysis in Myocardial Infarction 38 (TRITON-TIMI 38), prasugrel was associated with a higher incidence of any TIMI bleeding compared with clopidogrel, including major bleeding and fatal bleeding, leading regulators in many countries to restrict its indications (Wiviott et al. 2007). Ticagrelor, despite important benefits in terms of anti-ischemic efficacy, also caused more bleeding than clopidogrel. Although bleeding related to coronary artery bypass grafting (CABG) was not increased with ticagrelor, presumably one of the advantages of its reversible inhibition, this drug has been associated with an increased risk of spontaneous bleeding compared with clopidogrel, including fatal intracranial hemorrhage (Wallentin et al. 2009). These data underscore the importance of $P2Y_{12}$-dependant platelet activation not only for thrombosis but also for protective hemostasis and highlight the

challenges of uncoupling the risks and benefits associated with robust $P2Y_{12}$ inhibition among patients with atherothrombosis.

The goal of further reducing ischemic events without increasing the risk of bleeding is conceptually attractive. Nonhuman data indicate that hemostasis depends more on the effects of thrombin in the coagulation cascade rather than its cellular effects. While fibrinogen-deficient mice exhibit a severe bleeding phenotype, mice deficient in their primary platelet thrombin receptor (i.e., PAR-4) display reduced and delayed platelet activation, but no spontaneous bleeding (Hamilton et al. 2004). Similar data have been observed in nonhuman primates, which share with humans PAR-1 as their primary platelet thrombin receptor. Cynomolgus monkeys treated with SCH 530348, a potent PAR-1 antagonist described herein, showed no change in template bleeding times, clotting times, platelet count, coagulation times, and surgical blood loss compared with monkeys that received no active treatment. Notably, the administration of aspirin plus clopidogrel in this experimental model resulted in a marked prolongation of template bleeding time and surgical blood loss. However, co-administering SCH 530348 in addition to aspirin plus clopidogrel did not exacerbate the observed increases in bleeding time and blood loss (Chintala et al. 2008).

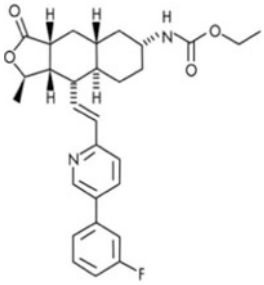
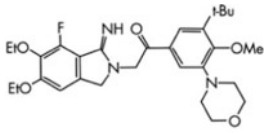
Additionally, multiple platelet activation pathways contribute to thrombosis. The redundancy of this process has provided the basis for combining antiplatelet agents (Gurbel and Tantry 2010). In a monkey model of thrombosis, the combined inhibition of the PAR-1 and $P2Y_{12}$ platelet activation with SCH 602539, an intravenous analog of vorapaxar, and cangrelor, a potent intravenous $P2Y_{12}$ receptor antagonist, had synergistic antithrombotic and antiplatelet effects (Chintala et al. 2010).

Finally, an important part of the rationale for TRA development rests on the persistence of thrombin generation in patients on dual antiplatelet therapy. The Clopidogrel in Unstable angina to prevent Recurrent Events (CURE) Coagulation substudy performed in patients admitted with non-ST elevation (NSTE)-ACS found that neither aspirin nor clopidogrel had an effect on blood markers of coagulation activity (Eikelboom et al. 2002). More importantly this study showed that thrombin generation was unaffected by clopidogrel + aspirin, persisted beyond the acute phase of ACS, and was associated with adverse clinical outcomes.

3 PARs and PAR-1 Inhibitors

Beyond being the main effector protease of the coagulation cascade, thrombin has several fundamental cellular actions. Acting via a specific class of surface platelet receptors—PARs—which are G-protein-linked members of the 7-transmembrane domain receptor superfamily (Dubois et al. 2004), this serine protease is a very potent platelet agonist—indeed the most potent yet described (Davì and Patrono 2007). Notably, PARs are not solely located on platelets. These receptors have also been identified in smooth muscle cells, endothelial cells, and fibroblasts, with the potential of broadening the overall implications of PAR-1 inhibition not only to

Table 1 Main characteristics of vorapaxar and atopaxar

Characteristic	Vorapaxar (SCH 530348)	Atopaxar (E5555)
Chemical structure		
Route of administration	Oral	Oral
Terminal half-life (h)	126–269	22–26
Metabolism	Hepatic, mainly via CYP3A4. <5% Renal	Mainly gastrointestinal
Clinical development stage	Phase III	Phase II

platelet antagonism but also to vascular regulatory actions (Coughlin 1999; Coughlin 2000). Thrombin activates PAR-1 by cleaving a peptide bond (Arg_{41}-Ser_{42}) in the receptor's extracellular domain. This cleavage discloses a new N-terminus of the receptor referred to as a tethered ligand. This "new tail" of the receptor interacts with a distinct domain of the cleaved receptor and, ultimately, causes its activation.

In human platelets, thrombin receptor signaling is mediated by two main PAR variants: PAR-1, the principal platelet thrombin receptor, and PAR-4. PAR-1 is activated by subnanomolar concentrations of thrombin, while PAR-4 requires higher concentration of the same agonist to be fully activated. Blocking antibodies directed against the interaction site of thrombin with PAR-1 have been shown to block receptor cleavage and platelet activation at low (1 nM), but not high (30 nM), concentrations of thrombin. In contrast, PAR-4-blocking antibodies had no measurable effect on thrombin-mediated platelet activation. However, combined PAR-1 and PAR-4 blockade markedly attenuates platelet activation, even at high thrombin concentrations. These observations suggest that PAR-1 and PAR-4 account for most, if not all, thrombin signaling in humans, PAR-1 is the primary platelet thrombin receptor and in absence of a functional PAR-1 (for example via pharmacological inhibition), thrombin can still activate platelets via PAR-4, but only at high concentrations (Kahn et al. 1999).

The first generation of PAR-1 antagonists was designed on the basis of the tethered ligand sequence and referred to as "peptidomimetics." These compounds had limited affinity for PARs. In addition, early generation PAR-1 inhibitors not only lacked high affinity binding but also possessed rapid dissociation rates from the receptor. Two non-peptide inhibitors, a natural himbacine derivative (vorapaxar) and a synthetic compound based on the bicyclic amidine motif (atopaxar), were first able to overcome the cellular kinetic limitations of early compounds (Table 1).

4 Vorapaxar

Vorapaxar (SCH 530348; Merck, USA) is a synthetic tricyclic 3-phenylpyridine structurally similar to himbacine, a natural antimuscarinic agent isolated from the barks of Galbulimima baccata (Chackalamannil et al. 2008). Vorapaxar, however, completely lacks the muscarinic M_2 antagonist activity typical of himbacine. Vorapaxar produce potent, selective, and reversible PAR-1 inhibition. This molecule, a first-in-class PAR-1 inhibitor, is orally active with rapid absorption and high bioavailability (>90%) after oral administration. Although reversible, vorapaxar dissociates from the PAR-1 receptor slowly, features that may account for its prolonged pharmacodynamic effect versus plasma concentration. The compound also has an exceptionally long terminal half-life, which ranges from 126 to 269 h (Becker et al. 2009). Functional assays performed in human platelet-rich plasma showed that the inhibition of α-thrombin and thrombin receptor-activating peptide (TRAP)-induced platelet aggregation was potent (IC_{50} of 47 nM and 25 nM, respectively), while no measurable antagonism was induced by other stimuli such as collagen, ADP, or PAR-4 agonist peptide. In addition, vorapaxar did not affect coagulation parameters (prothrombin time, activated partial thromboplastin time, or activated clotting time), and had no apparent interactions with factors involved in platelet adhesion (von Willebrand factor, collagen). All of these findings provide evidence of vorapaxar's selectivity for PAR-1.

Vorapaxar metabolism has been more clearly determined recently (Ghosal et al. 2011). This agent is extensively metabolized in humans. The parent compound (SCH 530348) is the major circulating drug-derived component after a single dose in plasma, but less than 2% of orally administered SCH 530348 is eliminated unchanged in the feces and none is eliminated in the urine. The major route of elimination for vorapaxar is via an amine metabolite (M19) formed by carbamate cleavage which is primarily mediated by the cytochrome P450 (CYP) 3A4 isoform. Minor amounts of mono- and dihydroxy-metabolites are also formed. After chronic administration of vorapaxar, another metabolite (M20; SCH 2046273) accumulates appreciably (Ghosal et al. 2011). This metabolite, which is equipotent to SCH530348 and has a similar half-life, represents about 23% of the parent compound after steady state of SCH530348 has been reached. The major enzymes generating M20 are CYP2J2 followed by CYP3A4. The relevance of CYP3A4 in the metabolism has been established and quantified by co-administration of vorapaxar with CYP3A4 inducers and inhibitors. Co-administration of vorapaxar with ketoconazole (a potent CYP3A4 inhibitor) nearly doubled its systemic exposure, while the co-administration with the potent CYP3A4 inducer rifampin halved systemic exposure. In contrast, vorapaxar has no significant renal clearance. A study performed in patients with end-stage renal disease showed no difference in overall exposure and bioavailability compared to normal subjects, and dialysis did not appear to affect elimination. A study in patients with various degree of hepatic dysfunction as measured by the Child-Pugh classification showed no difference in the pharmacokinetic profile of a single dose of 40 mg of vorapaxar compared with matched healthy volunteers.

Vorapaxar has been tested in a large phase I program including >500 healthy subjects. Overall, the phase 1 experience suggested that an initial loading dose (ranging from 10 to 40 mg), followed by a lower daily maintenance dose would be an appropriate strategy in acute-care settings. On the basis of pharmacokinetic modeling, mainly from the two largest phase 1 studies, three maintenance doses were selected for investigation in phase II: 0.5, 1, and 2.5 mg per day.

5 Phase II of Vorapaxar

The phase II program of vorapaxar consisted of three clinical trials—a large study in patients with stable coronary artery disease and two smaller studies in Japanese subjects. The Thrombin Receptor Antagonist-Percutaneous Coronary Intervention (TRA-PCI) tested the safety, tolerability, and efficacy of vorapaxar in 1,030 patients undergoing nonurgent cardiac catheterization with intent to perform percutaneous coronary revascularization (PCI) (Becker et al. 2009). The trial had two sequential randomizations, one for the loading dose and one for the maintenance dose (Fig. 1). Before coronary angiography, all patients were randomized 3:1 to receive one of three loading doses (10, 20, or 40 mg) or matching placebo. After this first randomization, only those patients who were actually underwent PCI (56% of the total cohort), representing the primary cohort of interest, were further randomized to a 60-day maintenance treatment with 0.5, 1 or 2.5 mg per day of vorapaxar, or matching placebo. During the index PCI, >90% of patients received an anti-thrombin agent (mostly unfractionated heparin or bivalirudin), while nearly every patient within the primary cohort was treated with aspirin (99%) and clopidogrel (97%). The incidence of the primary endpoint of TIMI major or minor bleeding was low overall and no differences were observed between placebo and the pooled vorapaxar groups, with an incidence of 3% in both the pooled treatment groups and the placebo group. The very low rate of TIMI major or minor bleeding prompted a decision to stop the trial short of the originally planned 1,600 patients. In addition, TIMI major/minor bleedings did not differ significantly in the secondary cohorts of patients who did not undergo PCI, i.e., those medically managed or undergoing coronary-artery bypass grafting. In the cohort of 76 patients who underwent coronary artery bypass grafting, sensitive indicators of bleeding in the surgical setting (such as the amount of chest tube drainage and administration of red blood cell transfusions), did not differ between placebo and vorapaxar. The composite endpoint of death, major cardiac adverse event, or stroke was numerically smaller in patients treated with any vorapaxar dose than with placebo (OR 0.67, 95% CI 0.33–1.34), a difference mainly driven by a reduction of peri-procedural MI in vorapaxar-treated patients. A dose–effect relationship was observed, with the 40 mg loading dose achieving the largest numerical reduction in the rate of peri-procedural MI.

The selection of the dose of vorapaxar to be tested in phase III was assisted by a prospective platelet substudy of TRA-PCI. This study showed that vorapaxar

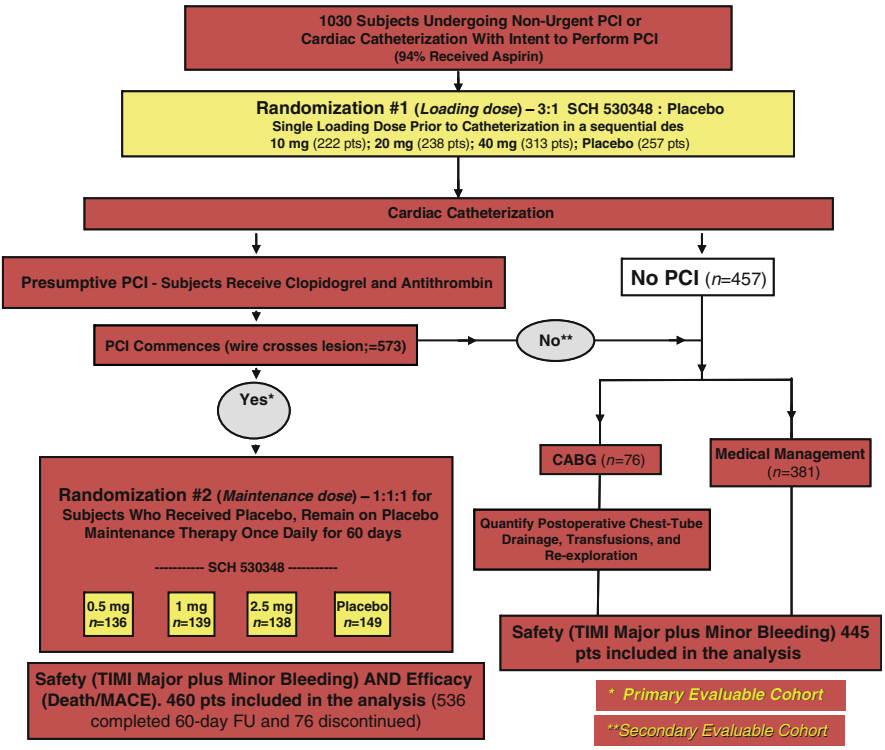


Fig. 1 TRA-PCI design

achieved a dose-dependent inhibition of TRAP-induced platelet aggregation. The 40 mg loading produced the most potent and predictable degree of platelet inhibition among the three loading doses tested, with two-thirds of patients achieving $\geq$80% after 1 h and almost all patients (96.3%) by 2 h. In contrast, only half (52.9%) of patients in the 20 mg arm achieved this level of platelet inhibition. Levels of inhibition were maintained at 30 and 60 days with 1.0 and 2.5 mg per day maintenance doses in all treated patients.

Findings similar to the TRA-PCI study were reported in a similarly designed phase II study conducted in 117 Japanese subjects admitted to the hospital with a NSTE-ACS (Goto et al. 2010a). In addition to exploring a different population compared with TRA-PCI, this study did *not* test the 10 mg loading dose and 0.5 mg maintenance dose of vorapaxar. The addition of vorapaxar to standard-of-care doses of aspirin, ticlopidine, and unfractionated heparin did not significantly increase the rate of TIMI major or minor bleeding in the prespecified primary cohort of 92 patients who underwent PCI. Despite the study's small sample size, the incidence of procedural MI in the PCI cohort was reduced with vorapaxar compared with placebo-treated patients: 16.9% vs. 42.9%, respectively ($P = 0.013$), corresponding to a 61% relative risk reduction. The last phase II study of vorapaxar

was conducted in 90 Japanese patients with a history of recent (>14 days but <1 year) ischemic stroke (Shinohara et al. 2012). These patients were randomized to vorapaxar (1 or 2.5 mg per day) or matching placebo in addition to low-dose aspirin (75–150 mg/day). The primary endpoint was the overall incidence of adverse events during the protocol-defined treatment phase (60 days). In this small study, only one TIMI minor bleed (decreased hematocrit) was observed in the placebo group while no TIMI major bleedings were recorded. Nonfatal stroke occurred in one patient allocated to placebo and one patient allocated to vorapaxar. There were no deaths or MIs.

6 Phase III of Vorapaxar

Vorapaxar has been tested in patients with acute and chronic atherothrombosis in two large phase III clinical trials: the Thrombin Receptor Antagonist for Clinical Event Reduction in Acute Coronary Syndrome (TRA•CER) trial and the Thrombin Receptor Antagonist in Secondary Prevention of Atherothrombotic Ischemic Events (TRA 2°P)-TIMI 50 trial, respectively. (The TRACER Executive Steering C 2009; Morrow et al. 2009). Both are long-term, event-driven trials with a minimum follow-up duration of 1 year that were designed with a number of similarities, including common endpoint definitions adjudicated according to the identical criteria (Fig. 2a, b). This will allow an integrated analysis of the overall vorapaxar phase III datasets, totaling almost 40,000 treated patients.

The hypothesis of the TRA•CER trial was that vorapaxar, administered as a 40 mg loading dose followed by a 2.5 mg maintenance dose in patients with high-risk NSTE-ACS, would reduce the incidence of cardiovascular death, MI, stroke, urgent coronary revascularization, or coronary ischemia requiring hospitalization. Because the phase II data suggested a possible benefit in reducing peri-procedural myocardial necrosis, study drug had to be administered at least 1 h before any coronary revascularization. The TRA 2°P trial has been investigating in >25,000 patients whether a once daily regimen of 2.5 mg per day of vorapaxar can reduce the occurrence of the primary composite endpoint of cardiovascular death, MI, urgent coronary revascularization, or stroke, in stable patients with established atherosclerosis involving the coronary, cerebral, or peripheral vascular systems.

In January 2011, the joint Data and Safety Monitoring Board for TRA•CER and TRA 2°P reported an excess of intracranial hemorrhage in patients with a history of stroke. As a consequence, study drug was discontinued in patients with a history of stroke in the TRA 2P-TIMI 50 trial, but remains ongoing for subjects with a history of MI or peripheral arterial disease. The TRA•CER trial was also stopped after reaching its prespecified number of primary endpoint events.

The results of the TRA•CER (Morrow et al. 2012) trials have been recently presented.

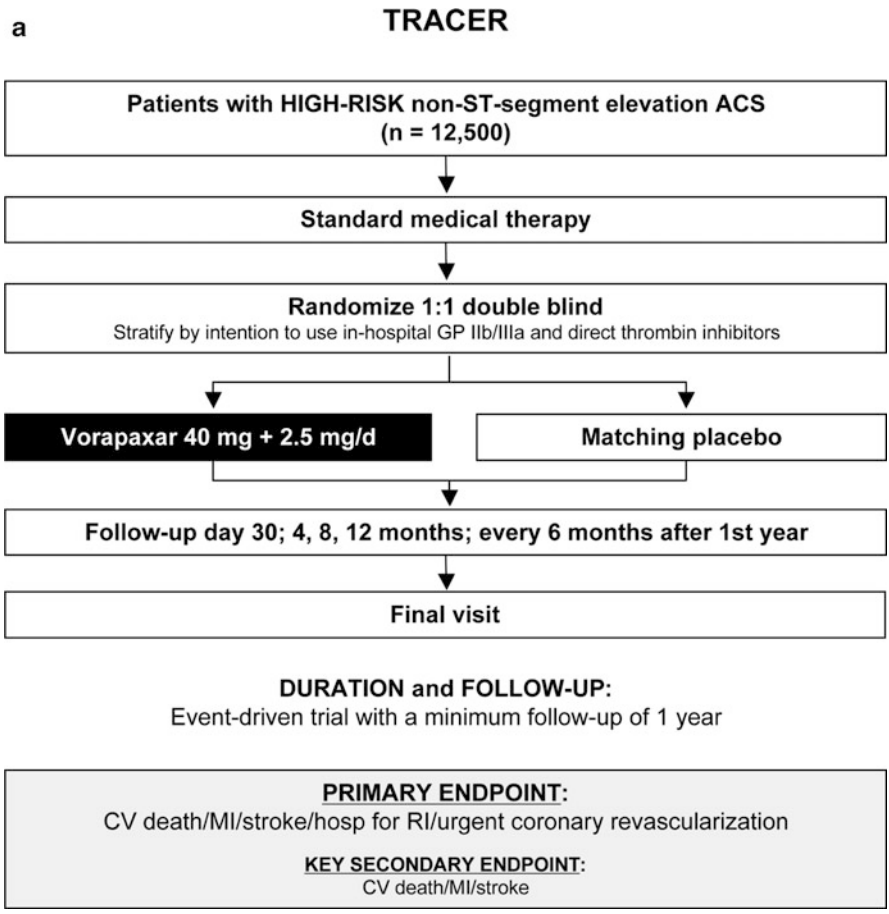


Fig. 2 (continued)

TRA•CER enrolled a total of 12,944 NSTE-ACS patients and showed that vorapaxar compared with placebo was associated with a non-significant ($P = 0.07$) 8% reduction in the HR of the primary quintuple endpoint and a nominally significant 11% reduction ($P = 0.02$) in the HR of the key secondary endpoint of cardiovascular death, MI, stroke. This difference was driven by a reduction of MI events in the vorapaxar treated patients (HR 0.88, 95% CI 0.79.0.98; $P = 0.02$), more evident on spontaneous MI. Vorapaxar however increased the risk of bleeding. Vorapaxar increased the rate of GUSTO moderate or severe bleeding, as compared with placebo (7.2% vs. 5.2%; hazard ratio, 1.35; 95% CI, 1.16 to 1.58; $P < 0.001$) (Table 4 and Fig. 2A). The rate of clinically significant TIMI bleeding was increased among patients treated with vorapaxar (20.2% vs. 14.6%; hazard ratio, 1.43; 95% CI, 1.31 to 1.57; $P < 0.001$) (Table 4 and Fig. 2B). The excess bleeding events continued to accrue during follow-up.

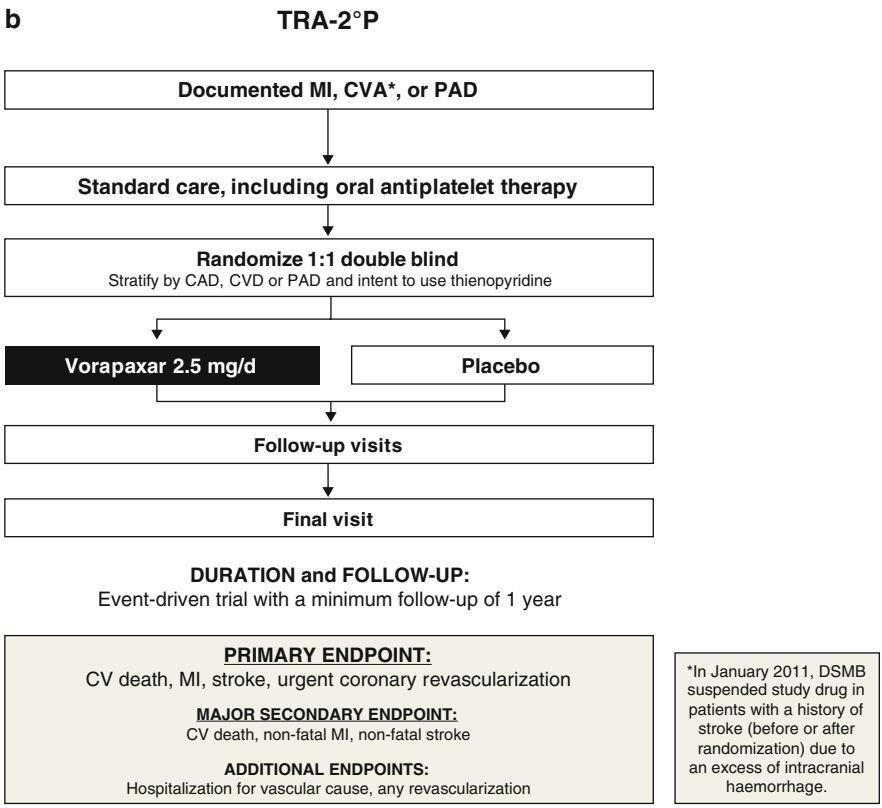


Fig. 2 Vorapaxar phase III program. (**a**) TRA-CER design and (**b**) TRA-2P design

On the basis of data from TRA•CER and before the lock of the database during blinded treatment period, the TRA 2°P steering committee reordered the hierarchy of efficacy analyses, defining as the primary end point the composite of cardiovascular death, myocardial infarction, or stroke. In over 26,000 patients who had a history of myocardial infarction, ischemic stroke, or peripheral arterial disease, vorapaxar significantly reduced the hazard of the newly defined primary end point of cardiovascular death, myocardial infarction, or stroke by 13% (HR 0.87; 95% CI 0.80 to 0.94; $P < 0.001$). The original primary end-point of cardiovascular death, myocardial infarction, stroke, or recurrent ischemia leading to revascularization was also significantly reduced (HR 0.88; 95% CI, 0.82 to 0.95; $P = 0.001$). The main bleeding endpoint (GUSTO moderate or severe bleeding) occurred in 4.2% of patients who received vorapaxar and 2.5% of those who received placebo (HR 1.66; 95% CI, 1.43 to 1.93; $P < 0.001$). Vorapaxar increased the risk of intracranial hemorrhage (1.0%, vs. 0.5% in the placebo group; $P < 0.001$).

Vorapaxar phase III program therefore suggested that this compound is effective at reducing ischemic outcomes in a broad population of patients with acute and

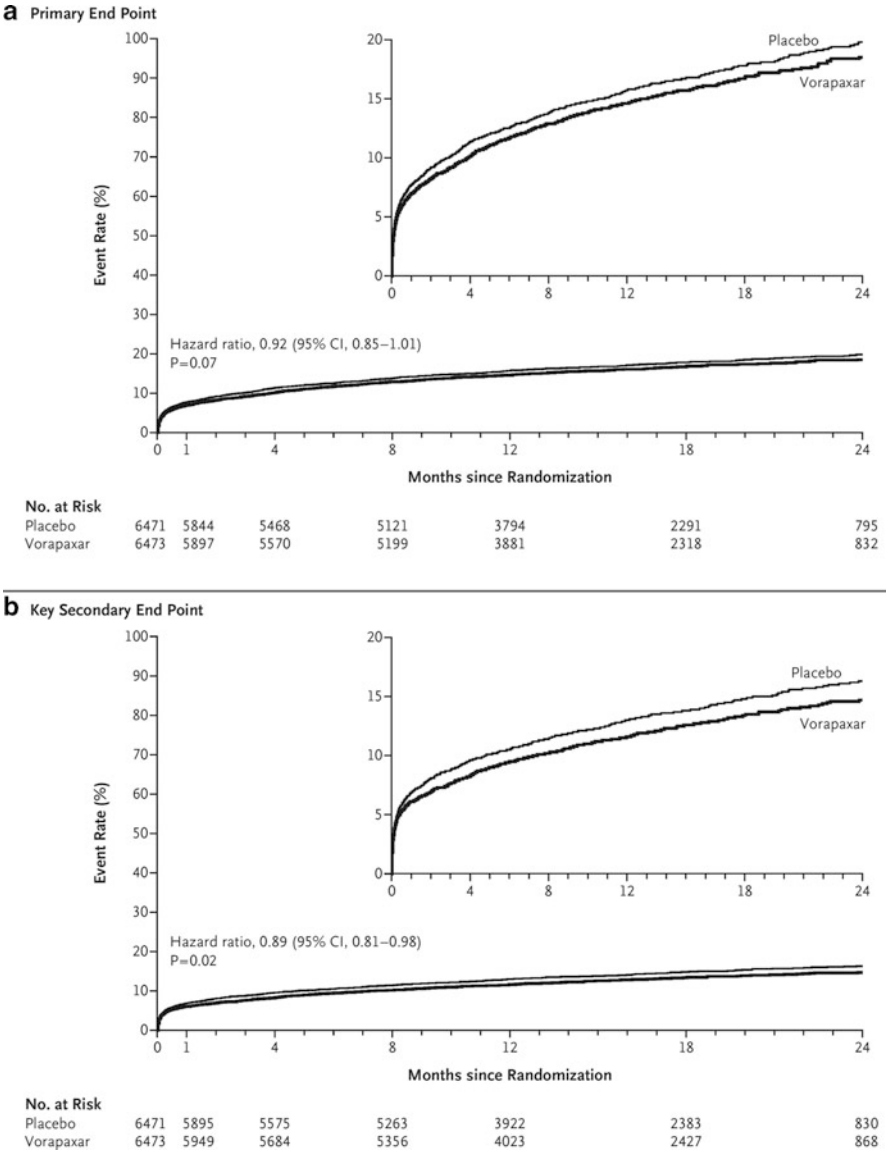


Fig. 3 Two-years Kaplan–Meier event rates with vorapaxar or placebo for the primary end point (cardiovascular death, myocardial infarction, stroke, recurrent ischemia with rehospitalization, or urgent coronary revascularization) (*Panel* **a**) and the key secondary efficacy end point (a composite of cardiovascular death, myocardial infarction, or stroke) (*Panel* **b**)

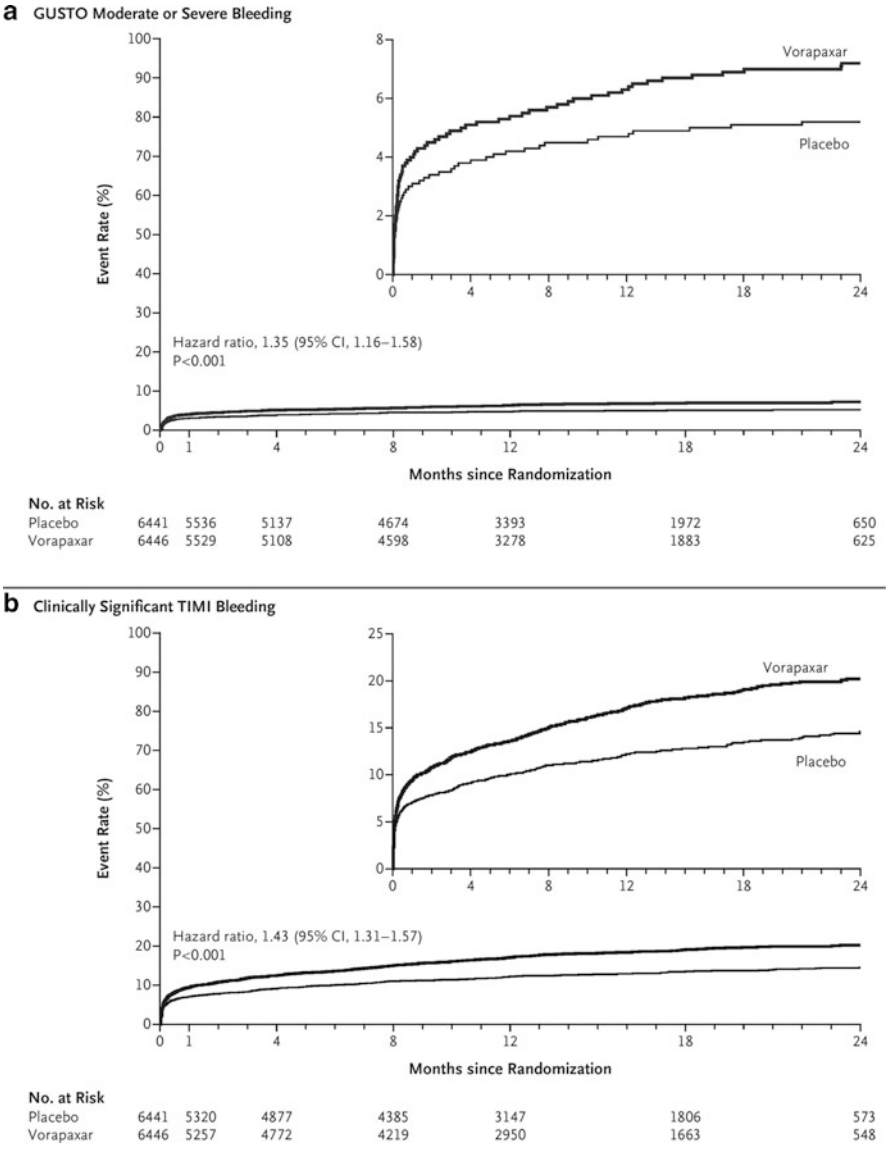


Fig. 4 Two-year Kaplan–Meier event rates with vorapaxar or placebo for Global Use of Strategies to Open Occluded Coronary Arteries (GUSTO) criteria for moderate or severe bleeding (*Panel* **a**) and for Thrombolysis in Myocardial Infarction (TIMI) criteria for clinically significant bleeding (*Panel* **b**)

chronic atherothrombosis in addition to aspirin (and often of a thienopyridine) but increased the risk of clinically important bleeding. Therefore the hypothesis that PAR-1 antagonism can mitigate thrombotic complications while preserving hemostasis was not proved in the population studied and further research will be needed to find the "sweet spot" between efficacy and safety, considering populations at lower risk of bleeding; different combinations of anti-thrombothic drug and dose and duration of treatments.

7 Atopaxar

Atopaxar (E5555) is another PAR-1 antagonist in advanced clinical testing. E5555 is an orally active, potent, and selective PAR-1 antagonist. Compared with vorapaxar, E5555 is more rapidly reversible (half-life of 22–26 h) (O'Donoghue et al. 2011) and has a primary gastrointestinal metabolism. The compound chemically defined as [1-(3-tert-butyl-4-methoxy-5-morpholinophenyl)-2-(5,6-diethoxy-7-fluoro-1-imino-1,3-dihydro-2H-isoindol-2-yl) ethanone hydrobromide)] is a small molecule based on the bicyclic amidine motif and has been developed by Eisai Co. Ltd (Tokyo, Japan).

Preclinical data suggested the potential benefit of atopaxar in the settings of acute and chronic atherothrombosis. The acute effects of atopaxar were evaluated in guinea pigs using a photochemically-induced thrombosis model. After oral administration, E5555 prolonged the time to arterial occlusion compared with controls without prolonging bleeding time, even at the highest dosages tested. E5555 showed potent and selective inhibitory effects on platelet aggregation induced by thrombin and TRAP with IC_{50} values of 0.13 and 0.097 μM, respectively, but had no effect on platelet aggregation induced by either ADP or collagen (Kogushi et al. 2011a). In a rat model of intimal thickening atopaxar selectively reduced aortic smooth muscle cell proliferation after balloon injury induced by thrombin and thrombin receptor-activating peptide (TRAP), but not proliferation induced by basic fibroblast growth factor (bFGF) (Kogushi et al. 2011b). These results suggested that, in addition to preventing thrombus formation atopaxar might attenuate restenosis following vascular intervention.

The ex vivo effects of atopaxar in humans have been reported. In blood samples taken from a group of healthy subjects ($n = 10$), patients with CAD treated with low-dose aspirin ($n = 10$), and patients with CAD treated with aspirin plus clopidogrel ($n = 10$), TRAP-induced platelet aggregation was almost completely inhibited at all tested concentrations of atopaxar (20, 50, and 100 mg/mL) without a clear concentration-dependent response (Serebruany et al. 2009). Available data suggest that this agent inhibits the binding of thrombin either at or very close to the tethered ligand-binding epitope site (Matsuoka et al. 2004) and attenuates thrombin-induced platelet aggregation with an IC value in humans of 0.064 μM (Kogushi et al. 2003). In its preliminary human testing, atopaxar reduced the release of CD-40

ligand from platelets (Kogushi et al. 2007)—a property suggesting that it might inhibit both platelet aggregation and activation in response to thrombin stimulation.

8 Phase II of Atopaxar

The safety and tolerability of atopaxar has been studied in a large phase II program—Lessons From Antagonizing the Cellular Effects of Thrombin Acute Coronary Syndromes (LANCELOT). This program included four distinct but similarly designed clinical trials with common endpoint definitions and protocol features: the Japanese studies (J-LANCELOT ACS and J-LANCELOT CAD) (Goto et al. 2010b), conducted in Japanese patients; and LANCELOT ACS (O'Donoghue et al. 2011) and CAD (Wiviott et al. 2011) conducted outside of Japan (Table 2).

J-LANCELOT were two randomized, double-blind, placebo-controlled trials conducted in patients with NSTE-ACS ($n = 241$) and high-risk CAD ($n = 263$), respectively. All patients were required to be taking aspirin (75–325 mg per day). Study duration was 12 weeks in the ACS study and 24 weeks in the CAD trial and all patients had to be followed for 4 weeks after the final dose. In the ACS study, patients randomized to atopaxar received a fixed loading dose of 400 mg daily or matching placebo. The primary endpoint was the occurrence of bleeding, which was classified according to the Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) scale (major and minor) and the Thrombolysis In Myocardial Infarction (TIMI) scale (major, minor, and minimal). Major adverse cardiovascular events (MACE), including cardiovascular death, MI, stroke, or recurrent ischemia were secondary endpoints related to efficacy. Overall, the incidence of bleeding was low. Three major (two in placebo, one in atopaxar 200 mg daily) and one minor CURE bleeding (atopaxar 200 mg daily) were observed in the ACS study and only two (one major, one minor both in atopaxar groups) in the CAD study. No TIMI major bleedings were observed, but nonmajor TIMI bleeding was numerically higher in the atopaxar 200 mg daily dose group. The incidence of MACE was low. No cardiovascular deaths were reported and 13 MACEs were observed in the ACS study (4 placebo, 9 in the pooled atopaxar groups; mainly recurrent ischemia with or without urgent revascularization) and 5 in the CAD study (3 placebo, 2 pooled atopaxar groups) with a numerical reduction in atopaxar-treated patients observed in both studies. The platelet substudy showed that, after stimulation with 15 μM of TRAP, platelet aggregation inhibition reached >90% with 100 mg and 200 mg of atopaxar, while the inhibition provided by the 50 mg dose was 50–60% in CAD patients and only 20–50% in ACS patients. In ACS patients treated with atopaxar, mean platelet inhibition reached >80% at 3–6 h after administration of the 400 mg loading dose. In both studies, atopaxar showed a significant dose-dependent increase in liver function abnormalities, especially in those on dual antiplatelet therapy, as well as a relative prolongation of the QTc interval.

Very similar was the design of the non-Japanese trials, LANCELOT ACS and LANCELOT CAD. In the LANCELOT ACS trial, 603 patients within 72 h of

Table 2 Key design features of the phase II Atopaxar program

	J-LANCELOT CAD	J-LANCELOT ACS	LANCELOT CAD	LANCELOT ACS
Patients (*N*)	263	241	720	603
Primary endpoint	Bleeding (CURE and TIMI scale)	Bleeding (CURE and TIMI scale)	Bleeding (CURE and TIMI scale)	Bleeding (CURE and TIMI scale)
Secondary endpoint	MACEs	MACEs	MACEs	MACEs
Loading dose	None	400 mg (for each atopaxar group) or MP	None	400 mg (for each atopaxar group) or MP
Maintenance dose	50, 100, or 200 mg or MP	50, 100, or 200 mg or MP	50, 100, or 200 mg or MP	50, 100, or 200 mg or MP
Follow-up (weeks)	24 + 4	12 + 4	24 + 4	12 + 4
Patients participating in the platelet substudy *N* (%)	80 (30.4)	42 (17.4)	80 (11.1)	63 (10.4)

MACEs (major adverse cardiovascular events) included cardiovascular death, myocardial infarction, stroke, and recurrent ischemia. Follow-up was 24 weeks in CAD trials and 12 weeks in ACS trials. Four additional weeks off-treatment were added to each of the four trials

non-ST-elevation ACS were randomized to 1–3 doses of atopaxar (50, 100, or 200 mg daily) or matching placebo. All patients in the atopaxar groups received a 400-mg loading dose. The incidence of CURE major bleeding, the primary endpoint, was numerically higher in the atopaxar group compared with the placebo group (1.8% vs. 0%; $P = 0.12$), while this difference was less striking for CURE major or minor bleeding (3.08% vs. 2.17%, respectively; $P = 0.63$). Bleeding was not increased in atopaxar-treated patients using the TIMI scale. The incidence of MACE (cardiovascular death, myocardial infarction, stroke, or recurrent ischemia) was similar between the atopaxar and placebo arms (8.03% vs. 7.75%; $P = 0.93$), but atopaxar significantly reduced the surrogate of ischemia on continuous ECG monitoring (Holter) at 48 h compared with placebo (relative risk, 0.67; $P = 0.02$). Dose-dependent trends for efficacy or safety were not apparent. Asymptomatic transaminase elevation and QTc prolongation were observed with the highest doses of atopaxar. Inhibition of platelet aggregation in response to 15 μmol/L TRAP was observed in the first 1–3 h after the loading dose in all active atopaxar treatment groups with a mean inhibition of platelet aggregation of 74%. At 3–6 h after the loading dose, >90% of subjects in the atopaxar groups had achieved >80% inhibition of platelet aggregation. During maintenance dosing, a trend toward greater dose-dependent inhibition of platelet aggregation was observed with increasing doses of drug. At the end of the on-treatment period of the study (week 12), mean inhibition of platelet aggregation was 66.5%, 71.5%, and 88.9% in the 50-, 100-, and 200-mg daily groups, respectively.

In LANCELOT CAD, 720 subjects with confirmed CAD underwent randomization and were treated for 24 weeks, plus 4 weeks of follow-up off-treatment. Subjects must have been receiving antiplatelet therapy with aspirin (75–325 mg daily) and/or a thienopyridine (clopidogrel or ticlopidine) for 1 month before screening. Subjects were randomly assigned at baseline to either placebo or 1 of 3 dosing regimens of atopaxar (50, 100, or 200 mg daily) in a 1:1:1:1 fashion. Key outcome measures were the proportion of subjects with any bleeding event meeting CURE or TIMI criteria through week 24 and MACE were secondary endpoints. More than 90% of patients were on aspirin and 39% of patients were on clopidogrel. There was a higher rate of any CURE bleeding with atopaxar than placebo (3.9% vs. 0.6%; $P = 0.03$), with a trend toward more bleeding across higher doses of atopaxar (P for trend $= 0.01$), but there was no difference in CURE major bleeding. Similar results were observed employing the TIMI scale. Escalating doses of atopaxar appeared to cause more overall bleeding in a dose-dependent fashion, but severe bleeding was infrequent in all treatment groups. The incidence of ischemia-related events was low overall and numerically reduced in patients randomized to atopaxar. A total of 80 subjects participated in the platelet substudy. Mean TRAP-induced inhibition of platelet aggregation (IPA) was dose-dependent. Seven days after the last dose mean IPA was almost 70% in patients receiving 200 mg daily of atopaxar, while it returned to baseline more quickly with 100 mg daily and 50 mg daily doses. Markers of inflammation, including high-sensitivity C-reactive protein, placental growth factor, and interleukin-6, were measured in the entire study population but no consistent trend for an anti-inflammatory effect was observed. Like the other

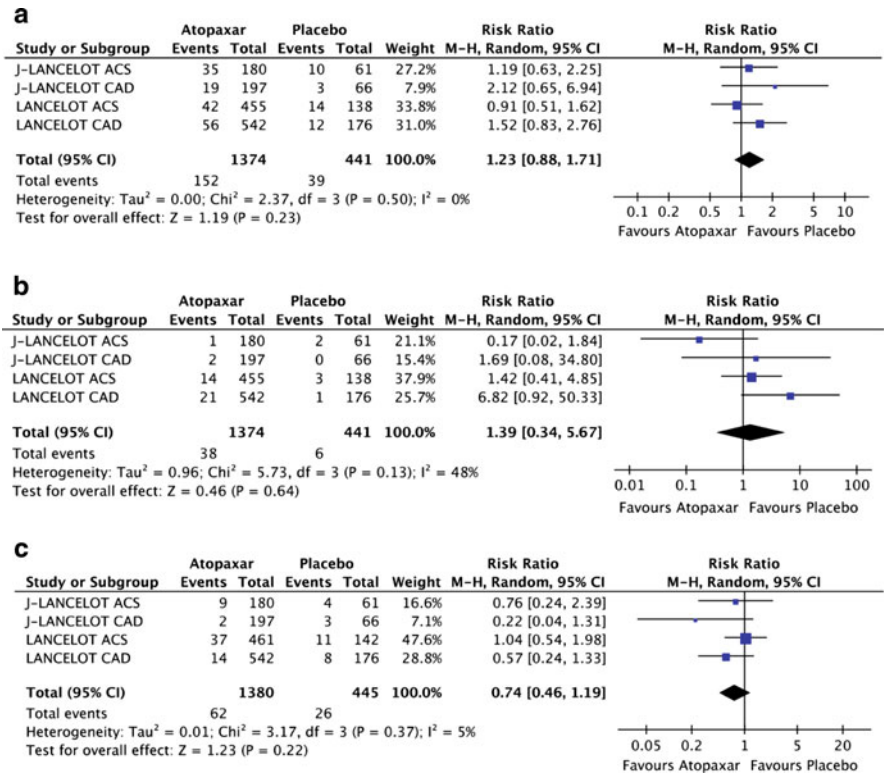


Fig. 5 LANCELOT program. (**a**) Incidence of any TIMI Bleed between pooled atopaxar doses (50, 100, and 200 mg) versus placebo. (**b**) Incidence of any CURE Bleed between pooled atopaxar doses (50, 100, and 200 mg) versus placebo. (**c**) Incidence of any MACE between pooled atopaxar doses (50, 100, and 200 mg) versus placebo. Random-effect meta-analysis of the four LANCELOT trials. Pooled estimates of risk ratio are calculated using the Mantel–Haenszel approach and presented as point estimates (95% confidence interval). For example, atopaxar (any dose) compared with placebo was associated with an increased risk of any TIMI bleeding of 23% (95% CI −12, +71), a difference that was not statistically significant ($P = 0.23$). Analyses were performed with RevMan 5.1, official software of the Cochrane Collaboration

LANCELOT trials, a transient elevation in liver transaminases and dose-dependent QTc prolongation without apparent complications was confirmed in higher-dose atopaxar treatment groups in LANCELOT CAD.

The main results of LANCELOT program are summarized in Fig. 5. In this figure we present a random-effect meta-analysis of the four trials on primary and key secondary endpoints. Overall, summary estimates of risk ratios directionally favor atopaxar on ischemia-related outcomes and placebo on bleeding-related endpoints without reaching a conventional level of statistical significance. The clinical utility of atopaxar and the significance of the safety signals for potential liver toxicity or malignant arrhythmias emerged in phase II will need to be considered and monitored closely if a large phase III program commences.

9 Conclusions

Despite important advancements in the pharmacological inhibition of $P2Y_{12}$-dependent platelet activation, recurrent ischemic events are common and bleeding tracks the intensity of $P2Y_{12}$ inhibition. PAR-1 inhibitors are a novel class of antiplatelet agents that inhibit thrombin-mediated platelet activation and were designed to complement current treatment options for patients with atherothrombosis. While pre-clinical data and observations from phase II investigations support the hypothesis that this pathway contributes to arterial thrombosis but may not be essential for protective haemostasis, the increase in clinically important bleeding including intracranial hemorrhage observed with vorapaxar indicate that this may not be true in selected groups of patients. Future research will be needed to establish potential mechanisms for this bleeding excess, especially in patients with a history of stroke (role of PAR-1 inhibition on top of dual antiplatelet therapy? Central nervous system-specific mechanisms?), and the role of these new antiplatelet agents in selected patients with acute and chronic atherothrombosis.

Knowledge Gaps

- It remains to be determined whether PAR-1 inhibition may be better tolerated in patients with either stable coronary heart disease or peripheral vascular disease in whom mono-platelet-directed therapy represents a common strategy.
- The large ongoing TRA•CER biomarker repository may help to identify a patient-specific profile or signature that portends a heightened or possibly prohibitive risk with antithrombotic therapy.

Key Messages

- Despite dual antiplatelet inhibition with aspirin and a $P2Y_{12}$ inhibitor in patients with cardiovascular disease, major adverse cardiovascular events are not uncommon and bleeding tracks the intensity of $P2Y_{12}$ inhibition.
- Thrombin, a highly potent platelet activator operating primarily via the PAR-1 receptor, may contribute to the residual risk of cardiovascular events.
- PAR-1 inhibitors (also called thrombin receptor antagonists) are a novel class of antiplatelet agents that selectively inhibit the PAR-1 receptor.
- Two compounds, vorapaxar (SCH530348) and atopaxar (E5555), are in advanced clinical development. Both are orally active, potent, and selective PAR-1 inhibitors. While vorapaxar has a very long half-life (>100 h) atopaxar is more rapidly reversible (22–26 h).

(continued)

- Phase II testing of these compounds yielded encouraging observations in terms of ischemic efficacy and did not raise overt safety concerns of bleeding risk.
- Vorapaxar, in a broad, unselected population of patients with NSTE-ACS and in secondary prevention of patients with a history of myocardial infarction, stroke, or PAD, reduced the risk of major ischemic outcomes, particularly MI, suggesting that thrombin-mediated platelet activation has a role in the residual risk of acute and chronic atherothrombotic complications. However, vorapaxar increased the risk of clinically important bleeding, including intracranial hemorrhage. Therefore the hypothesis that this PAR-1 antagonist can mitigate ischemic complications while preserving hemostasis was not proved in the population studied. Novel investigations will be needed to understand if a more favorable trade-off between efficacy and safety could be achieved in different populations, or with different durations, dose and combinations of anti-thrombothic.

References

Becker RC, Moliterno DJ, Jennings LK et al (2009) Safety and tolerability of SCH 530348 in patients undergoing non-urgent percutaneous coronary intervention: a randomised, double-blind, placebo-controlled phase II study. Lancet 373:919–928

Bhatt DL, Lincoff AM, Gibson CM et al (2009) Intravenous platelet blockade with cangrelor during PCI. N Engl J Med 361:2330–2341

Chackalamannil S, Wang Y, Greenlee WJ et al (2008) Discovery of a novel, orally active himbacine-based thrombin receptor antagonist (SCH 530348) with potent antiplatelet activity. J Med Chem 51:3061–3064

Chintala M, Vemulapalli S, Kurowski S, Sabin C, Reynolds D, Prevete K, Friedrichs G (2008) (2008) SCH 530348, a novel oral antiplatelet agent, demonstrated no bleeding risk alone or in combination with aspirin and clopidogrel in cynomolgus monkeys. Atheroscler Thromb Vasc Biol 28:e32–e149

Chintala M, Strony J, Yang B, Kurowski S, Li Q (2010) SCH 602539, a protease-activated receptor-1 antagonist, inhibits thrombosis alone and in combination with cangrelor in a Folts model of arterial thrombosis in cynomolgus monkeys. Arterioscler Thromb Vasc Biol 30:2143–2149

Coughlin SR (1999) How the protease thrombin talks to cells. Proc Natl Acad Sci USA 96:11023–11027

Coughlin SR (2000) Thrombin signalling and protease-activated receptors. Nature 407:258–264

Davì G, Patrono C (2007) Platelet activation and atherothrombosis. N Engl J Med 357:2482–2494

Dubois C, Steiner B, Meyer Reigner SC (2004) Contribution of PAR-1, PAR-4 and GPIbalpha in intracellular signaling leading to the cleavage of the beta3 cytoplasmic domain during thrombin-induced platelet aggregation. Thromb Haemost 91:733–742

Eikelboom JW, Weitz JI, Budaj A et al (2002) Clopidogrel does not suppress blood markers of coagulation activation in aspirin-treated patients with non-ST-elevation acute coronary syndromes. Eur Heart J 23:1771–1779

Ghosal A, Lu X, Penner N et al (2011) Identification of human liver cytochrome P450 enzymes involved in the metabolism of SCH 530348 (Vorapaxar), a potent oral thrombin protease-activated receptor 1 antagonist. Drug Metab Dispos 39:30–38

Goto S, Yamaguchi T, Ikeda Y, Kato K, Yamaguchi H, Jensen P (2010a) Safety and exploratory efficacy of the novel thrombin receptor (PAR-1) antagonist SCH 530348 for non-ST-segment elevation acute coronary syndrome. J Atheroscler Thromb 17:156–164

Goto S, Ogawa H, Takeuchi M, Flather MD, Bhatt DL (2010b) Double-blind, placebo-controlled Phase II studies of the protease-activated receptor 1 antagonist E5555 (atopaxar) in Japanese patients with acute coronary syndrome or high-risk coronary artery disease. Eur Heart J 31:2601–2613

Gurbel PA, Tantry US (2010) Combination antithrombotic therapies. Circulation 121:569–583

Hamilton JR, Cornelissen I, Coughlin SR (2004) Impaired hemostasis and protection against thrombosis in protease-activated receptor 4-deficient mice is due to lack of thrombin signaling in platelets. J Thromb Haemost 2:1429–1435

Harrington RA, Stone GW, McNulty S et al (2009) Platelet inhibition with cangrelor in patients undergoing PCI. N Engl J Med 361:2318–2329

Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR (1999) Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. J Clin Invest 103:879–887

Kogushi M, Kobayashi H, Matsuoka T et al (2003) Antithrombotic and bleeding time effects of E5555, an orally active protease-activated receptor-1 antagonist, in guinea pigs. Circulation 108:280, Abstract

Kogushi M, Yokohama H, Kitamura S et al (2007) Effects of E5555, a protease-activated receptor-1 antagonist, on the inflammatory markers in vitro. Presented at XXIst congress of the International Society on Thrombosis and Haemostasis, Geneva, Switzerland

Kogushi M, Matsuoka T, Kawata T et al (2011a) The novel and orally active thrombin receptor antagonist E5555 (Atopaxar) inhibits arterial thrombosis without affecting bleeding time in guinea pigs. Eur J Pharmacol 657:131–137

Kogushi M, Matsuoka T, Kuramochi H et al (2011b) Oral administration of the thrombin receptor antagonist E5555 (atopaxar) attenuates intimal thickening following balloon injury in rats. Eur J Pharmacol 666:158–164

Leonardi S, Rao SV, Harrington RA et al (2010) Rationale and design of the randomized, double-blind trial testing INtraveNous and Oral administration of elinogrel, a selective and reversible P2Y12-receptor inhibitor, versus clopidogrel to eVAluate Tolerability and Efficacy in nonurgent Percutaneous Coronary Interventions patients (INNOVATE-PCI). Am Heart J 160:65–72

Matsuoka T, Kogushi M, Kawata T et al (2004) Inhibitory effect of E5555, an orally active thrombin receptor antagonist, on intimal hyperplasia following balloon injury. J Am Coll Cardiol 43:68A

Morrow DA, Scirica BM, Fox KA et al (2009) Evaluation of a novel antiplatelet agent for secondary prevention in patients with a history of atherosclerotic disease: design and rationale for the Thrombin-Receptor Antagonist in Secondary Prevention of Atherothrombotic Ischemic Events (TRA 2°P)-TIMI 50 trial. Am Heart J 158:335.e3–341.e3

Morrow DA, Braunwald E, Bonaca MP et al (2012) Vorapaxar in the secondary prevention of atherothrombotic events. N Engl J Med 366(15):1404–1413

O'Donoghue ML, Bhatt DL, Wiviott SD et al (2011) Safety and tolerability of atopaxar in the treatment of patients with acute coronary syndromes/clinical perspective. Circulation 123:1843–1853

Patrono C, García Rodríguez LA, Landolfi R, Baigent C (2005) Low-dose aspirin for the prevention of atherothrombosis. N Engl J Med 353:2373–2383

Serebruany VL, Kogushi M, Dastros-Pitei D, Flather M, Bhatt DL (2009) The in-vitro effects of E5555, a protease-activated receptor (PAR)-1 antagonist, on platelet biomarkers in healthy volunteers and patients with coronary artery disease. Thromb Haemost 102:111–119

Shinohara Y, Goto S, Doi M, Jensen P (2012) Safety of the novel protease-activated receptor-1 antagonist Vorapaxar in Japanese patients with a history of ischemic stroke. J Stroke Cerebrovasc Dis 1(4):318–24

The Clopidogrel in Unstable Angina to Prevent Recurrent Events Trial Investigators (2001) Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med 345:494–502

The TRACER Executive Steering C (2009) The Thrombin Receptor Antagonist for Clinical Event Reduction in Acute Coronary Syndrome (TRA-CER) trial: study design and rationale. Am Hear J 158:327–334

Tricoci P, Huang Z, Held C et al (2012) Thrombin-receptor antagonist vorapaxar in acute coronary syndromes. N Engl J Med 366(1):20–33

Wallentin L, Varenhorst C, James S et al (2008) Prasugrel achieves greater and faster P2Y12receptor-mediated platelet inhibition than clopidogrel due to more efficient generation of its active metabolite in aspirin-treated patients with coronary artery disease. Eur Heart J 29:21–30

Wallentin L, Becker RC, Budaj A et al (2009) Ticagrelor versus clopidogrel in patients with acute coronary syndromes. N Engl J Med 361:1045–1057

Wiviott SD, Braunwald E, McCabe CH et al (2007) Prasugrel versus clopidogrel in patients with acute coronary syndromes. N Engl J Med 357:2001–2015

Wiviott SD, Flather MD, O'Donoghue ML et al (2011) Randomized trial of atopaxar in the treatment of patients with coronary artery disease/clinical perspective. Circulation 123:1854–1863

Thromboxane Receptors Antagonists and/or Synthase Inhibitors

Giovanni Davì, Francesca Santilli, and Natale Vazzana

Contents

G. Davì (✉) • F. Santilli • N. Vazzana
Internal Medicine, University of Chieti, Chieti, Italy
e-mail: gdavi@unich.it

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_11,

Abstract Atherothrombosis is the major cause of mortality and morbidity in Western countries. Several clinical conditions are characterized by increased incidence of cardiovascular events and enhanced thromboxane (TX)-dependent platelet activation. Enhanced TX generation may be explained by mechanisms relatively insensitive to aspirin. More potent drugs possibly overcoming aspirin efficacy may be desirable. Thromboxane synthase inhibitors (TXSI) and thromboxane receptor antagonists (TXRA) have the potential to prove more effective than aspirin due to their different mechanism of action along the pathway of TXA_2. TXSI prevent the conversion of PGH_2 to TXA_2, reducing TXA_2 synthesis mainly in platelets, whereas TXRA block the downstream consequences of TXA_2 receptors (TP) activation.

TXA_2 is a potent inducer of platelet activation through its interaction with TP on platelets. TP are activated not only by TXA_2, but also by prostaglandin (PG) D_2, PGE_2, $PGF_{2\alpha}$, PGH_2, PG endoperoxides (i.e., 20-HETE), and isoprostanes, all representing aspirin-insensitive mechanisms of TP activation. Moreover, TP are also expressed on several cell types such as macrophages or monocytes, and vascular endothelial cells, and exert antiatherosclerotic, antivasoconstrictive, and antithrombotic effects, depending on the cellular target.

Thus, targeting TP receptor, a common downstream pathway for both platelet and extraplatelet TXA_2 as well as for endoperoxides and isoprostanes, may be a useful antiatherosclerotic and a more powerful antithrombotic intervention in clinical settings, such as diabetes mellitus, characterized by persistently enhanced thromboxane (TX)-dependent platelet activation through isoprostane formation and low-grade inflammation, leading to extraplatelet sources of TXA_2. Among TXRA, terutroban is an orally active drug in clinical development for use in secondary prevention of thrombotic events in cardiovascular disease. Despite great expectations on this drug supported by a large body of preclinical and clinical evidence and pathophysiological rationale, the PERFORM trial failed to demonstrate the superiority of terutroban over aspirin in secondary prevention of cerebrovascular and cardiovascular events among ~20,000 patients with stroke. However, the clinical setting and the design of the study in which the drug has been challenged may explain, at least in part, this unexpected finding.

Drugs with dual action, such as dual TXS inhibitors/TP antagonist and dual COXIB/TP antagonists are currently in clinical development. The theoretical rationale for their benefit and the ongoing clinical studies are herein discussed.

Keywords Antiplatelet therapy • Atherothrombosis • Ischemic stroke • Platelet activation • Thromboxane biosynthesis • TP antagonists

1 Introduction

Atherosclerosis and its clinical manifestations (i.e., ischemic heart disease, cerebrovascular or peripheral artery disease) are major causes of mortality and morbidity in Western countries.

Conventional antiplatelet agents such as aspirin or clopidogrel are currently used in the prevention of cardiovascular events. However, more effective drugs with less bleeding or gastrointestinal complications are desirable.

Thromboxane (TX) A_2 is involved in a diverse range of physiological and pathophysiological processes, including thrombosis, asthma, myocardial infarction (MI), inflammation, acquired immunity, and atherogenesis. Thus, the stimulation of TX/endoperoxide receptors (TP) elicits diverse biological effects under both normal and pathological conditions. Stimulation of TP results in activation of different signaling cascades that regulate the cytoskeleton, cell adhesion, motility, nuclear transcription factors, proliferation, cell survival, and apoptosis (Nakahata 2008).

TXA_2 is the major product of the arachidonic acid (AA) metabolism in platelets that, in response to various stimuli, is produced via the consequent actions of cyclooxygenase (COX) and TX synthase (TXS). Through its interaction with TP receptors on platelets, TXA_2 is a potent inducer of platelet activation (Davì and Patrono 2007).

Furthermore, the activation of endothelial TP promotes the expression of adhesion molecules and favors adhesion and infiltration of monocytes/macrophages. TP receptors exhibit a wide distribution within the cardiovascular system; in fact, these receptors are membrane-bound G protein-coupled receptors (GPCR) found not only on platelets but also on circulating inflammatory cells, such as macrophages or monocytes, and in vascular endothelial cells, and smooth muscle cells (Meja et al. 1997; Miggin and Kinsella 1998).

Thus, antagonists of TP may have some advantages over aspirin as they not only block the effect of TXA_2 on platelets, but also inhibit other ligands such as prostaglandin (PG) endoperoxides and isoprostanes. Because of the wide distribution of TP receptors in platelets, in the vascular wall or in atherosclerotic plaques, TP antagonists inhibit also the effects of TXA_2 over TP receptors on vascular cells or in the plaque. Therefore, antagonists of TP receptors may not only have antiplatelet effects but also impact endothelial dysfunction and the inflammatory component of atherosclerosis (Chamorro 2009).

2 TP Receptors and Their Antagonism

2.1 Molecular and Cellular Biology

TP receptor is highly distributed in various tissues. In addition to platelets, endothelial cells, smooth muscle cells, glomerular mesangial cells, cardiac myocytes, and many other cells express the TP receptor (Fig. 1) (Nakahata 2008).

The multiple cellular signaling and regulatory mechanisms activated through TP have been only recently characterized. In particular, it has been reported that reactive oxidant species (ROS)-dependent upregulation of TP expression may increase TXA_2/isoprostane responses. This mechanism may significantly contribute

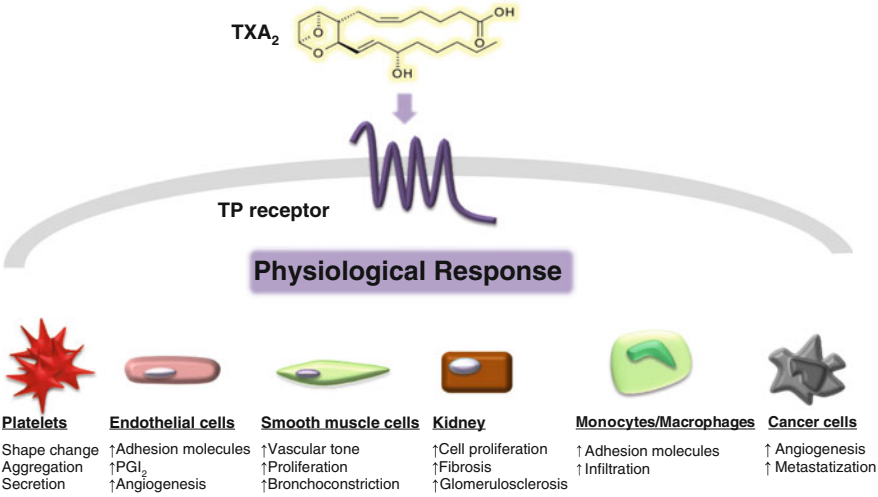


Fig. 1 TP receptor expression and functions. The thromboxane (TX) A_2 receptor (TP) is expressed in a variety of tissues and cells such as platelets, endothelial cells, vascular and bronchial smooth muscle cells, kidney, monocyte/macrophages, and cancer cells. TP activation in these cells is critically involved in the pathobiology of a wide range of diseases, including atherothrombosis, hypertension, renal disease, immune/inflammatory disease, and cancerogenesis. Abbreviations: *PGI_2* prostacyclin

to platelet activation and atherothrombosis in several clinical setting associated with enhanced oxidative stress, such as hypercholesterolemia, obesity, and type 2 diabetes mellitus (T2DM). In addition, posttranscriptional modifications of the receptor, such as phosphorylation or glycation, also determine TP internalization or ligand desensitization. Whether a TP antagonist may affect this receptorial cross-talk and these regulatory pathways is still unanswered.

TXA_2 is the major product of the AA metabolism in platelets that, in response to various stimuli, is produced via the consequent actions of COX and TXS (Davì and Patrono 2007). Low-dose aspirin inhibits platelet TXA_2 production through permanent inactivation of the COX activity of the enzyme prostaglandin G/H-synthase (Fig. 2), and it has clearly been shown to be cardioprotective in several clinical settings (Patrono et al. 2005b). However, many nucleated cells and tissues produce TXA_2 in response to proinflammatory signals and oxidative stress, which acts in an autocrine or paracrine manner as aspirin-insensitive TP agonists. TP receptors are activated not only by TXA_2, but also by PGD_2, PGE_2, $PGF_{2\alpha}$, PGH_2, PG endoperoxides (i.e., 20-HETE), and isoprostanes (Fig. 2).

By binding to TP receptor, these molecules activate several signaling cascades which regulate endothelial cell activation (i.e., adhesion molecules expression), vascular smooth muscle cell (VSMC) contraction, and platelet aggregation, thereby accelerating progression of atherosclerotic lesions (Dogne et al. 2005) (Fig. 3).

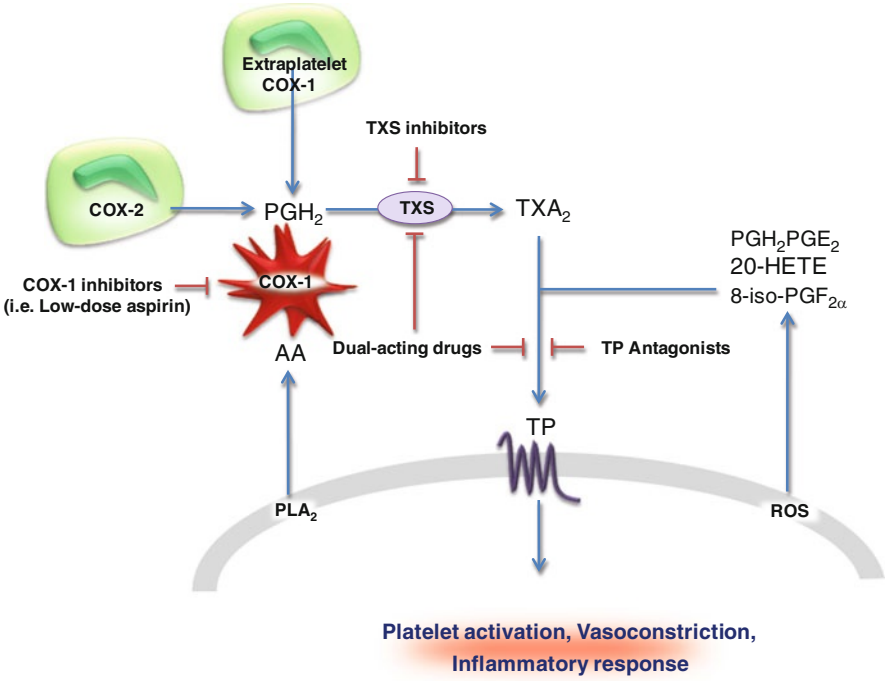


Fig. 2 Acting sites of drugs affecting the TXA_2/TP pathway. Low-dose aspirin is an irreversible inhibitor of the cyclooxygenase (COX) activity of platelet prostaglandin (PG) H_2 synthase-1. Thromboxane synthase (TXS) inhibitors (i.e., ozagrel) block the conversion of PGH_2 to TXA_2. However, TXS antagonism may increase PGH_2, which acts on TP as an agonist, thereby counteracting the antithrombotic efficacy. Dual-acting drugs (TXS inhibitors/TP antagonists: ridogrel, picotamide) and TP antagonists (terutroban) act downstream by blocking the activation of the thromboxane receptor (TP) by TXA_2 as well as other prostanoids, including PGH_2, PGE_2, endoperoxides, and F2-isoprostane. Abbreviations: *HETE* hydroxyeicosatetraenoic acid

In addition to platelets and endothelial cells, TP receptors are also expressed in other cell types involved in atherothrombosis, such as VSMCs (Miggin and Kinsella 1998), macrophages, and monocytes (Meja et al. 1997) (Fig. 1). More recently, it has been reported that TP is expressed in prostate cancer where it regulates cell motility and cytoskeleton reorganization, thus representing a novel target of antineoplastic interventions.

2.2 *TP Receptor Signaling in Platelets*

TXA_2 positively amplify platelet aggregation and is responsible for platelet aggregation in the presence of low concentrations of aggregatory stimuli, such as epinephrine and thrombin. This effect is fully reversed by aspirin or selective antibodies blocking TXS (Mehta et al. 1986).

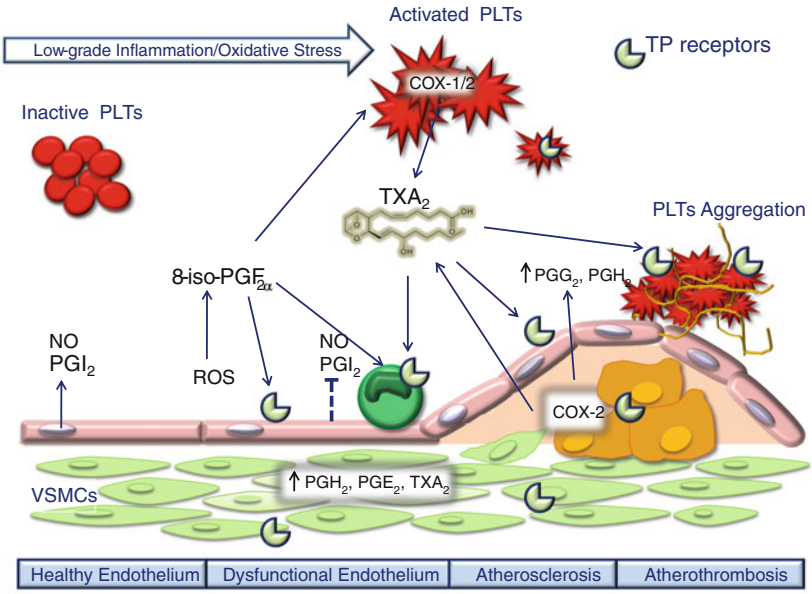


Fig. 3 The role of TXA_2/TP receptor pathway in atherothrombosis. Within the atherosclerotic plaque, the TP receptors are widely expressed on platelets (PLTs), endothelial cells, vascular smooth muscle cells (VSMCs), circulating monocytes, and resident macrophages. Stimulation of TP receptors by their ligands (TXA_2, prostaglandins, endoperoxides, and F2-isoprostanes) activates several signaling cascades which regulate endothelial cell activation (i.e., adhesion molecules expression), VSMCs contraction, and platelet aggregation, thereby accelerating progression of atherosclerotic lesions. Abbreviation: NO nitric oxide

Both TXA_2 and its precursor PGH_2 bind the TP receptor in platelets. TXA_2 is more potent than PGH_2 in initiating aggregation in platelet-rich plasma with EC50 of 66 ± 15 nM and 2.5 ± 1.3 mM, respectively. Conversely in washed platelets, PGH_2 is more potent than TXA_2 with EC50 values of 45 ± 2 and 163 ± 21 nM, respectively. However, whether PGH_2 may significantly contribute to the responses attributed to TXA_2 in vivo is still to be investigated (Mayeux et al. 1988).

In washed human platelets, $PGF_{2\alpha}$ and PGD_2 display lower affinity interactions to TP receptor (Mayeux et al. 1988). Individual prostanoid receptors display ~20–30% sequence identity and encode specific motifs common only to members of the subfamily of PG receptors. Given their structural similarities, PGs may activate more than one subtype of PG receptor. F2 isoprostanes are nonenzymatic, free radical-catalyzed products of AA relatively more stable than TXA_2. These molecules, in particular 8-iso-$PGF_{2\alpha}$, have been shown to bind TP and modulate the adhesive reactions and activation of platelets induced by low levels of other agonists.

2.3 TP Receptor Signaling in Vascular Endothelial and Smooth Muscle Cells

Increased vascular tone due to generation of prostanoids is a main feature of endothelial dysfunction. In fact, endothelium dysfunction is characterized by an increased production of prostanoids (i.e., TXA_2), which facilitate the penetration of macrophages in the vessel wall (Feletou et al. 2009). On endothelial cells, TXA_2 activates the expression of adhesion proteins, such as ICAM-1, VCAM-1, and endothelial leukocyte adhesion molecule-1 (ELAM-1) (Ishizuka et al. 1996). TP-receptor dependent expression of ICAM-1, VCAM-1, and ELAM-1 is mediated by protein kinase C (Ishizuka et al. 1998). TP activation also stimulates the expression of leukocytes adhesion molecules (LAM) on endothelial cells (Ashton et al. 2003). TP activation also promotes prostacyclin (PGI_2) production from endothelial cells through a negative feedback counterregulatory response (Cheng et al. 2002). In fact, PGI_2 attenuates platelet aggregation and VSMC contraction.

In several cardiovascular diseases, endothelial dysfunction is the result of the release of endothelium-derived contracting factors (EDCF) that counteract the vasodilator effect of nitric oxide (NO) produced by the endothelial NO synthase. These endogenous TP agonists produced by the vascular endothelium cause endothelium-dependent contraction and contribute to endothelial dysfunction, a key factor in atherogenesis. Endothelium-dependent contractions involve activation of COXs, production of ROS along with that of EDCFs, which diffuse toward the vascular smooth muscle cells and activate their TP.

Besides the activity of endothelial COX-1, the activation of TP-receptors on VSMCs is also relevant (Swinnen et al. 2009). TP-receptor activation stimulates VSMC proliferation and hypertrophy (Uehara et al. 1988), by potentiating the mitogenic effects of platelet derived growth factor (PDGF) and by increasing the synthesis and release of endogenous basic fibroblast growth factor (bFGF) (Ali et al. 1993).

3 Drugs Affecting TXA_2 Action: Other than COX Inhibitors

The dramatic success story of aspirin as antiplatelet drug had a paradoxically negative effect on the development of drugs that work by closely related but distinct mechanisms to that of aspirin (Fig. 2). TXS inhibitors and TP antagonists were perceived as too close to aspirin to compete effectively with this inexpensive and effective drug.

3.1 Inhibitors of Thromboxane Synthase

The inhibitors of TXS prevent the conversion of PGH_2 to TXA_2. These drugs reduce TXA_2 synthesis mainly in platelets and may improve TXA_2-mediated

pathophysiological conditions, such as thrombosis formation and thrombosis-related disorders (Dogne et al. 2004). TXS inhibitors also enhance vascular generation of PGI_2, which prevents platelet aggregation induced by all known agonists (FitzGerald et al. 1985). In fact, TXS inhibition leads to accumulation of PG endoperoxides in platelets that may be donated to endothelial prostacyclin synthase at sites of platelet–vascular interactions (endoperoxide "steal") (FitzGerald et al. 1985). Consistent with this hypothesis, increased PGI_2 generation in vivo has been reported after administration of several TXS inhibitors (FitzGerald et al. 1985). As PGI_2 may inhibit platelet activation by both TX-dependent and TX-independent mechanisms, it has been proposed that TXS inhibitors may be more effective than aspirin to prevent atherothrombosis.

Several TXS inhibitors—including dazoxiben, dazmagrel, pirmagrel, ozagrel, isbogrel, and furegrelate—have been tested in clinical settings associated with enhanced TX generation.

The greatest experience in human thrombotic disease has been gained with dazoxiben. However, the benefits of this molecule in patients with coronary heart disease were limited or absent (FitzGerald et al. 1985; Kiff et al. 1983; Thaulow et al. 1984).

Ozagrel has been used clinically since 1992 in Japan for the treatment of asthma. Treatment with ozagrel significantly reduced TX generation in patients with coronary or cerebrovascular disease (Uyama et al. 1985; Yui et al. 1984). However, this effect resulted into limited clinical benefit (Shikano et al. 1987).

It has been proposed that incomplete suppression of TX generation in vivo may partly account for the lack of clinical efficacy of these drugs. In addition, TXS inhibition increases PGI_2 generation as well as the formation of other prostanoids, including PGH_2 and endoperoxides, which act as TP agonists, thereby counteracting the reduction of TXA_2-mediated events (Nakahata 2008).

3.2 Dual TXS Inhibition/TP Antagonism

Combined TXS inhibitors and TP antagonist may theoretically overcome the limitations observed for TXS inhibitors. In fact, these drugs do not affect (or enhance) PGI_2 generation, while preventing TP activation by residual TX as well as by other agonists (Patscheke 1990).

Accordingly, the combined administration of a dual TXS inhibitor/TP antagonist gives stronger inhibition of platelet aggregation and prolongs bleeding time more than either drug alone or acetylsalicylic acid (Patrono 1990).

Ridogrel is a drug developed more than 20 years ago as a more potent antiplatelet agent than aspirin. Ridogrel is a TXA_2 inhibitor with additional TP antagonist properties that further enhance its antiaggregatory effects by diverting endoperoxide intermediates into the PGI_2 production pathway (Meadows and Bhatt 2007).

Ridogrel has been studied primarily as an adjunctive agent to thrombolytic therapy in acute MI. In 1993, animal studies showed that ridogrel limits MI size after mechanical coronary occlusion and reperfusion at doses enhancing coronary thrombolysis by streptokinase (Meadows and Bhatt 2007; Vandeplassche et al. 1993).

Thus, the Ridogrel Versus Aspirin Patency Trial (RAPT) was performed to compare the efficacy and safety of ridogrel with that of aspirin as conjunctive therapy for thrombolysis in patients with acute MI. However, despite positive results from initial pilot studies, the largest clinical study, the RAPT, failed to demonstrate any advantage with this agent over aspirin. In fact, in the study of 907 patients with acute MI, there was no difference in the primary end point of infarct vessel patency rate between those randomized to ridogrel (72.2%) or aspirin (75.5%). Despite ridogrel was not superior to aspirin in enhancing the fibrinolytic efficacy of streptokinase, it was more effective in preventing new ischemic events (The Ridogrel Versus Aspirin Patency Trial 1994).

In ulcerative colitis, local production of PGE_2, PGI_2, and TXA_2 has been demonstrated. The inflammatory infiltrate in ulcerative colitis consists of polymorphonuclear leukocytes, mononuclear leukocytes, and macrophages, all of which release considerable amounts of TXA_2. Although PGE_2 may have protective effects on intestinal mucosa, TXA_2 appears to promote the development of chronic inflammatory lesions in the bowel. Thus, an imbalance between the synthesis of cytoprotective prostaglandins, such as PGE_2, and of the pro-inflammatory TXA_2 may play a role in the development of chronic inflammation and mucosal damage in patients with ulcerative colitis.

Treatment with selective inhibitors of TXA_2 synthesis, including ridogrel, reduced the release of TXA_2, tissue damage, and the development of chronic inflammatory lesions in the colon. A pilot clinical trial in patients with chronic ulcerative colitis demonstrated that high-dose ridogrel (300 mg twice daily) significantly reduced colonic mucosal TXA_2 release, to 31% of basal levels, without significantly reducing the levels of protective PGE_2. However, two multicentre, randomized, double-blind studies failed to find significant differences in the primary efficacy outcome measure among two different doses of ridogrel and placebo. Thus, there was no clear indication in either trial of an effective dose of ridogrel in the treatment of ulcerative colitis (Tytgat et al. 2002).

Various mechanisms are likely responsible for the results observed with ridogrel in clinical trials, including potentially ineffective TP inhibition with the concentrations of ridogrel used in human studies. As such, there currently are no clinical indications for preferential use of ridogrel over aspirin.

Another drug with dual action (TXS inhibition/TP antagonism) is picotamide. In a double blind, randomized trial (ADEP), 2,304 patients with intermittent claudication were allocated to receive picotamide or placebo. However, the trial showed only a nonsignificant benefit of picotamide versus placebo in patients with peripheral artery disease (PAD) (Basili et al. 2010; Neri Serneri et al. 2004).

More recently, picotamide has been studied in diabetics with PAD randomized to receive picotamide or aspirin for 2 years (DAVID study). Overall mortality, the

predefined primary end point, was significantly lower among patients who received picotamide (3.0%) than in those who received aspirin (5.5%) with a relative risk ratio for picotamide versus aspirin of 0.55 (95% CI 0.31–0.98%). Conversely, the combined end point of mortality and morbidity has a slightly lower incidence in the picotamide group, but this difference does not reach statistical significance (Neri Serneri et al. 2004). The results of this study should be cautiously interpreted in the light of its limited statistical power and sample size. In fact, as confirmed by a recent meta-analysis comparing the efficacy of different antiplatelet treatments in patients with PAD, picotamide, like aspirin, is not associated with a statistically significant reduction in cardiovascular adverse events (Violi and Hiatt 2007). Conversely, a significant reduction in cardiovascular risk is observed with thienopyridines, suggesting that the presence of PAD may render platelet activation more critically dependent on ADP than on TXA_2 release.

4 Dual COXIB/TP Antagonists: A Possible New Twist in NSAID Pharmacology and Cardiovascular Risk

In the early 1990s, a new class of nonsteroidal anti-inflammatory drug (NSAID) became available (COX-2 inhibitors, or COXIBs). The gastrointestinal safety, dependent upon lack of COX-1 inhibition, coupled with the emerging evidence of cardiovascular hazard associated with COXIBs, leading to withdrawal of rofecoxib and valdecoxib, suggested that a potentially safer pharmacological approach should be combining the anti-inflammatory activity of COXIBs together with a cardioprotective component which might involve antagonism of TP receptors. This could be achieved by making a simple combination of existing drugs targeted against COX-2 or the TP receptor.

The possibility to combine powerful anti-inflammatory activity with TP antagonism within a single chemical entity provides the basis for a novel class of safer NSAIDs and to plan highly innovative studies of structure–activity relationships, chemical syntheses, and pharmacological investigations.

It has recently been demonstrated (Selg et al. 2007) that a traditional NSAID (diclofenac) and a selective COXIB (lumiracoxib) possess an additional activity: weak competitive antagonism at the TP receptor (Rovati et al. 2010). However, in light of the importance of maintaining a fine balance between TP receptor antagonism activity and COX-2 inhibition, the co-administration of two different molecules is not the best approach because it may result in significantly different pharmacokinetic profiles. Combining both activities into a single chemical entity represents a far better strategy (Selg et al. 2007): in fact, developing a compound with a more “balanced” pharmacological profile relative to these two activities may help evaluating if blocking the activity of TP might counterbalance the deleterious cardiovascular effects driven by the PGI_2 inhibition observed for COX-2 inhibitors.

4.1 TP Antagonists

Several TP antagonists have been developed since the early 1980s. Development of the earliest TP antagonists has been stopped because of their toxicity (or moderate activity) in clinical situations, whereas others have not been investigated for cardiovascular indication, and only the more recent TP antagonists reached clinical evaluation for their antithrombotic properties (Dogne et al. 2004).

Thus, many TP antagonists have been developed for the treatment of TP-mediated diseases, such as ifetroban, sulotroban, daltroban, linotroban, ramatoroban, and seratrodast. Among them, seratorodast is an orally active TP antagonist used clinically for the treatment of asthma in Japan since 1997 (Nakahata 2008).

However, evidence has been accumulated for a competitive TP antagonist, terutroban (S-18886), as a potential candidate for atherothrombosis treatment, for blocking atherosclerosis progression, and for transforming lesions towards a more stable phenotype.

Terutroban is an orally active TP antagonist in clinical development for use in secondary prevention of thrombotic events in cardiovascular disease. Terutroban has been developed as a highly specific, high-affinity TP antagonist. Binding studies show that the drug displaces the binding of [3H]-SQ29548 on human platelet membranes with a Ki value of 0.65 nmol/L, and the Kd value for binding of [3H]-S18886 to human platelet membranes averaged 0.96 nmol/L (Zuccollo et al. 2005). Unlike aspirin and clopidogrel that bind their respective receptor in an irreversible manner, terutroban has a reversible and dose-dependent antithrombotic effect within 96 h (Gaussem et al. 2005).

Escalating doses of terutroban (30 and 100 μg/kg/day) compared with that of aspirin (5 mg/kg/day) and clopidogrel (3 mg/kg/day) show a significant dose-dependent effect on platelet aggregation. When used at higher doses, terutroban is able to reduce collagen-dependent platelet aggregation, at least as well as clopidogrel. Terutroban reduces platelet deposition on low shear (212 s^{-1}) and high shear (1,690 s^{-1}) rate conditions (platelet thrombus formation in the Badimon perfusion chamber) at least as well as clopidogrel, whereas aspirin does not have any significant effect on platelet deposition. Thus, TP antagonists might be useful in clinical settings characterized by severe arterial injury and high shear rate, such as acute coronary syndromes and during and immediately after percutaneous transluminal coronary angioplasty (Chamorro 2009).

Pharmacokinetics and pharmacodynamics of terutroban have been studied in patients with PAD (Gaussem et al. 2005). Peak plasma levels are reached between 30 min and 2 h and the half life is 5.8–10 h. Maximal inhibition of U46619-induced platelet aggregation is achieved within 1 h, and this effect is maintained for at least 12 h. Over the range of studied doses, there is a predictable relation between plasma drug concentration and degree of platelet inhibition. Plasma concentrations above 10 ng/mL strongly inhibit U46619-induced platelet aggregation. These plasma concentrations are reached only by dosages higher than 10 mg/day (Gaussem et al. 2005).

The antithrombotic effects of increasing doses (1–30 mg/day) of terutroban have also been demonstrated in PAD (TAIPAD study) using a design based on the ex vivo evaluation of platelet aggregation. This effect was predictable, dose-dependent with maximal inhibition at 1 h, and lasted for approximately 48 h at the oral dose of 30 mg (Fiessinger et al. 2010).

Terutroban does not bind other prostanoid receptors, such as IP or DP receptors, and thus preserves antivasoconstrictive effects of their natural ligands, PGI_2 and PGD_2 (Chamorro 2009).

5 Pathophysiological Rationale for the Superiority of TP-Receptor Antagonists Over Aspirin

5.1 Advantages as an Antithrombotic Agent

An increased incidence of cardiovascular events and enhanced TX-dependent platelet activation characterize several clinical conditions, and aspirin is thought to be the best choice in these settings. However, enhanced TX generation may be explained by several mechanisms relatively insensitive to aspirin.

Monocytes and macrophages are the largest source of TXA_2 and are capable of newly synthesizing TXA_2 via their COX-2 pathway, which has a higher threshold of inhibition by aspirin than platelet COX-1. Thus, extraplatelet, nucleate sources of TXA_2 biosynthesis, possibly triggered by inflammatory stimuli, are less affected than platelet TXA_2 production by the once-daily regimen of administration and by the low dose administered, and may be an additional reason for the less than expected response to aspirin.

Moreover, in clinical settings characterized by enhanced platelet generation, younger reticulated platelets are increased, platelet COX-2 expression is up-regulated, and a consistent TX production may be driven by this enzymatic pathway relatively insensitive to aspirin (Guthikonda et al. 2007; Santilli et al. 2009).

Moreover, TP antagonists block all TP agonists; these include not only TXA_2 but also endoperoxide (i.e., 20-HETE) and several isoprostanes, nonenzymatic products of fatty acid oxidation formed under conditions of increased oxidative stress (Davì and Falco 2005) and which are not inhibited by aspirin (Fig. 2). Aspirin has no effect on isoprostanes and can actually increase endoperoxide (i.e., HETE) production by COX (Meade et al. 1993). Recently, it has been reported that the signaling mechanism of flow-induced constriction of human and rat cerebral arteries involves enhanced production of ROS, COX activity, and is mediated by 20-HETE via TP receptors (Toth et al. 2011).

Oxidative stress is responsible for enhanced peroxidation of AA to form biologically active F2-isoprostanes, such as 8-iso-$PGF_{2\alpha}$, that is widely recognized as a reliable marker of lipid peroxidation both in vitro and in vivo (Davì and Falco 2005; Patrono et al. 2005a).

Concentrations of 8-iso-$PGF_{2\alpha}$ in the range of 1 nmol/L to 1 μmol/L induce a dose-dependent increase in platelet shape change, calcium release from intracellular stores, and inositol phosphates (Patrono et al. 2005a). Moreover, 8-iso-$PGF_{2\alpha}$ causes dose-dependent, irreversible platelet aggregation in the presence of low concentrations of collagen, ADP, AA, and PGH_2/TXA_2 analogs that, when acting alone, fail to aggregate platelets (Patrono et al. 2005a). These effects are prevented by TP receptor antagonists and 8-iso-$PGF_{2\alpha}$ may cross-desensitize biochemical and functional responses to TX mimetics. These properties may be relevant to settings where platelet activation and enhanced free-radical formation coincide, such as in T2DM.

Patients with T2DM have a two- to fourfold increase in the risk of coronary artery disease, and patients with T2DM but without previous MI carry the same level of risk for subsequent acute coronary events as nondiabetic patients with previous MI (Schramm et al. 2008). Diabetic patients exhibit platelet "hyperreactivity" both in vitro and in vivo (Ferroni et al. 2004), (Davì et al. 1990) enhanced platelet regeneration (Watala et al. 2005), coupled with biochemical evidence of persistently enhanced TX-dependent platelet activation (Ferroni et al. 2004). Urinary TXA_2 metabolites, reflecting the whole biosynthesis of TXA_2 in the body by platelet and extraplatelet sources, are significantly higher in diabetes, with the absolute postaspirin values of 11-dehydro-TXB_2 in diabetics being comparable to nonaspirinated controls (Davì et al. 1990; Ferroni et al. 2004) and such residual TXA_2 biosynthesis despite low-dose aspirin treatment has been shown to be predictive of vascular events in high-risk settings, including diabetes (Eikelboom et al. 2002). Thus, elevated urinary 11-dehydro-TXB_2 levels identify patients who are relatively resistant to aspirin and who may benefit from alternative antiplatelet therapies or treatments that more effectively block in vivo TXA_2 production or activity.

Aspirin remains the cornerstone of antiplatelet prophylaxis, but appears to have limited benefit in diabetes (Baigent et al. 2009). TP antagonists, blocking the interaction of both aspirin-sensitive and aspirin-insensitive agonists should theoretically provide more potent protection against the anticipated detrimental effects of platelet activation (Fig. 4).

The hypothesis that hyperglycemia-induced oxidant stress in T2DM might trigger enhanced generation of 8-iso-$PGF_{2\alpha}$ and that this compound might, in turn, contribute to platelet activation is supported by the finding that 8-iso-$PGF_{2\alpha}$ formation is correlated with the rate of TXA_2 biosynthesis in this setting (Davì et al. 1999) and that intensive antidiabetic treatment is associated with a reduction in both urinary 8-iso-$PGF_{2\alpha}$ and TX metabolites excretion rates (Patrono et al. 2005a).

Thus, both COX-2-derived TXA_2 and F2-isoprostanes may act as aspirin-insensitive agonists of the platelet TP receptor, accounting for the less than complete inhibition of platelet aggregation in T2DM.

Another mechanism advocated in favor of TP antagonism, stands in its capacity to leave COX-1 and COX-2 or any pathway of prostanoid synthesis unaltered, thus preserving the cardioprotective eicosanoid PGI_2, an important homeostatic mechanism of endothelial thromboresistance triggered by platelet activation.

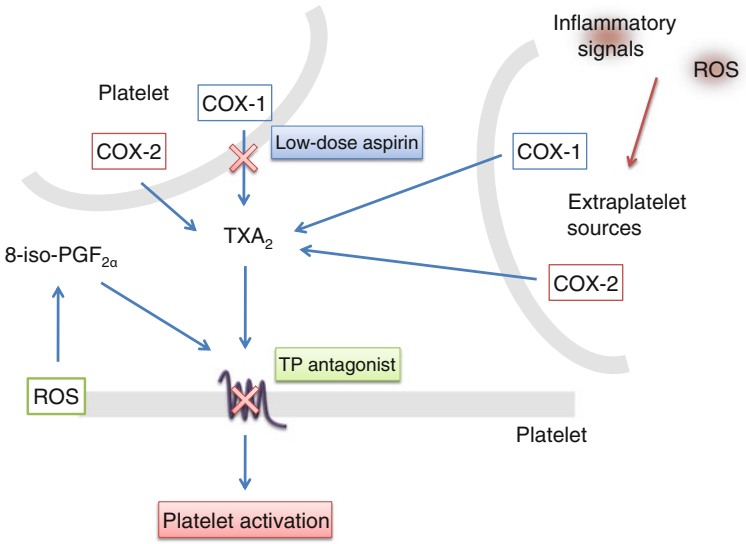


Fig. 4 Possible advantages of TP antagonism over aspirin. Low-dose aspirin irreversibly blocks TXA_2 generation by platelet COX-1. However, in high-risk patients, accelerated platelet turnover may increase platelet COX-1 recovery of TXA_2 biosynthesis as well as COX-2-generated TXA_2. In addition, enhanced oxidative stress and chronic inflammation further stimulated TXA_2 generation by extraplatelet COX-1 and COX-2. Finally, lipid peroxidation by reactive oxidant species (ROS) is able to generate biologically active F2-isoprostane. These molecules, in particular 8-iso-$PGF_{2\alpha}$, have been shown to bind TP and modulate the adhesive reactions and activation of platelets induced by low levels of other agonists. By blocking TP activation by TXA_2 and other aspirin-insensitive agonists, TP antagonists should theoretically provide more potent protection against the detrimental effects of platelet activation

In contrast, depression of COX-2-derived PGI_2 by traditional NSAIDS or COXIBs as well as by anti-inflammatory doses of aspirin removes a constraint on platelet COX-1-derived TXA_2 and other agonists that elevate blood pressure, promotes atherogenesis, and augments the thrombotic response to plaque rupture.

5.2 *Relevance of TP Receptors Inhibition in Atherosclerotic Disease*

Treatment with a TP antagonist, but not treatment with aspirin, inhibits atherogenesis in apo-E deficient mice (Cayatte et al. 2000), strongly suggesting that TP antagonists could be superior to aspirin in preventing atheroma. In New Zealand

white rabbits, terutroban induces regression of atherosclerotic lesions of the aorta detected by magnetic resonance imaging. The concomitant reduction in indexes of inflammation into the lesions, such as macrophages, apoptotic cells, matrix metalloproteinase-1, endothelin-1, suggests that selective inhibition of TP receptor may shift toward a more stable plaque phenotype (Chamorro 2009). Moreover, injury-induced vascular proliferation is enhanced in mice genetically deficient in the PGI_2 receptor and is reduced in mice lacking the TP receptor or treated with terutroban. Lack of both prostanoid receptors abolishes postinjury restenosis (Cheng et al. 2002).

Endogenous TP agonists are produced by the vascular endothelium, especially under pathological conditions, causing endothelium-dependent contraction and contributing to endothelial dysfunction. Therefore, TP antagonists may counteract endothelial dysfunction in diseases such as hypertension and diabetes (Feletou et al. 2010). In $apoE^{-/-}$ mice with streptozotocin-induced diabetes, terutroban reduces aortic atherosclerotic area, with improvement of endothelium-dependent relaxations to acetylcholine (Santilli et al. 2011). Thus, TP antagonism may attenuate inflammation and atherogenesis in experimental diabetes.

Administration of terutroban to coronary artery disease or to high-cardiovascular-risk patients on top of aspirin treatment improved endothelial function assessed by measuring flow-mediated dilatation (FMD) in the brachial artery (Belhassen et al. 2003; Lesault et al. 2011). The beneficial effect of terutroban on FMD was detectable after the first intake and persisted up to the end of the 2-week treatment period (Lesault et al. 2011). Thus, TP antagonists can inhibit prostanoid-mediated vasoconstriction associated with aging and/or cardiovascular risk factors related to increased oxidative stress and consequent up-regulation of COX-1 and/or induction of COX-2 (Giannarelli et al. 2010).

5.3 Ischemic Stroke: The Reasons of a Choice

The at least theoretical advantages on platelet inhibition and the actions far beyond its antithrombotic effect, including the antiproliferative and antiatherogenic properties, raised the hypothesis that TP-receptor antagonism could play a role in the clinical prevention of ischemic stroke. Preclinical findings supported this concept, indicating a greater beneficial effect of TP receptor inhibition over aspirin in a rat model of ischemic stroke (Gelosa et al. 2010). In a double-blind, parallel group study in patients with previous ischemic stroke and/or carotid stenosis, terutroban showed an antithrombotic activity superior to aspirin and similar to clopidogrel plus aspirin (Bal Dit Sollier et al. 2009). These encouraging data were the basis for undertaking the *Prevention of cerebrovascular and cardiovascular Events of ischemic origin with teRutroban in patients with a history oF ischemic strOke or tRansient ischaeMic attack* (PERFORM) study (Bousser et al. 2009).

This trial was designed to demonstrate the superiority of terutroban over aspirin in secondary prevention of cerebrovascular and cardiovascular events among ~20,000 patients with stroke. The trial (ISRCTN66157730) was recently halted on the basis of an interim analysis failing to support the superiority hypothesis, after 19,120 patients were randomly assigned, with a mean follow-up of 28·3 months (Bousser et al. 2011).

6 Great Expectations Disappointed: Did Terutroban Fail to Perform, or PERFORM Did Fail?

The PERFORM was stopped prematurely for futility after 19,120 patients were randomly assigned, with a mean follow-up of 28 months (Bousser et al. 2011). The investigators recorded no difference between terutroban and aspirin in the composite vascular primary end point, or any of the secondary or tertiary end points. However, the rate of minor bleeding was slightly increased with terutroban.

This apparent discrepancy versus the above-mentioned "great expectations" around terutroban, supported by a large body of preclinical and clinical evidence and pathophysiological rationale, draws attention and raises concerns about the interpretation of the encouraging preclinical data, as well as about the design of the clinical trial testing the superiority hypothesis of this drug versus aspirin.

6.1 Preliminary Data Revisited

The negative results of the PERFORM trial failed to come up to the expectations based on the rationale and the preliminary data supporting the superiority of terutroban over aspirin.

Both the anticipated superior antithrombotic effect and the peculiar antiatherogenic and antivasoconstrictive properties of TP antagonism need to be reconsidered in light of the clinical evidence.

Most of the data supporting a more potent antiplatelet effect of terutroban over aspirin relied upon ex vivo measurements of platelet function, such as optical aggregation to classical agonists and models of thrombus formation (Bal Dit Sollier et al. 2009; Fiessinger et al. 2010).

The apparent gap between these premises and the findings of PERFORM draws attention to the limitations of ex vivo measurements of platelet function in the characterization of platelet activation and inhibition in vivo: less than ideal intrasubject and intersubject variability, poor reproducibility on repeated measurements, variability of the TX-independent component of the different aggregation signals (Santilli et al. 2009).

Moreover, measurements of platelet function ex vivo provide an index of capacity that by no means reflects the extent of platelet activation and inhibition in vivo. Mechanism-based biochemical measurements would provide a more faithful estimation of the antiplatelet effect of aspirin.

As earlier mentioned, another war horse supporting the expected superiority of terutroban over aspirin stands in the preservation of the ability of the vasculature to synthesize the cardioprotective eicosanoid PGI_2, an important homeostatic mechanism of endothelial thromboresistance. However, the low-doses of aspirin (100 mg daily) employed vs. terutroban in the PERFORM trial have been previously shown to only marginally reduce systemic PGI_2 biosynthesis in heart failure and ischemic heart disease patients, counterbalanced by a profound reduction in TX biosynthesis (Santilli et al. 2010), consistent with the relative COX-1 selectivity achieved by a once-daily regimen of low-dose aspirin (Patrono et al. 2008) and the primary role of COX-2 in PGI_2 biosynthesis.

The antivasoconstrictive effect of terutroban, increasing blood flow through enhanced endothelium-dependent vasodilation, had the theoretical potential to affect the incidence of ischemic stroke in the PERFORM population, where traditional cardiovascular risk factors associated with endothelial dysfunction are highly prevalent. However, it has to be acknowledged that, despite several reports (Santos-Garcia et al. 2009) suggest an association, no data until now show conclusive evidence of a direct relation between endothelium-dependent contractions and the risk of ischemic stroke.

Similarly, the antiproliferative properties of terutroban, shown in a murine model of vascular injury-induced proliferation of the carotid artery, did not translate into a clinical benefit. This result might have been anticipated by the failure to prevent postcoronary angioplasty restenosis by previously developed TX-prostaglandin receptor antagonists, the CARPORT and the M-HEART (Savage et al. 1995; Serruys et al. 1991). However, none of these two studies were free of limitations, the first being an uncontrolled experience relying on a single measurement of an angiographic end point, the second including no measure of the degree of synthesis inhibition achieved by aspirin or of TP blockade by either antagonist. Moreover, the M-HEART based the superiority of terutroban over aspirin on the ability of terutroban to preserve the antiproliferative effect of PGI_2 biosynthesis during angiography, which was short lived and suppressed by the concurrent aspirin treatment in all patients undergoing PCI (Praticó et al. 2000).

The antiatherogenic properties of TP antagonists were considered as a relevant plus, stimulating the preferential recruitment of patients with an atherothrombotic cerebral ischemic event. However, even in this subgroup, no benefit was recorded for terutroban compared with aspirin in the PERFORM, possibly because their atheromatous lesions were already well advanced. This assumption is supported by a study performed in a murine model of atherogenesis, showing that TP antagonism inhibits initiation and early development of atherosclerotic lesions in mice, but failed to induce regression of established atherosclerotic disease (Egan et al. 2005). Thus, the clinical use of TP antagonists would be expected to be useful in the earlier stages of disease, rather than in reversing accumulated plaque burden in patients with diffuse, established atherosclerosis.

6.2 The PERFORM Design Revisited

A few lessons might be drawn by the comparison of PERFORM findings with similar trials of other antiplatelets vs. aspirin. PERFORM is the second largest secondary prevention trial of an antiplatelet drug undertaken so far in patients with cerebral ischemic events. The largest study, PROFESS (Sacco et al. 2008), compared aspirin plus extended-release dipyridamole and clopidogrel in 20,232 patients followed up for a mean of 30 months. This trial showed similar rates of recurrent stroke with aspirin plus extended-release dipyridamole and with clopidogrel. Failure to achieve the superiority goal in both trials raises concerns about the clinical setting in which selective TP receptor blockade might confer an advantage over low-dose aspirin. Ischemic stroke might be a difficult setting as compared to MI, but no trial so far is available to confirm or reject this speculative hypothesis.

Furthermore, the comparative analysis versus previously performed trials in the same setting unravels the likelihood that the event rate observed in the PERFORM might be someway lower than expected, thus affecting the statistical power of the study. Although recent trials (ESPRIT and CSPS 2) have shown similar rates of strokes with aspirin to those seen in PERFORM (Halkes et al. 2006; Shinohara et al. 2010), the stroke rate with aspirin in PERFORM should have been much higher because of the enrolment of several patients during the period of highest risk for recurrence (less than 3 months since the qualifying event) and because 52% of the qualifying strokes were due to the stroke subtype with the poorer prognosis, atherothrombosis (compared with about 30% in ESPRIT and CSPS 2). Even in the PROFESS trial, which, similar to the PERFORM, enrolled patients who had experienced an ischemic stroke for less than 90 days, the event rates were remarkably close to PERFORM, but the proportion of atherothrombotic infarcts was significantly higher (67% vs. 28%) in PERFORM.

The choice of the lag time after the qualifying event, similar to the PROFESS trial, might also have affected the effectiveness of the drug: in fact, patients could be enrolled after the index event in PERFORM much sooner than in other recent antiplatelet trials. While aspirin has proven benefits when given early after a stroke (Chen et al. 2000), the efficacy of this strategy for other antiplatelets has not been definitively proven.

The choice of the dose (30 mg once daily) has been an additional issue advocated to explain the drug failure to exert superior effects on vascular events than aspirin. However, the slight excess in minor bleeding with this dose suggests that, even if higher doses may be more appropriate on the efficacy side, this benefit could be offset by more hemorrhagic episodes, which seem proportional to the number of TX-prostaglandin receptors bound by the drug.

The duration of follow-up appears as another critical and still unanswered issue: given that only 15% of patients were followed up to or beyond 3 years, a difference in treatment effect might have emerged later, since additional potential longer term effects of terutroban cannot be excluded.

As previously shown for aspirin (Baigent et al. 2009), improving background risk-factor control could have blunted the ability of terutroban to outperform aspirin. Nowadays, many patients with a history of occlusive vascular events would have their risks of recurrence reduced substantially by statins, other modern drugs, and vascular procedures. If so, and if the other interventions approximately halve the recurrence risk, then the absolute benefit of adding an antiplatelet to these other methods might be only about half as great as that of giving an antiplatelet alone.

Finally, the observation that patients with a history of ischemic stroke before the qualifying event had a lower event rate with terutroban than with aspirin is worth of further attention. Although this finding could be attributable to chance, it is plausible that most of these patients would have been receiving aspirin before their PERFORM qualifying event, thus the switch to a different antiplatelet drug appears as a more effective strategy than continuation of aspirin despite having experienced an event while on aspirin. Lower than expected response to aspirin which translates into clinical failure might be an expected and relevant phenomenon in particular subgroups of patients, such as those with diabetes mellitus (representing 27% and 28% of each arm, respectively). Trials that randomly assign patients with a breakthrough event while on aspirin to a newer antiplatelet drug or to a different dosing regimen, rather than to the original aspirin dose, could provide insights into this issue. Perhaps terutroban could be tested for this specific issue or in clinical settings where a benefit is anticipated, such as diabetes mellitus. In the PERFORM, only about one out of five of the patient population had diabetes, thus potentially affecting the statistical power of the study to test the superiority hypothesis in diabetes.

7 Future Perspectives

As earlier mentioned, failure of the PERFORM does not preclude the possibility that TP receptor antagonist may be an effective tool in the prevention of vascular events in other clinical settings, such as diabetes, where the pathophysiological premises for a beneficial role look more sound. This might be worth of ad hoc trials, although the PERFORM findings are likely to discourage any other drug company from pursuing this drug target.

Moving from the cardiovascular setting, an increasing body of evidence provides the rationale for a role of TXS and TP receptor signaling in carcinogenesis, which may arise as a “rescue” setting for the clinical development of these drugs.

TXS signaling has been implicated in the development and progression of cancer, by acting on a range of tumor cell survival pathways, such as tumor cell proliferation, induction of apoptosis, invasion and metastasis, and tumor cell angiogenesis (Cathcart et al. 2010).

Increased expression of TXS and TP-β isoform has been found in the tissues of patients with bladder cancer. TP-β receptor overexpression in patients with bladder

cancer is associated with a poorer prognosis (Moussa et al. 2008). Studies in cell lines and mice have indicated a potential significant role of the TX signaling pathway in the pathogenesis of human bladder cancer. Stimulation of TP receptors is associated with a mitogenic response and in phosphorylation of several kinases. TP receptor antagonists augment in vitro and in vivo responses to cisplatin, by reducing cell proliferation in vitro, increasing the time of tumor onset, and reducing the rate of tumor growth in vivo (Moussa et al. 2008). These studies raise the possibility that the TP-β receptor could serve as a novel therapeutic target in bladder cancer and its presence and/or overexpression could be used as a predictor of prognosis and dictate therapy. Recently, increased tissue levels of the TP-β receptor in patients with bladder cancer have been found to be mirrored by increased urinary levels of TXB_2, the major metabolite of TXA_2, suggesting that patients with bladder cancer may be followed for progression or remission of their disease by quantitation of these substances in their urine (Moussa et al. 2011).

TXS is also overexpressed in nonsmall cell lung cancer (NSCLC), particularly in the adenocarcinoma subtype. Selective TXS inhibition prevents proliferation and induces apoptosis (Cathcart et al. 2011).

Clearly, further studies are needed to delineate the role of TXS and TP-β receptors in cancer and to address the challenge of their pharmacological inhibition through a clinical development program.

8 Conclusions and Implications for Clinical Usefulness of TP Antagonists

Several clinical conditions are characterized by increased incidence of cardiovascular events and enhanced TX-dependent platelet activation.

Aspirin is thought to be the best choice in these settings. However, the optimum regimen to suppress TX formation remains undefined. In fact, enhanced TX generation may be explained by mechanisms relatively insensitive to aspirin.

Extraplatelet, nucleate sources of TXA_2 biosynthesis, possibly triggered by inflammatory stimuli, and F2-isoprostane formation, reflecting ongoing in vivo oxidative stress, can activate platelets via the platelet TP receptor thus escaping inhibition of aspirin (Davì et al. 1990).

Thus, the relevance of the TP receptor in the pathogenesis of vascular diseases, particularly in diabetes, may be due to the fact that not only TXA_2, but other eicosanoids including HETEs and isoprostanes are produced to such an extent as to activate the TP receptor. Aspirin has no effect on isoprostanes which are formed nonenzymatically from AA, and aspirin can actually increase HETE production by COX (Meade et al. 1993).

In clinical settings characterized by enhanced platelet generation, younger reticulated platelets are increased, and platelet COX-2 expression is up-regulated and a consistent TX production may be driven by this enzymatic pathway, relatively insensitive to aspirin (Guthikonda et al. 2007; Santilli et al. 2009).

An antithrombotic intervention blocking TP may be required, as a common downstream pathway for both platelet and extraplatelet TXA_2 as well as for isoprostanes. Aspirin does not inhibit isoprostane formation. Moreover, intraplatelet or extraplatelet TX generation may be only partly inhibited by aspirin under certain pathological conditions, at least at the usual low doses given for cardiovascular protection.

Moreover, a TP antagonist has actions far beyond its antithrombotic effect exerted on platelets and can be attributed to direct effects on endothelial and smooth muscle cells within the blood vessel wall. These include effects on vascular adhesion molecules, NO synthase expression and function, oxidant production, and accumulation of extracellular matrix and advanced glycation end-products.

Thus, TP antagonists may represent an ideal tool to improve our knowledge on the pathophysiology of cardiovascular diseases and to improve our pharmacological "weapons" to counteract them in clinical settings, such as diabetes mellitus, characterized by persistent enhanced TXA_2-dependent platelet activation.

Knowledge Gaps

- The prevention of vascular events by TP receptor antagonists in clinical settings, such as diabetes, where the pathophysiological premises for a beneficial role look more sound needs to be defined.
- The hypothesis that TP-β receptor could serve as a novel therapeutic target in bladder cancer and its presence and/or overexpression could be used as a predictor of prognosis and dictate therapy needs to be tested.
- More in general, further studies are needed to delineate the role of TXS and TP-β receptors in cancer and to address the challenge of their pharmacological inhibition through a clinical development program.

Key Messages

- Thromboxane synthase (TXS) inhibitors and thromboxane receptor (TP) antagonists have the potential to prove more effective than aspirin due to their different mechanism of action along the pathway of TXA_2. TXS inhibitors prevent the conversion of PGH_2 to TXA_2, reducing TXA_2 synthesis mainly in platelets, whereas TP antagonists block the downstream consequences of TP activation.
- Targeting TP receptor, a common downstream pathway for both platelet and extraplatelet TXA_2 as well as for endoperoxides and isoprostanes, may be a useful antiatherosclerotic and a more powerful antithrombotic intervention in clinical settings, such as diabetes mellitus, characterized by persistently enhanced TX-dependent platelet activation through isoprostane formation and low-grade inflammation, leading to extraplatelet sources of TXA_2.

(continued)

- Despite great expectations on this drug supported by a large body of preclinical and clinical evidence and pathophysiological rationale, the PERFORM trial failed to demonstrate the superiority of terutroban over aspirin in secondary prevention of cerebrovascular and cardiovascular events among ~20,000 patients with stroke. However, the clinical setting and the design of the study in which the drug has been challenged, as well as a sometimes uncritical translation of preclinical data into a rationale for a clinical trial, may explain, at least in part, this unexpected finding.
- Drugs with dual action, such as dual TXS inhibitors/TP antagonist and dual COXIB/TP antagonists are currently in clinical development. Besides the theoretical rationale for their benefit, ongoing clinical studies are challenging their potential.

References

Ali S, Davis MG, Becker MW, Dorn GW 2nd (1993) Thromboxane A2 stimulates vascular smooth muscle hypertrophy by up-regulating the synthesis and release of endogenous basic fibroblast growth factor. J Biol Chem 268:17397–17403

Ashton AW, Ware GM, Kaul DK, Ware JA (2003) Inhibition of tumor necrosis factor alpha-mediated NFkappaB activation and leukocyte adhesion, with enhanced endothelial apoptosis, by G protein-linked receptor (TP) ligands. J Biol Chem 278:11858–11866

Baigent C, Blackwell L, Collins R, Emberson J, Godwin J, Peto R, Buring J, Hennekens C, Kearney P, Meade T, Patrono C, Roncaglioni MC, Zanchetti A (2009) Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. Lancet 373:1849–1860

Bal Dit Sollier C, Crassard I, Simoneau G, Bergmann JF, Bousser MG, Drouet L (2009) Effect of the thromboxane prostaglandin receptor antagonist terutroban on arterial thrombogenesis after repeated administration in patients treated for the prevention of ischemic stroke. Cerebrovasc Dis 28:505–513

Basili S, Raparelli V, Vestri A, Di Tanna GL, Violi F (2010) Comparison of efficacy of antiplatelet treatments for patients with claudication. A meta-analysis. Thromb Haemost 103:766–773

Belhassen L, Pelle G, Dubois-Rande JL, Adnot S (2003) Improved endothelial function by the thromboxane A2 receptor antagonist S 18886 in patients with coronary artery disease treated with aspirin. J Am Coll Cardiol 41:1198–1204

Bousser MG, Amarenco P, Chamorro A, Fisher M, Ford I, Fox K, Hennerici MG, Mattle HP, Rothwell PM (2009) Rationale and design of a randomized, double-blind, parallel-group study of terutroban 30 mg/day versus aspirin 100 mg/day in stroke patients: the prevention of cerebrovascular and cardiovascular events of ischemic origin with terutroban in patients with a history of ischemic stroke or transient ischemic attack (PERFORM) study. Cerebrovasc Dis 27:509–518

Bousser MG, Amarenco P, Chamorro A, Fisher M, Ford I, Fox KM, Hennerici MG, Mattle HP, Rothwell PM, de Cordoue A, Fratacci MD (2011) Terutroban versus aspirin in patients with cerebral ischaemic events (PERFORM): a randomised, double-blind, parallel-group trial. Lancet 377:2013–2022

Cathcart MC, Reynolds JV, O'Byrne KJ, Pidgeon GP (2010) The role of prostacyclin synthase and thromboxane synthase signaling in the development and progression of cancer. Biochim Biophys Acta 1805:153–166

Cathcart MC, Gately K, Cummins R, Kay E, O'Byrne KJ, Pidgeon GP (2011) Examination of thromboxane synthase as a prognostic factor and therapeutic target in non-small cell lung cancer. Mol Cancer 10:25

Cayatte AJ, Du Y, Oliver-Krasinski J, Lavielle G, Verbeuren TJ, Cohen RA (2000) The thromboxane receptor antagonist S18886 but not aspirin inhibits atherogenesis in apo E-deficient mice: evidence that eicosanoids other than thromboxane contribute to atherosclerosis. Arterioscler Thromb Vasc Biol 20:1724–1728

Chamorro A (2009) TP receptor antagonism: a new concept in atherothrombosis and stroke prevention. Cerebrovasc Dis 27(Suppl 3):20–27

Chen ZM, Sandercock P, Pan HC, Counsell C, Collins R, Liu LS, Xie JX, Warlow C, Peto R (2000) Indications for early aspirin use in acute ischemic stroke: a combined analysis of 40 000 randomized patients from the chinese acute stroke trial and the international stroke trial. On behalf of the CAST and IST collaborative groups. Stroke 31:1240–1249

Cheng Y, Austin SC, Rocca B, Koller BH, Coffman TM, Grosser T, Lawson JA, FitzGerald GA (2002) Role of prostacyclin in the cardiovascular response to thromboxane A2. Science 296:539–541

Davì G, Falco A (2005) Oxidant stress, inflammation and atherogenesis. Lupus 14:760–764

Davì G, Patrono C (2007) Platelet activation and atherothrombosis. N Engl J Med 357:2482–2494

Davì G, Catalano I, Averna M, Notarbartolo A, Strano A, Ciabattoni G, Patrono C (1990) Thromboxane biosynthesis and platelet function in type II diabetes mellitus. N Engl J Med 322:1769–1774

Davì G, Ciabattoni G, Consoli A, Mezzetti A, Falco A, Santarone S, Pennese E, Vitacolonna E, Bucciarelli T, Costantini F, Capani F, Patrono C (1999) In vivo formation of 8-iso-prostaglandin f2alpha and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation. Circulation 99:224–229

Dogne JM, de Leval X, Hanson J, Frederich M, Lambermont B, Ghuysen A, Casini A, Masereel B, Ruan KH, Pirotte B, Kolh P (2004) New developments on thromboxane and prostacyclin modulators part I: thromboxane modulators. Curr Med Chem 11:1223–1241

Dogne JM, Hanson J, Pratico D (2005) Thromboxane, prostacyclin and isoprostanes: therapeutic targets in atherogenesis. Trends Pharmacol Sci 26:639–644

Egan KM, Wang M, Fries S, Lucitt MB, Zukas AM, Pure E, Lawson JA, FitzGerald GA (2005) Cyclooxygenases, thromboxane, and atherosclerosis: plaque destabilization by cyclooxygenase-2 inhibition combined with thromboxane receptor antagonism. Circulation 111:334–342

Eikelboom JW, Hirsh J, Weitz JI, Johnston M, Yi Q, Yusuf S (2002) Aspirin-resistant thromboxane biosynthesis and the risk of myocardial infarction, stroke, or cardiovascular death in patients at high risk for cardiovascular events. Circulation 105:1650–1655

Feletou M, Verbeuren TJ, Vanhoutte PM (2009) Endothelium-dependent contractions in SHR: a tale of prostanoid TP and IP receptors. Br J Pharmacol 156:563–574

Feletou M, Cohen RA, Vanhoutte PM, Verbeuren TJ (2010) TP receptors and oxidative stress hand in hand from endothelial dysfunction to atherosclerosis. Adv Pharmacol 60:85–106

Ferroni P, Basili S, Falco A, Davì G (2004) Platelet activation in type 2 diabetes mellitus. J Thromb Haemost 2:1282–1291

Fiessinger JN, Bounameaux H, Cairols MA, Clement DL, Coccheri S, Fletcher JP, Hoffmann U, Turpie AG (2010) Thromboxane Antagonism with terutroban in Peripheral Arterial Disease: the TAIPAD study. J Thromb Haemost 8:2369–2376

FitzGerald GA, Reilly IA, Pedersen AK (1985) The biochemical pharmacology of thromboxane synthase inhibition in man. Circulation 72:1194–1201

Gaussem P, Reny JL, Thalamas C, Chatelain N, Kroumova M, Jude B, Boneu B, Fiessinger JN (2005) The specific thromboxane receptor antagonist S18886: pharmacokinetic and pharmacodynamic studies. J Thromb Haemost 3:1437–1445

Gelosa P, Ballerio R, Banfi C, Nobili E, Gianella A, Pignieri A, Brioschi M, Guerrini U, Castiglioni L, Blanc-Guillemaud V, Lerond L, Tremoli E, Sironi L (2010) Terutroban, a

thromboxane/prostaglandin endoperoxide receptor antagonist, increases survival in stroke-prone rats by preventing systemic inflammation and endothelial dysfunction: comparison with aspirin and rosuvastatin. J Pharmacol Exp Ther 334:199–205

Giannarelli C, Zafar MU, Badimon JJ (2010) Prostanoid and TP-receptors in atherothrombosis: is there a role for their antagonism? Thromb Haemost 104:949–954

Guthikonda S, Lev EI, Patel R, DeLao T, Bergeron AL, Dong JF, Kleiman NS (2007) Reticulated platelets and uninhibited COX-1 and COX-2 decrease the antiplatelet effects of aspirin. J Thromb Haemost 5:490–496

Halkes PH, van Gijn J, Kappelle LJ, Koudstaal PJ, Algra A (2006) Aspirin plus dipyridamole versus aspirin alone after cerebral ischaemia of arterial origin (ESPRIT): randomised controlled trial. Lancet 367:1665–1673

Ishizuka T, Suzuki K, Kawakami M, Hidaka T, Matsuki Y, Nakamura H (1996) Thromboxane A2 receptor blockade suppresses intercellular adhesion molecule-1 expression by stimulated vascular endothelial cells. Eur J Pharmacol 312:367–377

Ishizuka T, Kawakami M, Hidaka T, Matsuki Y, Takamizawa M, Suzuki K, Kurita A, Nakamura H (1998) Stimulation with thromboxane A2 (TXA2) receptor agonist enhances ICAM-1, VCAM-1 or ELAM-1 expression by human vascular endothelial cells. Clin Exp Immunol 112:464–470

Kiff PS, Bergman G, Atkinson L, Jewitt DE, Westwick J, Kakkar VV (1983) Haemodynamic and metabolic effects of dazoxiben at rest and during atrial pacing. Br J Clin Pharmacol 15:73S–77S

Lesault PF, Boyer L, Pelle G, Covali-Noroc A, Rideau D, Akakpo S, Teiger E, Dubois-Rande JL, Adnot S (2011) Daily administration of the TP receptor antagonist terutroban improved endothelial function in high-cardiovascular-risk patients with atherosclerosis. Br J Clin Pharmacol 71:844–851

Mayeux PR, Morton HE, Gillard J, Lord A, Morinelli TA, Boehm A, Mais DE, Halushka PV (1988) The affinities of prostaglandin H2 and thromboxane A2 for their receptor are similar in washed human platelets. Biochem Biophys Res Commun 157:733–739

Meade EA, Smith WL, DeWitt DL (1993) Differential inhibition of prostaglandin endoperoxide synthase (cyclooxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs. J Biol Chem 268:6610–6614

Meadows TA, Bhatt DL (2007) Clinical aspects of platelet inhibitors and thrombus formation. Circ Res 100:1261–1275

Mehta P, Mehta J, Lawson D, Krop I, Letts LG (1986) Leukotrienes potentiate the effects of epinephrine and thrombin on human platelet aggregation. Thromb Res 41:731–738

Meja KK, Barnes PJ, Giembycz MA (1997) Characterization of the prostanoid receptor(s) on human blood monocytes at which prostaglandin E2 inhibits lipopolysaccharide-induced tumour necrosis factor-alpha generation. Br J Pharmacol 122:149–157

Miggin SM, Kinsella BT (1998) Expression and tissue distribution of the mRNAs encoding the human thromboxane A2 receptor (TP) alpha and beta isoforms. Biochim Biophys Acta 1425:543–559

Moussa O, Ashton AW, Fraig M, Garrett-Mayer E, Ghoneim MA, Halushka PV, Watson DK (2008) Novel role of thromboxane receptors beta isoform in bladder cancer pathogenesis. Cancer Res 68:4097–4104

Moussa O, Ciupek A, Watson DK, Halushka PV (2011) Urinary thromboxane B(2) and thromboxane receptors in bladder cancer: opportunity for detection and monitoring. Prostaglandins Other Lipid Mediat 96(1–4):41–44

Nakahata N (2008) Thromboxane A2: physiology/pathophysiology, cellular signal transduction and pharmacology. Pharmacol Ther 118:18–35

Neri Serneri GG, Coccheri S, Marubini E, Violi F (2004) Picotamide, a combined inhibitor of thromboxane A2 synthase and receptor, reduces 2-year mortality in diabetics with peripheral arterial disease: the DAVID study. Eur Heart J 25:1845–1852

Patrono C (1990) Biosynthesis and pharmacological modulation of thromboxane in humans. Circulation 81:I12–I15, discussion I22–I23

Patrono C, Falco A, Davì G (2005a) Isoprostane formation and inhibition in atherothrombosis. Curr Opin Pharmacol 5:198–203

Patrono C, Garcia Rodriguez LA, Landolfi R, Baigent C (2005b) Low-dose aspirin for the prevention of atherothrombosis. N Engl J Med 353:2373–2383

Patrono C, Baigent C, Hirsh J, Roth G (2008) Antiplatelet drugs: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest 133:199S–233S

Patscheke H (1990) Thromboxane A2/prostaglandin H2 receptor antagonists. A new therapeutic principle. Stroke 21:IV139–IV142

Praticó D, Cheng Y, FitzGerald GA (2000) TP or not TP: primary mediators in a close runoff? Arterioscler Thromb Vasc Biol 20:1695–1698

Rovati GE, Sala A, Capra V, Dahlen SE, Folco G (2010) Dual COXIB/TP antagonists: a possible new twist in NSAID pharmacology and cardiovascular risk. Trends Pharmacol Sci 31:102–107

Sacco RL, Diener HC, Yusuf S, Cotton D, Ounpuu S, Lawton WA, Palesch Y, Martin RH, Albers GW, Bath P, Bornstein N, Chan BP, Chen ST, Cunha L, Dahlof B, De Keyser J, Donnan GA, Estol C, Gorelick P, Gu V, Hermansson K, Hilbrich L, Kaste M, Lu C, Machnig T, Pais P, Roberts R, Skvortsova V, Teal P, Toni D, Vandermaelen C, Voigt T, Weber M, Yoon BW (2008) Aspirin and extended-release dipyridamole versus clopidogrel for recurrent stroke. N Engl J Med 359:1238–1251

Santilli F, Rocca B, De Cristofaro R, Lattanzio S, Pietrangelo L, Habib A, Pettinella C, Recchiuti A, Ferrante E, Ciabattoni G, Davì G, Patrono C (2009) Platelet cyclooxygenase inhibition by low-dose aspirin is not reflected consistently by platelet function assays: implications for aspirin "resistance". J Am Coll Cardiol 53:667–677

Santilli F, Davì G, Basili S, Lattanzio S, Cavoni A, Guizzardi G, De Feudis L, Traisci G, Pettinella C, Paloscia L, Minuz P, Meneguzzi A, Ciabattoni G, Patrono C (2010) Thromboxane and prostacyclin biosynthesis in heart failure of ischemic origin: effects of disease severity and aspirin treatment. J Thromb Haemost 8:914–922

Santilli F, Mucci L, Davì G (2011) TP receptor activation and inhibition in atherothrombosis: the paradigm of diabetes mellitus. Intern Emerg Med 6:203–212

Santos-Garcia D, Blanco M, Serena J, Arias S, Millan M, Rodriguez-Yanez M, Leira R, Davalos A, Castillo J (2009) Brachial arterial flow mediated dilation in acute ischemic stroke. Eur J Neurol 16:684–690

Savage MP, Goldberg S, Bove AA, Deutsch E, Vetrovec G, Macdonald RG, Bass T, Margolis JR, Whitworth HB, Taussig A (1995) Effect of thromboxane A2 blockade on clinical outcome and restenosis after successful coronary angioplasty. Multi-Hospital Eastern Atlantic Restenosis Trial (M-HEART II). Circulation 92:3194–3200

Schramm TK, Gislason GH, Kober L, Rasmussen S, Rasmussen JN, Abildstrom SZ, Hansen ML, Folke F, Buch P, Madsen M, Vaag A, Torp-Pedersen C (2008) Diabetes patients requiring glucose-lowering therapy and nondiabetics with a prior myocardial infarction carry the same cardiovascular risk: a population study of 3.3 million people. Circulation 117:1945–1954

Selg E, Buccellati C, Andersson M, Rovati GE, Ezinga M, Sala A, Larsson AK, Ambrosio M, Lastbom L, Capra V, Dahlen B, Ryrfeldt A, Folco GC, Dahlen SE (2007) Antagonism of thromboxane receptors by diclofenac and lumiracoxib. Br J Pharmacol 152:1185–1195

Serruys PW, Rutsch W, Heyndrickx GR, Danchin N, Mast EG, Wijns W, Rensing BJ, Vos J, Stibbe J (1991) Prevention of restenosis after percutaneous transluminal coronary angioplasty with thromboxane A2-receptor blockade. A randomized, double-blind, placebo-controlled trial. Coronary Artery Restenosis Prevention on Repeated Thromboxane-Antagonism Study (CARPORT). Circulation 84:1568–1580

Shikano M, Ito T, Ogawa K, Satake T (1987) Effects of a selective thromboxane synthetase inhibitor (OKY-046) in patients with coronary artery disease during exercise. Jpn Heart J 28:663–674

Shinohara Y, Katayama Y, Uchiyama S, Yamaguchi T, Handa S, Matsuoka K, Ohashi Y, Tanahashi N, Yamamoto H, Genka C, Kitagawa Y, Kusuoka H, Nishimaru K, Tsushima M, Koretsune Y, Sawada T, Hamada C (2010) Cilostazol for prevention of secondary stroke (CSPS 2): an aspirin-controlled, double-blind, randomised non-inferiority trial. Lancet Neurol 9:959–968

Swinnen M, Vanhoutte D, Van Almen GC, Hamdani N, Schellings MW, D'Hooge J, Van der Velden J, Weaver MS, Sage EH, Bornstein P, Verheyen FK, VandenDriessche T, Chuah MK, Westermann D, Paulus WJ, Van de Werf F, Schroen B, Carmeliet P, Pinto YM, Heymans S (2009) Absence of thrombospondin-2 causes age-related dilated cardiomyopathy. Circulation 120:1585–1597

Thaulow E, Dale J, Myhre E (1984) Effects of a selective thromboxane synthetase inhibitor, dazoxiben, and of acetylsalicylic acid on myocardial ischemia in patients with coronary artery disease. Am J Cardiol 53:1255–1258

The Ridogrel Versus Aspirin Patency Trial (1994) Randomized trial of ridogrel, a combined thromboxane A2 synthase inhibitor and thromboxane A2/prostaglandin endoperoxide receptor antagonist, versus aspirin as adjunct to thrombolysis in patients with acute myocardial infarction. Circulation 89:588–595

Toth P, Rozsa B, Springo Z, Doczi T, Koller A (2011) Isolated human and rat cerebral arteries constrict to increases in flow: role of 20-HETE and TP receptors. J Cereb Blood Flow Metab 31:2096–2105

Tytgat GN, Van Nueten L, Van De Velde I, Joslyn A, Hanauer SB (2002) Efficacy and safety of oral ridogrel in the treatment of ulcerative colitis: two multicentre, randomized, double-blind studies. Aliment Pharmacol Ther 16:87–99

Uehara Y, Ishimitsu T, Kimura K, Ishii M, Ikeda T, Sugimoto T (1988) Regulatory effects of eicosanoids on thymidine uptake by vascular smooth muscle cells of rats. Prostaglandins 36:847–857

Uyama O, Nagatsuka K, Nakabayashi S, Isaka Y, Yoneda S, Kimura K, Abe H (1985) The effect of a thromboxane synthetase inhibitor, OKY-046, on urinary excretion of immunoreactive thromboxane B2 and 6-keto-prostaglandin F1 alpha in patients with ischemic cerebrovascular disease. Stroke 16:241–244

Vandeplassche G, Hermans C, Somers Y, Van de Werf F, de Clerck F (1993) Combined thromboxane A2 synthase inhibition and prostaglandin endoperoxide receptor antagonism limits myocardial infarct size after mechanical coronary occlusion and reperfusion at doses enhancing coronary thrombolysis by streptokinase. J Am Coll Cardiol 21:1269–1279

Violi F, Hiatt W (2007) A critical review of antiplatelet treatment in peripheral arterial disease. Intern Emerg Med 2:84–87

Watala C, Boncler M, Gresner P (2005) Blood platelet abnormalities and pharmacological modulation of platelet reactivity in patients with diabetes mellitus. Pharmacol Rep 57 (Suppl):42–58

Yui Y, Hattori R, Takatsu Y, Nakajima H, Wakabayashi A, Kawai C, Kayama N, Hiraku S, Inagawa T, Tsubojima M et al (1984) Intravenous infusion of a selective inhibitor of thromboxane A2 synthetase in man: influence on thromboxane B2 and 6-keto-prostaglandin F1 alpha levels and platelet aggregation. Circulation 70:599–605

Zuccollo A, Shi C, Mastroianni R, Maitland-Toolan KA, Weisbrod RM, Zang M, Xu S, Jiang B, Oliver-Krasinski JM, Cayatte AJ, Corda S, Lavielle G, Verbeuren TJ, Cohen RA (2005) The thromboxane A2 receptor antagonist S18886 prevents enhanced atherogenesis caused by diabetes mellitus. Circulation 112:3001–3008

Inhibitors of the Interaction Between von Willebrand Factor and Platelet GPIb/IX/V

Paolo Gresele and Stefania Momi

Contents

Abstract The formation of platelet-rich thrombi, a critical step in the pathogenesis of atherothrombotic events, is a multistep process involving several components, among which von Willebrand Factor (VWF) plays a central role. Ruptured atherosclerotic plaques expose subendothelial matrix proteins which bind VWF that represents a bridge between the injured blood vessel and activated platelets, playing

P. Gresele (✉)
Division of Internal and Cardiovascular Medicine, Department of Internal Medicine, University of Perugia, Via E. dal Pozzo, 06126 Perugia, Italy

Department of Internal Medicine, Section of Internal and Cardiovascular Medicine, University of Perugia, Perugia, Italy
e-mail: grespa@unipg.it

S. Momi
Division of Internal Medicine, Section of Internal and Cardiovascular Medicine, University of Perugia, Perugia, Italy

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_12,

a crucial role in platelet adhesion and aggregation, especially in conditions of high-shear rate. Due to these peculiarities, the binding of VWF to GPIbα is an attractive drug target. Here we summarize the present knowledge on the different classes of drugs targeting the VWF–GPIb interaction and we give an account of their level of clinical development. In particular, the following compounds are discussed: AJW200, an IgG4 humanized monoclonal antibody against VWF-A1; 82D6A3, a monoclonal antibody against VWF-A3; ALX-0081 and ALX-0681, bivalent humanized nanobodies targeting the VWF-A1 domain; ARC1779 and its advanced formulation ARC15105, second-generation aptamers that bind the VWF-A1 domain; h6B4-Fab, a murine monoclonal antibody, and GPG-290, a recombinant chimeric protein, both directed against GPIbα.

Keywords Glycoprotein Ib/IX/V • von Willebrand factor • Nanobody • Aptamer • Platelets • Thrombosis • Shear stress • Stroke • Acute coronary syndromes • Antiplatelet

Platelet activation plays a critical role in the pathogenesis of atherothrombotic events, such as acute coronary syndromes (ACS) or stroke. The formation of arterial thrombi is a multistep process involving several components, and among them von Willebrand Factor (VWF) appears to be crucial, especially in the first phases of the process. Rupture of an atherosclerotic plaque exposes collagen to which VWF is bound, starting platelet adhesion followed by platelet activation (Nilsson et al. 1957). VWF plays a particularly important role in platelet adhesion and aggregation under high-shear conditions (Nilsson et al. 1957; Nillson et al. 1957). Moreover, plasma levels of VWF are raised in disease states associated with endothelial activation and may increase the thrombotic potential, and indeed VWF levels are predictive of adverse cardiac events, including vascular death (Spiel et al. 2008). In view of the above-summarized role of VWF in thrombogenesis, the interaction between VWF and its platelet receptor, GPIb/IX/V, has been considered as a promising new target for antiplatelet therapy (Spiel et al. 2008).

1 von Willebrand Factor

1.1 Structure and Functions

VWF is a large adhesive, multimeric glycoprotein that fulfills two crucial roles in primary hemostasis: it functions as a bridge between subendothelial matrix and circulating platelets, and it acts as a carrier for blood clotting factor VIII, protecting it from rapid inactivation. The gene for VWF is located to the short arm of chromosome 12 and it comprises 180 kb and 52 exons. VWF is mainly synthesized by endothelial cells, where it is stored in Weibel-Palade bodies and secreted either constitutively or by exocytosis, but also by megakaryocytes. VWF is synthesized as

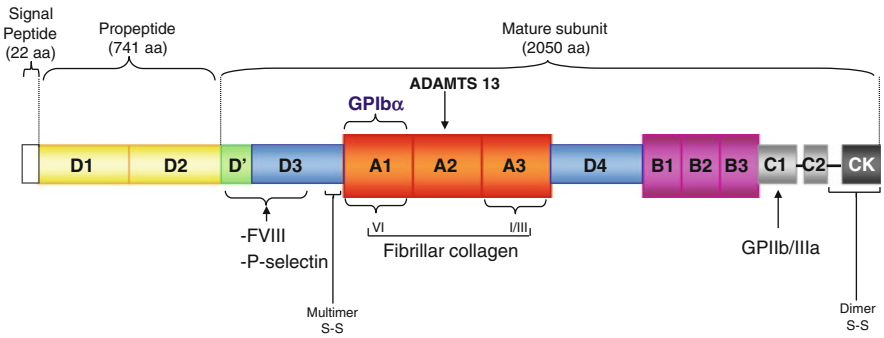


Fig. 1 Structure and domains of VWF

pre-pro-VWF, formed by a signal peptide of 22 aminoacids, a pro-peptide of 741 aminoacids and the mature subunit of 2,050 aminoacids, the latter consisting of four types of domains with specific functions (Fig. 1). The D′–D3 domain contains binding sites for FVIII and for P-selectin (Sadler 1998); P-selectin partially mediates the tethering of UL-VWF strings to the endothelial surface in flowing blood facilitating multimer degradation by ADAMTS-13 through the exposure of the cleavage site on VWF-A2 domain (Michaux et al. 2006); the A1 domain is the binding site for platelet GPIbα but it also contains binding sites for heparin, sulphated glycolipids and botrocetin, a lectin snake venom of Bothrops jararaca that elicits VWF binding to platelet GPIb leading to platelet agglutination (Sixma et al. 1991); the A3 domain is the major binding site for fibrillar collagen type I and III (Cruz et al. 1995); the A2 domain contains the cleavage site for the metalloproteinase ADAMTS-13; finally, the C1 domain is the binding site for GPIIb/IIIa (Beacham et al. 1992). In the endoplasmic reticulum proVWF dimerizes "tail-to-tail" through disulfide bonds between C-terminal cystine knot domains. Pro-VWF dimers are transported to the Golgi apparatus where a propeptide processing protease cleaves the propeptide (domains D1–D2); and additional disulfide bonds between D3 domains form "head to head" between D3 domains in the trans Golgi network resulting in multimers.

Weibel-Palade bodies of endothelial cells are the main storage granules for VWF wherefrom it can be secreted either constitutively or via a pathway regulated by secretagogues, including estrogens, histamine, thrombin, or fibrin (Wagner 1989, 1990; Claus et al. 2010). VWF stored within Weibel-Palade bodies is composed of the largest multimeric species, the UL-VWF (up to 10,000 kDa), usually absent from blood of normal individuals (Wagner and Marder 1984). In physiologic conditions, UL-VWF are in fact cleaved by ADAMTS-13 and disappear from the circulation; they can be detected only transiently in the circulation of normal individuals following treatment with I-deamino-8-D-arginine vasopressin (DDAVP), that induces their secretion from endothelial storage granules (Ruggeri et al. 1982). On the contrary, in microangiopathic disorders, such as thrombotic thrombocytopenic purpura (TTP) or haemolytic uremic

syndrome, UL-VWF accumulates in blood (Lowe and Werner 2005). Approximately 20% of VWF present in blood is stored in platelet α-granules (Harrison et al. 1993) and consists of UL-VWF multimers. Platelets activated at sites of vascular injury, mainly by thrombin, release their VWF content; therefore platelets and endothelial cells co-operate in the release of the most thrombogenic VWF multimers at a site of vascular wall damage. Moreover, when VWF is released acutely FVIII bound to it is detached by thrombin cleavage and made available for the clotting cascade (Nesheim et al. 1991).

In order to bind their ligands, the A1 and A3 domains of VWF are dependent on the fluid dynamic forces generated by blood flow. Shear stress, which expresses the forces produced by the sliding of different layers of blood inside vessels, is greater in arteries than in veins, and among different arteries it is higher in small arterioles of 10–50 μm, where it ranges from 500 to 5,000 s^{-1}, and highest in diseased arteries, such as coronary arteries with stenosis (Mailhac et al. 1994).

Above a shear rate of 1,500 s^{-1} platelet adhesion to a damaged surface is strictly dependent on the interaction between VWF A1 domain and GPIbα. In fact, in conditions of elevated shear stress, VWF exposes the A1 domain normally hidden by the folding of the molecule, and induces a sustained binding to platelet GPIbα stopping them via tether formation. Subsequently, the binding of plasma VWF C1 domain to platelet GPIIb/IIIa (Savage et al. 1996) on one side and to the exposed subendothelial collagen through its A3 domain on the other (Ruggeri et al. 1983) contributes to thrombus growth. UL-VWF multimers contain a large number of binding sites for GPIbα giving rise to spontaneous platelet aggregation and thrombosis (Moake et al. 1982). For this reason, UL-VWF must be actively removed from plasma. When released, UL-VWF are anchored to the surface of endothelial cells through P-selectin and stretched by fluid shear stress to an open conformation that exposes the A2 domain to cleavage by ADAMTS-13 (Schneider et al. 2007; Ruggeri 2003).

The binding of soluble VWF to GPIb-IX-V can be artificially induced in vitro by ristocetin (Giannini et al. 2007), a vancomycin-like antibiotic from *Nocardia lurida*, that binds to the proline-rich sequence, Glu-700 to Asp-709, C-terminal to the Cys-509–Cys-695 disulfide bond of the A1 domain, or by botrocetin that binds to the A1 domain of VWF (Girma et al. 1990).

The interaction of VWF A1 domain with platelet GPIbα is thought to play some role also under static conditions in vitro (Yamashita et al. 2004) or in venous-like blood flow conditions (Savage et al. 1992). Indeed, histology of thrombi from patients who died from pulmonary embolism revealed the presence of GPIIb/IIIa, VWF, and fibrin in close association (Takahashi et al. 2009).

2 GPIb and Platelet Activation

The GPIb/IX/V complex consists of leucine-rich repeat glycoproteins, GPIbα (130 kDa) and GPIbβ (22 kDa), that are disulfide-linked and non-covalently associated with GPIX (22 kDa) and GPV as a 2:2:2:1 complex. Under high-shear

conditions (1,000–10,000 s^{-1}) platelet–platelet interactions become progressively more VWF-dependent, with an important role of both GPIb and integrin $\alpha_{IIb}\beta_3$ in promoting the initial formation of platelet aggregates. Under pathologically high-shear conditions (>10,000 s^{-1}), like those found at sites of severe vessel narrowing, platelet aggregation is exclusively mediated by VWF–GPIb adhesive bonds (Jackson et al. 2009). The interaction between VWF and GPIb may trigger platelet activation (Du 2007). The mechanism through which the VWF–GPIbα interaction signals and contributes to subsequent platelet activation has not been completely defined yet. The cytoplasmic region of GPIbα is associated with filamin (also named actin-binding protein), calmodulin and 14-3-3ζ which provide links to signaling proteins such as PI3K, FAK, Src-related tyrosine kinases, GTPase-activating protein and tyrosine phosphatases (PTP1β and SHPTP10) (Du 2007). Topographical association of the GPIb/IX/V complex with other membrane proteins, such as GPVI, FcR γ-chain, $\alpha_2\beta_1$, FcγRIIA, most likely within specialized membrane microdomains known as lipid rafts, supports a crosslinking mechanism involved in GPIbα signaling (López et al. 2005). The engagement of GPIbα by immobilized VWF elicits activation signals, such as transient cytoplasmic Ca^{2+} elevations, protein phosphorylation (PLCγ2, ERK-1/2, Syk), TxA2 synthesis, ADP release and ultimately activation of $\alpha_{IIb}\beta_3$ (Rivera et al. 2009).

3 VWF/GPIb in Thrombotic Diseases

Plasma VWF is elevated in ACS, such as ST elevation myocardial infarction, and its early rise is predictive of a negative prognosis (Rivera et al. 2009; Montalescot et al. 1998; Ray et al. 2005). Reduced coronary flow was associated with high levels of VWF (Takahashi et al. 2009), and increased VWF levels, as well as low levels of ADAMTS-13, are associated with a high cardiovascular risk (Thompson et al. 1995; Bongers et al. 2009).

TTP is a rare (2–10 cases/million/year) microthrombotic disorder due to the accumulation in plasma of UL-VWF multimers generated by a deficiency, either inherited or acquired, of ADAMTS-13 (Remuzzi et al. 2002; Benz and Amann 2010; Levy et al. 2001; Tsai 2010).

Some polymorphisms of the gene coding for GPIbα have been associated with an increased risk of coronary artery disease in young individuals, like the threonine/methionine substitution at amino acid 145. The biological effect of the thr/met substitution remains to be determined, but it is thought to produce a change in the avidity of GPIb for VWF (Kunicki and Nugent 2002). Moreover, a T/C substitution in the region of the translation start site of the gene influences translation efficiency. The presence of the 5C allele (gene frequency of about 0.10 in various white populations) increases the mean level of GPIbα expressed on platelets (roughly, a 50% increase in homozygous individuals and a 33% increase in heterozygous

individuals), and there are data supporting an association between −5C and the severity of negative outcomes following acute myocardial infarction in young individuals (≤62 years old) (Kunichi and Nugent 2002). Moreover, a synergistic effect between −5C and Met145 in increasing the risk of stroke in younger individuals has been documented (Sonoda et al. 2001).

4 VWF/GPIb in Haemorrhagic Disorders

4.1 von Willebrand Disease

VWD is the most common inherited bleeding disorder, with an estimated prevalence of ~1%, but the disorder is often asymptomatic, thus the prevalence of clinically significant cases is estimated to be around one in 10,000 (Sadler et al. 2006; Lillicrap 2007). VWD is divided into three subtypes: Type 1 (partially quantitative defect), Type 2 (qualitative defects), and Type 3 (virtually complete absence of VWF). Type 2 can be further divided into 2A (caused by dominant loss-of-function mutations, usually in the A2 domain, and occasionally in the A1 domain), 2B (caused by dominant gain-of-function mutations, usually missense, in the A1 domain), 2M (caused by a mutations in the A1 domain leading to defective VWF binding to GPIbα), and 2N (caused by recessive mutations in the D′ to D3 domains which inactivate the binding of VWF to FVIII) (Federici 2009).

VWD encompasses a wide spectrum of disease severity, ranging from rare and mild bleeding symptoms to severe hemorrhagic episodes that are similar to those of severe hemophilia. The most common bleeding symptoms in VWD reflect impaired platelet adhesion, and include mucosal bleeding, especially epistaxis and menorrhagia. Life-threatening bleeding may rarely occur, especially in VWD type 3 or certain cases of VWD type 2 (Sadler et al. 2000; Federici et al. 2006).

4.2 Bernard Soulier Syndrome

Bernard Soulier syndrome (BSS) is a rare autosomal recessive, inherited platelet function disorder, associated with mild to severe mucocutaneous bleeding, due to a defect of platelet GPIb/IX/V (Lopez et al. 1998; Ware et al. 2000). In a recent paper an attempt to correlate genotype and phenotype in BSS patients has been made (Savoia et al. 2011). Bleeding diathesis did not correlate with thrombocytopenia, which was always moderate, and platelet GPIbα expression which was always severely impaired (Savoia et al. 2011)

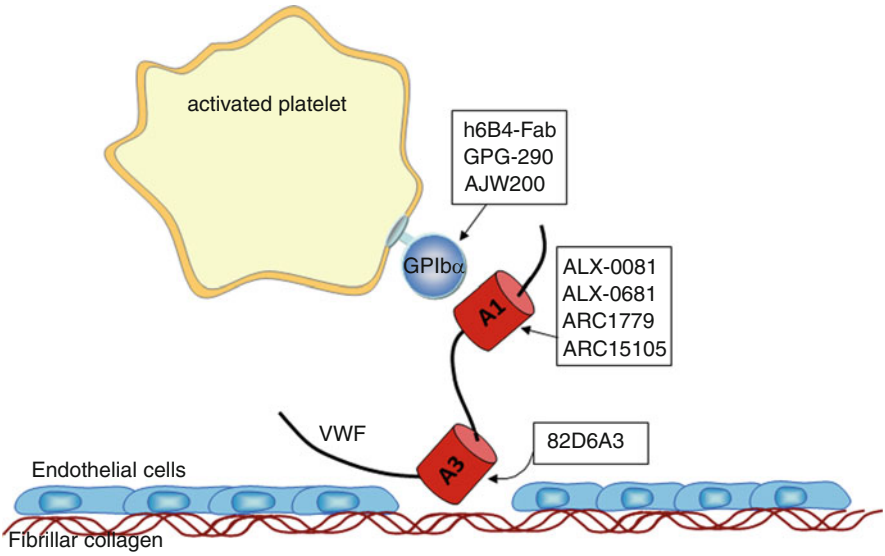


Fig. 2 Strategies aimed at interacting with the binding of VWF to GPIbα

5 Strategies to Inhibit the von Willebrand Factor–GPIb/IX/V Interaction

The VWF/GPIb interaction is a potentially attractive target for new antithrombotic agents. Indeed, since VWF has a role in platelet adhesion and activation essentially at high-shear stresses, it is expected that the blockade of VWF-mediated platelet activation would interfere more with platelet deposition in diseased arteries, such as stenosed coronary or cerebral arteries, than in healthy blood vessels, thus preventing thrombosis without affecting significantly physiologic haemostasis. Strategies aimed at interacting with the binding of VWF to GPIbα have involved the development of monoclonal antibodies, nanobodies or aptamers against VWF and GPIb (Fig. 2).

5.1 Monoclonal Antibodies Against VWF

5.1.1 AJW200

AJW200 is an IgG4 monoclonal antibody directed against the A1 domain of VWF, developed starting from the AJvW-2 murine monoclonal antibody, later humanized to minimize the immunological response when administered to humans (Fontayne et al. 2008). The precursor of AJW200, AJvW-2, was found to inhibit ristocetin- and botrocetin-induced aggregation, high-shear stress, but not low-shear stress-induced aggregation and adhesion to collagen type I of human platelets; moreover,

photochemically induced thrombosis of the carotid artery of guinea pigs was inhibited by AJvW-2 without concomitant prolongation of the bleeding time (Kageyama et al. 1997).

In Vitro Data

AJW200 selectively inhibits human (Kageyama et al. 2002a), canine (Kageyama et al. 2002b), and rabbit (Yamashita et al. 2003) platelet aggregation induced by ristocetin and botrocetin. AJW200 also suppresses high-shear stress (108 dyne/cm^2)-induced platelet aggregation, as measured by the cone-and-plate viscometer, with an IC_{50} of 1.0 $\pm$ 0.1 μg/ml. In contrast, low-shear stress (12 dyne/cm^2)-induced platelet aggregation is not affected by AJW200 up to a concentration of 80 μg/ml. Similarly, platelet adhesion to a type III collagen-coated surface was inhibited by AJW200 under high-shear stress conditions (1,500 s^{-1}) (IC_{50} 2.6 $\pm$ 0.2 μg/ml), but not at low-shear stress (360 s^{-1}) ($IC_{50} > 64$ μg/ml). AJW200 also inhibited high-shear stress-induced thrombin generation (Kageyama et al. 2002a).

Preclinical Studies

An inhibition of ristocetin-induced platelet aggregation sustained over 24 h, 6 days, and 2 weeks was observed after a single bolus injection of, respectively, 0.3, 1, and 3 mg/kg of AJW200 in cynomolgus monkeys. AJW200 did not affect template skin bleeding time at the dose of 0.3 mg/kg, while it significantly prolonged it at 1 and 3 mg/kg, although moderately as compared with the anti-GPIIb/IIIa abciximab (Kageyama et al. 2002a).

AJW200 inhibited thrombus formation in stenosed coronary arteries of beagle dogs without prolonging the bleeding time, showing a safer profile as compared with abciximab (Kageyama et al. 2002a). At least 50% occupancy of VWF (corresponding to 0.7 μg/ml of AJW200 in plasma) was required for the inhibition of cyclic flow variations in vivo, while the bleeding time was extensively prolonged when VWF occupancy reached 80–100% (corresponding to ~20 μg/ml of AJW200 in plasma) (Kageyama et al. 2002a). In a model of balloon injury-induced thrombosis of the iliac arteries in rabbits, a bolus of AJW200 (3 mg/kg) significantly reduced fibrin-rich thrombus formation and the subsequent neointimal growth (Kageyama et al. 2002b) and prevented occlusive thrombus formation (Yamashita et al. 2003).

Finally, AJW200 reduced the number and weight of iliac vein thrombi and pulmonary thromboemboli generated by positioning a polyethylene tube in the iliac veins of rabbits (Takahashi et al. 2009).

Clinical Studies

In a randomized, double blind, Phase I study, placebo or three doses of AJW200 (0.01, 0.03 or 0.05 mg/kg) were infused i.v. in 24 healthy male subjects. No significant adverse events were recorded and there was no evidence of immunogenicity. The maximum VWF occupancy obtained was 19.4, 51.0 and 62.4%, respectively, and correlated with plasma AJW200 concentrations. AJW200 produced a dose-dependent inhibition of RiCof at 1 h post-infusion (58 ± 22 vs. 110 ± 25% and 34 ± 15 vs. 116 ± 41%, at 0.03 and 0.05 mg/kg, respectively) and a prolongation of the PFA-100 closure time, an effect that appeared to be related to the baseline VWF level, without concomitantly prolonging the skin bleeding time (Machin et al. 2003).

5.1.2 Monoclonal Antibody Against the A3 Domain of VWF

82D6A3 is a monoclonal antibody against the A3 domain of human VWF that inhibits the interaction of VWF with fibrillar collagen type I and III (Hoylaerts et al. 1997). 82D6A3 binds the A3-domain of VWF but not of denatured or reduced VWF, suggesting that 82D6A3 does not recognize a linear epitope.

In Vitro Data

82D6A3 inhibited platelet adhesion under flow conditions at different shear rates. The inhibitory effect increased with increasing shear stresses, with no significant effect at 650 s^{-1}, a mild effect at 1,300 s^{-1}, and an almost complete inhibition at 2,600 s^{-1}, in agreement with the VWF dependence of the reaction (Vanhoorelbeke et al. 2003).

Preclinical Studies

The antithrombotic efficacy of 82D6A3 was evaluated in a modified Folts model in baboons. Baboons undergoing mechanical injury to the femoral artery were treated with an i.v. bolus of 82D6A3, at the doses of 100, 300, and 600 μg/kg, resulting in 58.3%, 100%, and 100% reduction, respectively, in cyclic blood flow variations. At the dose of 100 μg/kg 80% of the VWF-A3 domain was occupied, corresponding to a 30–36% ex vivo inhibition of VWF binding to collagen. 82D6A3 did not prolong the bleeding time up to 300 μg/kg mg/kg, even when 100% of VWF was occupied and 100% ex vivo inhibition of VWF-collagen binding was observed (Wu et al. 2002a). Under the same conditions, an anti-GPIIb/IIIa monoclonal antibody induced a strong prolongation of the bleeding time (Levy et al. 2001).

82D6A3 has been humanized by variable domain resurfacing and grafting on the constant region of a human IgG4 (Staelens et al. 2006) resulting in a h82D6A3 with an in vitro activity comparable to that of murine IgG, a step toward the use in humans. However, studies evaluating the antithrombotic activity of h82D6A3 in humans have not been reported yet.

5.2 *Nanobodies*

Despite significant clinical applications, monoclonal antibodies have several limitations that have opened the way to the development of smaller, more versatile antibodies. Nanobodies are naturally occurring antibodies devoid of light chains, initially discovered in the serum of dromedaries and then found in sera of all camelids; these antibodies, in addition to light chains, also lack the first constant domain of heavy chains (C_H1). The variable domains of these antibodies, referred to as V_nHs, represented the basis for the development of nanobodies, a new class of therapeutic proteins consisting of one or more single antigen-binding domains. Several nanobodies against various targets of potential therapeutic interest have been developed, including antithrombotic nanobodies (Siller-Matula et al. 2011).

5.2.1 ALX-0081

ALX-0081 is a first-in-class, bivalent humanized nanobody that binds with high avidity the A1 domain of VWF, thereby blocking the interaction between GPIbα and VWF under high-shear conditions (Van Bockenstaele et al. 2009). ALX-0081 consists of two identical building blocks, PMPaP2A2h1, bound to each other via a short linker. Bivalency is required to have a high affinity interaction with VWF A1 which translates in a potent inhibition of VWF binding to platelet GPIbα.

In Vitro Studies

ALX-0081 significantly inhibited ristocetin-induced binding of VWF to human platelets, as measured by ELISA, with an IC_{50} of 0.26 ± 0.04 nM (Markus et al. 2011). ALX-0081 (0.8 μg/ml) prevented platelet adhesion to collagen type III at high-shear rates (>1,500 s^{-1}) but not at low-shear rates (<500 s^{-1}). In the absence of VWF, ALX-0081 did not interfere with the platelet–collagen interaction (Ulrichts et al. 2011). The addition of ALX-0081 (0.1 to 3 μg/ml) to blood from patients with ACS treated with aspirin, clopidogrel, and unfractionated heparin, in which residual platelet activation was still observed, completely inhibited platelet adhesion under high-shear conditions (1,600 s^{-1}). Moreover, ALX-0081 inhibited ristocetin-induced aggregation (RIPA) of platelets from healthy individuals, at the concentration of 0.4 μg/ml, and from CAD patients, at the concentration of 0.8 μg/ml. The fact that lower concentrations of ALX-0081 were required in healthy volunteers to inhibit platelet function as compared to those required in CAD patients is probably explained by the higher VWF-Ag levels observed in CAD patients (Van Loon et al. 2011). Indeed, the effective ALX-0081 dose resulting in complete suppression of platelet adhesion to collagen under high shear ranged from 0.2 to 0.8 μg/ml and correlated with the levels of VWF in the plasma of patients

(Ulrichts et al. 2011). Finally, ALX-0081 dose-dependently prolonged the C/ADP closure time in the PFA-100 system (Van Loon et al. 2011).

Preclinical Studies

ALX-0081, fully active in humans, cross-reacts with VWF from primates and, partially, from guinea pigs, while it does not bind VWF from other rodents. Initial safety and efficacy studies in cynomologus monkeys showed that ALX-0081 (0.4 and 8 mg/kg, given by i.v. injection) inhibited ristocetin-induced platelet aggregation, for up to 48 h with the highest one (Ulrichts et al. 2011). The half life of a i.v. bolus of ALX-0081 ranged from 17 to 30 h. PK-PD analysis indicated that plasma levels exceeding 1 μg/ml of ALX-0081 resulted in complete RiCof inhibition.

The antithrombotic effect of ALX-0081 was tested in a Folt's model in the femoral arteries of baboons. Plasma levels of ALX-0081 between 0.3 and 0.5 μg/ml induced full inhibition of cyclic flow reductions. The effect of ALX-0081, abciximab, and clopidogrel on bleeding was evaluated by a surgical bleeding method. Mean blood loss was higher in animals treated with clopidogrel (10 mg/kg) and to an even greater extent in those treated with abciximab (20–500 μg/kg) than in animals receiving ALX-0081 (3–300 μg/kg) (1.6- to sixfold lower as compared with clopidogrel and abciximab, respectively). Comparing the doses required to prevent cyclic flow reductions and those increasing surgical bleeding, ALX-0081 showed a larger therapeutic window as compared with abciximab and clopidogrel (Ulrichts et al. 2011).

In a model of middle cerebral artery (MCA) thrombosis induced by photochemical injury in guinea pigs, ALX-0081 was compared with tirofiban, a GPIIb/IIIa inhibitor, and rtPA, a thrombolytic agent. ALX-0081 (5 mg/kg), administered immediately after the total occlusion of the MCA, restored blood perfusion; tirofiban (10 mg/kg i.v. bolus followed by 20 mg/kg/min for 2 h infusion) and rtPA (0.1 mg/kg i.v. bolus plus 0.9 mg/kg/min for 30 min infusion) were also effective. However, while treatment with ALX-0081 prevented brain damage, assessed by vital staining 24 h after the induction of injury, both tirofiban and rtPA were ineffective. The antithrombotic effect of ALX-0081 was not associated with intracerebral hemorrhage while intracranial bleeding was observed in tirofiban and rtPA-treated animals (Momi et al. 2011).

Clinical Studies

A Phase I study tested ALX-0081, given by i.v. infusions (1 h) at doses ranging from 0.5 mg to 12 mg, in 40 male healthy volunteers. ALX-0081 was well tolerated and appeared to be safe, with no bleeding and no immunogenic response. ALX-0081 displayed non-linear pharmacokinetic properties, following a two compartment model. Ristocetin-induced platelet agglutination was fully inhibited at doses $\geq$2 mg, corresponding to plasma concentrations of 400 ng/mL, 1 h post-dosing,

with a maximum duration of 12 h. A mild and transient reduction of FVIII and VWF in plasma was observed, fully reversible within 24 h.

A placebo-controlled, dose-escalating, Phase Ib study was carried out in 25 patients with stable angina undergoing PCI. Single escalating doses (2–9 mg) were followed by multiple dosing (four doses in 24 h for a total of 18 mg). A significant inhibition of ristocetin-induced platelet agglutination and ristocetin cofactor activity for 24 and 30 h was observed. Only mild and transient adverse events were reported and most of them seemed to be related to the PCI procedure; only minor bleedings were reported and these apparently did not differ between the treatment groups (Holz et al. 2009).

A Phase II, randomized, open-label study, designed to compare the safety, tolerability and biological effectiveness of ALX-0081 versus the GPIIb/IIIa inhibitor ReoPro in high risk PCI patients receiving standard treatment with acetylsalicylic acid plus clopidogrel and heparin is ongoing (http://clinicaltrials.gov/ct2/show/NCT01020383?term=ALX-0081&rank=1). Patients are randomly assigned to either ALX-0081 (four i.v. boluses, once every 6 h: the first of 6 mg, and the subsequent three doses of 4 mg) or ReoPro (0.25 mg/kg i.v. bolus followed by continuous i.v. infusion of 0.125 μg/kg/min for 12 h).

5.2.2 ALX-0081 Plus ALX-0681

ALX-0681 is a nanobody identical to ALX-0081, targeting the A1 domain of VWF but specifically formulated to be administered subcutaneously.

Clinical Studies

A Phase II, single-blind, randomized, placebo-controlled trial, involving 40 centers worldwide, designed to assess the efficacy and safety of anti-VWF nanobody as adjunctive treatment to plasma exchange in 110 patients with acquired TTP (TITAN Study) is ongoing (clinical trial identifier: NCT01151423). The dose regimen is 10 mg i.v. bolus of ALX-0081, prior to plasma exchange, followed by 10 mg ALX-0681 injected s.c. once or twice a day for 30 days; in the placebo-controlled group i.v. bolus injection prior to plasma exchange, followed by daily s.c. injection of placebo comparator, is administered.

Very preliminary results (5/110 patients) have been recently reported showing a significant shortening of the time to normalization of platelet count (primary end point of the study) as compared with plasma exchange alone, a reduction of UL-VWF in plasma and a sustained inhibition of ristocetin-induced platelet agglutination (Peyvandi et al. 2011). Final results of this trial are expected for 2013.

5.3 Aptamers

5.3.1 ARC 1779

Aptamers are nucleic acid molecules with high affinity and specificity for a selected target molecule, with an ability to fold into unique three-dimensional structures that promote target binding. Through conjugation with high molecular weight polyethylene glycol, aptamers are engineered to have some of the attributes of monoclonal antibodies and some of those of low molecular weight chemically synthesized drugs. ARC1779 is a nuclease-resistant aptamer conjugated to a 20 kDa polyethylene glycol at the 5′terminus. It binds with high affinity to the A1 domain of VWF and inhibits VWF-dependent platelet aggregation.

In Vitro Data

ARC1779 inhibits botrocetin-induced platelet aggregation with an IC_{50} of 344nM (Machin et al. 2003), VWF activity with an IC_{50} of 100nM, and shear dependent platelet function as assessed by the PFA-100 with an IC95 ~400nM, in blood from healthy volunteers as well as from ACS patients (Diener et al. 2009). Moreover, ARC1779 dose-dependently inhibits platelet adhesion in a parallel plate perfusion chamber under high-shear rate (1,500 s^{-1}), an effect completely absent at low-shear rate (Machin et al. 2003). In a model of human whole blood perfused at a high-shear rate (6,974 s^{-1}) over de-endothelized porcine arteries, ARC1779 significantly reduced platelet accumulation (Machin et al. 2003).

Preclinical Studies

In a carotid artery thrombosis model induced by electrical injury in cynomolgus monkeys, ARC1779 (i.v. bolus + infusion) inhibited the formation of occlusive thrombi (Machin et al. 2003). At the plasma concentration of 700 nM (9.1 μg/ml), effective in preventing thrombosis, only a mild prolongation of the bleeding time was observed (Machin et al. 2003).

The antithrombotic effects of ARC1779 were also determined in an ex vivo model in which blood from patients on double antiplatelet therapy with aspirin and clopidogrel labeled with ^{111}In was perfused over injured porcine aortic segments under high-shear rate. ARC1779 significantly reduced platelet adhesion at 75 and 250 nM (Diener et al. 2009).

Clinical Studies

A first in man phase I, randomized, double blind, placebo controlled, dose-escalating study was carried out in healthy volunteers testing the pharmacodynamic profile of ARC1779 administered either as an i.v. bolus or as an i.v. bolus followed

by a 4-h infusion (Spiel et al. 2009). ARC1779 induced complete inhibition of the PFA-100 C/ADP closure time with an EC_{50} of 2–3 μg/ml. These plasma concentrations of ARC1779 were achieved at C_{max} with doses as low as 0.1 mg/kg and were sustained after slow i.v. bolus administration of 1.0 mg/kg for at least 6 h. No bleeding manifestations were observed (Spiel et al. 2009).

In a phase II, randomized, cross-over, double-dummy, pilot study carried out in type 2B VWD patients, ARC1779 0.23 mg/kg plus a 4-hour continuous infusion of 0.001 mg/kg/min, that results in a steady state concentration of 4–5 μg/ml) completely blocked the VWF A1 domain, enhanced desmopressin-induced RiCo and FVIII activity and prevented the rapid consumption of VWF multimers together with agglutinated platelets that occurs in response to desmopressin in these patients (Jilma et al. 2010).

A prospective, open-label clinical trial with a partial cross-over design has been carried out to test the efficacy and safety of ARC1779, added to plasma exchange, in patients with TTP. Three different administration regimens were used: subcutaneous injections of 50 mg of ARC1779 on seven consecutive days, a low-dose infusion of ARC1779 (0.002 mg/kg/min) for 24–72 h, and a high-dose infusion (0.004–0.006 mg/kg/min) for up to 72 h. ARC1779 was well tolerated without any bleeding at concentrations spanning over three orders of magnitude.

Infusion of ARC1779 dose-dependently inhibited VWF-dependent platelet function and increased or stabilized platelet counts in congenital TTP. However, the tested doses, particularly the daily s.c. injections that did not reach therapeutically effective plasma concentrations, did not correct all clinical or laboratory features of TTP (Jilma-Stohlawetz et al. 2011).

More recently, the effect of treatment with ARC1779 on a surrogate marker of cerebral embolism, i.e., embolic signals assessed by trans-cranial Doppler Ultrasound, was evaluated in patients undergoing carotid endarterectomy. Subjects planned for carotid endarterectomy for symptomatic or asymptomatic carotid stenosis were randomized to placebo or ARC1779. The dose regimen for ARC1779 was 0.00015 mg/kg/min for 20 min, 0.003 mg/kg/min for 20 min, 0.006 mg/kg for 20 min followed by a continuous infusion at 0.0006 mg/kg/min. The administration of the study drug was begun 1 h before induction of anesthesia and continued for 3.5 h after skin closure. ARC1779 resulted in a rapid reduction in the frequency and mean intensity of embolic signals. Anemia was reported in the ARC1779 arm of the study (Markus et al. 2011).

5.3.2 ARC15105

ARC15105 is a second-generation VWF A1 domain-inhibitory aptamer; more specifically it is a 21 nucleotide all 2′OMe aptamer, conjugated to 40 kDa polyethylene glycol, with potency and pharmacokinetic characteristics suitable for chronic s.c. treatment. ARC15105 binds VWF with a KD of ~1 nM, is highly stable in human, monkey, and rat serum, with 87–99% of the intact aptamer remaining after

72 h. The 40 kDa PEGylated aptamer had a $t_{1/2}$ of 18 h in rats, ~66 h in monkeys with a bioavailability of almost 98%: allometric scaling estimates the human $t_{1/2}$ in approximately 217 h (Siller-Matula et al. 2011).

In Vitro Studies

ARC15105 inhibited platelet adhesion to collagen under arterial shear flow conditions in a perfusion chamber more effectively than ARC1779 (IC_{50}: 18.5 vs. 175 nM, $p < 0.001$). ARC15105 40nM inhibited by 93% platelet adhesion to denuded porcine aortas perfused under high-shear conditions with blood from healthy volunteers. Moreover, ARC15105 1 μM completely inhibited ristocetin-induced platelet agglutination but also reduced to some extent collagen, ADP, arachidonic acid, and TRAP-induced platelet aggregation (Siller-Matula et al. 2011), a finding not commented upon.

Finally, ARC15105 completely suppressed VWF activity, as measured by ELISA, in samples collected from patients with myocardial infarction (IC_{50}: 27 nM) (Siller-Matula et al. 2011).

5.4 GPIb Receptor Antagonists

GPIbα is the central component of the receptor complex formed by glycoproteins GPIbα, GPIbβ, GPV, and IX. Human platelets contain approximately 25,000 copies of the GPIb/IX/V complex. GPIbα anchors the complex to the cytoskeleton and harbors the VWF-binding function in its ~290 NH2-terminal residues (Huizinga et al. 2002).

A number of potent anti-GPIb inhibitory antibodies have been produced and extensively tested with respect to their in vitro effects on platelets under both static and flow conditions (Huizinga et al. 2002; Miller et al. 1991; Uff et al. 2002; Cauwenberghs et al. 2001).

5.4.1 h6B4-Fab

The murine monoclonal antibody 6B4 was raised against purified human GPIb. In particular, it recognizes the epitope mapped to the C-terminal flanking region of GPIbα (His1-Val289) (Cauwenberghs et al. 2001). A fully recombinant and humanized version of 6B4-Fab fragment (h6B4-Fab) was recently developed (Fontayne et al. 2006).

In Vitro Studies

The intact 6B4 IgG monoclonal antibody blocks dose-dependently the binding of GPIb to VWF, it inhibits ristocetin-induced platelet agglutination and platelet adhesion to human collagen type I, in a parallel plate perfusion chamber at a shear rate of 2,600 s^{-1} (Cauwenberghs et al. 2001). The MoAb 6B4 Fab fragment blocked ristocetin- and botrocetin-induced platelet aggregation with an IC_{50} of 1.2 ± 0.3 μg/ml and 2.0 ± 0.5 μg/ml, respectively (Cauwenberghs et al. 2000). Moreover, it inhibited more effectively than intact 6B4 platelet adhesion under shear rates of 650, 1,300, and 2,600 s^{-1} at the doses of 3.5 μg/ml, 1.1 μg/ml, and 0.5 μg/ml, respectively (Cauwenberghs et al. 2000).

Preclinical Studies

The injection of 100 μg/kg of intact 6B4 into baboons caused a rapid drop of the platelet count ($<30 \times 10^9$/l) within 10 min after injection, with a slow increase observed after 48 h. The same dose of the 6B4-Fab fragment induced a rapid decrease of platelet count to ~120–150 $\times 10^9$/l but after 24 h the number of circulating platelets was completely normalized (Cauwenberghs et al. 2001).

Pre-treatment of baboons with the 6B4 Fab fragment (80 and 160 μg/kg) reduced platelet deposition on a thrombogenic device (a polytetrafluoroethylene-silicon rubber arteriovenous shunt), by 43 and 65%, measured 15 min after treatment. No complete inhibition of platelet adhesion was observed, even at high doses, probably due to the medium shear rate used in these experiments (700 and 1,000 s^{-1}). Injection of 6B4 Fab fragment (110 μg/kg) in baboons 6 min after a thrombus was allowed to form did not affect platelet deposition indicating that, at least in this model, GPIb did not play a major role in platelet–platelet interactions (Cauwenberghs et al. 2001).

Moreover, the i.v. injection of a single 0.5 mg/kg bolus of h6B4-Fab significantly reduced, while two subsequent administrations, resulting in the cumulative doses of 1.5 and 2.5 mg/kg, completely abolished cyclic flow reductions of a stenosed femoral artery in baboons (Yamashita et al. 2003). Intravenous administration of 0.5 mg/kg of h6B4-Fab resulted in a plasma concentration of 6.3 ± 1.1 μg/ml, with a $t_{1/2}$ of 15.5 min. Plasma concentrations raised to 25.9 ± 3.5 μg/ml after an additive dose of 1 mg/kg. A plasma concentration of 10 μg/ml fully inhibited ristocetin-induced platelet agglutination (Fontayne et al. 2008).

The antithrombotic effect of h6B4-Fab was accompanied by an only mild prolongation of the skin template bleeding time and of bleeding loss from a standardized incision. No thrombocytopenia was observed (Fontayne et al. 2008).

Another study evaluated the anti-thrombotic effects of several doses of the 6B4-Fab fragments in combination with the anti-GPIIb/IIIa antibody MA-16N7C2 in baboons. Pre-treatment of baboons with a combination of 1.5 μg/ml of 6B4-Fab and 0.5 μg/mL of MA-16N7C2 inhibited ex vivo collagen-coated surface coverage by platelets by 76%, whereas 88% inhibition was achieved with 2.25 μg/ml 6B4-Fab

and 0.75 μg/ml of MA-16N7C2 as measured by a parallel plate perfusion chamber at the shear rate of 1,500 s^{-1}. 6B4-Fab (0.6 mg/kg) did not affect skin bleeding time in baboons while MA-16N7C2 (0.3 mg/kg) significantly prolonged it. The combination of the two antibodies (0.6 mg/kg of 6B4 plus 0.1 mg/kg of MA-16N7C2) completely abolished ristocetin-, ADP- and collagen-induced platelet aggregation and significantly reduced cyclic flow variations but, interestingly, did not further prolong the bleeding time as compared with the single drugs (Fontayne et al. 2008).

5.4.2 GPG-290

GPG-290 is a recombinant, chimeric protein containing the 290 amino-terminal amino acids of GPIba linked via a proline to a human IgG1 Fc produced and purified from Chinese hamster ovary (CHO) cells. GPG-290 is highly pure, stable, and well tolerated in animals and has a half-life of approximately 1.5 days.

Preclinical Studies

The antithrombotic effect of GPG-290, alone or in combination with clopidogrel, was evaluated in a canine model of electrolytic injury-induced thrombosis of the left circumflex coronary artery (Hennan et al. 2006).

GPG-290 (50, 100, and 500 μg/kg i.v.) dose-dependently prolonged the time to coronary artery occlusion. Template tongue bleeding time was unchanged after GPG-290 50 and 100 μg/kg while it was prolonged (>2.5-fold) after the administration of the highest dose, but only at the 1 h time point. Clopidogrel was administered orally in two dose regimens: a therapeutic dosing regimen of 4.3 mg/kg on day 2 followed by 1.1 mg/kg for the subsequent two days (the last dose was administered 1 h before the surgical procedure) and a loading dose regimen of 4.3 mg/kg 3 h before the procedure. Clopidogrel was effective in prolonging the time to thrombotic occlusion, but significant bleeding was observed after the loading dose (Hennan et al. 2006).

The combination of clopidogrel (therapeutic dosing regimen) with GPG-290 100 μg/kg further prolonged, although slightly, the time to artery occlusion as compared with the single drugs, and improved blood flow (Hennan et al. 2006). The combination of GPG-290 100 μg/kg with the loading dose of clopidogrel (4.3 mg/kg 3 h before the procedure) provided incremental protection against thrombosis, prolonging the occlusion time and reducing the number of occluded arteries, with no additional prolongation of the bleeding time as compared with clopidogrel alone (Wu et al. 2002b).

The antithrombotic effect of GPG-290 was also assessed in a Folt's model of stenosed coronary artery in dogs. GPG-290, at doses ranging from 25 to 100 μg/kg which correspond to plasma concentrations of 0.6–2.0 μg/ml, completely abolished cyclic flow variations in 67–100% of treated dogs, without prolonging the bleeding time. Moreover, GPG-290 had no-effect on plasma VWF antigen and VWF–collagen binding activity. Interestingly, the moderate prolongation of

the bleeding time (three to fourfold increase) induced by GPG-290 at the highest dose tested of 500 μg/kg (10 times the efficacious antithrombotic dose) was normalized by DDAVP (0.3 μg/kg over 5 min) (Wadanoli et al. 2007).

6 Conclusions

Given the pivotal role of VWF in mediating platelet adhesion under high-shear stress conditions, the inhibition of the GPIb–VWF axis is a potentially promising new strategy to widen the therapeutic window of antiplatelet therapy. The antithrombotic potential of drugs interfering in different ways with the GPIb–VWF interaction has been documented in animal models and in preliminary clinical studies in humans. Aptamers, monoclonal antibodies or nanobodies directed against the A1 domain of VWF, the A3 domain of VWF or against GPIb are expected to be tested soon for their therapeutic potential in acute coronary syndromes and in acute ischemic stroke. Whether the reduced bleeding risk associated with the inhibition of GPIb–VWF interaction, and the pre-eminent activity in conditions of elevated shear stress shown in several animal models and in preliminary clinical trials, will translate in enhanced clinical benefit remains to be established by large, prospective, multicenter clinical trials.

Knowledge Gaps

- A better definition of the bleeding/antithrombotic balance of inhibitors of the VWF/GPIb axis in vivo is needed.
- A precise definition of the degree of VWF blockade or GPIb occupancy required to obtain an antithrombotic effect but not inducing bleeding needs to be established.
- Combination studies with other antiplatelet/anticoagulant drugs (including the new oral anticoagulants) are warranted.
- For blockers of the VWF–collagen interaction (A3 domain inhibitors) further studies to exclude possible negative effects, due to the crucial physiologic role this interaction may have in platelet adhesion, are warranted.
- The potential variation of the effective concentration of VWF–GPIb blockade depending on the plasma VWF levels (e.g. higher in ACS patients as compared to healthy people), and its possible impact on the therapeutic effectiveness of this novel therapeutic approach, needs to be fully evaluated.
- Whether chronic inhibition of the VWF–GPIb interaction may have an effect on atherosclerosis progression remains to be established.

Key Messages

- VWF acts as a bridging element between damaged endothelial sites and the GPIb receptor on platelets.
- VWF plays a key role in platelet adhesion and aggregation, especially under high-shear conditions.
- In inflammatory and atherosclerotic conditions, chronically elevated levels of VWF are observed, that may contribute to an increased thrombotic tendency.
- In patients with TTP, ultra-large VWF multimers are present in plasma owing to a deficiency of the VWF-cleaving protease ADAMTS13.
- Inhibitors of the VWF/GPIb axis have been developed, including aptamers, nanobodies, Monoclonal antibodies.
- Several preliminary phase II trials have tested inhibitors of VWF or GPIb in TTP with promising results in terms of reduction of platelet activation, especially in condition of high-shear rate, and safety.
- Prospective clinical trials, evaluating the safety and efficacy of novel VWF inhibitors in cardiovascular disease, are ongoing.

References

Beacham DA, Wise RJ, Turci SM et al (1992) Selective inactivation of the Arg-Gly-Asp-Ser (RGDS) binding site in von Willebrand factor by site-directed mutagenesis. J Biol Chem 267:3409–3415

Benz K, Amann K (2010) Thrombotic microangiopathy: new insights. Curr Opin Nephrol Hypertens 19:242–247

Bongers TN, de Bruijne EL, Dipped DW et al (2009) Lower levels of ADAMTS13 are associated with cardiovascular disease in young patients. Atherosclerosis 207:250–254

Cauwenberghs N, Meiring M, Vauterin S et al (2000) Antithrombotic effect of platelet glycoprotein Ib-blocking monoclonal antibody Fab fragments in nonhuman primates. Arterioscler Thromb Vasc Biol 20:1347–1353

Cauwenberghs N, Vanhoorelbeke K, Vauterin S et al (2001) Epitope mapping of inhibitory antibodies against platelet glycoprotein Iba reveals interaction between the leucine-rich repeat N-terminal and C-terminal flanking region domains of glycoprotein Ibα. Blood 98:652–660

Claus RA, Bockmeyer CL, Sossdorf M et al (2010) The balance between von-Willebrand factor and its cleaving protease ADAMTS13: biomarker in systemic inflammation and development of organ failure? Curr Mol Med 10:236–248

Cruz MA, Yuan H, Lee JR et al (1995) Interaction of the von Willebrand factor (VWF) with collagen. Localization of the primary collagen-binding site by analysis of recombinant VWF A domain polypeptides. J Biol Chem 270:10822–10827

Diener JL, Danile Lagasse HA, Duerchmied D et al (2009) Inhibition of von Willebrand factor-mediated platelet activation and thrombosis by the anti von Willebrand factor A1-domain aptamer ARC1779. J Thromb Haemost 7:1155–1166

Du X (2007) Signaling and regulation of the glycoprotein Ib/IX/V complex. Curr Opin Hematol 14:262–269

Federici AB (2009) Classification of inherited von Willebrand Disease and implications in clinical practice. Thromb Res 124:S2–S6

Federici AB, Castaman G, Thompson A, Berntorp E (2006) Von Willebrand's disease: clinical management. Haemophilia 12:152–158

Fontayne A, Vanhoorelbeke K, Pareyn I et al (2006) Rational humanization of the powerful antithrombotic anti GPIba antibody: 6B4. Thromb Haemost 96:671–684

Fontayne A, Meiring M, Lamprecht S et al (2008) The humanized anti-glycoprotein Ib monoclonal antibody h6B4-Fab is a potent and safe antithrombotic in a high shear arterial thrombosis model in baboons. Thromb Haemost 100:670–677

Giannini S, Mezzasoma AM, Leone M, Gresele P (2007) Laboratory diagnosis and monitoring of desmopressin treatment of von Willebrand's disease by flow cytometry. Haematologica 92:1647–1654

Girma JP, Takahashi Y, Yoshioka A, Diaz J, Meyer D (1990) Ristocetin and botrocetin involve two distinct domains of von Willebrand factor for binding to platelet membrane glycoprotein Ib. Thromb Haemost 64:326–332

Harrison P, Cramer EM (1993) Platelet alpha-granules. Blood Rev 7:52–62

Hennan JK, Swillo RE, Morgan GA et al (2006) Pharmacologic inhibition of platelet VWF-GPIbα interaction prevents coronary artery thrombosis. Thromb Haemost 95:469–475

Holz J, Bartunek J, Barbato E et al (2009) ALX-0081 a novel anti-thrombotic: first results of a multiple dose phase 1 study in patients with stable angina undergoing PCI. J Thromb Haemost 7:PP-WE-416

Hoylaerts M, Yamamoto H, Nuyts K et al (1997) von Willebrand factor binds to native collagen VI primarily via its A1 domain. Biochem J 324:185–191

Huizinga EG, Tsuji S, Romijn RAP et al (2002) Structures of glycoprotein Ibα and its complex with von Willebrand factor A1 domain. Science 297:1176–1179

Jackson P, Nesbitt WS, Westein E (2009) Dynamics of platelet thrombus formation. J Thromb Haemost 7:17–20

Jilma B, Paulinska P, Jilma-Stohlawetz P et al (2010) A randomized pilot trial of the anti-von Willebrand factor aptamer ARC1779 in patients with type 2B von Willebrand disease. Thromb Haemost 104:563–570

Jilma-Stohlawetz P, Gilbert JC, Gorczyca ME, Knöbl P, Jilma B (2011) A dose ranging phase I/II trial of the von Willebrand factor inhibiting aptamer ARC1779 in patients with congenital thrombotic thrombocytopenic purpura. Thromb Haemost 106:539–547

Kageyama S, Yamamoto H, Nagano M, Arisaka H, Kayahara T, Yoshimoto R (1997) Anti-thrombotic effects and bleeding risk of AJvW-2, a monoclonal antibody against human von Willebrand factor. Br J Pharmacol 122:165–171

Kageyama S, Yamamoto H, Nakazawa J et al (2002a) Pharmacokinetics and pharmacodynamics of AJW200, a humanized monoclonal antibody to von Willebrand factor, in monkey. Arterioscler Thromb Vasc Biol 22:187–192

Kageyama S, Matsushita J, Yamamoto H (2002b) Effect of a humanized monoclonal antibody to von Willebrand factor in a canine model of coronary arterial thrombosis. Eur J Pharmacol 443:143–149

Kunicki T, Nugent D (2002) The influence of platelet glycoprotein polymorphisms on receptor function and risk for thrombosis. Vox Sang 83(Suppl 1):85–90

Levy GG, Nichols WC, Lian EC et al (2001) Mutations in a member of the ADAMTS13 gene family cause thrombotic thrombocytopenic purpura. Nature 413:488–494

Lillicrap D (2007) Von Willebrand disease-phenotype versus genotype: deficiency versus disease. Thromb Res 120:S11–S16

Lopez JA, Andrews RK, Afshar-Kharghan V et al (1998) Bernard-Soulier syndrome. Blood 91:4397–4418

Lopez JA, del Conde I, Shrimpton CN (2005) Receptors, rafts, and microvesicles in thrombosis and inflammation. J Thromb Haemost 3:1737–1744

Lowe EJ, Werner EJ (2005) Thrombotic thrombocytopenic purpura and hemolytic uremic syndrome in children and adolescent. Semin Thromb Hemost 31:717–730

Machin S, Clarke C, Ikemura O et al (2003) A humanized monoclonal antibody against VWF A1 domain inhibits VWF:RiCof activity and platelet adhesion in human volunteers. J Thromb Haemost (Suppl 1):OC328

Mailhac A, Badimon JJ, Fallon JT et al (1994) Effect of an eccentric severe stenosis on fibrin (ogen) deposition on severely damaged vessel wall in arterial thrombosis. Relative contribution of fibrinogen and platelets. Circulation 90:988–996

Markus HS, McCollum C, Imray C et al (2011) The von Willebrand inhibitor ARC1779 reduces cerebral embolization after carotid endarterectomy – a randomized trial. Stroke 42:2149–2153

Michaux G, Pullen TJ, Haberichter SL et al (2006) P-selectin binds to the D'-D3 domains of von Willebrand factor in Weibel-Palade bodies. Blood 107:3922–3924

Miller JL, Thiam-Cisse M, Drouet LO (1991) Reduction in thrombus formation by PG-1 $F(ab')_2$, an anti-guinea pig platelet glycoprotein Ib monoclonal antibody. Arterioscler Thromb Vasc Biol 11:1231–1236

Moake JL, Rudy CK, Troll JH et al (1982) Unusually large plasma factor VIII:von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. N Engl J Med 307:1432–1435

Momi S, Tantucci M, Van Roy M et al (2011) Selective blockade of the A1 domain of von Willebrand factor (VWF), prevents ischemic stroke in the guinea pig: comparison with the thrombolytic rtPA. J Thromb Haemost 9(Suppl 2):O-TU-098

Montalescot G, Philippe F, Ankri A et al (1998) Early increase of von Willebrand factor predicts adverse outcome in unstable coronary artery disease: beneficial effects of enoxaparin. French Investigators of the ESSENCE Trial. Circulation 98:294–299

Nesheim M, Pittman DD, Giles AR et al (1991) The effect of plasma von Willebrand factor on the binding of human factor VIII to thrombin-activated human platelets. J Biol Chem 266:17815–17820

Nillson IM, Blomback M, Jorpes E et al (1957) Von Willebrand's disease and its correction with human plasma fraction 1-0. Acta Med Scand 159:179–188

Nilsson IM, Blomback M, Von FI (1957) On an inherited autosomal hemorrhagic diathesis with antihemophilic globulin (AHG) deficiency and prolonged bleeding time. Acta Med Scand 159:35–57

Peyvandi F, Breems DA, Knoebl P et al (2011) First results of the Phase II TITAN trial: anti-von Willebrand factor Nanobody®as adjunctive treatment for patients with acquired thrombotic thrombocytopenic purpura. J Thromb Haemost 9(Suppl 2):SY-TH-027

Ray KK, Morrow DA, Gibson GM et al (2005) Predictors of the rise in VWF after ST elevation myocardial infarction: implications for treatment strategies and clinical outcome: an ENTIRE-TIMI 23 substudy. Eur Heart J 26:440–446

Remuzzi G, Galbusera M, Noris M et al (2002) Von Willebrand cleaving protease (ADAMTS13) is deficient in recurrent and familial thrombotic thrombocytopenic purpura and haemolytic uremic syndrome. Blood 100:778–785

Rivera J, Lozano ML, Navarro-Núñez L, Vicente V (2009) Platelet receptors and signaling in the dynamics of thrombus formation. Haematologica 94:700–711

Ruggeri ZM (2003) Von Willebrand factor: a matrix protein that tries to be soluble. Blood 101:2450

Ruggeri ZM, Mannucci PM, Lombardi R et al (1982) Multimeric composition of factor VIII/von Willebrand factor following administration of DDAVP: implications for pathophysiology and therapy of von Willebrand's disease subtypes. Blood 59:1272–1278

Ruggeri ZM, De Marco L, Gatti L et al (1983) Platelets have more than one binding site for von Willebrand factor. J Clin Invest 72:1–12

Sadler JE (1998) Biochemistry and genetics of von Willebrand factor. Annu Rev Biochem 67:395–424

Sadler JE, Mannucci PM, Berntorp E, et al. (2000) Impact, diagnosis and treatment of von Willebrand disease. Thromb Haemost 84:160–174

Sadler JE, Budde U, Eikelboom JC et al (2006) Update on the pathophysiology and classification of von Willebrand disease: a report of the subcommittee on von Willebrand factor. J Thromb Haemost 4:2103–2114

Savage B, Shattil SJ, Ruggeri ZM (1992) Modulation of platelet function through adhesion receptors. A dual role for glycoprotein IIb-IIIa (integrin alpha IIb beta 3) mediated by fibrinogen and glycoprotein Ib-von Willebrand factor. J Biol Chem 267:11300–11306

Savage B, Saldivar E, Ruggeri ZM (1996) Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. Cell 84:289–297

Savoia A, Pastore A, De Rocco D, et al. (2011) Clinical and genetic aspects of Bernard-Soulier syndrome: searching for genotype/phenotype correlations. Haematologica 96:417–423

Schneider SW, Nuschele S, Wixforth A et al (2007) Shear-induced unfolding triggers adhesion of von Willebrand factor fibers. Proc Natl Acad Sci USA 104:7899–7903

Siller-Matula JM, Mehri Y, Tanguay JF et al (2011) ARC15105 a potent antagonist of von Willebrand factor (VWF) platelet activation and adhesion. J Thromb Haemost 9(Suppl 2):O-MO-027

Sixma JJ, Schiphorst ME, Verweij CL et al (1991) Effect of deletion of the A1 domain of von Willebrand factor on its binding to heparin, collagen and platelets in the presence of ristocetin. Eur J Biochem 196:369–375

Sonoda A, Murata M, Ikeda Y, Fukuchi Y, Watanabe K (2001) Stroke and platelet glycoprotein Ib alpha polymorphisms. Thromb Haemost 85:573–574

Spiel AO, Gilbert JC, Jilma B (2008) von Willebrand factor in cardiovascular disease: focus on acute coronary syndromes. Circulation 117:1449–1459

Spiel AO, Mayr FB, Ladani N et al (2009) The aptamer ARC1779 is a potent and specific inhibitor of von Willebrand factor mediated ex vivo platelet function in acute myocardial infarction. Platelets 20:334–340

Staelens S, Hadders MA, Vauterin S, Platteau C et al (2006) Paratope determination of the antithrombotic antibody 82D6A3 based on the crystal structure of its complex with the von Willebrand factor A3-domain. J Biol Chem 281:2225–2231

Takahashi M, Yamashita A, Moriguchi-Goto S, et al. (2009) Critical role of von Willebrand factor and platelet interaction in venous thromboembolism. Histol Histopathol 24:1391–1398

Thompson SG, Kienast J, Pyke SD et al (1995) Hemostatic factors and risk of myocardial infarction or sudden death in patients with angina pectoris. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. N Engl J Med 332:635–641

Tsai HM (2010) Pathophysiology of thrombotic thrombocytopenic purpura. Int J Hematol 91:1–19

Uff S, Clemeston JM, Harrison T et al (2002) Crystal structure of the platelet glycoprotein Ibα N-terminal domain reveals an unmasking mechanism for receptor activation. J Biol Chem 38:35657–35663

Ulrichts H, Silence K, Schoolmester A et al (2011) Antithrombotic drug candidate ALX-0081 shows superior preclinical efficacy and safety compared to currently marketed antiplatelet drugs. Blood 118:757–765

Van Bockenstaele F, Holz JB, Revets H (2009) The development of nanobodies for therapeutic applications. Curr Opin Invest Drugs 10:1212–1224

Van Loon JE, de Jaegere PPT, Ulrichts H et al (2011) The in vivo effect of the new antithrombotic drug candidate ALX-0081 on blood samples of patients undergoing percutaneous coronary intervention. Thromb Haemost 106:165–171

Vanhoorelbeke K, Depraetere H, Romijn RA et al (2003) A consensus tetrapeptide selected by phage display adopts the conformation of a dominant discontinuous epitope of a monoclonal anti-VWF antibody that inhibits the von Willebrand factor-collagen interaction. J Biol Chem 278:37815–37821

Wadanoli M, Sako D, Shaw GD et al (2007) The von Willebrand factor antagonist (GPG-290) prevents coronary thrombosis without prolongation of bleeding time. Thromb Haemost 98:397–405

Wagner DD (1989) Storage and secretion of von Willebrand factor. In: Zimmerman TS, Ruggeri ZM (eds) Coagulation and bleeding disorders. The role of factor VIII and von Willebrand factor. Dekker, New York, pp 161–180

Wagner DD (1990) Cell biology of von Willebrand factor. Annu Rev Cell Biol 6:217–246

Wagner DD, Marder VJ (1984) Biosynthesis of von Willebrand protein by human endothelial cells: processing steps and their intracellular localization. J Cell Biol 99:2123–2130

Ware J, Russell S, Ruggeri ZM (2000) Generation and rescue of a murine model of platelet dysfunction: the Bernard-Soulier syndrome. Proc Natl Acad Sci USA 97:2803–2808

Wu D, Vanhoorelbeke K, Cauwenberghs N et al (2002a) Inhibition of the von Willebrand (VWF)-collagen interaction by an antihuman VWF monoclonal antibody results in abolition of in vivo arterial platelet thrombus formation in baboons. Blood 99:3623–3628

Wu D, Meiring M, Kotze HF et al (2002b) Inhibition of platelet glycoprotein Ib, glycoprotein IIb/IIIa, or both by monoclonal antibodies prevents arterial thrombosis in baboons. Arterioscler Thromb Vasc Biol 22:323–328

Yamashita A, Asasda Y, Sugimura H et al (2003) Contribution of von Willebrand factor to thrombus formation on neointima of rabbits stenotic iliac artery under high blood-flow velocity. Arterioscler Thromb Vasc Biol 23:1105–1110

Yamashita A, Furukoji E, Marutsuka K et al (2004) Increased vascular wall thrombogenicity combined with reduced blood flow promotes occlusive thrombus formation in rabbit femoral artery. Arterioscler Thromb Vasc Biol 24:2420–2424

Inhibitors of the Interactions Between Collagen and Its Receptors on Platelets

Hans Deckmyn, Simon F. De Meyer, Katleen Broos, and Karen Vanhoorelbeke

Contents

Abstract At sites of vascular injury, collagen-mediated platelet adhesion and activation have long been known as one of the first events in platelet-dependent thrombus formation. Studying patients with bleeding disorders that are caused by defective platelet adhesion to collagen resulted in the identification of several platelet collagen receptors, with glycoprotein VI and integrin α2β1 being the

H. Deckmyn (✉) • S.F. De Meyer • K. Broos • K. Vanhoorelbeke
Laboratory for Thrombosis Research, KU Leuven campus Kortrijk, Kortrijk, Belgium
e-mail: Hans.Deckmyn@kuleuven-kortrijk.be

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_13,

most important ones. Subsequent development of specific collagen receptor knock-out mice and various inhibitors of platelet binding to collagen have further proven the role of these receptors in haemostasis and thrombosis. The search for clinically applicable inhibitors for use as antithrombotic drug has led to the identification of inhibitory antibodies, soluble receptor fragments, peptides, collagen-mimetics and proteins from snake venoms or haematophagous animals. In experimental settings, these inhibitors have a good antithrombotic effect, with little prolongation of bleeding times, suggesting a larger therapeutic window than currently available antiplatelet drugs. However, at present, none of the collagen receptor blockers are in clinical development yet.

Keywords Platelet collagen receptors • Glycoprotein VI • Integrin α2β1 • Collagens

1 Introduction

Platelet recruitment at sites of vascular injury is crucial for normal haemostasis and efficient wound healing. At these sites, exposed collagen is one of the most thrombogenic substrates. Interaction of platelets with collagen leads to platelet activation, resulting in platelet shape change, generation of thromboxane A2 and secretion of granular content, with a.o. ADP, and activation of integrin αIIbβ3. These events result in the recruitment of additional platelets to the growing platelet plug and finally platelet aggregation.

Since the discovery of the effects of collagen on platelets already some 50 years ago by the pioneering studies of Hovig (1963) and Zucker and Borrelli (1961), the important role of platelet–collagen interactions in both haemostasis and thrombosis has been firmly established. Because of its prominent role in thrombogenesis, the platelet–collagen axis holds great promise as a therapeutic target to prevent platelet-dependent thrombus formation. However, not only do platelets interact with different types of collagen present in the vessel wall, they also possess several collagen receptors, making collagen-dependent thrombus formation an intricate interplay between different components. Nevertheless, the current knowledge, gathered using a.o. knockout mice and specific inhibitors, has allowed to assign relevant receptors and to define their potential to serve as targets for the development of new promising inhibitors.

In this chapter, a summary of these efforts is given with an estimate of the benefits and drawbacks of inhibition of the different platelet collagen receptors.

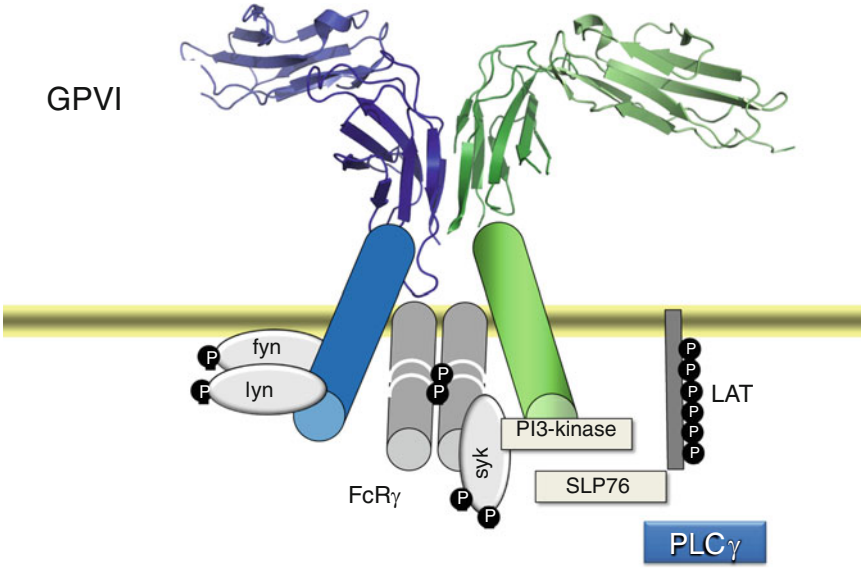


Fig. 1 Schematic representation of GPVI with the crystal structure of its collagen-binding domain (Horii et al. 2006) and the intracellular outside-in signalling interactors

2 Glycoprotein VI

A first well-studied collagen receptor on platelets is glycoprotein (GP) VI. Although GPVI has been identified on platelets in the late 1970s, it was only a decade later that its function as a collagen receptor was discovered via a patient with a mild bleeding problem (Sugiyama et al. 1987). In this patient, platelets were deficient in GPVI due to auto-antibodies against this glycoprotein, which resulted in defective collagen-induced platelet activation. Later, the development of collagen related peptides (CRP) and the use of convulxin, both powerful platelet activators via GPVI, helped in the characterization, purification and sequencing of the receptor (Clemetson et al. 1999). The role of GPVI–collagen interactions in physiological and pathophysiological thrombus formation was further elucidated by the development of GPVI knockout mice and of specific GPVI antagonists. These studies supported the concept that inhibiting GPVI–collagen interactions might be a good strategy to treat cardiovascular diseases.

2.1 Structure

GPVI is a 62-kDa glycoprotein that belongs to the Ig superfamily of surface receptors and is only expressed on megakaryocytes and platelets (Fig. 1). GPVI consists of a transmembrane complex formed by two extracellular immunoglobulin-

like domains, a mucin-like O-glycosylated stalk, a transmembrane region and a short cytoplasmic tail. The collagen-binding domain is comprised in the 2 Ig-like domains (Fig. 1). Surface expression of GPVI is dependent on its association with the FcRγ chain in the platelet membrane (Jandrot-Perrus et al. 2000) via a salt bridge between a positively charged arginine of GPVI and an aspartic acid residue of the FcRγ subunit. The FcRγ chain is a covalent-linked homodimer with each monomer bearing an immunoreceptor tyrosine-based activation motif (ITAM), which is essential for GPVI signalling in platelets (Fig. 1). Upon phosphorylation of the ITAM, the tyrosine-kinase Syk is recruited to the FcRγ chain via binding of its tandem SH2 domains to the two phosphotyrosines in the FcRγ chain. ITAM phosphorylation is mediated by the Scr family kinases Lyn and Fyn, a process that occurs specifically in lipid rafts, where Lyn and Fyn are concentrated. Apart from Lyn and Fyn, also other Src kinases could mediate activation (Quek et al. 2000). Activated Syk mediates a complex downstream signalling pathway, eventually leading to activation of phospholipase Cγ (Watson et al. 2005). Cleavage of phosphatidylinositol 4,5-bisphosphate by phospholipase Cγ then generates the second messengers inositol 1,4,5-trisphosphate and diacylglycerol that are responsible for raising Ca^{2+} levels and activating protein kinase C. In resting platelets, GPVI is partially or completely excluded from lipid rafts, but translocates to these rafts upon ligand engagement (Locke et al. 2002; Wonerow et al. 2002). A unique feature of GPVI in comparison to other ITAM receptors is the presence of a conserved proline-rich region (PxxP), which binds and activates the Src family kinases Lyn and Fyn (Suzuki-Inoue et al. 2002). This places the receptor in a "ready-to-go" state, which may be particularly important for rapid cell adhesion to collagen at high shear (Schmaier et al. 2009).

Recent evidence suggests the existence of both monomeric and dimeric receptor forms of GPVI, although it is believed that only the dimeric form has a unique conformation allowing high affinity binding to collagen (Arthur et al. 2007a; Berlanga et al. 2007; Jung et al. 2009). Cross-linking of two GPVI dimers was shown to be necessary for efficient activation (Jung et al. 2009). Crystallographic data revealed that the collagen-binding domain of dimeric GPVI adopts a fold with two parallel shallow grooves, of which the orientation and spacing match the dimensions of triple helices within an intact collagen fibre (Horii et al. 2006) (Fig. 1).

2.2 *Ligand Specificity*

For a long time, collagen has been considered the only physiological ligand for GPVI in the vasculature. The exact binding site of GPVI in collagen has not yet been fully elucidated. Using collagen peptides comprising both GPP and GPO triplets, it was shown that platelet and GPVI binding increases with GPO content

and that maximum interaction requires at least four contiguous GPO triplets (Smethurst et al. 2007). Later, it was found that GPVI interacts with specific loci in collagen type III but that GPVI-binding motifs in collagen type III are not solely determined by the location and number of OGP/GPO motifs (Jarvis et al. 2008).

During recent years, a number of other ligands and/or binding partners of GPVI, however, have been identified. Laminin is a major component of the basal lamina in the basement membrane, where collagen IV is not able to bind GPVI. Laminin has been shown to function as another ligand for GPVI, since GPVI can activate platelets that adhere to laminin through $\alpha_6\beta_1$ (Inoue et al. 2006). The exact physiologic role of laminin as a GPVI ligand is not yet clear, but mild injury could expose laminin, which could support platelet adhesion through GPVI. Also globular adiponectin, an adipocyte-derived cytokine, was shown to act as a ligand for GPVI (Riba et al. 2008). The interaction of GPVI with globular adiponectin stimulates platelet aggregation, which could contribute to unwanted platelet activation in diseased vessels. Another interesting observation is that GPVI plays a role in platelet adhesion to activated atherosclerotic endothelium without collagen exposure (Bultmann et al. 2010). Ligands that mediate these interactions are, however, still unknown.

2.3 Function

The main function of GPVI is triggering a potent signalling response via the associated FcRγ chains when the receptor binds to its main ligand collagen present in the damaged vessel wall. This allows stable platelet adhesion on exposed extracellular collagen, a.o. as a result of a shift of platelet integrins to a high affinity state and the release of secondary agonists such as ADP and thromboxane A_2, all of which reinforce further thrombus growth. Whether or not GPVI also exerts an adhesive function independent of signalling is not totally clear.

Depending on the severity of the vascular lesion, different subendothelial matrix components are exposed. More severe lesions result in exposure of platelets to tissue factor, leading to thrombin generation and strong platelet activation. This strong signal may obviate the need for GPVI signalling. Minor injury to the vessel wall, however, exposes subendothelial collagen fibres but not large amounts of tissue factor. In this scenario, collagen-induced platelet activation via GPVI (and/or $\alpha 2\beta 1$) may play a more dominant role in the process of thrombus formation and vessel repair (Dubois et al. 2006; Hechler et al. 2010). Whereas it is still unclear whether GPVI and $\alpha 2\beta 1$ function independently in their interaction with collagen, more and more evidence points towards a synergistic mechanism for optimal function.

More recently, several reports describe a crucial role for GPVI in a range of processes that go beyond the classical view of primary haemostasis, such as the closure of the ductus arteriosus in the heart after birth (Echtler et al. 2010) and the pathogenesis of rheumatoid arthritis (Boilard et al. 2010).

2.4 GPVI and Risk of Thrombosis and Bleeding in Humans

Given the importance of GPVI in thrombus formation, several studies have looked at the association between GPVI and thrombotic risk. Surface expression of GPVI was found to be elevated in patients with acute coronary syndrome and stroke, and was associated with a poor clinical outcome (Bigalke et al. 2006, 2008, 2009, 2010a, b). Some studies also described a positive association between gene polymorphisms in GPVI and cardiovascular incidents (Croft et al. 2001; Joutsi-Korhonen et al. 2003; Ollikainen et al. 2004). Interestingly, humans with GPVI defects are usually described as having a mild bleeding disorder although their platelets show impaired activation by collagen (Arthur et al. 2007b). Only two patients have been reported with a mild bleeding phenotype that could be directly attributed to mutations in the GPVI gene (Dumont et al. 2009; Hermans et al. 2009).

2.5 Mice with Genetically or Antibody Induced GPVI Depletion

The requirement of GPVI for normal collagen-dependent thrombus formation has been shown in genetically engineered mice that lack GPVI (Kato et al. 2003; Lockyer et al. 2006)or FcRγ chain (and as a consequence also GPVI) (Konishi et al. 2002) and GPVI-depleted mice (Massberg et al. 2003; Nieswandt et al. 2001a). GPVI depletion from platelets is an interesting observation, seen after administration of specific monoclonal anti-GPVI antibodies, but also in humans with anti-GPVI auto-antibodies. The exact mechanism of GPVI shedding is not yet fully understood but involvement of the sheddases ADAM17, ADAM10 and possibly a third GPVI-cleaving enzyme has been shown (Bender et al. 2010).

In a mouse model of ischemic stroke, GPVI depletion resulted in an improved outcome, indicating that GPVI is also involved in thrombotic stroke progression (Kleinschnitz et al. 2007). In other studies, however, GPVI seemed to be dispensable for thrombus formation, which was explained by differences in the extent of collagen exposure (Dubois et al. 2006; Mangin et al. 2006) or the presence of other components of the extracellular matrix that may elicit a thrombogenic response in the absence of GPVI (Konstantinides et al. 2006).

Remarkably, in these animal models, loss or inhibition of GPVI prevents arterial thrombus formation but causes only mildly prolonged bleeding times, which is consistent with the mild bleeding phenotype observed in humans with defective or absent GPVI. This suggests that GPVI could be a promising therapeutic target for prevention of arterial thrombotic diseases.

2.6 Inhibitors of GPVI In Vitro

Snake venoms. Although several snake venom metalloproteases have been found to interfere with GPVI function in vitro by either shedding GPVI (Wijeyewickrema

et al. 2007; Hsu et al. 2008) or direct binding to GPVI (Wang et al. 2005), they were not always GPVI specific (Hsu et al. 2008; Wang et al. 2005).

Inhibiting antibodies. Two human scFvs (10B12 and 1C3) specific for GPVI were obtained by screening two phage display libraries with the Ig-like ectodomains of human GPVI. 10B12 inhibited activation of platelets by CRP and collagen in aggregometry and thrombus formation by the latter in whole blood perfusion (Smethurst et al. 2004). Human platelet thrombus formation onto plaques in flowing blood was completely blocked by GPVI inhibition with the antibody 10B12 (Penz et al. 2005).

Another murine monoclonal antibody-derived Fab (9O12.2) specific for human GPVI blocks collagen-induced platelet aggregation and prevents platelet adhesion and thrombi formation under arterial flow conditions (Lecut et al. 2003). A humanized version of 9O12 has been generated (Muzard et al. 2009).

BLO8-1 is a human domain antibody that binds to an epitope within the collagen-binding domain of GPVI, blocking binding to collagen. As a result BLO8-1 prevented cross-linked collagen-related peptide-induced platelet and thrombus formation in whole blood under arterial shear conditions (Walker et al. 2009).

2.7 *Inhibitors of GPVI In Vivo*

Several drugs that target GPVI have been developed of which some have been studied in vivo. One approach is the development of monoclonal antibodies. JAQ1 is a monoclonal antibody directed against mouse GPVI that leads to irreversible depletion of GPVI on circulating platelets. As a result, platelet responses to collagen are abolished, which leads to a profound protection in a lethal mouse thromboembolism model induced by injection of a mixture of collagen and epinephrine (Nieswandt et al. 2001a). Mice treated with JAQ1 did not have severely prolonged bleeding times. Later, JAQ1-treated mice were shown to have a transiently reduced response to thrombin in terms of integrin activation, secretion, and procoagulant activity. This resulted in a protective effect against tissue factor-induced thromboembolism (Schulte et al. 2006). Similarly, the anti-human GPVI monoclonal antibody OM2 inhibited ex vivo collagen-induced platelet aggregation with only a slight prolongation of the bleeding time in cynomolgus monkeys (Matsumoto et al. 2006). The Fab fragment of OM4, another anti-human GPVI monoclonal antibody, cross-reacts with rat platelets without depleting platelet GPVI. It inhibited cyclic flow reductions in a modified Folts model in rat carotid artery without prolongation of the bleeding time (Li et al. 2007). Administration of the human GPVI-specific mouse mAbs mF1201 and mF1232 to monkeys caused GPVI immunodepletion with and without both significant thrombocytopenia and GPVI shedding, respectively (Takayama et al. 2008).

In addition to inhibition of GPVI by monoclonal antibodies, a soluble form of the receptor can be used, acting as a competitive inhibitor, thereby averting undesired effects on platelets. A fusion protein, consisting of the extracellular domain of GPVI and a human C-terminal Fc tag was constructed (Massberg et al. 2004). This dimeric soluble form of GPVI attenuated platelet adhesion to immobilized collagen in vitro and reduced platelet adhesion and aggregation at the injured carotid artery of mice (Massberg et al. 2004). Again, doses of the soluble form of GPVI sufficient to reduce platelet adhesion, only moderately prolonged tail bleeding times. However, upon comparison of the antithrombotic capacity of the soluble GPVI dimer with anti-GPVI antibodies in a carotid artery thrombosis model in mice, Grüner et al. could not confirm the antithrombotic effect of soluble dimeric GPVI-Fc, whereas anti-GPVI antibodies profoundly inhibited platelet adhesion and thrombus formation at the injured vessel wall (Gruner et al. 2005).

EXP3179 was originally identified as an active metabolite of the angiotensin II type 1 (AT1)-receptor antagonist Losartan (LOS), which is produced during the hepatic metabolization of LOS by the cytochrome-P450 pathway. However, Gorthusen et al. described that EXP3179 acts independently as an individual and selective inhibitor of GPVI. In a mouse model of carotid injury, EXP3179 significantly reduced platelet adhesion after acute vessel injury (Grothusen et al. 2007).

DZ-697b is a new orally active antiplatelet agent that inhibits collagen and VWF-induced platelet aggregation through blocking of FcRγ chain phosphorylation. In a guinea pig model, DZ-697b inhibited photochemically induced thrombosis with a lower risk of bleeding in comparison with aspirin. When given to healthy volunteers, DZ-697b caused dose dependently reduced ex vivo thrombus formation and platelet adhesion. Doses of DZ-697b with equal or greater antiplatelet potency than 300 mg clopidogrel resulted in significantly shorter bleeding time prolongations (Zafar et al. 2010).

3 Integrin α2β1

A second well-studied collagen receptor on platelets is integrin α2β1, also known as GPIa/IIa, VLA-2 or CD49b/CD29. Integrin α2β1 is after αIIbβ3 the second most abundant integrin receptor on platelets and is found on average in 1,500–4,000 copies on the platelet surface. Unlike other integrins, however, a significant variation in receptor density occurs in the healthy population (Furihata et al. 2002). α2β1 is also expressed on other cells, like endothelial cells (Kirchhofer et al. 1990) where it serves as a receptor for collagen, laminin and fibronectin. The physiological role of α2β1 in haemostasis is evident from patients with α2β1 deficiency (Handa et al. 1995; Nieuwenhuis et al. 1985) or an acquired anti-α2β1 antibody (Deckmyn et al. 1990) who all present with mild bleeding complications. Studies using α2 knockout

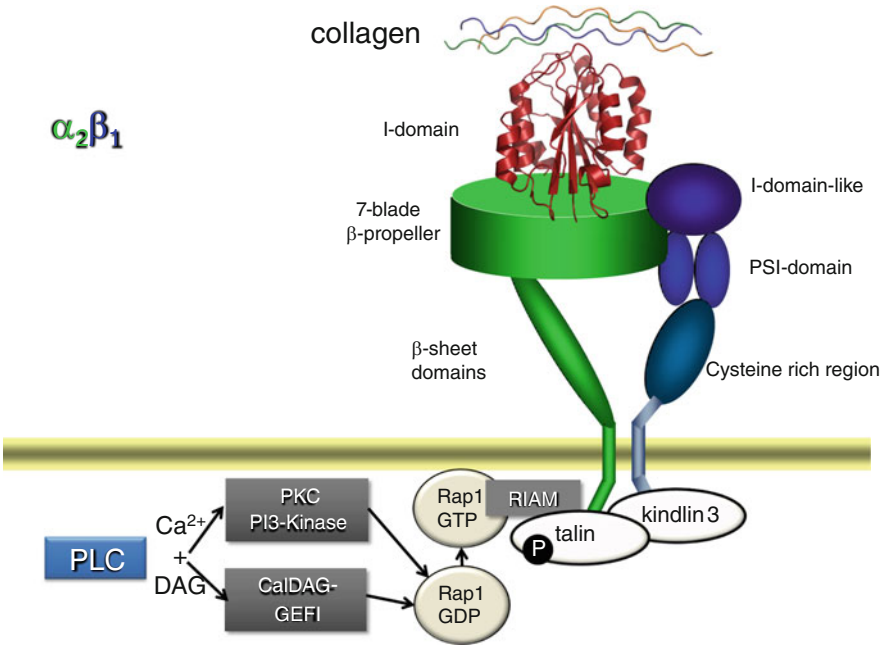


Fig. 2 Schematic representation of integrin α2β1 with the crystal structure of the α2 I-domain and a triple helical collagen peptide containing a critical GFOGER (Emsley et al. 2000) and the intracellular inside-out signalling interactors

and β1 null mice and specific α2β1 antagonists further established the role of α2β1 in thrombosis and haemostasis and supported the view that inhibiting α2β1-collagen interactions might be a good strategy to treat cardiovascular diseases.

3.1 Structure

All integrin receptors exist as αβ heterodimers consisting of a globular N-terminal head standing on two rigid stalks, the "legs," with membrane spanning helices ending in a short cytoplasmic tail (Fig. 2). The N-terminal region of the α2 subunit contains an additional ligand binding A- or I- (inserted) domain. Crystal structure analysis of this I-domain revealed that it adopts a "dinucleotide binding" or Rossman fold consisting of a central parallel β-sheet which contains at the C-terminal site a metal-ion (preferentially Mg^{2+}) dependent adhesion site (or MIDAS motif) surrounded by α-helices (Fig. 2). In comparison with the leukocyte integrin I domain, the α2 I-domain contains an additional helix, the C-helix, that is also involved in ligand binding (Emsley et al. 1997). Ligand binding induces conformational changes in the loop regions coordinating the metal ion and large movements

of several α-helices, as such creating a complementary surface groove that surrounds the collagen triple helix (Emsley et al. 1997).

Ligand affinity is allosterically regulated and mainly controlled by intracellular signalling (inside-out signalling), which requires binding of talin-1 and kindlin-3 to the β1 cytoplasmic domain. In the inactivated form, the α2-I domain adopts a "closed" conformation that contacts the sevenfold repeat of the α subunit (α-R) and the β1-I like domain and has a low affinity for collagen. Upon triggering, a switch-blade like action occurs which separates the tails and the α-R from the β1-I like domain, changing the integrin from a bent into an extended conformation (Emsley et al. 2000; Luo et al. 2007). These conformational changes release the intersubunit restraints on the α-I-domain, now allowing movement of the C-helix and occurrence of other structural changes. In this way, a high affinity collagen binding site is established (Emsley et al. 2004; Jung and Moroi 2000).

3.2 *Ligand Specificity*

In the extracellular matrix of blood vessels, collagens type I and III are the most abundant types of collagens and are the main ligands for α2β1. Initially, using synthetic collagen-derived peptides, the sequence GFOGER (with O for hydroxyproline) in collagen type I was identified as the highest affinity binding site for α2β1, but also the GLOGER sequence, which is conserved in both collagen type I and III was shown to be a high affinity binding site (Herr and Farndale 2009; Raynal et al. 2006). Later, the use of synthetic peptides that encompass the entire triple helical domain of collagen type III provided evidence for several other high, moderate and weak binding sites that might become more important upon platelet activation (Emsley et al. 2004; Raynal et al. 2006). Crystal structure analysis of the I-domain with the GFOGER triple helical peptide suggests that the GER triplet has a local destabilizing effect on the triple helical structure due to the lack of strong interchain electrostatic interactions allowing some side- and main-chain flexibility, which makes the GER a favoured binding site for the α2I-domain (Emsley et al. 2004).

3.3 *Function*

Where GPVI is one of the key players in collagen-induced intracellular signalling, platelet α2β1 mainly mediates firm adhesion to collagen and anchors the thrombi at the exposed collagen of the extracellular matrix. Flow experiments with receptor specific antibodies or synthetic collagen-derived triple helical peptides suggest that platelet GPIb receptor initially tethers platelets to VWF at the site of lesion (see chapter 13) allowing α2β1 to adhere to high-affinity collagen sequences which in turn supports the low affinity binding of GPVI. The latter induces a signalling cascade that regulates the switch of the α2β1 to the open conformation which

finally allows firm adhesion of the platelets to the exposed collagen. In the process of primary adhesion, the adhesive function of both GPIb and α2β1 partially overlap. Taken together, the interplay and crosstalk between all three platelet receptors is crucial for efficient thrombus formation (Pugh et al. 2010; Siljander et al. 2004).

3.4 α2β1 and Risk of Thrombosis and Bleeding in Humans

Also for α2β1, an association with thrombotic risk was investigated. Expression levels and functionality of α2β1 integrin vary 20-fold within the healthy population (Kunicki et al. 1993). These inherited differences in α2β1 density can be correlated to genetic polymorphisms in the α2 allele. High platelet expression levels of α2β1 result in an increased rate of platelet attachment to collagen I under high shear (Kritzik et al. 1998) and has been associated with acute coronary syndromes such as myocardial infarction (Moshfegh et al. 1999; Santoso et al. 1999) and stroke (Carlsson et al. 1999), especially in younger patients. Patients with α2β1 deficiency are diagnosed through mild bleeding complications and impaired responsiveness to collagen.

3.5 Mice with Genetically Induced α2 or β1 Depletion

Although one study reported that platelet adhesion and aggregation in α2-null mice and mice with Cre/loxP mediated loss of β1 is unaltered in a thrombosis model where the injury is induced by transient ligation of the common carotid artery (Gruner et al. 2003), most in vivo data point to a delayed platelet recruitment at the site of vascular injury combined with a decreased aggregate stability resulting in a reduced number of thrombi and thrombus size (He et al. 2003; Kuijpers et al. 2007). The rather limited effect on thrombus formation is in accordance with the lack of spontaneous bleeding or increase in bleeding time (Holtkotter et al. 2002; Nieswandt et al. 2001b), which can be explained by synergism between GPIb, α2β1 and GPVI.

3.6 Inhibitors of α2β1 In Vitro

Many molecules that inhibit platelet α2β1 binding to collagen in vitro have been identified demonstrating the role of α2β1-collagen interactions in thrombus formation. The molecules include snake venom proteins and related peptides (Eble et al. 2001; Tanjoni et al. 2010), a nucleoside derivative from an unknown fungus (Sato et al. 2006), a herbal extracted compound (Wu et al. 2008), a protein from hookworms (Chadderdon and Cappello 1999), but also antibodies (Coller et al.

1989; Estavillo et al. 1999; Saelman et al. 1994; Verkleij et al. 1998) and arylamide derivatives (Yin et al. 2006). The pharmacological properties, effects and specificities of all these inhibitors, however, strongly vary. The best characterized compounds will be discussed in the next section.

Snake venoms. The class P-III snake venom metalloprotease jararhagin (*Bothrops jararaca*) strongly inhibits collagen-induced platelet aggregation in vitro through binding to both the α2β1 receptor and the α2β1 recognition motif on collagen by its cysteine-rich and disintegrin-like domain, respectively (Moura-da-Silva et al. 2008). The inhibitory effect on α2β1 can be ascribed to proteolytical cleavage of the integrin β1 subunit by the metalloprotease (Kamiguti et al. 1997); however, this enzymatic activity is not a prerequisite to exert an inhibitory effect, as also the inactive enzyme, isolated domains and a jararhagin catalytic domain containing cyclic peptide (Pentikainen et al. 1999) competitively prevents collagen type I binding to the α2 I-domain. Similarly, NN-PF3, a metalloprotease from the Indian cobra (*Naja naja*) venom inhibits collagen-induced platelet aggregation in vitro through its interaction with the integrin α2 I-domain (Kumar et al. 2011). In addition, also EMS16, a snake venom protein belonging to the C-lectin protein family from *Echis multisquamatus* and Rhodocetin, from the snake *Calloselasma rhodostoma* (Eble et al. 2001, 2003; Eble and Tuckwell 2003) strongly and specifically inhibit collagen type I induced platelet aggregation through competition for the same binding site on the α2-I domain.

Herbal compound. The dried root of an Asian medical herb (*Salvia miltiorrhiza Bunge*), called Danshen, is used for its antiatherogenic and antithrombotic properties (Cheng 2007), for instance in the treatment of acute ischemic stroke (Han et al. 2008). One of its extracted compounds, named salvianolic acid B (SAB), has now been reported to inhibit platelet adhesion to collagen under flow through an α2β1-dependent mechanism. However, since SAB interferes with several biological processes, interacts with other drugs and needs high doses for antiplatelet activity, excludes further clinical development (Wu et al. 2008).

Inhibitory antibodies. A dozen of monoclonal antibodies targeting either the α2 or β1 integrin subunit have been described to block the adhesion of α2β1 to collagen in vitro. These antibodies all recognize a small region comprising respectively residues 173–259 on the α2I domain or residues 207–218 on the β1 domain (Estavillo et al. 1999; Saelman et al. 1994; Kamata et al. 1994). Several of these monoclonal antibodies, like the α2-specific 176D7, P1H5 and 6F1 inhibit platelet adhesion to collagen under flow (Coller et al. 1989; Estavillo et al. 1999; Saelman et al. 1994; Verkleij et al. 1998). The 6F1 antibody, however, fails to inhibit collagen-induced platelet aggregation, providing more evidence that α2β1 is allosterically regulated and rather supports platelet adhesion than playing a direct role in platelet aggregation.

3.7 *Inhibitors of α2β1 In Vivo*

Preclinical studies in mice allowed to evaluate the usefulness of α2β1 antagonists as antithrombotic drugs. By combining the 2,3-diaminopropionic acid (DAP) moiety

of known αIIbβ3 inhibitors with the benzenesulfonyl-propyl-phenylalanine of known α4β3 inhibitors, followed by further structural optimization, the group of DeGrado (Miller et al. 2009) developed the first α2β1 specific inhibitor that strongly blocks the α2β1-mediated platelet adhesion to collagen in vivo. In a murine $FeCl_3$ induced thrombosis model, i.v. administration of this compound, like aspirin, reduced clot formation by threefold. Computational modelling and in vitro assays using α2β1 mutants clearly indicated that this compound by binding to the β1-I-like domain interrupts the interaction with the α2I domain and thereby abolishes the allosteric affinity regulation of the integrin (Kapyla et al. 2007).

Although the ligand-binding site on the α2I domain is difficult to target by a small molecule as the molecule has to stabilize the closed conformation through constructive interactions with the C-helix and through coordination of the Mg^{2+} in the MIDAS, the group of Heino nevertheless selected, based on docking simulations, aromatic polyketides interacting with the MIDAS motif on the α2I domain and the C-helix, thereby stabilizing the α2β1 in the closed conformation (Nissinen et al. 2010). However, these compounds were not α2 specific. Based on this knowledge, a pharmacophore model was created enabling partial de novo design of an α2β1 selective inhibitor. The resulting sulphonamide derivative, BTT-3016, reduced thrombus formation in mice after laser-induced vascular injury through interfering with the α2 integrin unit. As also observed in α2-deficient mice, the tail bleeding time was slightly increased, but still not exceeding what is seen with aspirin (Nissinen et al. 2010).

As α2β1 inhibitors thus seem to only minimally interfere with platelet activation, whereas they also still allow platelet adhesion through other collagen receptors, they could turn out to be mild antithrombotics with limited risk for bleedings. Further clinical development will demonstrate their potential in the control of arterial thrombosis.

4 Other Platelet Receptors for Collagen

Besides the well-studied collagen receptors GPVI and α2β1, other (putative) collagen receptors on platelets (Table 1) have been studied and their role in thrombus formation in vitro and in vivo has been investigated. The early literature contains many additional putative collagen receptors such as GP61 and a 85/90-kDa platelet membrane protein but most of these have not been confirmed or have been eliminated in more recent studies.

One of the more extensively studied receptors is **GPVI** or CD36. GPIV was shown to recognize a multitude of ligands, including oxidized LDL, long-chain fatty acids, thrombospondin-1, fibrillar Aβ, the membrane of cells undergoing apoptosis (Hirano et al. 2003) and collagen (Diaz-Ricart et al. 1993). However others could not confirm that GPIV is also a collagen receptor as platelets deficient in GPIV, from otherwise healthy donors, aggregated normally to collagen (Daniel et al. 1994).

Table 1 Different platelet collagen receptors with their defined collagen-derived peptide ligands

Collagen type I	Ligand	Receptor
	not known	Collagen type I Specific receptor 65 kDa
	GXX'GER motifs with GFOGER	α2β1
Collagen	not known	Hsp47
	not known	GPV
	GPOGPOGPOGPO	GPVI
Collagen type III		
	GAOGLRGGAGPOG-PEGGKGAAGPOGPO	GPVI
	KOGEOGPK	Platelet Type III Collagen Binding Protein (TIIICBP) (kindlin-3)
	not known	Collagen type III Specific receptor 47 kDa
	GXRGQOGVMGFO	VWF

For **GPV** which is part of the GPIb/IX/V complex and initially known as a substrate for thrombin and ADAM17 upon platelet activation (Rabie et al. 2005), the evidence that it is a collagen receptor still stands. Indeed, platelets from GPV knockout mice which react normally with VWF and are more sensitive to thrombin, remarkably, adhere less to collagen type I under flow or static conditions. In addition, GPV deficient platelets have a reduced aggregation response towards collagen (Moog et al. 2001). In vivo, the net effect of GPV knockout is that the mice have a decreased tendency to form $FeCl_3$-induced occluding thrombi in mesenteric arteries and abnormal platelet interaction with the subendothelium (Moog et al. 2001). A monoclonal antibody V.3 against the extracellular domain of human GPV furthermore selectively inhibited collagen-induced aggregation of human or rat platelets, the latter was still true when tested ex vivo after injection in rats as a bolus (Moog et al. 2001). These data at present are standing alone and follow-up studies are clearly needed in order to allow estimation of the full impact of GPV as a collagen receptor.

Another receptor on platelets that seems to be able to interact with different collagen types was recently identified: by using a proteomic method to detect the enrichment of peripheral membrane proteins, heat-shock protein 47 (**Hsp47**) was found to be exposed on the surface of activated human platelets. Hsp47 was first identified as a collagen-binding chaperone protein in cells that secrete collagen (Miyaishi et al. 1992) but is also exposed on the surface of tumour cells where it may serve as a type of collagen receptor (Hebert et al. 1999). Moreover, an inhibitor of Hsp47 nearly abolished platelet aggregation induced by collagen fibrils (Kaiser et al. 2009). Also here further studies are required to investigate whether Hsp47 may be involved in platelet adhesion to collagen and in in vivo thrombus formation and thus would behave as a new collagen receptor or whether its main role in platelets resides in the stimulation of wound healing.

In addition to the above receptors for which no real preference for a certain collagen subtype was indicated, a number of receptors have been identified that are claimed to be specific for a certain collagen type. If this holds true, then platelets may well react differentially to different collagens present in the various layers of the blood vessel wall and therefore theoretically might be able by using appropriate receptors to adapt their reaction depending on the severity of the wound.

The group of TM Chiang has been persistent in its efforts to identify collagen type specific platelet receptors, and came up with both **a collagen type I and a collagen type III specific receptor** with M_r 65 and 47 kDa respectively. Both receptors were cloned, expressed and characterized and found to be specific indeed for their collagen type (Chiang et al. 1997, 2002). Antibodies against the receptors and peptides derived from the extracellular part of both receptors were able to completely block platelet adhesion to the respective collagens without effect on the other collagen or on other platelet agonists (Chiang et al. 1993; Chiang and Kang 1997; Zhu et al. 2007). Finally, a recombinant (6 kDa) hybrid peptide containing the active peptides of both types I and III collagen platelet receptors on a type III collagen platelet receptor backbone, and a chemically synthesized 30 amino acid peptide containing the active peptides of both receptors with a 12 amino acid residues linker (3 kDa), both inhibited $FeCl_3$-induced thrombosis in rat cremaster muscle vessels (Du et al. 2007), highlighting the physiological role of these receptors in thrombus formation. No data on the effect on bleeding times were provided. However, so far no patients have been identified that functionally lack one of these receptors nor have KO mice been generated. Hence, these currently available data are intriguing and clearly await independent confirmation and further evaluation in other animal models before any significant clinical application of these inhibitors can be considered.

Another collagen type III specific receptor: **platelet Type III Collagen Binding Protein (TIIICBP)** was identified by the group of Fauvel-Lafeve, starting with the observation that an octapeptide KOGEOGPK within the alpha(1)III-CB4 collagen fragment binds to platelets and specifically inhibits platelet aggregation, platelet contact and spreading on type III collagen and subendothelium under static and flow conditions (Monnet and Fauvel-Lafeve 2000). With this peptide as probe for ligand blotting a doublet of 68–72 kDa was identified. In a follow-up study, a monoclonal antibody was generated against the purified major 68 kDa protein, that inhibited platelet contact, spreading and aggregation induced by type III collagen and platelet interactions under flow conditions with type III collagen, endothelial cell matrix and the KOGEOGPK-peptide, without effects on platelet-type I collagen interactions (Monnet et al. 2001). As the KOGEOGPK sequence occurs in both human and murine collagen type III, with the position of the peptide in the α1 chain of collagen type III differing by only 1 amino acid (position 655–662 for human compared to position 654–661 for mouse collagen type III) it is clear that the mouse is a suitable model to determine the in vivo effect of the peptide. In a photochemically induced vascular thrombosis model, a protective effect of KOGEOGPK was demonstrated as the arterial thrombotic occlusion time was significantly prolonged when compared to vehicle and scrambled peptide controls

(Maurice et al. 2006). The effect in venous occlusion time was less prominent which was thought to be related to the lower expression levels of collagen type III in the venules compared to the arterioles. Also here no prolongation of the bleeding time was observed (Maurice et al. 2006) in line with studies using other collagen receptor antagonists. Very recently and quite unexpectedly the same group could show that TIIICBP presents high biochemical and functional similarities with kindlin-3, a member of a focal adhesion family of proteins, which in platelets is especially known as a key protein in the inside-out activation of integrins α2β1 and αIIbβ3. Indeed mass spectrometry surveys indicated that TIIICBP contains several peptides identical to kindlin-3, covering 41 % of the amino acid sequence and polyclonal antibodies against kindlin-3 recognized TIIICBP. And finally, anti-kindlin-3 antibodies inhibited platelet interactions with type III collagen under flow conditions similar to anti-TIIICBP antibodies and the KOGEOGPK peptide (Djaafri et al. 2011). All together suggesting that in platelets kindlin-3 not only affects integrin function, but that it also may be directly interacting with especially collagen III. How the intra- and extracellular actions of kindling-3 can be reconciled awaits further investigation.

5 Collagen Inhibitors

Thrombus formation cannot only be prevented by collagen receptor antagonists but also by proteins that interact with the binding site of these receptors in collagen. Although research on such collagen-binding proteins is less comprehensive than for the collagen receptor antagonists, proteins have been identified that bind to the binding sites of α2β1, GPVI or VWF in collagen and hence inhibit collagen-induced thrombus formation in vitro and in vivo.

Antibody cross-linking of and collagen binding to the Leukocyte Associated Ig-like Receptor-1 (LAIR-1) inhibit immune cell function in vitro (Lebbink et al. 2006). The soluble Ig-like receptor family member LAIR-2 binds to collagen and is a potent inhibitor of the interaction between LAIR-1 and collagen. Interestingly, the binding of LAIR-2 to collagen also prevented binding of collagen to glycoprotein VI and VWF. In this way, LAIR-2 interfered with thrombus formation in vitro under both low and high shear conditions (Lenting et al. 2010). The in vivo efficacy of LAIR-2 in preventing thrombus formation has not been demonstrated yet.

Calin, a partially purified protein product isolated from the saliva of the medicinal leech *Hirudo medicinalis*, inhibits both VWF binding and platelet adhesion to collagen under static and flow conditions (Harsfalvi et al. 1995). It was shown to contain 2 active proteins, one binding to the VWF-binding site in collagen and another to the α2β1 binding site (Depraetere et al. 1999). Calin furthermore dose dependently inhibited platelet-rich thrombus formation in the femoral vein of hamsters with no effect on bleeding times (Deckmyn et al. 1995). Later the component specifically interfering with the VWF–collagen interaction was cloned and named saratin (Barnes et al. 2001). The NMR structure of saratin shows that the

protein consists of one α-helix and 5 β-strands (ββαβββ) and that a distinct notch is present for binding to collagen as was observed for the I-domain in α2β1 (Gronwald et al. 2008). Topical application of saratin reduced platelet adhesion and development of intimal hyperplasia in a rat carotid endarterectomy model with no increase in bleeding time (Cruz et al. 2001). Saratin also reduced platelet deposition on human atherosclerotic plaques in a pig model (Vilahur et al. 2004).

From another leech, *Haementeria officinalis*, a protein (LAPP, leech antiplatelet protein) was isolated and cloned that inhibited both VWF–collagen and α2β1–collagen interactions under static and flow conditions (Connolly et al. 1992; Keller et al. 1992). It was suggested that VWF and α2β1 binding sites in collagen type I are distinct but close to each other and that recombinant LAPP (rLAPP) interacts with nearby GXR motifs (Verkleij et al. 1999) hence partly covering both epitopes. The extended conformation of the N-terminal region of LAPP would allow the protein to hinder the nearby VWF and α2β1 binding to collagen. Although rLAPP potently inhibited collagen-induced platelet aggregation, surprisingly, no antithrombotic effect of rLAPP could be demonstrated in vivo. Indeed, in a baboon thrombosis model where an arteriovenous shunt coated with collagen type I was mounted in the femoral artery, no in vivo inhibition of ^{111}In-labelled platelet deposition was observed (Schaffer et al. 1993).

Aegyptin, isolated from the salivary gland of the mosquito *Aedes aegypti*, binds to the VWF-binding sequence RGQOGVMG in collagen, and is consequently a potent inhibitor of the VWF–collagen interaction. However, aegyptin also recognizes $(GPO)_{10}$ and GFOGER sequences, although with lower affinity thereby also interfering with GPVI–collagen and α2β1–collagen interactions. The in vivo antithrombotic activity of aegyptin was clear in a laser-induced model of carotid injury in rats where a dose-dependent increase in occlusion time was measured after i.v. administration of aegyptin. The antithrombotic effect was not associated with a significant increase in blood loss after a tail cut compared to control animals injected with vehicle (Calvo et al. 2010). Future studies are needed to clarify whether the in vivo effect of aegyptin is mainly due to interference with the VWF–collagen interaction or whether blockage of collagen-GPVI and collagen α2β1 also plays a role.

6 Conclusion

The effects of collagen on platelets have been described already some 50 years ago. In the mean time numerous studies investigating patients with bleeding disorders due to defective collagen receptor–collagen interactions, identifying collagen receptors, developing specific collagen receptor knockout mice and using collagen receptor–collagen inhibitors undoubtedly proved that platelet–collagen interactions play a prominent role in haemostasis and thrombosis. Logically, the search for clinically applicable inhibitors has led to the identification of inhibitory antibodies, soluble receptor fragments, peptides, collagen-mimetics and proteins from snake

venoms or haematophagous animals. However, unfortunately so far no compounds are in clinical development.

Although many (putative) collagen receptors have been identified, it is clear that GPVI and α2β1 are the more prominent players and currently present the prime targets for the development of clinical applicable antithrombotic drugs. An ideal antiplatelet agent should be powerful without inducing major bleeding complications. In that way, targeting GPVI or α2β1 collagen interactions seems interesting because patients deficient in either one of these receptors only present with mild bleeding problems. In addition, blocking platelet adhesion might be more powerful than blocking platelet aggregation.

Since α2β1 is not exclusively expressed on platelets, blocking α2β1 likely would induce additional nonplatelet related effects and therefore might be less attractive as an antithrombotic strategy. Nevertheless, α2β1 antagonists inhibited thrombus formation in vivo without inducing major bleeding complications. However, more preclinical studies are needed to further evaluate their antithrombotic potential. The pharmacophore-based small molecules blocking α2β1 that in the mean have been developed raise hopes that eventually even orally available compounds may be generated that definitely will help to understand the potency of blocking α2β1 in the prevention of thrombosis.

From the current preclinical studies using GPVI antagonists, anti-GPVI antibodies seem to be really promising precursors of a new class of antithrombotic drugs as they have not only potent antithrombotic activities with minimal effects on bleeding time prolongations, they are in addition platelet specific, hence reducing the chances on unwanted side effects. Still, probably the best GPVI inhibitors to be used in patients should not induce GPVI depletion. Indeed, when in a worst-case scenario administration of anti-GPVI antibodies would result in bleeding complications, it clearly would be more difficult to reverse bleeding in patients with irreversibly GPVI-depleted platelets. However, as mice studies demonstrated that thrombus formation can also occur independently of GPVI depending on the severity of the lesion, further studies in animal models reproducing human disease are needed to evaluate the full potential of blocking GPVI in cardiovascular diseases in man.

In conclusion, blocking GPVI– or α2β1–collagen interactions are interesting new ways to treat cardiovascular diseases. Indeed from preclinical studies it is clear that inhibiting platelet–collagen interactions results in a potent antithrombotic effect without major bleeding complications in line with the observations in patients deficient in GPVI and α2β1. However, more studies using animal models that mimic human disease are warranted before the first clinical trial can be organized and the clinical potential of GPVI or α2β1 antagonists can be fully explored.

Knowledge Gaps

- The contribution of the other putative collagen receptors (the collagen type I specific receptor, the two collagen type III specific receptors, GPV and Hsp47) to platelet-collagen interaction needs confirmation.
- It is unclear whether a permanent depletion of GPVI by, e.g. antibody-induced shedding would be a more effective and safer antiplatelet strategy than a reversible inhibition of GPVI.
- Further studies in clinically relevant animal models are needed to firmly establish the usefulness of this new class of inhibitors for treatment of cardiovascular disease.

Key Messages

- Platelet collagen interactions play an important role in haemostasis and thrombosis
- Many (putative) collagen receptors have been identified
- GPVI and $\alpha 2\beta 1$ are the main platelet collagen receptors
- Studies in knockout mice and studies using GPVI or $\alpha 2\beta 1$ antagonists highlight the potency of GPVI and $\alpha 2\beta 1$ inhibitors as precursors of a new class of antithrombotic drugs
- As expression of GPVI is restricted to MK/platelets this represents a prime antithrombotic target

References

Arthur JF, Dunkley S, Andrews RK (2007b) Platelet glycoprotein VI-related clinical defects. Br J Haematol 139:363–372

Arthur JF, Shen Y, Kahn ML, Berndt MC, Andrews RK, Gardiner EE (2007a) Ligand binding rapidly induces disulfide-dependent dimerization of glycoprotein VI on the platelet plasma membrane. J Biol Chem 282:30434–30441

Barnes CS, Krafft B, Frech M, Hofmann UR, Papendieck A, Dahlems U, Gellissen G, Hoylaerts MF (2001) Production and characterization of saratin, an inhibitor of von Willebrand factor-dependent platelet adhesion to collagen. Semin Thromb Hemost 27:337–348

Bender M, Hofmann S, Stegner D, Chalaris A, Bosl M, Braun A, Scheller J, Rose-John S, Nieswandt B (2010) Differentially regulated GPVI ectodomain shedding by multiple platelet-expressed proteinases. Blood 116:3347–3355

Berlanga O, Bori-Sanz T, James JR, Frampton J, Davis SJ, Tomlinson MG, Watson SP (2007) Glycoprotein VI oligomerization in cell lines and platelets. J Thromb Haemost 5:1026–1033

Bigalke B, Stellos K, Weig HJ, Geisler T, Seizer P, Kremmer E, Potz O, Joos T, May AE, Lindemann S, Gawaz M (2009) Regulation of platelet glycoprotein VI (GPVI) surface

expression and of soluble GPVI in patients with atrial fibrillation (AF) and acute coronary syndrome (ACS). Basic Res Cardiol 104:352–357

Bigalke B, Stellos K, Geisler T, Kremmer E, Seizer P, May AE, Lindemann S, Melms A, Luft A, Gawaz M (2010a) Expression of platelet glycoprotein VI is associated with transient ischemic attack and stroke. Eur J Neurol 17:111–117

Bigalke B, Stellos K, Geisler T, Lindemann S, May AE, Gawaz M (2010b) Glycoprotein VI as a prognostic biomarker for cardiovascular death in patients with symptomatic coronary artery disease. Clin Res Cardiol 99:227–233

Bigalke B, Lindemann S, Ehlers R, Seizer P, Daub K, Langer H, Schonberger T, Kremmer E, Siegel-Axel D, May AE, Gawaz M (2006) Expression of platelet collagen receptor glycoprotein VI is associated with acute coronary syndrome. Eur Heart J 27:2165–2169

Bigalke B, Geisler T, Stellos K, Langer H, Daub K, Kremmer E, Seizer P, May AE, Lindemann S, Gawaz M (2008) Platelet collagen receptor glycoprotein VI as a possible novel indicator for the acute coronary syndrome. Am Heart J 156:193–200

Boilard E, Nigrovic PA, Larabee K, Watts GF, Coblyn JS, Weinblatt ME, Massarotti EM, Remold-O'Donnell E, Farndale RW, Ware J, Lee DM (2010) Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. Science 327:580–583

Bultmann A, Li Z, Wagner S, Peluso M, Schonberger T, Weis C, Konrad I, Stellos K, Massberg S, Nieswandt B, Gawaz M, Ungerer M, Munch G (2010) Impact of glycoprotein VI and platelet adhesion on atherosclerosis–a possible role of fibronectin. J Mol Cell Cardiol 49:532–542

Calvo E, Tokumasu F, Mizurini DM, McPhie P, Narum DL, Ribeiro JM, Monteiro RQ, Francischetti IM (2010) Aegyptin displays high-affinity for the von Willebrand factor binding site (RGQOGVMGF) in collagen and inhibits carotid thrombus formation in vivo. FEBS J 277:413–427

Carlsson LE, Santoso S, Spitzer C, Kessler C, Greinacher A (1999) The alpha2 gene coding sequence T807/A873 of the platelet collagen receptor integrin alpha2beta1 might be a genetic risk factor for the development of stroke in younger patients. Blood 93:3583–3586

Chadderdon RC, Cappello M (1999) The hookworm platelet inhibitor: functional blockade of integrins GPIIb/IIIa (alphaIIbbeta3) and GPIa/IIa (alpha2beta1) inhibits platelet aggregation and adhesion in vitro. J Infect Dis 179:1235–1241

Cheng TO (2007) Cardiovascular effects of Danshen. Int J Cardiol 121:9–22

Chiang TM, Cole F, Woo-Rasberry V (2002) Cloning, characterization, and functional studies of a 47-kDa platelet receptor for type III collagen. J Biol Chem 277:34896–34901

Chiang TM, Kang AH (1997) A synthetic peptide derived from the sequence of a type I collagen receptor inhibits type I collagen-mediated platelet aggregation. J Clin Invest 100:2079–2084

Chiang TM, Rinaldy A, Kang AH (1997) Cloning, characterization, and functional studies of a nonintegrin platelet receptor for type I collagen. J Clin Invest 100:514–521

Chiang TM, Seyer JM, Kang AH (1993) Collagen-platelet interaction: separate receptor sites for types I and III collagen. Thromb Res 71:443–456

Clemetson JM, Polgar J, Magnenat E, Wells TN, Clemetson KJ (1999) The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcalphaR and the natural killer receptors. J Biol Chem 274:29019–29024

Coller BS, Beer JH, Scudder LE, Steinberg MH (1989) Collagen-platelet interactions: evidence for a direct interaction of collagen with platelet GPIa/IIa and an indirect interaction with platelet GPIIb/IIIa mediated by adhesive proteins. Blood 74:182–192

Connolly TM, Jacobs JW, Condra C (1992) An inhibitor of collagen-stimulated platelet activation from the salivary glands of the Haementeria officinalis leech. I. Identification, isolation, and characterization. J Biol Chem 267:6893–6898

Croft SA, Samani NJ, Hampton KK, Steeds RP, Channer KS, Daly ME (2001) Novel platelet membrane glycoprotein VI dimorphism is a risk factor for myocardial infarction. Circulation 104:1459–1463

Cruz CP, Eidt J, Drouilhet J, Brown AT, Wang Y, Barnes CS, Moursi MM (2001) Saratin, an inhibitor of von Willebrand factor-dependent platelet adhesion, decreases platelet aggregation and intimal hyperplasia in a rat carotid endarterectomy model. J Vasc Surg 34:724–729

Daniel JL, Dangelmaier C, Strouse R, Smith JB (1994) Collagen induces normal signal transduction in platelets deficient in CD36 (platelet glycoprotein IV). Thromb Haemost 71:353–356

Deckmyn H, Chew SL, Vermylen J (1990) Lack of platelet response to collagen associated with an autoantibody against glycoprotein Ia: a novel cause of acquired qualitative platelet dysfunction. Thromb Haemost 64:74–79

Deckmyn H, Stassen JM, Vreys I, Van Houtte E, Sawyer RT, Vermylen J (1995) Calin from Hirudo medicinalis, an inhibitor of platelet adhesion to collagen, prevents platelet-rich thrombosis in hamsters. Blood 85:712–719

Depraetere H, Kerekes A, Deckmyn H (1999) The collagen-binding leech products rLAPP and calin prevent both von Willebrand factor and alpha2beta1(GPIa/IIa)-I-domain binding to collagen in a different manner. Thromb Haemost 82:1160–1163

Diaz-Ricart M, Tandon NN, Carretero M, Ordinas A, Bastida E, Jamieson GA (1993) Platelets lacking functional CD36 (glycoprotein IV) show reduced adhesion to collagen in flowing whole blood. Blood 82:491–496

Djaafri I, Maurice P, Labas V, Vinh J, Lemesle M, Arbeille B, Legrand C, Mourah S, Fauvel-Lafeve F (2011) Platelet type III collagen binding protein (TIIICBP) presents high biochemical and functional similarities with kindlin-3. Biochimie 94(2):416–426

Du H, Zawaski JA, Gaber MW, Chiang TM (2007) A recombinant protein and a chemically synthesized peptide containing the active peptides of the platelet collagen receptors inhibit ferric chloride-induced thrombosis in a rat model. Thromb Res 121:419–426

Dubois C, Panicot-Dubois L, Merrill-Skoloff G, Furie B, Furie BC (2006) Glycoprotein VI-dependent and -independent pathways of thrombus formation in vivo. Blood 107:3902–3906

Dumont B, Lasne D, Rothschild C, Bouabdelli M, Ollivier V, Oudin C, Ajzenberg N, Grandchamp B, Jandrot-Perrus M (2009) Absence of collagen-induced platelet activation caused by compound heterozygous GPVI mutations. Blood 114:1900–1903

Eble JA, Beermann B, Hinz HJ, Schmidt-Hederich A (2001) alpha 2beta 1 integrin is not recognized by rhodocytin but is the specific, high affinity target of rhodocetin, an RGD-independent disintegrin and potent inhibitor of cell adhesion to collagen. J Biol Chem 276:12274–12284

Eble JA, Tuckwell DS (2003) The alpha2beta1 integrin inhibitor rhodocetin binds to the A-domain of the integrin alpha2 subunit proximal to the collagen-binding site. Biochem J 376:77–85

Eble JA, Bruckner P, Mayer U (2003) Vipera lebetina venom contains two disintegrins inhibiting laminin-binding beta1 integrins. J Biol Chem 278:26488–26496

Echtler K, Stark K, Lorenz M, Kerstan S, Walch A, Jennen L, Rudelius M, Seidl S, Kremmer E, Emambokus NR, von Bruehl ML, Frampton J, Isermann B, Genzel-Boroviczeny O, Schreiber C, Mehilli J, Kastrati A, Schwaiger M, Shivdasani RA, Massberg S (2010) Platelets contribute to postnatal occlusion of the ductus arteriosus. Nat Med 16:75–82

Emsley J, Knight CG, Farndale RW, Barnes MJ (2004) Structure of the integrin alpha2beta1-binding collagen peptide. J Mol Biol 335:1019–1028

Emsley J, Knight CG, Farndale RW, Barnes MJ, Liddington RC (2000) Structural basis of collagen recognition by integrin alpha2beta1. Cell 101:47–56

Emsley J, King SL, Bergelson JM, Liddington RC (1997) Crystal structure of the I domain from integrin alpha2beta1. J Biol Chem 272:28512–28517

Estavillo D, Ritchie A, Diacovo TG, Cruz MA (1999) Functional analysis of a recombinant glycoprotein Ia/IIa (Integrin alpha(2)beta(1)) I domain that inhibits platelet adhesion to collagen and endothelial matrix under flow conditions. J Biol Chem 274:35921–35926

Furihata K, Nugent DJ, Kunicki TJ (2002) Influence of Platelet Collagen Receptor Polymorphisms on Risk for Arterial Thrombosis. Arch Pathol Lab Med 126:305–309

Gronwald W, Bomke J, Maurer T, Domogalla B, Huber F, Schumann F, Kremer W, Fink F, Rysiok T, Frech M, Kalbitzer HR (2008) Structure of the leech protein saratin and characterization of its binding to collagen. J Mol Biol 381:913–927

Grothusen C, Umbreen S, Konrad I, Stellos K, Schulz C, Schmidt B, Kremmer E, Teebken O, Massberg S, Luchtefeld M, Schieffer B, Gawaz M (2007) EXP3179 inhibits collagen-dependent platelet activation via glycoprotein receptor-VI independent of AT1-receptor antagonism: potential impact on atherothrombosis. Arterioscler Thromb Vasc Biol 27:1184–1190

Gruner S, Prostredna M, Koch M, Miura Y, Schulte V, Jung SM, Moroi M, Nieswandt B (2005) Relative antithrombotic effect of soluble GPVI dimer compared with anti-GPVI antibodies in mice. Blood 105:1492–1499

Gruner S, Prostredna M, Schulte V, Krieg T, Eckes B, Brakebusch C, Nieswandt B (2003) Multiple integrin-ligand interactions synergize in shear-resistant platelet adhesion at sites of arterial injury in vivo. Blood 102:4021–4027

Han JY, Fan JY, Horie Y, Miura S, Cui DH, Ishii H, Hibi T, Tsuneki H, Kimura I (2008) Ameliorating effects of compounds derived from Salvia miltiorrhiza root extract on microcirculatory disturbance and target organ injury by ischemia and reperfusion. Pharmacol Ther 117:280–295

Handa M, Watanabe K, Kawai Y, Kamata T, Koyama T, Nagai H, Ikeda Y (1995) Platelet unresponsiveness to collagen: involvement of glycoprotein Ia- IIa (alpha 2 beta 1 integrin) deficiency associated with a myeloproliferative disorder. Thromb Haemost 73:521–528

Harsfalvi J, Stassen JM, Hoylaerts MF, Van Houtte E, Sawyer RT, Vermylen J, Deckmyn H (1995) Calin from Hirudo medicinalis, an inhibitor of von Willebrand factor binding to collagen under static and flow conditions. Blood 85:705–711

Hebert C, Norris K, Della CR, Reynolds M, Ordonez J, Sauk JJ (1999) Cell surface colligin/Hsp47 associates with tetraspanin protein CD9 in epidermoid carcinoma cell lines. J Cell Biochem 73:248–258

He L, Pappan LK, Grenache DG, Li ZZ, Tollefsen DM, Santoro SA, Zutter MM (2003) The contributions of the alpha(2)beta(1) integrin to vascular thrombosis in vivo. Blood 102:3652–3657

Hechler B, Nonne C, Eckly A, Magnenat S, Rinckel JY, Denis CV, Freund M, Cazenave JP, Lanza F, Gachet C (2010) Arterial thrombosis: relevance of a model with two levels of severity assessed by histologic, ultrastructural and functional characterization. J Thromb Haemost 8:173–184

Hermans C, Wittevrongel C, Thys C, Smethurst PA, Van GC, Freson K (2009) A compound heterozygous mutation in glycoprotein VI in a patient with a bleeding disorder. J Thromb Haemost 7:1356–1363

Herr AB, Farndale RW (2009) Structural insights into the interactions between platelet receptors and fibrillar collagen. J Biol Chem 284:19781–19785

Hirano K, Kuwasako T, Nakagawa-Toyama Y, Janabi M, Yamashita S, Matsuzawa Y (2003) Pathophysiology of human genetic CD36 deficiency. Trends Cardiovasc Med 13:136–141

Holtkotter O, Nieswandt B, Smyth N, Muller W, Hafner M, Schulte V, Krieg T, Eckes B (2002) Integrin alpha 2-deficient mice develop normally, are fertile, but display partially defective platelet interaction with collagen. J Biol Chem 277:10789–10794

Horii K, Kahn ML, Herr AB (2006) Structural basis for platelet collagen responses by the immune-type receptor glycoprotein VI. Blood 108:936–942

Hovig T (1963) Aggregation of rabbit blood platelets produced in vitro by saline extract of tendons. Thromb Diath Haemorrh 143:248–263

Hsu CC, Wu WB, Huang TF (2008) A snake venom metalloproteinase, kistomin, cleaves platelet glycoprotein VI and impairs platelet functions. J Thromb Haemost 6:1578–1585

Inoue O, Suzuki-Inoue K, McCarty OJ, Moroi M, Ruggeri ZM, Kunicki TJ, Ozaki Y, Watson SP (2006) Laminin stimulates spreading of platelets through integrin alpha6beta1-dependent activation of GPVI. Blood 107:1405–1412

Jandrot-Perrus M, Busfield S, Lagrue AH, Xiong X, Debili N, Chickering T, Le Couedic JP, Goodearl A, Dussault B, Fraser C, Vainchenker W, Villeval JL (2000) Cloning, characterization, and functional studies of human and mouse glycoprotein VI: a platelet-specific collagen receptor from the immunoglobulin superfamily. Blood 96:1798–1807

Jarvis GE, Raynal N, Langford JP, Onley DJ, Andrews A, Smethurst PA, Farndale RW (2008) Identification of a major GpVI-binding locus in human type III collagen. Blood 111:4986–4996

Joutsi-Korhonen L, Smethurst PA, Rankin A, Gray E, Ijsseldijk M, Onley CM, Watkins NA, Williamson LM, Goodall AH, de Groot PG, Famdale RW, Ouwehand WH (2003) The low-frequency allele of the platelet collagen signaling receptor glycoprotein VI is associated with reduced functional responses and expression. Blood 101:4372–4379

Jung SM, Moroi M (2000) Signal-transducing mechanisms involved in activation of the platelet collagen receptor integrin alpha(2)beta(1). J Biol Chem 275:8016–8026

Jung SM, Tsuji K, Moroi M (2009) Glycoprotein (GP) VI dimer as a major collagen-binding site of native platelets: direct evidence obtained with dimeric GPVI-specific Fabs. J Thromb Haemost 7:1347–1355

Kaiser WJ, Holbrook LM, Tucker KL, Stanley RG, Gibbins JM (2009) A functional proteomic method for the enrichment of peripheral membrane proteins reveals the collagen binding protein Hsp47 is exposed on the surface of activated human platelets. J Proteome Res 8:2903–2914

Kamata T, Puzon W, Takada Y (1994) Identification of putative ligand binding sites within I domain of integrin alpha 2 beta 1 (VLA-2, CD49b/CD29). J Biol Chem 269:9659–9663

Kamiguti AS, Markland FS, Zhou Q, Laing GD, Theakston RD, Zuzel M (1997) Proteolytic cleavage of the beta1 subunit of platelet alpha2beta1 integrin by the metalloproteinase jararhagin compromises collagen-stimulated phosphorylation of pp72. J Biol Chem 272:32599–32605

Kapyla J, Pentikainen OT, Nyronen T, Nissinen L, Lassander S, Jokinen J, Lahti M, Marjamaki A, Johnson MS, Heino J (2007) Small molecule designed to target metal binding site in the alpha2I domain inhibits integrin function. J Med Chem 50:2742–2746

Kato K, Kanaji T, Russell S, Kunicki TJ, Furihata K, Kanaji S, Marchese P, Reininger A, Ruggeri ZM, Ware J (2003) The contribution of glycoprotein VI to stable platelet adhesion and thrombus formation illustrated by targeted gene deletion. Blood 102:1701–1707

Keller PM, Schultz LD, Condra C, Karczewski J, Connolly TM (1992) An inhibitor of collagen-stimulated platelet activation from the salivary glands of the Haementeria officinalis leech. II. Cloning of the cDNA and expression. J Biol Chem 267:6899–6904

Kirchhofer D, Languino LR, Ruoslahti E, Pierschbacher MD (1990) Alpha 2 beta 1 integrins from different cell types show different binding specificities. J Biol Chem 265:615–618

Kleinschnitz C, Pozgajova M, Pham M, Bendszus M, Nieswandt B, Stoll G (2007) Targeting platelets in acute experimental stroke: impact of glycoprotein Ib, VI, and IIb/IIIa blockade on infarct size, functional outcome, and intracranial bleeding. Circulation 115:2323–2330

Konishi H, Katoh Y, Takaya N, Kashiwakura Y, Itoh S, Ra C, Daida H (2002) Platelets activated by collagen through immunoreceptor tyrosine-based activation motif play pivotal role in initiation and generation of neointimal hyperplasia after vascular injury. Circulation 105:912–916

Konstantinides S, Ware J, Marchese P, mus-Jacobs F, Loskutoff DJ, Ruggeri ZM (2006) Distinct antithrombotic consequences of platelet glycoprotein Ibalpha and VI deficiency in a mouse model of arterial thrombosis. J Thromb Haemost 4:2014–2021

Kritzik M, Savage B, Nugent DJ, Santoso S, Ruggeri ZM, Kunicki TJ (1998) Nucleotide polymorphisms in the alpha2 gene define multiple alleles that are associated with differences in platelet alpha2 beta1 density. Blood 92:2382–2388

Kuijpers MJ, Pozgajova M, Cosemans JM, Munnix IC, Eckes B, Nieswandt B, Heemskerk JW (2007) Role of murine integrin alpha2beta1 in thrombus stabilization and embolization: contribution of thromboxane A2. Thromb Haemost 98:1072–1080

Kumar MS, Girish KS, Vishwanath BS, Kemparaju K (2011) The metalloprotease, NN-PF3 from Naja naja venom inhibits platelet aggregation primarily by affecting alpha2beta1 integrin. Ann Hematol 90:569–577

Kunicki TJ, Orchekowski R, Annis D, Honda Y (1993) Variability of integrin alpha 2 beta 1 activity on human platelets. Blood 82:2693–2703

Lebbink RJ, de RT, Adelmeijer J, Brenkman AB, van Helvoort JM, Koch M, Farndale RW, Lisman T, Sonnenberg A, Lenting PJ, Meyaard L (2006) Collagens are functional, high affinity ligands for the inhibitory immune receptor LAIR-1. J Exp Med 203:1419–1425

Lecut C, Feeney LA, Kingsbury G, Hopkins J, Lanza F, Gachet C, Villeval JL, Jandrot-Perrus M (2003) Human platelet glycoprotein VI function is antagonized by monoclonal anti body-derived Fab fragments. J Thromb Haemost 1:2653–2662

Lenting PJ, Westerlaken GH, Denis CV, Akkerman JW, Meyaard L (2010) Efficient inhibition of collagen-induced platelet activation and adhesion by LAIR-2, a soluble Ig-like receptor family member. PLoS One 5:e12174

Li H, Lockyer S, Concepcion A, Gong X, Takizawa H, Guertin M, Matsumoto Y, Kambayashi J, Tandon NN, Liu Y (2007) The Fab fragment of a novel anti-GPVI monoclonal antibody, OM4, reduces in vivo thrombosis without bleeding risk in rats. Arterioscler Thromb Vasc Biol 27:1199–1205

Locke D, Chen H, Liu Y, Liu C, Kahn ML (2002) Lipid rafts orchestrate signaling by the platelet receptor glycoprotein VI. J Biol Chem 277:18801–18809

Lockyer S, Okuyama K, Begum S, Le S, Sun B, Watanabe T, Matsumoto Y, Yoshitake M, Kambayashi J, Tandon NN (2006) GPVI-deficient mice lack collagen responses and are protected against experimentally induced pulmonary thromboembolism. Thromb Res 118:371–380

Luo BH, Carman CV, Springer TA (2007) Structural basis of integrin regulation and signaling. Annu Rev Immunol 25:619–647

Mangin P, Yap CL, Nonne C, Sturgeon SA, Goncalves I, Yuan Y, Schoenwaelder SM, Wright CE, Lanza F, Jackson SP (2006) Thrombin overcomes the thrombosis defect associated with platelet GPVI/FcRgamma deficiency. Blood 107:4346–4353

Massberg S, Konrad I, Bultmann A, Schulz C, Munch G, Peluso M, Lorenz M, Schneider S, Besta F, Muller I, Hu B, Langer H, Kremmer E, Rudelius M, Heinzmann U, Ungerer M, Gawaz M (2004) Soluble glycoprotein VI dimer inhibits platelet adhesion and aggregation to the injured vessel wall in vivo. FASEB J 18:397–399

Massberg S, Gawaz M, Gruner S, Schulte V, Konrad I, Zohlnhofer D, Heinzmann U, Nieswandt B (2003) A crucial role of glycoprotein VI for platelet recruitment to the injured arterial wall in vivo. J Exp Med 197:41–49

Matsumoto Y, Takizawa H, Nakama K, Gong X, Yamada Y, Tandon NN, Kambayashi J (2006) Ex vivo evaluation of anti-GPVI antibody in cynomolgus monkeys: dissociation between anti-platelet aggregatory effect and bleeding time. Thromb Haemost 96:167–175

Maurice P, Pires V, Amant C, Kauskot A, Da NS, Sonnet P, Rochette J, Legrand C, Fauvel-Lafeve F, Bonnefoy A (2006) Antithrombotic effect of the type III collagen-related octapeptide (KOGEOGPK) in the mouse. Vascul Pharmacol 44:42–49

Miller MW, Basra S, Kulp DW, Billings PC, Choi S, Beavers MP, McCarty OJ, Zou Z, Kahn ML, Bennett JS, DeGrado WF (2009) Small-molecule inhibitors of integrin alpha2beta1 that prevent pathological thrombus formation via an allosteric mechanism. Proc Natl Acad Sci U S A 106:719–724

Miyaishi O, Sakata K, Matsuyama M, Saga S (1992) Distribution of the collagen binding heat-shock protein in chicken tissues. J Histochem Cytochem 40:1021–1029

Monnet E, Fauvel-Lafeve F (2000) A new platelet receptor specific to type III collagen. Type III collagen-binding protein. J Biol Chem 275:10912–10917

Monnet E, Depraetere H, Legrand C, Deckmyn H, Fauvel-Lafeve F (2001) A monoclonal antibody to platelet type III collagen-binding protein (TIIICBP) binds to blood and vascular cells, and inhibits platelet vessel-wall interactions. Thromb Haemost 86:694–701

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 (2001) Platelet glycoprotein V binds to collagen and participates in platelet adhesion and aggregation. Blood 98:1038–1046

Moshfegh K, Wuillemin WA, Redondo M, Lammle B, Beer JH, Liechti-Gallati S, Meyer BJ (1999) Association of two silent polymorphisms of platelet glycoprotein Ia/IIa receptor with risk of myocardial infarction: a case-control study. Lancet 353:351–354

Moura-da-Silva AM, Ramos OH, Baldo C, Niland S, Hansen U, Ventura JS, Furlan S, Butera D, la-Casa MS, Tanjoni I, Clissa PB, Fernandes I, Chudzinski-Tavassi AM, Eble JA (2008) Collagen binding is a key factor for the hemorrhagic activity of snake venom metalloproteinases. Biochimie 90:484–492

Muzard J, Bouabdelli M, Zahid M, Ollivier V, Lacapere JJ, Jandrot-Perrus M, Billiald P (2009) Design and humanization of a murine scFv that blocks human platelet glycoprotein VI in vitro. FEBS J 276:4207–4222

Nieswandt B, Brakebusch C, Bergmeier W, Schulte V, Bouvard D, Mokhtari-Nejad R, Lindhout T, Heemskerk JW, Zirngibl H, Fassler R (2001b) Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. EMBO J 20:2120–2130

Nieswandt B, Schulte V, Bergmeier W, Mokhtari-Nejad R, Rackebrandt K, Cazenave JP, Ohlmann P, Gachet C, Zirngibl H (2001a) Long-term antithrombotic protection by in vivo depletion of platelet glycoprotein VI in mice. J Exp Med 193:459–469

Nieuwenhuis HK, Akkerman JW, Houdijk WP, Sixma JJ (1985) Human blood platelets showing no response to collagen fail to express surface glycoprotein Ia. Nature 318:470–472

Nissinen L, Pentikainen OT, Jouppila A, Kapyla J, Ojala M, Nieminen J, Lipsanen A, Lappalainen H, Eckes B, Johnson MS, Lassila R, Marjamaki A, Heino J (2010) A small-molecule inhibitor of integrin alpha2 beta1 introduces a new strategy for antithrombotic therapy. Thromb Haemost 103:387–397

Ollikainen E, Mikkelsson J, Perola M, Penttila A, Karhunen PJ (2004) Platelet membrane collagen receptor glycoprotein VI polymorphism is associated with coronary thrombosis and fatal myocardial infarction in middle-aged men. Atherosclerosis 176:95–99

Pentikainen O, Hoffren AM, Ivaska J, Kapyla J, Nyronen T, Heino J, Johnson MS (1999) "RKKH" peptides from the snake venom metalloproteinase of Bothrops jararaca bind near the metal ion-dependent adhesion site of the human integrin alpha(2) I-domain. J Biol Chem 274:31493–31505

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 (2005) Human atheromatous plaques stimulate thrombus formation by activating platelet glycoprotein VI. FASEB J 19:898–909

Pugh N, Simpson AM, Smethurst PA, de Groot PG, Raynal N, Farndale RW (2010) Synergism between platelet collagen receptors defined using receptor-specific collagen-mimetic peptide substrata in flowing blood. Blood 115:5069–5079

Quek LS, Pasquet JM, Hers I, Cornall R, Knight G, Barnes M, Hibbs ML, Dunn AR, Lowell CA, Watson SP (2000) Fyn and Lyn phosphorylate the Fc receptor gamma chain downstream of glycoprotein VI in murine platelets, and Lyn regulates a novel feedback pathway. Blood 96:4246–4253

Rabie T, Strehl A, Ludwig A, Nieswandt B (2005) Evidence for a role of ADAM17 (TACE) in the regulation of platelet glycoprotein V. J Biol Chem 280:14462–14468

Raynal N, Hamaia SW, Siljander PRM, Maddox B, Peachey AR, Fernandez R, Foley LJ, Slatter DA, Jarvis GE, Farndale RW (2006) Use of synthetic peptides to locate novel integrin alpha(2) beta(1)-binding motifs in human collagen III. J Biol Chem 281:3821–3831

Riba R, Hughes CE, Graham A, Watson SP, Naseem KM (2008) Globular adiponectin induces platelet activation through the collagen receptor GPVI-Fc receptor gamma chain complex. J Thromb Haemost 6:1012–1020

Saelman EU, Nieuwenhuis HK, Hese KM, de Groot PG, Heijnen HF, Sage EH, Williams S, McKeown L, Gralnick HR, Sixma JJ (1994) Platelet adhesion to collagen types I through VIII under conditions of stasis and flow is mediated by GPIa/IIa (alpha 2 beta 1-integrin). Blood 83:1244–1250

Santoso S, Kunicki TJ, Kroll H, Haberbosch W, Gardemann A (1999) Association of the platelet glycoprotein Ia C807T gene polymorphism with nonfatal myocardial infarction in younger patients. Blood 93:2449–2453

Sato S, Futaki F, Fukuchi N, Kaida K, Hiraga M, Kobaru S, Tsuji T (2006) A new nucleoside derivative, AJP117510, as an inhibitor of integrin alpha2beta1-collagen binding. J Antibiot (Tokyo) 59:251–253

Schaffer LW, Davidson JT, Siegl PK, Gould RJ, Nutt RF, Brady SF, Connolly TM (1993) Recombinant leech antiplatelet protein prevents collagen-mediated platelet aggregation but not collagen graft thrombosis in baboons. Arterioscler Thromb 13:1593–1601

Schmaier AA, Zou Z, Kazlauskas A, Emert-Sedlak L, Fong KP, Neeves KB, Maloney SF, Diamond SL, Kunapuli SP, Ware J, Brass LF, Smithgall TE, Saksela K, Kahn ML (2009) Molecular priming of Lyn by GPVI enables an immune receptor to adopt a hemostatic role. Proc Natl Acad Sci USA 106:21167–21172

Schulte V, Reusch HP, Pozgajova M, Varga-Szabo D, Gachet C, Nieswandt B (2006) Two-phase antithrombotic protection after anti-glycoprotein VI treatment in mice. Arterioscler Thromb Vasc Biol 26:1640–1647

Siljander PRM, Munnix ICA, Smethurst PA, Deckmyn H, Lindhout T, Ouwehand WH, Farndale RW, Heemskerk JWM (2004) Platelet receptor interplay regulates collagen-induced thrombus formation in flowing human blood. Blood 103:1333–1341

Smethurst PA, Onley DJ, Jarvis GE, O'Connor MN, Knight CG, Herr AB, Ouwehand WH, Farndale RW (2007) Structural basis for the platelet-collagen interaction: the smallest motif within collagen that recognizes and activates platelet Glycoprotein VI contains two glycine-proline-hydroxyproline triplets. J Biol Chem 282:1296–1304

Smethurst PA, Joutsi-Korhonen L, O'Connor MN, Wilson E, Jennings NS, Garner SF, Zhang Y, Knight CG, Dafforn TR, Buckle A, Ijsseldijk MJ, de Groot PG, Watkins NA, Farndale RW, Ouwehand WH (2004) Identification of the primary collagen-binding surface on human glycoprotein VI by site-directed mutagenesis and by a blocking phage antibody. Blood 103:903–911

Sugiyama T, Okuma M, Ushikubi F, Sensaki S, Kanaji K, Uchino H (1987) A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia. Blood 69:1712–1720

Suzuki-Inoue K, Tulasne D, Shen Y, Bori-Sanz T, Inoue O, Jung SM, Moroi M, Andrews RK, Berndt MC, Watson SP (2002) Association of Fyn and Lyn with the proline-rich domain of glycoprotein VI regulates intracellular signaling. J Biol Chem 277:21561–21566

Takayama H, Hosaka Y, Nakayama K, Shirakawa K, Naitoh K, Matsusue T, Shinozaki M, Honda M, Yatagai Y, Kawahara T, Hirose J, Yokoyama T, Kurihara M, Furusako S (2008) A novel antiplatelet antibody therapy that induces cAMP-dependent endocytosis of the GPVI/Fc receptor gamma-chain complex. J Clin Invest 118:1785–1795

Tanjoni I, Evangelista K, la-Casa MS, Butera D, Magalhaes GS, Baldo C, Clissa PB, Fernandes I, Eble J, Moura-da-Silva AM (2010) Different regions of the class P-III snake venom metalloproteinase jararhagin are involved in binding to alpha2beta1 integrin and collagen. Toxicon 55:1093–1099

Verkleij MW, Ijsseldijk MJ, Heijnen-Snyder GJ, Huizinga EG, Morton LF, Knight CG, Sixma JJ, de Groot PG, Barnes MJ (1999) Adhesive domains in the collagen III fragment alpha1(III)CB4 that support alpha2beta1- and von Willebrand factor-mediated platelet adhesion under flow conditions. Thromb Haemost 82:1137–1144

Verkleij MW, Morton LF, Knight CG, de Groot PG, Barnes MJ, Sixma JJ (1998) Simple collagen-like peptides support platelet adhesion under static but not under flow conditions: interaction via alpha2 beta1 and von Willebrand factor with specific sequences in native collagen is a requirement to resist shear forces. Blood 91:3808–3816

Vilahur G, Duran X, Juan-Babot O, Casani L, Badimon L (2004) Antithrombotic effects of saratin on human atherosclerotic plaques. Thromb Haemost 92:191–200

Walker A, Pugh N, Garner SF, Stephens J, Maddox B, Ouwehand WH, Farndale RW, Steward M (2009) Single domain antibodies against the collagen signalling receptor glycoprotein VI are inhibitors of collagen induced thrombus formation. Platelets 20:268–276

Wang WJ, Shih CH, Huang TF (2005) Primary structure and antiplatelet mechanism of a snake venom metalloproteinase, acurhagin, from Agkistrodon acutus venom. Biochimie 87:1065–1077

Watson SP, Auger JM, McCarty OJ, Pearce AC (2005) GPVI and integrin alphaIIb beta3 signaling in platelets. J Thromb Haemost 3:1752–1762

Wijeyewickrema LC, Gardiner EE, Moroi M, Berndt MC, Andrews RK (2007) Snake venom metalloproteinases, crotarhagin and alborhagin, induce ectodomain shedding of the platelet collagen receptor, glycoprotein VI. Thromb Haemost 98:1285–1290

Wonerow P, Obergfell A, Wilde JI, Bobe R, Asazuma N, Brdicka T, Leo A, Schraven B, Horejsi V, Shattil SJ, Watson SP (2002) Differential role of glycolipid-enriched membrane domains in glycoprotein VI- and integrin-mediated phospholipase Cgamma2 regulation in platelets. Biochem J 364:755–765

Wu YP, Zhao XM, Pan SD, Guo dA, Wei R, Han JJ, Kainoh M, Xia ZL, de Groot PG, Lisman T (2008) Salvianolic acid B inhibits platelet adhesion under conditions of flow by a mechanism involving the collagen receptor alpha2beta1. Thromb Res 123:298–305

Yin H, Gerlach LO, Miller MW, Moore DT, Liu D, Vilaire G, Bennett JS, DeGrado WF (2006) Arylamide derivatives as allosteric inhibitors of the integrin alpha2beta1/type I collagen interaction. Bioorg Med Chem Lett 16:3380–3382

Zafar MU, Ibanez B, Choi BG, Vorchheimer DA, Pinero A, Jin X, Sharma RK, Badimon JJ (2010) A new oral antiplatelet agent with potent antithrombotic properties: comparison of DZ-697b with clopidogrel a randomised phase I study. Thromb Haemost 103:205–212

Zhu J, Cole F, Woo-Rasberry V, Fang XR, Chiang TM (2007) Type I and type III collagen-platelet interaction: inhibition by type specific receptor peptides. Thromb Res 119:111–119

Zucker MB, Borrelli J (1961) Platelet viscous metamorphosis produced by connective tissue particles. Fed Proc 20:61

Intracellular Signaling as a Potential Target for Antiplatelet Therapy

Patrick Andre

Contents

P. Andre (✉)
Portola Pharmaceuticals Inc, 270 E. Grand Avenue, Suite 22 South San Francisco, CA 94080, USA

Merck Research Laboratories, 126 E. Lincoln Avenue, Rahway, NJ 07065, USA
e-mail: Patrick.andre@merck.com

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_14,

Abstract Three classes of inhibitors of platelet aggregation have demonstrated substantial clinical benfits. Aspirin acts by irreversibly inhibiting COX-1 and therefore blocking the synthesis of proaggregatory thromboxane A $_2$ (TxA_2). The indirect acting (ticlopidine, clopidogrel, prasugrel) and the direct acting (ticagrelor) antagonists of $P2Y_{12}$ block the thrombus stabilizing activity of ADP. Parenteral GP IIb-IIIa inhibitors directly block platelet-platelet interactions. Despite well-established benefits, all antiplatelet agents have important limitations: increased bleeding and gastrointestinal toxicities (aspirin), high incidence of thrombotic thrombocytopenic purpura (ticlopidine), potentially nonresponders (clopidogrel), severe bleeding (prasugrel, GP IIb-IIIa antagonists) and "complicated" relationships with aspirin ticagrelor). In this chapter, we present the genetic and pharmacological evidence that supports the development and expectations associated with novel antiplatelet strategies directed at intrasignaling pathways.

Keywords Platelet • Agonist • Signaling • Kinase • Thrombus stability

Three classes of inhibitors of platelet aggregation have demonstrated substantial clinical benefits, significantly improving short- and long-term outcomes of patients with ACS, thrombotic stroke, vascular intervention, and other indications which involve platelet-rich thrombi. Aspirin acts by irreversibly inhibiting COX-1 and therefore blocking the synthesis of proaggregatory thromboxane A_2 (TxA_2). The indirect acting (ticlopidine, clopidogrel, prasugrel) and the direct acting (ticagrelor) antagonists of $P2Y_{12}$ block the thrombus stabilizing activity of ADP. Parenteral GP IIb-IIIa inhibitors directly block platelet–platelet interactions.

Despite well-established benefits, all antiplatelet agents have important limitations. Aspirin is only a weak inhibitor of platelet function and high doses have been associated with increased bleeding and gastrointestinal toxicities. The use of ticlopidine has been hampered by a high incidence of thrombotic thrombocytopenic purpura. The effect of clopidogrel relies on the patient's ability to generate an active metabolite while prasugrel has the potential to cause severe bleeding complications. Ticagrelor also showed benefits but at the expense of increased bleeding and demonstrated a complicated interaction with aspirin. Finally, parenteral GP IIb-IIIa antagonists are useful only in the acute setting and can severely compromise primary hemostasis.

In this chapter, we present the genetic and pharmacological evidence that supports the development and expectations associated with novel antiplatelet strategies directed at intrasignaling pathways. We briefly review the inside-out (I-O.) and outside-in (O-I.) signaling pathways that promote platelet aggregation and thrombosis in order to identify key players with potential ubiquitous activities that could represent targets for novel drug therapies. Three of the I-O. signaling pathways leading to the activation of GP IIb-IIIa (the receptor mediating the final step in aggregation) and the outside-in (O-I.) signaling downstream of GP IIb-IIIa will be the main focus. The I-O. signaling pathways are activated in response to the engagement of glycoprotein receptors expressed on the platelet surface. These include, but are not limited to: GPVI, which mediates collagen-induced platelet activation; GPIbα, the key receptor that triggers recruitment of platelets onto vWF-bound collagen or on already activated platelets at high shear rates; and G-protein-coupled receptors (GPCRs) which include receptors for ADP, thrombin, TxA_2, and epinephrine. We also discuss the existence of negative regulators of platelet function and evaluate whether they could constitute a novel addition to the armametum used in the fight against cardiovascular diseases.

We finish by presenting the current landscape and the challenges facing the development of future pharmacological agents that will target intraplatelet signaling pathways.

1 Key Intracellular Proteins of Platelet Signaling Pathways Promoting Platelet Aggregation/Thrombosis

1.1 Key Signaling Molecules of the GPVI Signaling Pathway (Fig. 1, Top Panel)

After engagement of GPVI by its cognate ligands (collagen, CRP, convulxin), the two YXXL motifs of the FcRγ ITAM are phosphorylated by SFKs (Src family kinases, Fyn and Lyn). This phosphorylation provides docking sites for the Syk-SH2 domains resulting in a structural alteration of the autoinhibited state of Syk, activation of its kinase function, and autophosphorylation on several residues. In parallel, activated (phosphorylated) Syk separates from the ITAM domain and recruits key signaling proteins to its phosphorylated tyrosine residues. These events facilitate the binding of the SH2 domains of VAV, p85α subunit of PI-3K, and PLCγ. Phosphorylation of and signaling through adapter proteins (e.g., linker for activation of T cells (LAT); SLP-76) lead to the formation of a complex comprising LAT, SLP-76, Bruton tyrosine kinase (Btk), Gads, and PLC-γ, which in turn activate PLC-γ. The Syk signaling pathway also has a positive feedback loop

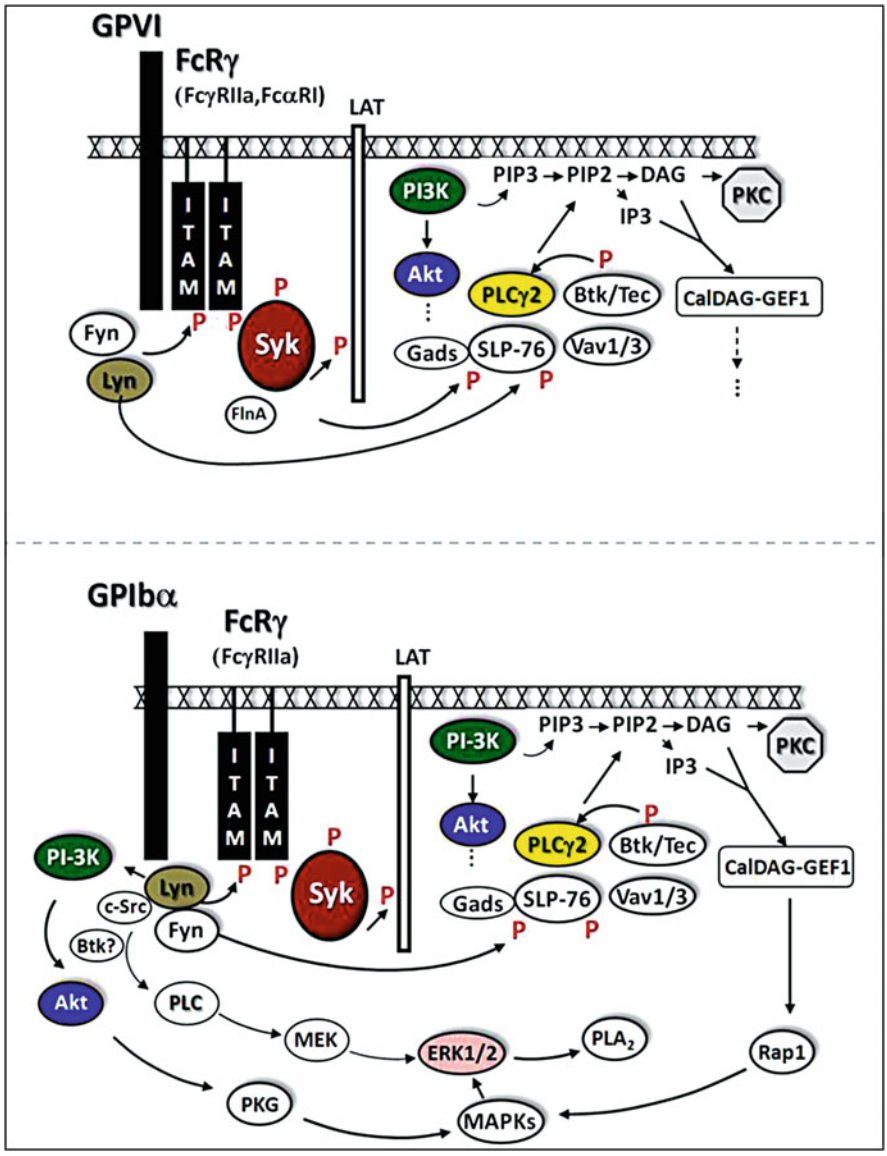


Fig. 1 Key signaling players of the GPVI (*top panel*) and GPIbα (*bottom panel*) pathways leading to platelet activation

originating from phosphorylation events (i.e., autophosphorylation of the tyrosine residues in its linker region, phosphorylation of ITAM tyrosine residues) that contribute to a sustained activation. PLC-γ2 hydrolyzes membrane-bound PIP2 to generate inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG). The

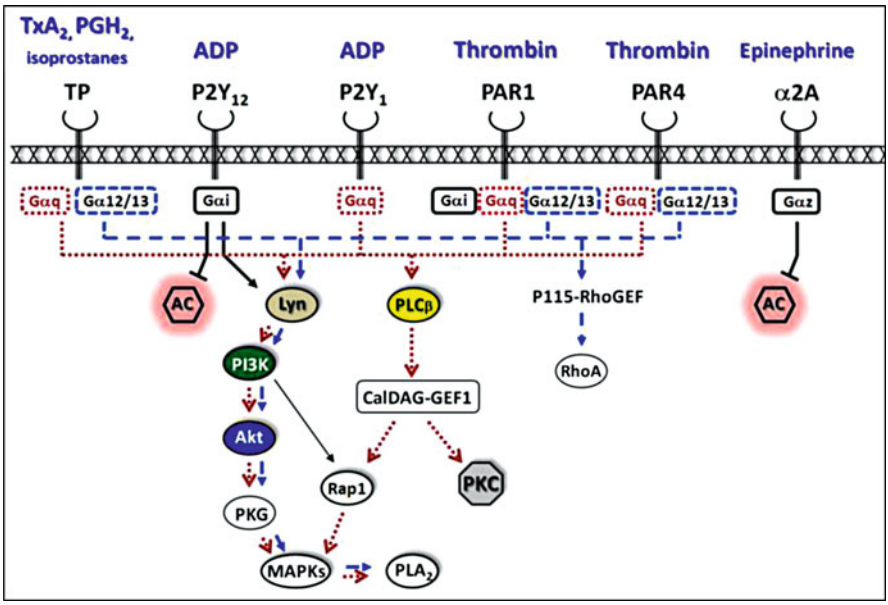


Fig. 2 Key signaling players of GPCR signaling pathways

former releases cytosolic calcium from internal stores, the latter activates protein kinase C. Activation of PLCγ2 ultimately leads to the synthesis of thromboxane A_2 (TxA_2), the release of granule contents, and activation of GP IIb-IIIa.

1.2 Key Signaling Molecules of the GPIbα Signaling Pathway (Fig. 1, Lower Panel)

GPIbα interaction with vWF under arterial shear rates is known to trigger at least two platelet responses. It facilitates the recruitment of platelets as well as induces platelet activation signaling events necessary for activation of the glycoprotein receptor GP IIb-IIIa. In addition, it can also sensitize platelets to low-thrombin concentrations; however, the relative contribution of this event to arterial thrombosis remains to be established. The cytoplasmic domain of GPIbα interacts with the 107 Src family kinase, Lyn as well as PI-3k. Lyn is key to the activation of PI-3K which in turn leads to the activation of Akt [a pathway also involved in Gas6-receptor signaling (Angelillo-Scherrer et al. 2005)]. Engagement of GPIbα also leads to the activation of cGMP-dependent protein kinase (PKG), p38 MAPK, and extracellular signal-regulated kinase (ERK). Additionally, the GPIbα complex is associated with ITAM receptors (e.g., FcRγ, FcγRIIA). This signaling pathway (also common to GPVI) is believed to contribute to granule secretion and O-I. signaling.

1.3 Key Signaling Molecules of GPCR Signaling Pathways (Fig. 2)

GPCR play a crucial role in the extension phase of thrombosis. GPCRs activate G proteins leading to the activation of key effectors such as adenylyl cyclase, PLC, PI-3K, and p115-RhoGEF. In brief, some platelet agonists of GPCRs activate phospholipase Cβ through Gαq. This leads to the formation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). The subsequent Ca^{2+} mobilization from intracellular stores combined with DAG activates protein kinase C (PKC). Other agonists (e.g., thrombin, ADP, TxA_2) activate platelets via activation of phosphoinositide 3-kinase (PI-3K). As a result, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ levels increase and this leads to recruitment of Akt. Stimulation by GPCR agonists coupled to $G_{\alpha 12}$ and $G_{\alpha 13}$ leads to platelet shape change, while that through $G_{\alpha i}$-coupled GPCRs suppresses cAMP synthesis by inhibition of adenylyl cyclase as well as PI-3K activation.

1.4 Key Signaling Molecules of the Outside-In Signaling Pathway

I-O.-mediated activation of GP IIb-IIIa is followed by a GP IIb-IIIa-mediated O-I. signaling that is responsible for platelet spreading, granule secretion, stable adhesion, and clot retraction. Targeting outside-in signaling to treat arterial thrombosis has been proposed by Law and colleagues who showed that DiYf mice (expressing an $\alpha_{IIb}\beta_3$ in which the tyrosines in the integrin cytoplasmic tyrosine motif have been mutated to phenylalanines) are selectively impaired in outside-in signaling, aggregation, and clot-retraction and have a pronounced tendency to rebleed (Law et al. 1999a). c-Src activation subsequent to coupling of $G_{\alpha 13}$ with the cytoplasmic domain of the β_3 subunit is key to the phosphorylation of the two tyrosines and resultant outside-in signaling. C-Src also phosphorylates and activates 190 Rho GTPase-activating protein. Members of the Src family kinases have also been shown to activate Syk in a FcγRIIA-dependent manner (Boylan et al. 2008) which leads to the activation of $PLC\gamma_2$ (see above).

1.5 Summary

Platelet agonists activate a complex signalosome involving sequential and parallel pathways which often utilize common partners and are influenced by multiple positive and negative feedback loops.

2 Genetic and Pharmacologic Modulations of Intrasignaling Pathways: Impact on Platelet Function, Thrombosis, and Primary Hemostasis (Table 1)

At least 20 kinases including their isoforms have been identified that significantly contribute to platelet aggregation and thrombosis. Genetic and pharmacological evidence of their critical roles are presented below.

2.1 Akt

2.1.1 Akt-1

Akt-1-deficiency is associated with impaired platelet aggregation in response to collagen and low concentrations of thrombin (Yin et al. 2008b). Akt-1-deficient platelets are also characterized by an overall reduction in granule release. In accordance with its ubiquitous role in multiple platelet signaling pathways, animals lacking Akt-1 have significantly longer tail bleeding times (Chen et al. 2004).

2.1.2 Akt-2

Defects in granule secretion, platelet aggregation, and thrombus formation in vivo also characterize mice deficient in Akt-2 (Woulfe et al. 2004). The platelet defect is mostly apparent following activation induced by thrombin and TxA_2 but in contrast to deficiency of Akt-1 isoform, it is not associated with an impairment of primary hemostasis. Yin and collaborators have also shown that genetic deletion or pharmacological modulation of Akt1 and Akt2 diminish vWF-induced cGMP elevation, platelet spreading on vWF (but not on immobilized fibrinogen) as well as platelet aggregation (botrocetin-induced), and stable adhesion on vWF under arterial shear rates (Yin et al. 2008b).

2.2 Btk

Quek et al. reported that Bruton's tyrosine kinase (Btk) has a significant role in collagen and collagen-related peptide (CRP) but not thrombin-signaling pathways leading to platelet aggregation, dense granule secretion, as well as calcium mobilization (Quek et al. 1998). Liu et al. reported that Btk is essential for botrocetin-induced platelet aggregation and GPIbα-dependent arterial thrombus formation in vivo (Liu et al. 2006). The defect associated with Btk-deficiency is further

Table 1 Genetic and pharmacological evidence for the participation of signaling partners in platelet function and thrombosis

Genetic deletion	Target	Effects	Bleeding compared to WT	References	Inhibitor	Effects	Bleeding compared to WT	References
Akt1	GPIbα	Block. of I-O. signaling		Yin et al. (2008b)	SH6	Block. GPIbα-response in vitro		Yin et al. (2008b)
	PAR, GPVI	Block. of I-O. signaling, aggregation and thrombosis	>	Chen et al. (2004)				
Akt2	GPIbα	Block. of I-O. signaling		Yin et al. (2008b)				
	PAR, TP	Block. of I-O. signaling, aggregation and thrombosis	=	Woulfe et al. (2004)				
Btk*	GPIbα	Inh. of thrombosis		Liu et al. (2006)				
Mutants	GPVI	Block. I-O. signaling		Liu et al. (2006)				
Patients	GPVI	Affect I-O. signaling	=	Quek et al. (1998), Atkinson et al. (2003)				
CalDAG-GEFI	GPVI	Inh. of I-O. signaling	>	Crittenden et al. (2004), Stefanini et al. (2009)				
		Inh. of immune thrombocytopenia		Stolla et al. (2011)				
$cPLA_2$		Inh. of thrombosis		Bonventre et al. (1997)	VRV-PL-IIIB	Inh. $P2Y_{12}$-mediated aggregation		Prasad et al. (1996)
cSrc	GP IIb-IIIa	Block. of O-I. signaling	>	Obergfell et al. (2002),				

				Ablooglu et al. (2009)			
Fyn	GPVI	Inh. of Akt I-O. signaling		Kim et al. (2009)			
	GPIbα	Affect 2nd wave of agg.	>	Yin et al. (2008a)			
	GP IIb-IIIa	Defects in O-I. signaling		Reddy et al. (2008)			
LAT	GPVI	Inh. of I-O. signaling Inh. of thrombosis	>	Pasquet et al. (1999), Munnix et al. (2005), Kalia et al. (2008)			
Lyn	GPIbα	Block. of I-O. signaling		Liu et al. (2006)			
	GPVI	Enhances degranulation		Chari et al. (2009b)			
p38MAPK dominant negative	GPIbα	Inh. of I-O. signaling		Li et al. (2006)	SB203580	Block. GPIbα-response	Minuz et al. (2002), Canobbio et al. (2004), Flevaris et al. (2009)
	GPVI					Block. TP-response and clot retraction	
						Block. platelet granule secretion	
PI-3K(s)					Wort. LY294002	Block. GPIbα-mediated I-O. signaling and O-I. signaling	Kim et al. (2009), Gilio et al. (2009)
α	GPVI	Inh. of I-O. signaling	=	Watanabe et al. (2003)	PIK-75*	Affects GPVI-Akt signaling	Bird et al. (2011), Kim et al. (2009)
β	GPVI	Block. of I-O. signaling	=		TGX-221		

(continued)

Table 1 (continued)

Genetic deletion	Target	Effects	Bleeding compared to WT	References	Inhibitor	Effects	Bleeding compared to WT	References
				Canobbio et al. (2009)		Affect GPVI-Akt signaling		
β	$P2Y_{12}$, TP	Inh. of I-O. signaling		Canobbio et al. (2009)	TGX-221*	Block. $P2Y_{12}$-Akt signaling, aggregation	>	Garcia et al. (2010)
β	GP IIb-IIIa	Inh. of O.I. signaling		Canobbio et al. (2009)				
β	PAR	Inh. of clot retraction		Schoenwaelder et al. (2010)	TGX-221	Inh. clot retraction		Schoenwaelder et al. (2010)
γ	$P2Y_{12}$	Inh. I-O sig. thrombosis	=	Canobbio et al. (2009)	AS-252424			
γ	GPVI	No effects		Kim et al. (2009)	IC-87114	No effect on GPVI-Akt signaling		Kim et al. (2009)
δ	$P2Y_{12}$	Inh. of I-O. signaling		Hirsch et al. (2001)				
δ	GPVI	No effects		Senis et al. (2005), Gilio et al. (2009)				
PKC(s)					Go6976	Block. GPVI, GPIbα-resp. to alboagg. A		Crosby and Poole (2003)
α	GPVI, PAR	Inh. of thrombosis, Block. of δ granule secretion	=	Konopatskaya et al. (2009)	GF109203X			Chari et al. (2009a)
α	GP IIb-IIIa				d(V1-1)-TAT			
β	GP IIb-IIIa	Block. of O-I. signaling		Buensuceso et al. (2005)				

θ	GPVI	Block. of O-I. signaling		Soriani et al. (2006)	Go6983pan	Enhances Gz induced PI-3K Akt pathway	
	GPVI PAR	Inh. of thrombosis		Pula et al. (2006), Nagy et al. (2009)	RACK	Inh. of platelet aggregation	kim et al. (2011)
δ	GPVI	Enhances filopodia generation		Pula et al. (2006)			
δ	PAR	Inh. of I-O. signaling		Chari et al. (2009a)	Go6983pan	Enhances GPVI-dpdt δ granule secretion	Chari et al. (2009a)
		Signaling enhanced			Rottlerin	Enhances response to alboagg. A Decreases PAR4-dpdt δ gr. secretion	Crosby and Poole (2003)
PLCβ	GPVI	Inh. of thrombosis		Lian et al. (2005)			
PLCγ2	GPVI	Partial inh. of thrombosis	>	Munnix et al. (2005), Nonne et al. (2005), Kalia et al. (2008)			
PLD1	GPIbα	Inh. of thrombosis, I-O. signaling	=	Elvers et al. (2010)			
Rap1b	GPVI	Inh. of thrombosis, I-O. and O-I. signaling	>	Chrzanowska-Wodnicka et al. (2005)			
	$P2Y_{12}$	No effects	>	Gilio et al. (2009), Wang et al. (2009)			

(continued)

Table 1 (continued)

Genetic deletion	Target	Effects	Bleeding compared to WT	References	Inhibitor	Effects	Bleeding compared to WT	References
SLP76	GPIbα	Block. I-O.signaling		Liu et al. (2006)				
	GPVI	Block. I-O., O-I. signaling		Clements et al. (1999), Gross et al. (1999)				
	GPVI	Inh. of thrombosis		Bezman et al. (2008)				
Syk radiation chimeras	GPVI, CLEC-2	Block. of I-O. signaling		Liu et al. (2006), Poole et al. (1997)	Piceatannol	Block. GPVI-response		Mayanglambam et al. (2010)
					Curcumin	Block. GPVI-response		Spalton et al. (2009a)
	FcRγ, FcγRIIA				R406	Block. FcRγ-response	=	Podolanczuk et al. (2009)
	GPIbα							
	$P2Y_{12}$	Affect I-O. signaling	=	Law et al. (1999b), Obergfell et al. (2002)	PRT060318, PRT062607	Block. FcRγ, GPVI-response	=	Reilly et al. (2011)
	GPIIb-IIIa	Affect O-I. signaling						
Vav1,3	GPVI	Inh. of I-O. signaling		Pearce et al. (2004)				

Alboagg., alboaggregin A; Block., blockage; Inh., inhibition; I-O., inside-out; O-I., outside-in; Wort., wortmannin; agg, agrgegation

amplified by a lack of Tec, although responses to ADP and outside-in signaling remain unaffected (Atkinson et al. 2003). Interestingly, XLA patients (deficient in Btk) do not exhibit a platelet-dependent bleeding disorder despite inhibition of the collagen-signaling pathway (Oda et al. 2000).

2.3 cSrc

Beta3 knock-in mice lacking the three C-terminal β3 residues (thus blunting the interaction of GP IIb-IIIa with c-Src) have defects in platelet spreading on fibrinogen, were protected from arterial thrombosis, and exhibited prolonged bleeding in a tail bleeding assay (Ablooglu et al. 2009).

2.4 CalDAG-GEFI

CalDAG-GEFI$^{-/-}$ platelets aggregate normally in response to phorbol ester but fail to aggregate when stimulated with nearly all tested GPCR-dependent and GPCR-independent agonists (Crittenden et al. 2004; Bergmeier et al. 2007). The marked defect in GP IIb-IIIa-mediated platelet aggregation is also accompanied by a defect in granule secretion (Crittenden et al. 2004; Cifuni et al. 2008) as a result of a decrease in TxA_2 synthesis. Mice lacking CalDAG-GEFI are protected from vascular occlusion in multiple arterial thrombosis models. This is accompanied, however, by increased bleeding. While this may be viewed as a potential drawback for chronic therapy, the relatively limited cellular distribution of CalDAG-GEFI (Kawasaki et al. 1998; Crittenden et al. 2004) may be advantageous in that a submaximal pharmacological modulation by a specific inhibitor may provide acceptable therapeutic index.

2.5 Fyn

Fyn-knockout platelets exhibit a partial inhibition of the vWF/botrocetin-induced secondary-wave of platelet aggregation (Yin et al. 2008a) and are also characterized by a defect in O.I. signaling including delayed spreading on fibrinogen and a tendency to rebleed after the first hemostatic plug (Reddy et al. 2008).

2.6 LAT

LAT-deficiency results in a significant reduction in tyrosine phosphorylation of PLCγ and decreased GP IIb-IIIa activation and alpha-granule secretion (Pasquet

et al. 1999). Kalia and colleagues showed that $LAT^{-/-}$ mice have a significant impairment of arterial thrombosis, but this is accompanied by an increase in volume of blood loss in a tail bleeding time model (Kalia et al. 2008).

2.7 Lyn

Lyn-deficiency enhances GPVI-mediated δ granule secretion but impairs that mediated by PARs, indicating that Lyn acts as a negative regulator on the collagen-signaling pathway (Chari et al. 2009b). In contrast, the genetic deletion of Lyn blunts the TxA_2-dependent second wave of platelet aggregation in response to botrocetin as well as firm spreading on a vWF-coated surface (Yin et al. 2008a), indicating a key role for Lyn downstream GPIbα engagement by its ligand.

2.8 P38MAPK

Using a dominant negative mutant of p38MAPK, Li and colleagues established that the kinase plays an important role in the activation of GPIIb-IIIa induced by vWF (-ristocetin) and thrombin (Li et al. 2006). Flevaris et al. also showed that granule secretion by low dose collagen is inhibited by a small molecule p38MAPK inhibitor (Flevaris et al. 2009).

2.9 PI-3K

2.9.1 PI-3Kα

Watanabe and collaborators showed that platelet aggregation induced by collagen and CRP (but not by ADP, thrombin, U-46,619, PMA, A23187, or botrocetin) is impaired in knockout mice (Watanabe et al. 2003). Interestingly, genetic deletion was not accompanied by enhanced bleeding.

2.9.2 PI-3Kβ

Garcia and coworkers have suggested that PI-3Kβ alone, but not other isoforms, is key to the $P2Y_{12}$-Akt signaling pathway promoting platelet aggregation (Garcia et al. 2010). Using genetically engineered mice and isoform-selective inhibitors, Schoenwaelder et al. have reported that PI-3Kβ plays a major role in regulating thrombin-stimulated fibrin clot retraction (Schoenwaelder et al. 2010), while Kim and colleagues showed that GPVI-mediated platelet aggregation and secretion were

inhibited by TGX-221, an inhibitor of PI-3Kβ (Kim et al. 2009). While the genetic deletion has not been associated with enhanced bleeding, a recent study has suggested that the therapeutic index (bleeding versus antithrombotic activity) of PI-3Kβ antagonists may not be as wide as originally expected (Bird et al. 2011).

2.9.3 PI-3Kδ and PI-3Kγ

Gilio and colleagues extended the observations made with the α and β isoforms, demonstrating that PI-3Kα and β have nonredundant roles in GPVI-mediated platelet activation and thrombus formation while the δ and γ isoforms did not have any major function (Gilio et al. 2009). Canobbio and colleagues found that PI-3Kγ-deficiency impaired ADP-induced platelet aggregation (Canobbio et al. 2009), a defect leading to resistance to thromboembolism (Hirsch et al. 2001).

2.10 PKC

2.10.1 PKCα

Konopatskaya et al. have demonstrated that platelets deficient in the α isoform have defective aggregation in response to CRP or thrombin, impaired thrombotic process, and impaired granule secretion with no impact on bleeding (Konopatskaya et al. 2009).

2.10.2 PKCβ

Buensuceso et al. found that PKCβ-deficiency is accompanied by a defect in outside-in signaling (poor adhesion of platelets to immobilized fibrinogen and defect in dense granule secretion) but still have a normal inside-out pathway (Buensuceso et al. 2005).

2.10.3 PKCθ

Soriani et al. reported that mice deficient in PKCθ fail to spread on immobilized fibrinogen but have a normal inside-out signaling pathway (Soriani et al. 2006). Nagy et al. observed that both genetic deletion and pharmacologic targeting affect GPVI- and PAR-mediated aggregation and thrombosis (Nagy et al. 2009).

2.10.4 PKCδ

Pula et al. showed that PKCδ acts as a negative regulator of filopodia formation in a VASP-dependent manner (Pula et al. 2006). Crosby and Poole demonstrated that rottlerin, a specific PKCδ inhibitor, enhanced the GPIbα- and GPVI-mediated responses to alboaggregin A (Crosby and Poole 2003) as well as act as a positive regulator of PAR-mediated dense granule release (Murugappan et al. 2004; Chari et al. 2009b).

2.11 PLA2

cPLA2$^{-/-}$ mice were shown to have smaller infarct size in a middle cerebral artery thrombosis model (Bonventre et al. 1997).

2.12 PLC

2.12.1 PLCγ2

Deficiency has been associated with a defect in GPVI-induced inside-out activation (Suzuki-Inoue et al. 2003), collagen-induced thrombus formation under arterial shear rates, collagen-dependent coagulation, a relative defect in thrombus formation in vivo as a function of lesion severity, and an increase in volume of blood loss (Munnix et al. 2005; Nonne et al. 2005; Kalia et al. 2008).

2.12.2 PLCβ

PLCβ$^{-/-}$ platelets failed to assemble filamentous actin, were defective in intracellular calcium mobilization and granule secretion, were unable to form stable platelet aggregates, and spread poorly on fibrinogen (Lian et al. 2005).

2.13 PLD1

PLD1$^{-/-}$ mice had a defect in the vWF-GPIbα pathway altering arterial thrombosis under high shear rates with no impairment of primary hemostasis.

2.14 Rap1b

Chrzanowska-Wodnicka et al. were the first group to show that both the inside-out (aggregation of Rap1b-deficient platelets was reduced in both GPCR-dependent and -independent agonist pathways) and outside-in signaling pathways were severely compromised in the genetically engineered mice. The platelet defect was associated with a significant inhibition of arterial thrombosis but also a mild bleeding diathesis (Chrzanowska-Wodnicka et al. 2005).

Further analyses by Wang et al. demonstrated a significant role for Rap1b in mediating convulxin-, but not ADP- or thrombin-induced α2β1 activation. Rap1b-deficient platelet had normal adhesion and spreading onto immobilized collagen (Wang et al. 2009).

2.15 SLP76

Stimulation by CRP as well as activation through the FcγRIIA receptor stimulated tyrosine phosphorylation of SLP-76. Mice with SLP76$^{-/-}$ platelets had a pronounced defect in both collagen- and GP IIb-IIIa-signaling (Clements et al. 1999; Judd et al. 2000, 2002). Deficiency was associated with a severe defect in tyrosine phosphorylation of PLCγ2 following CRP stimulation (Gross et al. 1999).

2.16 Syk

Syk is a key downstream effector of GPVI which mediates platelet adhesion and activation to vascular collagen [reviewed in (Watson et al. 2005)] as well as of the C-type lectin receptor CLEC-2 (Severin et al. 2011; Watson et al. 2005; Suzuki-Inoue et al. 2006; Spalton et al. 2009b). It is also a principal player in signaling pathways of GPIbα and GPIIb-IIIa (Obergfell et al. 2002; Gibbins 2004; Watson et al. 2005; Boylan et al. 2008), which are known to be involved in the growth of arterial thrombi. Syk has been implicated in platelet signaling events following engagement of multiple membrane immune receptors such as FcγRIIA, FcRγ, and FcαRI (Yanaga et al. 1995; Gibbins et al. 1996; Poole et al. 1997; Qian et al. 2008) as well as Sema4D (Wannemacher et al. 2010).

Studies in Syk or FcRγ gene-deficient mice have generated inconsistent data (Poole et al. 1997; Liu et al. 2008). Similarly, preliminary data obtained with R406, a small molecule inhibitor of Syk, have been somewhat contradictory as full inhibition of basophil activation in whole blood were not accompanied by antiplatelet activity in PRP (Braselmann et al. 2006). Using a highly specific small molecule inhibitor (PRT060318), it was demonstrated that inhibition of Syk

kinase activity significantly affected thrombosis in multiple thrombosis models and animals species (Reilly et al. 2011; Andre et al. 2011), but was not accompanied by changes in coagulation parameters or by a prolongation of the bleeding time.

2.17 Vav1/3

Pearce et al. have shown that platelets deficient in both Vav1 and Vav3 had a marked reduction in functional responses to GPVI activation by CRP and collagen which was associated with a decreased tyrosine phosphorylation of PLCγ2. Vav1 and Vav3 were shown to have important but redundant roles in GPVI signaling.

3 Key Intracellular Partners of Platelet Signaling Pathways Inhibiting Platelet Aggregation/Thrombosis

PECAM-1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) that partially counteracts the ITAM motif that promotes activation downstream of FcRγ, FcγRIIA, or FcεRI. The activation/phosphorylation of ITIMs upon clustering of PECAM-1 leads to the recruitment and activation of tyrosine phosphatases (SHP-1/SHP-2) which decreases the strength of the signal coming from the ITAM motif. In general, activation of platelets (e.g., by collagen and thrombin) induces the phosphorylation of tyrosine residues in PECAM-1 [for review see Gibbins (2002)]. Using knockout mice, Patil et al. have shown that PECAM-1 inhibited platelet responses to collagen as PECAM-$1^{-/-}$ platelets displayed an enhanced response to GPVI signaling (Patil et al. 2001). This phenotype has been attributed to the interaction of the PECAM-1-associated SHP-2 with p85, resulting in a reduction in PI-3K signaling (Moraes et al. 2010). These in vitro findings have been confirmed in vivo in a carotid artery $FeCl_3$-thrombosis model by Falati et al. who showed that PECAM-1-deficient mice had an accelerated thrombotic process in response to vascular injury (Falati et al. 2006).

4 Challenges and Current Development

A strategy targeting intraplatelet signaling pathways is both interesting and challenging. While the signaling pathways of the main receptors have been relatively well documented, there are still major unknowns, platelet specific signaling pathways are sparse and many hurdles face the development of agents that will

need to cross the plasma membrane and keep specificity for the target of interest inside the platelet.

4.1 Knowledge Gaps

Most platelet signaling events have been studied in isolated systems, with a single platelet agonist, despite the fact that thrombotic events are the summation of multiple platelet signalosomes. The impact of the modulation of a specific target should be undertaken in a physiological (and pathophysiological) environment using novel technologies allowing monitoring of the kinetics of thrombosis in real time under variable and defined shear rate conditions. Similarly, often modulation of the platelet signalosome is performed in acute models of thrombosis which blunt our understanding of potential on- or off-target effects beyond inhibition of the platelet function and does not evaluate possible long-term activities. It is crucial to understand how modulation of platelet function will impact overall homeostasis and whether a safe therapeutic window exists.

4.2 Identification of Highly Specific Agents

While there is numerous genetic evidence emphasizing the important contribution to platelet function (and in some cases to arterial thrombosis) of each of the kinases mentioned above, the pharmacological evidence has been sparse as only a limited number of highly specific pharmacological agents exist. This is likely explained by the fact that genetic validation of a target is seen as an early, relatively "simple" event when compared with the extraordinarily complicated task of developing a selective drug.

Several properties are usually sought for an inhibitor to climb the drug development ladder (Table 2). First, the target of interest should have a clear impact on arterial thrombosis, an expression level limited to the platelet and a minimal involvement in primary hemostasis. Second, the agent should be highly specific for the signaling target of interest, requiring testing against a panel of both closely structurally and functionally related proteins. This can represent a significant hurdle: in one example, if one looks at the kinome (most of the identified platelet targets are kinases), specificity has to be tested against several hundred candidates. Additionally, it is desirable that the agent should also be well behaved with respect to pharmacokinetics properties and drug–drug interactions (see Table 2).

An example of a paradigm utilized for the development of a small molecule inhibitor of platelet signaling is illustrated on Fig. 3.

In addition, drugs need to demonstrate activity in multiple experimental models and across multiple animal species. Since the activity of the agent can be affected

Table 2 Drug profile

Factors	Comments
• Activity	• Preferentially limited to the arterial compartment • Distinguishes thrombosis from hemostasis and offers a superior therapeutic index over Standard of Care
• Tissue distribution (target)	• Preferentially limited to the platelet compartment
• Volume of distribution (drug)	• Small: to reduce potential unwanted on- and off-target activities
• Bioavailability, protein binding, half-life, Peak-to-trough ratio	• Ideal combination in order to provide a well-behaved coverage throughout the dosing cycle
• MoA, Irreversible	• (+) Sustained inhibition (−) Bleeding risks
• MoA, Reversible	• (+) Ease of use, reduced bleeding risks (−) Compliance
• Drug–drug interaction	• Minimal
• Metabolism	• If possible, no dependency on hepatic metabolism in order to provide broad and predictable coverage
• Formulation	• If possible intravenous and oral formulation for treatment and prevention
• PD monitoring	• Reproducible, predictive, ease of use
• Toxicity	• Minimal

MoA mechanism of action

by species specificity (possible differences in signaling pathways between species) which can also dictate pharmacokinetic profile, drug candidates are often abandoned at the preclinical stage. Another key factor requiring consideration is the duration and cost associated with the development of an antiplatelet agent. It usually takes more than 10 years from discovery to market and tens of thousands of patients to demonstrate the clinical benefits of effective antiplatelet agents and combination therapies.

4.3 Current Landscape

Despite all these hurdles, some targets have been identified by pharmaceutical companies, the modulation of which is likely to offer antithrombotic and anti-inflammatory benefits (Table 3).

4.3.1 Syk Inhibition

Thrombocytopenia and thrombosis induced by antibodies reactive with heparin-platelet factor 4 complexes (HIT model) or in chronic refractory immune thrombocytopenia patients are inhibited by inhibition of Syk (Reilly et al. 2011; Podolanczuk et al. 2009). R406 is in phase 2 for the treatment of refractory ITP. Interestingly, since Syk is also highly expressed in mast cells, monocytes, macrophages, neutrophils, and B-cells and since its pharmacological inhibition

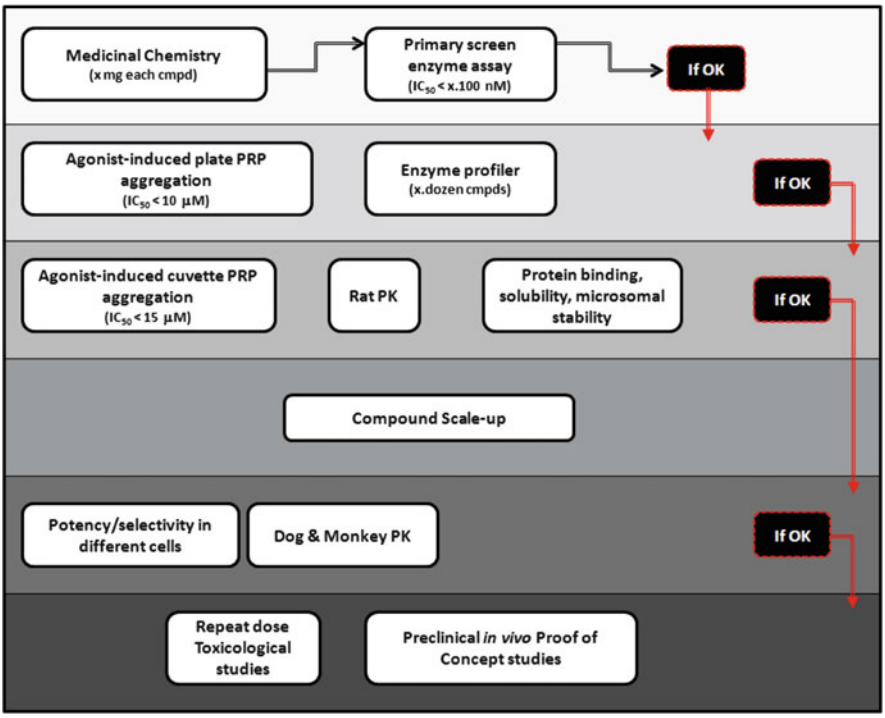


Fig. 3 Template of an early drug discovery paradigm for drugs targeting intraplatelet signaling pathways. Each small molecule synthesized follows the paradigm and must consecutively show: potency, specificity at the enzyme level, potency, and specificity at the cellular level (without and with plasma proteins) before going into pharmacokinetic studies and further characterization. If acceptable, scale-up is performed for use in pharmacokinetic studies in larger animal species, cellular specificity and activity studies, and safety and toxicological studies. Additionally, other tests are required in order to ensure the development of safe pharmacological agents. These include, but are not limited to hERG binding (to test for potential cardiotoxic activity) + patch clamp assays, dog telemetry, purkinje fiber

does not severely compromise the immune function, diseases as diverse as allergic asthma, rheumatoid arthritis, restenosis, and atherosclerosis (all of which are known to involve both leukocytes and platelets) are being considered. As an example, preclinical data showed that modulation of Syk impaired inflammatory cell accumulation, cellular proliferation, and significantly inhibit atherogenesis in both $LDLR^{-/-}$ and $Apo\text{-}e^{-/-}$ mice (Hilgendorf et al. 2011; Andre et al. 2011).

4.3.2 PKC Inhibition

A PKCδ inhibitor (KAI-9803) is being developed for the reduction of infarct size in AMI patient and also as adjunct to primary percutaneous coronary intervention.

Table 3 Current agents in development

Pathway/company	Title	Phase	Drug	Indication
p38 MAPK				
GSK plc	A randomized, double-blind, placebo-controlled study to evaluate the safety of 12 weeks of dosing With GW-856553 and its effects on inflammatory markers, infarct size, and cardiac function in subjects with MI without ST-segment elevation	2	GW-856553	CAD
Vertex Pharm. Inc	A phase I, ascending dose, safety and pharmacokinetic study of VX-702 in patients with acute coronary syndrome undergoing PCI	1	VX-702	CAD
Phospholipase inhibitor				
Anthera Pharm. Inc	Phospholipase levels and serological markers of atherosclerosis 2: an examination of once daily (QD) dosing of A-002 in subjects with stable CAD	2	A-002	CAD
GSK plc	A double-blind, placebo-controlled, parallel study to evaluate effects of repeat doses of rilapladib on platelet aggregation in healthy male volunteers	1	SB-659032	Athero.
GSK plc	A phase II, randomized, dose-ranging study of darapladib in patients with CHD	2	Darapladib	CAD
GSK plc	A multicenter, randomized, double-blind, placebo-controlled, parallel-group, dose-ranging study of SB-480848, an oral lipoprotein-associated phospholipase A2 (Lp-PLA2) inhibitor, in subjects with stable CHD or CHD-risk equivalent to examine chronic inhibition of Lp-PLA2, effects on circulating biomarkers associated with CVRisk, safety and tolerability over 12 weeks	2	Darapladib	Athero Heart disease, CAD
Protein kinase C (PKC)				
KAI Pharm. Inc	Inhibition of delta-protein kinase C for the reduction of infarct size in AMI	2b	KAI-9803	CVD

KAI Pharm. Inc	Intracoronary KAI-9803 for injection as an adjunct to primary PCI for acute ST-elevation MI	1/2	KAI-9803	MI
Spleen tyrosine kinase (SYK)				
AstraZeneca plc	A phase II, open-label, efficacy and safety, ascending-dose, pilot study of R-935788 for the treatment of adult refractory ITP	2	R-935788	ITP
Phosphoinositide 3-kinase inhibitor (PI3K)				
AstraZeneca plc	A randomized, open-label, single-center, phase I, crossover study to evaluate the effect of AZD-6482, compared with clopidogrel, on bleeding time in healthy volunteers receiving low-dose ASA	1	AZD-6482	Thrombosis
AstraZeneca plc	A randomized, double-blind, placebo-controlled, phase I study to assess the tolerability, safety, pharmacokinetic, and pharmacodynamic properties of AZD-6482, alone and coadministered with ASA, after single ascending intravenous doses to healthy male subjects	1	AZD-6482	Thrombosis
TargeGen Inc	A phase I/II, multicenter, randomized, double-blind, placebo controlled, prospective study to evaluate the safety and potential efficacy of single, increasing doses of TG-100-115 in subjects undergoing PCI for acute anterior ST elevation myocardial infarction	1/2	TG-100-115	MI

4.3.3 PI-3K Inhibition

Two agents are being tested in human. AZD-6482 is in phase 1 clinical trial to evaluate its safety, pharmacokinetic profile, and pharmacodynamic properties. TG-100-115 is being tested in a phase 1/2 prospective study in patients undergoing PCI for acute anterior ST elevation MI.

4.3.4 p38-MAPK Inhibition

There are currently two p38-MAPK inhibitors (GW-856553, VX-702) undergoing development as potential anti-inflammatory/ thrombotic agents in subjects with MI without ST-Segment elevation and in ACS patients undergoing PCI.

Time will tell whether these new strategies will provide substantial benefits over current therapies. One risk, however, is a use of traditional clinical development plan (involving large platelet trials such as those performed in ACS and CHD patients) that may dictate their fate independently of their clinical potential in niche indications.

5 Conclusions

Current antiplatelet therapies do not provide optimal protection and are often associated with a poor therapeutic window. Novel agents could provide additional significant protection. The biggest challenge is to identify a specific pathway and develop a specific inhibitor which will reduce the incidence of thrombotic events and their deleterious effects without impairing primary hemostasis. This challenge is further complicated by the fact that the current antiplatelet therapies are well-established standard of care, necessitating the design of large, costly clinical trials to demonstrate superiority as a result of a low event rate.

Several lines of evidence suggest, however, that better agents may be on the horizon. Over the last 15 years, multiple and diverse genetic approaches in mice have shed some light on the signaling pathways leading to thrombus growth and stabilization. Particularly interesting are the antithrombotic phenotypes associated with genetic deletion but which also lack activity on primary hemostasis. While there are only a few strategies actively being pursued by the pharmaceutical industry, it is reasonable to believe that one will lead towards a better and safer antiplatelet agent.

Knowledge Gaps

- Most platelet signaling events have been studied in isolated systems, with a single platelet agonist, despite the fact that thrombotic events are the summation of multiple platelet signalosomes.
- The impact of the modulation of a specific target should be undertaken in a physiological (and pathophysiological) environment using novel technologies allowing monitoring of the kinetics of thrombosis in real time under variable and defined shear rate conditions.
- Often modulation of the platelet signalosome is performed in acute models of thrombosis which blunt our understanding of potential on- or off-target effects beyond inhibition of the platelet function and does not evaluate possible long-term activities.
- It is crucial to understand how modulation of platelet function will impact overall homeostasis and whether a safe therapeutic window exists.

Key Messages

- Genetic and pharmacological modulation of platelet intracellular signaling pathways can significantly affect arterial thrombosis
- Targets exist that may offer improved therapeutic window over current antiplatelet strategies:
  - Modulation of Akt2, Btk, PI3K-α, β, γ, PKCα, PLD1, and Syk affect thrombosis with minimal impact on primary hemostasis
- Novel strategies are needed to fight thrombo-inflammatory diseases
  - Targets exist that may offer dual inhibition (e.g., Syk)
  - Novel models are required to study thrombosis on an inflammation background
- Development of novel anti-thrombotics is hampered by the cost of clinical trials
  - Novel development strategies, choice of clinical indications could reduce cost and allow for faster regulatory approval

References

Ablooglu AJ, Kang J, Petrich BG et al (2009) Antithrombotic effects of targeting alphaIIbbeta3 signaling in platelets. Blood 113:3585–3592

Andre P, Morooka T, Sim D et al (2011) Critical role for Syk in responses to vascular injury. Blood 118(18):5000–5010

Angelillo-Scherrer A, Burnier L, Flores N et al (2005) Role of Gas6 receptors in platelet signaling during thrombus stabilization and implications for antithrombotic therapy. J Clin Invest 115 (2):237–246

Atkinson BT, Ellmeier W, Watson SP (2003) Tec regulates platelet activation by GPVI in the absence of Btk. Blood 102:3592–3599

Bergmeier W, Goerge T, Wang HW et al (2007) Mice lacking the signaling molecule CalDAG-GEFI represent a model for leukocyte adhesion deficiency type III. J Clin Invest 117: 1699–1707

Bezman NA, Lian L, Abrams CS et al (2008) Requirements of SLP76 tyrosines in ITAM and integrin receptor signaling and in platelet function in vivo. J Exp Med 205:1775–1788

Bird JE, Smith PL, Bostwick JS et al (2011) Bleeding response induced by anti-thrombotic doses of a phosphoinositide 3-kinase (PI3K)-beta inhibitor in mice. Thromb Res 127:560–564

Bonventre JV, Huang Z, Taheri MR et al (1997) Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase A2. Nature 390:622–625

Boylan B, Gao C, Rathore V et al (2008) Identification of FcgammaRIIa as the ITAM-bearing receptor mediating alphaIIbbeta3 outside-in integrin signaling in human platelets. Blood 112:2780–2786

Braselmann S, Taylor V, Zhao H et al (2006) R406, an orally available spleen tyrosine kinase inhibitor blocks fc receptor signaling and reduces immune complex-mediated inflammation. J Pharmacol Exp Ther 319:998–1008

Buensuceso CS, Obergfell A, Soriani A et al (2005) Regulation of outside-in signaling in platelets by integrin-associated protein kinase C beta. J Biol Chem 280:644–653

Canobbio I, Reineri S, Sinigaglia F et al (2004) A role for p38 MAP kinase in platelet activation by von Willebrand factor. Thromb Haemost 91:102–110

Canobbio I, Stefanini L, Cipolla L et al (2009) Genetic evidence for a predominant role of PI3Kbeta catalytic activity in ITAM- and integrin-mediated signaling in platelets. Blood 114:2193–2196

Chari R, Getz T, Nagy B Jr et al (2009a) Protein kinase C[delta] differentially regulates platelet functional responses. Arterioscler Thromb Vasc Biol 29:699–705

Chari R, Kim S, Murugappan S et al (2009b) Lyn, PKC-delta, SHIP-1 interactions regulate GPVI-mediated platelet-dense granule secretion. Blood 114:3056–3063

Chen J, De S, Damron DS et al (2004) Impaired platelet responses to thrombin and collagen in AKT-1-deficient mice. Blood 104:1703–1710

Chrzanowska-Wodnicka M, Smyth SS, Schoenwaelder SM et al (2005) Rap1b is required for normal platelet function and hemostasis in mice. J Clin Invest 115:680–687

Cifuni SM, Wagner DD, Bergmeier W (2008) CalDAG-GEFI and protein kinase C represent alternative pathways leading to activation of integrin alphaIIbbeta3 in platelets. Blood 112:1696–1703

Clements JL, Lee JR, Gross B et al (1999) Fetal hemorrhage and platelet dysfunction in SLP-76-deficient mice. J Clin Invest 103:19–25

Crittenden JR, Bergmeier W, Zhang Y et al (2004) CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation. Nat Med 10:982–986

Crosby D, Poole AW (2003) Physical and functional interaction between protein kinase C delta and Fyn tyrosine kinase in human platelets. J Biol Chem 278:24533–24541

Elvers M, Stegner D, Hagedorn I et al (2010) Impaired alpha(IIb)beta(3) integrin activation and shear-dependent thrombus formation in mice lacking phospholipase D1. Sci Signal 3:ra1

Falati S, Patil S, Gross PL et al (2006) Platelet PECAM-1 inhibits thrombus formation in vivo. Blood 107:535–541

Flevaris P, Li Z, Zhang G et al (2009) Two distinct roles of mitogen-activated protein kinases in platelets and a novel Rac1-MAPK-dependent integrin outside-in retractile signaling pathway. Blood 113:893–901

Garcia A, Kim S, Bhavaraju K et al (2010) Role of phosphoinositide 3-kinase beta in platelet aggregation and thromboxane A2 generation mediated by Gi signalling pathways. Biochem J 429:369–377

Gibbins JM (2002) The negative regulation of platelet function: extending the role of the ITIM. Trends Cardiovasc Med 12:213–219

Gibbins JM (2004) Platelet adhesion signalling and the regulation of thrombus formation. J Cell Sci 117:3415–3425

Gibbins J, Asselin J, Farndale R et al (1996) Tyrosine phosphorylation of the Fc receptor gamma-chain in collagen- stimulated platelets. J Biol Chem 271:18095–18099

Gilio K, Munnix IC, Mangin P et al (2009) Non-redundant roles of phosphoinositide 3-kinase isoforms alpha and beta in glycoprotein VI-induced platelet signaling and thrombus formation. J Biol Chem 284:33750–33762

Gross BS, Lee JR, Clements JL et al (1999) Tyrosine phosphorylation of SLP-76 is downstream of Syk following stimulation of the collagen receptor in platelets. J Biol Chem 274:5963–5971

Hilgendorf I, Eisele S, Remer I et al (2011) The oral spleen tyrosine kinase inhibitor fostamatinib attenuates inflammation and atherogenesis in low-density lipoprotein receptor-deficient mice. Arterioscler Thromb Vasc Biol 31:1991–1999

Hirsch E, Bosco O, Tropel P et al (2001) Resistance to thromboembolism in PI3Kγ-deficient mice. FASEB J 15:2019–2021

Judd BA, Myung PS, Leng L et al (2000) Hematopoietic reconstitution of SLP-76 corrects hemostasis and platelet signaling through alpha IIb beta 3 and collagen receptors. Proc Natl Acad Sci USA 97:12056–12061

Judd BA, Myung PS, Obergfell A et al (2002) Differential requirement for LAT and SLP-76 in GPVI versus T cell receptor signaling. J Exp Med 195:705–717

Kalia N, Auger JM, Atkinson B et al (2008) Critical role of FcR gamma-chain, LAT, PLCgamma2 and thrombin in arteriolar thrombus formation upon mild, laser-induced endothelial injury in vivo. Microcirculation 15:325–335

Kawasaki H, Springett GM, Toki S et al (1998) A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia. Proc Natl Acad Sci USA 95:13278–13283

Kim S, Mangin P, Dangelmaier C et al (2009) Role of phosphoinositide 3-kinase beta in glycoprotein VI-mediated Akt activation in platelets. J Biol Chem 284:33763–33772

Kim S, Kim S, Kim J et al (2011) PKC inhibitors RO 31-8220 and Gö 6983 enhance epinephrine-induced platelet aggregation in catecholamine hypo-responsive platelets by enhancing Akt phosphorylation. BMB Rep 44:140–145

Konopatskaya O, Gilio K, Harper MT et al (2009) PKCalpha regulates platelet granule secretion and thrombus formation in mice. J Clin Invest 119:399–407

Law DA, DeGuzman FR, Heiser P et al (1999a) Integrin cytoplasmic tyrosine motif is required for outside-in alphaIIbbeta3 signalling and platelet function. Nature 401:808–811

Law DA, Nannizzi-Alaimo L, Ministri K et al (1999b) Genetic and pharmacological analyses of Syk function in alphaIIbbeta3 signaling in platelets. Blood 93:2645–2652

Li Z, Zhang G, Feil R et al (2006) Sequential activation of p38 and ERK pathways by cGMP-dependent protein kinase leading to activation of the platelet integrin alphaIIb beta3. Blood 107:965–972

Lian L, Wang Y, Draznin J et al (2005) The relative role of PLCbeta and PI3Kgamma in platelet activation. Blood 106:110–117

Liu J, Fitzgerald ME, Berndt MC et al (2006) Bruton tyrosine kinase is essential for botrocetin/VWF-induced signaling and GPIb-dependent thrombus formation in vivo. Blood 108:2596–2603

Liu J, Joglekar M, Ware J et al (2008) Evaluation of the physiological significance of botrocetin/von Willebrand factor in vitro signaling. J Thromb Haemost 6:1915–1922

Mayanglambam A, Dangelmaier CA, Thomas D et al (2010) Curcumin inhibits GPVI-mediated platelet activation by interfering with the kinase activity of Syk and the subsequent activation of PLCgamma2. Platelets 21:211–220

Minuz P, Gaino S, Zuliani V et al (2002) Functional role of p38 mitogen activated protein kinase in platelet activation induced by a thromboxane A2 analogue and by 8-iso-prostaglandin F2alpha. Thromb Haemost 87:888–898

Moraes LA, Barrett NE, Jones CI et al (2010) Platelet endothelial cell adhesion molecule-1 regulates collagen-stimulated platelet function by modulating the association of phosphatidylinositol 3-kinase with Grb-2-associated binding protein-1 and linker for activation of T cells. J Thromb Haemost 8:2530–2541

Munnix IC, Strehl A, Kuijpers MJ et al (2005) The glycoprotein VI-phospholipase Cgamma2 signaling pathway controls thrombus formation induced by collagen and tissue factor in vitro and in vivo. Arterioscler Thromb Vasc Biol 25:2673–2678

Murugappan S, Tuluc F, Dorsam RT et al (2004) Differential role of protein kinase C delta isoform in agonist-induced dense granule secretion in human platelets. J Biol Chem 279:2360–2367

Nagy B Jr, Bhavaraju K, Getz T et al (2009) Impaired activation of platelets lacking protein kinase C-theta isoform. Blood 113:2557–2567

Nonne C, Lenain N, Hechler B et al (2005) Importance of platelet phospholipase Cgamma2 signaling in arterial thrombosis as a function of lesion severity. Arterioscler Thromb Vasc Biol 25:1293–1298

Obergfell A, Eto K, Mocsai A et al (2002) Coordinate interactions of Csk, Src, and Syk kinases with [alpha]IIb[beta]3 initiate integrin signaling to the cytoskeleton. J Cell Biol 157:265–275

Oda A, Ikeda Y, Ochs HD et al (2000) Rapid tyrosine phosphorylation and activation of Bruton's tyrosine/Tec kinases in platelets induced by collagen binding or CD32 cross-linking. Blood 95:1663–1670

Pasquet JM, Gross B, Quek L et al (1999) LAT is required for tyrosine phosphorylation of phospholipase cgamma2 and platelet activation by the collagen receptor GPVI. Mol Cell Biol 19:8326–8334

Patil S, Newman DK, Newman PJ (2001) Platelet endothelial cell adhesion molecule-1 serves as an inhibitory receptor that modulates platelet responses to collagen. Blood 97:1727–1732

Pearce AC, Senis YA, Billadeau DD et al (2004) Vav1 and vav3 have critical but redundant roles in mediating platelet activation by collagen. J Biol Chem 279:53955–53962

Podolanczuk A, Lazarus AH, Crow AR et al (2009) Of mice and men: an open-label pilot study for treatment of immune thrombocytopenic purpura by an inhibitor of Syk. Blood 113:3154–3160

Poole A, Gibbins JM, Turner M et al (1997) The Fc receptor gamma-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. EMBO J 16:2333–2341

Prasad BN, Kemparaju K, Bhatt KG et al (1996) A platelet aggregation inhibitor phospholipase A2 from Russell's viper (Vipera russelli) venom: isolation and characterization. Toxicon 34:1173–1185

Pula G, Schuh K, Nakayama K et al (2006) PKCdelta regulates collagen-induced platelet aggregation through inhibition of VASP-mediated filopodia formation. Blood 108:4035–4044

Qian K, Xie F, Gibson AW et al (2008) Functional expression of IgA receptor Fc{alpha}RI on human platelets. J Leukoc Biol 84(6):1492–1500

Quek LS, Bolen J, Watson SP (1998) A role for Bruton's tyrosine kinase (Btk) in platelet activation by collagen. Curr Biol 8:1137–1140

Reddy KB, Smith DM, Plow EF (2008) Analysis of Fyn function in hemostasis and alphaIIbbeta3-integrin signaling. J Cell Sci 121:1641–1648

Reilly MP, Sinha U, Andre P et al (2011) PRT-060318, a novel Syk inhibitor, prevents heparin-induced thrombocytopenia and thrombosis in a transgenic mouse model. Blood 117: 2241–2246

Schoenwaelder SM, Ono A, Nesbitt WS et al (2010) Phosphoinositide 3-kinase p110 beta regulates integrin alpha IIb beta 3 avidity and the cellular transmission of contractile forces. J Biol Chem 285:2886–2896

Senis YA, Atkinson BT, Pearce AC et al (2005) Role of the p110delta PI 3-kinase in integrin and ITAM receptor signalling in platelets. Platelets 16:191–202

Severin S, Pollitt AY, Navarro-Nunez L et al (2011) Syk-dependent phosphorylation of CLEC-2: a novel mechanism of hem-immunoreceptor tyrosine-based activation motif signaling. J Biol Chem 286:4107–4116

Soriani A, Moran B, de Virgilio M et al (2006) A role for PKCtheta in outside-in alpha(IIb)beta3 signaling. J Thromb Haemost 4:648–655

Spalton JC, Mori J, Pollitt AY et al (2009) The novel Syk inhibitor R406 reveals mechanistic differences in the initiation of GPVI and CLEC-2 signaling in platelets. J Thromb Haemost 7:1192–1199

Stefanini L, Roden RC, Bergmeier W (2009) CalDAG-GEFI is at the nexus of calcium-dependent platelet activation. Blood 114:2506–2514

Stolla M, Stefanini L, Andre P et al (2011) CalDAG-GEFI deficiency protects mice in a novel model of Fc{gamma}RIIA-mediated thrombosis and thrombocytopenia. Blood 118(4):1113–1120

Suzuki-Inoue K, Inoue O, Frampton J et al (2003) Murine GPVI stimulates weak integrin activation in PLCgamma2-/- platelets: involvement of PLCgamma1 and PI3-kinase. Blood 102:1367–1373

Suzuki-Inoue K, Fuller GL, Garcia A et al (2006) A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. Blood 107:542–549

Wang Z, Holly SP, Larson MK et al (2009) Rap1b is critical for glycoprotein VI-mediated but not ADP receptor-mediated alpha2beta1 activation. J Thromb Haemost 7:693–700

Wannemacher KM, Zhu L, Jiang H et al (2010) Diminished contact-dependent reinforcement of Syk activation underlies impaired thrombus growth in mice lacking Semaphorin 4D. Blood 116:5707–5715

Watanabe N, Nakajima H, Suzuki H et al (2003) Functional phenotype of phosphoinositide 3-kinase p85alpha-null platelets characterized by an impaired response to GP VI stimulation. Blood 102:541–548

Watson SP, Auger JM, McCarty OJ et al (2005) GPVI and integrin alphaIIb beta3 signaling in platelets. J Thromb Haemost 3:1752–1762

Woulfe D, Jiang H, Morgans A et al (2004) Defects in secretion, aggregation, and thrombus formation in platelets from mice lacking Akt2. J Clin Invest 113:441–450

Yanaga F, Poole A, Asselin J et al (1995) Syk interacts with tyrosine-phosphorylated proteins in human platelets activated by collagen and cross-linking of the Fc gamma-IIA receptor. Biochem J 311:471–478 [published erratum appears in Biochem J 1996 Feb 1; 313(Pt 3):1047]

Yin H, Liu J, Li Z et al (2008a) Src family tyrosine kinase Lyn mediates VWF/GPIb-IX-induced platelet activation via the cGMP signaling pathway. Blood 112:1139–1146

Yin H, Stojanovic A, Hay N et al (2008b) The role of Akt in the signaling pathway of the glycoprotein Ib-IX induced platelet activation. Blood 111:658–665

Novel Targets for Platelet Inhibition

Kathleen Freson and Chris Van Geet

Contents

K. Freson (✉)
Center for Molecular and Vascular Biology, University of Leuven, Herestraat 49, 3000 Leuven, Belgium
e-mail: Kathleen.Freson@med.kuleuven.be

C. Van Geet
Center for Molecular and Vascular Biology, University of Leuven, Herestraat 49, 3000 Leuven, Belgium

Department of Pediatrics, University of Leuven, Herestraat 49, 3000 Leuven, Belgium

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_15,

Abstract Atherothrombosis often underlies coronary artery disease, stroke, and peripheral arterial disease. Antiplatelet drugs have come to the forefront of prophylactic treatment of atherothrombotic disease. Dual antiplatelet therapy of aspirin plus clopidogrel—the current standard—has benefits, but it also has limitations with regard to pharmacologic properties and adverse effects with often severe bleeding complications. For these reasons, within the last decade or so, the investigation of novel antiplatelet targets has prospered. Target identification can be the result of large-scale genomic or proteomic studies, functional genomics in animal models, the genetic analysis of patients with inherited bleeding disorders, or a combination of these techniques.

Keywords Proteomics • Functional genomics • Inherited bleeding disorder • Genome-wide association studies • Antiplatelet drugs

1 Introduction

Apart from the central beneficial role platelets play in hemostasis, they are also involved in atherothrombotic diseases (including acute coronary syndromes, ischemic strokes, and symptomatic peripheral arterial disease). The goal of antiplatelet drugs is to reduce the development and clinical expression of these diseases. The current and experimental antiplatelet drugs as intensively discussed in the previous chapters are mainly developed against the major platelet receptors or enzymes that have been studied extensively over the last decades for their major role as regulators of platelet adhesion, aggregation, and/or secretion (Bennett and Mousa 2001; Siller-Matula et al. 2010; Bennett 2001; Xiang et al. 2008). Though most of the established and novel antiplatelet agents are highly effective as inhibitor of platelet activation, problems as resistance, drug–drug interaction, discontinuation, monitoring, and safety have also appeared. Due to these problems in the current antiplatelet drugs, the search for novel targets is still warranted. We here review methods that contribute to the identification of some important novel targets of platelet function such as proteomic approaches, studies of patients with inherited bleeding disorders, large-scale genomic studies, and functional genomics in animal models. These studies can identify platelet function modulators in patients but also in healthy subjects having a platelet hypo- or hyperfunction without a clinical presentation of bleeding or thrombosis. This chapter will focus on some novel targets that were recently identified using each of these approaches. A thorough understanding of

these targets and their underlying pathways that regulate platelet function will eventually allow the development of novel antiplatelet agents. They can include intrinsic targets expressed by the platelets or can indirectly modulate platelet function as plasma components or via expression by other cells.

2 Identification of Novel Targets via Platelet Proteomics

2.1 General Concept

Recent large-scale proteomic approaches, including especially the two-dimensional difference gel electrophoresis (2D-DIGE) method, offer a huge potential for an almost complete overview of proteins present in a cell or a subcellular system and they result in differential protein expression patterns between two samples in terms of protein quantification, posttranslational modifications, localization and interactions of thousands of gene products at a time. Proteomics represents one of the best tools to approach platelet biology, as both genomic and transcriptomic analysis are hampered by the absence of DNA and the very low level of mRNA in the anucleated platelet (Lindemann and Gawaz 2007). Most of the proteomic approaches involve the study of platelets from normal subjects to reveal changes in proteins due to platelet activation and to identify components of some agonist-related signaling pathways such as for thrombin (Tucker et al. 2009), TRAP (Piersma et al. 2009), or a monoclonal anti-GPVI antibody (Schulz et al. 2010). Moreover, the proteomic strategy leads to insights in protein changes by posttranslational modification such as phosphorylation under basal and activated conditions (Premsler et al. 2011; Qureshi et al. 2009; Marcus et al. 2000; Zahedi et al. 2008). In addition, proteomics was also used to specifically study some subcellular part of the platelets such as the releasate (Coppinger et al. 2004), alpha granules (Maynard et al. 2007), dense granules (Hernandez-Ruiz et al. 2007), microparticles (Garcia et al. 2005), and membranes (Foy and Maguire 2007; Moebius et al. 2005; Lewandrowski et al. 2009). Figure 1 summarizes these proteomic approaches. Still only few proteomic studies deal with patient samples although recently some studies identified platelet protein changes in acute coronary syndrome (Parguiña et al. 2010), hypertension (Sacristan et al. 2008), diabetes (Springer et al. 2009), platelet-related bleeding disorder (Maurer-Spurej et al. 2008), storage pool disease (Di Michele et al. 2011), and gray platelet disorder (Maynard et al. 2010). The proteomic approach to study the pathophysiology of disorders usually results in changes in a relatively large number of proteins that correlate with the disease or it unravels pathways that seem to be involved in the disease. However, these studies do in general not identify specific candidate proteins that are causative for the disease. In general, proteomics might serve as a tool to identify novel platelet proteins and their abundance and thereby select for future research targets and potential novel antiplatelet drugs.

We here focus on three studies that identified novel antiplatelet targets using different proteomic techniques though some other examples based on similar assays

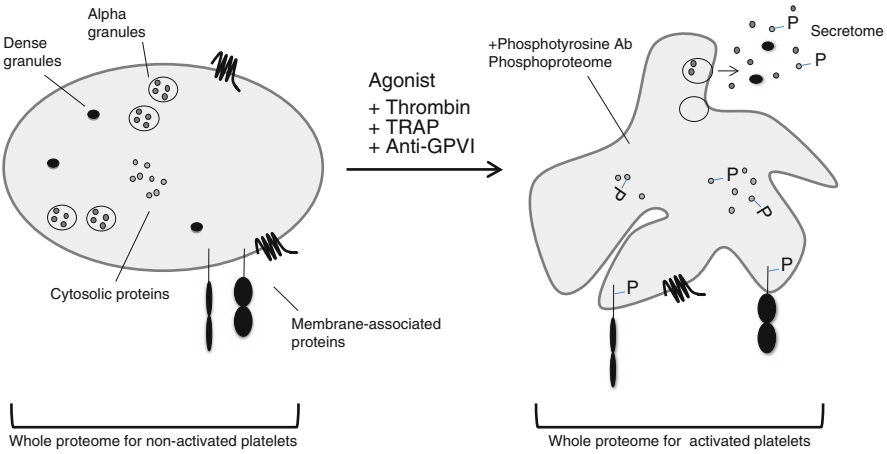


Fig. 1 Proteomic studies of quiescent (*left*) and activated (*right*) platelets. Proteomic studies involved whole platelet lysates or subcellular structures such as cytosolic, membrane-fraction, tyrosine phosphorylated proteins, and the releasate (secretome)

can be found in literature. These studies were selected based upon the existence of at least some confirmation experiments that illustrate the functional importance of the novel identified target in platelet function and the fact that a different proteomic approach was used to identity the target.

2.2 *Tyrosine Phosphorylated Immunoglobulin Receptor: G6B*

The immunoglobulin receptor G6B was identified in two proteomics studies. The initial study identified this receptor using parallel protein separation steps of a preextracted platelet membrane fraction using one-dimensional SDS-PAGE and benzyldimethyl-n-hexadecylammonium chloride/SDS prior to mass spectrometry analysis by nano scale liquid chromatography and mass spectrometry (Moebius et al. 2005). The second study used a receptor enrichment protocol with subsequent liquid chromatography and tandem mass spectrometry (Senis et al. 2009a, b). Their additional experiments further showed the expression of a G6b-B isoform with two immunoreceptor tyrosine-based inhibitory motifs (ITAM) and its association with SHP-1 in activated platelets pointing to a possible role in limiting platelet activation. A comparative gene expression analysis between platelets and other blood cells moreover identified G6b as a platelet-specific gene while flow cytometry confirmed G6b protein expression on platelet membranes (Macaulay et al. 2007). Moreover, G6b cross-linking via a polyclonal antibody resulted in inhibition of platelet aggregation induced by ADP and CRP (Newland et al. 2007) and this inhibition was shown to be calcium independent. An ITAM defective mutant G6b receptor lacks its inhibitory action and the hypothesis was formed that G6b-B

inhibits platelet function via the inhibition of the constitutive signaling through Src and Syk and not via interference with SHP-1 or SHP-2 (Mori et al. 2008). It was therefore suggested that G6b, identified as a novel inhibitory platelet receptor, could be an interesting novel antithrombotic target.

2.3 *Actin-Binding Adaptor: HIP-55 in Thrombin Activated Platelets*

In an attempt to identify changes in the membrane-associated proteome of thrombin-activated platelets, a comparative analysis was performed using biotinylated surface proteins that are isolated with avidin affinity chromatography before separation with liquid phase IEF and SDS-PAGE followed by mass spectrometry identification of differentially expressed proteins (Tucker et al. 2009). Novel proteins and proteins that are translocated to the membrane after activation were identified such as HIP-55 and again the G6b receptor. HIP-55 as ubiquitously expressed actin-binding adaptor was shown in T cells to interact with ZAP-70, a tyrosine kinase member of the Syk family, that phosphorylates LAT (Han et al. 2003). Interestingly, HIP-55 deficient mice platelets present with reduced fibrinogen binding after thrombin stimulation (Tucker et al. 2009), validating HIP-55 as novel regulator of platelet function.

2.4 *Aldose Reductase-AR and Disulfide Isomerase: ERp57 in GPVI Signaling*

A proteomic comparison using 2D-DIGE and mass spectrometry was performed to display changes in proteins after GPVI activation of platelets using a monoclonal anti-GPVI antibody (Schulz et al. 2010). This method analyzed cytoplasmic platelet extracts and was performed for seven biological replicates. Two of eight differentially expressed proteins identified as being modulated by GPVI were further characterized. Aldose reductase (AR) abundance and activity was increased after GPVI activation and AR inhibitors dose-dependently reduced platelet aggregation induced by the anti-GPVI antibody. ERp57 is released after GPVI activation and belongs to the disulfide isomerase family of which the other member protein disulfide isomerase (PDI) was shown to regulate tissue factor (TF) decryption as initial activation step in coagulation (Reinhardt et al. 2008). Interestingly, GPVI activation was shown to increase platelet-dependent TF activation that could be suppressed by an anti-ERp57 antibody. This 2D-DIGE approach therefore identified novel players in platelet GPVI activation and helped to identify novel antiplatelet target. A similar approach could be applied for stimulation of other platelet receptors such as GPIIb/IIIa, P2Y12, GPIbalpha, and others.

3 Identification of Novel Targets via Studies in Patients with Inherited Platelet Disorders

3.1 General Concept

By studying the etiology, molecular biology, and pathophysiology of inherited platelet disorders (IPDs), DNA variants, genes, and pathways will be discovered that place pointers at new regulators of the function and/or formation of platelets. This knowledge will be relevant for improved prevention and treatment of bleeding in these patients but can also be useful to identify novel targets for antiplatelet therapy. The advantage of such an approach is that molecules can be targeted from which it is known that they are clinically relevant. To prove that defective gene products are the real cause of the bleeding, these target molecules mostly have been confirmed by functional genomics (see further). For the most common IPDs such as Glanzmann Thrombastenia and Bernard–Soulier syndrome, the causative defective platelet receptors GPIIb/IIIa and GPIbalpha have already been developed as target for useful antiplatelet drugs (see previous chapters). Also patients with defects in GPVI, TXA2R, and P2Y12 have a bleeding disorder and again inhibition of these receptors proved to be a useful successful antiplatelet therapy approach. Therefore, in this part of the chapter, we focus on some more novel but very rare but genetically proven IPDs that results from gene defects in novel genes that could be again a novel target for antiplatelet drug development. We specifically focus on diseases in the stimulatory G protein (Gs) pathway that is responsible for cAMP formation, an essential component that inhibits platelet activation (Noé et al. 2010a). Figure 2 shows the cAMP regulation and signaling pathways in platelets. We will discuss a disease caused by overexpression of the Gs-coupled receptor ligand PACAP, mutants of Gsalpha and its large variant XLalphas, and a mutation of RGS2, that all result in bleeding (PACAP, XLalphas) or a thrombotic tendency (Gsalpha, RGS2).

3.2 Bleeding in Patients with PACAP Overexpression

The Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) was originally isolated from ovine hypothalamic extracts as stimulator of cAMP formation in rat anterior pituitary cells (Miyata et al. 1989; Vaudry et al. 2009). This neuropeptide is highly homologous to the vasoactive intestinal peptide (VIP). PACAP is believed to act as a hormone, a neurohormone, a neurotransmitter, and a trophic factor in different peripheral tissues. PACAP38 is a ligand for three G protein-coupled receptors: the PACAP-specific PAC1 receptor, which is coupled to several transduction systems and mainly expressed in the central nervous system, and the PACAP/VIP-indifferent VPAC1 and VPAC2 receptors, which are primarily

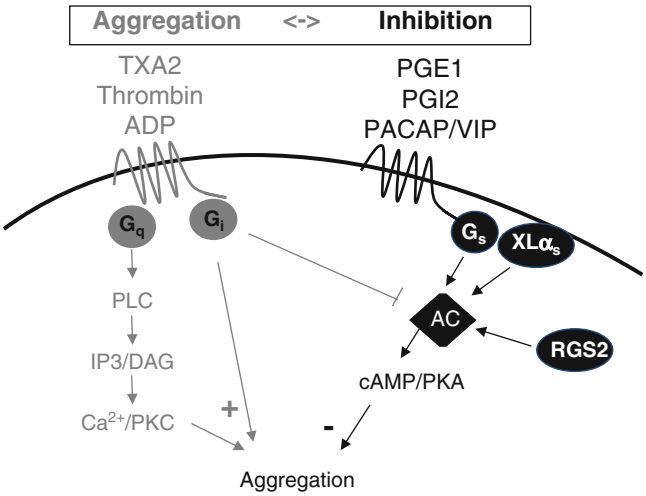


Fig. 2 Schema of the platelet aggregation-inhibition test to study defects in cAMP signaling. Thromboxane A2 (TXA2), ADP, thrombin, PGE2, PGI2, and PACAP bind to GPCRs on the platelet surface. Different agonists for receptors coupled to Gqα or Giα subunits stimulate platelet aggregation and shape change. Gqα activates PLC, which stimulates the production of IP3 and DAG, with the consequent stimulation of PKC and release of intracellular calcium. The presence of high levels of cytosolic Ca^{2+} is needed for integrin αIIbß3 activation and stable platelet aggregation. Giα inhibits the production of cAMP by adenylyl cyclase (AC) while Gsα and its isoform XLαs stimulated cAMP production. High levels of cAMP inhibit platelet aggregation via multiple mechanisms

coupled to adenylyl cyclase and widely distributed in peripheral tissues (Vaudry et al. 2009). PACAP has presently been investigated in endocrine glands, central nervous system, respiratory system, cardiovascular system, and gastrointestinal tract. The active PACAP peptides are mainly expressed in testis and brain but they can cross the blood–brain barrier (Banks et al. 1993) and are stably transported in plasma via coupling to ceruloplasmin (Tams et al. 1999). One study reports the expression of VPAC1(VIPR1) on human megakaryocytes and platelets and VIP binding was shown to be present on platelet membranes (Park et al. 1996) which also suggests a role for VIP in megakaryopoiesis and platelet function. A physiological role for PACAP and its Gs-coupled receptor VPAC1 was described for platelet function and formation based upon studies in patients with a platelet-based bleeding phenotype (Freson et al. 2004). FISH analysis in these two related patients with an unbalanced reciprocal translocation t(18;20)(p11.21; p13) revealed three copies of the PACAP gene on 18p (Freson et al. 2004). Increased PACAP transcript levels were found in their skin fibroblasts and ELISA showed an elevated PACAP concentration in their plasma. These patients suffer from multiple neurological, gastrointestinal, and endocrinological problems. In addition, they also have a severe bleeding tendency with repetitive epitaxis and a markedly prolonged Ivy bleeding time due to thrombopathy combined with mild thrombocytopenia (70–100 $\times$ 10^9 platelets/L blood). Platelet aggregation with all standard platelets agonists was

strongly reduced in these patients and also a decreased platelet secretion of ATP and beta-thromboglobulin was present. The VPAC1 receptor in platelets and fibroblasts is coupled to adenylyl cyclase activation for cAMP formation after stimulation with PACAP or VIP (Peeters et al. 2008). Accordingly, highly increased basal cAMP levels in the patients' fibroblasts and platelets have been found, providing an explanation for the reduced platelet aggregation in these patients as cAMP is a strong inhibitor for platelet function. The defect in platelet function and formation described for these patients has been phenocopied in a transgenic mouse model with a megakaryocyte-specific PACAP overexpression (Freson et al. 2004). These mice have high PACAP plasma values, have a prolonged tail bleeding time, and a decreased platelet aggregation. Interestingly, PACAP knockout mice had an increased platelet activity (Freson et al. 2004).

3.3 Inhibitors of cAMP: Studies from Gsalpha/RGS2 Defective Patients

Intracellular cAMP levels are regulated via the Gs and Gi alpha subunits of heterotrimeric G proteins, which couple to adenylyl cyclase to respectively stimulate or inhibit cAMP production (Fig. 1). Binding of a ligand to its G protein-coupled receptor activates these G proteins. An increase in Gs function, or alternatively a defective Gi signaling, can be a risk factor for bleeding, while a loss of Gs function can result in a prothrombotic state. Platelet Gs activity can be easily studied by using the platelet inhibition-aggregation test, which is based on the inhibition of platelet aggregation upon stimulation with different Gsα agonists such as PGI_2 (Freson et al. 2001). With this test, a platelet Gs hyperfunction has been identified in a group of patients having a paternally inherited polymorphism in the GNASXL gene that codes for XLαs (Freson et al. 2003). The "extra large" variant of Gsalpha or XLαs can mimic Gs functions as the activation of adenylyl cyclase. The mutated variant of XLαs is associated with elevated inducible platelet Gs activity and high levels of cAMP after Gs-coupled receptor activation, and therefore leads to an increased trauma-related bleeding tendency. In contrast, a Gs hypofunction is also relevant for platelet function since a compound heterozygous Gsα deficient patient was described with a thrombotic phenotype due to absence of inhibition of platelet aggregation by Gs stimulation that resulted in platelet hyperreactivity (Freson et al. 2008).

Regulator of G protein signaling (RGS) proteins accelerates the rate of inactivation of G protein-mediated signaling. One of the RGS proteins, RGS2, inhibits Gs signaling by interacting directly with adenylyl cyclase and thereby reducing cAMP levels. Three related patients with a heterozygous mutation in the RGS2 gene were described (Noé et al. 2010b). They present with decreased platelet Gs function and the mutation was shown to result in an increased ability of RGS2 to inhibit adenylyl cyclase. As such, this mutation leads to a reduced production of cAMP after stimulation of the Gs pathway in platelets of the carriers.

4 Identification of Novel Targets via Large-Scale Genetic Studies in Healthy Subjects

4.1 General Concept

Interindividual responsiveness of platelets to different agonists is highly variable but reproducible over time as shown in different large cohorts studies of normal subjects and this for all agonists and different platelet function methods (O'Donnell et al. 2001; Yee et al. 2005, 2006; Panzer et al. 2006; Jones et al. 2009). It was therefore assumed that platelet function differences in normal subjects are inheritable and genomic approaches can be used to find gene variants that are causative for these relatively small functional differences without causing bleeding phenotypes. These causative genes would also be good candidates for further antiplatelet drug development. In this part, we will discuss the candidate gene association using a functional genomics approach and the recent genome wide association study (GWAS) that studied quantitative traits for small changes in platelet number and/or MPV as indirect markers for platelet functional differences. Table 1 was added to inform the reader with some details on the identified novel targets based on the available knowledge concerning their potential role in platelets as described in the Online Mendelian Inheritance in Man (OMIM) database.

4.2 Candidate Gene Association Studies Using a Genomics Approach and Functional Platelet Testing

The availability of high-throughput, cost-effective, genomic sequencing enabled a more objective study of larger cohort sizes and the simultaneous evaluation of many SNPs. Through the comparison of 1,327 SNPs in a selected group of 97 candidate genes, Jones et al. (2009) identified 17 SNPs that account for 47 % of the variation in platelet reactivity induced by either ADP or CRP. This study was performed in 500 healthy individuals of Northern European ancestry. Their platelet reactivity was measured by fibrinogen binding to activated GPIIb/IIIa and the second step in platelet activation was determined by measuring P-selection expression on the platelet membrane, after activation with ADP and CRP. Via this approach two distinct signaling pathways were analyzed, being GPVI signaling as an early event that acts via the FcRγ/ITAM pathway and ADP that amplifies platelet reactivity in a later stage through the G protein-coupled receptors, P2Y1 and P2Y12. The 97 candidate genes were selected as having a known involvement in these two pathways. An associated with platelet reactivity to ADP and/or CRP was found for platelet receptor genes CD36, GP6, ITGA2, PEAR1, P2Y12; adaptor proteins FCERG1 and GNAZ; kinases JAK2, MA2K2, MAP2K4; and intracellular signaling proteins ITPR1 and VAV3. Another study has shown that a variant in the promoter region of PEAR1 (platelet endothelial aggregation receptor 1) is

Table 1 Novel targets identified by genomic approaches

Gene	Target	Known function in platelets Based on OMIM NCBI database[a]
Functional genomic approaches—direct targets of platelet function		
CD36	Leukocyte differentiation antigen	MIM173510. Receptor (GPV) for thrombospondin in platelets
GP6	GlycoproteinVI	MIM605546. Collagen receptor involved in platelet aggregation. Compound heterozygosity for mutations lead to mild bleeding phenotype
ITGA2	Integrin alpha2	MIM192974. Collagen receptor involved in platelet adhesion. Knockout mice have no bleeding phenotype but their platelets and fibroblasts have decreased adhesion to collagen
PEAR1	Platelet endothelial aggregation receptor 1	MIM610278. Expressed on platelets and endothelial cells. Involved in platelet contact-induced activation
P2Y12	Purinergic receptor P2Y, G protein-coupled, 12	MIM600515. ADP receptor. Compound heterozygous mutations lead to bleeding phenotype
FCER1G	Fc fragment of IgE, high affinity I, receptor for gamma subunit	MIM147139. Fcgamma associates with GPVI on platelet membranes
GNAZ	Guanine nucleotide-binding protein alpha Z	MIM139160. G protein subunit that is activated by epinephrine activation of the alpha2A-adrenergic receptor on platelets
JAK2	Janus kinase 2	MIM147796. Downstream of TPO signaling pathway. Somatic mutations lead to thrombocytosis
MAP2K2	Mitogen-activated protein kinase kinase 2	MIM601263. Involved in MAPK pathway, function in platelets unknown
MAP2K4	Mitogen-activated protein kinase kinase 4	MIM601335. Involved in MAPK pathway, function in platelets unknown
ITPR1	Inositol 1,4,5-triphosphate receptor, type 1	MIM147265. Involved in calcium release but exact function in platelets not known
VAV3	VAV3 oncogen	MIM605541. Guanine exchange factor for rho and Rac family. Role in platelet activation by collagen
ADRA2A	Alpha2A adrenergic receptor	MIM104210. Activated by epinephrine to induce aggregation in platelets
PIK3CG	Phosphatidylinositol 3-kinase, catalytic, gamma	MIM601232. Involved in the PI3K signaling pathway, exact role in platelets not studied
JMJD1C	Jumonji domain-containing protein 1C	MIM604503. Thyroid hormone-dependent transcription factor with unknown function in platelets
MRVI1	Murine retrovirus integration site 1, homolog of	MIM604673. Regulation of intracellular calcium with unknown function in platelets
SHH	Sonic hedgehog	MIM600725. Involved in limb and brain development with unknown role in platelets

(continued)

Table 1 (continued)

Gene	Target	Known function in platelets Based on OMIM NCBI database[a]
COMMD7	COMM domain containing 7	Not in OMIM. Role in NFkB pathway. Studies in zebrfish show a modest effect of gene depletion on thrombus formation
LRRFIP1	Leucine-rich repeat in fli1-interacting protein 1	MIM603256. Positive regulator of thrombus formation as studied in zebrafish
Genome wide association studies—MPV/platelet count regulators as indirect targets of platelet function		
WDR66	WD repeat domain 66	Not in OMIM. Unknown function
ARHGEF3	Rho guanine nucleotide exchange factor 3	MIM612115. Guanine nucleotide exchange factor for RhoA and RhoB. Highly expressed in platelets but exact role unknown, gene depletion in zebrafish results in thrombocytopenia
TAOK1	TAO kinase 1	MIM610266. Involved in JNK signaling. Role in platelets not known
DNM3	Dynamin 3	MIM611445. Involved in actin-mediated processes such as membrane budding with unknown function in platelets
CD226	Cd229 antigen	MIM605397. Member of the immunoglobulin superfamily with unknown role in platelets
TPM1	Tropomyosin 1	MIM191010. Structural role in actin filaments of myofibrils and stress fibers with unknown role in platelets
SIRPA (PTPNS1)	Signal regulatory protein alpha or protein tyrosine phosphatase, nonreceptor type, substrate 1	MIM602461. Can bind to SHP-1 and SHP-2 but unknown role in platelets
EHD4	EH domain-containing 4	MIM605892. Regulation of endosomal transport with unknown role in platelets
BAK1	Bcl2 antagonist killer 1	MIM600516. Promotes apoptosis via inhibition of Bcl2. Apoptosis is known to play a role in the final stage of megakaryopoiesis but the exact role of BAK1 is not yet defined in this process

[a]http://www.ncbi.nlm.nih.gov/omim/

associated with increased platelet aggregability (Herrera-Galeano et al. 2008). It was described before via standard in vitro platelet functional studies that PEAR1 is an epidermal growth factor repeat-containing transmembrane receptor that is involved in the platelet contact-induced activation that signals secondary to GPIIb/IIIa mediated platelet–platelet contacts (Nanda et al. 2005). More recently, PEAR1 was also one of the candidate genes identified via another genome-wide meta-analysis that studied gene association with platelet aggregation responses to

ADP, epinephrine, and collagen (Johnson et al. 2010). In this study, 2.5 millions SNPs were screened in two cohort of European ancestry (2,753 subjects from the Framingham heart study and 1,238 subjects from the genetic study of Atherosclerosis risk). Seven loci were identified as modifiers for platelet aggregation responses, being GP6, PEAR1, ADRA2A, PIK3CG, JMJD1C, MRVI1, and SHH. Finally, transcription profiling in human platelets was used to study genome-wide platelet RNA expression in 37 subjects representing a normal range of platelet responsiveness and 63 genes had transcript levels that were correlated with variation in platelet response to ADP and/or CRP (Goodall et al. 2010). Six from these 63 genes were further screened in 4,235 cases with myocardial infarction and 6,379 controls and an association with MI was found for COMM domain-containing protein 7 (COMMD7) and leucine-rich repeat interacting protein 1 (LRRFIP1).

4.3 Genome-Wide Association Study

Currently, there is no GWAS study available that directly studied platelet function. Since platelet function is indirectly modified with changes in platelet count and volume, we here refer to three recently available GWAS studies performed for platelet count and volume (Meisinger et al. 2009; Soranzo et al. 2009a, b). The first GWAS study evaluated Affymetrix 500 K gene Chip data in KORA F3 ($n = 1{,}644$) and replicated in three large cohorts. MPV was found to be associated with three common SNPs in or nearby WDR66, ARHGEF3, and TAOK1 (Meisinger et al. 2009). The Affymetrix 500 K gene Chip data was also used in the second GWAS study for the UKBS-CC1 ($n = 1{,}221$) cohort and replicated in four other cohorts to identify one intergenic locus on 7q22.3 that was associated with MPV (Soranzo et al. 2009a). Finally, the third GWAS study used a larger number of subjects from essentially the same population cohorts and found 12 quantitative traits that were associated with MPV of which 8 were not present in the first 2 GWAS studies (Soranzo et al. 2009b) and these include DNM3, CD226, JMJDIC, TPM1, SIRPA, EHD4, BAK1, and JAK2. However, functional biological data are now needed to define the role of these novel MPV regulators in platelet formation and function.

5 Characterization of Novel Targets via Functional Genomics in Animal Models

5.1 General Concept

Functional genomics in animal models have been used to focus on the role of a single gene product and/or its regulatory pathway. Candidate proteins as regulators of platelet function are usually selected based on knowledge of their platelet-specific

expression pattern or as being identified in an important modulator of platelet function, such as described earlier for HIP-55. Mice are still the most used general animal model for functional genomics although recently, a transgenic zebrafish model with GFP-labeled thrombocytes became available to easily study their formation and function (Lin et al. 2005). Functional genomic approaches in mice and zebrafish resulted in numerous gene depletion studies with description of a disturbed platelet function as the only phenotype although usually in combination with other phenotypes. It will, of course, be impossible to give a complete overview of the large amount of functional genomic studies in mice but in this part of the chapter we discuss: (1) some of the initial studies that described knockout mice with a bleeding phenotype in more detail concerning the role of the novel target in platelet function and (2) we grouped some more recent knockout studies in mice according to the role of the target in modifying platelet receptor function, intracellular signaling, and/or secretion (Table 2 for overview of all models). We also specifically focus on studies describing a defective platelet function but normal thrombopoiesis. The last part deals with the few studies that used zebrafish as a model to characterize the effect of gene depletion on platelet function. All these animal studies thereby identified a potential novel candidate for further experimental antiplatelet drug development.

5.2 Mouse Model for Ecto-Nucleotidase: CD39/NTPDaseI

The extracellular nucleotide P2-receptor effects on platelets, endothelium, and leukocytes are mainly mediated by the dominant ecto-nucleotidase CD39/NTPDaseI that is expressed by endothelium, monocytes, and vascular smooth muscle cells. CD39 hydrolyzes ATP and ADP into AMP and blocks platelet aggregation responses to ADP (Enjyoji et al. 1999; Pinsky et al. 2002). CD39 deficient mice are characterized by disordered thromboregulation with heightened susceptibility to inflammatory vasculary reactions and high levels of tissue fibrin. Paradoxically, these mice also have a bleeding phenotype with P2Y1 desensitization (Enjyoji et al. 1999). On the other hand, CD39 overexpression by transgenesis, adenoviral vectors, or the use of pharmacological soluble CD39 derivatives has major potential beneficial effects on the platelet and endothelial cell activation in a setting of vascular inflammation (Dwyer et al. 2004; Marcus et al. 2003).

5.3 Mouse Models for Growth Arrest-Specific Gene 6: Gas6 and Its Receptors Tyro3, Axl, and Mer

The growth arrest-specific gene 6 (Gas6) is a member of the vitamin K-dependent protein family, related to protein S but it lacks a loop that is necessary for anticoagulation activity. Gas6 deficient mice or mice treated with Gas6 antibodies

Table 2 Mice knockout studies to characterize novel antiplatelet targets

Knockout target	Prolonged bleeding (Yes/No)	In vitro platelet test	In vivo models
Platelet receptor function			
Tyro3, Axl, Mer receptors and ligand GAS6	No	Reduced aggregation/ secretion to low dose agonists	Protection against arterial thrombosis
Leptin receptor and ligand leptin	na	Decreased intracellular phosphorylation	Protection against arterial thrombosis
CD148 receptor	Yes	Defective Syl and GPCR signaling	Protection against arterial thrombosis
Alpha2A adrenergic receptor	Yes	Reduced epinephrine aggregation	Protection against arterial thrombosis
CalDAG-GEFI for beta 1/2/3 integrin activation	Yes	Strongly reduced but not absent aggregation	Protection against arterial thrombosis
Kindlin-3 for beta1/3 integrin activation	Yes	Absent aggregation	Protection against arterial thrombosis
TSSC6 interacts with integrin	Yes	Reduced aggregation and spreading on fibrinogen	Protection against arterial thrombosis
CIB1 interacts with integrin	Yes	Reduced spreading on fibrinogen	Protection against arterial thrombosis
Platelet intracellular signaling			
PI3Kgamma	No	Reduced aggregation with ADP, reduced AKT phosphorylation	Protection against arterial thrombosis
AKT1 downstream effector of PI3K	Yes	Reduced aggregation and spreading	na
PKCalpha	No	Reduced aggregation and secretion	Protection against arterial thrombosis
JNK1 acts on PKC	Yes	Reduced aggregation and secretion	na
Rab1b	Yes	Reduced aggregation	Protection against arterial thrombosis
Rac1	Yes	Reduced aggregation and P-selectin expression	Protection against arterial thrombosis
Platelet secretion			
ILK	Yes	Reduced aggregation, normal dense granules, defective alpha granule secretion	na

(continued)

Table 2 (continued)

Knockout target	Prolonged bleeding (Yes/No)	In vitro platelet test	In vivo models
RanBP10	Yes	Decreased alpha and dense granule markers	na
VAMP8	na	Reduced ADP aggregation and dense granule secretion	Protection against arterial thrombosis

Na not available

are protected from venous and arterial thrombosis but do not suffer from spontaneous bleeding with normal tail bleeding times (Angelillo-Scherrer et al. 2001). It was further shown that platelet aggregation and ATP secretion were decreased in response to different low dose agonists but had normal hematological counts. A subsequent study showed that loss of any of the three Gas6 receptors on platelets, i.e.,Tyro3, Axl, or Mer, also protected mice against thrombosis (Angelillo-Scherrer et al. 2001). Initial platelet aggregation was normal but the secondary secretion-dependent step and outside-in signaling was inhibited (Angelillo-Scherrer et al. 2005), resulting in impaired stabilization of aggregates. Indeed, Gas6 activates PI3K and Akt and induces tyrosine phosphorylation of beta3 integrin. A more recent study showed that under flow conditions, absence of Gas6 provoked gradual platelet disaggregation and GPIIbIIIa inactivation (Cosemans et al. 2010). It was suggested that the ADP-P2Y12 and Gas6-Tyro3/Axl/Mer activation pathways synergize to achieve persistent GPIIbIIIa activation and platelet aggregation.

5.4 Mouse Models for Leptin and Leptin Receptor

Another circulating plasma factor that alters platelet function is the obesity related satiety factor leptin. Platelets express the leptin receptor and ob/ob mice that lack leptin have a decreased thrombotic response to arterial injury (Konstantinides et al. 2001). Similar findings are obtained in leptin receptor deficient db/db mice. Another study showed that leptin time- and dose-dependently phosphorylated different intracellular signaling pathways in platelets (Dellas et al. 2007). Leptin receptor antagonists might therefore be useful in the care of obese individuals to also reduce their cardiovascular complications (Schäfer et al. 2004).

5.5 Mouse Models for Platelet Receptor Function Defects

Different steps in platelet activation are regulated by a whole set of G protein-coupled, tyrosine kinase-linked, and integrin receptors. Knockout mice are useful to unravel their more specific contribution to the overall serial steps in platelet

activation responses. The only receptor-like protein tyrosine phosphatase receptor described to date in platelets is CD148. CD148 deficient mice have a bleeding tendency and present with defective arterial thrombosis due to decreased basal and agonist-stimulated Src family kinase (SFK) activity but also a reduced G protein-coupled receptor responses (Senis et al. 2009a, b).

The alpha(2A)-adrenergic receptor (α2A-AR) is activated by epinephrine in platelets and is coupled to Gz. Variable tail bleeding times were described in α2A-AR knockout mice and their platelets have a defect in the epinephrine-induced platelet aggregation though flow experiments show normal adhesion on collagen (Pozgajová et al. 2006). However, three different in vivo thrombosis models illustrate the important role of α2A-AR in thrombus stabilization. The α2A-AR deficient mice are protected from lethal pulmonary thromboembolism induced by injection of collagen/epinephrine, and a twofold decrease in embolus formation was observed in a model of FeCl(3)-induced injury in mesenteric arterioles and vessel occlusion after mechanical injury was significantly reduced with embolization of the initially formed thrombi.

Finally, we discuss different mice models that identified novel molecular players important for GPIIb/IIIa (integrin alpha2beta3) receptor signaling. Mice that lack the calcium and diacylglycerol-regulated guanine nucleotide exchange factor (CalDAG-GEFI) have a disturbed regulation of the three beta integrins (Bergmeier et al. 2007). In addition to their immune problems caused by defects in neutrophil migration, a complete inhibition of arterial thrombosis was observed. This mouse model represents a phenotype comparable as observed for patients with leukocyte adhesion deficiency type III (LAD3), a syndrome characterized by Glanzmann thrombasthenia-like bleeding problems and impaired adhesion of leukocytes to inflamed endothelia due to Kindlin3 mutations. Mice defective for Kindlin-3 (or FERMT3) also present with a disturbed primary hemostasis and neutrophl binding and spreading on beta2 integrin-dependent ligands (Moser et al. 2009). The FERM domain of Kindlin-3 is required for the activation of integrins beta1 and beta3 on platelets. GPIIb/IIIa outside-in signaling is impaired in mice defective for the hematopoietic-specific tetraspanin superfamily member, TSSC6 (Goschnick et al. 2006). TSSC6 is expressed on the platelet surface, interacts with GPIIb/IIIa, and is upregulated after thrombin stimulation, indicative for an intracellular pool of TSSC6. TSSC6 knockout mice have increased tail bleeding times with increased numbers of rebleeds as possible indication of unstable thrombi. More detailed platelet function studies show an impaired clot retraction, reduced platelet aggregation with low dose of PAR4 and collagen, and a decreased platelet spreading on fibrinogen though their platelet have a normal inside-out GPIIb/IIIa signaling as detected via FITC-fibrinogen and JON/A binding. Finally, an in vivo vascular injury model showed unstable arterial thrombi in TSSC6 knockout mice. The last modifier of GPIIb/IIIa function is CIB1 that was shown to directly bind to this receptor. CIB1 deficiency also resulted in defective outside-in signaling with increased tail bleeding times and a rebleeding phenotype (Naik et al. 2009). Platelet spreading on fibrinogen is again impaired and FeCl(3)-induced injury was delayed.

5.6 *Mouse Models for Intracellular Signaling*

An inexhaustible number of intracellular molecules will regulate platelet activation and therefore for this section we focused on mice models that show the importance of phosphoinositide 3-kinases (PI3K), protein kinase C (PKC), and some small GTPases in their regulation of platelet function. Platelet aggregation with ADP but not collagen or thrombin is impaired in PI3Kgamma deficient mice and ADP stimulation also resulted in a decreased PKB/AKT phosphorylation (Hirsch et al. 2001). Though these mice do not present with an increased bleeding time, mice were protected from ADP-induced thromboembolic vascular occlusion. Also PI3Kp110beta plays an important role in platelet activation as specific inhibitors of this PI3K isoform prevent the formation of stable GPIIb/IIIa adhesion contacts that lead to a defective platelet thrombus formation (Jackson et al. 2005). Injection of these inhibitors in mice eliminates occlusive thrombus formation without a prolongation of the bleeding time, which makes them attractive as target for further antithrombotic drug development. AKT1 is one of the major downstream effectors of PI3K activation. AKT1 deficient mice exhibit reduced platelet aggregation and spreading after activation with different agonists (Chen et al. 2004). Though AKT1 is not the predominant AKT isoform in platelets, its absence results almost in a complete abolishment of AKT phosphorylation after thrombin activation. In addition, thrombin-induced release of alpha and dense granules is decreased and fibrinogen binding in response to collagen and thrombin is reduced though GPIIb/IIIa is normally expressed. These platelet dysfunctions lead to prolongation of the bleeding times in AKT1 knockout mice.

Different steps in platelet activation as integrin activation, secretion, aggregation, and spreading are mediated via different PKC isoforms and broad-spectrum PKC inhibitors have been shown to reduce secretion and aggregation. Platelets from PKCalpha deficient mice show defects in secretion and aggregation and in vivo testing showed a reduced thrombus formation though bleeding times are normal (Harper and Poole 2010). Absence of PKCbeta and PKCtheta decrease platelet spreading while PKCdelta inhibits filopodia formation with subsequent inhibition of aggregation and thrombus formation. Interestingly, the c-jun NH2-terminal kinase 1 (JNK1) leads to GPIIb/IIIa activation and aggregation via a mechanism involving PKC. JNK1 knockout mice have longer bleeding times and perfusion on collagen experiments shows an about 50 % reduction in thrombus formation (Adam et al. 2010). In vitro tests further showed that JNK1 deficient platelets have an impaired aggregation and secretion to low doses of agonists.

There are more than a hundred proteins in the Ras superfamily and based on structure, sequence, and function similarities, this superfamily is divided into eight main subfamilies (including Rap and Rho members) that share a common essential GTPase and nucleotide exchange activity. Platelets express multiple Ras members and mouse models with disturbed platelet function exist for Rap1b and Rac1. Rab1b is an abundant small GTPase in platelets that becomes activated upon stimulation with different agonists. Rap1b defective mice have a bleeding phenotype due to decreased aggregation responses to different agonists with decreased

activation of GPIIbIIIa (Chrzanowska-Wodnicka et al. 2005). In addition, Rap1b mice are protected against arterial thrombosis as also studied in vivo. Rac1 is a member of the Rho GTPase subfamily and is expected to have an important role in the actin cytoskeleton and cell adhesion. Rac1 depletion was obtained by gene targeting and by using the Rac1 small molecule inhibitor NSC23766 (Akbar et al. 2007). Both models resulted in a reduced P-selectin expression and platelet aggregation after activation with ADP, thrombin, and u46619 and bleeding time of Rac1 knockouts or mice treated with NSC23766 were prolonged.

5.7 Mouse Models for Secretion Defects

Platelet secretion is the second step in platelet activation and involves the release of alpha and dense granules. Numerous genes involved in the formation of granules have been described. An highly informative Hermansky–Pudlack syndrome (HPS) database is available online (liweilab.genetics.ac.cn/HPSD) and provides an integrated, annotatory, and curative dataset for the recently cloned human and mouse HPS genes, as well as the genes responsible for HPS related syndromes, such as Chediak–Higashi syndrome (CHS) (Li et al. 2006). Platelet dense granule has been studied in most HPS mouse models and found to be defective as cause of their bleeding phenotype. Since HPS genes are also involved in regulation of secretion in other tissues as the retina and melanocytes, it will be more difficult to develop these as drug target. In addition to these HPS mice models, some recent reports describe some other mice with a defective platelet secretion.

Integrin-linked kinase (ILK) has been described as important regulator of cell survival, growth, differentiation, and adhesion. ILK associates with integrins and conditional knockout mice for ILK in platelets have an increased bleeding time (Tucker et al. 2008). They present with a reduced aggregation, fibrinogen binding, and thrombus formation under arterial flow. ILK-deficient platelets have a normal dense granule release but their alpha granules secretion is defective.

Degranulation is also defective in RanBP10 deficient mice as cause of their bleeding problems (Kunert et al. 2009). RanBP10 is a tubulin-binding protein that is concentrated along polymerized microtubules in mature proplatelet-forming megakaryocytes. RanBP10 knockout mice still have normal platelet counts and normal megakaryocytes though structural studies have shown disorganized microtubule localization with abnormal granule release as this process depends on the internal contraction of the microtubule marginal coil. Dense and alpha granule markers, CD63 and CD62P, are decreased after platelet stimulation as studied by flow cytometry.

Endobrevin or VAMP8 is a SNARE protein that mediates membrane fusion events that are essential for granule cargo release. VAMP8 deficient mice have a retarded but not absent thrombus formation after laser-induced vascular injury (Graham et al. 2009). They also have an abnormal ADP-induced platelet aggregation and reduced dense granule release. This study shows that dense granule release is important in the earliest phase of thrombus formation and validated the platelet secretion machinery as potential target for antiplatelet therapy.

5.8 *CD41-GFP Transgenic Zebrafish Models for Rapidly Characterizing Novel Platelet Proteins*

Since the first description of the CD41 transgenic zebrafish line having GFP-labeled thrombocytes in 2005, some studies appeared that used these fish to rapidly screen genes with an unknown function in platelets (Lin et al. 2005). Thrombocytes in fish are nucleated and appear 42 h postfertilization (hpf) as a nonmobile population in an area between the dorsal aorta and the caudal vein while they enter circulation at 48 hpf. We only focus here on the characterization of genes that modulate platelet function without affecting their formation. A recent study characterized 5 novel platelet membrane proteins via morpholino-induced depletion in this transgenic zebrafish model and subsequently analyzed their thrombocyte function via a laser-induced arterial thrombosis model (O'Connor et al. 2009). Four resulted in altered arterial thrombosis with a role for DCBLD2 and ESAM in inhibition and BAMBI and LRRC32 in promotion of thrombus formation.

Discoidin, cub, and LCCL domain-containing protein 2 (DCBLD2) has a structure that is homologs to the semaphoring-binding neuropilins and being expressed in megakaryocytes and platelets could have an important role in signaling. ESAM is expressed on platelet alpha granules, plays a role in vascular permeability at endothelial cell tight junctions, and knockout mice form larger thrombi in an in vivo thrombosis model. BAMBI encodes a protein related to type I receptors of the transforming growth factor-beta1 and knockout mice develop normally though their platelet function was not studied. Finally, LRRC32 is structurally related to GPIbalpha and GPV and therefore predicted to operate as part of a multiprotein complex, probably with a role in adhesion. LRR-rich receptors form homotypic interactions and it was suggested that LRRC32 could be involved in platelet–platelet and platelet–endothelial cell interactions.

Another study applied the zebrafish model to study the role of Mlck1a, a zebrafish ortholog for one of the three human myosine light chain kinases (MLCK), to study its role in thrombocyte function (Tournoij et al. 2010). Mlck1a morpholino-induced depletion resulted in impaired morphology changes of thrombocyte adhesion to fibrinogen and a retarded thrombus formation after vessel wall damage using laser irradiation. The MLCK subtype Mlck1a was therefore suggested to play a role in platelet shape change and in thrombus formation.

6 Conclusions and Future Directions

Platelets are central mediators in the development of atherothrombosis. The current antiplatelet therapy involves the use of aspirin and different antagonists for GPIIb/IIIa and P2Y12 improved survival and reduced adverse cardiac events in patients with vascular disease. However, despite their clinical effectiveness, these drugs are still limited by increasing risk for major and minor bleeding, other

nonbleeding site effects, a relative lack of efficacy, and drug resistance in some subjects. Therefore, the identification and design of more efficacious and safer antiplatelet targets is still highly warranted. This chapter provides some methods that have been applied to unravel novel candidates and pathways that play essential roles in platelet function. Some agents act directly, whereas others indirectly modulate platelet reactivity. Platelet proteomics and genomics studies can be applied to identify regulators of platelet function in diseased samples but also to identify small differences of altered platelet response in the healthy population. Not only these large-scale genomics and proteomics approaches but also the identification of genes that are causative for the bleeding phenotype in patients with inherited platelet disorders resulted in a wealth of novel potential candidates that play essential roles in platelet function and can therefore be further developed as antiplatelet drug. Functional genomic approaches in animal models such as mice knockouts or zebrafish could be helpful in the further functional clarification of the role of these novel targets in platelet function and formation. Future treatment of acute vascular events and chronic therapy for secondary and primary prevention of vascular disease will likely change over time from our current treatment by using safer and more affective agents.

Knowledge Gaps

- Recent large-scale platelet proteomics and genomics studies have generated extensive lists of proteins and genes that are important as regulators of platelet formation and/or function.
- The challenge ahead will be to apply easy system biology approaches (such as in vitro functional platelet studies or zebrafish models) to rapidly select and functionally characterize the most interesting candidates before initiating validation studies in small or larger animal models.

Key Messages

- Current and experimental antiplatelet drugs are effective as inhibitors of platelet aggregation, adhesion, and/or secretion with a positive impact on the risk of arterial thrombosis though still cannot be dissociated from increased risk of bleeding.
- Characteristics of novel targets as novel antiplatelet drug should be studied extensively in terms of bleeding problems, resistance, drug–drug interactions, and side effects.

- Identification methods for novel targets are:
  - Proteomics of complete or subcellular parts from platelets
  - Functional genomics via animal models
  - Genetic studies in patients with inherited bleeding disorders
  - Genomic approaches

Acknowledgments This work was supported by the 'Excellentie financiering KULeuven' (EF/05/013), by research grants from the FWO-Vlaanderen-Belgium (G.0453.05, G.0743.09, and G.0124.02), and from the Research Council of the University of Leuven (Onderzoeksraad K.U. Leuven, Belgium) (GOA/2009/13).
C.VG is holder of a clinical-fundamental research mandate of the Fund for Scientific Research-Flanders (F.W.O.-Vlaanderen, Belgium) and of the Bayer and Norbert Heimburger (CSL Behring) Chairs.

References

Adam F, Kauskot A, Nurden P, Sulpice E, Hoylaerts MF, Davis RJ, Rosa JP, Bryckaert M (2010) Platelet JNK1 is involved in secretion and thrombus formation. Blood 115:4083–4092

Akbar H, Kim J, Funk K, Cancelas JA, Shang X, Chen L, Johnson JF, Williams DA, Zheng Y (2007) Genetic and pharmacologic evidence that Rac1 GTPase is involved in regulation of platelet secretion and aggregation. J Thromb Haemost 5:1747–1755

Angelillo-Scherrer A, de Frutos P, Aparicio C, Melis E, Savi P, Lupu F, Arnout J, Dewerchin M, Hoylaerts M, Herbert J, Collen D, Dahlbäck B, Carmeliet P (2001) Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. Nat Med 7:215–221

Angelillo-Scherrer A, Burnier L, Flores N, Savi P, DeMol M, Schaeffer P, Herbert JM, Lemke G, Goff SP, Matsushima GK, Earp HS, Vesin C, Hoylaerts MF, Plaisance S, Collen D, Conway EM, Wehrle-Haller B, Carmeliet P (2005) Role of Gas6 receptors in platelet signaling during thrombus stabilization and implications for antithrombotic therapy. J Clin Invest 115:237–246

Banks WA, Kastin AJ, Komaki G, Arimura A (1993) Passage of pituitary adenylate cyclase activating polypeptide1-27 and pituitary adenylate cyclase activating polypeptide1-38 across the blood-brain barrier. J Pharmacol Exp Ther 267:690–696

Bennett JS, Mousa S (2001) Platelet function inhibitors in the year 2000. Thromb Haemost 85:395–400

Bennett JS (2001) Novel platelet inhibitors. Annu Rev Med 52:161–184

Bergmeier W, Goerge T, Wang HW, Crittenden JR, Baldwin AC, Cifuni SM, Housman DE, Graybiel AM, Wagner DD (2007) Mice lacking the signaling molecule CalDAG-GEFI represent a model for leukocyte adhesion deficiency type III. J Clin Invest 117:1699–1707

Chen J, De S, Damron DS, Chen WS, Hay N, Byzova TV (2004) Impaired platelet responses to thrombin and collagen in AKT-1-deficient mice. Blood 104:1703–1710

Chrzanowska-Wodnicka M, Smyth SS, Schoenwaelder SM, Fischer TH, White GC 2nd (2005) Rap1b is required for normal platelet function and hemostasis in mice. J Clin Invest 115:680–687

Coppinger JA, Cagney G, Toomey S, Kislinger T, Belton O, McRedmond JP, Cahill DJ, Emili A, Fitzgerald DJ, Maguire PB (2004) Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions. Blood 103:2096–2104

Cosemans JM, Van Kruchten R, Olieslagers S, Schurgers LJ, Verheyen FK, Munnix IC, Waltenberger J, Angelillo-Scherrer A, Hoylaerts MF, Carmeliet P, Heemskerk JW (2010) Potentiating role of Gas6 and Tyro3, Axl and Mer (TAM) receptors in human and murine platelet activation and thrombus stabilization. J Thromb Haemost 8:1797–1808

Dellas C, Schäfer K, Rohm IK, Lankeit M, Leifheit M, Loskutoff DJ, Hasenfuss G, Konstantinides SV (2007) Leptin signalling and leptin-mediated activation of human platelets: importance of JAK2 and the phospholipases Cgamma2 and A2. Thromb Haemost 98(106):3–71

Di Michele M, Thys C, Waelkens E, Overbergh L, D'Hertog W, Mathieu C, De Vos R, Peerlinck K, Van Geet C, Freson K (2011) An integrated proteomics and genomics analysis to unravel a heterogeneous platelet secretion defect. J Proteomics 74:902–913

Dwyer KM, Robson SC, Nandurkar HH, Campbell DJ, Gock H, Murray-Segal LJ, Fisicaro N, Mysore TB, Kaczmarek E, Cowan PJ, d'Apice AJ (2004) Thromboregulatory manifestations in human CD39 transgenic mice and the implications for thrombotic disease and transplantation. J Clin Invest 113:1440–1446

Enjyoji K, Sévigny J, Lin Y, Frenette PS, Christie PD, Esch JS 2nd, Imai M, Edelberg JM, Rayburn H, Lech M, Beeler DL, Csizmadia E, Wagner DD, Robson SC, Rosenberg RD (1999) Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. Nat Med 5:1010–1017

Foy M, Maguire PB (2007) Recent advances in the characterisation of the platelet membrane system by proteomics. Curr Pharm Des 13:2647–2655

Freson K, Hoylaerts MF, Jaeken J, Eyssen M, Arnout J, Vermylen J, Van Geet C (2001) Genetic variation of the extra-large stimulatory G protein alpha-subunit leads to Gs hyperfunction in platelets and is a risk factor for bleeding. Thromb Haemost 86:733–738

Freson K, Jaeken J, Van Helvoirt M, de Zegher F, Wittevrongel C, Thys C, Hoylaerts MF, Vermylen J, Van Geet C (2003) Functional polymorphisms in the paternally expressed XLalphas and its cofactor ALEX decrease their mutual interaction and enhance receptor-mediated cAMP formation. Hum Mol Genet 12:1121–1130

Freson K, Hashimoto H, Thys C, Wittevrongel C, Danloy S, Morita Y, Shintani N, Tomiyama Y, Vermylen J, Hoylaerts MF, Baba A, Van Geet C (2004) The pituitary adenylate cyclase-activating polypeptide is a physiological inhibitor of platelet activation. J Clin Invest 113:905–912

Freson K, Izzi B, Jaeken J, Van Helvoirt M, Thys C, Wittevrongel C, de Zegher F, Van Geet C (2008) Compound heterozygous mutations in the GNAS gene of a boy with morbid obesity, thyroid-stimulating hormone resistance, pseudohypoparathyroidism, and a prothrombotic state. J Clin Endocrinol Metab 93:4844–4849

Garcia BA, Smalley DM, Cho H et al (2005) The platelet microparticle proteome. J Proteome Res 4:1516–1521

Goodall AH, Burns P, Salles I, Macaulay IC, Jones CI, Ardissino D, de Bono B, Bray SL, Deckmyn H, Dudbridge F, Fitzgerald DJ, Garner SF, Gusnanto A, Koch K, Langford C, O'Connor MN, Rice CM, Stemple D, Stephens J, Trip MD, Zwaginga JJ, Samani NJ, Watkins NA, Maguire PB, Ouwehand WH, Bloodomics Consortium (2010) Transcription profiling in human platelets reveals LRRFIP1 as a novel protein regulating platelet function. Blood 116:4646–4656

Goschnick MW, Lau LM, Wee JL, Liu YS, Hogarth PM, Robb LM, Hickey MJ, Wright MD, Jackson DE (2006) Impaired "outside-in" integrin alphaIIbbeta3 signaling and thrombus stability in TSSC6-deficient mice. Blood 108:1911–1918

Graham GJ, Ren Q, Dilks JR, Blair P, Whiteheart SW, Flaumenhaft R (2009) Endobrevin/VAMP-8-dependent dense granule release mediates thrombus formation in vivo. Blood 114:1083–1090

Han J, Kori R, Shui JW, Chen YR, Yao Z, Tan TH (2003) The SH3 domain-containing adaptor HIP-55 mediates c-Jun N-terminal kinase activation in T cell receptor signaling. J Biol Chem 278:52195–52202

Harper MT, Poole AW (2010) Diverse functions of protein kinase C isoforms in platelet activation and thrombus formation. J Thromb Haemost 8:454–462

Hernandez-Ruiz L, Valverde F, Jimenez-Nuñez MD, Ocaña E, Sáez-Benito A, Rodríguez-Martorell J, Bohórquez JC, Serrano A, Ruiz FA (2007) Organellar proteomics of human platelet dense granules reveals that 14-3-3zeta is a granule protein related to atherosclerosis. J Proteome Res 6:4449–4457

Herrera-Galeano JE, Becker DM, Wilson AF, Yanek LR, Bray P, Vaidya D, Faraday N, Becker LC (2008) A novel variant in the platelet endothelial aggregation receptor-1 gene is associated with increased platelet aggregability. Arterioscler Thromb Vasc Biol 28:1484–1490

Hirsch E, Bosco O, Tropel P, Laffargue M, Calvez R, Altruda F, Wymann M, Montrucchio G (2001) Resistance to thromboembolism in PI3Kgamma-deficient mice. FASEB J 15:2019–2021

Jackson SP, Schoenwaelder SM, Goncalves I, Nesbitt WS, Yap CL, Wright CE, Kenche V, Anderson KE, Dopheide SM, Yuan Y, Sturgeon SA, Prabaharan H, Thompson PE, Smith GD, Shepherd PR, Daniele N, Kulkarni S, Abbott B, Saylik D, Jones C, Lu L, Giuliano S, Hughan SC, Angus JA, Robertson AD, Salem HH (2005) PI 3-kinase p110beta: a new target for antithrombotic therapy. Nat Med 11:507–514

Johnson AD, Yanek LR, Chen MH, Faraday N, Larson MG, Tofler G, Lin SJ, Kraja AT, Province MA, Yang Q, Becker DM, O'Donnell CJ, Becker LC (2010) Genome-wide meta-analyses identifies seven loci associated with platelet aggregation in response to agonists. Nat Genet 42:608–613

Jones CI, Bray S, Garner SF, Stephens J, de Bono B, Angenent WG, Bentley D, Burns P, Coffey A, Deloukas P, Earthrowl M, Farndale RW, Hoylaerts MF, Koch K, Rankin A, Rice CM, Rogers J, Samani NJ, Steward M, Walker A, Watkins NA, Akkerman JW, Dudbridge F, Goodall AH, Ouwehand WH, Bloodomics Consortium (2009) A functional genomics approach reveals novel quantitative trait loci associated with platelet signaling pathways. Blood 114:1405–1416

Konstantinides S, Schäfer K, Koschnick S, Loskutoff DJ (2001) Leptin-dependent platelet aggregation and arterial thrombosis suggests a mechanism for atherothrombotic disease in obesity. J Clin Invest 108:1533–1540

Kunert S, Meyer I, Fleischhauer S, Wannack M, Fiedler J, Shivdasani RA, Schulze H (2009) The microtubule modulator RanBP10 plays a critical role in regulation of platelet discoid shape and degranulation. Blood 114:5532–5540

Lewandrowski U, Wortelkamp S, Lohrig K, Zahedi RP, Wolters DA, Walter U, Sickmann A (2009) Platelet membrane proteomics: a novel repository for functional research. Blood 114: e10–e19

Li W, He M, Zhou H, Bourne JW, Liang P (2006) Mutational data integration in gene-oriented files of the Hermansky-Pudlak Syndrome database. Hum Mutat 27:402–407

Lin HF, Traver D, Zhu H, Dooley K, Paw BH, Zon LI, Handin RI (2005) Analysis of thrombocyte development in CD41-GFP transgenic zebrafish. Blood 106:3803–3810

Lindemann S, Gawaz M (2007) The active platelet: translation and protein synthesis in an anucleate cell. Semin Thromb Hemost 33:144–150

Macaulay IC, Tijssen MR, Thijssen-Timmer DC, Gusnanto A, Steward M, Burns P, Langford CF, Ellis PD, Dudbridge F, Zwaginga JJ, Watkins NA, van der Schoot CE, Ouwehand WH (2007) Comparative gene expression profiling of in vitro differentiated megakaryocytes and erythroblasts identifies novel activatory and inhibitory platelet membrane proteins. Blood 109:3260–3269

Marcus K, Immler D, Sternberger J, Meyer HE (2000) Identification of platelet proteins separated by two-dimensional gel electrophoresis and analyzed by matrix assisted laser desorption/ionization-time of flight-mass spectrometry and detection of tyrosine-phosphorylated proteins. Electrophoresis 21:2622–2636

Marcus AJ, Broekman MJ, Drosopoulos JH, Islam N, Pinsky DJ, Sesti C, Levi R (2003) Metabolic control of excessive extracellular nucleotide accumulation by CD39/ecto-nucleotidase-1: implications for ischemic vascular diseases. J Pharmacol Exp Ther 305:9–16

Maurer-Spurej E, Kahr WH, Carter CJ, Pittendreigh C, Cameron M, Cyr TD (2008) The value of proteomics for the diagnosis of a platelet-related bleeding disorder. Platelets 19:342–351

Maynard DM, Heijnen HF, Horne MK, White JG, Gahl WA (2007) Proteomic analysis of platelet alpha-granules using mass spectrometry. J Thromb Haemost 5:1945–1955

Maynard DM, Heijnen HF, Gahl WA, Gunay-Aygun M (2010) The α-granule proteome: novel proteins in normal and ghost granules in gray platelet syndrome. J Thromb Haemost 8:1786–1796

Meisinger C, Prokisch H, Gieger C, Soranzo N, Mehta D, Rosskopf D, Lichtner P, Klopp N, Stephens J, Watkins NA, Deloukas P, Greinacher A, Koenig W, Nauck M, Rimmbach C, Völzke H, Peters A, Illig T, Ouwehand WH, Meitinger T, Wichmann HE, Döring A (2009) A genome-wide association study identifies three loci associated with mean platelet volume. Am J Hum Genet 84:66–71

Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, Culler MD, Coy DH (1989) Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochem Biophys Res Commun 164:567–574

Moebius J, Zahedi RP, Lewandrowski U, Berger C, Walter U, Sickmann A (2005) The human platelet membrane proteome reveals several new potential membrane proteins. Mol Cell Proteomics 4:1754–1761

Mori J, Pearce AC, Spalton JC, Grygielska B, Eble JA, Tomlinson MG, Senis YA, Watson SP (2008) G6b-B inhibits constitutive and agonist-induced signaling by glycoprotein VI and CLEC-2. J Biol Chem 283:35419–35427

Moser M, Bauer M, Schmid S, Ruppert R, Schmidt S, Sixt M, Wang HV, Sperandio M, Fässler R (2009) Kindlin-3 is required for beta2 integrin-mediated leukocyte adhesion to endothelial cells. Nat Med 15:300–305

Naik MU, Nigam A, Manrai P, Millili P, Czymmek K, Sullivan M, Naik UP (2009) CIB1 deficiency results in impaired thrombosis: the potential role of CIB1 in outside-in signaling through integrin alpha IIb beta 3. J Thromb Haemost 7:1906–1914

Nanda N, Bao M, Lin H, Clauser K, Komuves L, Quertermous T, Conley PB, Phillips DR, Hart MJ (2005) Platelet endothelial aggregation receptor 1 (PEAR1), a novel epidermal growth factor repeat-containing transmembrane receptor, participates in platelet contact-induced activation. J Biol Chem 280:24680–24689

Newland SA, Macaulay IC, Floto AR, de Vet EC, Ouwehand WH, Watkins NA, Lyons PA, Campbell DR (2007) The novel inhibitory receptor G6B is expressed on the surface of platelets and attenuates platelet function in vitro. Blood 109:4806–4809

Noé L, Di Michele M, Giets E, Thys C, Wittevrongel C, De Vos R, Overbergh L, Waelkens E, Jaeken J, Van Geet C, Freson K (2010a) Platelet Gs hypofunction and abnormal morphology resulting from a heterozygous RGS2 mutation. J Thromb Haemost 8:1594–1603

Noé L, Peeters K, Izzi B, Van Geet C, Freson K (2010b) Regulators of platelet cAMP levels: clinical and therapeutic implications. Curr Med Chem 17:2897–2905

O'Connor MN, Salles II, Cvejic A, Watkins NA, Walker A, Garner SF, Jones CI, Macaulay IC, Steward M, Zwaginga JJ, Bray SL, Dudbridge F, de Bono B, Goodall AH, Deckmyn H, Stemple DL, Ouwehand WH, Bloodomics Consortium (2009) Functional genomics in zebrafish permits rapid characterization of novel platelet membrane proteins. Blood 113:4754–4762

O'Donnell CJ, Larson MG, Feng D, Sutherland PA, Lindpaintner K, Myers RH, D'Agostino RA, Levy D, Tofler GH, Framingham Heart Study (2001) Genetic and environmental contributions to platelet aggregation: the Framingham heart study. Circulation 103:3051–3056

Panzer S, Höcker L, Koren D (2006) Agonists-induced platelet activation varies considerably in healthy male individuals: studies by flow cytometry. Ann Hematol 85:121–125

Parguiña AF, Grigorian-Shamajian L, Agra RM, Teijeira-Fernández E, Rosa I, Alonso J, Viñuela-Roldán JE, Seoane A, González-Juanatey JR, García A (2010) Proteins involved in platelet signaling are differentially regulated in acute coronary syndrome: a proteomic study. PLoS One 5:e13404

Park SK, Olson TA, Ercal N, Summers M, O'Dorisio MS (1996) Characterization of vasoactive intestinal peptide receptors on human megakaryocytes and platelets. Blood 87:4629–4635

Peeters K, Stassen JM, Collen D, Van Geet C, Freson K (2008) Emerging treatments for thrombocytopenia: increasing platelet production. Drug Discov Today 13:798–806

Piersma SR, Broxterman HJ, Kapci M, de Haas RR, Hoekman K, Verheul HM, Jiménez CR (2009) Proteomics of the TRAP-induced platelet releasate. J Proteomics 72:91–109

Pinsky DJ, Broekman MJ, Peschon JJ, Stocking KL, Fujita T, Ramasamy R, Connolly ES Jr, Huang J, Kiss S, Zhang Y, Choudhri TF, McTaggart RA, Liao H, Drosopoulos JH, Price VL, Marcus AJ, Maliszewski CR (2002) Elucidation of the thromboregulatory role of CD39/ectoapyrase in the ischemic brain. J Clin Invest 109:1031–1040

Pozgajová M, Sachs UJ, Hein L, Nieswandt B (2006) Reduced thrombus stability in mice lacking the alpha2A-adrenergic receptor. Blood 108:510–514

Premsler T, Lewandrowski U, Sickmann A, Zahedi RP (2011) Phosphoproteome analysis of the platelet plasma membrane. Methods Mol Biol 728:279–290

Qureshi AH, Chaoji V, Maiguel D, Faridi MH, Barth CJ, Salem SM, Singhal M, Stoub D, Krastins B, Ogihara M, Zaki MJ, Gupta V (2009) Proteomic and phospho-proteomic profile of human platelets in basal, resting state: insights into integrin signaling. PLoS One 4:e7627

Reinhardt C, von Brühl ML, Manukyan D, Grahl L, Lorenz M, Altmann B, Dlugai S, Hess S, Konrad I, Orschiedt L, Mackman N, Ruddock L, Massberg S, Engelmann B (2008) Protein disulfide isomerase acts as an injury response signal that enhances fibrin generation via tissue factor activation. J Clin Invest 118:1110–1122

Sacristan D, Marques M, Zamorano J et al (2008) Modifications by Olmesartan medoxomil treatment of the platelet protein profile of moderate hypertensive patients. Proteomics Clin Appl 2:1300–1312

Schäfer K, Halle M, Goeschen C, Dellas C, Pynn M, Loskutoff DJ, Konstantinides S (2004) Leptin promotes vascular remodeling and neointimal growth in mice. Arterioscler Thromb Vasc Biol 24:112–117

Schulz C, Leuschen NV, Fröhlich T, Lorenz M, Pfeiler S, Gleissner CA, Kremmer E, Kessler M, Khandoga AG, Engelmann B, Ley K, Massberg S, Arnold GJ (2010) Identification of novel downstream targets of platelet glycoprotein VI activation by differential proteome analysis: implications for thrombus formation. Blood 115:4102–4110

Senis YA, Antrobus R, Severin S, Parguiña AF, Rosa I, Zitzmann N, Watson SP, García A (2009a) Proteomic analysis of integrin alphaIIbbeta3 outside-in signaling reveals Src-kinase-independent phosphorylation of Dok-1 and Dok-3 leading to SHIP-1 interactions. J Thromb Haemost 7:1718–1726

Senis YA, Tomlinson MG, Ellison S, Mazharian A, Lim J, Zhao Y, Kornerup KN, Auger JM, Thomas SG, Dhanjal T, Kalia N, Zhu JW, Weiss A, Watson SP (2009b) The tyrosine phosphatase CD148 is an essential positive regulator of platelet activation and thrombosis. Blood 113:4942–4954

Siller-Matula JM, Krumphuber J, Jilma B (2010) Pharmacokinetic, pharmacodynamic and clinical profile of novel antiplatelet drugs targeting vascular diseases. Br J Pharmacol 159:502–517

Soranzo N, Rendon A, Gieger C, Jones CI, Watkins NA, Menzel S, Döring A, Stephens J, Prokisch H, Erber W, Potter SC, Bray SL, Burns P, Jolley J, Falchi M, Kühnel B, Erdmann J, Schunkert H, Samani NJ, Illig T, Garner SF, Rankin A, Meisinger C, Bradley JR, Thein SL, Goodall AH, Spector TD, Deloukas P, Ouwehand WH (2009a) A novel variant on chromosome 7q22.3 associated with mean platelet volume, counts, and function. Blood 113:3831–3837

Soranzo N, Spector TD, Mangino M, Kühnel B, Rendon A, Teumer A, Willenborg C, Wright B, Chen L, Li M, Salo P, Voight BF, Burns P, Laskowski RA, Xue Y, Menzel S, Altshuler D, Bradley JR, Bumpstead S, Burnett MS, Devaney J, Döring A, Elosua R, Epstein SE, Erber W, Falchi M, Garner SF, Ghori MJ, Goodall AH, Gwilliam R, Hakonarson HH, Hall AS, Hammond N, Hengstenberg C, Illig T, König IR, Knouff CW, McPherson R, Melander O, Mooser V, Nauck M, Nieminen MS, O'Donnell CJ, Peltonen L, Potter SC, Prokisch H, Rader DJ, Rice CM, Roberts R, Salomaa V, Sambrook J, Schreiber S, Schunkert H, Schwartz SM, Serbanovic-Canic J, Sinisalo J, Siscovick DS, Stark K, Surakka I, Stephens J, Thompson JR, Völker U, Völzke H, Watkins NA, Wells GA, Wichmann HE, Van Heel DA, Tyler-Smith C,

Thein SL, Kathiresan S, Perola M, Reilly MP, Stewart AF, Erdmann J, Samani NJ, Meisinger C, Greinacher A, Deloukas P, Ouwehand WH, Gieger C (2009b) A genome-wide meta-analysis identifies 22 loci associated with eight hematological parameters in the HaemGen consortium. Nat Genet 41:1182–1190

Springer DL, Miller JH, Spinelli SL, Pasa-Tolic L, Purvine SO, Daly DS, Zangar RC, Jin S, Blumberg N, Francis CW, Taubman MB, Casey AE, Wittlin SD, Phipps RP (2009) Platelet proteome changes associated with diabetes and during platelet storage for transfusion. J Proteome Res 8:2261–2272

Tams JW, Johnsen AH, Fahrenkrug J (1999) Identification of pituitary adenylate cyclase-activating polypeptide1-38-binding factor in human plasma, as ceruloplasmin. Biochem J 341:271–276

Tournoij E, Weber GJ, Akkerman JW, de Groot PG, Zon LI, Moll FL, Schulte-Merker S (2010) Mlck1a is expressed in zebrafish thrombocytes and is an essential component of thrombus formation. J Thromb Haemost 8:588–595

Tucker KL, Sage T, Stevens JM, Jordan PA, Jones S, Barrett NE, St-Arnaud R, Frampton J, Dedhar S, Gibbins JM (2008) A dual role for integrin-linked kinase in platelets: regulating integrin function and alpha-granule secretion. Blood 112:4523–4531

Tucker KL, Kaiser WJ, Bergeron AL, Hu H, Dong JF, Tan TH, Gibbins JM (2009) Proteomic analysis of resting and thrombin-stimulated platelets reveals the translocation and functional relevance of HIP-55 in platelets. Proteomics 9:4340–4354

Vaudry D, Falluel-Morel A, Bourgault S, Basille M, Burel D, Wurtz O, Fournier A, Chow BK, Hashimoto H, Galas L, Vaudry H (2009) Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. Pharmacol Rev 61:283–357

Xiang YZ, Kang LY, Gao XM, Shang HC, Zhang JH, Zhang BL (2008) Strategies for antiplatelet targets and agents. Thromb Res 123:35–49

Yee DL, Sun CW, Bergeron AL, Dong JF, Bray PF (2005) Aggregometry detects platelet hyperreactivity in healthy individuals. Blood 106:2723–2729

Yee DL, Bergeron AL, Sun CW, Dong JF, Bray PF (2006) Platelet hyperreactivity generalizes to multiple forms of stimulation. J Thromb Haemost 4:2043–2050

Zahedi RP, Lewandrowski U, Wiesner J, Wortelkamp S, Moebius J, Schütz C, Walter U, Gambaryan S, Sickmann A (2008) Phosphoproteome of resting human platelets. J Proteome Res 7:526–534

Variability of Platelet Indices and Function: Acquired and Genetic Factors

Giovanni de Gaetano, Iolanda Santimone, Francesco Gianfagna, Licia Iacoviello, and Chiara Cerletti

Contents

Abstract Each individual has an inherent variable risk of bleeding linked to genetic or acquired abnormal platelet number or platelet dysfunction. In contrast, it is less obvious that the variability of platelet phenotypes (number, mean platelet volume, function) may contribute to the variable individual risk of thrombosis. Interindividual variability of platelet indices or function may be either due to acquired factors, such as age, sex, metabolic variables, smoke, dietary habits, and ongoing inflammation, or due to genetic factors. Acquired variables explain a small portion of the heterogeneity of platelet parameters. Genetic factors, instead, appear

G. de Gaetano (✉) • I. Santimone • F. Gianfagna •
L. Iacoviello • C. Cerletti
Research Laboratories, Fondazione di Ricerca e Cura "Giovanni Paolo II", Università Cattolica, Largo Gemelli, 1, 86100 Campobasso, Italy
e-mail: giovanni.degaetano@moli-sani.org

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_16,

to play a major role, although a consistent portion of such a genetic variance has not yet been attributed to any specific genetic factor, possibly due to the high number of DNA loci potentially involved and to the limited effect size of each individual SNP. A portion of variance remains thus unexplained, also due to variability of test performance. A major contradiction in present platelet knowledge is, indeed, the difficulty to reconcile the universally accepted importance of platelet indices or function and the lack of reliable platelet parameters in cardiovascular risk prediction models. Trials on antiplatelet drugs were generally designed to select a homogeneous sample, whose results could be applied to an "average subject," tending to exclude the deviation/extreme values. As the current indications for antiplatelet treatment in primary or secondary prevention of ischemic vascular disease still derive from the results of such clinical trials where platelet function and its variability was not investigated, we cannot at present rely upon any current platelet test to either initiate, or monitor, or modify or stop treatment with any antiplatelet drug. Evidence is, however, increasing that traditional platelet aggregometry and other more recently developed platelet function assays could be useful to optimize antiplatelet therapy and to predict major adverse cardiac events.The observation of interindividual differences in platelet response to antiplatelet drugs has enlarged the spectrum and the possible clinical relevance of the variability of platelet indices or function. The development of "personalized medicine" will benefit from the concepts discussed in this chapter.

Keywords Platelet count • Platelet indices • Platelet volume (mean) • Platelet function • Variability • Antiplatelet drugs • Aspirin • Clopidogrel • Genetic factors • Personalized medicine

A sower went out to sow. And as he sowed, some seed fell on the path, and birds came and ate it up. Some fell on rocky ground, where it had little soil. It sprang up at once because the soil was not deep, and when the sun rose it was scorched, and it withered for lack of roots. Some seed fell among thorns, and the thorns grew up and choked it. But some seed fell on rich soil, and produced fruit, a hundred or sixty or thirtyfold (Matthew 13, 3–8)

On that night two people will be sleeping in the same bed, but only one will be taken. The other will be left. Two women will be together grinding wheat, but only one will be taken. The other will be left. Two men will be in the same field, but only one will be taken. The other will be left (Luke 17, 34–36)

1 Introduction

The variability of platelet number and volume or of platelet function within a population is long known and there are a number of clinical conditions characterized by thrombocytopenia or thrombocytosis or of variants affecting platelet function, in

particular, those leading to clinical hemorrhagic phenotypes, such as Glazmann thrombasthenia or von Willebrand disease (Nurden and Nurden 2011). On the other hand, the concept that pro-thrombotic platelet phenotypes may also vary is less obvious and has not been fully developed so far. While platelet function defects that lead to bleeding are known to be caused by a single genetic defect, thrombosis in the scenery of cardiovascular disease is a multifactorial phenomenon recognizing multiple pathogenetic factors. Therefore, while it is accepted that each individual has an inherent risk of bleeding, it is less obvious that the variability of platelet phenotypes may contribute to the individual risk of thrombosis.

More recently, the observation of interindividual differences in platelet response to antiplatelet drugs has enlarged the spectrum and the possible clinical relevance of platelet variability (Quinn and Topol 2001; Faraday et al. 2007a). Attention to this crucial issue started with the clinical observation that the well-established efficacy of antiplatelet treatment in the prevention of recurrent thrombosis and cardiovascular disease, based on large controlled trial results (Antithrombotic Trialists' Collaboration 2002), was restricted to a limited portion of the treated populations. The occurrence of treatment failure in a multifactorial condition, such as thrombosis, is not surprising (Patrono 2003). The novelty of the research approach developed in the past few years consists in the search for a possible correlation between platelet phenotypes—either basal or on antiplatelet treatment—and clinical outcomes (de Gaetano and Cerletti 2003).

The issue of variability of platelet function in response to aspirin, initially referred to as "aspirin resistance" but lately extended also to clopidogrel, became rapidly fashionable and stimulated a large amount of research aimed at correctly defining and detecting platelet variability (de Gaetano and Cerletti 2003; Patrono 2003). Interindividual variability of platelet indices or function may be due either to acquired factors, such as age, sex, metabolic variables, smoke, dietary habits, ongoing inflammation, or to genetic factors. In turn, this variability may affect the efficacy and safety of antiplatelet drugs used in the treatment and prevention of thrombosis.

In this chapter, we shall discuss interindividual variability of major platelet indices and of functional response to agonists and/or to antiplatelet agents, in relation to its possible clinical relevance and to major acquired or genetic factors contributing to their variability.

2 Variability of Platelet Count and Mean Platelet Volume

2.1 Clinical Relevance

Besides the widely used platelet count, mean platelet volume (MPV) is the most commonly measured platelet index. An inverse correlation between platelet count and MPV was frequently reported and could be explained by the need to maintain constant platelet functional mass. However, platelet count and MPV both increase

during stimulated thrombopoiesis, suggesting that they may be regulated, at least partially, by independent mechanisms (Garg et al. 1971; Thompson and Jakubowski 1988; Smith et al. 2002; De Luca et al. 2010).

Early studies suggested the use of MPV as a potential marker of platelet reactivity (Bath and Butterworth 1996; van der Loo and Martin 1999; Bath et al. 2004). Larger platelets might have greater pro-thrombotic potential (Chu et al. 2010), being denser and containing more alpha granules, dense granules, and lysosomes, which release pro-thrombotic factors (Hendra et al. 1988; Thompson and Jakubowski 1988; White 2007). They are metabolically and enzymatically more active than smaller platelets, with increased thromboxane (Tx) A_2 biosynthesis and glycoprotein (GP) IIb/IIIa receptor expression (Giles et al. 1994; Kamath et al. 2001). Larger platelets also show greater aggregability in response to ADP and decreased inhibition of aggregation by prostacyclin in vitro (Karpatkin et al. 1978; Jakubowski et al. 1985); they are also associated with increased reticulated platelets, an independent predictor of poor response to dual antiplatelet therapy (Guthikonda et al. 2008).

Epidemiologic studies suggest that both platelet count and MPV are related to the risk of cardiovascular disease. Indeed, vascular and nonvascular deaths have been associated to platelet count (Thaulow et al. 1991; van der Bom et al. 2009), while MPV has been associated to cardiovascular risk in a recent meta-analysis (Chu et al. 2010).

Previous investigators had observed a correlation between MPV and certain cardiovascular risk factors (Thaulow et al. 1991). A recent systematic review and meta-analysis concluded that MPV was associated with acute myocardial infarction, restenosis following angioplasty and mortality following myocardial infarction (MI) (Chu et al. 2010). In the largest study to date (Martin et al. 1991), Martin and colleagues reported that after a myocardial infarction, MPV is an independent risk factor for recurrent ischemia or death at 2 years of follow-up. Of note, a recent study found that statin, namely rosuvastatin, has MPV-reducing properties, an effect not correlated to changes in lipid values, suggesting a novel pleiotropic, possibly platelet-mediated, mechanism of cardiovascular benefit by this drug (Coban and Afacan 2008).

Moreover, MPV was associated with development and prognosis of MI and with cardiovascular intermediate phenotypes, such as diabetes mellitus, hypertension, hypercholesterolemia, and obesity (Chu et al. 2010). MPV could therefore be a novel common mechanism contributing to the risk of cardiovascular disease.

In view of the increasing importance of MPV as a marker or a predictor of cardiovascular risk, an effort should be made to standardize instruments and methods to measure it. MPV values may be influenced, for instance, by the anticoagulant used for blood collection and the time delay from sampling to analysis; in particular, EDTA may induce platelet swelling. The discordant results between different, and even the same, cell counters limit at present a wide clinical usefulness of MPV. Standards for this measure should also be established (Turner-Stokes et al. 1991).

Table 1 Platelet counts and prevalence of thrombocytopenia and thrombocytosis in men and women from Molise and Ogliastra regions

	Molise $n = 19{,}211$		Ogliastra (Sardinia) $n = 12{,}517$	
	Men	Women	Men	Women
Age (years)	55 ± 12	56 ± 12	44 ± 21	46 ± 21
Platelet count (10^9/L)	235 ± 59	261 ± 64	240 ± 65	260 ± 67
Thrombocytopenia *n* (%)	412 (4.7)	211 (2.2)	271 (4.9)	234 (3.3)
Thrombocytosis *n* (%)	100 (1.5)	259 (2.8)	87 (1.6)	196 (2.8)

Age and platelet count are reported as mean ± SD
Data extracted from Santimone et al. (2011) and Biino et al. (2011)

2.2 *Acquired Factors*

Both platelet count and MPV appear to be determined by environmental factors for a minor extent, while genetic factors account for a higher portion of variance. Platelet count may vary according to age, gender, endogenous variables, and ethnicity (Bain 1996; Segal and Moliterno 2006; Biino et al. 2011; Santimone et al. 2011). In the Ogliastra population, a Sardinia geographic isolate, including subjects with a large age range, Biino et al. (2011) showed that platelet count progressively decreases with increasing age, with a consequent increased number of thrombocytopenias and a decreased number of thrombocytosis in the elderly (Table 1). Our group (Santimone et al. 2011) took advantage of a large adult population-based survey, performed in the Molise region of Italy (Iacoviello et al. 2007; Centritto et al. 2009), to measure platelet indices and to study the association of these parameters with a number of biochemical, environmental, and clinical variables.

As reported in Table 1, despite the average younger age of the Ogliastra population, the parameters measured were comparable with those of the Molise population. Moreover, in both settings women had higher platelet count, lower prevalence of thrombocytopenia but higher of thrombocytosis. Figure 1 shows a significant decline of platelet count with age in both men and women from these two different populations. Such a decline may reflect a reduction in hematopoietic stem cell reserve during aging or a survival advantage in those subjects with lower platelet counts.

MPV increased with age (up to79 years) in men from Molise, while it remained constant in women (Santimone et al. 2011). Previous studies yielded contrasting results on the association between MPV and age, but a sex-specific analysis was not performed in any of them (Huczek et al. 2005; Berger et al. 2010; Braekkan et al. 2010). As women had not only higher platelet count but also larger MPV values than men, a hormonal influence in the regulation of both indices might be proposed. The process by which megakaryocytes proceed to proplatelet formation and platelet production is indeed under the influence of autocrine estrogen (Nagata et al. 2003). This is reinforced by the observation that estrogen-receptor antagonists inhibit

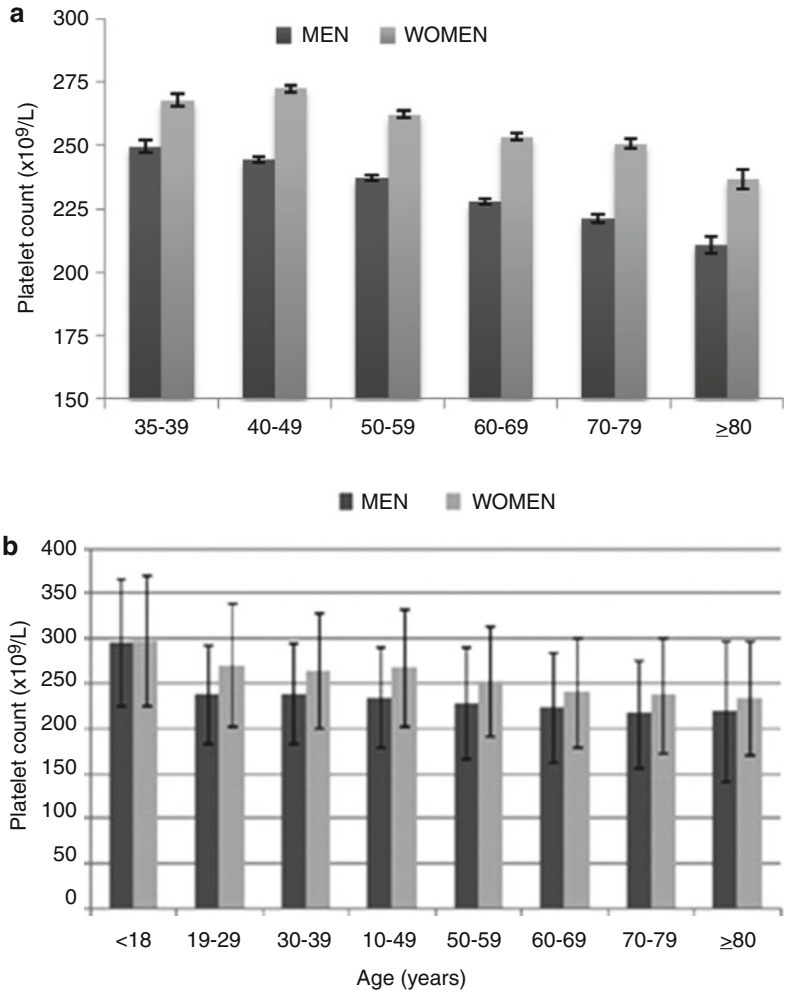


Fig. 1 Platelet count by age and sex in men and women from Molise (**a**) and Sardinia (**b**) regions. The difference between men and women holds at any age range, in both populations. For further details, see Santimone et al. (2011) and Biino et al. (2011). Modified with publisher permission from Santimone et al. (2011) and Biino et al. (2011)

platelet production in vivo (Segal and Moliterno 2006). This interpretation, however, is difficult to reconcile with the observation that differences between men and women persisted for both platelet indices at all age ranges, as well as after the menopause (Bain 1996; Santimone et al. 2011).

Variables such as total, HDL, or LDL cholesterol, glucose, triglycerides, body mass index (BMI), smoking habit, systolic or diastolic blood pressure, and antiplatelet drug use were all associated in a statistically significant manner to platelet count and/or to MPV (Fig. 2a, b), but each variable only explained by itself

less than 0.5 % of the variability in either platelet index (Santimone et al. 2011). Thus, a large number of nongenetic variables only explain a small proportion of the heterogeneity in platelet indices. The acquired determinants that explained most of the variability of both platelet indices were age, sex, white blood cell count, C-Reactive Protein and D-dimers, underlining an interesting relation between platelets, inflammation and activation of blood coagulation (Santimone et al. 2011).

As statistical differences in a given parameter among different groups might be due to the play of chance, platelet counts from the Moli-Sani population (Santimone et al. 2011) were compared among 12 groups formed according to individual zodiac signs: as shown in Fig. 3, no difference was apparent among any zodiacal group, supporting a real value of the statistical differences observed in other subgroups.

A significant variation in platelet number and in the prevalence of thrombocytopenia and thrombocytosis had been observed among the Ogliastra villages,

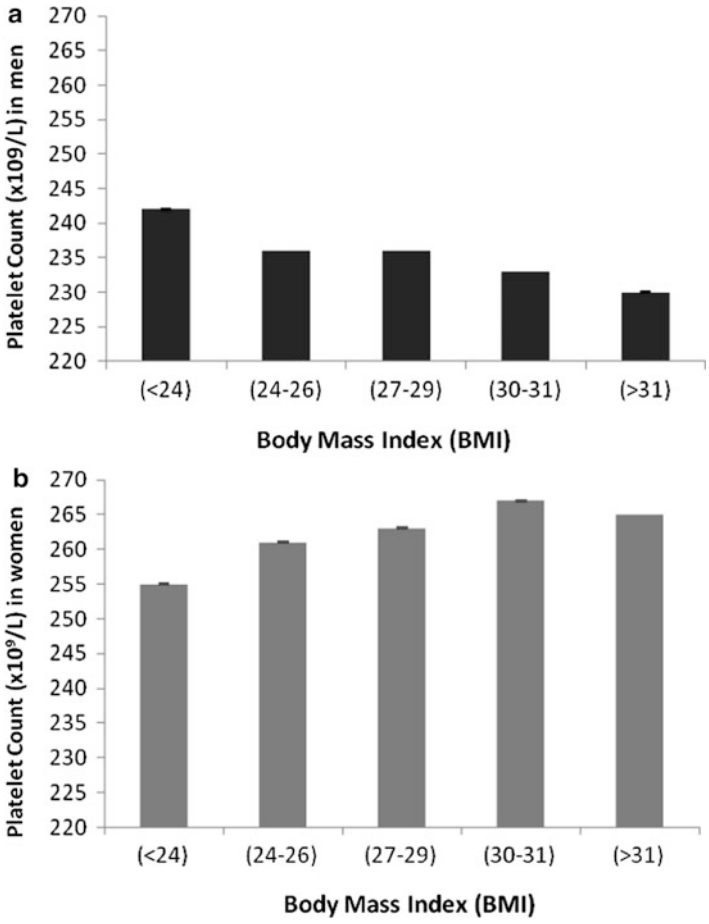


Fig. 2 (continued)

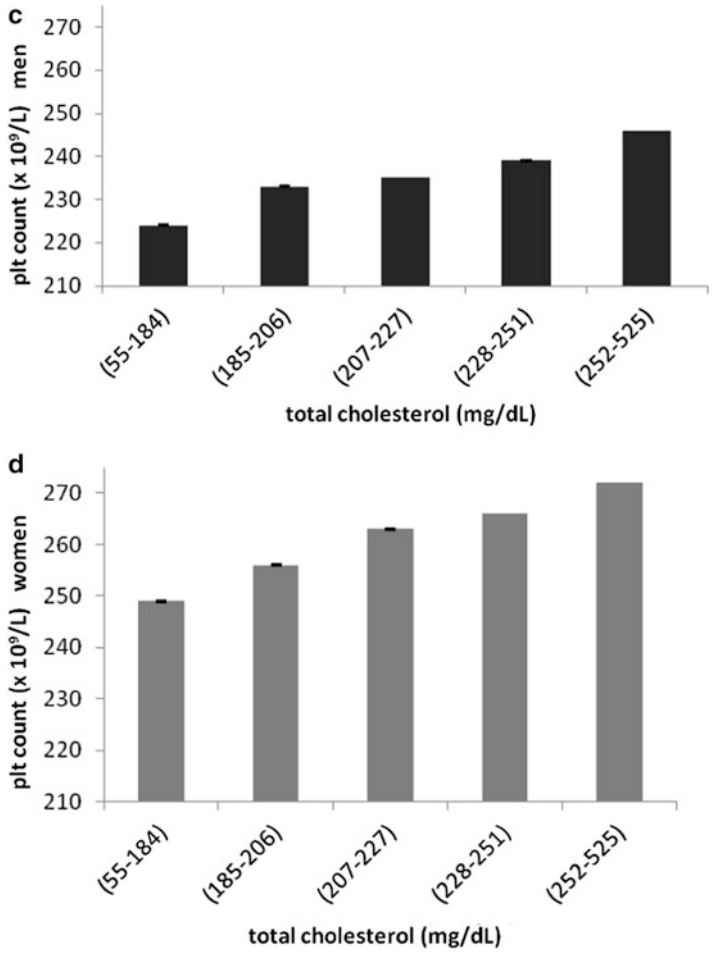


Fig. 2 Platelet count stratified for BMI (**a**, **b**) and total cholesterol (**c**, **d**) values in men (**a** and **c**) and women (**b** and **d**). Mean values are age adjusted. *Vertical bars* represent 95 % confidence intervals

considered to be genetic isolates (Biino et al. 2011). Our group (Santimone et al. 2011) confirmed a significant different distribution of platelet indices across Molise villages that cannot however be considered as genetic isolates (Fig. 4). In the absence of genetic analyses (still to be performed), this variation could not be explained by differences in other platelet indices determinants, or liver disorders or cancer distribution or the altitude above the sea of the villages or their distance from the recruitment centre.

Ethnicity had been considered a major factor in the variability of platelet indices (Bain 1996; Segal and Moliterno 2006). Our findings, together with Biino's data (Biino et al. 2011), indicate that there is a micro-heterogeneity in platelet

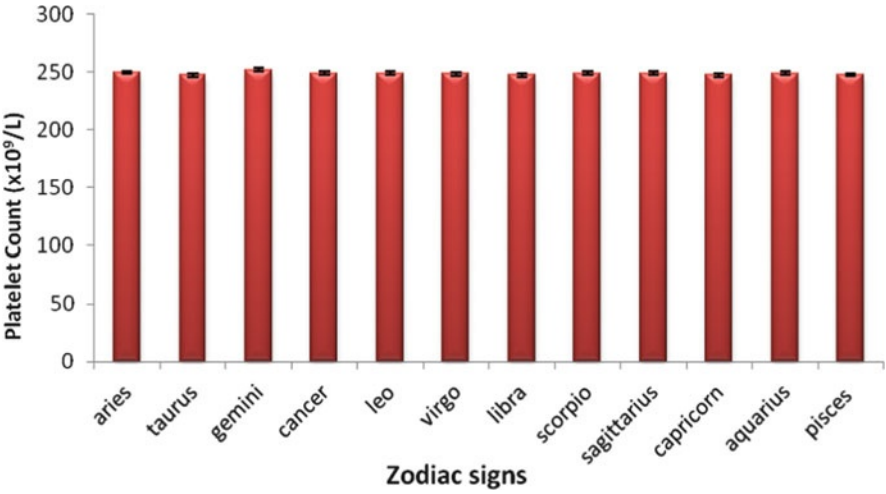


Fig. 3 Platelet count stratified for zodiac signs to have an estimate of chance effect

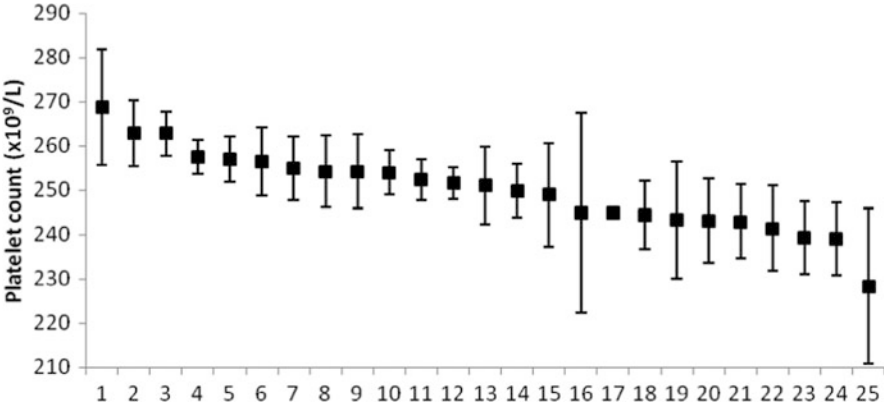


Fig. 4 Platelet count in 25 Molise villages. Mean values are age and sex adjusted. *Vertical bars* represent 95 % confidence intervals. Code for the Molise villages: Gildone = 1; Mirabello Sannitico = 2; Baranello = 3; Frosolone = 4; Ripalimosani = 5; Campodipietra = 6; Busso = 7; Toro = 8; Gambatesa = 9; Vinchiaturo = 10; Ferrazzano = 11; Bojano = 12; Petrella Tifernina = 13; Fossalto = 14; Riccia = 15; Molise = 16; Campobasso = 17; Matrice = 18; San Giovanni in Galdo = 19; Oratino = 20; Sepino = 21; Montagano = 22; Spinete = 23; Cercemaggiore = 24; Sant'Angelo Limosano = 25. Number of subjects from each Molise village: Gildone = 84; Mirabello Sannitico = 258; Baranello = 551; Frosolone = 968; Ripalimosani = 36; Campodipietra = 41; Busso = 278; Toro = 219; Gambatesa = 207; Vinchiaturo = 65; Ferrazzano = 641; Bojano = 1,086; Petrella Tifernina = 186; Fossalto = 378; Riccia = 105; Molise = 28; Campobasso = 10,439; Matrice = 239; San Giovanni in Galdo = 81; Oratino = 158; Sepino = 205; Montagano = 156; Spinete = 213; Cercemaggiore = 210; Sant'Angelo Limosano = 46. Modified with publisher permission from Santimone et al. (2011)

parameters even within the same general population formed by apparently ethnically homogeneous subjects living in the same country and even in the same region. The understanding of the regulation of platelet indices at a general population level, in genetic isolates or in different ethnic groups, is still limited and should be further investigated.

2.3 Genetic Factors

Genetics may greatly contribute to variation in platelet count and MPV (Lindemann et al. 1977; Whitfield and Martin 1985; Garner et al. 2000). Various studies indeed reported that inherited components explain a large part of the variability of platelet indices in normal people (Bray et al. 2007; Traglia et al. 2009; Kunicki and Nugent 2010; Vohnout et al. 2011). Heritability of both platelet count and MPV is high, consistently with that of other hemochrome parameters: variance component studies, aimed at dissecting phenotypic variance in genetic and environmental factors, reported heritability of 54–86 % for platelet number and 55–88 % for MPV, with Caucasians having lower values than African Americans. With the only exception of the Caucasian population (Bray et al. 2007a), the heritability of MPV is very high (>70 %), suggesting a strong genetic impact for this index. This is strengthened by the fact that MPV is only poorly conditioned by known environmental factors, at variance with platelet count, which appears to be mainly influenced by household effects (Johnson 2011).

During the last years, to better discriminate the DNA loci responsible for the phenotypic variability, both platelet count and MPV were the object of several genome-wide association studies (GWAS). These studies, aiming at identifying associations between a phenotype and thousands or even millions of single nucleotide polymorphisms (SNP)s in large samples, abandoned the concept of candidate gene studies, with the advantage to have a broad view of genetic factors but the disadvantage to deal with false positive results, due to the enormous number of associations tested. The majority of the GWAS signals were in fact located to noncoding regions, suggesting a major role for mechanisms that alter phenotypes at the level of transcriptional or posttranscriptional modifications, once excluded the potentially false positive results (Gianfagna et al. 2012a). However, a recent meta-analysis of GWAS identified 12 loci linked to MPV in regions near the genes with potential involvement in pathways plausibly related to the phenotype (Soranzo et al. 2009a), some of which were previously found to be involved in hematopoiesis (Table 2). Despite some plausibility related to the function of genes near the associated loci, the SNPs identified in this meta-analysis only explain 8.6 % of MPV variance, while heritability reaches even 88 % in variance component analyses (Soranzo et al. 2009a).

This suggests that a large number of SNPs could be involved, each exerting a relatively low effect, perhaps with different impact in different populations. The same meta-analysis also identified other three loci—only associated with platelet

Table 2 Genes lying in region associated with MPV and platelet count

Phenotype	Gene function	Gene	Other associated phenotypes
MPV	Cell adhesion	SIRPA	
		CD226	
	Transcriptional activation	WDR66	
		JMJD1C	PRP epinephrine (Johnson et al. 2010)
	Intracellular signaling	PIK3CG	PRP epinephrine (Johnson et al. 2010)
		ARHGEF3	
		TAOK1	PRP ADP (Johnson et al. 2010)
	Protein transport and endocytosis	DNM3	Collagen[a] stimulated platelet P-selectin (Goodall et al. 2010)
		EHD3	
		BET1L	
	Actin–myosin interaction and cell motility	TPM1	
	Transmembrane and coiled-coil domain family 2	TMCC2	
Platelet count	Intracellular signaling	SH2B3	Myocardial infarction (Soranzo et al. 2009a)
	Megakaryocyte maturation	JAK2	
	Apoptosis	BAK1	

Data extracted from Soranzo et al. (2009a), Goodall et al. (2010) and Johnson et al. (2010)
PRP Epinephrine and PRP ADP: platelet aggregation in platelet-rich plasma, induced by epinephrine or ADP, respectively
[a]Association with DNM3 transcript levels

count—that were involved in intracellular signaling (SH2B3), megakaryocyte maturation (JAK2), and apoptosis (BAK1). Furthermore, among the loci associated with MPV, three were also associated with platelet count, with the SNP alleles having a consistent opposite effect on either MPV or platelet count (raising the former, while decreasing the latter). According to conditional analyses, all SNPs appeared to exert their main effects through MPV (Soranzo et al. 2009a). This fact confirms the major impact of genetic regulation for MPV and suggests that a large portion of the platelet count heritability could be explained by loci regulating MPV. Our data from the Moli-family study suggest a high shared genetic regulation between platelet count and MPV, with genetic correlation coefficients of -0.54 ($p < 0.00001$) (Iacoviello et al. 2009).

Genetic results could help explain the link between platelet count/volume parameters (as markers of platelet activation) and platelet function or clinical events. In fact, associations were found between SNPs linked to either MPV or platelet count and platelet function or clinical events. SNPs in regions near the PIK3CG and JMJD1C genes were associated with platelet activation by collagen-related peptide and epinephrine (Soranzo et al. 2009b; Johnson et al. 2010), as well as DNM3 transcript levels were associated with platelet aggregation in platelet-rich plasma (PRP) (Goodall et al. 2010). More interestingly, SNPs in the 12q24 region (spanning 1.6 Mb and harboring 15 genes including SH2B3), associated with increased platelet count, were found to be also linked to MI occurrence in replication studies. Similarly, PHACTR1 locus (encoding for a phosphatase regulator), associated with early MI in a previous GWAS (Kathiresan et al. 2009), was recently

found to be also associated with platelet count (Ferreira et al. 2009). Genetic factors being involved both in platelet count or volume and in platelet function or cardiovascular risk could help dealing with the nature of the association between platelet count or volume and cardiovascular disease, i.e., whether increased MPV could be one of the causes or mainly a consequence (marker) of thrombotic events.

3 Variability of Platelet Function

3.1 Clinical Relevance

The variability in platelet activation has been systematically examined by relatively few studies only. Historically, platelet function testing was used to detect inherited or acquired platelet function disorders and its primary use remains for this purpose in routine practice to our days (Cattaneo 2003). More recently, platelet function techniques have been extended beyond the diagnosis of platelet function defects.

Numerous platelet function assays are currently available for the clinician's use, but the evidence of their clinical utility is still debated (Michelson et al. 2006). Although no single platelet function assay has been sufficiently studied to become part of standard clinical care (de Gaetano et al. 2008), the results so far available suggest that establishing meaningful correlations between platelet function tests and clinical outcomes appears to be a reasonably achievable goal. The great number and technical variety of platelet tests underline the complexity of platelet function, but also provides an integrated view of different biochemical pathways underlying the expression of normal or abnormal platelet function. In the early 1960s, platelet function testing advanced with the development of Born's aggregometer (Born 1962), a device that measured changes in the optical density of plasma during platelet aggregation. Although Born's aggregometer was envisioned to be used as an in vitro or an ex vivo tool for platelet pathways and drug discovery, it became the "gold standard" for investigating platelet disorders and the effect of drugs as inhibitors of platelet aggregation (Weiss et al. 1968; Vermylen et al. 1971). This occurred despite the fact that there was a lack of research into the clinical applicability of aggregometry. In small-scale trials using aggregometry, methodological differences in platelet function studies among different laboratories make it difficult to compare results (Hayward and Eikelboom 2007; Seyfert et al. 2007). This emphasizes again the need for consensus and standardization of assays before any clinical utility of platelet function tests be achieved (Michelson et al. 2006; Cattaneo et al. 2010). Besides aggregometry, many techniques in different settings were developed to measure platelet aggregation or release of granule content or glycoprotein expression characterizing platelet function. The availability of different techniques may provide information on the different steps of platelet activation cascades, potentially involved in atherothrombosis, to be integrated in a comprehensive evaluation. Platelets can be activated in vitro and, possibly, in vivo by

agonists, which trigger different activation pathways and therefore reveal different aspects of platelet function and variability. In some in vitro studies platelet agonists have been used in combination, inducing activation responses different from that elicited by each isolated stimulus (Rao et al. 1981; Cerletti et al. 1986; Di Minno et al. 1986; May et al. 1997).

Using different agonists and a broad range of concentrations, Yee et al. (2005) observed substantial interindividual variability in platelet aggregation in healthy subjects. They found that submaximal agonist concentrations identify small populations of individuals with platelets at increased reactivity; moreover, platelets with enhanced reactivity to one agonist showed enhanced reactivity to others, a phenomenon associated with female sex and higher fibrinogen levels. These data should encourage to further use the aggregometry (or other platelet function tests) to better define the platelet response phenotype in vitro and to assess the individual's risk for thrombosis.

3.2 Acquired Factors

Besides the laboratory test used, other issues that may cause platelet function variability in a population include blood drawing techniques, timing of blood collection, anticoagulant chosen, PRP preparation, type and concentration of agonists used to induce aggregation, as well as drug use and patient compliance to treatments (Michelson et al. 2006; de Gaetano and Cerletti 2007; Seyfert et al. 2007; de Gaetano et al. 2008; Femia et al. 2012). Interindividual platelet variation may also rely upon expression of receptors and adhesive surface proteins (usually measured by flow cytometric analyses). For instance, platelets with a high expression of GPVI receptors or Ca^{2+} signaling proteins may be more prone to phosphatidyl serine (PS) exposure than platelets with lower expression levels (Munnix et al. 2009). Furthermore, differences in the expression of signal desensitization proteins or in proteins involved in signal transduction may be related to an altered functional response (Hardy et al. 2005; Jones et al. 2009).

Maturing megakaryocytes show heterogeneity in Ca^{2+} signaling properties (Den Dekker et al. 2003) and aging platelets appear to change their surface characteristics by clustering and shedding of GPIb-V-IX (Hoffmeister et al. 2003). In addition, prolonged platelet storage results in reduced metabolic activity and gradual decrease in responsiveness (Curvers et al. 2004), while the response variation to phosphatidyl serine (PS)-exposing and non-PS-exposing platelets is maintained (Cauwenberghs et al. 2007).

A decreased platelet aggregatory response and platelet degranulation, detected as fibrinogen binding and P-selectin expression, in response to ADP and thrombin, was reported with increasing age in healthy adults (Knight et al. 1997). On the other hand, in comparison to adult, neonatal platelets showed hyposensitivity to stimulation with some agonists, such as thrombin or TRAP, but not with ADP, suggesting that the lower response of neonatal platelets is only limited to selected agonists.

Although reported in several papers, the effect of smoking on platelet function, including the proteomic pattern, is still controversial, possibly due to heterogeneity in platelet function tests used (Della Corte et al. 2012).

3.3 Genetic Factors

Studies in siblings, twins, and families with history of premature coronary artery disease suggest that endogenous and environmental factors only partially explain the interindividual variability in aggregation responses, which show high level of heritability for some platelet response phenotypes (Gaxiola et al. 1984; O'Donnell et al. 2001; Johnson et al. 2010). In the large population-based Framingham Heart Study, O'Donnell et al. (2001) demonstrated that platelet aggregation induced by epinephrine, or ADP, and collagen-induced lag phase in PRP, are determined by a number of covariates (age, sex, blood lipids, BMI, diabetes, alcohol use, estrogen status, antihypertensive drugs, and diastolic blood pressure) for 6 %, 7 %, and 4 % of variance, respectively. More recently, in families with a history of premature coronary artery disease (CAD), Bray et al. (2007a) confirmed these values in 687 Caucasian subjects: known cardiovascular risk factors added to the models showed a modest total contribution, which was never more than 16 % in any platelet phenotype, while the impact of genetic factors appeared to be higher.

As already mentioned, in the family-based study of Bray et al. (2007a), the total contribution of genetic factors was quite high with respect to known cardiovascular risk factors added to the models. The highest heritability resulted for platelet aggregation in PRP stimulated by ADP, epinephrine, or arachidonic acid (higher in African Americans than in Caucasians, >66 % vs. 36 %–42 %, respectively) and for PFA-100 closure time (60.9 % vs. 23.7 %), followed by platelet lag phase after collagen stimulation in PRP (50 % in both ethnic groups), whole blood platelet aggregation to ADP or high-dose collagen or arachidonic acid (28 %–58 %), and TxA_2 release (0 %–39 %). These high values are not overestimated by the inclusion in heritability of unmeasured household effect, since correlation analyses in spouse pairs gave lower values (O'Donnell et al. 2001). Furthermore, a recent study on extended families, each composed of 2–16 households, failed to show any impact of shared household factors on PFA-100 closure time (Iacoviello et al. 2009).

Most attempts to identify genetic differences influencing platelet function in normal persons relied on a candidate gene approach, mainly focusing on loci putatively regulating number and function of platelet surface receptors and their ligands (Kunicki and Nugent 2010). These results were not always replicated in independent populations, nor confirmed by meta-analyses. Candidate gene association studies were limited by SNP selection, since the number of polymorphisms throughout the DNA is enormous and even a set of well-selected SNPs could not represent the underlying sequence variation within its haplotype block.

More recently, several studies have applied genome-wide scans in large population identifying many loci associated with platelet function (Johnson et al. 2010;

Mathias et al. 2010; Guerrero et al. 2011). Johnson et al. (2010), in two European ancestry samples with replication in one African ancestry sample, identified regions strongly associated with platelet response to ADP (near the following genes: PEAR1, a receptor involved in cell–cell interactions; MRVI1, involved in intracellular signal pathways; SHH, involved in megakaryocyte differentiation), to epinephrine (ADRA2A, the primary receptor for epinephrine on platelet; PEAR1, PIK3CG, JMJD1C) and collagen lag time (GP6, the receptor mediating collagen response in platelets). Further loci in a region near genes plausibly involved in platelet function were found associated with some parameters with borderline statistical significance.

Some of the genes identified by Johnson et al. (2010) were previously found to be associated to platelet activation in candidate gene studies (ADRA2, GP6 and PEAR1) or even to other related traits as MPV (PIK3CG and JMJD1C) or venous thrombosis (GP6) in GWAS (Johnson 2011). The large number of loci associated with phenotypes in GWAS resulting distant from known genes, as well as the findings from the increasing transcriptomic technologies, suggest to search the reasons of phenotype variability in mechanisms driven by mRNAs. Therefore, GWAS results should be integrated with those from transcriptome profiling studies (Johnson 2011). Kondkar et al. (2010) identified 290 genes up- or down-regulated in platelets exhibiting hyper-responsiveness to low-dose epinephrine in aggregometry, including a member of v-SNARE proteins, previously found to be involved in the release of granule contents (Ren et al. 2007). Goodall et al. (2010) identified 63 transcripts as correlated with platelet responses to ADP and/or a collagen mimetic, several of which are transcribed from loci associated at borderline statistical significance in GWAS for platelet aggregation (Johnson et al. 2010). Human platelets express 284 miRNAs, with 74 miRNAs differentially expressed between subjects grouped according to platelet aggregation by epinephrine. Three miRNA knocked down the expression of related proteins, leading to reduced platelet activation when the PRKAR2B transcript is implicated; the latter is a cAMP-dependent protein kinase involved in signal transduction (Nagalla et al. 2011). Results from studies on endothelial cells and leukocytes should also be taken into account, due to their interactions with platelets and their consequent shared effects on phenotypes/disease.

A recent study identified a significant association between levels of platelet reactivity and SNPs in the 9p21 region, a region previously associated (and validated) with cardiovascular disease and several inflammatory disorders (Musunuru et al. 2010). STAT1 expression, a transcription factor playing a major role in driving the pro-inflammatory responses leading to endothelial cell activation and vascular damage was found to regulate the long mRNA ANRIL transcription present in this region in a vascular cell type (Sikorski et al. 2011). This finding suggests a link between CAD genetic susceptibility and the response to inflammatory signaling (Harismendy et al. 2011), perhaps mediated by platelet and leukocyte cross-talk. These results of a preliminary linkage analysis from the Moli-family study suggested the 9p21 region being also involved in platelet–leukocyte interaction, following platelet activation (Gianfagna et al. 2012b).

Platelet surface P-selectin, assessed by flow cytometry, is the most used marker of platelet activation. Variance component analysis from the Moli-family study population showed that basal P-selectin levels are determined to about 40 % by shared unknown environmental factors, while the addition of several covariates does not explain more than 6 % of variance. Its heritability was instead high (39.5 %) (Gianfagna et al. 2012b). Platelet activation-induced P-selectin surface expression was previously investigated in candidate gene studies (Jones et al. 2009; Freedman 2007), reporting high heritability values. Whereas the preceding studies focused on surface receptor genes, others encoding relevant proteins in signaling cascades downstream of these receptors were studied by the Bloodomics Consortium (Jones et al. 2009). The effects of 97 candidate genes involved in platelet signaling pathways were evaluated on four platelet functional phenotypes: P-selectin expression and fibrinogen binding to activated GPIIb/IIIa, in response to either ADP or collagen. Fifteen genes resulted to be associated with platelet activation, accounting for 30–74 % of the total variation in these four phenotypes, with variation in GP6 accounting for up to 40 % of the variation in GPVI signaling (Jones et al. 2009). These genes include cell surface receptor genes (CD36, GP6, ITGA2, PEAR1, and P2RY12), adapter proteins (FCERG1 and GNAZ, kinases JAK2, MA2K2, MAP2K4, and MAPK14), and intracellular signaling molecules (ITPR1 and VAV3) (Jones et al. 2009). Interestingly, PEAR1, previously found to be associated with platelet aggregation by GWAS, was identified as a regulator of both collagen and ADP signaling. This candidate gene study supports the suggestion that a comprehensive and well-performed selection of multiple SNPs from a set of plausibly related candidate genes could explain most of unexplained variance due to genetics.

There are apparently no studies investigating heritability or variance components of mechanisms underlying the interaction with leukocytes following platelet activation. Beyond their role in hemostasis and thrombosis, platelets are also involved in the onset of atherosclerosis, representing an important link between inflammation and atherothrombogenic processes. Platelets interact with inflammatory cells including leukocytes and endothelium, leading to leukocyte recruitment towards the vascular wall, initiating extravasation of circulating white cells and foam cell generation. Higher levels of circulating heterotypic aggregates are found in cardiovascular disease and in some inflammatory conditions (Totani and Evangelista 2010), suggesting they might be a marker of inflammation-mediated damage. The Moli-family study showed that mixed conjugate levels had noteworthy levels of heritability, with stimulated levels showing higher heritability than basal levels (Gianfagna et al. 2012b). Heritability due to the 9p21 region could be of relevance for stimulated mixed cell conjugate levels, as mentioned earlier, with nominally significant empirical p values ($p = 0.01$). These findings suggest the importance of studying the phases related to heterotypic aggregates formation after in vitro stimulus.

Other platelet function assays include unstimulated circulating biomarkers of vascular inflammation, as indirect markers of platelet activation, directly secreted by platelets and/or other cells. Variability in P-selectin soluble levels appears to be interesting, due to its association with cardiovascular events (Reiner et al. 2008) and to results related to SNPs in the same gene (SELP), showing an association with

circulating and surface P-selectin levels and carotid intima-media thickness. Furthermore, high heritability for transcript levels of SELP gene in platelets was previously reported (Freedman 2007; Barbalic et al. 2010).

Future studies are warranted to identify the key genetic variants that regulate platelet function to explain the missing heritability, to have better risk prediction models, and to lay the groundwork for rational pharmacogenetic approaches. It remains to be seen if any of these loci provide new therapeutic targets or contribute to a deeper understanding of common or rare conditions of thrombosis or hemostasis defects (Johnson 2011).

4 Variability of Platelet Response to Drugs

4.1 Clinical Relevance

Despite the efficacy of antiplatelet therapy in preventing cardiovascular events, shown by hundreds of controlled clinical trials and confirmed by several large meta-analyses (Antithrombotic Trialists' Collaboration 2002), many patients still experience ischemic events while on standardized antiplatelet treatments. This has spurred a new debate on the variability of platelet response, the optimal monitoring of antiplatelet therapy, and the relationship between ex vivo measurements and clinical outcomes (Patrono 2003; de Gaetano and Cerletti 2003, 2007; Michelson et al. 2006; de Gaetano et al. 2008).

The concept of a variable response to aspirin initially referred to as "aspirin resistance" was born together with the first observation that aspirin could interfere with factors of the hemostatic system (de Gaetano 2001). Indeed as early as 1966, Quick (1966) proposed an "aspirin tolerance test" based on the observation that not all patients with von Willebrand disease showed similar prolongation of the bleeding time after aspirin. When, few years later, the inhibitory effect of aspirin on platelet aggregation was described (Weiss et al. 1968), it was obvious that only some parameters of platelet function such as the second wave of aggregation induced by ADP or adrenaline, but not others, such as the aggregating response to thrombin, could be prevented by aspirin (O'Brien 1968). Shortly thereafter, Smith and Willis (1971) reported that aspirin suppresses platelet prostaglandin production, but not platelet aggregation by thrombin.

Despite the lack of any standardization of this technique, aggregation studies had allowed to establish already in 1968 that in some subjects doses of aspirin as low as 150 mg, much lower than the anti-inflammatory doses used at that time, were able to fully suppress platelet aggregation and serotonin release for several days; concomitantly aspirin, but not salicylate, was found to be active, suggesting the role of irreversible acetylation in the mechanism of the long-lasting action of aspirin (O'Brien 1968; Smith and Willis 1971; de Gaetano et al. 1985; de Gaetano 2001). The observation that other nonsteroidal anti-inflammatory drugs were

platelet inhibitors comparable to aspirin, though short lasting, suggested the critical role of a putative platelet common site of inhibition for all these compounds (Smith and Willis 1971; Livio et al. 1982; de Gaetano et al. 1985). Platelet aggregation tests cast doubts on the clinical usefulness of selective TxA2-synthase inhibitors, while suggesting the potential benefit of the Tx receptor antagonists (Bertelé et al. 1981, 1984; Gresele et al. 1991).

The existence of "responders" and "nonresponders" to antiplatelet drugs other than aspirin is not a recent finding either. In 1974, indomethacin, a nonsteroidal anti-inflammatory drug given to patients with chronic glomerulonephritis, reportedly induced a variable inhibition of platelet aggregation tests: stronger inhibition of platelet function was significantly associated with higher reduction of urinary excretion of protein and fibrinogen/fibrin-related antigen (de Gaetano et al. 1974). Few years later, variability of response to TxA_2-synthase inhibitors was reported by several groups (Heptinstall and Fox 1983; Bertelé et al. 1984; Gresele et al. 1988). The possibility that salicylate, accumulating in blood during chronic administration of aspirin, could interfere with the antiplatelet effect of the parent compound and induce a variable response to the latter, was also proposed (de Gaetano 2001; de Gaetano et al. 1985). The interaction at the level of the binding site of cyclooxygenase-1 (COX-1) between nonsteroidal anti-inflammatory drugs, such as indomethacin or ibuprofen, and aspirin was also documented (Livio et al. 1982; de Gaetano et al. 1985; Catella-Lawson et al. 2001; de Gaetano 2001). Functional and modeling studies recently demonstrated the interactions of different plant-derived polyphenols (gallic acid, resveratrol, quercetin) at the platelet COX-1 level and their possible interaction with aspirin on platelet function (Crescente et al. 2009).

4.2 Aspirin

4.2.1 Variability of Platelet Response to Aspirin

The observation that TxA_2 generation in platelets could be substantially inhibited by any dose of aspirin and the availability of an easy and reliable method for measuring TxB_2 in serum formed by spontaneous blood clotting in vitro (Patrignani et al. 1982) focused the attention of clinical investigators on a biochemical parameter endowed with relatively low variability rather than on the evaluation of the multifaceted and variable platelet function.

In the Eighties, to the question "Should we treat with aspirin or other antiplatelet drugs a patient with coronary heart disease or stroke?" the common answer was to rely upon the results of clinical trials and meta-analyses on the antithrombotic clinical efficacy of antiplatelet drugs (de Gaetano 2001; de Gaetano and Cerletti 2007). To contribute to thrombosis—it was argued—platelets need not hyper-aggregate, it is sufficient that they simply aggregate. This view was shared by many investigators, as shown by the fact that no platelet marker was utilized to predict the risk of future cardiovascular events in different clinical conditions (van de Loo 1989; Cortellaro

et al. 1992). For several decades a serious limitation of clinical trials on antiplatelet drugs was that they were performed on patients enrolled without any previous measurement of their basal platelet function, or of their platelet response to the drug under trial, as measured by laboratory tests. Thus clinical end points could not be correlated to any laboratory parameter of platelet function.

Thus, on the basis of the almost complete and reproducible suppression of platelet TxA_2, its biochemical target, the concept prevailed that aspirin is a strong inhibitor of platelet function, rather than a weak and variable antithrombotic agent, as suggested by the complex interplay of different platelet functional pathways, only partially and variably inhibited by this drug. As an example, the experimental observation that—despite an almost complete suppression of TxA_2 formation by a low dose aspirin—platelet aggregation could be fully restored by combined stimuli (Rao et al. 1981; Cerletti et al. 1986; Di Minno et al. 1986; May et al. 1997) was not given sufficient attention at clinical level. Controlled clinical trials and meta-analyses confirmed that aspirin fails indeed to prevent about four-fifths of recurrent serious vascular events among high-risk patients (Antithrombotic Trialists' Collaboration 2002, 2009).

The apparent discrepancy between studies of "aspirin resistance" based on platelet function test measurements and the results of clinical trials of aspirin prophylaxis in high-risk patients initially pointed to the limitations of platelet function studies (Patrono 2003). Such an apparent discrepancy was subsequently reconciled by acknowledging the biological and clinical relevance of the variability of platelet function and the potential limitations of randomized clinical trials when extrapolated to individuals (de Gaetano and Cerletti 2003). Trials were compared to a kind of "epidemiological night" where all patients look black. The favorable, statistically significant results of clinical trials on aspirin derive in fact from a relatively small number of "responders," while the majority of patients do not get any benefit from the drug and are in fact "nonresponders" ("resistant") (de Gaetano et al. 2003).

The question was therefore addressed whether platelet response variability to antiplatelet therapy—rather than being a methodological limitation (Kunicki 2002; Patrono 2003; Santilli et al. 2009)—could be used instead to predict clinical outcomes and to modulate antiplatelet drug treatment for better achieving prevention or treatment of thrombosis in otherwise "nonresponder" subjects (Kawasaki et al. 2000; de Gaetano and Cerletti 2003).

An effort was thus made in the last few years to improve the prediction of risk of vascular events, therapeutic response, and clinical outcomes by available platelet function tests. Early evidence was obtained that "aspirin resistance" – better defined at present as residual on-treatment platelet response measured by platelet function tests – significantly correlated with the lack of clinical response in patients with cardiovascular disease (Snoep et al. 2007; Crescente et al. 2008a, b; Krasopoulos et al. 2008; Reny et al. 2008; Sofi et al. 2008). Advantage was mainly taken from some newly developed point-of-care tests of platelet function. These tests are not only better standardized than previous techniques, such as the aggregometry, but

Table 3 Main proposed mechanisms of aspirin "resistance"

Bioavailability of aspirin
Noncompliance
Insufficient aspirin dosage
Accumulation of salicylate, preventing the access of aspirin to COX-1 binding site
Concurrent intake of short-lasting nonsteroidal anti-inflammatory drugs, preventing the long-lasting effect of aspirin and of proton pump inhibitors, reducing aspirin bioavailability
Concurrent intake of dietary polyphenols, either preventing or potentiating the effect of aspirin
Platelet function
Accelerated platelet turnover, introducing into blood stream newly formed, nonaspirinated platelets
Variable expression of COX-2 in (newly formed) platelets
Increased platelet sensitivity to ADP and collagen
Polymorphisms
Polymorphisms of platelet collagen receptor
Polymorphisms of COX-1, COX-2, TxA_2-synthase, or other arachidonate metabolism enzymes
Polymorphisms of the platelet fibrinogen receptor GpIIbIIIa
Factor XIII Val34Leu polymorphism, leading to variable inhibition of FXIII activation by low dose aspirin
Platelet interactions with other blood cells and cell-derived products
Inadequate blockade of red cell-induced platelet activation
Transcellular arachidonate metabolism between aspirinated platelets and vascular cells
Monocyte-macrophage derived TxA_2
COX-1/COX-2-catalyzed vascular PGI_2 as regulator of platelet TxA_2 or vascular tissue plasminogen activator (tPA) release
Other factors
Increased levels of norepinephrine (excessive physical exercise, mental stress)
Smoking
Oxidant stress and biosynthesis of 8-iso-$PGF_{2\alpha}$, a bioactive product of arachidonate nonenzymatic peroxidation
Interaction of aspirin with acetylcholine-mediated nitric oxide antiplatelet and vasodilatory effect

Modified from de Gaetano and Cerletti (2003)

can also be performed at or near a patient bedside without the need of a high degree of technical experience (de Gaetano et al. 2008).

These observations go in the same direction of a previous study suggesting the clinical relevance of "aspirin-resistant" TxA_2 biosynthesis (as measured by urinary Tx metabolite excretion) in relation to the occurrence of major vascular events in high-risk patients (Eikelboom et al. 2002). The lively debate on platelet response variability has renewed interest to identify and characterize possible mechanisms accounting for the apparent failure of aspirin to prevent healthy individuals or patients at different vascular risk from occurrence of vascular events. A number of possible mechanisms were proposed (Table 3) (de Gaetano and Cerletti 2003) and some of them are presently a matter of intensive investigation.

Although these mechanisms cannot be discussed here, they clearly reflect the fact that individual response to aspirin is variable and may depend on the interaction of several variables. Much attention has been focused on variability in the ability of aspirin to inhibit COX-1 and TxA_2-dependent platelet activation, the major molecular target and biochemical mechanism of action of aspirin. In the presence of sufficient aspirin dosing and compliance, failure to inhibit COX-1-dependent platelet activation tests appears to be rather uncommon. The main problem related to aspirin variability is due to the fact that platelets may be activated along pathways that are only partially dependent on or even fully independent of COX-1 (Frelinger et al. 2006; Ohmori et al. 2006; Faraday et al. 2007b; Crescente et al. 2011), as suggested by data on prevalence and association with cardiovascular events of non-COX-1-dependent platelet pathways.

Different meta-analyses reported high prevalence of residual on-treatment platelet response to aspirin at population level. According to definitions used in the original studies, 28 % of subjects taking aspirin were classified as aspirin "nonresponders," with a wide range from 0 % to 57 % depending on the methods used, varying time points, different cut-off values, and variable concentrations of the stimulating agents (Krasopoulos et al. 2008). Six different platelet function assays were used. Among aspirin-treated subjects, lower prevalence of hyporesponsiveness for COX-1 dependent pathways was found (approximately 6 % for arachidonic acid stimulation, while a higher number of subjects appeared to be less responder to stimulation of COX-1-independent pathways (PFA-100, 27–33 %) (Crescente et al. 2008a; Krasopoulos et al. 2008). Data from a meta-analysis of 20 studies, totaling 2,930 patients, indicate that in patients with cardiovascular disease taking aspirin at doses 75–325 mg daily, nonfatal or fatal cardiovascular events occurred in 39 % of patients with residual on-treatment platelet response to antiplatelet drugs compared to 16 % of aspirin sensitive patients (OR = 3.85, 95 % CI 3.08–4.80) by various assays (Krasopoulos et al. 2008). As for prevalence data, residual platelet activation in aspirin-treated patients, independent of COX activity, and residual activation in the collagen pathway was associated with an eightfold excess in the occurrence of future cardiovascular morbidity (Frelinger et al. 2006; Ohmori et al. 2006). Also the PFA-100 closure time has been associated with outcome in aspirin-treated cardiovascular patients: in a systematic review of eight studies, comprising 847 subjects, aspirin nonresponders, as identified by PFA-100, were more likely to have vascular events than aspirin responders (relative risk (RR) 1.63; 95 % CI 1.16–2.28) (Krasopoulos et al. 2008); this finding was confirmed in a larger meta-analysis of 19 studies comprising 3,003 patients (Crescente et al. 2008b). Given the prevalence and the conferred risk, the potential impact of residual on-treatment platelet response to antiplatelet drugs is large. New guidelines on cardiovascular prevention and therapy are being developed to afford this problem, considering the suggested determinants of single or combined drug hyporesponsiveness, reviewed later.

Administration to aspirin-resistant patients of omega-3 fatty acids seems to improve response to aspirin and effectively reduces platelet reactivity, similarly to increasing the aspirin dose (Lev et al. 2010). Resveratrol, a plant-derived

polyphenol content in several food, including red wine, added in vitro to platelet-rich plasma from high-risk cardiac patients under chronic aspirin treatment, inhibited residual platelet aggregation induced by collagen or epinephrine (Stef et al. 2006). In a double-blind, crossover study in aspirin-treated patients with coronary artery disease, the consumption of flavonoid-rich purple grape juice for 14 days, although not providing additive antiplatelet effects, suppressed platelet-dependent inflammatory indices, such as plasma sCD40L levels, linked to cardiovascular disease (Albers et al. 2004). These studies suggest that nutritional compounds of different chemical nature may interfere with platelet response to aspirin and partially explain interindividual variability to antiplatelet response.

4.2.2 Acquired Factors

In a variance component analysis of platelet function, the contribution of cardiovascular risk factors and polygenic heritability to variable platelet function, both at baseline and in response to aspirin, was assessed (Faraday et al. 2007b). This study also allowed to get an estimate of the impact of preaspirin test results on the variability of platelet response to this drug. The total variance attributable to measured covariates in each postaspirin platelet phenotype was modest, ranging from <1 % to 13 %. Platelet responsiveness to aspirin was a highly heritable trait (27 %–77 %). Phenotypes indirectly related to COX-1 (both pre- and postaspirin) were more strongly and consistently heritable, with the exception of PFA closure time, but direct COX-1 related phenotypes were not. Which unmeasured variables might be responsible for the remaining variance portion was not determined. Methodologic variability of the test, that is difficult to estimate, could explain a relatively high portion of this unexplained variance. In this study, as aspirin was correctly dosed and adequate compliance was carefully checked, total variance did not include the expected effect of noncompliance, which has instead to be considered when we refer to other studies.

In the study of Faraday et al. (2007b) in each postaspirin platelet phenotype, age and sex substantially contributed to phenotypic variance among cardiac risk factor covariates. Depending on the specific aspirin response phenotype, age accounted for up to 7 % of variance in whites and 3 % in blacks, and sex accounted for up to 11 % in whites and 7 % in blacks; age and sex were only modestly related to postaspirin treatment by COX-1-dependent platelet function. However, these covariates were more strongly related to non-COX-1 function testing.

Reduced platelet suppression after aspirin therapy was associated with female sex for indirect COX-1 phenotypes. In a large primary-prevention trial in women, the observed cardioprotective benefits from low-dose aspirin therapy were not statistically significant (Ridker et al. 2005). Furthermore, other studies observed associations between female sex and higher platelet reactivity, even after aspirin therapy (Yee et al. 2005; Becker et al. 2006). Moreover, an association between genetic variations in platelet GPIbα and GPVI and the risk of coronary heart disease events in postmenopausal women taking hormone therapy was reported (Bray et al.

2007b). These results are attractive, providing bases for explanation of variability of platelet response to aspirin and as a potential cause for the failure of aspirin sometimes reported in primary prevention studies in women (Lev et al. 2010). However, a meta-analysis found no difference between men and women in the prevalence of "aspirin resistance" studied by PFA-100 (Crescente et al. 2008a).

Cardiovascular risk factors have been shown to be associated with platelet response to aspirin, but in the study of Faraday et al. (2007b) the contribution appears to be small. In each postaspirin platelet phenotype, other covariates accounted for <2 % of phenotypic variance, except for the contribution of the von Willebrand factor levels at the PFA-100 phenotype (2 % in whites, 7 % in blacks). When baseline variables are added to the model, they accounted for up to 38 % of the variance for postaspirin pathways indirectly related to COX-1, while for ≤ 2 % in postaspirin phenotypes directly related to COX-1, as reported in Table 4a, b (Faraday et al. 2007b). The factors with the major impact in variance determination for postaspirin phenotypes were the baseline preaspirin measurement of platelet phenotype.

Aspirin "nonresponder" subjects more likely appear to be diabetic (Di Minno et al. 1986; Sacco et al. 2003; Angiolillo et al. 2004; Marcucci et al. 2007; Takahashi et al. 2007), as well as primary prevention of cardiovascular events by aspirin may not be effective in diabetes (Sacco et al. 2003; Belch et al. 2008). Residual on-treatment platelet response to aspirin in diabetes is a complex interplay between a number of factors: accelerated platelet turnover might be a major factor in "aspirin resistance" originally reported in diabetic patients by Di Minno et al. (1986). Subgroups of risk factor exposures showed significant differences among diabetics and nondiabetics, while subjects exposed or not to smoking, hypertension and dyslipidemia had comparable values of platelet response to aspirin in PFA-100 (Crescente et al. 2008a).

4.2.3 Genetic Factors

Response of platelets to aspirin appears to be heritable too. Although some differences in heritability were observed between black and white subjects, the overall pattern was inconsistent (Freedman 2007). Studies on aspirin pharmacogenetics tried to identify individuals with different sensitivity to the drug, allowing to recognize possible genetic regulations of variability of platelet response to aspirin and atherothrombotic events under aspirin treatment (Fitzgerald and Pirmohamed 2011).

Genetic variation in the molecules responsible for platelet activation is an important potential contributor to the adequacy of the antiplatelet action of aspirin. Thus far, small candidate gene studies examining the relation between platelet function and specific gene variants in pathways directly and indirectly related to COX-1 have not provided consistent evidence for a gene–aspirin response relation, while only recently GWAS results became available (Faraday et al. 2007b).

COX-1 gene region appears to be highly polymorphic: C50T, the most extensively studied polymorphism in candidate gene studies, gave however conflicting results.

Table 4 Determinants of variability of postaspirin platelet phenotypes, directly or indirectly related to COX-1 pathways, in Whites (a) and in Blacks (b)

Phenotype		$h^2 \pm$ SE* (p)	Age r^2 (p)	Sex r^2 (p)	All covariates r^2 (p)	Preaspirin r^2 (p)
(a)						
Direct COX-1 pathways	TxB2	0.378 ± 0.071 (<0.0001)	0	0.036 (<0.0001)	0.046 (<0.0001)	0.003 (0.0720)
	Tx-M	0.194 ± 0.076 (0.0102)	0.012 (0.0008)	0.003 (0.0930)	0.059 (<0.0001)	0.026 (<0.0001)
Indirect COX-1 pathways	PRP collagen	0.284 ± 0.071 (<0.0001)	0.048 (<0.0001)	0.008 (0.0006)	0.259 (<0.0001)	0.198 (<0.0001)
	PRP lag time collagen	0.217 ± 0.084 (0.0098)	0.030 (<0.0001)	0.003 (0.0610)	0.135 (<0.0001)	0.098 (<0.0001)
	WB collagen	0.268 ± 0.068 (<0.0001)	0.019 (<0.0001)	0.012 (0.0002)	0.132 (<0.0001)	0.096 (<0.0001)
	PRP ADP	0.206 ± 0.075 (0.0060)	0.011 (<0.0001)	0.023 (0.0001)	0.262 (<0.0001)	0.221 (<0.0001)
	WB ADP	0.207 ± 0.068 (0.0023)	0	0.020 (<0.0001)	0.406 (<0.0001)	0.378 (<0.0001)
	PRP epinephrine	0.242 ± 0.067 (0.0003)	0.046 (<0.0001)	0.002 (0.1154)	0.297 (<0.0001)	0.248 (<0.0001)
	PFA CT*	0.168 ± 0.152 (0.2686)	0.001 (0.5140)	0.031 (0.0002)	0.251 (<0.0001)	0.202 (<0.0001)
	βTG release	0.306 ± 0.096 (0.0015)	0	0	0.366 (<0.0001)	0.360 (<0.0001)

(b)

Direct COX-1 pathways	TxB2	0.144 ± 0.120 (0.2302)	0.001 (0.3408)	0	0.013 (0.6205)	0.003 (0.0747)
	Tx-M	0.333 ± 0.149 (0.0252)	0.028 (0.8061)	0	0.021 (0.2070)	0.004 (0.7078)
Indirect COX-1 pathways	PRP collagen	0.311 ± 0.110 (0.0047)	0.002 (0.0058)	0	0.276 (<0.0001)	0.006 (<0.0001)
	PRP lag time collagen	0.230 ± 0.153 (0.1330)	0	0	0.098 (<0.0001)	0
	WB collagen	0.251 ± 0.106 (0.0179)	0.008 (0.6208)	0.002 (0.0276)	0.086 (<0.0001)	0.251 (<0.0001)
	PRP ADP	0.128 ± 0.128 (0.3192)	0.005 (0.0349)	0	0.262 (<0.0001)	0.085 (<0.0001)
	WB ADP	0.155 ± 0.103 (0.1317)	0	0.007 (<0.0001)	0.386 (<0.0001)	0.073 (<0.0001)
	PRP epinephrine	0.129 ± 0.113 (0.2557)	0.005 (0.1037)	0.010 (0.9082)	0.311 (<0.0001)	0.244 (<0.0001)
	PFA CT*	0	0	0.018 (0.4268)	0.295 (<0.0001)	0.359 (<0.0001)
	βTG release	0.356 ± 0.213 (0.0949)	0.003 (0.2688)	0	0.300 (<0.0001)	0.292 (<0.0001)

h^2 and r^2 indicate the contribution of heritability and measured covariates, respectively, to the variability in postaspirin platelet phenotypes, adjusted for baseline phenotypes

All covariates: age, sex, hypertension, currently smoking, BMI, diabetes mellitus, low density lipoprotein cholesterol, fibrinogen, von Willebrand Factor (*PFA only), and baseline platelet phenotypes

Preaspirin = baseline phenotype

COX-1 cyclooxygenase-1, *PRP* platelet-rich plasma, *WB* whole blood, *TxB_2* thromboxane B_2, *Tx-M* urinary 11-dehydro thromboxane B_2, *PFA* platelet function analyzer, *βTG* beta thromboglobulin

Data extracted from Faraday et al. (2007b)

Some studies have shown no association with aspirin response and studies which have shown an association with lower response to aspirin were not correlated with clinical outcomes (Lepäntalo et al. 2006; Takahashi et al. 2007; Clappers et al. 2008).

Many studies focused on polymorphisms of different platelet membrane glycoproteins in small groups of patients "nonresponder" to aspirin in terms of platelet aggregation response (Macchi et al. 2002): platelets from individuals homozygous for the Pl(A1) allele (encoding for the GPIIIa) appeared indeed to be less sensitive to the inhibitory action of low-dose (160 mg) aspirin. The relationship between GPIIIa and aspirin antiplatelet mechanism is, however, obscure. Conflicting results have been reported with the P2Y1 and P2Y12 platelet receptor genes and GPVI/GPIIIa genes summarized in a systematic review (Goodman et al. 2008): 31 studies analyzing 50 polymorphisms in 11 genes demonstrated that only the PLA1/A2 polymorphism in the GpIIIa gene was significantly associated with variability of platelet response to aspirin in healthy subjects (OR 2.36; 95 % CI 1.24–4.48; $p = 0.009$). However, the effect size was reduced considerably by combining healthy subjects with patients with cardiovascular disease (OR 1.14; 95 % CI 0.84–1.54; $p = 0.40$). In addition, the observed association between platelet response to aspirin and the PLA1/A2 polymorphism was only seen when measuring platelet aggregation; when PFA-100 was used instead, a not statistically significant association could be found in many studies between this polymorphism and modified aspirin sensitivity (Goodman et al. 2008).

In many studies polymorphisms in COX-1, GPIa, P2Y1, or P2Y12 were not associated with any significant variability of platelet response to aspirin, probably due to inadequate assessment of phenotype and risk factors, or because the contribution of each single gene variant to the overall phenotypic variance was too low and/or the population size too small. Other genes codifying for proteins involved in other pathways may be important in modulating the response to aspirin and therefore genotyping strategies will need to reflect this, for example by using pathway pharmacogenetics or GWAS (Fitzgerald and Pirmohamed 2011).

Results from Faraday et al. (2007b) suggest a genetic cause for differences among individuals in aspirin responsiveness and support the search for specific genes and gene–gene interactions that determine platelet responsiveness to aspirin therapy. Genome wide linkage (GWL) and association analyses were performed on the same sample through analysis of 37 agonist-induced platelet phenotypes, collected before and after 14 days of treatment with 81 mg/day aspirin. Eleven patterns of similar platelet function phenotypes, pooled with a principal component analysis, showed suggestive evidence for linkage across 14 regions in the genome, with postaspirin treatment phenotypes showing higher linkage than preaspirin. Furthermore, 30 SNPs resulted significant according to the false discovery rate threshold, some of these lying in region found to be linked to phenotypes in previous GWL analyses or being related to pathways known to be involved in platelet function. Future studies have to increase sample sizes and improve the phenotype selection by considering newly suggested platelet phenotypes with roles in atherothrombosis, such as platelet–leukocyte cross talk (Cerletti et al. 2011).

4.3 Clopidogrel

4.3.1 Variability of Platelet Response to Clopidogrel

Clopidogrel is a second-generation thienopyridine. It is absorbed as a pro-drug and converted to its active thiol metabolite by hepatic cytochrome (CYP) P450 enzymes. A regimen including both aspirin and clopidogrel has been used in an attempt to improve the antithrombotic prevention of secondary stroke or myocardial infarction, but this has yielded contradictory results. Although aspirin variability remains an uncontrolled variable on the basis of genetic polymorphisms, noncompliance, and drug interactions, recent evidence suggests that reduced responsiveness to clopidogrel is the main cause of the reduced efficacy of dual antiplatelet therapy (Peace et al. 2008).

Mean prevalence of clopidogrel nonresponsiveness was around 20 %, while the prevalence of dual nonresponsiveness to both aspirin and clopidogrel was below 10 % (Neubauer et al. 2011). Among patients with CAD, who underwent percutaneous coronary intervention, risk of death and/or thrombotic recurrences increased by about sixfolds in those with a poor response to clopidogrel treatment (data from a meta-analysis of 14 studies recruiting 4,564 subjects) (Sofi et al. 2010).

The pro-drug clopidogrel is absorbed via ATP-binding cassette efflux transporters located in the apical membrane of the intestinal mucosa encoded by the multidrug resistance gene MDR (ABCB1) (Taubert et al. 2006). The majority of the absorbed pro-drug is inactivated by hepatic esterases and lacks any antiplatelet activity. Less than 15 % of the absorbed pro-drug is further metabolized by two sequential oxidative steps through the hepatic CYP450 system, to generate the active thiol metabolite, which targets and irreversibly inhibits the ADP $P2Y_{12}$ receptor. Among the CYP isoenzymes, so far identified as having a relevant metabolic role, the polymorphically expressed CYP2C19 affects both metabolic steps of clopidogrel active metabolite generation and therefore plays a dominant role in this process (Kazui et al. 2010).

The clopidogrel response variability, hypothesized more than a decade ago on theoretical grounds (de Gaetano et al. 2002), was first described by Gurbel et al. (2003) followed by several other confirmatory studies, recently reviewed by Cattaneo (2004) and by Bernlochner et al. (2011).

Criteria to define clopidogrel nonresponse are numerous and discrepant, some with obvious limits, and use either relative or absolute differences in platelet aggregation before and after clopidogrel or arbitrary cut points or 2 standard deviations from the mean to define low responders. It is therefore often difficult to establish a consensus from literature data on the incidence of clopidogrel nonresponse (Bonello et al. 2010). Factors that impact on clopidogrel response variability are either acquired or genetic.

4.3.2 Acquired Factors

Increasing the clopidogrel loading dose from 300 mg to 600 mg does not decrease the phenomenon of interindividual variability. High values of BMI and inflammatory biomarkers or, as in the case of variability in platelet response to aspirin, diabetes mellitus have also been associated with enhanced baseline platelet activation and higher platelet aggregation values on clopidogrel therapy (Cattaneo 2004; Bonello et al. 2010; Bernlochner et al. 2011). Several comorbidities have been found to influence the antiplatelet efficacy of clopidogrel. Patients of older age with renal insufficiency also suffer significantly more often from high residual on-treatment platelet reactivity with standard dual antiplatelet therapy. Patients with acute coronary syndrome or left ventricular dysfunction also consistently showed reduced clopidogrel responsiveness, most likely due to decreased drug metabolism or impaired enteric absorption of the pro-drug (Cattaneo 2004; Bonello et al. 2010; Bernlochner et al. 2011). The same mechanism, in opposite direction, has been suggested to explain the greater inhibition of platelet aggregation by clopidogrel observed in smokers vs. nonsmokers, as cigarette smoking induces cytochrome P450 (CYP1A2), which converts clopidogrel into its active metabolite (Bliden et al. 2008). Cigarette smoking seems to positively modify the beneficial effect of clopidogrel on both angiographic and clinical outcomes (Desai et al. 2009). Interference with clopidogrel metabolism by other drugs that are frequently given to patients with atherosclerosis, such as atorvastatin or proton pump inhibitors, can increase the number of patients who are resistant to clopidogrel, although this is still a controversial issue (Cattaneo 2004; Bonello et al. 2010; Bernlochner et al. 2011). These data highlight the contribution of environmental factors to the interpatient variability in response to clopidogrel.

4.3.3 Genetic Factors

The primary cause of variability in responsiveness to clopidogrel lies in its pharmacokinetics, being largely dependent on the amount of active metabolite generated. Recent studies have clearly shown that several gene polymorphisms are involved in the variability of clopidogrel responsiveness, with an important influence on clinical outcomes (Campo et al. 2011). Multiple gene variants resulting from SNPs in different enteric and hepatic genes, involved in the clopidogrel absorption and metabolism process, have been associated with variation in response to clopidogrel: Table 5 lists the polymorphisms reported to strongly influence clopidogrel response (Campo et al. 2011). Many studies focused on the influence of SNPs in specific CYP450 hepatic isoenzymes, involved in the hepatic two-step sequential bioactivation of clopidogrel.

Several studies demonstrated that, among various alleles of CYP2C19, the *2 allele is associated with attenuated response to clopidogrel in a gene dose-dependent manner (homozygote vs. heterozygote allele carriage) with a consequent higher risk for ischemic events including stent thrombosis in CYP2C19*2 carriers (Cattaneo 2004; Bonello et al. 2010; Bernlochner et al. 2011; Campo et al. 2011).

Table 5 Gene polymorphisms with a strong influence on clopidogrel response

Allele	Cytogenetic location	Nucleotide change	Functional effect	Influence on clinical outcome
ABCB1*2	chr7 q21.12	C3435T	Increased intestinal efflux of pro-drug	TT vs. C carriers HR 2; 95 % CI 1.3–3.1 for the risk of death, MI, stroke T carriers vs. CC HR 1.7; 95 % CI 1.2–2.7 for the risk of death, MI, stroke
CYP2C19*2	chr10 q23.33	G19 154A	Lack of enzyme activity	A carriers vs. GG HR 1.7; 95 % CI 1.1–2.8 for the risk of death, MI, stroke A carriers vs. GG OR 2.5; 95 % CI 1.1–5.5 for the risk of death, MI, stroke
CYP2C19*3	chr10 q23.33	G636A	Inactive enzyme metabolism	A carriers vs. GG HR 1.98; 95 % CI 1.1–3.5 for the risk of death, MI, stroke
CYP2C19*17	chr10 q23.33	C806T	Increased enzyme function	T carriers vs. CC HR 2; 95 % CI 1.1–5.3 for the risk of bleeding complications T carriers vs. CC OR 3.3; 95 % CI 1.3–8.1 for the risk of bleeding complications
CYP2C9*3	chr10 q23.33	A42 614C	Reduced enzyme function	C carriers vs. AA OR 3.3; 95 % CI 1.1–10 for the risk of death, MI, stroke
PON1 Q192R	chr7 q21.3	A576G	Increased enzyme function	AA vs. G carriers HR 3.6; 95 % CI 1.6–7.9 for the risk of death, MI, stroke

Notes: *CYP* cytochrome P450, *chr* chromosome, *PON1* Paraoxonase-1, *MI* myocardial infarction, *HR* hazard ratio
Modified from Campo et al. (2011)

Another genetic variant of clopidogrel responsiveness is the ABCB1 gene, which encodes for MDR1, the protein responsible for the intestinal absorption of orally administered clopidogrel. Patients with variant alleles of ABCB1 polymorphism suffer from a higher ischemic event rate in comparison with those with the wild-type genotype. Against this finding, a genetic substudy of the PLATO trial was unable to show any significant influence of SNPs of the ABCB1 gene on outcomes of treatment with clopidogrel or ticagrelor in acute coronary syndrome patients (Wallentin et al. 2010).

Recently, Bouman et al. (2010) postulated a new gene variant within the paraoxonase gene, encoding for the paraoxonase-1 (PON1) enzyme, to be the principal factor in clopidogrel bioactivation and to be the major determinant of clopidogrel response variability. Furthermore, this group questioned the previously indicated involvement of CYP2C19*2 in clopidogrel efficacy since in the same patients they did not find any evidence for the contribution of CYP2C19 to clopidogrel metabolism.

An important role in modulating clopidogrel pharmacokinetics and pharmacodynamics is given by the P2RY12 and the ITGB3 genes, which encode for the

platelet $P2Y_{12}$ and GPIIbIIIa receptors, respectively (Fontana et al. 2003); however, so far, neither polymorphisms of the P2RY12 nor of the ITGB3 gene have been linked to clopidogrel efficacy or clinical outcome (Bernlochner et al. 2011).

Shuldiner et al. (2009) performed a GWAS of clopidogrel response in 429 healthy Amish people. Thirteen SNPs within and flanking the CYP2C18-2C19-2C9-2C8 cluster on chromosome 10q24 were strongly associated with reduced clopidogrel response, with CYP2C19*2 variant accounting for most of the signals. In a replication study involving 227 cardiovascular disease patients undergoing nonemergent percutaneous coronary interventions, carriers of the CYP2C19*2 allele had higher rates of cardiovascular events at 1-year follow-up: the CYP2C19*2 genotype was estimated to account for approximately 12 % of the clopidogrel response variability (Shuldiner et al. 2009).

5 Conclusions

Variability of platelet count, volume, and function appears to be a key issue in current biomedical research and its impact in public health may be strong, mainly due to high impact in cardiovascular event determination in the presence or in the absence of antiplatelet drugs.

Investigating the reasons underlying this heterogeneity, acquired variables explain a small portion of the heterogeneity of platelet parameters. Considering the single factors, age and sex result to be the major determinants, explaining a relatively large portion of phenotypic variance, while lifestyles have a limited impact. Genetic factors, instead, appear to play a major role, although a consistent portion of such a genetic variance has not yet been attributed to any specific genetic factor. This apparent inconsistency is mainly due to the high number of DNA loci potentially involved in the various molecular platelet pathways and to the limited effect size of each individual SNP that in GWAS studies could not be found indeed to be associated with any platelet phenotype. A portion of variance remains thus unexplained, probably also due to other unknown variables such as diet, or to variability of test performance.

The identification of pathways underlying variability could provide new targets to modulate platelet function. To this aim, a comprehensive view is provided by investigation of multiple phenotypes, related to both platelet parameters and disease outcomes, which in some cases share the same genetic determinants, thus helping speculate the plausibility of their suggested involvement. Platelet function is actually analyzed through several tests not globally investigating all various underlying pathways. Then, we need yet to discover new platelet phenotypes, whose heterogeneity could be more strongly associated with diseases. The main contradiction in present platelet knowledge is, in fact, the difficulty to reconcile the universally accepted importance of platelet function and the lack of reliable platelet parameters in CV risk prediction models.

Finding biomarkers of clinically determinant platelet parameters (as drug hyporesponsiveness and, as noted earlier, baseline predrug platelet pathways) could be

helpful to develop better risk prediction models and to recognize subjects who need different *intervention schedules*, providing the background for new pharmacogenetic approaches.

A novel understanding of the role of platelets and the variability of their number, volume, and function in thrombosis pathogenesis has currently been developed in respect to the Eighties, when it was argued that to contribute to thrombosis platelets need not be larger or hyper-aggregate but simply aggregate. The search of individuals with variable platelet parameters, different sensitivity to platelet agonists and/or antiplatelet drugs, or diverse clinical outcomes is now fully justified by the evidence discussed in this chapter.

Trials are generally designed to select a homogeneous sample, whose results may be applied to an "average subject," tending to exclude the deviation/extreme values. Results from subgroups of trial samples are limited and are considered unreliable due to false discovery selection; moreover, trials on subjects with characteristics associated with variability of platelet function are still few. Therefore, an effort should be made to integrate epidemiological results into trial designing to move towards the so-called personalized medicine.

As new functional and biochemical assays become available to platelet researchers, the understanding of platelet physiology and pharmacodynamics associated with antiplatelet therapy will rapidly increase and new antiplatelet agents and additional tests to monitor platelet response will hopefully be available to clinicians in the near future.

In conclusion, as the current indications for antiplatelet treatment in primary or secondary prevention of ischemic vascular disease still derive from the results of clinical trials where platelet function was not investigated, we cannot rely upon any platelet test to either initiate, or monitor, or modify or stop treatment with any antiplatelet drug. Evidence is, however, increasing that traditional platelet aggregometry and other more recently developed platelet function assays could be useful to optimize antiplatelet therapy and to predict major adverse cardiac events.

Knowledge Gaps

- Will the normal range of platelet number routinely be defined in a distinct way for different populations? for gender and age?
- Will platelet number be considered as an easy marker of thrombotic risk? Will it be considered as part of any individual's characteristics?
- Will mean platelet volume be measured as a reliable, reproducible, and easy marker of thrombotic tendency and/or of the risk of other diseases such as aspirin-sensitive colon cancer?
- Will the decision to start or to modify or to stop a therapy with any antiplatelet drug be based—even not exclusively—on the results of platelet function tests?
- Will clusters of genes or of genes and noncoding DNA be identified to be associated with variability of platelet number, or mean platelet volume or platelet function?

(continued)

- Will epigenetics better explain the contribution of DNA variability on platelet variability?
- Will mechanisms other than inhibition of platelet cyclo-oxygenase be discovered as contributing to the overall antithrombotic effect of aspirin and explain the variability of therapeutic effect of this drug among individuals?
- Will any antiplatelet drug be administered to an individual on the basis of his/her bleeding risk, easily identified by a genetic analysis and/or a laboratory test?
- Will platelet function be better defined if studied in conjunction with other blood and/or vascular cells? In particular, will platelet–leukocyte interactions be routinely measured as a reliable marker of thrombotic and/or inflammatory and/or cancer risk?

Key Messages

- While it is accepted that each individual has an inherent risk of bleeding linked to some platelet dysfunction, it is less obvious that the variability of platelet phenotypes (number, mean platelet volume, function) may contribute to the individual risk of thrombosis.
- During the last decade, "aspirin resistance" was a fashionable expression to describe the poor response of platelets from some healthy individuals or patients to the inhibitory effect of aspirin: it was wrong indeed, but stimulated a large amount of new knowledge on the variability of platelet response to either stimuli or drugs or both.
- The observation of interindividual differences in platelet response to antiplatelet drugs has enlarged the spectrum and the possible clinical relevance of platelet variability.
- Interindividual variability of platelet indices or function may be due to either acquired factors, such as age, sex, metabolic variables, smoke, dietary habits, ongoing inflammation, or genetic factors, and may affect the efficacy and safety of antiplatelet drugs used in the treatment and prevention of thrombosis.
- The development of "personalized medicine" will benefit from the concepts discussed in this chapter.

Acknowledgements This review was prepared in the frame of a research project funded by the Italian Ministry of Research (MIUR, decreto N. 1588).
Thanks are due to Maria Benedetta Donati for her constructive criticism.
The authors declare no conflict of interest.

References

Albers AR, Varghese S, Vitseva O et al (2004) The anti-inflammatory effects of purple grape juice consumption in subjects with stable coronary artery disease. Arterioscler Thromb Vasc Biol 24:e179–e180

Angiolillo DJ, Fernández-Ortiz A, Bernardo E et al (2004) High clopidogrel loading dose during coronary stenting: effects on drug response and interindividual variability. Eur Heart J 25:1903–1910

Antithrombotic Trialists' (ATT) Collaboration, Baigent C, Blackwell L, Collins R et al (2009) Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. Lancet 373:1849–1860

Antithrombotic Trialists' Collaboration (2002) Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. BMJ 324:71–86

Bain BJ (1996) Ethnic and sex differences in the total and differential white cell count and platelet count. J Clin Pathol 49:664–666

Barbalic M, Dupuis J, Dehghan A et al (2010) Large-scale genomic studies reveal central role of ABO in sP-selectin and sICAM-1 levels. Hum Mol Genet 19:1863–1872

Bath PM, Butterworth RJ (1996) Platelet size: measurement, physiology and vascular disease. Blood Coagul Fibrinolysis 7:157–161

Bath P, Algert C, Chapman N et al (2004) Association of mean platelet volume with risk of stroke among 3134 individuals with history of cerebrovascular disease. Stroke 35:622–626

Becker DM, Segal J, Vaidya D et al (2006) Sex differences in platelet reactivity and response to low-dose aspirin therapy. J Am Coll Cardiol 295:1420–1427

Belch J, MacCuish A, Campbell I, Prevention of Progression of Arterial Disease and Diabetes Study Group, Diabetes Registry Group, Royal College of Physicians Edinburgh et al (2008) The prevention of progression of arterial disease and diabetes (POPADAD) trial: factorial randomised placebo controlled trial of aspirin and antioxidants in patients with diabetes and asymptomatic peripheral arterial disease. Br Med J 337:a1840

Berger JS, Eraso LH, Xie D et al (2010) Mean platelet volume and prevalence of peripheral artery disease, the National Health and Nutrition Examination Survey, 1999–2004. Atherosclerosis 213:586–591

Bernlochner I, Byrne RA, Kastrati A et al (2011) The future of platelet function testing to guide therapy in clopidogrel low and enhanced responders. Expert Rev Cardiovasc Ther 9:999–1014

Bertelé V, Cerletti C, Schieppati A et al (1981) Inhibition of thromboxane synthetase does not necessarily prevent platelet aggregation. Lancet 1:1057–1058

Bertelé V, Falanga A, Tomasiak M et al (1984) Pharmacologic inhibition of thromboxane synthetase and platelet aggregation: modulatory role of cyclooxygenase products. Blood 63:1460–1466

Biino G, Balduini CL, Casula L et al (2011) Analysis of 12, 517 inhabitants of Sardinian geographic isolate reveals that propensity to develop mild thrombocytopenia during ageing and to present mild, transient thrombocytosis in youth are new genetic traits. Haematologica 96:96–101

Bliden KP, Dichiara J, Lawal L et al (2008) The association of cigarette smoking with enhanced platelet inhibition by clopidogrel. J Am Coll Cardiol 52:531–533

Bonello L, Tantry US, Marcucci R, Working Group on High On-Treatment Platelet Reactivity et al (2010) Consensus and future directions on the definition of high on-treatment platelet reactivity to adenosine diphosphate. J Am Coll Cardiol 56:919–933

Born GV (1962) Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 194:927–929

Bouman HJ, Schomig E, van Werkum JW et al (2010) Paraoxonase-1 is a major determinant of clopidogrel efficacy. Nat Med 17:110–116

Braekkan SK, Mathiesen EB, Njølstad I et al (2010) Mean platelet volume is a risk factor for venous thromboembolism: the Tromsø Study, Tromsø, Norway. J Thromb Haemost 8:157–162
Bray PF, Mathias RA, Faraday N et al (2007a) Heritability of platelet function in families with premature coronary artery disease. J Thromb Haemost 5:1617–1623
Bray PF, Howard TD, Vittinghoff E et al (2007b) Effect of genetic variations in platelet glycoproteins Ibalpha and VI on the risk for coronary heart disease events in postmenopausal women taking hormone therapy. Blood 109:1862–1869
Campo G, Miccoli M, Tebaldi M et al (2011) Genetic determinants of on-clopidogrel high platelet reactivity. Platelets 22:399–407
Catella-Lawson F, Reilly MP, Kapoor SC et al (2001) Cyclooxygenase inhibitors and the antiplatelet effects of aspirin. N Engl J Med 345:1809–1817
Cattaneo M (2003) Inherited platelet-based bleeding disorders. J Thromb Haemost 1:1628–1636
Cattaneo M (2004) Aspirin and clopidogrel: efficacy, safety, and the issue of drug resistance. Arterioscler Thromb Vasc Biol 24:1980–1987
Cattaneo M, Cerletti C, Harrison P et al (2010) Final report of the Working Party on LTA Standardization. In: 56th annual SSC meeting of the International Society on thrombosis and haemostasis, Cairo, Egypt
Cauwenberghs S, van Pampus E, Curvers J et al (2007) Hemostatic and signaling functions of transfused platelets. Transfus Med Rev 21:287–294
Centritto F, Iacoviello L, di Giuseppe R et al (2009) Dietary patterns, cardiovascular risk factors and C reactive protein in a healthy Italian population. Nutr Metab Cardiovasc Dis 19:697–706
Cerletti C, Carriero MR, de Gaetano G (1986) Platelet-aggregation response to single or paired aggregating stimuli after low-dose aspirin. N Engl J Med 314:316–318
Cerletti C, Tamburrelli C, Izzi B et al (2011) Platelet-leukocyte interactions in thrombosis. Thromb Res. 129:263–266
Chu SG, Becker RC, Berger PB et al (2010) Mean platelet volume as a predictor of cardiovascular risk: a systematic review and meta-analysis. J Thromb Haemost 8:148–156
Clappers N, van Oijen MG, Sundaresan S et al (2008) The C50T polymorphism of the cyclooxygenase-1 gene and the risk of thrombotic events during low-dose therapy with acetyl salicylic acid. Thromb Haemost 100:70–75
Coban E, Afacan B (2008) The effect of rosuvastatin treatment on the mean platelet volume in patients with uncontrolled primary dyslipidemia with hypolipidemic diet treatment. Platelets 19:111–114
Cortellaro M, Boschetti C, Cofrancesco E et al (1992) The PLAT Study: hemostatic function in relation to atherothrombotic ischemic events in vascular disease patients. Principal results. PLAT Study Group. Progetto Lombardo Atero-Trombosi (PLAT) Study Group. Arterioscler Thromb 12:1063–1070
Crescente M, Di Castelnuovo A, Iacoviello L et al (2008a) Response variability to aspirin as assessed by platelet function analyzer (PFA-100): a systematic review. Thromb Haemost 99:14–26
Crescente M, Di Castelnuovo A, Iacoviello L et al (2008b) PFA-100 closure time to predict cardiovascular events in aspirin-treated cardiovascular patients: a metaanalysis of 19 studies comprising 3003 patients. Thromb Haemost 99:1129–1131
Crescente M, Jessen G, Momi S et al (2009) Interactions of gallic acid, resveratrol, quercetin and aspirin at the platelet cyclooxygenase-1 level. Functional and modelling studies. Thromb Haemost 102:336–346
Crescente M, Mezzasoma AM, Del Pinto M et al (2011) Incomplete inhibition of platelet function as assessed by the platelet function analyzer (PFA-100) identifies a subset of cardiovascular patients with high residual platelet response while on aspirin. Platelets 22:179–187
Curvers J, van Pampus ECM, Feijge MAH et al (2004) Decreased responsiveness and development of activation markers of platelets stored in plasma. Transfusion 44:49–58
de Gaetano G (2001) Historical overview of the role of platelets in hemostasis and thrombosis. Haematologica 86:349–356

de Gaetano G, Cerletti C (2003) Aspirin resistance: a revival of platelet aggregation tests? J Thromb Haemost 1:2048–2050
de Gaetano G, Cerletti C (2007) Platelet function, antiplatelet therapy and clinical outcomes: to test or not to test? J Thromb Haemost 5:1835–1838
de Gaetano G, Vermylen J, Donati MB et al (1974) Indomethacin and platelet aggregation in chronic glomerulonephritis: existence of non-responders. Br Med J 2:301–303
de Gaetano G, Cerletti C, Dejana E et al (1985) Pharmacology of platelet inhibition in humans. Implications of the salicylate-aspirin interaction. Circulation 72:1185–1193
de Gaetano G, Cerletti C, Iacoviello L (2002) Pharmacogenetics as a new antiplatelet strategy. In: Gresele P, Page CP, Fuster V, Vermylen J (eds) Platelets in thrombotic and non thrombotic disorders. Cambridge University Press, Cambridge, pp 964–977
de Gaetano G, Cerletti C, Iacoviello L et al (2003) The epidemiological night where all patients are black: will pharmacogenetics shed some light? Thromb Res 112:273–274
de Gaetano G, Crescente M, Cerletti C (2008) Current concepts about inhibition of platelet aggregation. Platelets 19:565–570
De Luca G, Venegoni L, Iorio S et al (2010) Platelet distribution width and the extent of coronary artery disease: results from a large prospective study. Platelets 21:508–514
Della Corte A, Tamburrelli C, Crescente M et al (2012) Platelet proteome in healthy volunteers who smoke. Platelets 23:91–105
Den Dekker E, van Abel M, van der Vuurst H et al (2003) Cell-to-cell variability in the differentiation program of human megakaryocytes. Biochim Biophys Acta 1643:85–94
Desai NR, Mega JL, Jiang S et al (2009) Interaction between cigarette smoking and clinical benefit of clopidogrel. J Am Coll Cardiol 53:1273–1278
Di Minno G, Silver MJ, Cerbone AM et al (1986) Trial of repeated low-dose aspirin in diabetic angiopathy. Blood 68:886–891
Eikelboom JW, Hirsh J, Weitz JI et al (2002) Aspirin-resistant thromboxane biosynthesis and the risk of myocardial infarction, stroke, or cardiovascular death in patients at high risk for cardiovascular events. Circulation 105:1650–1655
Faraday N, Becker DM, Becker LC (2007a) Pharmacogenomics of platelet responsiveness to aspirin. Pharmacogenomics 8:1413–1425
Faraday N, Yanek LR, Mathias R et al (2007b) Heritability of platelet responsiveness to aspirin in activation pathways directly and indirectly related to cyclooxygenase-1. Circulation 115:2490–2496
Femia EA, Pugliano M, Podda G et al (2012) Comparison of different procedures to prepare platelet-rich plasma for studies of platelet aggregation by light transmission aggregometry. Platelets 23:7–10
Ferreira MA, Hottenga JJ, Warrington NM et al (2009) Sequence variants in three loci influence monocyte counts and erythrocyte volume. Am J Hum Genet 85:745–749
Fitzgerald R, Pirmohamed M (2011) Aspirin resistance: effect of clinical, biochemical and genetic factors. Pharmacol Ther 130:213–225
Fontana P, Dupont A, Gandrille S et al (2003) Adenosine diphosphate-induced platelet aggregation is associated with P2Y12 gene sequence variations in healthy subjects. Circulation 108:989–995
Freedman JE (2007) Heritability, platelet function, and aspirin: a link established but cause unknown. Circulation 115:2468–2470
Frelinger AL, Furman MI, Linden MD et al (2006) Residual arachidonic acid-induced platelet activation via an adenosine diphosphate-dependent but cyclooxygenase-1- and cyclooxygenase- 2-independent pathway. Circulation 113:2888–2896
Garg SK, Amorosi EL, Karpatkin S (1971) Use of the megathrombocyte as an index of megakaryocyte number. N Engl J Med 284:11–17
Garner C, Tatu T, Reittie JE et al (2000) Genetic influences on F cells and other hematologic variables: a twin heritability study. Blood 95:342–346

Gaxiola B, Friedl W, Propping P (1984) Epinephrine-induced platelet aggregation. A twin study. Clin Genet 26:543–548

Gianfagna F, Cugino D, Santimone I et al (2012a) From candidate genes to genome-wide association studies in cardiovascular disease. Thromb Res 129(3):320–324, PMID: 22154244

Gianfagna F, Tamburrelli C, Vonhout B, Moli-family Study Investigators et al (2012b) Heritability, genetic correlation and linkage to 9p21 region of mixed platelet-leukocytes conjugates in families with and without early myocardial infarction. Nutr Metab Cardiovasc Dis. May 25. [Epub ahead of print]

Giles H, Smith RE, Martin JF (1994) Platelet glycoprotein IIb–IIIa and size are increased in acute myocardial infarction. Eur J Clin Invest 24:69–72

Goodall AH, Burns P, Salles I et al (2010) Transcription profiling in human platelets reveals LRRFIP1 as a novel protein regulating platelet function. Blood 116:4646–4656

Goodman T, Ferro A, Sharma P (2008) Pharmacogenetics of aspirin resistance: a comprehensive systematic review. Br J Clin Pharmacol 66:222–232

Gresele P, Blockmans D, Deckmyn H et al (1988) Adenylate cyclase activation determines the effect of thromboxane synthase inhibitors on platelet aggregation in vitro. Comparison of platelets from responders and nonresponders. J Pharmacol Exp Ther 246:301–307

Gresele P, Deckmyn H, Nenci GG et al (1991) Thromboxane synthase inhibitors, thromboxane receptor antagonists and dual blockers in thrombotic disorders. Trends Pharmacol Sci 12:158–163

Guerrero JA, Rivera J, Quiroga T et al (2011) Novel loci involved in platelet function and platelet count identified by a genome-wide study performed in children. Haematologica 96:1335–1343

Gurbel PA, Bliden KP, Hiatt BL et al (2003) Clopidogrel for coronary stenting: response variability, drug resistance, and the effect of pretreatment platelet reactivity. Circulation 107:2908–2913

Guthikonda S, Alviar CL, Vaduganathan M et al (2008) Role of reticulated platelets and platelet size heterogeneity on platelet activity after dual antiplatelet therapy with aspirin and clopidogrel in patients with stable coronary artery disease. J Am Coll Cardiol 52:743–749

Hardy AR, Conley PB, Luo J et al (2005) P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms. Blood 105:3552–3560

Harismendy O, Notani D, Song X et al (2011) 9p21 DNA variants associated with coronary artery disease impair interferon-γ signalling response. Nature 470:264–268

Hayward CP, Eikelboom J (2007) Platelet function testing: quality assurance. Semin Thromb Hemost 33:273–282

Hendra TJ, Oswald GA, Yudkin JS (1988) Increased mean platelet volume after acute myocardial infarction relates to diabetes and to cardiac failure. Diabetes Res Clin Pract 5:63–69

Heptinstall S, Fox SC (1983) Human blood platelet behaviour after inhibition of thromboxane synthetase. Br J Clin Pharmacol 15(Suppl 1):31S–37S

Hoffmeister KM, Felbinger TW, Falet H et al (2003) The clearance mechanism of chilled blood platelets. Cell 112:87–97

Huczek Z, Kochman J, Filipiak KJ et al (2005) Mean platelet volume on admission predicts impaired reperfusion and longterm mortality in acute myocardial infarction treated with primary percutaneous coronary intervention. J Am Coll Cardiol 46:284–290

Iacoviello L, Bonanni A, Costanzo S et al (2007) The Moli-Sani Project, a randomized, prospective cohort study in the Molise region in Italy; design, rationale and objectives. Ital J Public Health 4:110–118

Iacoviello L, Vohnout B, Gianfagna F et al (2009) Genetic regulation of inflammation-mediated haemostasis activation: a family-based approach. J Thromb Haemost 7(Suppl 2):S710

Jakubowski JA, Adler B, Thompson CB et al (1985) Influence of platelet volume on the ability of prostacyclin to inhibit platelet aggregation and the release reaction. J Lab Clin Med 105:271–276

Johnson AD (2011) The genetics of common variation affecting platelet development, function and pharmaceutical targeting. J Thromb Haemost 9(Suppl 1):246–257

Johnson AD, Yanek LR, Chen MH et al (2010) Genome-wide meta-analyses identifies seven loci associated with platelet aggregation in response to agonists. Nat Genet 42:608–613
Jones CI, Bray S, Garner SF, Bloodomics Consortium et al (2009) A functional genomics approach reveals novel quantitative trait loci associated with platelet signaling pathways. Blood 114:1405–1416
Kamath S, Blann AD, Lip GY (2001) Platelet activation: assessment and quantification. Eur Heart J 22:1561–1571
Karpatkin S, Khan Q, Freedman M (1978) Heterogeneity of platelet function. Correlation with platelet volume. Am J Med 64:542–546
Kathiresan S, Voight BF, Purcell S et al (2009) Genomewide association of early-onset myocardial infarction with single nucleotide polymorphisms and copy number variants. Nat Genet 41:334–341
Kawasaki T, Ozeki Y, Igawa T et al (2000) Increased platelet sensitivity to collagen in individuals resistant to low-dose aspirin. Stroke 31:591–595
Kazui M, Nishiya Y, Ishizuka T et al (2010) Identification of the human cytochrome P450 enzymes involved in the two oxidative steps in the bioactivation of clopidogrel to its pharmacologically active metabolite. Drug Metab Dispos 38:92–99
Knight CJ, Panesar M, Wright C et al (1997) Altered platelet function detected by flow cytometry. Effects of coronary artery disease and age. Arterioscler Thromb Vasc Biol 17:2044–2053
Kondkar AA, Bray MS, Leal SM et al (2010) VAMP8/endobrevin is overexpressed in hyperreactive human platelets: suggested role for platelet microRNA. J Thromb Haemost 8:369–378
Krasopoulos G, Brister SJ, Beattie WS et al (2008) Aspirin "resistance" and risk of cardiovascular morbidity: systematic review and meta-analysis. Br Med J 336:195–198
Kunicki TJ (2002) The influence of platelet collagen receptor polymorphisms in hemostasis and thrombotic disease. Arterioscler Thromb Vasc Biol 22:14–20
Kunicki TJ, Nugent DJ (2010) The genetics of normal platelet reactivity. Blood 116:2627–2634
Lepäntalo A, Mikkelsson J, Reséndiz JC et al (2006) Polymorphisms of COX-1 and GPVI associate with the antiplatelet effect of aspirin in coronary artery disease patients. Thromb Haemost 95:253–259
Lev EI, Solodky A, Harel N et al (2010) Treatment of aspirin-resistant patients with omega-3 fatty acids versus aspirin dose escalation. J Am Coll Cardiol 55:114–121
Lindemann JP, Kang KW, Christian JC (1977) Genetic variance of erythrocyte parameters in adult male twins. Clin Genet 12:73–76
Livio M, Del Maschio A, Cerletti C et al (1982) Indomethacin prevents the long-lasting inhibitory effect of aspirin on human platelet cyclo-oxygenase activity. Prostaglandins 23:787–796
Macchi L, Christiaens L, Brabant S et al (2002) Resistance to aspirin in vitro is associated with increased platelet sensitivity to adenosine diphosphate. Thromb Res 107:45–49
Marcucci R, Gori AM, Paniccia R et al (2007) Residual platelet reactivity is associated with clinical and laboratory characteristics in patients with ischemic heart disease undergoing PCI on dual antiplatelet therapy. Atherosclerosis 195:e217–e223
Martin JF, Bath PM, Burr ML (1991) Influence of platelet size on outcome after myocardial infarction. Lancet 338:1409–1411
Mathias RA, Kim Y, Sung H et al (2010) A combined genome-wide linkage and association approach to find susceptibility loci for platelet function phenotypes in European American and African American families with coronary artery disease. BMC Med Genomics 3:22
May JA, Heptinstall S, Cole AT et al (1997) Platelet responses to several agonists and combinations of agonists in whole blood: a placebo controlled comparison of the effects of a once daily dose of plain aspirin 300 mg, plain aspirin 75 mg and enteric coated aspirin 300 mg, in man. Thromb Res 88:183–192
Michelson AD, Frelinger AL 3rd, Furman MI (2006) Current options in platelet function testing. Am J Cardiol 98:4N–10N
Munnix IC, Cosemans JM, Auger JM et al (2009) Platelet response heterogeneity in thrombus formation. Thromb Haemost 102:1149–1156

Musunuru K, Post WS, Herzog W et al (2010) Association of single nucleotide polymorphisms on chromosome 9p21.3 with platelet reactivity: a potential mechanism for increased vascular disease. Circ Cardiovasc Genet 3:445–453

Nagalla S, Shaw C, Kong X et al (2011) Platelet microRNA-mRNA coexpression profiles correlate with platelet reactivity. Blood 117:5189–5197

Nagata Y, Yoshikawa J, Hashimoto A et al (2003) Proplatelet formation of megakaryocytes is triggered by autocrine-synthesized estradiol. Genes Dev 17:2864–2869

Neubauer H, Kaiser AF, Endres HG et al (2011) Tailored antiplatelet therapy can overcome clopidogrel and aspirin resistance–the BOchum CLopidogrel and Aspirin Plan (BOCLA-Plan) to improve antiplatelet therapy. BMC Med 9:3

Nurden A, Nurden P (2011) Advances in our understanding of the molecular basis of disorders of platelet function. J Thromb Haemost 9(Suppl 1):76–91

O'Brien JR (1968) Effects of salicylates on human platelets. Lancet 1:779–783

O'Donnell CJ, Larson MG, Feng D, Framingham Heart Study et al (2001) Genetic and environmental contributions to platelet aggregation. The Framingham Heart Study. Circulation 103:3051–3056

Ohmori T, Yatomi Y, Nonaka T et al (2006) Aspirin resistance detected with aggregometry cannot be explained by cyclooxygenase activity: involvement of other signaling pathway(s) in cardiovascular events of aspirin-treated patients. J Thromb Haemost 4:1271–1278

Patrignani P, Filabozzi P, Patrono C (1982) Selective cumulative inhibition of platelet thromboxane production by low-dose aspirin in healthy subjects. J Clin Invest 69:1366–1372

Patrono C (2003) Aspirin resistance: definition, mechanisms and clinical read-outs. J Thromb Haemost 1:1710–1713

Peace AJ, Tedesco AF, Foley DP et al (2008) Dual antiplatelet therapy unmasks distinct platelet reactivity in patients with coronary artery disease. J Thromb Haemost 6:2027–2034

Quick AJ (1966) Salicylates and bleeding: the aspirin tolerance test. Am J Med Sci 252:265–269

Quinn MJ, Topol EJ (2001) Common variations in platelet glycoproteins: pharmacogenomic implications. Pharmacogenomics 2:341–352

Rao GH, Reddy RK, White JG (1981) Low dose aspirin, platelet function and prostaglandin synthesis: influence of epinephrine and alpha adrenergic blockade. Prostaglandins Med 6:485–494

Reiner AP, Carlson CS, Thyagarajan B et al (2008) Soluble P-selectin, SELP polymorphisms, and atherosclerotic risk in European-American and African-African young adults: the Coronary Artery Risk Development in Young Adults (CARDIA) Study. Arterioscler Thromb Vasc Biol 28:1549–1555

Ren Q, Barber HK, Crawford GL et al (2007) Endobrevin/VAMP-8 is the primary v-SNARE for the platelet release reaction. Mol Biol Cell 18:24–33

Reny JL, De Moerloose P, Dauzat M et al (2008) Use of the PFA-100 closure time to predict cardiovascular events in aspirin-treated cardiovascular patients: a systematic review and meta-analysis. J Thromb Haemost 6:444–450

Ridker PM, Cook NR, Lee IM et al (2005) A randomized trial of low-dose aspirin in the primary prevention of cardiovascular disease in women. N Engl J Med 352:1293–1304

Sacco M, Pellegrini F, Roncaglioni MC, PPP Collaborative Group et al (2003) Primary prevention of cardiovascular events with low-dose aspirin and vitamin E in type 2 diabetic patients: results of the Primary Prevention Project (PPP) trial. Diabetes Care 26:3264–3272

Santilli F, Rocca B, De Cristofaro R, Lattanzio S et al (2009) Platelet cyclooxygenase inhibition by low-dose aspirin is not reflected consistently by platelet function assays: implications for aspirin "resistance". J Am Coll Cardiol 53:667–677

Santimone I, Di Castelnuovo A, De Curtis A, MOLI-SANI Project Investigators et al (2011) White blood cell count, sex and age are major determinants of heterogeneity of platelet indices in an adult general population: results from the MOLI-SANI project. Haematologica 96:1180–1188

Segal JB, Moliterno AR (2006) Platelet counts differ by sex, ethnicity, and age in the United States. Ann Epidemiol 16:123–130

Seyfert UT, Haubelt H, Vogt A et al (2007) Variables influencing Multiplate whole blood impedance platelet aggregometry and turbidimetric platelet aggregation in healthy individuals. Platelets 18:199–206

Shuldiner AR, O'Connell JR, Bliden KP et al (2009) Association of cytochrome P450 2C19 genotype with the antiplatelet effect and clinical efficacy of clopidogrel therapy. J Am Coll Cardiol 302:849–857

Sikorski K, Czerwoniec A, Bujnicki JM et al (2011) STAT1 as a novel therapeutical target in pro-atherogenic signal integration of IFNγ, TLR4 and IL-6 in vascular disease. Cytokine Growth Factor Rev 22:211–219

Smith JB, Willis AL (1971) Aspirin selectively inhibits prostaglandin production in human platelets. Nat New Biol 231:235–237

Smith NM, Pathansali R, Bath PM (2002) Altered megakaryocyte-platelet-haemostatic axis in patients with acute stroke. Platelets 13:113–120

Snoep JD, Hovens MM, Eikenboom JC et al (2007) Association of laboratory-defined aspirin resistance with a higher risk of recurrent cardiovascular events: a systematic review and meta-analysis. Arch Intern Med 167:1593–1599

Sofi F, Marcucci R, Gori AM et al (2008) Residual platelet reactivity on aspirin therapy and recurrent cardiovascular events – a meta-analysis. Int J Cardiol 128:166–171

Sofi F, Marcucci R, Gori AM et al (2010) Clopidogrel non-responsiveness and risk of cardiovascular morbidity. An updated meta-analysis. Thromb Haemost 103:841–848

Soranzo N, Spector TD, Mangino M et al (2009a) A genome-wide meta-analysis identifies 22 loci associated with eight hematological parameters in the HaemGen consortium. Nat Genet 41:1182–1190

Soranzo N, Rendon A, Gieger C et al (2009b) A novel variant on chromosome 7q22.3 associated with mean platelet volume, counts, and function. Blood 113:3831–3837

Stef G, Csiszar A, Lerea K et al (2006) Resveratrol inhibits aggregation of platelets from high-risk cardiac patients with aspirin resistance. J Cardiovasc Pharmacol 48:1–5

Takahashi S, Ushida M, Komine R et al (2007) Increased basal platelet activity, plasma adiponectin levels, and diabetes mellitus are associated with poor platelet responsiveness to in vitro effect of aspirin. Thromb Res 119:517–524

Taubert D, von Beckerath N, Grimberg G et al (2006) Impact of P-glycoprotein on clopidogrel absorption. Clin Pharmacol Ther 80:486–501

Thaulow E, Erikssen J, Sandvik L et al (1991) Blood platelet count and function are related to total and cardiovascular death in apparently healthy men. Circulation 84:613–617

Thompson CB, Jakubowski JA (1988) The pathophysiology and clinical relevance of platelet heterogeneity. Blood 72:1–8

Totani L, Evangelista V (2010) Platelet-leukocyte interactions in cardiovascular disease and beyond. Arterioscler Thromb Vasc Biol 30:2357–2361

Traglia M, Sala C, Masciullo C et al (2009) Heritability and demographic analyses in the large isolated population of Val Borbera suggest advantages in mapping complex traits genes. PLoS One 4:e7554

Turner-Stokes L, Jones D, Patterson KG et al (1991) Measurement of haematological indices of chronic rheumatic disease with two newer generation automated systems, the H1 and H6000 (Technicon). Ann Rheum Dis 50:583–587

van de Loo JC (1989) Predictive value of factors of the hemostatic system in screening procedures for coronary artery disease. Ric Clin Lab 19:333–338

van der Bom JG, Heckbert SR, Lumley T et al (2009) Platelet count and the risk for thrombosis and death in the elderly. J Thromb Haemost 7:399–405

van der Loo B, Martin JF (1999) A role for changes in platelet production in the cause of acute coronary syndromes. Arterioscler Thromb Vasc Biol 19:672–679

Vermylen J, de Gaetano G, Verstraete M (eds) (1971) Round-the-table conference on normal and modified platelet aggregation, Leuven-Brussels, 25–26 Sept 1970. Acta Medica Scandinavica Suppl 525

Vohnout B, Gianfagna F, Lorenzet R, Moli-family Study Investigators et al (2011) Genetic regulation of inflammation-mediated activation of haemostasis: family based approaches in population studies. Nutr Metab Cardiovasc Dis 21:857–861

Wallentin L, James S, Storey RF et al (2010) Effect of CYP2C19 and ABCB1 single nucleotide polymorphisms on outcomes of treatment with ticagrelor versus clopidogrel for acute coronary syndromes: a genetic substudy of the PLATO trial. Lancet 376:1320–1328

Weiss HJ, Aledort LM, Kochwa S (1968) The effect of salicylates on the hemostatic properties of platelets in man. J Clin Invest 47:2169–2180

White JG (2007) Platelet pathology in carriers of the X-linked GATA-1 macrothrombocytopenia. Platelets 18:620–627

Whitfield JB, Martin NG (1985) Genetic and environmental Influences on the size and number of cells in the blood. Genet Epidemiol 2:133–144

Yee DL, Sun CW, Bergeron AL et al (2005) Aggregometry detects platelet hyperreactivity in healthy individuals. Blood 106:2723–2729

Small RNAs as Potential Platelet Therapeutics

Leonard C. Edelstein and Paul F. Bray

Contents

Abstract MicroRNAs (miRNAs) are 21-23 nucleotide RNAs that regulate more than 60% of mammalian protein coding genes. miRNAs play critical roles in hematopoiesis and megakaryocyte function and development. Platelets, in addition to possessing functional miRNA processing machinery, have miRNA levels that have been correlated with platelet reactivity, and these miRNAs have been shown to target mRNAs that encode proteins that alter platelet function. There are potential uses of platelet miRNA as biomarkers and therapeutic agents. Due to the ability of platelets to release miRNA-containing microparticles at sites of activation, including angiogenic regions, tumors, and atherosclerotic plaques, there is the possibility of engineering platelets to deliver miRNA-based therapies to these sites. Cellpreferential expression of miRNAs could be exploited to restrict

L.C. Edelstein • P.F. Bray (✉)
The Cardeza Foundation for Hematologic Research and the Department of Medicine, Jefferson Medical College, Thomas Jefferson University, Curtis Building, Room 324, 1015 Walnut Street, Pennsylvania, PA 19107, USA
e-mail: paul.bray@jefferson.edu

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_17,

transgene expression in hematopoietic stem cell gene therapy to the desired lineage, including megakaryocytes and platelets. Finally, manipulation of gene expression in stored platelets may allow more effective platelet storage. Although much work remains to be done, there is great potential in miRNA-based platelet therapies.

Keywords miRNA • Platelet • Hematopoiesis • Megakaryocyte • Microparticles • Gene therapy

1 Introduction

Over the last 20 years it has become increasingly clear that a large amount of genetic information is stored in noncoding RNAs, the best studied of which is microRNA (miRNA). MiRNAs are 21–23 nucleotide regulatory RNAs expressed in multicellular organisms, from viruses to plants to humans (Bartel 2004). MiRNAs regulate most (>60 %) mammalian protein coding genes primarily by repressing gene expression. Current estimates from MiRBase version 17 predict 1,733 mature human miRNAs (Griffiths-Jones et al. 2008), although not all of these have been validated. Some miRNAs are expressed ubiquitously, but many are specific for tissue and/or developmental stage (Wienholds et al. 2005). Cell miRNA content is highly variable from 1 to 10,000 copies (Chen et al. 2005).

Altered miRNA levels and function impact on human disease. MiRNAs have been shown to play important roles in cancer, angiogenesis, vascular disease, and apoptosis (Nana-Sinkam and Croce 2011). In addition, miRNAs have the potential to regulate aberrant biological functions as both therapeutics and as targets of therapy. The focus of this chapter is on miRNAs, although most of the therapeutic principles to be discussed also apply to small interfering RNAs (siRNA). In this chapter, we will review: (1) the biology of miRNAs and their role in megakaryocytes (MKs) and platelets, (2) the clinical relevance of miRNAs as biomarkers, targets, therapeutics, and tissue-specific restrictors of genes expression, (3) the potential of platelets and platelet-derived microparticles to act as delivery vehicles of miRNAs, and (4) the possible role of miRNAs in regulating protein translation in stored platelets and platelet lifespan. Although there has been an explosion of information about the biology of miRNAs in the past decade, the clinical therapeutic utility of small interfering RNAs is rather speculative.

2 miRNA Biogenesis and Function

The steps of "canonical" miRNA biogenesis are shown in Fig. 1. Approximately 70 % of miRNA genes reside within larger protein coding genes; the remainder are located in intergenic regions. MiRNA genes are usually transcribed by RNA polymerase II, and the resulting large primary transcript (pri-miRNA) is capped and poly

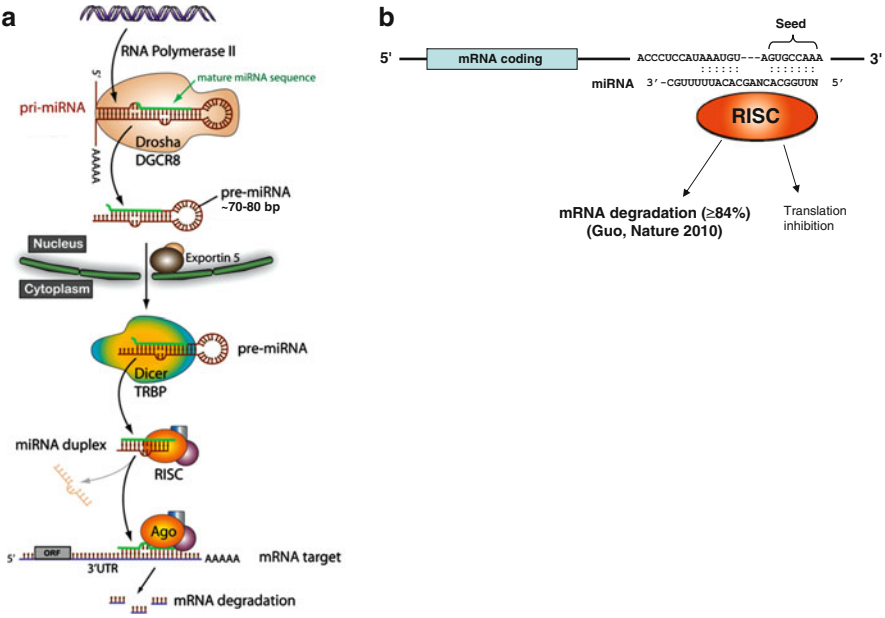


Fig. 1 MicroRNA biogenesis and function. (**a**) The canonical miRNA biosynthesis pathway. Note that exceptions to many of these steps have been described. Drosha, an RNase type III endonuclease; DGCR8, DiGeorge syndrome critical region 8; Dicer, another RNase type III endonuclease; TRBP, TAR RNA-binding protein; RISC, RNA-induced silencing complex; Ago, Argonaute 2; ORF, open reading frame. (**b**) The seed region of the miRNA (bp 2–8) bind to complementary sites on the 3′ UTR of target mRNAs where it predominantly not only causes a decrease in mRNA levels, but may also inhibit translation

adenylated (Cai et al. 2004; Lee et al. 2004). The type III RNase Drosha cleaves the pri-miRNA into a 60–70 bp hairpin-containing pre-miRNA, which is transported out of the nucleus by Exportin 5 and Ran GTPase. Once in the cytoplasm, another type III RNAse, Dicer, removes the hairpin leaving a miRNA/miRNA* duplex. The miRNA* strand is removed, leaving a mature miRNA. The mature miRNA is loaded into the RNA-induced silencing complex (RISC), where it directs cleavage of target RNAs via Argonaute 2 (Ago2) (reviewed in Kim et al. 2009). Targeting is accomplished by perfect complementation of nucleotides 2–8, the 5′ "seed" region of the miRNA, with complementary sequences in the targeted mRNA (Fig. 1b). These target sites are usually, but not always, located within the 3′ untranslated region (UTR) of the mRNA. 3′ to the seed region in the miRNA, base pairing is imperfect with the target mRNA. Importantly, one miRNA typically targets many mRNAs and most mRNAs contain binding sites for multiple miRNAs. Usually, miRNA control is not all or none, but rather a fine tuning of gene expression levels. mRNA, protein, and miRNA are often in highly regulated relationships. In addition, there many examples of miRNAs indirectly altering gene expression by suppressing the expression of a transcription factor (Bartel 2004).

The importance of miRNA processing and function is demonstrated by genetic and acquired diseases that are caused by altered miRNA synthesis and targeting, the best studied being cancer. The mechanisms include polymorphisms in the 3′UTR of target mRNAs that disrupt miRNA binding, miRNA "decoys" that compete with binding sites for the available supply of miRNAs, and defects in the miRNA biogenesis machinery that alters miRNA levels (Sethupathy and Collins 2008; Bandiera et al. 2010; Poliseno et al. 2010).

3 Function of miRNAs in Megakaryocytes and Platelets

Considerable evidence has accumulated supporting the critical role of the miRNA machinery in hematopoiesis (Garzon and Croce 2008), including erythropoiesis, granulocytopoiesis/monocytopoiesis, and lymphopoiesis. Studies utilizing $CD34^+$ hematopoietic stem cells (HSCs) or MK-like cell lines have defined a role for miRNAs in megakaryocytopoiesis and MK function (reviewed in Edelstein and Bray 2011). Among the best-characterized MK miRNAs are (1) miR-155, which decreases in abundance in $CD34^+$ cells induced to differentiate into MKs and which prevents differentiation when overexpressed in K562 cells, and (2) miR-150, which increases in cord blood derived $CD34^+$ cells during differentiation into MKs and enhances differentiation when overexpressed.

Platelets possess miRNAs and the machinery with which to process them, including Dicer, TRBP2, and Ago2 (Landry et al. 2009; Nagalla et al. 2011). The first human platelet miRNA profiling study was performed in 2008 by Bruchova et al. to test for differentially expressed miRNAs in patients with polycythemia vera (Bruchova et al. 2008). Our group has investigated associations between miRNAs and platelet reactivity, both to understand megakaryocyte/platelet gene expression better and to identify the potential biomarkers for thrombotic risk. There is a well-known inter-individual variation in human platelet reactivity, which likely contributes to the risk of pathologic bleeding and thrombosis. Platelets of differing reactivity have different levels of *VAMP8* mRNA (Kondkar et al. 2010), which encodes a protein facilitating platelet granule release. A SNP in the *VAMP8* 3′ UTR is associated with coronary artery disease and is near a binding site for miR-96. Intriguingly, miR-96 is associated with platelet reactivity and knocks down *VAMP8* expression. In addition, bioinformatic approaches have identified miRNA–mRNA pairs in platelets that, when tested in cells, demonstrated novel regulators of platelet reactivity (Nagalla et al. 2011). This "pairs" approach led to the identification of a functional role for the regulatory subunit of protein kinase A (*PRKAR2B*) in platelets. Platelet *miR-200b* is differentially expressed between hyperreactive and hyporeactive platelets, and knocks down *PRKAR2B* expression leading to reduced platelet reactivity.

4 miRNAs as Biomarkers

MiRNAs possess several properties that make them particularly suitable as biomarkers. MiRNAs are readily detected in many body fluids such as blood, plasma, tears, breast milk, bronchial lavage, and colostrum by microarrays, quantitative real-time PCR (qPCR), and deep sequencing (Weber et al. 2010). Most importantly, miRNAs are extremely stable and show little degradation to repeated freeze–thaw cycles or a wide pH range (Chen et al. 2008). miRNA levels predict disease activity and outcome in lymphoma, leukemia, myelodysplasia, and some myeloproliferative disease patients (Garzon and Croce 2008). MiRNAs are globally repressed in cancer cells and miRNA profiles have been used to identify tumor type and subtype, the primary site of cancers of unknown origin, prognosis, and responsiveness to drug therapy. In particular, miRNA signatures have been identified for breast carcinoma, hepatocellular carcinoma, papillary thyroid carcinoma, and lung cancer (Takamizawa et al. 2004; Calin et al. 2005; Lu et al. 2005; Nana-Sinkam and Croce 2011). miRNAs have also been characterized as diagnostic markers of cardiovascular disease (Ye et al. 2008). Circulating levels of miR-126, -17, -92a, and -155 are lower in patients with coronary artery disease and the plasma levels of miR-1, -133a, -208a, and -499 are increased in patients with acute myocardial infarction.

Only recently have platelet miRNAs been considered as biomarkers for platelet reactivity. Nagalla et al. performed platelet miRNA profiling on a cohort 19 healthy donors and showed that the platelet aggregation response could be predicted by as few as 7 miRNAs (miR-19b, -34b, -190, -320a, -320b, -320c, and -320d) (Nagalla et al. 2011). Although these intriguing findings need confirmation in other cohorts, these data provide candidate biomarkers for studies of patients with platelet-mediated disorders of thrombosis and hemostasis.

5 Approaches for miRNA Therapeutics

There are two general therapeutic strategies involving miRNAs: (1) overexpression of an exogenous miRNA to knock down unwanted gene expression, or (2) overexpression of an inhibitor of miRNAs to reverse the malevolent effects of disease-producing miRNAs. Most reports with miRNA manipulation have used inhibitors. These inhibitors are antisense oligonucleotides (ASOs) or anti-miRNA oligonucleotides (AMOs), which are short, complimentary antisense molecules that bind to miRNAs. To increase stability and specificity of AMOs, antagomirs that are 2′-*O*-methyl cholesterol conjugated have also been developed (Krutzfeldt et al. 2005). A third generation of AMOs are locked nucleic acids (LNAs) in which the ribose of one or more nucleotides contains an extra methylene bridge to "lock" the nucleic acid in a conformation that stabilizes the duplexes formed with miRNAs (Kurreck 2003). Another approach is the use of miRNA decoys or "sponges" that act as competitive inhibitors. These miRNA sponges are transgenes expressed

containing multiple, tandem binding sites for a microRNA of interest (Ebert et al. 2007). Advantages of miRNA sponges are that they can block multiple miRNAs and can be expressed by genetically modified cells, unlike the chemically modified antagomirs and LNAs.

6 Modification of miRNA Expression in Disease Models

To date, miRNA manipulation has not been used in patients. Although numerous technical hurdles would need to be overcome before platelet miRNA-based therapies could become a reality, modification of platelets can be accomplished by ex vivo viral transduction of $CD34^+$ hematopoietic stem cells and subsequent differentiation into megakaryocytes and platelets (Gillitzer et al. 2005). Alternatively, platelets can be directly transfected with small noncoding RNA (Hong et al. 2011). A large body of cell biology evidence and xenograph transplant models support the potential for using miRNA expression-modifying therapeutics to treat pathologic conditions. Doebele et al. reported that intravenous antagomirs-17 and -20 increased the number of perfused vessels invading matrigel plugs implanted in mice (Doebele et al. 2010). Systemic administration of anti-miR-132 nanoparticles decreased tumor burden and angiogenesis in an orthotopic xenograft mouse model of human breast carcinoma, MDA-MB 231 (Anand et al. 2010). Ma and colleagues reported that intravenous administration of antagomir-10b suppressed the formation of lung metastases in a mouse mammary carcinoma model (Ma et al. 2010). Antagomir-21 blocked interstitial fibrosis and improved myocardial dysfunction in a mouse model of heart failure, and antagomir -92a promoted angiogenesis and improved function of ischemic myocardium in a mouse model of myocardial ischemia (Thum et al. 2008; Bonauer et al. 2009). Based on these types of experiments, it is reasonable to believe that manipulating miRNAs in platelets may benefit disorders of platelet function or number. Alternatively, platelets could be used to deliver miRNAs or miRNA inhibitors to specific anatomic sites.

7 Platelets as Delivery Vehicles for miRNA-Based Therapies

Platelets are intimately involved in the physiology and pathophysiology of hemostasis, thrombosis, wound repair, inflammation, atherosclerosis, and cardiovascular disease. For example, besides their critical role in hemostasis and thrombosis, platelets (1) interact with tumor cells and stimulate angiogenic tumor growth by releasing VEGF and microparticles (MPs) (Kim et al. 2002, 2004; Gillitzer et al. 2005), (2) facilitate the spread of tumor metastasis (Verheul et al. 1997; Wartiovaara et al. 1998), (3) mediate atherosclerosis by monocyte recruitment to inflamed endothelium and release of growth factors (e.g., PDGF and TGFβ) (Slupsky et al. 1998), and (4) enhance inflammation by the delivery of

inflammatory chemokines (e.g., CD40L, RANTES, IL-1β, macrophage chemotactic peptide-3).

There is compelling rationale to consider the possibility of utilizing genetically modified platelets to deliver miRNAs or miRNA inhibitors to sites where platelets localize, secrete granule contents, and shed MPs. First, platelets are a large source of miRNAs (Landry et al. 2009; Nagalla et al. 2011). Second, small interfering RNAs are mobile and can be transferred via cell-to-cell contact (Molnar et al. 2010). Third, MPs contain abundant miRNAs and can transfer microRNAs to fibroblasts in vitro (Hunter et al. 2008; Yuan et al. 2009). Perhaps P-selectin bearing platelet MPs could target miRNAs to PSGL-1 expressing leukocytes at the sites of vessel injury, inflammation, or tumorigenesis. Finally, a similar strategy has been used by the Poncz laboratory to engineer platelets that ectopically deliver factor VIII to the sites of hemorrhage (Greene et al. 2010). There is also a pharmacogenetic rationale for regulating miRNA levels in disease, because interferon has been shown to be more effective in patients with hepatocellular carcinoma who had low levels of miR-26 (Ji et al. 2009). Therefore, modification of platelets to deliver miRNAs or miRNA inhibitors may be a useful approach in treating a variety of disease processes. Examples in which this approach might have clinical potential include the targeted delivery of miRNAs that (1) knock down platelet P2Y12 in disorders of pathologic thrombosis, (2) knock down fibrinolytic factors in hemorrhagic disorders, (3) knock down mediators of apoptosis to lengthen the lifespan of transfused platelets, or (4) knock down IL-1β to reduce inflammation.

8 Utilization of Tissue-Specific miRNA Expression to Avoid Off-Target Effects in Gene Therapy

The ability of miRNAs to inhibit gene expression makes them a potential tool to restrict transgene expression to specific cell types when expression in other cells would cause undesired consequences. For example, Qiao and colleagues took advantage of the liver-restricted expression of miR-122. The liver is often a site of ectopic expression of gene-therapy viruses and these investigators reasoned that the presence of miR-122 could prevent virally delivered transgene expression in the liver. Multiple miR-122 target sites were engineered into the 3′-UTR of transgenes carried by adeno-associated virus vectors and found that the presence of the target sites reduced liver expression of luciferase and β-galactosidase 50-fold and 70-fold, respectively (Qiao et al. 2011). Hematologic gene therapies that transduce hematopoietic stem cells (HSCs) ex vivo and then reintroduce them into the patient typically desire tissue-specific expression. Knowing the repertoire of miRNAs expressed in different hematopoietic cell lineages would permit the construction of transgene expression vectors with miRNA binding sites that would favor tissue-specific expression by effects on translation (thus complementing the existing "tissue-specific"(sic) promoter effects on transcription). For example, certain types of thrombocytopenias can be traced to defects in the RUNX1 transcription

factor; however, overexpression of this factor in lymphocytic cell types could lead to transformation (Geiss et al. 2008). Therefore, it would be prudent to restrict RUNX1 overexpression to the MK lineage. A similar strategy could be used when the goal is to load megakaryocytes and platelets (but not other cells) with therapeutic miRNA or miRNA inhibitors.

9 Regulation of Protein Translation in Stored Platelets

Human platelets are harvested and stored in Blood Banks for subsequent transfusion; storage is at room temperature for up to 5 days. Storage times longer than 5 days are associated with reduced platelet survival and function, as well as an increased risk of bacterial overgrowth. The latter problem could be reduced by storage at 4 °C, but cold-induced GPIbα clustering results in clearance by the hepatic macrophage complement type 3 receptor (Hoffmeister et al. 2003). Not only do platelets remain metabolically active in storage, but they also retain the capacity to translate mRNA proteins (Thon and Devine 2007). This raises the intriguing possibility that selected platelet proteins could be knocked down during storage. Hong et al. established proof-of-principle that small inhibitory RNAs could be introduced into platelets and knock down target mRNA (Hong et al. 2011). Thus, technological advances may allow the manipulation of gene expression in stored platelets in a manner that could enhance platelet reactivity, increase lifespan, or permit storage at 4 °C.

10 Conclusion

The importance of miRNAs in normal and pathologic biology has become clear in the recent years. miRNAs have been shown to have a role in hemostasis/thrombosis, immunology, malignancy, liver disease, lung disease, and cardiac disease. High stability of miRNAs makes them especially useful biomarkers. The ability of miRNAs to modify gene expression also makes them attractive potential therapeutics, and their tissue-specific expression may facilitate novel vectors for gene therapy with reduced off-target effects. Negative or positive modification of mRNA expression may be accomplished with miRNAs or miRNA inhibitors, respectively. Platelets are well suited to deliver miRNA-expression modifying agents because they contain high levels of miRNAs and they circulate and release their contents at areas of vessel injury, tumor growth, and inflammation. In addition, platelets stored in Blood Banks are attractive targets for miRNA-based therapies because there is little risk of oncogenic transformation. Although we are just beginning to understand the role of miRNAs in platelet and megakaryocyte biology, there is great potential for novel approaches to manipulate gene expression via miRNAs in the disorders of hemostasis and thrombosis.

Knowledge Gaps

- The study of microRNAs in megakaryocyte and platelet biology and therapeutics is in its infancy.
- The forced regulation of platelet miRNA levels on platelet function and number has not been studied.
- The forced regulation of platelet miRNA levels in platelets in storage is unknown.

Key Messages

- Altered miRNA expression can cause human disease.
- MiRNAs are critical regulators of hematopoiesis, including megakaryacytopoiesis.
- Platelets possess functional miRNA processing machinery.
- Emerging evidence suggests levels of selected platelet miRNA can predict platelet reactivity.
- MiRNA levels can be manipulated both positively using genetic vectors, and negatively utilizing chemically modified oligonucleotides or decoys.
- Manipulation of miRNA expression in megakaryocytes or platelets has the potential to (a) deliver therapeutic miRNAs to the sites where platelets become activated, (b) obtain hematopoietic lineage- specific transgene expression, and (c) enhance platelet lifespan in storage.

References

Anand S, Majeti BK, Acevedo LM et al (2010) MicroRNA-132-mediated loss of p120RasGAP activates the endothelium to facilitate pathological angiogenesis. Nat Med 16:909–914

Bandiera S, Hatem E, Lyonnet S et al (2010) microRNAs in diseases: from candidate to modifier genes. Clin Genet 77:306–313

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297

Bonauer A, Carmona G, Iwasaki M et al (2009) MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. Science 324:1710–1713

Bruchova H, Merkerova M, Prchal JT (2008) Aberrant expression of microRNA in polycythemia vera. Haematologica 93:1009–1016

Cai X, Hagedorn CH, Cullen BR (2004) Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA 10:1957–1966

Calin GA, Ferracin M, Cimmino A et al (2005) A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med 353:1793–1801

Chen C, Ridzon DA, Broomer AJ et al (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 33:e179

Chen X, Ba Y, Ma L et al (2008) Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 18:997–1006
Doebele C, Bonauer A, Fischer A et al (2010) Members of the microRNA-17-92 cluster exhibit a cell-intrinsic antiangiogenic function in endothelial cells. Blood 115:4944–4950
Ebert MS, Neilson JR, Sharp PA (2007) MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. Nat Methods 4:721–726
Edelstein LC, Bray PF (2011) MicroRNAs in platelet production and activation. Blood 117:5289–5296
Garzon R, Croce CM (2008) MicroRNAs in normal and malignant hematopoiesis. Curr Opin Hematol 15:352–358
Geiss GK, Bumgarner RE, Birditt B et al (2008) Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat Biotechnol 26:317–325
Gillitzer A, Peluso M, Laugwitz KL et al (2005) Retroviral infection and selection of culture-derived platelets allows study of the effect of transgenes on platelet physiology ex vivo and on thrombus formation in vivo. Arterioscler Thromb Vasc Biol 25:1750–1755
Greene TK, Wang C, Hirsch JD et al (2010) In vivo efficacy of platelet-delivered, high specific activity factor VIII variants. Blood 116:6114–6122
Griffiths-Jones S, Saini HK, van Dongen S et al (2008) miRBase: tools for microRNA genomics. Nucleic Acids Res 36:D154–D158
Hoffmeister KM, Felbinger TW, Falet H et al (2003) The clearance mechanism of chilled blood platelets. Cell 112:87–97
Hong W, Kondkar AA, Nagalla S et al (2011) Transfection of human platelets with short interfering RNA. Clin Transl Sci 4:180–182
Hunter MP, Ismail N, Zhang X et al (2008) Detection of microRNA expression in human peripheral blood microvesicles. PLoS One 3:e3694
Ji J, Shi J, Budhu A et al (2009) MicroRNA expression, survival, and response to interferon in liver cancer. N Engl J Med 361:1437–1447
Kim CW, Lee HM, Lee TH et al (2002) Extracellular membrane vesicles from tumor cells promote angiogenesis via sphingomyelin. Cancer Res 62:6312–6317
Kim HK, Song KS, Chung JH et al (2004) Platelet microparticles induce angiogenesis in vitro. Br J Haematol 124:376–384
Kim VN, Han J, Siomi MC (2009) Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 10:126–139
Kondkar AA, Bray MS, Leal SM et al (2010) VAMP8/endobrevin is overexpressed in hyperreactive human platelets: suggested role for platelet microRNA. J Thromb Haemost 8:369–378
Krutzfeldt J, Rajewsky N, Braich R et al (2005) Silencing of microRNAs in vivo with 'antagomirs'. Nature 438:685–689
Kurreck J (2003) Antisense technologies. Improvement through novel chemical modifications. Eur J Biochem 270:1628–1644
Landry P, Plante I, Ouellet DL et al (2009) Existence of a microRNA pathway in anucleate platelets. Nat Struct Mol Biol 16:961–966
Lee Y, Kim M, Han J et al (2004) MicroRNA genes are transcribed by RNA polymerase II. EMBO J 23:4051–4060
Lu J, Getz G, Miska EA et al (2005) MicroRNA expression profiles classify human cancers. Nature 435:834–838
Ma L, Reinhardt F, Pan E et al (2010) Therapeutic silencing of miR-10b inhibits metastasis in a mouse mammary tumor model. Nat Biotechnol 28:341–347
Molnar A, Melnyk CW, Bassett A et al (2010) Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. Science 328:872–875
Nagalla S, Shaw C, Kong X et al (2011) Platelet microRNA-mRNA coexpression profiles correlate with platelet reactivity. Blood 117:5189–5197
Nana-Sinkam SP, Croce CM (2011) MicroRNAs as therapeutic targets in cancer. Transl Res 157:216–225

Poliseno L, Salmena L, Zhang J et al (2010) A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. Nature 465:1033–1038
Qiao C, Yuan Z, Li J et al (2011) Liver-specific microRNA-122 target sequences incorporated in AAV vectors efficiently inhibits transgene expression in the liver. Gene Ther 18:403–410
Sethupathy P, Collins FS (2008) MicroRNA target site polymorphisms and human disease. Trends Genet 24:489–497
Slupsky JR, Kalbas M, Willuweit A et al (1998) Activated platelets induce tissue factor expression on human umbilical vein endothelial cells by ligation of CD40. Thromb Haemost 80:1008–1014
Takamizawa J, Konishi H, Yanagisawa K et al (2004) Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 64:3753–3756
Thon JN, Devine DV (2007) Translation of glycoprotein IIIa in stored blood platelets. Transfusion 47:2260–2270
Thum T, Gross C, Fiedler J et al (2008) MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature 456:980–984
Verheul HM, Hoekman K, Luykx-de Bakker S et al (1997) Platelet: transporter of vascular endothelial growth factor. Clin Cancer Res 3:2187–2190
Wartiovaara U, Salven P, Mikkola H et al (1998) Peripheral blood platelets express VEGF-C and VEGF which are released during platelet activation. Thromb Haemost 80:171–175
Weber JA, Baxter DH, Zhang S et al (2010) The microRNA spectrum in 12 body fluids. Clin Chem 56:1733–1741
Wienholds E, Kloosterman WP, Miska E et al (2005) MicroRNA expression in zebrafish embryonic development. Science 309:310–311
Ye Z, Yu X, Cheng L (2008) Lentiviral gene transduction of mouse and human stem cells. Methods Mol Biol 430:243–253
Yuan A, Farber EL, Rapoport AL et al (2009) Transfer of microRNAs by embryonic stem cell microvesicles. PLoS One 4:e4722

Pharmacological Modulation of the Inflammatory Actions of Platelets

Richard Amison, Clive Page, and Simon Pitchford

Contents

Abstract Patients with inflammatory diseases often exhibit a change in platelet function, with these alterations being clearly distinct from the well-characterized role of platelets in haemostasis and thrombosis. It has recently been revealed that platelets can behave as innate inflammatory cells in immune responses with roles in leukocyte recruitment, migration into tissues, release of cytotoxic mediators, and in tissue remodelling following injury.

Platelets exhibit a wide range of receptors for mediators involved in the inflammatory pathway and the immune response (Fig. 1). These include purinergic receptors, selectins, integrins, toll-like receptors, immunoglobulins, and chemokine receptors, but the precise role platelets play in the inflammatory process is still under investigation. Nevertheless, given that many of these receptors are distinct from those involved in thrombosis and haemostasis, this raises the real possibility of targeting these receptors to regulate inflammatory diseases without compromising haemostasis.

R. Amison • C. Page (✉) • S. Pitchford
Sackler Institute of Pulmonary Pharmacology, King's College London, 150 Stamford Street, Room 5.44, 5th Floor, Franklin-Wilkins Building, Waterloo Campus, London SE1 9NH, UK
e-mail: clive.page@kcl.ac.uk

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_18,

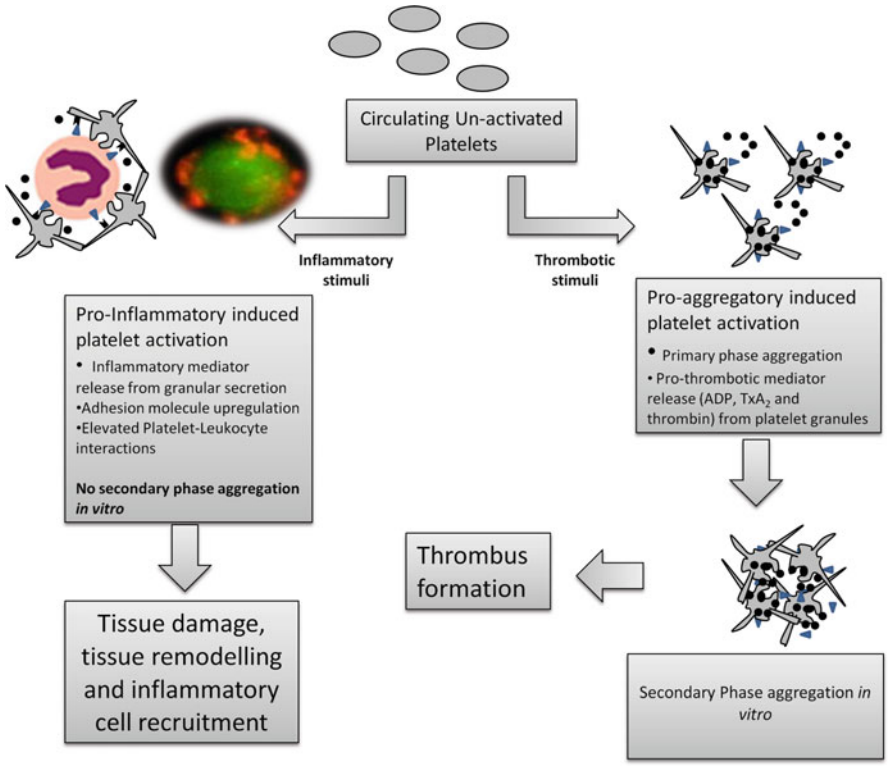


Fig. 1 Dichotomy of platelet function. It is possible for platelets to become activated by either pro-thrombotic or pro-inflammatory mediators depending on the activating stimuli. Therefore, the final function of platelets is dependent on the type of activating stimuli

Keywords Platelets • Inflammation • Pharmacology • P-selectin • $P2Y_1$ • $P2Y_{12}$ • Phosphodiesterase • NF-κB • Glycoprotein IIb-IIIa

1 A Dichotomy in Platelet Function

Platelets have primarily been associated with their role in haemostasis and thrombosis. However, over the past decade, the role of platelets in inflammatory disorders has become much more apparent with their actions on both the innate and adaptive immune responses gaining more attention (von Hundelshausen and Weber 2007). In particular, platelets have been implicated in inflammatory disorders such as allergic inflammation (asthma, allergic rhinitis, and eczema), chronic obstructive pulmonary disease (COPD), inflammatory bowel disease (IBD), atherosclerosis, inflammation associated with cardiovascular disease, rheumatoid arthritis (RA), and immune responses involved in host defence, for example bacteria and parasites (Pitchford 2007; Semple and Freedman 2010). Due to evolutionary traits, it has been hypothesized that it is the requisite role platelets play in haemostasis and

therefore host defence that links them intimately with inflammatory processes (Pitchford 2007). The processes involved in both haemostasis and innate immune responses have separated such that they now appear distinct (Pitchford 2007). Nevertheless, platelets retain a requisite role in both, such that platelets behave in a dichotomous nature in vivo. Thus, depending on the type of stimulus involved (either pro-thrombotic or pro-inflammatory), platelets may induce either a thrombotic response or an inflammatory response. The type of stimulus therefore dictating the ultimate function of the platelet (Fig. 1) (Maccia et al. 1977; Thompson et al. 1984; Page 1993). It is this dichotomy in platelet function that may ultimately lead to novel therapeutic options targeting the actions of platelets in inflammation that is separate and distinct from current pharmacological therapies used to inhibit the platelet activation contribution to thrombosis.

2 How Do Platelets Contribute to Inflammatory Disorders?

Platelets can contribute to inflammatory processes through various mechanisms. These mechanisms include migration into inflamed tissue and release of substances that destroy or modulate organ function directly; leukocyte 'priming' for efficient tissue recruitment; a contribution to chronic inflammatory events resulting in changes to tissue architecture; and stimulating the adaptive immune response by acting as a bridge via direct involvement in the innate immune response (Fig. 2) (Pitchford 2007).

A major involvement of platelets to the inflammatory response is their participation in the tissue recruitment of leukocytes. In conditions leading to thrombosis, the ability of platelets to adhere to damaged endothelial cells initiating further activation of both platelets and endothelial cells through interactions between integrin $\alpha_{11b}\beta_3$ on platelets and adhesive proteins on the endothelium such as von Willebrand factor (vWF), fibronectin, and fibrinogen is well documented (von Hundelshausen and Weber 2007). Once activated, mediators such as cytokines, chemokines, histamine, 5-HT, and matrix metallaproteases (MMPs) are released from platelets leading to the formation of a clot (von Hundelshausen and Weber 2007). Conversely, it has now been widely documented in patients with various inflammatory diseases such as asthma, COPD, atherosclerosis, and RA that circulating platelet–leukocyte complexes occur via adhesion molecule interactions that do not lead to platelet aggregation (Fig. 3) (Arber et al. 1991; Ferroni et al. 2000; Pitchford et al. 2003, 2005; Joseph et al. 2001; Bunescu et al. 2004; Neumann et al. 1997; Ott et al. 1996; Irving et al. 2002; Sarma et al. 2002; Huo et al. 2003). It is believed this phenomenon 'primes' resting circulating leukocytes for efficient recruitment to inflamed tissue. In this regard, experimental models of disease have provided evidence for a profound and virtually absolute requirement of platelets in pulmonary neutrophil, eosinophil, and lymphocyte recruitment in models of allergic and non-allergic pulmonary inflammation (Pitchford et al. 2003, 2005; Kornerup et al. 2010); neutrophil and monocyte recruitment in atherosclerosis (Arber et al. 1991; Neumann et al. 1997; Hayward et al. 1999); neutrophil and monocyte recruitment to synovial joints in RA (Schmitt-Sody et al. 2005); and

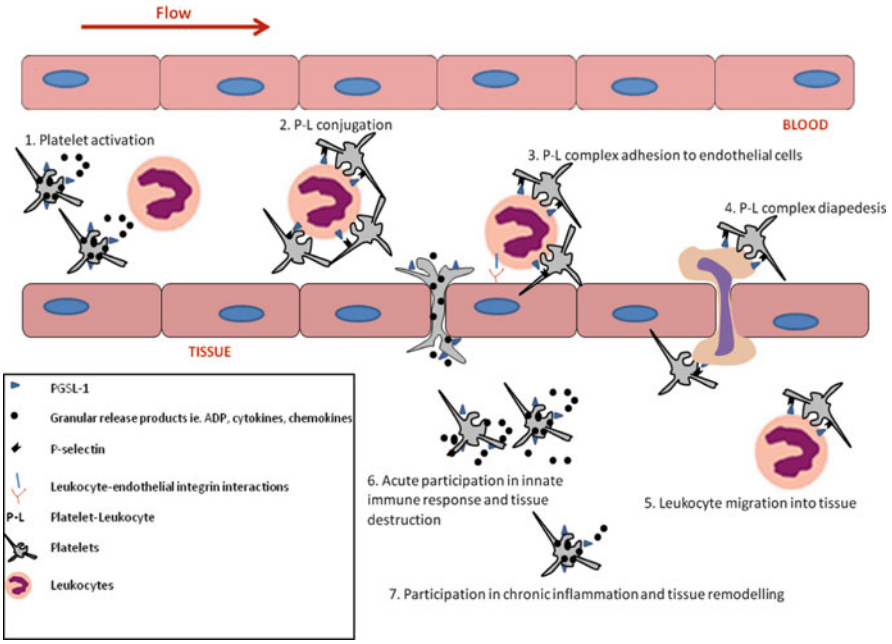


Fig. 2 Contribution of platelets to inflammatory processes. Platelet activation results in granular secretion of inflammatory mediators and upregulation of adhesion molecules. Expression of P-selectin and PGSL-1 triggers platelet–leukocyte (P–L) conjugation eventually targeting the P–L complex to the endothelium for firm adhesion through integrin activation. This allows the P–L complex to migrate across the endothelium into damaged tissue

neutrophil recruitment in acute lung injury (ALI) (Zarbock et al. 2006). This phenomenon requires intact platelets expressing mediators on the cell surface with platelet P-selectin or the counter ligand PSGL-1 of particular importance as disruption of these selectin-mediated events reduces the subsequent expression of integrins on the surface of the leukocytes (Pitchford et al. 2003, 2005; Huo et al. 2003; Kornerup et al. 2010; Zarbock et al. 2006; Schober et al. 2002; Diacovo et al. 1996a, b). Selectin-mediated rolling is thus an essential step towards firm cell–cell adhesion with endothelium directed by β_2 integrins, eventually leading to leukocyte diapedesis into tissue.

As previously described, the release of inflammatory mediators such as ADP, 5-HT, PF-4, RANTES, etc. from platelets has been widely documented. However during allergic reactions, metabolites of arachidonic acid and phospholipid metabolism are also released from platelets (Vargaftig et al. 1981). For example, TXA_2, a potent vasoconstrictor exhibits an increase in synthesis following co-operative interactions between platelets and macrophages/eosinophils (Maghni et al. 1993). The platelet-specific enzyme 12-lipoxygenase is also responsible for the production of Hydroxyeicosatetraenoic acid (12-HETE) which exhibits chemotactic activity for eosinophils (Goeztl et al. 1977; Marcus et al. 1984). Interestingly, neutrophils

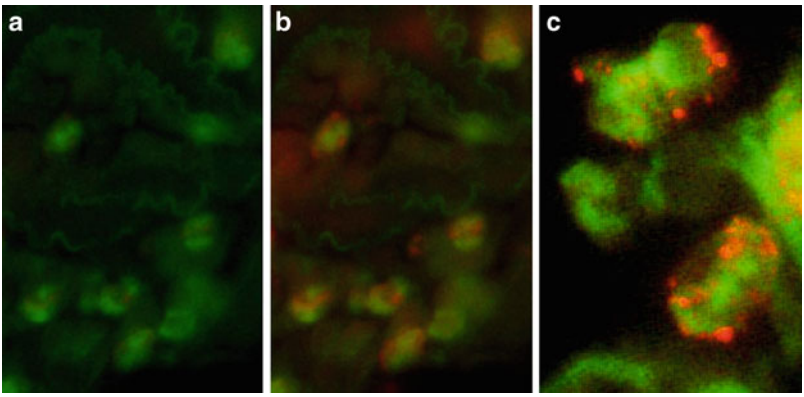


Fig. 3 Identification of platelet–eosinophil complexes taken from allergen-sensitized mice after allergen exposure. Lung samples taken from allergen-challenged mice were stained with rat anti-MBP for eosinophils (*green fluorescence*) and goat anti-CD41 for platelets (*red fluorescence*). (**a**) Image with *green fluorescence* filter only. (**b**) *Red* and *green fluorescence* filters combined, showing eosinophils complexed with platelets. (**c**) Individual platelets (*red*) attached to eosinophils (*green*) (Pitchford et al. 2005)

take up 12-HETE resulting in the production of 20-diHETE. 20-diHETE is a neutrophil chemoattractant which neutrophils are unable to synthesise in independently but require activated platelets to provide 12-HETE for 20-diHETE synthesis (Marcus et al. 1984, 1987, 1988). Furthermore, 12-HETE also stimulates leukocyte 5-lipoxygenase contributing to leukotriene production (Maclouf et al. 1982). The leukotrienes consist of the cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4) and LTB_4. These are potent inflammatory mediators, inducing bronchospasm, mucus hypersecretion and increased vascular permeability (Lewis et al. 1990; Maclouf and Murphy 1988; Piacentini and Kaliner 1991), and also increase hyper-responsiveness in asthmatics (Vargaftig et al. 1981). LTC_4 and LTD_4 induce eosinophil infiltration into the lungs (Foster and Chan 1991; Diamant et al. 1997), whilst LTB_4 acts as a non-specific chemoattractant (Seeds et al. 1995). LTA_4 produced by leukocytes can be converted to LTC_4 by platelets via glutathione-s-transferase via intimate contact of the two cell types through P-selectin interactions (Maclouf and Murphy 1988). Thus, it has been demonstrated that platelets and neutrophils cooperate in a synergistic manner with regards to the metabolism of aracadonic acid.

Current dogma would suggest that platelet participation in tissue damage and influencing repair processes is indirect and secondary to platelet 'priming' of leukocyte recruitment. Nevertheless, platelets have been observed to undergo diapedesis in sections of lung from asthmatic patients visualized under electron microscopy, and lungs (including BAL fluid) from allergen sensitized and challenged mice, rabbits, and guinea pigs suggesting platelets are also contributing to the pathogenesis of respiratory diseases in a more direct manner (Jeffery et al. 1989; Beasley et al. 1989; Pitchford et al. 2008; Metzger et al. 1987; Lellouch-Tubiana et al. 1988). Recently, we have quantified platelet migration through inflamed tissue in vivo in

allergic animals and reported that platelets undergo chemotaxis to allergen via an IgE-mediated process in vitro (Pitchford et al. 2008). This migratory response to allergen was shown to begin before significant leukocyte recruitment and this correlates with evidence suggesting platelets can be recruited to the lungs immediately following allergen exposure in an experimental animal model (Pitchford et al. 2008; Yoshida et al. 2002). When allergen is administered intravenously, platelet accumulation is an event that precedes histamine release from mast cells, suggesting allergen may directly activate platelets, and platelets were shown to remain in the lung for long periods (Yoshida et al. 2002). Evidence is now accumulating that platelet migration also occurs in other diseases, with platelets, and platelet microparticles accumulating in synovial fluid of the joints of RA, and transmigration occurring into the vascular wall after periods of ischaemia (Boilard et al. 2010; Endresen and Forre 1992; Farr et al. 1984; Kraemer et al. 2010). In vitro, platelets have been reported to undergo chemotaxis towards f-MLP and SDF-1α (Kraemer et al. 2010; Czapiga et al. 2005). It is therefore feasible that platelet recruitment to sites of inflammation is directed by a number of chemokines, since stromal cell-derived factor-1 (SDF-1, CXCL12), macrophage derived chemokine (MDC, CCL22), and TARC (CCL17) can activate platelets via their receptors CXCR4, CCR1, CCR3, and CCR4 via an ADP-dependent process (Abi-Younes et al. 2000, 2001; Kowalska et al. 2000; Clemetson et al. 2000).

Therefore, the ability of platelets to invade tissue is an intriguing and novel aspect of platelet function, since platelets contain a formidable array of inflammatory mediators and cytotoxic compounds within their granules and those generated from platelet membranes that are capable of inducing tissue damage and subsequent repair processes (tissue remodelling) directly. Examples include the formation of free radicals and reactive oxygen species (ROS), cationic proteins (PCPs), and platelet basic proteins (PBPs). Interestingly, platelets that release free radicals do not aggregate, and platelet aggregation itself inhibits free radical production, thus further revealing the dichotomy in platelet function (Joseph 1995). A consequence of persistent, chronic inflammation is alteration to tissue structure and function. It is evident that platelets contribute to these processes in atherosclerosis, causing neointimal thickening of the damaged artery (Ross 1999). In athereosclerosis, the seriousness of chronic inflammatory events leading to changes in the architecture is apparent since the main cause of mortality is vascular wall rupture with a 70–90 % mortality rate following rupture due to this chronic inflammation (Liu et al. 2011). In this regard, matrix metalloproteinases (MMPs) released from platelet granules degrade extracellular matrix proteins contributing to a decrease in thrombus stability, eventually leading to the rupture of the atherosclerotic plaque. As such, through inhibiting the release of pro-inflammatory mediators such as MMPs, anti-platelet therapy holds a potentially novel mechanism for cardiac protection in addition to well-characterized anti-thrombotic actions (Liu et al. 2011).

We have demonstrated that mice subjected to a chronic period (8 weeks) of aerosolized allergen exhibit similar structural changes within the lungs to those of asthmatic individuals, with remodelling events occurring in the distal airways of OVA-sensitized mice, but not in sham-sensitized mice (Pitchford et al. 2004).

Changes to airway architecture observed were thickening of epithelium and smooth muscle, and also increased reticular-fibre deposition in the extracellular matrix. The depletion of platelets resulted in the virtual abolition of these remodelling processes which was not apparent with long-term treatment of dexamethasone (Pitchford et al. 2004). Platelets may therefore contribute to a favourable micro-environment for wound repair by releasing cellular mitogens such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), transforming growth factor β (TGFβ), and vascular endothelial growth factor (VEGF) amongst other mediators that also have mitogenic effects, such as TxA_2 (Rendu and Brohard-Bohn 2002). Recent evidence also reveals platelet involvement in stem cell and progenitor cell recruitment, differentiation, and proliferation within damaged tissues, and this characteristic of platelet function might also contribute to the inflammatory response depending on the severity of the lesion (Jin et al. 2006; Massberg et al. 2006; Zernecke et al. 2005).

Finally, one further implication of the ability of platelets to migrate into inflamed tissue is the possibility of platelets acting as a 'bridge' between the innate and the adaptive immune response (von Hundelshausen and Weber 2007; Pitchford 2007; Semple and Freedman 2010). To this end platelets possess an array of receptors involved in antigen recognition, for example: the toll-like receptors (TLRs) that recognize molecules that are broadly shared by pathogens, but distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns (PAMPs). Platelets express functional TLR2, 4, and 9, which are involved in the recognition of diacylated and tri-acylated lipopeptides, LPS, and bacterial or viral DNA, respectively (Cognasse et al. 2005). Furthermore, platelets display functional receptors for numerous immunoglobulins such as FcγRI, FcγRII, FcγRIII; FcεRI, FcγRII; FcαRI/CD89, strongly indicating that platelets can play an important role in the development of the adaptive immune response (von Hundelshausen and Weber 2007; Pitchford 2007; Semple and Freedman 2010). Indeed, platelets express CD40L, and when in contact with T-lymphocytes via CD40–CD40L interactions can induce many cell-mediated inflammatory and immune responses (Sallusto et al. 1998; Henn et al. 1998; Danese et al. 2004). Activated platelets can also mediate IgM to IgG isotype switching, a crucial event in humoral immunity via CD40L (Elzey et al. 2003). Platelet CD40L can also lead to the activation of endothelial cells to have a pro-inflammatory phenotype, contributing to the inflammatory cell recruitment observed in atherosclerosis (Danese et al. 2004). Such CD40–CD40L interactions may also be important in providing a bridge between tissue trauma and acquired immunity, since platelets activated by thrombin induce the activation and maturation of primary bone marrow dendritic cells into mature antigen presenting cells (APCs) (Czapiga et al. 2004). Stimulation via this pathway leads to IL-12 production and the surface expression of CD80 and CD83 on dendritic cells (Czapiga et al. 2004).

The summarized evidence earlier therefore reveals an extraordinary breadth of function by which platelets can mediate inflammation, both directly, via invasion of inflamed tissue; and indirectly, via the recruitment of leukocytes. The evidence also reveals a diverse range of disease states by which the inflammatory actions of

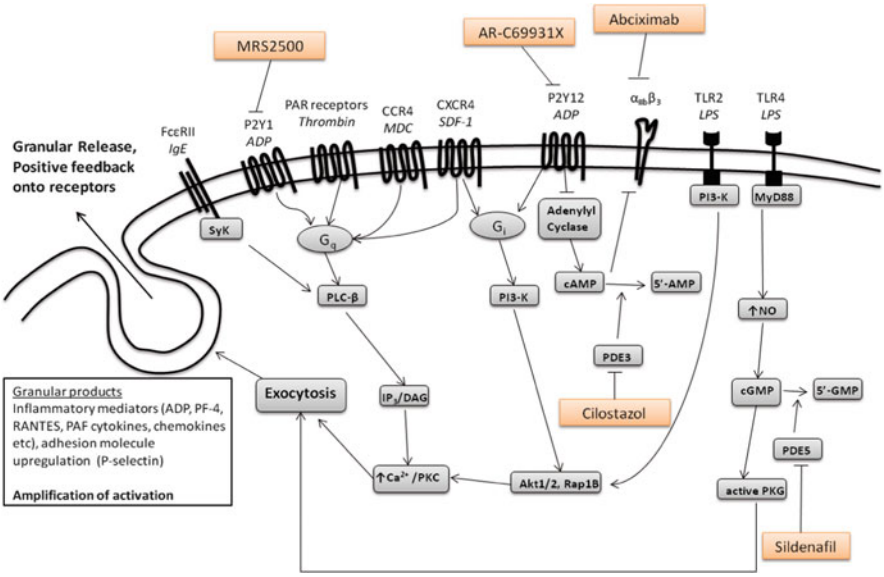


Fig. 4 Platelet receptors involved in inflammation

platelets may modulate disease pathogenesis: from allergy and autoimmune diseases, through to infections, in anatomically distinct organs. It is interesting to note that some anti-platelet drugs are in use clinically with actions that are known to affect the inflammatory pathways in which platelets are involved. An example is clopidogrel, which is used in the treatment of thrombosis, which has beneficial effects on atherosclerosis. A new generation of $P2Y_1$ and $P2Y_{12}$ antagonists has since been developed, but their anti-inflammatory actions are unknown. P-selectin antagonists, on the other hand, have been developed for their anti-inflammatory properties, and the translation pre-clinically from atherosclerosis to other inflammatory diseases has been more forthcoming. In the future, drug development may focus on the recent increase in understanding of platelet-dependent mechanisms that control certain inflammatory pathways (Fig. 4), and therefore exploit differences in platelet activation in inflammation compared to thrombosis.

3 Purinergic Receptor Modulation

The surface of platelets exhibit three distinct classes of purinergic receptors (Woulfe 2005). The $P2X_1$ receptor is a ligand-gated cation channel activated by ATP triggering rapid calcium influx. In addition, the $P2Y_1$ and $P2Y_{12}$ receptors are G protein-coupled receptors (GPCRs) activated by ADP and coupled to G proteins G_q and G_i, respectively (Gachet 2006). Activation of $P2Y_1$ signalling through the α subunit of G_q mobilizes calcium release from intracellular stores and activates

Protein Kinase C (PKC), mediating granular release and shape change, initiating a transient ADP-induced platelet aggregation. The $P2Y_{12}$ receptor is, however, coupled to G_i. Activation of this receptor can contribute to $P2Y_1$-mediated Ca^{2+} mobilization through PI3-K activation via the β/γ subunits, whilst also mediating integrin activation through inhibition of cyclic adenosine monophosphate (cAMP) formation (Gachet 2006; Hardy et al. 2004). $P2Y_1$ is further coupled to other signalling pathways, for example Rho Kinase, but the role of these proteins has not yet been elucidated (Gachet 2006; Hardy et al. 2004). Activation of platelet integrins, particularly $\alpha_{IIb}\beta_3$ is critical for full platelet aggregation, as such $P2Y_{12}$ activation leads to full platelet aggregation through $\alpha_{IIb}\beta_3$. Interestingly however, if full platelet aggregation to ADP is to occur, co-activation of both the $P2Y_1$ and $P2Y_{12}$ receptors is required, whilst $P2Y_{12}$ activation is also required for full aggregation by other platelet agonists (Nylander et al. 2004). The role of the platelet purinergic receptors in platelet aggregation has been well characterized; however, their potential roles in inflammation are now receiving much greater attention. As previously discussed, the expression of platelet P-selectin appears to be critical in the conjugation of platelets and leukocytes and the main driving force in inflammatory cell recruitment (Pitchford et al. 2005; Kornerup et al. 2010; Hayward et al. 1999; Schmitt-Sody et al. 2005; Zarbock et al. 2006; Schober et al. 2002; Diacovo et al. 1996a, b). Activation of both the $P2Y_1$ and $P2Y_{12}$ receptors appear to increase P-selectin expression on the surface of platelets as well as platelet–leukocyte conjugation (Leon et al. 2003, 2004; Straub et al. 2011), indicating that purinergic receptors might contribute to the inflammatory phenotype of platelets. A variety of specific purinergic antagonists exist including the $P2Y_1$-specific antagonists MRS2179, MRS2500, and MRS2279 all of which mimic ATP and the $P2Y_{12}$ specific antagonists clopidogrel, MRS2395, AR-C66096, and AR-C69931X.

Platelets are also activated by chemokines, for example, the CC chemokines MDC and thymus and activation-regulated chemokine (TARC). Both have been shown to trigger platelet activation through the CCR4 receptor, whilst SDF-1 triggers platelet activation through the CXCR4 receptor (Abi-Younes et al. 2000, 2001; Kowalska et al. 2000; Clemetson et al. 2000). Gear et al. (2001) described an exaggeration of this effect by ADP which demonstrated significantly elevated surface P-selectin following stimulation with SDF-1 and MDC (Gear et al. 2001). This potentiation of platelet activation by chemokines in combination with low levels of ADP appears to be $P2Y_1$ dependent, but not $P2Y_{12}$ dependent, as antagonism with A2P5P strongly inhibited aggregation, yet treatment with AR-C69931MX had no effect (Storey et al. 2000). Using $P2Y_1$ and $P2Y_{12}$ receptor antagonists as well as knockout mice, the blockade of P2Y receptors has illustrated inhibition of a variety of inflammatory parameters, including elevated P-selectin expression and platelet–leukocyte conjugation (Leon et al. 2003; Storey et al. 2000; Zerr et al. 2011). Whilst the effect of $P2Y_1$ antagonism on chemokine-induced P-selectin expression and platelet–leukocyte conjugation has yet to be investigated, it is possible that ADP signalling through the $P2Y_1$ receptor may be critical in the initial stages of platelet activation in an

inflammatory setting. Furthermore, recent studies have demonstrated the ability of the $P2Y_1$ receptor antagonist MRS2179 to attenuate allergen-induced pulmonary eosinophil and lymphocyte recruitment in a murine model of allergic inflammation (Pitchford and Page 2006).

Clopidogrel is a specific $P2Y_{12}$ antagonist and a well-known anti-platelet agent which has been prescribed for its anti-thrombotic actions. Recently, evidence indicating potential anti-inflammatory actions have prompted new research. In mice clopidogrel was able to reduce the capacity of platelets to adhere to polymorphonuclear cells (PMNs) and stimulate inflammatory functions (Evangelista et al. 2005). Clopidogrel inhibited P-selectin expression, platelet–leukocyte conjugation, and platelet-dependent production of ROS. This was also the case in human platelets (Evangelista et al. 2005) in agreement with data produced with AR-C69931MX, but not aspirin, confirming that this effect was mediated by ADP receptors (Storey et al. 2002). This decrease in inflammatory functions, and particularly P-selectin expression has also been observed in a rabbit model of early atherosclerosis, where clopidogrel treatment significantly decreased atherosclerosis progression as well as levels of P-selectin, ICAM-1, VCAM-1, and MCP-1, all of which are involved in tissue leukocyte recruitment (Li et al. 2007). The anti-inflammatory effects of clopidogrel seen in vivo are associated with clinical benefits since in aspirin-treated patients with stable coronary artery disease, patients co-treated with clopidogrel demonstrated a 64 % decrease in ADP-induced P-selectin expression (Perneby et al. 2007). The effectiveness of clopidogrel compared to aspirin was also investigated in a clinical trial entitled 'Clopidogrel Versus Aspirin in Patients as Risk of Ischaemic Events (CAPRIE)' (CS Committee 1996). This trial concluded that clopidogrel showed an 8.7 % reduction in relative risk in the occurrence of vascular death, ischemic stroke, or myocardial infarction in comparison to aspirin (CS Committee 1996). It may be that the anti-inflammatory components associated with $P2Y_{12}$ receptor blockade by clopidogrel accounts for the observed reduction in risk (Evangelista et al. 2005; Palmerini et al. 2010; Heitzer et al. 2006; Willerson et al. 2009; Patti et al. 2011; Antonio et al. 2009); however, it is important to note that this study analysed mainly stable patients (CS Committee 1996). What is clear through a variety of studies is that clopidogrel has repeatedly shown anti-inflammatory effects resulting in decreased P-selectin expression and circulating platelet–leukocyte complexes in patients with acute coronary syndromes (Palmerini et al. 2010; Heitzer et al. 2006; Patti et al. 2011; Antonio et al. 2009). All of the research on the inflammatory nature of ADP stimulation of P2Y receptors triggering granular release and its subsequent pro-inflammatory contributions implicates a significant role of P2Y antagonists inhibiting these inflammatory events which contributes to disorders such as atherosclerosis, allergic inflammation, rheumatoid arthritis, and other cardiovascular disorders. Further research will inform as to whether $P2Y_1$ or $P2Y_{12}$ make better targets for inhibiting the activation of platelets in inflammation without affecting the necessary purinergic stimulation of platelets during haemostasis.

4 Modulation of Platelet Adhesion Molecules

The platelet adhesion molecule P-selectin and its counter ligand PGSL-1 on leukocytes are responsible for mediating the interactions between platelets, leukocytes, and endothelial cells targeting leukocytes for recruitment from the vasculature into damaged tissue (Pitchford et al. 2005; Kornerup et al. 2010; Hayward et al. 1999; Schmitt-Sody et al. 2005; Zarbock et al. 2006; Schober et al. 2002; Diacovo et al. 1996a, b; Mayadas et al. 1993).

Activation of platelets triggers granule secretion and upregulation of P-selectin and also PSGL-1. P-selectin/PGSL-1 and PSGL-1/L-selectin interactions promote platelet–leukocyte conjugation leading to 'inside-out' signalling and activation of leukocytes as demonstrated by elevated leukocyte CD11b and VLA-4 expression (Pitchford et al. 2003). These interactions vastly increase the efficiency of leukocyte rolling allowing firm adhesion, paving the way for migration of leukocytes into damaged tissue (Pitchford et al. 2005; Kornerup et al. 2010; Hayward et al. 1999; Schmitt-Sody et al. 2005; Zarbock et al. 2006; Schober et al. 2002; Diacovo et al. 1996a, b; Mayadas et al. 1993). The importance of these adhesive interactions in leukocyte recruitment has thus made both P-selectin and PGSL-1 attractive targets for therapeutic manipulation in the treatment of inflammation. These methods involve a range of different compounds from blocking antibodies, competitive small molecular antagonists (Bimosiamose) that mimic the tetrasaccharide Sialyl Lewis X (sLeX), a cell surface epitope essential for selectin/selectin ligand binding, and inhibitors of enzymes involved in the synthesis of sLeX.

The most extensive evidence for the importance of P-selectin comes from animal studies using blocking antibodies for P-selectin. Blockade of P-selectin with the blocking antibody RB40.34 significantly attenuated allergen-induced eosinophil recruitment in a murine model of allergic inflammation (Pitchford et al. 2005), whilst the same antibody has also shown efficacy in an in vivo model of ischaemia (Mayadas et al. 1993). The concept of P-selectin blocking antibodies has been advanced towards the clinic with the production of a humanized antibody (mEP.SC7), although the efficacy of mEP.SC7 has not been widely reported (He et al. 1998).

Bimosiamose (TBC1269) is a biphenol chemical pan-selectin antagonist (but with higher affinity for P-selectin compared to L- and E-selectin) that competes with sLeX for selectin binding. Bimosiamose reproducibly inhibits leukocyte recruitment in a range of in vivo assays by 50–60 % (Kogan et al. 1998; Onai et al. 2003; Hicks et al. 2005). This efficacy in in vivo models of inflammation has led to the demonstration that bimosiamose has significant activity in phase IIa asthma and COPD trials, providing important clinical proof of concept for small molecule anti-(P) selectin strategies (Kirsten et al. 2011; Beeh et al. 2006). Other small molecule inhibitors have also been investigated for P-selectin antagonism. Thus, high throughput screening has identified quinoline salicylic acids as a class of compounds which also antagonize P-selectin by competing with the sLeX moieties on P-selectin ligands (Kaila et al. 2007). This class of antagonist demonstrated efficacy in a rat adjuvant-induced arthritis model (Kaila et al. 2007). Two quinoline

salicylic acid derivatives: PSI-697 and PSI-421 are orally bioavailable and have been demonstrated in vivo to significantly inhibit inflammation and induce reopening of the vascular lumen occurring in models of deep vein thrombosis, without affecting coagulation (Bedard et al. 2008; Myers et al. 2007; Meier et al. 2008), showing promising development in anti-P-selectin therapy.

Blockade of PGSL-1 has also produced similar outcomes to those seen through P-selectin inhibition. Treatment with rPGSL-Ig (a recombinant immunoglobulin chimera form of PGSL-1) inhibits P-selectin-mediated platelet-neutrophil adhesion (Perneby et al. 2007). rPGSL-Ig decreased neointimal hyperplasia following balloon injury, with quantitative histological examination revealing a 70 % decrease in macrophage infiltration compared to control tissues (Wang et al. 2001). Separate from cardiovascular disease, a rPSGL-1 antibody has been shown to ameliorate cell accumulation, TNF-α levels, and joint severity in a murine model of RA (Sumariwalla et al. 2004). In this regard, targeting PSGL-1 may offer a more important target compared to targeting P-selectin since it is a ligand for all three selectins.

Lastly, an alternative to the direct disruption of PSGL-1/P-selectin interaction with bimosiamose or quinoline salicylic acid derivatives is the inhibition of enzymes involved in PSGL-1 biosynthesis. A particularly suitable target is beta-1-4-galactosyltransferase-1 (B4GalT1). B4GalT1 is essential for the biosynthesis of the terminal tetrasaccharide sLeX on PSGL-1. B4GalT1-deficient mice show impaired PSGL-1 biosynthesis and reduced acute and chronic inflammatory responses. Reflecting the central role of B4GalT1 for PSGL-1 biosynthesis, inhibition of the enzyme with a disaccharide decoy substrate successfully reduced selectin-mediated cellular adhesion and diminished cellular recruitment in vivo (Asano et al. 2003; Brown et al. 2009). Recently, attempts have been made to create small molecule inhibitors with better pharmacokinetic profiles (Pesnot et al. 2010).

5 NF-κB and Its Role in Platelet Activation

In its inactive form, NF-κB exists as a cytoplasmic complex tightly bound with inhibitory proteins belonging to the IκB family. NF-κB is activated following IκB phosphorylation, in the case of platelets; platelet activation is responsible for initiating IκB phosphorylation. Phosphorylation and subsequent ubiquitination of the IκB subunit frees NF-κB and allows its translocation to the nucleus where it binds to target genes and initiates gene transcription. Malaver et al. (2009) demonstrated through immunofluorescence that human platelets express NF-κB, and that following platelet activation with thrombin, IκB was phosphorylated with greater than 50 % being subsequently degraded (Malaver et al. 2009).

Whilst platelets do not have nuclei, reports have identified transcription factors expressed by platelets for steroid receptors and the peroxisome proliferator activated receptor (PPAR), of which platelets express all three subtypes: α, β, and γ (Khetawat et al. 2000; Akbiyik et al. 2004; Ali et al. 2006; Steinhubl et al. 2007). Selective agonists for all three PPAR receptors (fenofibrate: PPARα;

GW0742 and L165041: PPARβ; and rosiglitazone: PPARγ) are capable of inhibiting platelet activation (Steinhubl et al. 2007). Recently, PPARγ agonists acting on platelets have been shown to have anti-inflammatory properties, inhibiting platelet release of CD40L, and TxA_2 production (Ali et al. 2006). Through these receptors, the binding of prednisolone can inhibit platelet activation (Steinhubl et al. 2007). These examples indicate the ability of transcription factors to affect platelet functions.

NF-κB is also responsible for the expression of many pro-inflammatory molecules including monocyte chemoattractant protein-1 (MCP-1) which is responsible for the recruitment of monocytes to sites of tissue injury, IL-8 and CD40 ligand (CD40L) (Henn et al. 2001). CD40L is upregulated following platelet activation and interacts with CD40 on endothelial cells resulting in the production and release of pro-inflammatory cytokines/chemokines (i.e. IL-1, IL-6, TNFα, and MCP-1) and adhesion molecules enabling the recruitment of leukocytes to the endothelium. As such the targeting and inhibition of NF-κB expression demonstrates a potentially useful target for treating platelet-derived components of the inflammatory response (Cyrus et al. 2002). Aspirin is also capable of reducing NF-κB expression and in turn pro-inflammatory mediators such as IL-1, TNFα, and MCP-1. In agreement with the anti-inflammatory benefits of NF-κB inhibition, treatment with low-dose aspirin significantly reduced the extent of vascular inflammation and improved the stability of atherosclerotic plaques (Schneider 2011).

Pre-treatment with two separate NF-κB inhibitors BAY 11-7082 (prevents IκB phosphorylation) and Ro 106-9920 (selectively inhibits IκB ubiquitination) produced a variety of effects leading to potential anti-inflammatory targeting of NF-κB in platelets. Upon activation through inside-out signalling, the platelet integrin $\alpha_{IIb}\beta_3$ undergoes a conformation change. This change exposes the activated fibrinogen receptor recognized by the mAb PAC-1 which is a marker of platelet activation. Pre-treatment with either BAY 11-7082 or Ro 106-9920 significantly decreased both PAC-1 and soluble fibrinogen binding (Malaver et al. 2009).

6 Oral Glycoprotein IIb–IIIa Antagonists: Pro-inflammatory?

Glycoprotein IIb/IIIa (GP IIb/IIIa) is the most abundant receptor on the platelet surface. Currently, three inhibitors of GPIIb/IIIa (GPIs) are approved for clinical use; these are abciximab, eptifibatide, and ticrofiban, all of which prevent the formation of interplatelet bridges and aggregates (Yousuf and Bhatt 2011; Bhatt and Topol 2000). These GPIs have shown significant clinical benefits in patients with both stable and unstable acute coronary syndromes for the reduction of periprocedural MI, with the incidence of MI decreasing, and in the specific case of abciximab, an increase in patient survival (Boersma et al. 2002). Furthermore analysis of over 30,000 patients revealed that GPI treatment reduced the incidence of mortality/MI by 9 % compared to vehicle

controls, completing the data profile, with only a 1 % increase in risk of absolute bleeding being observed (Chew et al. 2001).

The use of oral GPIs in the clinic was dealt a significant blow following large-scale trials of four oral GPIs as these studies showed a 31 % increase in mortality combined with a significant increase in major bleeding; this increased mortality has also been largely attributed to an incomplete inhibition of the GP IIb/IIIa receptor over the course of 24 h (Klinkhardt et al. 2002). Research into this identified a potential pro-inflammatory profile of GPIs. Human ex vivo studies actually demonstrated that treatment with abciximab resulted in a 30 % increase in P-selectin expression following ADP stimulation unlike clopidogrel which conversely demonstrated a 30–50 % decrease in P-selectin expression. Furthermore abciximab also demonstrated a significant increase in platelet–leukocyte conjugate formation compared to a significant reduction in platelet–leukocyte conjugation in clopidogrel-treated groups (Caron et al. 2002). Further studies showed elevated P-selectin positive platelets induced by *N*-formyl-methionyl-leucyl-phenylalanine (f-MLP) and elevated leukocyte CD11b expression induced by collagen. This increased expression of CD11b and P-selectin was significantly inhibited by the GP IIb/IIIa MAb RFGP56 whereas the non-peptide inhibitor SR121566, the active metabolite of the orally available pro-drug SR121787 contributed to the pro-inflammatory profile (Li et al. 2000).

However, other studies demonstrated that whilst GPIs inhibited platelet aggregation they had minimal effect on P-selectin expression and platelet–neutrophil conjugation (Caron et al. 2002). In addition, the GPI MK-852 promoted increased P-selectin, CD40L, and tissue factor expression coupled with an increase in platelet–leukocyte conjugation (Zhao et al. 2003). All of these factors are known to be pro-inflammatory, therefore it is reasonable to believe that the GPI-induced increase in expression of P-selectin, CD40L, TF, and platelet leukocyte conjugates is contributing to the overall negative clinical effects associated with GPI treatment. The combined results of the examined clinical trials and the emergence of more effective and more potent purinergic antagonists such as Cangrelor (AR-C69931X) and Ticagrelor (AR-C66096) have limited the prospects of clinical use of GPIs in coronary diseases and their potential use in disorders with inflammatory backgrounds suggesting that purinergic antagonism would be more favourable.

7 Phosphodiesterase Inhibitors as an Anti-platelet Therapy

Under normal conditions Phosphodiesterases (PDEs) catalyse the hydrolysis of cAMP and cGMP to inactive 5′-AMP and 5′-GMP. This limits intracellular levels allowing regulation of cAMP/cGMP signalling (Bender and Beavo 2006; Gresele et al. 2011). Under normal conditions cAMP acts as a ‘brake’ on platelet integrin activation, therefore hydrolysis of cAMPs by PDEs would allow integrin activation and in turn allow platelet activation. This would highlight a potential avenue for

PDE inhibitors such as dipyridamole and cilostazol as both an anti-thrombotic and anti-inflammatory mediator in platelet function (Kariyazono et al. 2001). Platelets express three different PDE isoforms; these are PDE2, PDE3, and PDE5, with PDE3 and PDE2 hydrolysing cAMP and PDE5/PDE2 hydrolysing cGMP. This would suggest optimal targeting of PDE3 and PDE2 by PDE inhibitors for modulation of platelet function.

The effects of a PDE3-specific inhibitor cilostazol on platelet function has demonstrated potential anti-inflammatory capabilities and a number of studies have shown that treatment with cilostazol inhibited platelet aggregation as well as P-selectin expression in a dose-dependent manner (Ito et al. 2004; Matsunaga et al. 2009). Furthermore, cilostazol treatment actually inhibited platelet conjugation with both PMNs and monocytes in a dose-dependent manner through inhibition of platelet activation and decreased P-selectin expression (Ito et al. 2004; Matsunaga et al. 2009).

In Crohn's disease (CD), one of the main characteristics is an accumulation of inflammatory cells in the affected regions of the gastrointestinal tract. It has been demonstrated that cilostazol reduces monocyte recruitment through inhibition of platelet–monocyte interactions (Ito et al. 2004; Matsunaga et al. 2009). In SAMP1/Yit mice, significant inflammation was induced in the ileum, characterized by increases in intestinal weight and monocyte infiltration and pre-treatment with cilostazol significantly attenuated monocyte infiltration whilst also reducing this increased intestinal weight, thus providing a mechanism by which the main inflammatory characteristics of CD can be alleviated (Matsunaga et al. 2009).

This ability of PDE inhibitors to modulate platelet function, and particularly platelet leukocyte interactions through inhibition of P-selectin expression, provides a further mechanism by which anti-platelet agents might be used to treat the pro-inflammatory effects of platelets in inflammatory disorders such as CD, IBD, and asthma.

8 Conclusion

Platelets can contribute to inflammatory disorders through the release of pro-inflammatory mediators, i.e. reactive oxygen species, cationic proteins, cytokines, chemokines, histamine, growth factors, and MMPs from their granules. In addition, surface adhesion molecules such as P-selectin and CD40L are upregulated, contributing to platelet–leukocyte conjugation and promoting leukocyte rolling, firm adhesion, and eventually cell migration into the tissues. Modulation of platelet function through targeting of surface receptors, platelet selectins, or through targeting of intracellular mechanisms (e.g. NF-κB inhibitors, PDE inhibitors) might inhibit platelet activation and prevent granular secretion, platelet migration, platelet–leukocyte conjugation, and ultimately leukocyte recruitment into damaged tissues. These mechanisms of platelet modulation have therefore shown benefits in pre-clinical models inflammatory linked disorders such as asthma, COPD, IBD, RA, atherosclerosis, and CD.

Knowledge Gaps

- The molecular mechanism underlying the ability for platelets to become activated and contribute to inflammation without proceeding to secondary aggregation and thrombus formation remains unclear.
- If the molecular mechanisms can be identified anti-platelet agents targeting the inflammatory profile could be developed without affecting bleeding.

Key Messages

Within this chapter the following key points are discussed

- Platelets have been implicated in both thrombosis and inflammation with distinct outcomes demonstrating a dichotomy of platelet function.
- A predominant role for platelets in inflammation is the formation of circulating platelet–leukocyte complexes promoting leukocyte diapedesis into damaged tissue.
- Platelets express a variety of different receptors triggering signalling cascades contributing to platelet activation and adhesion molecule expression (e.g. P-selectin). Targeting of these receptors and downstream signalling proteins provides a potential mechanism to target platelets as an anti-inflammatory treatment.

References

Abi-Younes S, Sauty A, Mach F et al (2000) The stromal cell-derived factor-1 chemokine is a potent platelet agonist highly expressed in atherosclerotic plaques. Circ Res 86:131–139

Abi-Younes S, Si-Tahar M, Luster AD (2001) The CC chemokines MDC and TARC induce platelet activation via CCR4. Thromb Res 101:279–289

Adelroth E, Morris MM, Hargreave FE, O'Byrne PM (1986) Airway responsiveness to leukotrienes C_4 and D_4 and to methacholine in patients with asthma and normal controls. N Engl J Med 315:480–484

Akbiyik F, Ray DM, Gettings KF et al (2004) Human bone marrow megakaryocytes and platelets express PPARgamma, and PPARgamma agonists blunt platelet release of CD40 ligand and thromboxanes. Blood 104:1361–1368

Ali FY, Davidson SJ, Moraes LA et al (2006) Role of nuclear receptor signaling in platelets: antithrombotic effects of PPARβ. FASEB J 20:326–328

Antonio MJ, Mahla E, Bliden KP et al (2009) Effect of long-term clopidogrel treatment on patient function and inflammation in patients undergoing coronary artery stenting. Am J Cardiol 103:1545–1550

Arber N, Berliner S, Pras E et al (1991) Heterotypic leukocyte aggregation in the peripheral blood of patients with leukaemia, inflammation and stress. Nouv Rev Fr Hematol 33:251–255

Asano M, Nakae S, Kotani N et al (2003) Impaired selectin-ligand biosynthesis and reduced inflammatory responses in beta- 1,4-galactosyltransferase-I-deficient mice. Blood 102:1678–1685

Beasley R, Roche WR, Roberts JA, Holgate ST (1989) Cellular events in the bronchi in mild asthma and after bronchial provocation. Am Rev Respir Dis 139:806–817

Bedard PW, Clerin V, Sushkova N et al (2008) Characterization of the novel P-selectin inhibitor PSI-697 [2-(4-chlorobenzyl)-3-hydroxy-7,8,9,10-tetrahydrobenzo[h] quinoline-4-carboxylic acid] in vitro and in rodent models of vascular inflammation and thrombosis. J Pharmacol Exp Ther 324:497–506

Beeh KM, Beier J, Meyer M et al (2006) Bimosiamose, an inhaled small-molecule pan-selectin antagonist, attenuates late asth matic reactions following allergen challenge in mild asthmatics: a randomized, double-blind, placebo-controlled clinical cross-over-trial. Pulm Pharmacol Ther 19:233–241

Bender AT, Beavo JA (2006) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. Pharmacol Rev 58:488–520

Bhatt D, Topol EJ (2000) Current role of platelet glycoprotein IIb/IIIa inhibitors in acute coronary syndromes. JAMA 284:1549–1558

Boersma E, Harrington RA, Moliterno DJ et al (2002) Platelet glycoprotein IIb/IIIa inhibitors in acute coronary syndromes: a meta-analysis of all major randomised clinical trials. Lancet 359:189–198

Boilard E, Nigrovic PA, Larabee K et al (2010) Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. Science 327:580–583

Brown JR, Yang F, Sinha A et al (2009) Deoxygenated disaccharide analogs as specific inhibitors of β1-4-galactosyltransferase 1 and selectin-mediated tumour metastasis. J Biol Chem 284:4952–4959

Bunescu A, Seideman P, Lenkei R et al (2004) Enhanced Fcγ receptor I, $\alpha_M\beta_2$ integrin receptor expression by monocytes and neutrophils in rheumatoid arthritis: interaction with platelets. J Rheumatol 31:2347–2355

Caron A, Théorêt JF, Mousa SA, Merhi Y (2002) Anti-platelet effects of GPIIb/IIIa and P-selectin antagonism, platelet activation, and binding to neutrophils. J Cardiovasc Pharmacol 40:296–306

Chew DP, Bhatt DL, Sapp S, Topol EJ (2001) Increased mortality with oral platelet glycoprotein IIb/IIIa antagonists: a meta analysis of phase III multicenter randomised trials. Circulation 103: L201–L206

Clemetson KJ, Clemetson JM, Proudfoot AE et al (2000) Functional expression of CCR1, CCR3, CCR4, and CXCR4 chemokine receptors on human platelets. Blood 96:4046–4054

Cognasse F, Hamzeh H, Chavarin P et al (2005) Evidence of Toll-like receptor molecules on human platelets. Immunol Cell Biol 83:196–198

CS Committee (1996) A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. Lancet 348:1329–1339

Cyrus T, Sung S, Zhao L et al (2002) Effect of low-dose aspirin on vascular inflammation, plaque stability, and atherogenesis in low-density lipoprotein receptor-deficient mice. Circulation 106:1282–1287

Czapiga M, Kirk AD, Lekstrom-Himes J (2004) Platelets deliver costimulatory signals to antigen-presenting cells: a potential bridge between injury and immune activation. Exp Hematol 32:135–139

Czapiga M, Gao JL, Kirk A, Lekstrom-Himes J (2005) Human platelets exhibit chemotaxis using functional N-formyl peptide receptors. Exp Hematol 33:73–84

Danese S, De La Motte C, Rivera Reyes BM et al (2004) T cells trigger CD40-dependent platelet activation and granular RANTES release: a novel pathway for immune response. J Immunol 172:2011–2015

Diacovo TG, Puri KD, Warnock RA et al (1996a) Platelet-mediated lymphocyte delivery to high endothelial venules. Science 273:252–255

Diacovo TG, Roth SJ, Buccola JM et al (1996b) Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. Blood 88:146–157

Diamant Z, Hilterman JT, Van Rensen EL (1997) The effect of inhaled leukotriene D_4 and methacholine on cell differentials in sputum from patients with asthma. Am J Respir Crit Care Med 155:1247–1253

Elzey BD, Tian J, Jensen RJ et al (2003) Platelet mediated modulation of adapted immunity. A communication link between innate and adaptive immune compartments. Immunity 19:9–19

Endresen GK, Forre O (1992) Human platelets in synovial fluid. A focus on the effects of growth factor on the inflammatory responses in rheumatoid arthritis. Clin Exp Rheumatol 10:181–187

Evangelista V, Manarini S, Dell'Elba G et al (2005) Clopidogrel inhibits platelet-leukocyte adhesion and platelet- dependent leukocyte activation. Thromb Haemost 94:568–577

Farr M, Wainwright A, Salmon M et al (1984) Platelets in the synovial fluid of patients with rheumatoid arthritis. Rheumatol Int 4:13–17

Ferroni P, Basili S, Martini F et al (2000) Soluble P-selectin as a marker of platelet hyperactivity in patients with chronic obstructive pulmonary disease. J Investig Med 48:21–27

Foster A, Chan CC (1991) Peptide leukotriene involvement in pulmonary eosinophil migration upon antigen challenge in the actively sensitised guinea pig. Int Arch Allergy Appl Immunol 96:279–284

Gachet C (2006) Regulation of platelet functions by P2 receptors. Ann Rev Pharmacol Toxicol 46:277–300

Gear AR, Suttitanamongkol S, Viisoreanu D (2001) Adenosine diphosphate strongly potentiates the ability of the chemokines MDC, TARC, and SDF-1 to stimulate platelet function. Blood 97:937–945

Goeztl EJ, Woods JM, Gorman RR (1977) Stimulation of human eosinophil and neutrophul polymorphonuclear leukocyte chemotaxis and random migration by 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid. J Clin Invest 59:179–183

Gresele P, Momi S, Falcinelli E (2011) Anti-platelet therapy: phosphodiesterase inhibitors. Br J Clin Pharmacol 72:634–646

Hardy AR, Jones ML, Mundell SJ, Poole AW (2004) Reciprocal cross-talk between P2Y1 and P2Y12 receptors at the level of calcium signaling in human platelets. Blood 104:1745–1752

Hayward R, Campbell B, Shin YK et al (1999) Recombinant soluble P-selectin glycoprotein ligand-1 protects against myocardial is chemic reperfusion injury in cats. Cardiovasc Res 41:65–76

He XY, Xu Z, Melrose J et al (1998) Humanization and pharmacokinetics of a monoclonal antibody with a specificity for both E- and P-selectin. J Immunol 160:1029–1035

Heitzer T, Rudolph V, Schwedhelm E et al (2006) Clopidogrel improves systemic endothelial nitric oxide bioavailability in patients with coronary artery disease: evidence for antioxidant and antinflammatory effects. Arterioscler Thromb Vasc Biol 26:1648–1652

Henn V, Slupsky JR, Grafe M et al (1998) CD40L on activated platelets platelets triggers an inflammatory reaction on endothelial cells. Nature 391:591–594

Henn V, Steinbach S, Büchner K et al (2001) The inflammatory action of CD40 ligand (CD154) expressed on activated human platelets is temporally limited by coexpressed CD40. Blood 98:1047–1054

Hicks AE, Abbitt KB, Dodd P et al (2005) The anti-inflammatory effects of a selectin ligand mimetic, TBC-1269, are not a result of competitive inhibition of leukocyte rolling in vivo. J Leukoc Biol 77:59–66

Huo Y, Schober A, Forlow B et al (2003) Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. Nat Med 9:61–67

Irving PM, Macey MG, Feakins RM et al (2002) Platelet-leucocyte aggregates form in the mesenteric vasculature in patients with ulcerative colitis. Eur J Gastroenterol Hepatol 20:283–289

Ito H, Miyakoda G, Mori T (2004) Cilostazol inhibits platelet-leukocyte interaction by suppression of platelet activation. Platelets 15:293–301

Jeffery PK, Wardlaw AJ, Nelson FC et al (1989) Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. Am Rev Respir Dis 140:1745–1753

Jin DK, Shido K, Kopp HG et al (2006) Cytokine mediated deployment of SDF-1 induces revascul;arization through recruitment of CXCR4 hemangiocytes. Nat Med 12:557–567

Joseph M (1995) The generation of free radicals by blood platelets. In: Joseph M (ed) Immunopharmacology of platelets. Academic Press, London, pp 209–225

Joseph JE, Harrison P, Mackie IJ et al (2001) Increased circulating platelet-leukocyte complexes and platelet activation in patients with antiphospholipid syndrome, systemic lupus erthematosus and rheumatoid arthritis. Br J Haematol 115:451–459

Kaila N, Janz K, DeBernardo S et al (2007) Synthesis and biological evaluation of quinoline salicylic acids as P-selectin antagonists. J Med Chem 50:21–39

Kariyazono H, Nakamura K, Shinkawa T et al (2001) Inhibition of platelet aggregation and the release of P-selectin from platelets by cilostazol. Thromb Res 101:445–453

Khetawat G, Faraday N, Nealen ML et al (2000) Human megakaryocytes and platelets contain the estrogen receptor β and androgen receptor (AR): testosterone regulates AR expression. Blood 95:2289–2296

Kirsten A, Watz H, Kretschmar G et al (2011) Efficacy of the pan-selectin antagonist Bimosiamose on ozone-induced airway inflammation in healthy subjects–a double blind, randomized, placebo-controlled, cross-over clinical trial. Pulm Pharmacol Ther 24:555–558

Klinkhardt U, Graff J, Harder S (2002) Clopidogrel, but not abciximab, reduces platelet leukocyte conjugates and P-selectin expression in a human ex vivo in vitro model. Clin Pharmacol Ther 71:176–185

Kogan TP, Dupré B, Bui H et al (1998) Novel synthetic inhibitors of selectin-mediated cell adhesion: synthesis of 1,6-bis[3-(3-carboxymethylphenyl)-4-(2-alpha-D- mannopyranosyloxy)phenyl]hexane (TBC1269). J Med Chem 41:1099–1111

Kornerup KN, Salmon GP, Pitchford SC et al (2010) Circulating platelet-neutrophil complexes are important for subsequent neutrophil activation and migration. J Appl Physiol 109:758–767

Kowalska MA, Ratajczak MZ, Majka M et al (2000) Stromal cell-derived factor-1 and macrophage-derived chemokine: 2 chemokines that activate platelets. Blood 96:50–57

Kraemer BF, Borst O, Gehring EM et al (2010) PI3 kinase-dependent stimulation of platelet migration by stromal cell-derived factor 1 (SDF-1). J Mol Med 88:1277–1288

Lehmberg J, Beck J, Baethmann A, Uhl E (2006) Effect of P-selectin inhibition on leukocyte-endothelium interaction and survival after global cerebral ischemia. J Neurol 253:357–363

Lellouch-Tubiana A, Lefort J, Simon MT et al (1988) Eosinophil recruitment into guinea pig lungs after PAF-acether and allergen administration. Modulation by prostacyclin, platelet depletion, and selective antagonists. Am Rev Respir Dis 137:948–954

Leon C, Ravanat C, Freund M et al (2003) Differential involvement of the P2Y1 and P2Y12 receptors in platelet procoagulant activity. Arterioscler Thromb Vasc Biol 23:1941–1947

Leon C, Alex M, Klocke A et al (2004) Platelet ADP receptors contribute to the initiation of intravascular coagulation. Blood 103:594–600

Lewis RA, Austen KF, Soberman RJ (1990) Leukotrienes and other products of the 5-lipoxygenase pathway: biochemistry and relation to pathobiology in human disease. N Engl J Med 323:645–655

Li N, Hu H, Lindqvist M et al (2000) Platelet-leukocyte cross talk in whole blood. Arterioscler Thromb Vasc Biol 20:2702–2708

Li M, Zhang Y, Ren H et al (2007) Effect of clopidogrel on the inflammatory progression of early atherosclerosis in rabbits model. Atherosclerosis 194:348–356

Liu Y, Xia-Ming G, Fang L et al (2011) Novel role of platelets in mediating inflammatory responses and ventricular rupture or remodeling following myocardial infarction. Arterioscler Thromb Vasc Biol 31:834–841

Maccia CA, Gallagher JS, Ataman G et al (1977) Platelet thrombopathy in asthmatic patients with elevated immunoglobulin E. J Allergy Clin Immunol 59:101–108

Maclouf J, Murphy RC (1988) Transcellular metabolism of neutrophil-derived leukotriene A_4 by human platelets: a potential source of leukotriene C_4. J Biol Chem 263:174–181

Maclouf J, De Laclos BF, Borgeat P (1982) Stimulation of leukotriene biosynthesis in human blood leukocytes by platelet derived 12-hydroperoxy-icosatetraenoic acid. Proc Natl Acad Sci USA 79:6042–6046

Maghni K, Carrier J, Cloutier S, Sirois P (1993) Cell-cell interactions between platelets, macrophages, eosinophils and natural killer cells in thromboxane A2 biosynthesis. J Lipid Mediat 6:321–332

Malaver E, Romaniuk MA, D'Atri LP et al (2009) NF-kappaB inhibitors impair platelet activation responses. J Thromb Haemost 7:1333–1343

Marcus AJ, Safier LB, Ullman HL (1984) 12S,20-dihydroxyicosatetraenoic acid: a new eicosanoid synthesised by neutrophils from 12S-hydroxyicosatetraenoic acid produced by thrombin- or collagen-stimulated platelets. Proc Natl Acad Sci USA 81:903–907

Marcus AJ, Safier LB, Ullman HL et al (1987) Studies on the mechanism of omega-hydroxylation of platelet 12- hydroxyicosatetraenoic acid (12-HETE) by unstimulated neutrophils. J Clin Invest 79:179–187

Marcus AJ, Safier LB, Ullman HL (1988) Platelet-neutrophil interactions. J Biol Chem 263:2223–2229

Massberg S, Konrad I, Schurzinger K et al (2006) Platelets secrete stromal cell-derived factor 1α and recruit bone marrow derived progenitor cells to arterial thrombi in vivo. J Exp Med 205:1221–1233

Matsunaga H, Hokari R, Higashiyama M et al (2009) Cilostazol, a specific PDE-3 inhibitor, ameliorates chronic ileitis via suppression of interaction of platelets with monocytes. Am J Physiol Gastrointest Liver Physiol 297:G1077–G1084

Mayadas TN, Johnson RC, Rayburn H et al (1993) Leukocyte rolling and extravasation are severely compromised in P-selectin-deficient mice. Cell 74:541–554

Meier TR, Myers DD Jr, Wrobleski SK et al (2008) Prophylactic P-selectin inhibition with PSI-421 promotes resolution of venous thrombosis without anticoagulation. Thromb Haemost 99:343–51

Metzger WJ, Sjoerdsma K, Richerson HB et al (1987) Platelets in bronchoalveolar lavage from asthmatic patients and allergic rabbits with allergen-induced late phase responses. Agents Actions Suppl 21:151–159

Myers DD Jr, Wrobleski SK, Longo C et al (2007) Resolution of venous thrombosis using a novel oral small-molecule inhibitor of P-selectin (PSI-697) without anticoagulation. Thromb Haemost 97:400–407

Neumann FJ, Marx N, Gawaz M et al (1997) Induction of cytokine expression in leukocytes by binding of thrombin stimulated platelets. Circulation 95:2387–2394

Nylander S, Mattsson C, Ramström S, Lindahl TL (2004) Synergistic action between inhibition of P2Y12/P2Y1 and P2Y12/thrombin in ADP- and thrombin-induced human platelet activation. Br J Pharmacol 142:1325–1331

Onai Y, Suzuki J, Nishiwaki Y et al (2003) Blockade of cell adhesion by small molecule selectin antagonist attenuates myocardial ischaemia/ reperfusion injury. Eur J Pharmacol 481:217–225

Ott I, Neumann FJ, Gawaz M et al (1996) Increased neutrophil-platelet adhesion in patients with unstable angina. Circulation 94:1239–1246

Page CP (1993) Platelets. In: Holgate ST, Church MK (eds) Allergy illustrated. Gower Medical Publishing, London, pp 8–18

Palmerini T, Barozzi C, Tomasi L et al (2010) A randomised study comparing the antiplatelet and antinflammatory effect of clopidogrel 150 mg/day versus 75 mg/day in patients with ST-segment elevation acute myocardial infarction and poor responsiveness to clopidogrel: results from the DOUBLE study. Thromb Res 125:309–314

Patti G, Grieco D, Dicuonzo G et al (2011) High versus standard clopidogrel maintenance dose after percutaneous coronary intervention and effects on platelet inhibition, endothelial function, and inflammation results of the ARMYDA-150 mg (antiplatelet therapy for reduction of myocardial damage during angioplasty) randomized study. J Am Coll Cardiol 57:771–778

Perneby C, Wallén NH, Hofman-Bang C et al (2007) Effect of clopidogrel treatment on stress-induced platelet activation and myocardial ischemia in aspirin-treated patients with stable coronary artery disease. Thromb Haemost 98:1316–1322

Pesnot T, Jørgensen R, Palcic MM, Wagner GK (2010) Structural and mechanistic basis for a new mode of glycosyltransferase inhibition. Nat Chem Biol 6:321–323

Piacentini GL, Kaliner MA (1991) The potential roles of leukotrienes in bronchial asthma. Am Rev Respir Dis 143:S96–S99

Pitchford SC (2007) Novel uses for anti-platelet agents as anti-inflammatory drugs. Br J Pharmacol 152:987–1002

Pitchford SC, Page CP (2006) MRS2179, a P2Y1 antagonist suppresses leukocyte recruitment in a murine model of allergic inflammation. Proc Am Thorac Soc 3:A340

Pitchford SC, Yano H, Lever R et al (2003) Platelets are essential for leukocyte recruitment in allergic inflammation. J Allergy Clin Immunol 112:109–118

Pitchford SC, Riffo-Vasquez Y, Sousa A et al (2004) Platelets are necessary for airway wall remodelling in a murine model of chronic allergic inflammation. Blood 103:639–647

Pitchford SC, Momi S, Giannini S et al (2005) Platelet P-selectin is required for pulmonary eosinophil and lymphocyte recruitment in a murine model of allergic inflammation. Blood 105:2074–2081

Pitchford SC, Momi S, Baglioni S et al (2008) Allergen induces the migration of platelets to lung tissue in allergic asthma. Am J Respir Crit Care Med 177:604–612

Rendu R, Brohard-Bohn B (2002) Platelets organelles. In: Gresele P, Page C, Fuster V, Vermylen J (eds) Platelets in thrombotic and non-thrombotic disorders. Cambridge University Press, Cambridge, pp 104–112

Ross R (1999) Atheroschlerosis: an inflammatory disease. N Engl J Med 340:115–126

Sallusto F, Lanzavecchia A, Mackay CR (1998) Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses. Immunol Today 19:568–574

Sarma J, Laan CA, Alam S et al (2002) Increased platelet binding to circulating monocytes in acute coronary syndromes. Circulation 105:2166–2171

Schmitt-Sody M, Klose A, Gottschalk O et al (2005) Platelet-endothelial cell interactions in murine antigen-induced arthritis. Rheumatology 44:885–889

Schneider DJ (2011) Anti-platelet therapy: glycoprotein IIb-IIIa antagonists. Br J Clin Pharmacol 72:672–682

Schober A, Manka D, Von Hundelshausen P et al (2002) Deposition of platelet RANTES triggering monocyte recruitment requires P-selectin and is involved in neointima formation after arterial injury. Circulation 106:1523–1529

Seeds EAM, Kilfeather S, Okiji S, Page CP (1995) Role of lipoxygenase metabolites in PAF and antigen induced bronchial hyperresponsiveness and eosinophil infiltration. Eur J Pharmacol 293:369–376

Semple JW, Freedman J (2010) Platelets and innate immunity. Cell Mol Life Sci 67:499–511

Steinhubl SR, Badimon JJ, Bhatt DL et al (2007) Clinical evidence for anti-inflammatory effects of antiplatelet therapy in patients with atherothrombotic disease. Vasc Med 12:113–122

Storey RF, Sanderson HM, White AE et al (2000) The central role of the P(2T) receptor in amplification of human platelet activation, aggregation, secretion and procoagulant activity. Br J Haematol 110:925–934

Storey RF, Judge HM, Wilcox RG, Heptinstall S (2002) Inhibition of ADP-induced P-selectin expression and platelet-leukocyte conjugate formation by clopidogrel and the P2Y12 receptor antagonist AR-C69931MX but not aspirin. Thromb Haemost 88:488–494

Straub A, Krajewski S, Hohmann JD et al (2011) Evidence of platelet activation at medically used hypothermia and mechanistic data indicating ADP as a key mediator and therapeutic target. Arterioscler Thromb Vasc Biol 31:1607–1616

Sumariwalla PF, Malfait AM, Feldmann M (2004) P-selectin glycoprotein ligand 1 therapy ameliorates established collagen-induced arthritis in DBA/1 mice partly through the suppression of tumour necrosis factor. Clin Exp Immunol 136:67–75

Suttitanamongkol S, Gear AR (2001) ADP receptor antagonists inhibit platelet aggregation induced by the chemokines SDF-1, MDC and TARC. FEBS Lett 490:84–87

Thompson CB, Jakubowski JA, Quinn PG et al (1984) Platelet size and age determine platelet function independently. Blood 63:1372–1375

Vargaftig BB, Lefort J, Wal F, Chignard M (1981) Role of the metabolites of arachidonic in platelet-dependent and independent experimental bronchoconstriction. Bull Eur Physiopathol Resp 17:723

von Hundelshausen P, Weber C (2007) Platelets as immune cells bridging. Inflammation and cardiovascular disease. Circ Res 100:27–40

Wang K, Zhou Z, Zhou X et al (2001) Prevention of intimal hyperplasia with recombinant soluble P-selectin glycoprotein ligand-immunoglobulin in the porcine coronary artery balloon injury model. J Am Coll Cardiol 38:577–582

Willerson JT, Cable G, Yeh ET et al (2009) PROCLAIM: pilot study to examine the effects of clopidogrel on inflammatory markers in patients with metabolic syndrome receiving low-dose aspirin. Tex Heart Inst J 36:530–539

Woulfe DS (2005) Platelet G protein-coupled receptors in hemostasis and thrombosis. J Thromb Haemost 3:2193–2200

Yoshida A, Ohba M, Wu X et al (2002) Accumulation of platelets in the lung and liver and their degranulation following antigen-challenge in sensitized mice. Br J Pharmacol 137:146–152

Yousuf O, Bhatt DL (2011) The evolution of antiplatelet therapy in cardiovascular disease. Nat Rev Cardiol 8:547–559

Zarbock A, Singbartl K, Ley K (2006) Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation. J Clin Invest 116:3211–3219

Zernecke A, Schober A, Bot I et al (2005) SDF-1α/CXCR4 axis is instrumental in neo-intimal hyperplasia and recruitment of smooth muscle progenitor cells. Circ Res 96:784–791

Zerr M, Hechler B, Freund M et al (2011) Major contribution of the P2Y1 receptor in purinergic regulation of TNF{alpha}-induced vascular inflammation. Circulation 123:2404–2413

Zhao L, Bath PMW, May J et al (2003) P-selectin, tissue factor and CD40 ligand expression on platelet-leucocyte conjugates in the presence of a GPIIb/IIIa antagonist. Platelets 14:473–480

Part III
Therapy

The Role of Laboratory Monitoring in Antiplatelet Therapy

Marco Cattaneo

Contents

Abstract In the last decade, several studies reported a high inter-individual variability in the pharmacological response to antiplatelet drugs. Suboptimal response to aspirin, as determined by specific tests (serum thromboxane B_2), is rare and, when present, it appears to be caused by poor compliance in most instances. In contrast, studies that used specific tests to measure the pharmacological effect of clopidogrel showed a wide variability of response, with about 1/3 of treated subjects who are very poor responders. Inter-individual difference in the extent of metabolism of clopidogrel to its active metabolite by cytochrome P450

M. Cattaneo (✉)
Unità di Medicina 3 – Ospedale San Paolo, Dipartimento di Scienze della Salute, Università degli Studi di Milano, Via di Rudinì 8, 20142 Milano, Italy
e-mail: marco.cattaneo@unimi.it

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_19,

isoforms is the most relevant cause of the observed inter-individual variability in platelet inhibition. Tailored treatment based on laboratory monitoring of platelet function has been proposed as a solution to poor responsiveness to clopidogrel. However, we still need to identify the ideal laboratory test and to answer basic questions on its clinical utility and cost-effectiveness, before monitoring clopidogrel therapy can be recommended in clinical practice.

Keywords Acute coronary syndromes • Aspirin • Clopidogrel • Platelet function • Prasugrel • Ticagrelor

1 Introduction

Antiplatelet drugs are widely used to decrease the risk of major adverse cardiovascular events (MACE). Since their introduction in clinical practice, they have been administered to patients at standard doses, without monitoring their pharmacological effects by means of laboratory tests. However, in the last few years, several studies revealed inter-individual response variability to aspirin and the thienopyridine drug clopidogrel (Cattaneo 2007a). Because poor responders are not adequately protected from MACE, it has been proposed that antiplatelet therapy should be tailored to each individual patient, based on the results of platelet function tests (Cattaneo 2007a). Although this approach is often considered a desirable evolution of modern medicine, which ideally should be tailored based on individual needs, it is in fact an old remedy (of yet unproven efficacy, in the case of antiplatelet therapy) to the problem of response variability to antithrombotic drugs. The aim of personalized medicine is to optimize the risk/benefit and the cost/benefit ratios of health care, by identifying *"a priori"*, for each individual patient, the best possible management, including the choice between treatment with the most effective and safe drug and no treatment at all (many patients would not develop clinical events even if left untreated). In contrast, tailored treatment, based on the results of laboratory tests, simply aims at adjusting *"a posteriori"* the dose of a drug with unpredictable and variable bioavailability. Treatment with vitamin K antagonists and with unfractionated heparin has always been tailored to the individual patient, based on laboratory monitoring, because the bioavailability of these drugs is unpredictable and highly variable (Duxbury and Poller 2001). However, laboratory monitoring is expensive, increases the workload of health personnel, may be inaccurate, is uncomfortable for patients, and may decrease patients' adherence to treatment (Baroletti and Dell'Orfano 2010). For the above reasons, treatment with anticoagulant drugs is progressively disposing of laboratory monitoring, thanks to the introduction in the clinical practice of new drugs with very good and predictable bioavailability, such as low molecular weight heparins which do not need laboratory monitoring, and have progressively replaced unfractionated heparin. Therefore, it appears that antiplatelet therapy is heading towards an opposite direction compared to anticoagulant therapy.

In conclusion, given its many drawbacks, laboratory monitoring of antiplatelet therapy should be taken in consideration as a means to solve the problem of the high inter-individual variability of pharmacological response to the drug, only if easier solutions are unavailable, such as the use of alternative drugs with more uniform and predictable bioavailability, and with favourable profiles in terms of risk/benefit and cost/benefit ratios.

This chapter will focus on the issue of response variability to aspirin and clopidogrel.

2 How to Measure the Pharmacological Response to Antiplatelet Drugs

Aspirin and the thienopyridines are the most widely used antiplatelet agents in the clinical practice. Aspirin irreversibly inhibits the cyclooxygenase-1 (COX-1)-dependent production of thromboxane A_2 (TxA_2), while the thienopyridines (ticlopidine, clopidogrel, prasugrel) irreversibly inhibit the platelet $P2Y_{12}$ receptor for adenosine diphosphate (ADP). Their good antithrombotic effect, in spite of the fact that they inhibit a single pathway of platelet activation, is explained by the fact that both the TxA_2 pathway and the ADP pathway contribute to the amplification of platelet activation and are essential for the full aggregation response of platelets (Cattaneo 2007a).

Many studies used various techniques to measure platelet function ex vivo in order to evaluate the degree of its inhibition by antiplatelet treatment and, in some instances, to predict the risk of atherothrombotic events (Cattaneo 2007a). Although this approach is justified and rationale, one should be aware that the relative importance of the TxA_2 and $P2Y_{12}$ pathways in platelet activation varies considerably among different subjects and with the type of laboratory test used. Therefore, the finding of high, residual platelet reactivity in vitro in patients on aspirin or clopidogrel may not necessarily imply that these patients are resistant to treatment, especially when platelet function is measured by laboratory tests that are not specific for the effect of the antiplatelet drug on its pharmacological target. Therefore, only the use of specific tests that measure the pharmacological effect of the antiplatelet drug will clarify whether their platelet hyper-reactivity is due to insufficient pharmacological effect of the drug or to other causes (Cattaneo 2007a).

3 Inter-individual Variability of Response to Aspirin

3.1 Laboratory Tests to Measure the Response to Aspirin

Many laboratory tests have been used to monitor the antiplatelet effect of aspirin (Table 1). Light transmission aggregometry (LTA) is a poorly standardized

Table 1 Main laboratory tests that have been used to measure the degree of inhibition of platelet function by aspirin

- Serum thromboxane B_2
- Urinary 11-dehydro thromboxane B_2
- Light transmission aggregometry
- Impedance aggregometry
- VerifyNow-aspirin
- Plateletworks
- Arachidonic acid-stimulated formation of platelet-leucocyte hetero-aggregates, expression of activated αIIbβ3, or expression of P-selectin on the platelet surface (flow cytometry)
- Thromboelastography Platelet Mapping System
- IMPACT cone-and-plate(let) analyzer
- PFA-100

For details about these tests, see Cattaneo (2007a) and NCI-NHGRI Working Group on Replication in Association Studies et al. (2007)

technique, which is sensitive to several variables (Cattaneo 2007a; Cattaneo et al. 2009). Even when arachidonic acid (AA), the precursor of TxA_2, is used as platelet agonist, the results obtained with this technique may overestimate the prevalence of poor responders to aspirin (Cattaneo 2007a). Methods that measure directly the capacity of platelets to synthesize TxA_2 are preferable. Of these, the urinary levels of the TxB_2 metabolite, 11-dehydrothromboxane B_2 represent a time-integrated index of TxA_2 biosynthesis in vivo (Cattaneo 2007a). However, about 30 % of the urinary metabolite derives from extra-platelet sources (Cattaneo 2007a, b) and this fraction can increase in pathological conditions, such as in inflammatory diseases (Cattaneo 2007a, b). Therefore, the method is not highly specific for monitoring the effects of aspirin on platelet COX-1. In contrast, serum TxB_2 reflects the total capacity of platelets to synthesize TxA_2, of which it is a stable metabolite. Because the contribution of other blood cells to its synthesis is marginal, serum TxB_2 is the most specific test to measure the pharmacological effect of aspirin on platelets (Cattaneo 2007a, b).

Comparison of different laboratory methods usually showed very weak or no correlation in published studies (Cattaneo 2007b) indicating that they are sensitive to different parameters. Usually, the number of individuals with residual, significant TxB_2 production on aspirin was extremely low, while the prevalence of individuals with no inhibition of platelet function measured by other, less specific tests tended to be much higher (Cattaneo 2007a, b).

Studies that measured serum TxB_2 to assess the response to aspirin revealed that the prevalence of poor responders is extremely low (Cattaneo 2007a, b; Frelinger et al. 2006) with the possible exceptions of patients with recent CABG surgery (Brambilla et al. 2010) and patients with myeloproliferative neoplasms (Dragani et al. 2010) in whom high levels of serum TxB_2 despite aspirin treatment can be occasionally measured. Although TxB_2 production by platelets from aspirin-treated diabetic patients may be slightly higher than that by normal, aspirin-treated platelets, the degree of its inhibition by aspirin is usually still extremely good (Pulcinelli et al. 2009; Fontana et al. 2010).

3.2 Causes of Altered Pharmacological Response to Aspirin

Lack of compliance is probably the most frequent and plausible cause of insufficient inhibition of COX-1 by aspirin (Table 2) (Frelinger et al. 2006; Cattaneo 2004; Schwartz et al. 2005; Hennekens et al. 2010).

As yet, data for the extent of genetic contribution to the pharmacological response to aspirin are inconclusive, with investigators reporting conflicting results (Goodman et al. 2007). Increased platelet turnover in some disease states could account for a more rapid recovery of COX-1-dependent platelet function within the 24 h interval between two consecutive administrations of the drug (Table 2) (Henry et al. 2011). An increase in aspirin dose by means of a second daily administration was associated with a significant reduction in TxA2-dependent platelet reactivity or serum TxB2 levels in diabetic patients (Capodanno et al. 2011; Spectre et al. 2011)

Competition of aspirin with other non-steroidal anti-inflammatory drugs, such as ibuprofen, can prevent aspirin access at Ser529 of COX-1 and, as a consequence, its irreversible acetylation and inactivation of the enzyme (Table 2) (Catella-Lawson et al. 2001).

3.3 Clinical Consequences of Altered Pharmacological Response to Aspirin

Impaired inhibition of urinary excretion of Tx metabolites has been associated with increased incidence of MACE, and interpreted as due to insufficient inhibition of platelet COX-1 (Eikelboom et al. 2008). However, considering that atherothrombosis is an inflammatory disease, a possible, alternative interpretation of these results is that high urinary levels of 11-dehydrothromboxane B_2 reflect an increased generation of COX-2-dependent prostaglandins and thromboxanes by monocytes/macrophages in severely inflamed atherosclerotic plaques, which are at high risk of thrombotic complications (Cattaneo 2009).

The degree of inhibition of MACE by aspirin in diabetic patients does not seem to be significantly lower than in non-diabetic patients, both in primary (Antithrombotic Trialists' (ATT) Collaboration et al. 2009) and secondary prevention (Ferreiro and Angiolillo 2011), indicating that the slightly lower degree of inhibition of TxB_2 production by aspirin that has been occasionally described in diabetic platelets, compared to non-diabetic platelets, is not biologically and clinically relevant.

Frelinger et al. reported that a direct measure (serum $TXB_2 < 3.1$ ng/mL) but not indirect measures of residual platelet COX-1 function are associated with subsequent MACE in aspirin-treated patients (Frelinger et al. 2009). However, given a potential for bias based on the method used to define the cut-off for high residual serum TXB_2, the need to adjust for covariables to show a significant association between serum TXB_2 and subsequent MACE, and the

Table 2 Main variables affecting the pharmacodynamic response to aspirin

• Lack of compliance
• Under-dosing
• Interaction of other non-steroidal anti-inflammatory drugs with cyclooxygenase-1
• Increased platelet turnover
• Increased expression of platelet cyclooxygenase-2

failure of indirect assays of residual platelet COX-1 function to confirm an association with MACE, the link between residual platelet COX-1 function as reported by serum $TXB_2 < 3.1$ ng/mL and MACE should be further verified (Frelinger et al. 2009).

3.4 Laboratory Monitoring of Aspirin Therapy

Because insufficient response to aspirin is extremely rare and its proper treatment, if any, is unknown, it is usually recommended that, other than in research studies, the response to aspirin in treated patients should not be tested (Cattaneo 2004, a, b; Michelson et al. 2005). The most important factor contributing to these recommendations is that no published studies addressed the clinical effectiveness of altering therapy on the basis of the results of laboratory tests. Moreover, it is known that increasing aspirin dosage would not be very effective in decreasing the incidence of MACE, but is associated with increased risk of bleeding (Patrono et al. 2005).

4 Inter-individual Variability of Response to Clopidogrel

4.1 Laboratory Tests to Measure the Response to Inhibitors of $P2Y_{12}$

Several laboratory tests have been used to measure the degree of inhibition of platelet function by clopidogrel (Table 3).

ADP-induced platelet aggregation measured by LTA may overestimate the prevalence of poor responders to $P2Y_{12}$ inhibitors, because ADP induces platelet aggregation by interacting with both its platelet receptors, $P2Y_1$ and $P2Y_{12}$ (Cattaneo 2011). The platelet aggregation-based assay VerifyNow *$P2Y_{12}$* and the flow cytometry-based assay Platelet VASP® (which measures the $P2Y_{12}$-dependent inhibition by ADP of phosphorylation of vasodilator-stimulated phosphoprotein, VASP), are more specific assays for measuring the effects of thienopyridines and other drugs inhibiting the platelet $P2Y_{12}$ receptor (Cattaneo 2007a).

Table 3 Main variables affecting the pharmacodynamics response to clopidogrel

• Lack of compliance
• Reduced absorption (e.g. in carriers of the TT3435 mutation of ABCB1, encoding for P-glycoprotein)
• Loss-of-function or gain-of-function mutations of CYP2C19 (and other CYP isoforms)
• Interaction of other drugs (proton pump inhibitors, lipophilic statins, calcium channel blockers)
• Age
• High body mass index
• Diabetes mellitus
• Renal insufficiency in diabetes mellitus
• Pre-existent variability in platelet response to ADP
• Increased platelet turnover (theoretical)
• Tobacco smoking (heightened response)

4.2 Inter-individual Variability in the Pharmacological Response to Clopidogrel

The clinical utility of clopidogrel is hampered by the high inter-individual variability of inhibition of $P2Y_{12}$-dependent platelet function, which is mostly caused by the variable bioavailability of its active metabolite (Cattaneo 2007a, 2011; Bonello et al. 2010): about 25 % of treated patients display suboptimal response to the drug (Cattaneo 2007a, 2011; Bonello et al. 2010).

Like the other thienopyridines, clopidogrel is a pro-drug that needs to be metabolized to an active metabolite in order to exert its pharmacological effect. The formation of the active metabolite of clopidogrel involves a two-step process, which is regulated by isoforms of the hepatic cytochrome P450 (CYP): CYP2C19, CYP1A2 and CYP2B6 are responsible for the first metabolic step, whereas CYP2C19, CYP2C9, CYP2B6 and CYP3A are responsible for the second step (Fig. 1) (Duxbury and Poller 2001; Baroletti and Dell'Orfano 2010). Loss-of-function (e.g. CYP2C19*2, CYP2C19*3) and gain-of-function (e.g. CYP2C19*17) genotypes are associated with variable degrees of production of the active metabolite and, hence, of the pharmacodynamic response to the drug (Bonello et al. 2010; Cattaneo 2010). However, it has been hypothesized that the impaired pharmacodynamic response to clopidogrel in association with CYP2C19*2 may be due to an imbalance in the formation of pro-inflammatory and anti-inflammatory cytokines, which could contribute to altered platelet aggregability, rather than to impairment of the formation of the active metabolite of clopidogrel (Bouman et al. 2011a). The same authors identified paraoxonase-1 (PON-1) as the crucial enzyme for clopidogrel bioactivation (Bouman et al. 2011b), but this finding was not confirmed by several subsequent studies (Hulot et al. 2011; Camps et al. 2011; Dansette et al. 2011; Cuisset et al. 2011; Fontana et al. 2011; Trenk et al. 2011; Sibbing et al. 2011).

Variable levels of active metabolite generation and/or of pharmacodynamic response to clopidogrel are also associated with (Table 3): (1) limited intestinal absorption, which is associated with the homozygous 3435C→T mutation of ABCB1, a gene encoding for the efflux pump P-glycoprotein, a key protein

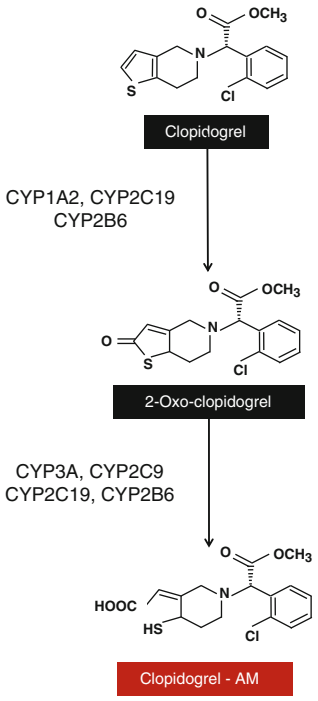


Fig. 1 Metabolism of clopidogrel. Schematic representation of the main enzymatic pathways involved in the metabolism of clopidogrel.
CYP = cytochrome P450 isoenzyme; clopidogrel-AM: clopidogrel active metabolite

Table 4 Main laboratory tests that have been used to measure the degree of inhibition of platelet function by clopidogrel

- Light transmission aggregometry
- Impedance aggregometry
- VerifyNow-P2Y$_{12}$
- Plateletworks
- Platelet VASP®
- ADP-stimulated formation of platelet-leucocyte hetero-aggregates, expression of activated αIIbβ3, or expression of P-selectin on the platelet surface (flow cytometry)
- Thromboelastography Platelet Mapping System
- IMPACT cone-and-plate(let) analyzer

For details about these tests, see Cattaneo (2007a) and NCI-NHGRI Working Group on Replication in Association Studies et al. (2007)

involved in thienopyridine absorption; (2) interaction with other drugs, including some proton pump inhibitors (PPI), calcium channel blockers and lipophilic statins, which are metabolized by CYP2C19 and CYP3A isoenzymes; (3) stimulation of CYP1A2 activity by tobacco smoking; (4) pre-existent variability in platelet response to ADP (Table 4) (Bonello et al. 2010; Cattaneo 2010). Other variables that influence the response to clopidogrel include advanced age, high body mass index and diabetes mellitus, renal insufficiency in diabetes mellitus, which are associated with decreased response to the drug (Table 4) (Bonello et al. 2010;

Cattaneo 2010). Needless to say, noncompliance is to be considered an obvious and frequent cause of poor response to clopidogrel (Cattaneo 2004).

4.3 Clinical Consequences of the Inter-individual Variability of Response to Clopidogrel

Several independent studies demonstrated an association between suboptimal generation of the active metabolite of clopidogrel, decreased inhibition of platelet function, presence of enzyme polymorphisms and clinical outcomes (Bonello et al. 2010; Cattaneo 2010; Campo et al. 2011; Brar et al. 2011). However, no study has yet associated all of these parameters in the same patient population, and some uncertainties still persist. For instance, no clear negative association with clinical outcomes of the co-administration of clopidogrel with drugs that potentially interfere with its metabolism has been documented so far, despite their negative interaction with the pharmacodynamic response to the drug (Bates et al. 2011; Chen et al. 2011). This is true not only for lipophilic statins and calcium channel blockers (Bonello et al. 2010; Bates et al. 2011), but also for omeprazole and other proton pump inhibitors (PPI) interfering with CYP2C19. The evidence of the negative interaction of some PPIs with the pharmacodynamic response to clopidogrel activity (Chen et al. 2011; Gilard et al. 2006, 2008; Angiolillo et al. 2010), albeit controversial (Gremmel et al. 2010), and the demonstration in observational studies, case–control studies and of post-hoc analyses of some randomized clinical trials that the risk of MACE was higher in patients on combined treatment with clopidogrel and PPI, compared with patients on clopidogrel not in combined treatment with PPI (Hulot et al. 2010; Siller-Matula et al. 2010; Ho et al. 2009), prompted drug-regulating authorities to issue a warning for the use of these drugs in combination with clopidogrel (Abraham et al. 2010). However, the existence of this negative interaction stemming from randomized clinical trials was less evident (Hulot et al. 2010; O'Donoghue et al. 2009; Ray et al. 2010; Rassen et al. 2009; Simon et al. 2011; Hsiao et al. 2011). Moreover, the only randomized controlled trial that prospectively tested the interaction between omeprazole and clopidogrel, which was terminated prematurely due to bankruptcy of the sponsor, failed to show that the co-administration of the two drugs increases the incidence of MACE (Bhatt et al. 2010). A third, more recent meta-analysis indicated an obvious discrepancy between the negative results of randomized clinical trials and the positive results of observational studies, concerning the clinical consequences of the negative pharmacological interaction of PPI with clopidogrel (Chen et al. 2011).

The association between poor clinical outcomes of patients on treatment with clopidogrel and the presence of loss-of-function mutations of CYP has been demonstrated in observational and intervention studies. However, it must be emphasized that the testing and validation of statistical hypotheses in genetic epidemiology is not an easy task. Ioannidis et al. showed that significant

between-study heterogeneity is frequent, and that the results of the first studies (usually suggesting a strong genetic defect) correlate only modestly with subsequent research on the same association (Ioannidis et al. 2001). As a matter of fact, three meta-analyses, which included the early published studies, demonstrated an increased risk of MACE and, particularly, of stent thrombosis in carriers of either one or two mutated CYP2C19*2 alleles (Hulot et al. 2010; Mega et al. 2010a; Sofi et al. 2011), while three more recent meta-analyses did not indicate a substantial or consistent influence of loss-of-function CYP2C19 gene polymorphisms on the clinical efficacy of clopidogrel (Bauer et al. 2011; Zabalza et al. 2011; Holmes et al. 2011). The different results of these last meta-analyses are likely due to the fact that they included studies published after the year 2010 (which showed weaker effects compared to the previous ones) (Bauer et al. 2011; Zabalza et al. 2011) and extracted only data of pre-specified clinical events that conformed to unbiased and standardized definitions (Bauer et al. 2011). Moreover, the meta-analysis by Zabalza et al. showed that a significant association between the loss-of-function alleles and a higher risk of cardiovascular outcomes was demonstrated by small size studies (<500 patients), whereas no significant effect was observed in the pooled analysis of studies with a sample size >500 patients (Zabalza et al. 2011). The overestimation of the effect size in small genetic association studies is well known and could be related to different factors such as spurious association due to biased publication of positive results in these types of studies (NCI-NHGRI Working Group on Replication in Association Studies et al. 2007).

Finally, the question remains open as to whether the association that was found in some studies is explained by impaired clopidogrel metabolism by carriers of the mutation, or by pleiotropic effects, with negative impact on cardiovascular outcomes, independently of the administration of clopidogrel. This latter hypothesis is corroborated by the results of a retrospective analysis of two trials comparing clopidogrel to placebo in patients with acute coronary syndromes and in patients with atrial fibrillation, which showed that the protective effect of clopidogrel as compared with placebo is consistent, irrespective of CYP2C19 loss-of-function carrier status (Paré et al. 2010).

In patients treated with clopidogrel, ABCB1 3435C→T genotype was significantly associated with the risk of cardiovascular death, myocardial infarction, or stroke in the TRITON TIMI-38 trial, which compared clopidogrel and prasugrel in patients with acute coronary syndromes undergoing PCI (Mega et al. 2010b).

In conclusion, there still are contrasting reports in the literature, linking negative interactions with the pharmacodynamic response to the drug and negative interactions with clinical outcomes. These uncertainties are likely due to inaccuracies and lack of standardization of the methodological approaches that have been used to detect “non-responders” to clopidogrel, and to uncertainties on how to interpret their results, stemming from the absence of universally accepted and validated cut-off values of platelet function: indeed, it would appear unquestionable from a logical standpoint that an antithrombotic drug (and any drug in general) that is unable to hit its pharmacological target is also expected to be unable to be fully clinically effective. Therefore, independently of the uncertainties

stemming from the published studies, efforts aimed at obtaining a good inhibition of $P2Y_{12}$-dependent platelet function in all treated patients are justified. The most commonly proposed and widely tested approach has been to tailor treatment with clopidogrel to each individual patient, based on the results of platelet function tests, used to monitor the individual pharmacodynamic response to the drug.

4.4 Laboratory Monitoring of Clopidogrel Therapy

In order to be implemented in the clinical practice, laboratory monitoring of clopidogrel therapy should undergo the same validation process that was undertaken for laboratory monitoring of VKA, which included: (a) identification of the most sensitive and specific laboratory test to identify accurately those patients who exhibit an abnormal response to the drug; (b) identification of a "therapeutic window", within which both the risk of thrombosis and that of bleeding are minimized; (c) standardization of both the pre-analytical and the analytical conditions of the laboratory test; (d) identification of efficacious, safe and cost-effective treatments for patients whose values fall outside the "therapeutic window".

(a) *Identification of the most sensitive and specific laboratory test to identify accurately those patients who exhibit an abnormal response to the drug*. Several tests have been used to monitor the pharmacological effect of clopidogrel (Table 4) (Cattaneo 2007a; Michelson 2009). Many studies compared the performance of different laboratory tests for evaluating the pharmacological effects of clopidogrel: all of them demonstrated that the degree of agreement among different tests is unacceptably low (Cuisset et al. 2010; Gremmel et al. 2009; Paniccia et al. 2007, 2010; Breet et al. 2010; Madsen et al. 2010; von Beckerath et al. 2010; Gaglia et al. 2011): the same patients could be considered poor responders by one test and good responders by another test, and vice versa. Therefore, because no single laboratory test has been shown to be more accurate than others in measuring the degree of inhibition of ADP/$P2Y_{12}$-dependent platelet function by clopidogrel, the equivalent of the prothrombin time, which is the gold standard for monitoring VKA therapy, for treatment with clopidogrel has not been identified yet.

(b) *Identification of the "therapeutic window"*. Inhibition of the haemostatic system by anticoagulant or antiplatelet drugs not only decreases the risk of thrombosis, but also inevitably increases the risk of bleeding. Therefore, when monitoring an antithrombotic treatment, it is necessary to identify not only a cut-off value for the degree of inhibition of haemostasis below which protection from MACE is suboptimal, but also the cut-off value above which the risk of bleeding is high. Indeed, reduction of the risk of bleeding is of paramount clinical importance, because severe bleeding complicating antithrombotic therapy have negative consequences, not only because they

may be fatal, disabling, and expose the patients to the risks that are associated with blood transfusion: they are also associated with poor prognosis of the patients, whose risk of death is increased during follow-up (Mehran et al. 2010; Eikelboom et al. 2006; Pocock et al. 2010). The cut-off values of this therapeutic window for the degree of anticoagulation that can be achieved with VKA have been validated and are universally accepted by all laboratories worldwide. In contrast, only recently this important clinical issue has started to be addressed for some tests of platelet function used for tailoring clopidogrel therapy (Bonello et al. 2010; Campo et al. 2011; Sibbing et al. 2010), and the cut-off values for both the risk of thrombosis (see later) and the risk of bleeding are far from being universally accepted.

(c) *Standardization of both the pre-analytical and the analytical conditions of the laboratory test*. Standardization of laboratory tests is indispensable to obtain comparable results in different laboratories, which is an essential requirement for the implementation of laboratory monitoring in the clinical practice. An example of the importance of laboratory standardization is provided by the evolution of VKA monitoring with the prothrombin time (PT). Monitoring problems arose from the introduction, especially in the USA, of some poorly responsive commercial tissue extracts for use as tissue extract thromboplastin reagent in the PT (Duxbury and Poller 2001). More oral anticoagulant drug was then needed to prolong the test to the required therapeutic targets, with a resultant increase in bleeding (Duxbury and Poller 2001). It was not until 1983 that the problem was resolved thanks to the PT standardization scheme of the World Health Organization, using the international normalized ratio (INR), which allowed the widespread adoption of "low-dose warfarin" regimen, leading to improved effectiveness and safety of VKA (Duxbury and Poller 2001).

Many pre-analytical and analytical variables affect the results of light transmission aggregometry, the standardization of which poses several problems, despite some attempts have been done, especially in the last few years (Cattaneo et al. 2009; Hayward et al. 2010). As a consequence, the results obtained within one laboratory can hardly be compared to those obtained in a different laboratory: therefore, any attempt to define a universal therapeutic window of platelet aggregation to monitor clopidogrel treatment would be pointless.

Analytical variables may be more easily standardized for other laboratory techniques, especially for those relying on the use of commercially available kits that need no or very limited handling of blood samples, such as the VerifyNow $P2Y_{12}$ and the Platelet VASP® assay. However, also for these techniques pre-analytical variables need to be standardized.

Many studies showed that platelet function in patients under treatment with clopidogrel is a rather unstable phenotype, with up to about 50 % of treated patients switching from the category of "poor responders" to that of "good responders" and vice versa at different times since the start of therapy (Campo et al. 2011; Arméro et al. 2010; Jaitner et al. 2011; Aleil et al. 2008).

This variability of the results of platelet function tests casts doubts about their accuracy and points to the need of repeated testing during the follow-up of the patients, with the consequence of increasing the costs and complexity of patient management. In addition, there is no clear information on when platelet function testing provides the best prediction of clinical events. Campo et al. recently showed that platelet reactivity at day 30 since the start of treatment best predicts both ischemic and bleeding events (Campo et al. 2011): one wonders if an earlier information would be more clinically useful.

The time elapsed since the last intake of clopidogrel and blood sampling is also expected to affect the results of any platelet function test, especially in patients with coronary artery disease whose increased platelet turnover accelerates the entry of newly formed, non-inhibited platelets into the circulation.

Finally, the time of the day in which blood is sampled may significantly affect the results, because platelet function follows a circadian rhythm, being highest at midmorning (Tofler et al. 1987). Consistently with this hypothesis, Kozinsky et al. showed that, among patients under maintenance treatment with 75 mg/day clopidogrel, which was administered at 8:00 a.m., the highest prevalence of "poor responders" (20.3 %) was observed at 10:00 a.m. compared to 8.5 % at 6:00 a.m. and 10.2 % both at 2:00 p.m. and 7:00 p.m. ($p < 0.02$) (Kozinski et al. 2011).

(d) *Identification of efficacious, safe and cost-effective treatments for patients whose values fall outside the "therapeutic window"*. Preliminary experiments of tailored clopidogrel treatment, based on the results of laboratory tests, gave results that are incompletely satisfactory. Bonello et al. identified patients with acute coronary syndromes scheduled for percutaneous coronary intervention (PCI), who were considered resistant to a loading dose of 600 mg clopidogrel, based on the results of the Platelet VASP® assay (Bonello et al. 2008, 2009). These patients were randomized to undergo Platelet VASP®-guided additional loading doses of 600 mg clopidogrel until they reached adequate inhibition of $P2Y_{12}$ function, or no further treatment (control group). Some patients in the Platelet VASP®-guided group achieved this goal after repeated clopidogrel doses, but about 10 % of them were still resistant after a total of 2,400 mg clopidogrel (32 pills!) (Bonello et al. 2008, 2009). Although the rate of MACE (Bonello et al. 2008) or stent thrombosis (Bonello et al. 2009) at 30 days was lower in the VASP-guided group than in the control group, it appears that the tailored treatment approach that was used in this study is far from ideal, because it is cumbersome, time consuming, expensive, and, most importantly, not effective in all patients, despite many interventions to correct the dose of the drug. In addition, one wonders whether resistant patients who, after many loading doses of clopidogrel did eventually display an adequate response to the drug, maintained a satisfactory inhibition of platelet function when given the much lower daily maintenance dose of clopidogrel that were administered afterwards. This concern is substantiated by the results of another study, which showed how difficult may be to override clopidogrel resistance by increasing the maintenance doses of the drug. Some patients were still resistant to

maintenance doses of 300 mg clopidogrel daily, which could not be continued, due to the occurrence of severe side effects (stomach discomfort and joint pain) (Pena et al. 2009). It was only after the administration of regular maintenance doses of prasugrel that these patients exhibited adequate inhibition of $P2Y_{12}$-dependent platelet function (Pena et al. 2009). In conclusion, those by Bonello et al. are important prove-of-concept studies, because they demonstrated that the improvement of the pharmacological response to clopidogrel may improve its clinical efficacy, but the management strategy that were employed in these studies can hardly be transferred in the clinical practice.

Mostly based on the aforementioned knowledge gaps (see "Knowledge Gaps"), and in compliance with the rules of *Evidence Based Medicine*, guidelines of Scientific Societies and a recent consensus paper concluded that until the results of large-scale trials of personalized antiplatelet therapy are available, the routine use of platelet function measurements in the care of patients with cardiovascular disease cannot be recommended (Bonello et al. 2010; Holmes et al. 2010; Task Force on Myocardial Revascularization of the European Society of Cardiology (ESC) and the European Association for Cardio-Thoracic Surgery (EACTS) et al. 2010).

Our incompetence on personalized antiplatelet treatment has been recently testified by the negative results of GRAVITAS, the first, large randomized prospective trial testing the efficacy and safety of tailored clopidogrel treatment in patients undergoing PCI (Price et al. 2011a). GRAVITAS enrolled 5,429 patients with coronary artery disease (predominantly, albeit not uniquely, with stable disease), who were treated with a loading dose of clopidogrel (600 mg) before undergoing PCI with stent implantation. Platelet function was measured by the VerifyNow $P2Y_{12}$ test, 12–24 h after PCI, which identified 2,214 patients with high on-treatment platelet reactivity (Platelet Reactivity Units [PRU] $\geq$ 230). These patients were randomized to standard treatment (daily maintenance dose of 75 mg clopidogrel) or to high-dose clopidogrel (additional loading dose, plus daily maintenance dose of 150 mg) for 6 months. The primary efficacy end-point was the incidence of cardiovascular death, acute myocardial infarction or stent thrombosis, the safety end-point was the incidence of severe or moderate bleeding, based on the GUSTO definition, while the pharmacodynamic end-point was the prevalence of persistent high on-treatment platelet reactivity during the follow-up of the study. The results of the GRAVITAS trial showed that treatment with high-dose clopidogrel of patients with high on-treatment platelet reactivity after a loading dose of the drug did not reduce the incidence of MACE compared with the standard dose (2.3 % in both groups, $P = 0.97$), nor did it increase the incidence of bleeding (1.4 % and 2.3 %, $P = 0.1$) (Price et al. 2011a). The prevalence of high on-treatment platelet reactivity during follow-up decreased significantly more in the high-dose group (−62 %), compared to the standard dose group (−49 %, $P < 0.001$). Moreover, the incidence of the primary efficacy end-point among patients treated with standard dose clopidogrel was not significantly different from that of a group of 586 patients, randomly chosen among the 3,215 who showed a satisfactory inhibition of platelet function after the loading dose of clopidogrel,

and were treated with standard dose of the drug (Price et al. 2011a). These last findings emphasize the dubious association between the results of platelet function tests in patients on treatment with clopidogrel and clinical outcomes, which I have mentioned in a previous part of this review.

In conclusion, (1) the negative results of the GRAVITAS trial confirm and emphasize that, in compliance with the rules of *Evidence Based Medicine*, no treatment of proven efficacy and safety should be replaced by new treatments, even if theoretically more rational, before their efficacy, safety and favourable cost–benefit ratio has been proven; (2) the adoption of the personalized treatment strategy that has been tested in the GRAVITAS trial would cause (and has already caused, in the Institutions that already adopted it) an unjustified expenditure of resources, without being of any benefit to the patients.

Within the intense debate that followed the publication of the results of the GRAVITAS trial, many criticisms were raised about its design, which included the type of enrolled patients, the choice of the platelet function test, the timing and/or the frequency of their performance, the type of pharmacological intervention and others (Gurbel and Tantry 2011, http://www.theheart.com]. Even the choice of the cut-off value of PRU has been criticized, despite the fact that it was chosen, based on the results of previous observational studies (Price et al. 2011a): this is a further demonstration that the "therapeutic window" of clopidogrel has not been clearly validated and universally accepted yet. A post-hoc analysis of the results of the GRAVITAS trial showed that the choice of a different cut-off value (PRU = 208), according to the results of a recent observational study (Campo et al. 2010) would have allowed a more accurate identification of poor responders to clopidogrel and, possibly, the therapeutic success that was missed by the GRAVITAS trial (Price et al. 2011b). However, the same authors who identified in PRU $\geq$ 208 the best cut-off value (Campo et al. 2010), in a later publication indicated that PRU $\geq$ 235 best predicts the risk of cardiovascular events (Campo et al. 2011), while a very recent meta-analysis identified PRU $\geq$ 230 as the best cut-off value (Brar et al. 2011), adding to the confusion on this very important issue. Moreover, the results of RECLOSE 2-ACS, a recently published large, prospective observational study (Parodi et al. 2011), deny that a more accurate identification of poor responders to clopidogrel would be in itself sufficient to secure the success of treating poor responders with high dose of clopidogrel. Indeed, despite the fact that the laboratory test (platelet aggregation induced by ADP, studied with LTA) could predict the risk of MACE of poor responders, the improvement of the pharmacological response to high doses (up to 300 mg daily maintenance dose) of clopidogrel (or to ticlopidine, up to 1,000 mg daily) was not associated with a reduction of the incidence of MACE (Parodi et al. 2011). The criticisms that have been raised to the GRAVITAS trial, the amendments that have subsequently proposed by its authors and the therapeutic failure of RECLOSE 2-ACS further emphasize our uncertainties and, as a consequence, the prematurity and incorrectness of tailoring clopidogrel treatment based on laboratory tests in the clinical practice. Other trials of tailored

clopidogrel treatment based on laboratory monitoring are ongoing and their results are expected in the near future (Bonello et al. 2010).

4.5 *Alternative Approaches to the Problem of Response Variability to Clopidogrel*

As an alternative to laboratory monitoring with platelet function tests, the identification of carriers of loss-of-function mutations of CYP through genotyping can be considered. In March 2010, the US Food and Drug Administration (FDA) added a "boxed warning" to the label of clopidogrel including a reference to patients who do not effectively metabolize the drug and therefore may not receive the full benefits on the basis of their genetic characteristics (FDA Drug Safety Communication: reduced effectiveness of Plavix (clopidogrel) in patients who are poor metabolizers of the drug n.d.), without giving indications on how to manage these patients. More recently the American College of Cardiology Foundation and the American Heart Association have published a consensus document addressing this FDA warning, which states that the role of genetic testing and the clinical implications and consequences of this testing remains to be determined (Bonello et al. 2008). Therefore, waiting for the results of some ongoing studies (Epstein and Teagarden 2010), genetic testing should not be performed in the clinical practice. Based on the findings of observational studies, it is difficult to foresee that this approach will be very successful, because it has been demonstrated that CYP2C19*2 accounts for only 5–12 % of the variability of response to clopidogel (Hochholzer et al. 2010; Shuldiner et al. 2009; Bouman et al. 2011c). In addition, the sensitivity of CYP2C19 genotyping for poor responders to clopidogrel is as low as about 40 % (Hochholzer et al. 2010; Aleil et al. 2009).

Other potential strategies of personalized treatment (serial testing, combined patient genotyping and serial testing, use of the new $P2Y_{12}$ inhibitors instead of high-dose clopidogrel in low responders) might prove effective (Price et al. 2011a; Gurbel and Tantry 2011). However, (1) serial testing with or without genotyping will increase the overall cost of treatment, possibly offsetting the advantage of using the cheaper drug clopidogrel instead of the new, more expensive $P2Y_{12}$ inhibitors; (2) tailored antiplatelet treatment should not be considered an achievement of modern medicine to be pursued by any means, but, rather, a potential solution to the problem of hyporesponsiveness to clopidogrel: it is quite obvious that not having to face the problem would be preferable; (3) the use of the new $P2Y_{12}$ antagonists prasugrel or ticagrelor instead of clopidogrel would markedly lessen the problem of hyporesponsiveness, because they effectively inhibit platelet function in the vast majority of patients (Cattaneo 2010), although some degree of variability of response is observed also with these drugs (Michelson et al. 2009; Bonello et al. 2011; Gurbel et al. 2010), as could be easily predicted. Both prasugrel and ticagrelor increase the incidence of bleeding, but it is mainly to be ascribed to

the fact that most patients treated with these drugs display a good inhibition of platelet function and are therefore protected from thrombosis and exposed to the risk of bleeding (as opposed to only about 70 % of patients treated with clopidogrel) (Cattaneo 2010). Accordingly, it has been clearly demonstrated that patients displaying good response to clopidogrel are at higher risk of bleeding than those who are nonresponsive (Cattaneo 2010). In other words, if all patients were to respond well to clopidogrel (which is the aim of tailored treatment with clopidogrel) they would have a lower incidence of MACE and a higher incidence of bleeding: exactly like prasugrel- or ticagrelor-treated patients. The rate of bleeding complications is related to the degree of inhibition of platelet function, rather than to the type of drug used to cause it. The use of the new $P2Y_{12}$ inhibitors in all patients, without testing, might prove more effective and cost-effective than personalized treatment: this hypothesis should be tested in controlled studies. While we await the results of additional controlled studies, personalized treatment should not yet be implemented in clinical practice.

5 Conclusion

- Inter-individual variability in the pharmacological response to antiplatelet drugs has been reported in some studies.
- Suboptimal response to aspirin, as determined by specific tests (serum thromboxane B_2), appears to be very rare and, in most instances, caused by poor compliance.
- Therefore, there is no need to monitor aspirin treatment.
- In contrast, studies that used specific tests to measure the pharmacological effect of clopidogrel showed a wide variability of response, with significant proportions of subjects (about one-third) who are very poor responders.
- Genetic and environmental factors influencing the absorption and/or the extent of metabolism of clopidogrel to its active metabolite account for the observed variability of response to clopidogrel.
- Tailored treatment based on the results of laboratory tests of platelet function has been proposed as a solution to poor responsiveness to clopidogrel.
- Although it is often considered a desirable evolution of modern medicine, which ideally should be personalized based on individual needs, tailored treatment based on laboratory tests is actually an old remedy (of yet unproven efficacy, in the case of antiplatelet therapy) to the problem of response variability to antithrombotic drugs with unpredictable bioavailability.
- When possible, the use of alternative drugs with more uniform and predictable bioavailability, and with favourable profiles in terms of risk/benefit and cost/benefit ratios should be preferred.
- We still need to identify and standardize the ideal laboratory test to be implemented for laboratory monitoring of clopidogrel treatment, and to answer basic questions on its clinical utility and cost-effectiveness.

- The only randomized clinical trial that tested the safety and efficacy of tailored clopidogrel treatment (GRAVITAS), based on the results of the VerifyNow $P2Y_{12}$ test, failed to demonstrate an advantage of tailored treatment compared to treatment with standard doses of the drug.
- While we await the results of additional controlled studies, laboratory monitoring of clopidogrel treatment should not yet be implemented in clinical practice.

Knowledge Gaps

- The most accurate test to monitor clopidogrel treatment has not been identified yet
- No universally accepted cut-off values for the risk of thrombosis and the risk of bleeding have been identified yet
- Lack of standardization of pre-analytical and analytical variables of laboratory tests
- Efficacious, safe and cost-effective treatments for patients whose values fall outside the "therapeutic window" are unknown

Key Messages

- Inter-individual variability in the pharmacological response to antiplatelet drugs has been reported in some studies.
- Suboptimal response to aspirin, as determined by specific tests is very rare.
- Therefore, there is no need to monitor aspirin treatment.
- The response to clopidogrel is variable: about one-third of patients are very poor responders.
- Genetic and environmental factors influencing the absorption and/or the extent of metabolism of clopidogrel to its active metabolite account for the observed variability of response to clopidogrel.
- Tailored treatment based on the results of laboratory tests of platelet function has been proposed as a solution to poor responsiveness to clopidogrel.
- Tailored treatment based on laboratory tests is an old remedy (of yet unproven efficacy, in the case of antiplatelet therapy) to the problem of response variability to antithrombotic drugs with unpredictable bioavailability.
- The use of alternative drugs with more uniform and predictable bioavailability, and with favourable profiles in terms of risk/benefit and cost/benefit ratios should be preferred.
- We still need to identify and standardize the ideal laboratory test to be implemented for laboratory monitoring of clopidogrel treatment.

(continued)

- The only randomized clinical trial that tested the safety and efficacy of tailored clopidogrel treatment based on laboratory tests failed to demonstrate an advantage of tailored treatment compared to treatment with standard doses of the drug.
- While we await the results of additional controlled studies, laboratory monitoring of clopidogrel treatment should not yet be implemented in clinical practice.

References

Abraham NS, Hlatky MA, Antman EM et al (2010) ACCF/ACG/AHA 2010 Expert Consensus Document on the concomitant use of proton pump inhibitors and thienopyridines: a focused update of the ACCF/ACG/AHA 2008 expert consensus document on reducing the gastrointestinal risks of antiplatelet therapy and NSAID use: a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents. Circulation 122:2619–2633

Aleil B, Jacquemin L, De Poli F et al (2008) Clopidogrel 150 mg/day to overcome low responsiveness in patients undergoing elective percutaneous coronary intervention: results from the VASP-02 (Vasodilator-Stimulated Phosphoprotein-02) randomized study. JACC Cardiovasc Interv 1:631–638

Aleil B, Léon C, Cazenave JP, Gachet C (2009) CYP2C19*2 polymorphism is not the sole determinant of the response to clopidogrel: implications for its monitoring. J Thromb Haemost 7:1747–1749

Angiolillo DJ, Gibson CM, Cheng S et al (2010) Differential effects of omeprazole and pantoprazole on the pharmacodynamics and pharmacokinetics of clopidogrel in healthy subjects: randomized, placebo-controlled, crossover comparison studies. Clin Pharmacol Ther 89:65–74

Antithrombotic Trialists' (ATT) Collaboration, Baigent C, Blackwell L, Collins R et al (2009) Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. Lancet 373:1849–1860

Arméro S, Camoin Jau L, Omar Aït Mokhtar O et al (2010) Intra-individual variability in clopidogrel responsiveness in coronary artery disease patients under long term therapy. Platelets 21:503–507

Baroletti S, Dell'Orfano H (2010) Medication adherence in cardiovascular disease. Circulation 121:1455–1458

Bates ER, Lau WC, Angiolillo DJ (2011) Clopidogrel-drug interactions. J Am Coll Cardiol 57:1251–1263

Bauer T, Bouman HJ, van Werkum JW et al (2011) Impact of CYP2C19 variant genotypes on clinical efficacy of antiplatelet treatment with clopidogrel: systematic review and meta-analysis. BMJ 343:d4588

Bhatt DL, Cryer BL, Contant CF et al (2010) Clopidogrel with or without omeprazole in coronary artery disease. N Engl J Med 363:1909–1917

Bonello L, Camoin-Jau L, Arques S et al (2008) Adjusted clopidogrel loading doses according to vasodilator-stimulated phosphoprotein phosphorylation index decrease rate of major adverse cardiovascular events in patients with clopidogrel resistance: a multicenter randomized prospective study. J Am Coll Cardiol 51:1404–1411

Bonello L, Camoin-Jau L, Armero S et al (2009) Tailored clopidogrel loading dose according to platelet reactivity monitoring to prevent acute and subacute stent thrombosis. Am J Cardiol 103:5–10

Bonello L, Tantry US, Marcucci R et al (2010) Consensus and future directions on the definition of high on-treatment platelet reactivity to adenosine diphosphate. J Am Coll Cardiol 56:919–933
Bonello L, Pansieri M, Mancini J, Bonello R et al (2011) High on-treatment platelet reactivity after prasugrel loading dose and cardiovascular events after percutaneous coronary intervention in acute coronary syndromes. J Am Coll Cardiol 58:467–473
Bouman HJ, Schömig E, van Werkum JW et al (2011a) Paraoxonase-1 and clopidogrel efficacy – reply. Nat Med 17:1042–1044
Bouman HJ, Schömig E, van Werkum JW et al (2011b) Paraoxonase-1 is a major determinant of clopidogrel efficacy. Nat Med 17:110–116
Bouman HJ, Harmsze AM, van Werkum JW et al (2011c) Variability in on-treatment platelet reactivity explained by CYP2C19*2 genotype is modest in clopidogrel pretreated patients undergoing coronary stenting. Heart 97:1239–1244
Brambilla M, Parolari A, Camera M et al (2010) Effect of two doses of aspirin on thromboxane biosynthesis and platelet function in patients undergoing coronary surgery. Thromb Haemost 103:516–524
Brar SS, Ten Berg J, Marcucci R et al (2011) Impact of platelet reactivity on clinical outcomes after percutaneous coronary intervention a collaborative meta-analysis of individual participant data. J Am Coll Cardiol 58:1945–1954
Breet NJ, van Werkum JW, Bouman HJ et al (2010) Comparison of platelet function tests in predicting clinical outcome in patients undergoing coronary stent implantation. JAMA 303:754–762
Campo G, Fileti L, de Cesare N (2010) Long-term clinical outcome based on aspirin and clopidogrel responsiveness status after elective percutaneous coronary intervention: a 3T/2R (tailoring treatment with tirofiban in patients showing resistance to aspirin and/or resistance to clopidogrel) trial substudy. J Am Coll Cardiol 56:1447–1455
Campo G, Parrinello G, Ferraresi P et al (2011) Prospective evaluation of on-clopidogrel platelet reactivity over time in patients treated with percutaneous coronary intervention relationship with gene polymorphisms and clinical outcome. J Am Coll Cardiol 57:2474–2483
Camps J, Joven J, Mackness B et al (2011) Paraoxonase-1 and clopidogrel efficacy. Nat Med 17:1041–1042
Capodanno D, Patel A, Dharmashankar K et al (2011) Pharmacodynamic effects of different aspirin dosing regimens in type 2 diabetes mellitus patients with coronary artery disease. Circ Cardiovasc Interv 4:180–187
Catella-Lawson F, Reilly MP, Kapoor SC et al (2001) Cyclooxygenase inhibitors and the antiplatelet effects of aspirin. N Engl J Med 345:1809–1817
Cattaneo M (2004) Aspirin and clopidogrel. Efficacy, safety and the issue of drug resistance. Arterioscler Thromb Vasc Biol 24:1980–1987
Cattaneo M (2007a) Resistance to antiplatelet drugs: molecular mechanisms and laboratory detection. J Thromb Haemost 5(Suppl 1):230–237
Cattaneo M (2007b) Laboratory detection of 'aspirin resistance': what test should we use (if any)? Eur Heart J 28:1673–1675
Cattaneo M (2009) Letter by cattaneo regarding article, "incomplete inhibition of thromboxane biosynthesis by acetylsalicylic acid: determinants and effect on cardiovascular risk". Circulation 119:e594
Cattaneo M (2010) New P2Y(12) inhibitors. Circulation 121:171–179
Cattaneo M (2011) The platelet $P2Y_{12}$receptor for adenosine diphosphate: congenital and drug-induced defects. Blood 117:2102–2112
Cattaneo M, Hayward CP, Moffat KA et al (2009) Results of a worldwide survey on the assessment of platelet function by light transmission aggregometry: a report from the platelet physiology subcommittee of the SSC of the ISTH. J Thromb Haemost 7:1029
Chen M, Wei JF, Xu YN et al (2011) A meta-analysis of impact of proton pump inhibitors on antiplatelet effect of clopidogrel. Cardiovasc Ther [Epub ahead of print]

Cuisset T, Frere C, Poyet R et al (2010) Clopidogrel response: head-to-head comparison of different platelet assays to identify clopidogrel non responder patients after coronary stenting. Arch Cardiovasc Dis 103:39–45
Cuisset T, Morange PE, Quilici J et al (2011) Paraoxonase-1 and clopidogrel efficacy. Nat Med 17:1039
Dansette PM, Rosi J, Bertho G, Mansuy D (2011) Paraoxonase-1 and clopidogrel efficacy. Nat Med 17:1040–1041
Dragani A, Pascale S, Recchiuti A et al (2010) The contribution of cyclooxygenase-1 and -2 to persistent thromboxane biosynthesis in aspirin-treated essential thrombocythemia: implications for antiplatelet therapy. Blood 115:1054–1061
Duxbury BM, Poller L (2001) The oral anticoagulant saga: past, present, and future. Clin Appl Thromb Hemost 7:269–275
Eikelboom JW, Mehta SR, Anand SS et al (2006) Adverse impact of bleeding on prognosis in patients with acute coronary syndromes. Circulation 114:774–782
Eikelboom JW, Hankey GJ, Thom J et al (2008) Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management and Avoidance (CHARISMA) Investigators. Incomplete inhibition of thromboxane biosynthesis by acetylsalicylic acid: determinants and effect on cardiovascular risk. Circulation 118:1705–1712
Epstein RS, Teagarden JR (2010) Comparative effectiveness research and personalized medicine: catalyzing or colliding? PharmacoEconomics 28:905–913
FDA Drug Safety Communication: reduced effectiveness of Plavix (clopidogrel) in patients who are poor metabolizers of the drug (n.d.) http://www.fda.gov/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/ucm190836.htm. Accessed 3 Aug 2010
Ferreiro JL, Angiolillo DJ (2011) Diabetes and antiplatelet therapy in acute coronary syndrome. Circulation 123:798–813
Fontana P, Berdagué P, Castelli C et al (2010) Clinical predictors of dual aspirin and clopidogrel poor responsiveness in stable cardiovascular patients from the ADRIE study. J Thromb Haemost 8:2614–2623
Fontana P, James R, Barazer I et al (2011) Relationship between paraoxonase-1 activity, its Q192R genetic variant and clopidogrel responsiveness in the ADRIE study. J Thromb Haemost 9:1664–1666
Frelinger AL 3rd, Furman MI, Linden MD et al (2006) Residual arachidonic acid-induced platelet activation via an adenosine diphosphate-dependent but cyclooxygenase-1- and cyclooxygenase-2-independent pathway: a 700-patient study of aspirin resistance. Circulation 113:2888–2896
Frelinger AL 3rd, Li Y, Linden MD et al (2009) Association of cyclooxygenase-1-dependent and -independent platelet function assays with adverse clinical outcomes in aspirin-treated patients presenting for cardiac catheterization. Circulation 120:2586–2596
Gaglia MA, Torguson R, Pakala R et al (2011) Correlation between light transmission aggregometry, VerifyNow P2Y12, and VASP-P platelet reactivity assays following percutaneous coronary intervention. J Interv Cardiol 24(6):529–534
Gilard M, Arnaud B, Le Gal G et al (2006) Influence of omeprazol on the antiplatelet action of clopidogrel associated to aspirin. J Thromb Haemost 4:2508–2509
Gilard M, Arnaud B, Cornily JC et al (2008) Influence of omeprazole on the antiplatelet action of clopidogrel associated with aspirin: the randomized, double-blind OCLA (Omeprazole CLopidogrel Aspirin) study. J Am Coll Cardiol 51:256–260
Goodman T, Sharma P, Ferro A (2007) The genetics of aspirin resistance. Int J Clin Pract 61:826–834
Gremmel T, Steiner S, Seidinger D et al (2009) Comparison of methods to evaluate clopidogrel-mediated platelet inhibition after percutaneous intervention with stent implantation. Thromb Haemost 101:333–339
Gremmel T, Steiner S, Seidinger D et al (2010) The influence of proton pump inhibitors on the antiplatelet potency of clopidogrel evaluated by 5 different platelet function tests. J Cardiovasc Pharmacol 56:532–539

Gurbel PA, Tantry US (2011) An initial experiment with personalized antiplatelet therapy: the GRAVITAS trial. JAMA 305:1136–1137

Gurbel PA, Bliden KP, Butler K et al (2010) Response to ticagrelor in clopidogrel nonresponders and responders and effect of switching therapies: the RESPOND study. Circulation 121:1188–1199

Hayward CP, Moffat KA, Raby A et al (2010) Development of North American consensus guidelines for medical laboratories that perform and interpret platelet function testing using light transmission aggregometry. Am J Clin Pathol 134:955–963

Hennekens CH, Schneider WR, Hebert PR et al (2010) Hypothesis formulation from subgroup analyses: nonadherence or nonsteroidal anti-inflammatory drug use explains the lack of clinical benefit of aspirin on first myocardial infarction attributed to "aspirin resistance". Am Heart J 159:744–748

Henry P, Vermillet A, Boval B et al (2011) 24-hour time-dependent aspirin efficacy in patients with stable coronary artery disease. Thromb Haemost 105:336–344

Ho PM, Maddox TM, Wang L et al (2009) Risk of adverse outcomes associated with concomitant use of clopidogrel and proton pump inhibitors following acute coronary syndrome. JAMA 301:937–944

Hochholzer W, Trenk D, Fromm MF et al (2010) Impact of cytochrome P450 2C19 loss-of-function polymorphism and of major demographic characteristics on residual platelet function after loading an maintenance treatment with clopidogrel in patients undergoing elective coronary stent placement. J Am Coll Cardiol 55:2427–2434

Holmes DR Jr, Dehmer GJ, Kaul S et al (2010) ACCF/AHA clopidogrel clinical alert: approaches to the FDA "boxed warning": a report of the American College of Cardiology Foundation Task Force on clinical expert consensus documents and the American Heart Association endorsed by the Society for Cardiovascular Angiography and Interventions and the Society of Thoracic Surgeons. J Am Coll Cardiol 56:321–341

Holmes MV, Perel P, Shah T et al (2011) *CYP2C19* genotype, clopidogrel metabolism, platelet function, and cardiovascular events. A systematic review and meta-analysis. JAMA 306:2704–2714

Hsiao FY, Mullins CD, Wen YW et al (2011) Relationship between cardiovascular outcomes and proton pump inhibitor use in patients receiving dual antiplatelet therapy after acute coronary syndrome. Pharmacoepidemiol Drug Saf 20:1043–1049

Hulot JS, Collet JP, Silvain J et al (2010) Cardiovascular risk in clopidogrel-treated patients according to cytochrome P450 2C19*2 loss-of-function allele or proton pump inhibitor coadministration: a systematic meta-analysis. J Am Coll Cardiol 56:134–143

Hulot JS, Collet JP, Cayla G et al (2011) CYP2C19 but not PON1 genetic variants influence clopidogrel pharmacokinetics, pharmacodynamics, and clinical efficacy in post-myocardial infarction patients. Circ Cardiovasc Interv 4:422–428

Ioannidis JP, Ntzani EE, Trikalinos TA, Contopoulos-Ioannidis DG (2001) Replication validity of genetic association studies. Nat Genet 29:306–309

Jaitner J, Stegherr J, Morath T et al (2011) Stability of the high on-treatment platelet reactivity phenotype over time in clopidogrel-treated patients. Thromb Haemost 105:107–112

Kozinski M, Bielis L, Wisniewska-Szmyt J et al (2011) Diurnal variation in platelet inhibition by clopidogrel. Platelets 22:579–587

Madsen EH, Saw J, Kristensen SR et al (2010) Long-term aspirin and clopidogrel response evaluated by light transmission aggregometry, VerifyNow, and thrombelastography in patients undergoing percutaneous coronary intervention. Clin Chem 56:839–847

Mega JL, Simon T, Collet JP et al (2010a) Reduced-function CYP2C19 genotype and risk of adverse clinical outcomes among patients treated with clopidogrel predominantly for PCI: a meta-analysis. JAMA 304:1821–1830

Mega JL, Close SL, Wiviott SD et al (2010b) Genetic variants in ABCB1 and CYP2C19 and cardiovascular outcomes after treatment with clopidogrel and prasugrel in the TRITON-TIMI 38 trial: a pharmacogenetic analysis. Lancet 376:1312–1319

Mehran R, Pocock SJ, Nikolsky E et al (2010) A risk score to predict bleeding in patients with acute coronary syndromes. J Am Coll Cardiol 55:2556–2566

Michelson AD (2009) Methods for the measurement of platelet function. Am J Cardiol 103(3 Suppl):20A–26A

Michelson AD, Cattaneo M, Eikelboom JW, Platelet Physiology Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis, Working Group on Aspirin Resistance et al (2005) Aspirin resistance: position paper of the Working Group on Aspirin Resistance. J Thromb Haemost 3:1309–1311

Michelson AD, Frelinger AL 3rd, Braunwald E et al (2009) Pharmacodynamic assessment of platelet inhibition by prasugrel vs. clopidogrel in the TRITON-TIMI 38 trial. Eur Heart J 30:1753–1763

NCI-NHGRI Working Group on Replication in Association Studies, Chanock SJ, Manolio T, Boehnke M et al (2007) Replicating genotype-phenotype associations. Nature 447:655–660

O'Donoghue ML, Braunwald E, Antman EM et al (2009) Pharmacodynamic effect and clinical efficacy of clopidogrel and prasugrel with or without a proton-pump inhibitor: an analysis of two randomised trials. Lancet 374:989–997

Paniccia R, Antonucci E, Gori AM et al (2007) Different methodologies for evaluating the effect of clopidogrel on platelet function in high-risk coronary artery disease patients. J Thromb Haemost 5:1839–1847

Paniccia R, Antonucci E, Maggini N et al (2010) Comparison of methods for monitoring residual platelet reactivity after clopidogrel by point-of-care tests on whole blood in high-risk patients. Thromb Haemost 104:287–292

Paré G, Mehta SR, Yusuf S et al (2010) Effects of CYP2C19 genotype on outcomes of clopidogrel treatment. N Engl J Med 363:1704–1714

Parodi G, Marcucci R, Valenti R et al (2011) High residual platelet reactivity after clopidogrel loading and long-term cardiovascular events among patients with acute coronary syndromes undergoing PCI. JAMA 306:1215–1223

Patrono C, García Rodríguez LA et al (2005) Low-dose aspirin for the prevention of atherothrombosis. N Engl J Med 353:2373–2383

Pena A, Collet JP, Hulot JS et al (2009) Can we override clopidogrel resistance? Circulation 119:2854–2857

Pocock SJ, Mehran R, Clayton TC et al (2010) Prognostic modeling of individual patient risk and mortality impact of ischemic and hemorrhagic complications: assessment from the Acute Catheterization and Urgent Intervention Triage Strategy trial. Circulation 121:43–51

Price MJ, Berger PB, Teirstein PS et al (2011a) Standard- vs high-dose clopidogrel based on platelet function testing after percutaneous coronary intervention: the GRAVITAS randomized trial. JAMA 305:1097–1105

Price MJ, Angiolillo DJ, Teirstein PS et al (2011b) Platelet reactivity and cardiovascular outcomes after percutaneous coronary intervention: a time-dependent analysis of the Gauging Responsiveness with a VerifyNow P2Y12 assay: Impact on Thrombosis and Safety (GRAVITAS) trial. Circulation 124:1132–1137

Pulcinelli FM, Biasucci LM, Riondino S et al (2009) COX-1 sensitivity and thromboxane A2 production in type 1 and type 2 diabetic patients under chronic aspirin treatment. Eur Heart J 30:1279–1286

Rassen JA, Choudhry NK, Avorn J, Schneeweiss S (2009) Cardiovascular outcomes and mortality in patients using clopidogrel with proton pump inhibitors after percutaneous coronary intervention or acute coronary syndrome. Circulation 120:2322–2329

Ray WA, Murray KT, Griffin MR et al (2010) Outcomes with concurrent use of clopidogrel and proton-pump inhibitors: a cohort study. Ann Intern Med 152:337–345

Schwartz KA, Schwartz DE, Ghosheh K et al (2005) Compliance as a critical consideration in patients who appear to be resistant to aspirin after healing of myocardial infarction. Am J Cardiol 95:973–975

Shuldiner AR, O'Connell JR, Bliden KP et al (2009) Association of cytochrome P450 2C19 genotype with the antiplatelet effect and clinical efficacy of clopidogrel therapy. JAMA 302:849–857

Sibbing D, Steinhubl SR, Schulz S et al (2010) Platelet aggregation and its association with stent thrombosis and bleeding in clopidogrel-treated patients: initial evidence of a therapeutic window. J Am Coll Cardiol 56:317–318

Sibbing D, Koch W, Massberg S et al (2011) No association of paraoxonase-1 Q192R genotypes with platelet response to clopidogrel and risk of stent thrombosis after coronary stenting. Eur Heart J 32:1605–1613

Siller-Matula JM, Jilma B, Schrör K et al (2010) Effect of proton pump inhibitors on clinical outcome in patients treated with clopidogrel: a systematic review and meta-analysis. J Thromb Haemost 8:2624–2641

Simon T, Steg PG, Gilard M et al (2011) Clinical events as a function of proton pump inhibitor use, clopidogrel use, and cytochrome P450 2C19 genotype in a large nationwide cohort of acute myocardial infarction: results from the French Registry of Acute ST-Elevation and Non-ST-Elevation Myocardial Infarction (FAST-MI) registry. Circulation 123:474–482

Sofi F, Giusti B, Marcucci R et al (2011) Cytochrome P450 2C19*2 polymorphism and cardiovascular recurrences in patients taking clopidogrel: a meta-analysis. Pharmacogenomics J 11:199–206

Spectre G, Arnetz L, Östenson CG et al (2011) Twice daily dosing of aspirin improves platelet inhibition in whole blood in patients with type 2 diabetes mellitus and micro- or macrovascular complications. Thromb Haemost 106:491–499

Task Force on Myocardial Revascularization of the European Society of Cardiology (ESC) and the European Association for Cardio-Thoracic Surgery (EACTS), European Association for Percutaneous Cardiovascular Interventions (EAPCI), Wijns W, Kolh P, Danchin N et al (2010) Guidelines on myocardial revascularization. Eur Heart J 31:2501–2555

Tofler GH, Brezinski D, Schafer AI et al (1987) Concurrent morning increase in platelet aggregability and the risk of myocardial infarction and sudden cardiac death. N Engl J Med 316:1514–1518

Trenk D, Hochholzer W, Fromm MF et al (2011) Paraoxonase-1 Q192R polymorphism and antiplatelet effects of clopidogrel in patients undergoing elective coronary stent placement. Circ Cardiovasc Genet 4:429–436

von Beckerath N, Sibbing D, Jawansky S et al (2010) Assessment of platelet response to clopidogrel with multiple electrode aggregometry, the VerifyNow P2Y12 analyzer and platelet Vasodilator-Stimulated Phosphoprotein flow cytometry. Blood Coagul Fibrinolysis 21:46–52

Zabalza M, Subirana I, Sala J et al (2011) Meta-analyses of the association between cytochrome CYP2C19 loss- and gain-of-function polymorphisms and cardiovascular outcomes in patients with coronary artery disease treated with clopidogrel. Heart 98:100–108

Antiplatelet Agents in Ischemic Heart Disease

Christopher H. May and A. Michael Lincoff

Contents

C.H. May • A.M. Lincoff (✉)
Department of Cardiovascular Medicine, Cleveland Clinic, The Cleveland Clinic Foundation, 9500 Euclid Avenue – J2-3, Cleveland, OH 44195, USA
e-mail: lincofa@ccf.org

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_20,

Keywords ADP inhibitors • GP IIb/IIIa inhibitors • Aspirin • Ischemic heart disease • Antiplatelet agents

Antiplatelet agents comprise a critical component in the multi-modality treatment of ischemic heart disease. In addition to anticholesterol, beta-blocking, and angiotensin-converting enzyme inhibiting agents as well as mechanical intervention, platelet inhibition has resulted in improved outcomes for patients with ischemic heart disease. Platelet aggregation and resultant thrombus formation is a multi-step process with several points of potential intervention. As this pathway has been elucidated, more opportunities to intervene pharmacologically have become apparent.

The antiplatelet agent acetylsalicylic acid (ASA) has long been used medicinally; however, only within the last few decades has it been shown to be an effective treatment for patients with coronary artery disease. More recently, glycoprotein IIb/IIIa inhibitors were investigated and found to be useful in inhibiting platelet aggregation. Likewise, the $P2Y_{12}$ blockers, which are ADP receptor antagonists, have also proven beneficial in this patient population. The focus of this chapter will be to briefly review the characteristics of the various classes as well as explore these agents in context of the major trials which have supported their use in patients with ischemic heart disease, including the acute setting such as unstable angina (UA), non-ST-elevation myocardial infarction (NSTEMI), and ST-elevation myocardial infarction (STEMI), as well as in patients with chronic ischemic heart disease.

1 Introduction

1.1 Acetylsalicylic Acid

ASA, also called aspirin, is one of the most commonly used medicines in the world. Today, there is a large body of evidence supporting its use in patients with ischemic heart disease, and it is indeed a cornerstone of treatment in this patient population. Aspirin exerts its effect by irreversibly inhibiting the enzyme cyclooxygenase-1 (COX-1) thus preventing the formation of thromboxane A_2, a promoter of platelet aggregation and a potent vasoconstrictor (see Patrono and Rocca 2012). The most common adverse effect seen with aspirin is gastrointestinal toxicity including irritation and occult blood loss. Intracranial hemorrhage is a more serious but very rare complication of aspirin use. In some of the major trials investigating aspirin use, other major bleeding rates increase with an increasing aspirin dose. Rarely, some patients may develop an aspirin hypersensitivity which can manifest in variety of ways including airway obstruction and hyper-responsiveness to urticaria, angioedema, and anaphylactic shock.

1.2 $P2Y_{12}$ Blockers

Another target for inhibiting platelet activation is the adenosine diphosphate (ADP) receptor located on the surface of platelets. When platelets are stimulated, ADP is released which binds to ADP receptors, promoting platelet aggregation (see Bernlochner and Sibbing 2012). There are three ADP receptor inhibitors which have been routinely used in clinical practice—ticlopidine, clopidogrel, and prasugrel. These agents bind irreversibly to the ADP receptor and thus mitigate thrombus formation. Ticagrelor, a novel agent which was recently approved for use, is a non-thienopyridine, reversible inhibitor of this receptor. Their effectiveness in ischemic heart disease will be explored below. The most common complication of this class of medications includes bleeding. Rare but significant complications also include hematologic abnormalities such as neutropenia and thrombotic thrombocytopenic purpura. In current practice, clopidogrel and prasugrel have supplanted the use of ticlopidine given its relatively increased risks of precipitating hematologic abnormalities.

1.3 Glycoprotein IIb/IIIa Inhibitors

With the discovery of the glycoprotein IIb/IIIa receptor in the early 1980s, a new target for preventing platelet aggregation was realized. During platelet activation, this receptor undergoes structural changes, increasing its affinity for fibrinogen thus facilitating platelet aggregation by cross-linking to other IIb/IIIa receptors on neighboring platelets (see Hook and Bennett 2012). Blocking this receptor inhibits this process, a major contributor to the ischemic complications of coronary artery disease. Three GP IIb/IIIa receptor inhibitors have been approved for use in this patient population—abciximab, eptifibatide, and tirofiban. As with other anticoagulants, the major complication from using these agents is bleeding. As experience with these agents has increased, bleeding complications have been mitigated by using various strategies including using smaller sheaths for vascular access, early removal of the sheath, and weight-adjusted heparin dosing. In addition, clinically significant thrombocytopenia is a consideration when using these agents, particularly abciximab.

2 Antiplatelet Agents in Chronic Ischemic Heart Disease and Elective Coronary Revascularization

Antiplatelet agents are a crucial component in the therapy of patients with chronic heart disease. These agents have proven efficacious in patients with known coronary artery disease regardless if they have suffered an acute coronary syndrome

(ACS). In addition, for patients undergoing elective coronary revascularization, these agents decrease the risk of periprocedural myocardial infarction as well as reduce short and longer term mortality. This section will explore the use of antiplatelet medications in the setting of chronic ischemic heart disease and in elective coronary revascularization.

2.1 Aspirin

The issue of aspirin therapy for primary prevention in patients without known ischemic heart disease will be discussed in a subsequent chapter. The use of aspirin indefinitely in patients who have experienced an ACS will be explored here. Aspirin also plays a significant role in improving outcomes in patients with chronic ischemic heart disease but who have not experienced a myocardial infarction. The Swedish angina pectoris aspirin trial (SAPAT) randomized 2,035 patients with stable angina and no history of myocardial infarction to aspirin 75 mg daily versus placebo (Juul-Moller et al. 1992). The primary outcome of myocardial infarction and sudden death was decreased in the aspirin group compared to placebo (4.0% vs. 6.1%, RRR of 34%, NNT of 48, $p = 0.003$). These data were combined with six other trials for a combined patient population of 2,920 by the Antithrombotic Trialists' Collaboration to explore the use of antiplatelet therapy in patients with stable angina. Of those in the treatment group, there was a 33% odds reduction of major vascular events when compared to controls (9.9% vs. 14.1%) (Antithrombotic Trialists' Collaboration 2002). Thus, aspirin is an important component of treating patients with ischemic heart disease who have not suffered a myocardial infarction. The optimal dosing of aspirin in these patients for efficacy and avoidance of bleeding is 75–81 mg daily (Campbell et al. 2007). With regards to patients who are undergoing elective PCI, aspirin has been shown to significantly decrease periprocedural myocardial infarctions and thus can be recommended in this setting as well (Schwartz et al. 1988).

2.2 $P2Y_{12}$ Blockers

Clopidogrel is the ADP antagonist which has been most studied in patients with chronic ischemic heart disease. The Clopidogrel versus Aspirin in Patients at Risk of Ischemic Events (CAPRIE) trial, a randomized controlled trial of 19,185 patients that compared clopidogrel to aspirin, was the first study which investigated clopidogrel's use as an antiplatelet agent in this patient population (CAPRIE Steering Committee 1996). CAPRIE enrolled patients with recent myocardial infarction, ischemic stroke, or symptomatic peripheral artery disease and randomized them to clopidogrel 75 mg daily versus aspirin 325 mg daily. The primary outcome was cardiovascular death, myocardial infarction, or ischemic stroke. Those patients in the clopidogrel group had a significantly lower annual

risk of 5.32% of meeting this endpoint compared with 5.83% in the aspirin group (RRR of 8.7%, $p = 0.043$). This suggests that clopidogrel is at least as effective as aspirin in monotherapy and may be used, for example, in patients who are intolerant to aspirin. More compelling data exist, however, showing benefit of dual antiplatelet therapy with both aspirin and clopidogrel relative to aspirin alone in multiple settings of patients with coronary artery disease. The Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance (CHARISMA) trial addressed the role of dual antiplatelet therapy in patients with stable ischemic heart disease (Bhatt et al. 2006). This randomized controlled trial enrolled 15,603 patients with cardiovascular disease (documented coronary artery disease, cerebrovascular disease, or symptomatic peripheral artery disease) or multiple risk factors to clopidogrel 75 mg daily plus low dose aspirin (75–162 mg daily) versus placebo and low dose aspirin. Patients were followed for a median of 2.25 years and the primary endpoint was a composite of cardiovascular death, myocardial infarction, or stroke. There was no significant difference in meeting the primary endpoint between the two treatment groups (6.8% in experimental group vs. 7.3% in controls, $p = 0.22$). However, a prespecified subgroup analysis of patients with cardiovascular disease which consisted of 12,531 patients out of the total population suggested that they benefited with regards to the primary outcome from dual antiplatelet therapy (6.9% clopidogrel vs. 7.9% with placebo, relative risk 0.88, $p = 0.046$) while those in the multiple risk factor group (3,284 patients) did not. A subsequent analysis of the CHARISMA data which looked exclusively at higher risk patients with history of prior myocardial infarction, history of prior ischemic stroke, or symptomatic peripheral artery disease, indicated a significant reduction in the primary endpoint in patients on clopidogrel (7.3% vs. 8.8%, $p = 0.01$) in addition to a significant reduction in the secondary endpoint (11.4% vs. 13.2%, $p = 0.008$) (Bhatt et al. 2007). As in the overall trial, there was a statistically significant increase in moderate bleeding but no significant increase in fatal bleeding or intracranial hemorrhage. Taken together, these studies suggest a benefit of dual platelet therapy in higher risk patients; however, there is no clear evidence to add clopidogrel to patients with stable cardiovascular disease. More studies are needed to refine the exact patient population that would benefit most from dual antiplatelet therapy in patients with chronic ischemic heart disease.

2.3 Glycoprotein IIb/IIIa Inhibitors

Given the efficacy of intravenous GP IIb/IIIa inhibitors in the setting of ACS and percutaneous intervention (PCI), it would be reasonable to surmise a role for oral agents in patients with chronic heart disease. However, multiple trials involving a wide array of patient populations (elective PCI, presenting with ACS, use in secondary prevention) have shown oral GP IIb/IIIa inhibitors to be associated with increased mortality; thus, there is no clinical application for these agents (Topol et al. 2003; Chew et al. 2001).

To the contrary, intravenous GP IIb/IIIa inhibitors have proven efficacious in the setting of patients undergoing elective PCI. This was first demonstrated by the Evaluation of 7E3 for the Prevention of Ischemic Complications (EPIC) investigators where 2,099 high risk patients (patients with acute myocardial infarction, unstable angina, or high risk anatomy) scheduled for coronary angioplasty or atherectomy were randomized to placebo, abciximab bolus, or abciximab bolus plus a 12 h infusion (Anonymous 1994). Ischemic complications (death, myocardial infarction, or urgent revascularization) were reduced by abciximab bolus plus infusion (12.8% vs. 8.3%) but not by bolus only dosing. The benefit of abciximab was also demonstrated by the Evaluation in PTCA to Improve Long-Term Outcome with Abciximab GP IIb/IIIa Blockade (EPILOG) trial with 2,792 patients undergoing PCI randomized to abciximab with standard-dose heparin, abciximab with low-dose heparin, and placebo with standard-dose heparin (EPILOG Investigators 1997). In these lower risk patients (patients with ACS were excluded) abciximab with low-dose, weight-adjusted heparin markedly improve cardiovascular outcomes while reducing bleeding events. Likewise, the Evaluation of Platelet IIb/IIIa Inhibitor for Stenting (EPISTENT) trial confirmed abciximab's beneficial effect in those patients undergoing stenting procedures with a 50% reduction in ischemic complications in those patients receiving abciximab and stenting versus those who underwent stenting with placebo (EPISTENT Investigators 1998). Similar findings were also found by the Enhanced Suppression of the Platelet IIb/IIIa Receptor with Integrilin Therapy (ESPRIT) investigators who showed that pretreatment with eptifibatide prior to coronary stenting significantly reduces ischemic complications at 48 h and 30 days by 35% (ESPRIT Investigators 2000).

Taken in totality, glycoprotein IIb/IIIa inhibitors played an important role in the reduction of ischemic events and likely long term mortality in the era prior to pretreatment with clopidogrel before elective stenting. Indeed, a study which combined the results of the abciximab trials showed a 25–30% reduction in long term mortality in patients who received abciximab while undergoing PCI (Anderson et al. 2001). However, in the modern era with clopidogrel pretreatment prior to elective PCI, the data do not support the use of abciximab in low to intermediate risk patients. This was the focus of the Intracoronary Stenting and Antithrombotic Regimen-Rapid Early Action for Coronary Treatment (ISAR-REACT), a 2,159 patient trial that randomized low to intermediate risk patients undergoing PCI to either abciximab or placebo with all patients receiving 600 mg of clopidogrel at least 2 h prior to the procedure (Kastrati et al. 2004). There was no difference in ischemic outcomes between these two groups at 30 days, suggesting abciximab was of little additional benefit in this patient group. Likewise, the Randomized Evaluation in PCI Linking Angiomax to Reduced Clinical Events (REPLACE-2) trial randomized 6,010 patients undergoing elective or urgent PCI to either monotherapy with bivalirudin or heparin and a GP IIb/IIIa inhibitor (either abciximab or eptifibatide) (Lincoff et al. 2003). All patients received clopidogrel; over half received it more than 2 h prior to the procedure. At 30 days, bivalirudin treatment was found to be noninferior to heparin plus GP IIb/IIIa inhibition in the prevention of acute ischemic endpoints; moreover, the patients in the bivalirudin arm had significantly lower rates of bleeding (2.4% vs. 4.1%, $p < 0.001$). On

follow-up, there was no difference in clinical outcomes between the two treatment regimens at 6 months and 1 year. Thus in contemporary practice, there is no apparent benefit of adding a GP IIb/IIIa inhibitor to the therapy of patients who are undergoing elective PCI.

Of note, there is little comparative data between the various GP IIb/IIIa inhibitors. The Do Tirofiban and ReoPro Give Similar Efficacy (TARGET) trial showed that patients undergoing PCI (either electively or for UA or NSTEMI but not STEMI) had better outcomes with abciximab compared to tirofiban (Topol et al. 2001). This advantage of abciximab was significant for the patients who presented with ACS, but there was no difference between the two agents when used in patients undergoing stenting for other reasons. There is a suggestion that the doses of tirofiban used in this trial were too low which could account for the difference noted.

3 Antiplatelet Agents in Unstable Angina and Non-ST-Segment Elevation Myocardial Infarction

Antiplatelet agents play a key role in the treatment of patients with ACS. In this section, the use of ASA, GP IIb/IIIa inhibitors, and $P2Y_{12}$ blockers and their complementary roles in platelet inhibition will be explored in the context of patients presenting with unstable angina (UA) or non-ST segment elevation myocardial infarction (NSTEMI).

3.1 Aspirin

The role of aspirin in patients with UA or NSTEMI has been assessed in four randomized, controlled trials, enrolling a combined 3096 patients (Lewis et al. 1983; Cairns et al. 1985; Theroux et al. 1988; The RISC Group 1990). These studies revealed a consistent decrease in mortality and nonfatal myocardial infarction in patients who received aspirin in both the short-term and longer term. Taken together, these studies showed a 50% reduction in the rate of vascular events with a NNT of 17 in patients treated with aspirin (Bassand et al. 2007). In addition, a large meta-analysis performed by the Antithrombotic Trialists' Collaboration involving over 135,600 patients explored the effects of antiplatelet therapy, the majority of which was aspirin, among patients at high risk of vascular events (Antithrombotic Trialists' Collaboration 2002). In the subset of patients with unstable angina, antiplatelet therapy produced a 46% reduction in cardiovascular death, myocardial infarction, and stroke. Among all high risk patients evaluated in this analysis, antiplatelet therapy reduced these major vascular events by about 25%. Among the patients with acute myocardial infarction, antiplatelet therapy allowed for 38 fewer events per 1,000 patients treated for 1 month. This analysis also suggested a long-term daily dose of 75–150 mg was equally effective as higher doses. The initial dose of aspirin in patients presenting with UA or NSTEMI should

be 162–325 mg; it should be chewed and administered as soon as feasible. Patients should subsequently be maintained indefinitely on a 75–150 mg daily dose.

3.2 *P2Y$_{12}$ Blockers*

There are four P2Y$_{12}$ blockers which have been used to treat patients with UA or NSTEMI—ticlopidine, clopidogrel, prasugrel, and ticagrelor. Ticlopidine has a relatively increased risk of precipitating neutropenia or thrombotic thrombocytopenic purpura and is thus of historical interest only since its use has largely been supplanted by the other agents with their improved hematologic safety profile and more rapid onset of action.

Clopidogrel's efficacy in the setting of non-ST elevation ACS was evaluated in the Clopidogrel in Unstable Angina to Prevent Recurrent Events trial (CURE), a randomized controlled trial of 12,562 patients (Yusuf et al. 2001). In CURE, patients treated with aspirin and clopidogrel (300 mg loading dose, plus 75 mg daily) had improved outcomes compared to patients receiving aspirin and placebo. There was a relative risk reduction of 18.4% in the clopidogrel group compared to placebo at 12 months for the primary endpoint of cardiovascular death, myocardial infarction, or stroke. In addition, clopidogrel's benefit occurred early with a 34% risk reduction of cardiovascular death, myocardial infarction, severe ischemia, or stroke at 24 h. Of note, there was a significant increase in major bleeding associated with clopidogrel use; however, there was not a significant increase in life threatening bleeding.

In patients that proceed to PCI, clopidogrel also provides a long-term benefit in combination with aspirin. The Clopidogrel for Reduction of Events During Observation (CREDO) trial showed that patients who had a loading dose of 300 mg prior to PCI followed by 75 mg for 1 year had improved outcomes when compared to those who only took 75 mg daily for four weeks (with no loading dose and no long-term therapy) (Steinhubl et al. 2002). Only bare mental stents were used for stenting. At 1 year, patients in the long-term clopidogrel arm had a 26.9% relative risk reduction in a combined endpoint of death, myocardial infarction, or stroke ($p = 0.02$). In addition, the investigators noted that in a prespecified subset of patients that received clopidogrel at least 6 h prior to PCI had a 38.6% relative risk reduction ($p = 0.051$) for this same endpoint at 28 days but no reduction if the treatment was less than 6 h prior to PCI. While these data are not conclusive, they do suggest a benefit with clopidogrel loading at least 6 h prior to PCI. A subanalysis of the aforementioned CURE trial also indicated similar benefit to clopidogrel use in patients undergoing PCI (Mehta et al. 2001).

The question of optimal dosing of clopidogrel in patients with ACS referred for an invasive strategy was addressed by the CURRENT-OASIS 7 trial, a randomized controlled trial involving 25,086 patients (Mehta et al. 2010). The experimental group received a 600 mg loading dose, followed by 150 mg daily for six days, followed by 75 mg daily. The control group received a 300 mg loading dose

followed by the conventional maintenance dose of 75 mg daily. The composite primary endpoint of cardiovascular death, myocardial infarction, or stroke at 30 days was not significantly reduced by high-dose clopidogrel in the overall trial population. However, in those patients actually undergoing PCI (69% of the study population), those patients who received the higher dose had a significantly reduced rate of the primary outcome (3.9% vs. 4.5%), although the authors note that this could be due to chance given it was one of the multiple subgroup analyses performed. Moreover, high dose clopidogrel provided a significant reduction in the secondary outcome of stent thrombosis among those who underwent PCI (1.6% vs. 2.3%, a relative risk reduction of 30%). These data taken together with those from much smaller trials including ALBION (Montalescot et al. 2006), ARMYDA-2 (Patti et al. 2005), and ISAR-CHOICE (von Beckerath et al. 2005) suggest an optimal loading dose of 600 mg. After loading, it is reasonable for ACS patients who undergo PCI to receive clopidogrel 150 mg daily for six days, provided they are not at high risk for bleeding, which can be then followed by maintenance therapy of 75 mg daily. Ideally, clopidogrel should be administered at least 6 h prior to PCI to allow for optimal platelet inhibition.

Prasugrel is a novel thienopyridine with a more rapid onset of action and more platelet inhibition when compared to clopidogrel. The Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel (TRITON-TIMI 38) was thus performed to compare prasugrel to clopidogrel in patients with ACS who proceeded to PCI (Wiviott et al. 2007). In this 13,608 patient trial (10,074 with UA or NSTEMI), the combined endpoint of cardiovascular death, myocardial infarction, or stroke was reduced in patients treated with prasugrel compared to those with clopidogrel (9.9% vs 12.1%, RRR of 18.1 with NNT of 46. $p < 0.001$). There were significant reductions in the individual endpoints of myocardial infarction, urgent revascularization, and stent thrombosis in patients treated with prasugrel. However, there was a significant increase in both nonfatal and fatal bleeding in the prasugrel group, particularly in patients with a history of stroke or transient ischemic attack (TIA). Although overall mortality was unchanged between the two experimental groups, the study was not powered to detect a difference. Thus prasugrel may play an important role in patients at high risk for ischemic events (such as patients with diabetes), although its use should be avoided in patients at higher risk of bleeding (particularly elderly patients, those with history of stroke or TIA, or those with low body weight). In addition, prasugrel may be an effective alternative for patients who demonstrate clopidogrel resistance. Further studies will need to be performed to better delineate its optimal role in patients with ischemic heart disease.

Ticagrelor, unlike the thienopyridines, is a reversible inhibitor of the ADP receptor. It has a more rapid onset and higher degree of platelet inhibition when compared to clopidogrel. It was compared to clopidogrel in the multicenter, randomized Platelet inhibition and patient Outcomes (PLATO) trial (Wallentin et al. 2009). The trial enrolled 18,624 patients with ACS and randomized them to ticagrelor (180 mg loading dose followed by 90 mg twice daily) or clopidogrel (300–600 mg loading dose followed by 75 mg daily). The primary endpoint of

cardiovascular death, myocardial infarction, or stroke by 12 months occurred in 9.8% of patients treated with ticagrelor compared to 11.7% of patients in the clopidogrel arm (RRR of 16% in favor of ticagrelor, $p < 0.001$). Of note, patients with UA or NSTEMI made up 60% of the patient population with a relatively equal distribution between treatment and control groups. Although there was no significant difference between the two treatment groups in major or fatal bleeding, patients in the ticagrelor arm experienced increased major bleeding not due to coronary artery bypass grafting (4.5% vs. 3.8%, $p = 0.03$). In contrast to prasugrel in the TRITON-TIMI 38 trial, ticagrelor in PLATO was associated with significant reduction in vascular mortality (4.0 vs. 5.1%, $p < 0.001$), suggesting that the lack of incremental bleeding risk allowed late advantages of reduced ischemic events to become manifest.

In summary, ADP inhibitors are an important component in the treatment of patients with UA or NSTEMI; however, the ideal setting and patient population for each agent needs to be further characterized. Additional studies such as the ongoing Targeted Platelet Inhibition to Clarify the Optimal Strategy to Medically Manage Acute Coronary Syndromes (TRILOGY ACS) study, a 10,000 patient study which compares prasugrel and aspirin to clopidogrel and aspirin in NSTEMI patients who are medically managed, will be helpful in clarifying each drug's role (Chin et al. 2010).

3.3 Glycoprotein IIb/IIIa Inhibitors

The efficacy of GP IIb/IIIa inhibitors was first exhibited in the aforementioned EPIC trial where the patient population consisted of patients with ACS as well as high risk elective PCI. Subsequently, the GP IIb/IIIa inhibitors, including tirofiban, eptifibatide, lamifiban, and abciximab, were examined in six randomized trials in patients with UA and NSTEMI (Anonymous 1998a, b, c, d; Simoons et al. 2001; Global Organization Network (PARAGON)-B Investigators 2002). Of note, no trial required patients to undergo revascularization with several of them actively discouraging PCI. In general, acute events were reduced among patients receiving these agents; abciximab was not associated with improved outcomes in the trial which explored its use, possibly due to inconsistent levels of platelet inhibition on long-term infusion and because patients rarely proceeded to PCI. A meta-analysis combining the results of these six trials showed that death or myocardial infarction was decreased at 30 days with the use of glycoprotein IIb/IIIa inhibitors (10.8% vs. 11.8%; OR of 0.91, $p = 0.015$) (Boersma et al. 2002). Of note, these trials occurred prior to routine use of ADP inhibition.

More recently, trials have evaluated the role of GP IIb/IIIa blockade in contemporary practice with invasive strategies and thienopyridine treatment among patients with UA/NSTEMI. The Intracoronary Stenting and Antithrombotic Regimen: Rapid Early Action for Coronary Treatment 2 (ISAR-REACT 2) trial randomized patients presenting with UA/NSTEMI to abciximab or placebo at the time of PCI (Kastrati

et al. 2006). All patients were pretreated with aspirin and clopidogrel 600 mg at least 2 h prior to coronary revascularization. The composite endpoint of death, myocardial infarction, or urgent target vessel revascularization was significantly reduced at 30 days in the abciximab group (8.9% vs. 11.9%, RRR of 25%, $p = 0.03$). Notably, this finding contrasted with that of the preceding ISAR-REACT trial where benefit was not observed with abciximab among stable patients undergoing elective PCI, suggesting that routine GP IIb/IIIa inhibition should be reserved for high risk patients with unstable ischemic syndromes. However, the Acute Catheterization and Urgent Intervention Triage Strategy (ACUITY) trial provided evidence that patients treated with bivalirudin (with provisional GP IIb/IIIa inhibitor in approximately 9% of patients) rather than heparin with routine use of GP IIb/IIIa blockade may experience a similar benefit with regards to ischemic events yet have significantly less major bleeding (Stone et al. 2006). Therefore, in today's practice with thienopyridine pretreatment, bivalirudin may be the preferred agent given its improved safety profile while providing a similar reduction in thrombotic adverse events.

Two trials have investigated the question of optimal timing of initiating GP IIb/IIIa blockade. The ACUITY timing trial consisted of 9,207 patients presenting with UA or NSTEMI randomized to receive upstream (at the time of hospital admission) or deferred (at the time of PCI) GP IIb/IIIa inhibitor (Stone et al. 2007). There was no difference in the composite endpoint of death, myocardial infarction, or unplanned revascularization at 30 days between the two groups. In the Early Glycoprotein IIb/IIIa Inhibition in Non-ST Segment Elevation Acute Coronary Syndrome (EARLY ACS), 9,492 patients were randomized to early initiation of eptifibatide versus delayed, selective administration (Giugliano et al. 2009). The early strategy was not superior to selective use of eptifibatide after angiography; in fact, only 39% of patients in the delayed arm even received eptifibatide. In both of these trials bleeding events were increased for the early administration groups. Thus taken together, these trials show no benefit of routinely using a GP IIb/IIIa inhibitor upfront in patients treated with both aspirin and an ADP inhibitor. GP IIb/IIIa inhibitors can therefore be reserved for patients with refractory symptoms or those perceived to be at high risk (pronounced ST-segment depression, diabetes, or significantly elevated troponin levels). Similarly, patients who are selected for a noninvasive approach should be treated with aspirin and clopidogrel with GP IIb/IIIa inhibition considered in those patients who have refractory symptoms on these therapies and subsequently proceed to diagnostic angiography.

4 Antiplatelet Agents in ST Elevation Myocardial Infarction

Antiplatelet agents are a mainstay in the treatment of patients who present with STEMI. In addition to timely mechanical reperfusion, antiplatelet therapies play a critical role in optimizing patient outcomes. Below, we will review the major clinical trials supporting their use in patients who present with STEMI.

4.1 Aspirin

The Second International Study of Infarct Survival (ISIS-2) group provided the original trial that showed a clear benefit of aspirin therapy in patients with acute myocardial infarction (Anonymous 1988). This study examined the use of aspirin, streptokinase, both agents, or neither in 17,187 patients who presented with ACS, the majority of whom had ST elevation. Aspirin at 160 mg daily for 30 days decreased mortality at five weeks by 23% ($2p < 0.00001$); a 1 h infusion of streptokinase had a similar effect with vascular mortality decreased by 25%. The two agents together had an additive effect, reducing vascular death by 42% in those patients who received both (8.0% in experimental vs. 13.2% in placebo, NNT 20, $2p < 0.0001$). Thus the importance of aspirin in patients with STEMI was established, becoming a mainstay of therapy in this patient group. These data from ISIS-2 were combined with other trials in the large meta-analysis performed by the Antithrombotic Trialists' Collaboration. This group showed that in the subgroup of patients presenting with suspected acute myocardial infarction (19,288 patients from 15 trials), those who received antiplatelet therapy for 1 month had 38 fewer major vascular events (cardiovascular death, nonfatal myocardial infarction, or stroke) per 1,000 patients (Antithrombotic Trialists' Collaboration 2002). More specifically, there were 23 fewer cardiovascular deaths per 1,000 treated patients ($p < 0.0001$), 13 fewer nonfatal myocardial infarctions per 1,000 treated patients ($p < 0.0001$), and two fewer strokes ($p < 0.02$). This benefit far exceeded the risk of major bleeding events (estimated at 1–2 per 1,000 treated patients). In this same meta-analysis, patients with a history of myocardial infarction who were allocated to antiplatelet therapy for a mean duration of 27 months experienced 36 fewer major vascular events per 1,000 treated patients (14 fewer cardiovascular deaths ($p = 0.0006$), 18 fewer nonfatal myocardial infarctions ($p < 0.0001$), and five fewer strokes ($p = 0.002$)).

In the modern era, patients routinely progress to cardiac catheterization to receive PCI and stent implantation. Current guidelines recommend the use of 162–325 mg of aspirin for 1 month in patients who receive bare mental stents, 3 months in those who receive a sirolimus eluting stent, and 6 months for those who receive a paclitaxel eluting stent; patients should subsequently take 75–162 mg daily indefinitely. However, more recent data from the CURRENT-OASIS 7 trial described previously suggest a lower aspirin dose is acceptable as there was no significant difference in cardiovascular death, myocardial infarction, or stroke between the high dose (300–325 mg daily) and low dose (75–100 mg daily) groups at 30 days (Mehta et al. 2010). Approximately 58% of the patients in both the high and low dose groups received bare metal stents while 42% received a drug eluting stent. In addition, there was no significant difference between these two groups with regards to major bleeding events. There was, however, a significant difference in minor bleeding with 5.0% of patients in the high dose experiencing minor bleeding compared to 4.3% in the low dose group ($p = 0.04$) as well as a small increase in gastrointestinal bleeding in the high dose group (0.4% vs. 0.2%, $p = 0.04$). Thus, in patients considered to be at high risk for bleeding, a lower dose of aspirin after PCI would be reasonable.

4.2 $P2Y_{12}$ Blockers

In patients presenting with STEMI, clopidogrel and prasugrel are the contemporary ADP inhibitors employed in their treatment. Both agents produce additive benefits when used in combination with aspirin and play an important role in platelet inhibition in the acute setting as well as long-term by preventing stent thrombosis for those patients that proceed to PCI. The use of clopidogrel in this patient group is well established while the role of the novel agent prasugrel has been defined by one large trial among patients treated with invasive management.

Clopidogrel's effectiveness in patients with STEMI has been explored in two randomized controlled trials. The Clopidogrel as Adjunctive Reperfusion Therapy (CLARITY-TIMI 28) trial consisted of 3,491 patients who presented within 12 h of presentation with STEMI (Sabatine et al. 2005). Patients were randomized to clopidogrel (300 mg loading dose followed by 75 mg daily) or placebo; all patients received aspirin, a fibrinolytic agent, and heparin or low molecular weight heparin (in patients who received a fibrin-specific lytic, which was 69% of the total population). Patients were scheduled to undergo angiography 2–8 days after starting the trial medication. The primary endpoint was a combined endpoint consisting of occluded infarct-related artery on angiography, death, or recurrent myocardial infarction prior to angiography. The addition of clopidogrel reduced the composite endpoint from 21.7% in the control group to 15.0% in the experimental group (RRR of 30.9%, NNN = 15, $p < 0.001$). Of note, benefit was driven primarily by a reduction in infarct-related artery occlusion (18.4% in placebo vs. 11.7% with clopidogrel, $p = < 0.001$) and less so by recurrent myocardial infarction (3.6% in placebo vs. 2.5% with clopidogrel, $p = 0.08$). There was no significant increased risk of bleeding in those patients who were treated with clopidogrel when compared to placebo. Additionally, clopidogrel significantly reduced cardiovascular death, recurrent myocardial infarction, and need for urgent revascularization at 30 days when compared to placebo (RRR of 17.7%, $p = 0.03$).

Although CLARITY-TIMI 28 did not specifically reveal a mortality benefit with clopidogrel in patients with STEMI, the Clopidogrel Metoprolol Myocardial Infarction (COMMIT) trial did show an all-cause mortality benefit (Chen et al. 2005). COMMIT was a much larger randomized control trial that consisted of 45,852 patients presenting within 24 h of ACS (42,683 with STEMI or LBBB) that were randomized to clopidogrel 75 mg daily vs. placebo. All patients received aspirin 162 mg daily. Other therapies including fibrinolysis (54% of patients), anticoagulation primarily with heparin (75% of patients), and PCI (<5%) were at the discretion of the treating physician. Treatment continued until discharge, death, or 28 days of hospitalization. All-cause mortality, one of the primary outcomes, was significantly reduced in the clopidogrel arm compared to placebo (7.5% vs 8.1%, RRR of 7.4%, $p = 0.03$). A composite of death, reinfarction, or stroke was also significantly reduced in patients on clopidogrel (9.2% vs. 10.1%, RRR of 8.9%, $p = 0.002$). There was no significant difference in major bleeding rates in patients on clopidogrel when compared to controls, even in those of advanced age or in

patients who received fibrinolytics. When considering these two trials together, there is an apparent benefit to adding clopidogrel to the treatment of patients who present with STEMI and no evidence of a worsening safety profile with respect to major bleeding, even in the setting of fibrinolytic use. Given the brief treatment duration in both of these trials, these data do not provide evidence to support the long-term use of clopidogrel in this specific setting; however, benefit in the long-term can be inferred from data from longer term trials such as CURE. There remains some uncertainty regarding the safety of a loading dose of clopidogrel in elderly patients receiving fibrinolysis. In CLARITY, a loading dose was used, but patients over 75 years of age were excluded from enrollment. In COMMIT, age was not an exclusion criteria; however, no loading dose was administered. Thus, current guidelines do not recommend a loading dose of clopidogrel for patients over 75 years of age who receive fibrinolytic therapy.

A subanalysis of the Harmonizing Outcomes With Revascularization and Stents in Acute Myocardial Infarction (HORIZONS-AMI) trial suggested that patients with STEMI treated with primary PCI may have improved outcomes with a 600 mg loading dose of clopidogrel compared with 300 mg although this comparison was nonrandomized and is thus considered "hypothesis-generating" (Dangas et al. 2009). Specifically, the higher loading dose was associated with lower 30 day mortality, recurrent myocardial infarction, and definite or probable stent thrombosis. There was no difference in bleeding rates between the two groups.

As described previously, prasugrel was studied in TRITON-TIMI 38, a trial of over 13,600 patients comparing clopidogrel to prasugrel in patients with ACS who undergo PCI. A subgroup analysis consisting of the 3,534 patients with STEMI demonstrated that those randomized to prasugrel (60 mg loading dose followed by 10 mg daily) had a significantly reduced risk of meeting the combined primary endpoint of cardiovascular death, myocardial infarction, or stroke when compared to patients treated with clopidogrel (300 mg loading dose followed by 75 mg daily) (Montalescot et al. 2009). This was observed at 30 days (6.5% in prasugrel versus 9.5% in clopidogrel, RRR of 31.6% with NNT of 34, $p = 0.0017$) and persisted at 15 months (10% in prasugrel vs. 12.4% in clopidogrel, $p = 0.0205$). The secondary endpoint of cardiovascular death, myocardial infarction, and urgent revascularization was also significantly reduced at both time points in patients treated with prasugrel. Of note, there was no difference between major bleeding unrelated to CABG at 30 days or 15 months in this subgroup of patients; therefore, prasugrel may be a more effective treatment with overall improved outcomes in this specific group of patients presenting with STEMI although caution should be maintained in patients at high risk of bleeding (elderly, history of stroke, or low body weight).

As noted previously, ticagrelor was investigated in the PLATO trial. Patients presenting with STEMI made up approximately 38% of the population with a relatively equal distribution between treatment and control groups. Overall results favored ticagrelor as described previously. Of note, patients on ticagrelor who received coronary stents had a lower rate of definite stent thrombosis compared to those on clopidogrel (1.3% vs. 1.9%, RRR of 32%, $p = 0.009$).

For those patients who proceed to PCI and stenting for ACS, a $P2Y_{12}$ blocker should be prescribed at discharge. According to current guidelines, patients should maintain ADP inhibitor therapy for at least 1 year, regardless if they have received a bare metal or drug eluting stent. However, some clinicians prefer to maintain therapy beyond 1 year given observational data suggesting an increased risk of very late stent thrombosis in patients who stop ADP inhibition at 1 year. The ongoing Dual Antiplatelet Therapy Study (DAPT) proposes to answer this question (Mauri et al. 2010). In this study, patients who are 12 months from stent implantation and are free from death, myocardial infarction, stroke, repeat revascularization, stent thrombosis, and major bleeding will be randomized to an additional 18 months of thienopyridine (either clopidogrel or prasugrel) versus placebo. This trial anticipates enrolling around 20,000 patients (15,000 with DES and 5,000 with BMS) and will be helpful in guiding long-term dual antiplatelet therapy.

4.3 Glycoprotein IIb/IIIa Inhibitors

Abciximab pharmacotherapy has been extensively studied in the setting of STEMI in multiple trials (Brener et al. 1998; Neumann et al. 2000; Montalescot et al. 2001; Stone et al. 2002; Antoniucci et al. 2003). A meta-analysis combining over 27,100 patients from 11 randomized trials of abciximab in STEMI affirmed abciximab was associated with a 30 day mortality benefit (2.4% vs. 3.4%, $p = 0.047$) as well as 6–12 month mortality (4.4% vs. 6.2%, $p = 0.01$) benefit in patients undergoing PCI but not in patients who underwent fibrinolysis (De Luca et al. 2005). There was no increase in major bleeding in patients who underwent PCI; however, there was a significant increase in major bleeding in those patients who received a fibrinolytic. Another smaller meta-analysis suggests the benefits from abciximab in patients presenting with STEMI and undergoing PCI may last to 36 months (Montalescot et al. 2007).

There have been several small trials comparing the different GP IIb/IIIa inhibitors in patients with STEMI; however, they were all too small to assess meaningful clinical endpoints. For example, the Eptifibatide Versus Abciximab in Primary PCI for Acute Myocardial Infarction (EVA-AMI) trial randomized 427 patients presenting with STEMI to either abciximab or eptifibatide (Zeymer et al. 2010). The use of eptifibatide was noninferior compared to abciximab for the primary endpoint of ST resolution 1 h post-PCI, and there was no significant difference between the two groups with regards to the combined endpoint of death, myocardial infarction, and target vessel revascularization. Furthermore, a meta-analysis combining the results of this trial plus five others involving a total of 2,197 patients compared abciximab versus eptifibatide and tirofiban in PCI for patients presenting with STEMI (De Luca et al. 2009). This study showed no difference in outcomes between abciximab versus tirofiban or eptifibatide with regards to TIMI flow, ST-segment resolution, or 30-day mortality or reinfarction.

Larger trials will be needed to differentiate between the various agents with regards to significant clinical events.

The HORIZONS-AMI study evaluated whether the benefits of bivalirudin observed for lower risk patients in REPLACE-2 and ACUITY (similar protection against ischemic events with reduced bleeding) could be achieved in patients with STEMI undergoing primary PCI (Stone et al. 2008). This trial randomized 3,602 patients with STEMI to heparin plus a GP IIb/IIIa inhibitor or to monotherapy with bivalirudin. Those in the bivalirudin arm had significantly less all-cause mortality (2.1% vs. 3.1%, $p = 0.047$) as well as lower rates of cardiac mortality at 30 days (1.8% vs. 2.9%, $p = 0.03$). As with the other trials of this agent, bivalirudin reduced the risk of major bleeding relative to heparin plus GP IIb/IIIa inhibition (4.9% vs. 8.4%, $p < 0.001$). On long-term follow-up, benefits persisted to 3 years, including lower rates of reinfarction, cardiac mortality, and all-cause mortality (Stone et al. 2011). Thus, while GP IIb/IIIa inhibitors have played an important role in improving outcomes for patients presenting with STEMI, there is now compelling evidence that bivalirudin may be the treatment of choice over GP IIb/IIIa inhibitors (at least when combined with heparin) in current practice.

5 Future Directions

Antiplatelet agents comprise a crucial component of the medicinal armamentarium employed to treat patients with coronary artery disease. The ability to inhibit platelet aggregation at multiple different steps has greatly contributed to the improved outcomes of patients with acute and chronic ischemic heart disease. The common thread among many of these trials is exploring the combination of therapies that optimize outcomes while minimizing complications such as bleeding. As novel agents are approved for use, their therapeutic role in patients with ischemic heart disease will need to be investigated. For example, in patients with UA/NSTEMI who are managed medically, is there a role for prasugrel? This is the goal of the ongoing TRILOGY ACS study described previously. Another important question is the optimal duration of ADP inhibition in patients who receive drug-eluting stents. Although the rate of very late stent thrombosis is low, the catastrophic potential of this event makes it an important area of investigation. The aforementioned DAPT trial aims to clarify the role of long-term ADP blockade with patients in the experimental group receiving 2.5 years of thienopyridine compared to 1 year in the control group. Moreover, the role of intravenous $P2Y_{12}$ inhibitors, such as cangrelor, remains to be seen. The initial two trials that explored its use in patients undergoing PCI were stopped early as the interim analysis showed a lack of efficacy (Harrington et al. 2009; Bhatt et al. 2009). However, given its short duration of action, it may yet play a role in bridging patients who require $P2Y_{12}$ inhibition to surgery; this is the subject of ongoing studies. In summary, the outcomes of patients with ischemic heart disease have been significantly improved

with the use of antiplatelet agents. However, further studies are needed to define more precisely the exact patient population that would most benefit from each of these agents.

Knowledge Gaps

- Further delineation of specific patient populations that most benefit from specific therapies.
- Efficacy of incorporating information on individual genetic differences into $P2Y_{12}$ inhibitor selection.
- Optimal duration of ADP inhibition to avoid very late stent thrombosis in patients who receive drug-eluting stents.
- The role of prasugrel in patients with medically managed UA/NSTEMI.
- The role of prasugrel or ticagrelor in patients receiving fibrinolytics for STEMI.
- The role of intravenous $P2Y_{12}$ inhibitors in bridging patients who require $P2Y_{12}$ inhibition to surgery or other procedure.

Key Messages

- Aspirin is a key component of therapy in all stages of ischemic heart disease.
- Clopidogrel has been widely studied and its efficacy has been demonstrated in many different clinical settings.
- Prasugrel should be considered over clopidogrel in patients who present with ACS and do not have a prior history of TIA or stroke.
- Ticagrelor appears to be superior to clopidogrel in patients who present with ACS who are managed either invasively or noninvasively.
- In ACS patients undergoing PCI in whom bivalirudin is used, GP IIb/IIIa inhibitors can be used provisionally in higher risk patients.

Appendix

Recommended antiplatelet therapy for chronic ischemic heart disease

	Secondary prevention	Prior to elective PCI
Aspirin	75–81 mg daily indefinitely[a]	300–325 mg loading dose for those not on aspirin (or on lower daily dose), then 162–325 mg daily for 1 month for BMS[b] 162–325 mg daily for 3 months for SES7[b] 162–325 mg daily for 6 months for PES[b], followed by 75–162 mg daily indefinitely
Clopidogrel	75 mg daily indefinitely in patients intolerant to aspirin 75 mg daily combined with aspirin up to 1 year after hospitalization for ACS	600 mg loading dose at least 2 h (preferably 24 h) prior to PCI, then 75 mg daily for at least 1 month for BMS 75 mg daily 1 year for DES
GP IIb/IIIa inhibitors	None	Use is generally limited to patients with higher risk, those who have not received pretreatment with clopidogrel, or those with a higher risk angiographic result[c]

[a]May also consider this dose in patients with known coronary artery disease but no history of myocardial infarction

[b]Consider lower dose of 75–162 mg daily in patients at high risk of bleeding

[c]Alternatives to GP IIb/IIIa inhibitor include heparin (when pretreated with clopidogrel) or bivalirudin (pretreated or not pretreated with clopidogrel)

BMS bare metal stent, *SES* sirolimus-eluting stent, *PES* paclitaxel eluting stent, *DES* drug-eluting stent

Recommended antiplatelet therapy for unstable angina and non-ST segment elevation myocardial infarction

	Conservative strategy	Invasive strategy	
Aspirin	162–325 mg loading dose, followed by 75–162 mg daily indefinitely	162–325 mg loading dose, followed by 162–325 mg daily for 1 month for BMS[a] 162–325 mg daily for 3 months for SES[a] 162–325 mg daily for 6 months for PES[a], followed by 75–162 mg daily indefinitely	
ADP inhibitors	*Clopidogrel* 300 mg loading dose followed by 75 mg daily for at least 1 month, ideally for 1 year *Prasugrel* Not currently recommended, under investigation *Ticagrelor* 180 mg loading dose, followed by 90 mg twice daily for at least 1 year[d]	*Clopidogrel* 600 mg loading dose, followed by 75 mg for at least 1 month for BMS (ideally 1 year) or 75 mg 1 year for DES[b], OR *Prasugrel* (if patient is undergoing PCI)[c] 60 mg loading dose, followed by 10 mg daily for at least 1 year, OR *Ticagrelor* 180 mg loading dose, followed by 90 mg twice daily for at least 1 year[d]	
GP IIb/IIIa inhibitors	Eptifibatide or tirofiban use is reserved for refractory symptoms or in patients not receiving an ADP inhibitor. Abciximab use is not recommended *Eptifibatide*: 180 mcg/kg IV bolus, followed by 2 mcg/kg/min; reduce to 1 mcg/kg/min if CrCl < 50 mL/min *Tirofiban*: 25 mcg/kg IV bolus, then 0.15 mcg/kg/min; reduce by 50 % if CrCl < 30 mL/min	*Upstream* Eptifibatide or Tirofiban for patients who did not receive ADP inhibitor prior to PCI Consider GP IIb/IIIa inhibitor, in addition to aspirin and ADP inhibitor, in high risk patients (elevated troponin levels, diabetes, or significant ST-segment depression)	*At time of PCI* GP IIb/IIIa inhibitors (abciximab, eptifibatide, or tirofiban) can be administered to patients with higher risk angiographic features or in patients with thrombotic complications *Abciximab*: 0.25 mg/kg IV bolus, then 0.125 mcg/kg/min up to 12 h *Eptifibatide*: 180 mcg/kg IV bolus followed by second 180 mcg/kg IV bolus after 10 min. 2.0 mcg/kg/min should be started after the first bolus; reduce rate by 50 % in patients with CrCl < 50 mL/min. Continue for 12–18 h *Tirofiban*: 25 mcg/kg IV bolus, then 0.1 mcg/kg/min; reduce rate by 50 % in patients with CrCl < 30 mL/min. Continue for 18 h
		GP IIb/IIIa inhibition may be omitted if bivalirudin is used as the anticoagulant and at least 300 mg of clopidogrel was given more than 6 h prior to PCI	

[a]Consider lower dose of 75–162 mg daily in patients at high risk of bleeding

[b]In patients with a low risk of bleeding, an alternative regimen consisting of a 600 mg loading dose, then 150 mg daily for 6 days, followed by 75 mg for at least 1 month for BMS (ideally 1 year) or 75 mg 1 year for DES may be better

[c]Contraindicated in patients with history of TIA or stroke. Avoid in patients age >75 and dose adjust in patients <60 kg

[d]When ticagrelor is prescribed, a low aspirin dose of 75–100 mg daily should be used

BMS bare metal stent, *SES* sirolimus-eluting stent, *PES* paclitaxel eluting stent, *DES* drug-eluting stent

Recommended antiplatelet therapy for ST-segment myocardial infarction

	Receiving fibrinolysis	Undergoing primary PCI
Aspirin	162–325 mg loading dose, followed by 162–325 mg daily for 1 month for BMS[a] 162–325 mg daily for 3 months for SES[a] 162–325 mg daily for 6 months for PES[a], followed by 75–162 mg daily indefinitely	162–325 mg loading dose, followed by 162–325 mg daily for 1 month for BMS[a] 162–325 mg daily for 3 months for SES[a] 162–325 mg daily for 6 months for PES[a], followed by 75–162 mg daily indefinitely
ADP inhibitors	*Clopidogrel* Age <75: 300 mg loading dose followed by 75 mg daily for at least 14 days, ideally for 1 year. Age ≥75: 75 mg daily with no loading dose for at least 14 days, ideally for 1 year *Prasugrel* Not recommended; has not been studied *Ticagrelor* Not recommended; has not been studied	*Clopidogrel* 600 mg loading dose, followed by 75 mg for at least 1 month for BMS (ideally 1 year) or 75 mg 1 year for DES[b], OR *Prasugrel*[c] 60 mg loading dose, followed by 10 mg daily for at least 1 year , OR *Ticagrelor* 180 mg loading dose, followed by 90 mg twice daily for at least 1 year[d]
GP IIb/IIIa inhibitors	Of uncertain benefit	At time of PCI with heparin used as the anticoagulant, either: *Abciximab*: 0.25 mg/kg IV bolus, followed by 0.125 mcg/kg/min up to 12 h *Eptifibatide*: 180 mcg/kg IV bolus followed by another 180mcg/kg IV bolus after 10 min. 2.0 mcg/kg/min should be started after the first bolus; reduce rate by 50 % in patients with CrCl < 50 mL/min. Continue for 12–18 h *Tirofiban*: 25 mcg/kg IV bolus followed by 0.1 mcg/kg/min; reduce rate by 50 % in patients with CrCl < 30 mL/min. Continue for 18 h If bivalirudin is used as the anticoagulant, GP IIb/IIIa inhibitors can be used provisionally for ischemic complications, angiographic complications, or high-risk features

[a]Consider lower dose of 75–162 mg daily in patients at high risk of bleeding

[b]In patients with a low risk of bleeding, an alternative regimen consisting of a 600 mg loading dose, then 150 mg daily for six days, followed by 75 mg for at least 1 month for BMS (ideally 1 year) or 75 mg 1 year for DES may be better

[c]Preferred therapy over clopidogrel. Contraindicated in patients with history of TIA or stroke. Avoid in patients age >75 and dose adjust in patients <60 kg

[d]When ticagrelor is prescribed, a low aspirin dose of 75–100 mg daily should be used

BMS bare metal stent, *SES* sirolimus-eluting stent, *PES* paclitaxel eluting stent, *DES* drug-eluting stent

References

Anderson KM et al (2001) Long-term mortality benefit with abciximab in patients undergoing percutaneous coronary intervention. J Am Coll Cardiol 37:2059–2065

Anonymous (1988) Randomised trial of intravenous streptokinase, oral aspirin, both, or neither among 17,187 cases of suspected acute myocardial infarction: ISIS-2. ISIS-2 (Second International Study of Infarct Survival) Collaborative Group. Lancet 332:349–360

Anonymous (1994) Use of a monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty. The EPIC investigation. N Engl J Med 330:956–961

Anonymous (1998) Inhibition of the platelet glycoprotein IIb/IIIa receptor with tirofiban in unstable angina and non-Q-wave myocardial infarction. Platelet Receptor Inhibition in Ischemic Syndrome Management in Patients Limited by Unstable Signs and Symptoms (PRISM-PLUS) Study Investigators. N Engl J Med 338:1488–1497

Anonymous (1998) A comparison of aspirin plus tirofiban with aspirin plus heparin for unstable angina. Platelet Receptor Inhibition in Ischemic Syndrome Management (PRISM) Study Investigators. N Engl J Med 338:1498–1505

Anonymous (1998) International, randomized, controlled trial of lamifiban (a platelet glycoprotein IIb/IIIa inhibitor), heparin, or both in unstable angina. The PARAGON Investigators. Platelet IIb/IIIa Antagonism for the Reduction of Acute coronary syndrome events in a Global Organization Network. Circulation 97:2386–2395

Anonymous (1998) Inhibition of platelet glycoprotein IIb/IIIa with eptifibatide in patients with acute coronary syndromes. The PURSUIT Trial Investigators. Platelet Glycoprotein IIb/IIIa in Unstable Angina: Receptor Suppression Using Integrilin Therapy. N Engl J Med 339:436–443

Antithrombotic Trialists' Collaboration (2002) Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. BMJ 324:71–86

Antoniucci D et al (2003) A randomized trial comparing primary infarct artery stenting with or without abciximab in acute myocardial infarction. J Am Coll Cardiol 42:1879–1885

Bassand JP et al (2007) Guidelines for the diagnosis and treatment of non-ST-segment elevation acute coronary syndromes. Task Force for Diagnosis and Treatment of Non-ST-Segment Elevation Acute Coronary Syndromes of European Society of Cardiology. Eur Heart J 28:1598–1660

Bernlochner I, Sibbing D (2012) Thienopyridines and other ADP-receptor antagonists. In: Gresele P, Born GVR, Patrono C, Page CP (eds) Antiplatelet agents. Springer, Heidelberg

Bhatt DL et al (2006) Clopidogrel and aspirin versus aspirin alone for the prevention of atherothrombotic events. N Engl J Med 354:1706–1717

Bhatt DL et al (2007) Patients with prior myocardial infarction, stoke, or symptomatic peripheral arterial disease in the CHARISMA trial. J Am Coll Cardiol 49:1982–1988

Bhatt DL et al (2009) Intravenous platelet blockade with cangrelor during PCI. N Engl J Med 361:2330–2341

Boersma E et al (2002) Platelet glycoprotein IIb/IIIa inhibitors in acute coronary syndromes: a meta-analysis of all major randomised clinical trials. Lancet 359:189–198

Brener SJ et al (1998) Randomized, placebo-controlled trial of platelet glycoprotein IIb/IIIa blockade with primary angioplasty for acute myocardial infarction. ReoPro and Primary PTCA Organization and Randomized Trial (RAPPORT) Investigators. Circulation 98:734–741

Cairns JA et al (1985) Aspirin, sulfinpyrazone, or both in unstable angina. Results of a Canadian multicenter trial. N Engl J Med 313:1369–1375

Campbell C et al (2007) Aspirin dose for the prevention of cardiovascular disease: a systematic review. JAMA 297:2018–2024

CAPRIE Steering Committee (1996) A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). Lancet 348:1329–1339

Chen ZM et al (2005) Addition of clopidogrel to aspirin in 45,852 patients with acute myocardial infarction: randomised placebo-controlled trial. Lancet 366:1607–1621

Chew DP et al (2001) Increased mortality with oral platelet glycoprotein IIb/IIIa antagonists: a meta-analysis of the phase III multicenter randomized trials. Circulation 103:201–206
Chin CT et al (2010) Study design and rationale of a comparison of prasugrel and clopidogrel in medically managed patients with unstable angina/non-ST-segment elevation myocardial infarction: the TaRgeted platelet Inhibition to cLarify the Optimal strateGy to medicallY manage Acute Coronary Syndromes (TRILOGY ACS) trial. Am Heart J 160:16–22
Dangas G et al (2009) Role of clopidogrel loading dose in patients with ST-segment elevation myocardial infarction undergoing primary angioplasty: results from the HORIZONS-AMI (harmonizing outcomes with revascularization and stents in acute myocardial infarction) trial. J Am Coll Cardiol 54:1438–1446
De Luca G et al (2005) Abciximab as adjunctive therapy to reperfusion in acute ST-segment elevation myocardial infarction: a meta-analysis of randomized trials. JAMA 293:1759–1765
De Luca G et al (2009) Benefits from small molecule administration as compared with abciximab among patients with ST-segment elevation myocardial infarction treated with primary angioplasty: a meta-analysis. J Am Coll Cardiol 53:1668–1673
EPILOG Investigators (1997) Platelet glycoprotein IIb/IIIa receptor blockade and low-dose heparin during percutaneous coronary revascularization. N Engl J Med 336:1689–1696
EPISTENT Investigators (1998) Randomised placebo-controlled and balloon-angioplasty-controlled trial to assess safety of coronary stenting with use of a platelet glycoprotein-IIb/IIIa blockade. Lancet 352:87–92
ESPRIT Investigators (2000) Novel dosing regimen of eptifibatide in planned coronary stent implantation (ESPRIT): a randomised, placebo-controlled trial. Lancet 356:2037–2044
Giugliano RP et al (2009) Early versus delayed, provisional eptifibatide in acute coronary syndromes. N Engl J Med 360:2176–2190
Global Organization Network (PARAGON)-B Investigators (2002) Randomized, placebo-controlled trial of titrated intravenous lamifiban for acute coronary syndromes. Circulation 105:316–321
Harrington RA et al (2009) Platelet inhibition with cangrelor in patients undergoing PCI. N Engl J Med 361:2318–2329
Hook KM, Bennett JS (2012) Glycoprotein IIb/IIIa antagonists. In: Gresele P, Born GVR, Patrono C, Page CP (eds) Antiplatelet agents. Springer, Heidelberg
ISIS-2 (Second International Study of Infarct Survival) Collaborative Group (1988) Randomised trial of intravenous streptokinase, oral aspirin, both, or neither among 17,187 cases of suspected acute myocardial infarction: ISIS-2. Lancet 332:349–360
Juul-Moller S et al (1992) Double-blind trial of aspirin in primary prevention of myocardial infarction in patients with stable chronic angina pectoris. The Swedish Angina Pectoris Aspirin Trial (SAPAT) Group. Lancet 340:1421–1425
Kastrati A et al (2004) A clinical trial of abciximab in elective percutaneous coronary intervention after pretreatment with clopidogrel. N Engl J Med 350:232–238
Kastrati A et al (2006) Abciximab in patients with acute coronary syndromes undergoing percutaneous coronary intervention after clopidogrel pretreatment: the ISAR-REACT 2 randomized trial. JAMA 295:1531–1538
Lewis HD Jr et al (1983) Protective effects of aspirin against acute myocardial infarction and death in med with unstable angina. Results of a Veterans Administration Cooperative Study. N Engl J Med 309:396–403
Lincoff AM et al (2003) Bivalirudin and provisional glycoprotein IIb/IIIa blockade compared with heparin and planned glycoprotein IIb/IIIa blockade during percutaneous coronary intervention: REPLACE-2 randomized trial. JAMA 289:853–863
Mauri L et al (2010) Rationale and design of the dual antiplatelet therapy study, a prospective, multicenter, randomized, double-blind trial to assess the effectiveness and safety of 12 versus 30 months of dual antiplatelet therapy in subjects undergoing percutaneous coronary intervention with either drug-eluting stent or bare metal stent placement for the treatment of coronary artery lesions. Am Heart J 160:1035–1041

Mehta SR et al (2001) Effects of pretreatment with clopidogrel and aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: the PCI-CURE study. Lancet 358:527–533

Mehta SR et al (2010) Dose comparisons of clopidogrel and aspirin in acute coronary syndromes. N Engl J Med 363:930–942

Montalescot G et al (2001) Platelet glycoprotein IIb/IIIa inhibition with coronary stenting for acute myocardial infarction. N Engl J Med 344:1895–1903

Montalescot G et al (2006) A randomized comparison of high clopidogrel loading doses in patients with non-ST-segment elevation acute coronary syndromes: the ALBION (Assessment of the Best Loading Dose of Clopidogrel to Blunt Platelet Activation, Inflammation, and Ongoing Necrosis) trial. J Am Coll Cardiol 48:931–938

Montalescot G et al (2007) Abciximab in primary coronary stenting of ST-elevation myocardial infarction: a European meta-analysis on individual patients' data with long-term follow-up. Eur Heart J 28:443–449

Montalescot G et al (2009) Prasugrel compared with clopidogrel in patients undergoing percutaneous coronary intervention for ST-elevation myocardial infarction (TRITON-TIMI 38): double-blind, randomised controlled trial. Lancet 373:723–731

Neumann FJ et al (2000) Effect of glycoprotein IIb/IIIa receptor blockade with abciximab on clinical and angiographic restenosis rate after the placement of coronary stents following acute myocardial infarction. J Am Coll Cardiol 35:915–921

Patrono C, Rocca B (2012) Aspirin and other COX-1 inhibitors. In: Gresele P, Born GVR, Patrono C, Page CP (eds) Antiplatelet agents. Springer, Heidelberg

Patti G et al (2005) Randomized trial of high loading dose of clopidogrel for reduction of periprocedural myocardial infarction in patients undergoing coronary intervention: results from the ARMYDA-2 (Antiplatelet therapy for Reduction of MYocardial Damage during Angioplasty) study. Circulation 111:2099–2106

Sabatine MS et al (2005) Addition of clopidogrel to aspirin and fibrinolytic therapy for myocardial infarction with ST-segment elevation. N Engl J Med 253:1179–1189

Schwartz MD et al (1988) Aspirin and dipyridamole in the prevention of re-stenosis after percutaneous transluminal coronary angioplasty. N Engl J Med 318:1714–1719

Simoons ML et al (2001) Effect of glycoprotein IIb/IIIa receptor blocker abciximab on outcome in patients with acute coronary syndromes without early coronary revascularisation: the GUSTO IV-ACS randomised trial. Lancet 357:1915–1924

Steinhubl SR et al (2002) Early and sustained dual oral antiplatelet therapy following percutaneous coronary intervention: a randomized controlled trial. JAMA 288:2411–2420

Stone GW et al (2002) Comparison of angioplasty with stenting, with or without abciximab, in acute myocardial infarction. N Engl J Med 346:957–966

Stone GW et al (2006) Bivalirudin for patients with acute coronary syndromes. N Engl J Med 355:2203–2216

Stone GW et al (2007) Routine upstream initiation vs deferred selective use of glycoprotein IIb/IIIa inhibitors in acute coronary syndromes: the ACUITY Timing trial. JAMA 297:591–602

Stone GW et al (2008) Bivalirudin during primary PCI in acute myocardial infarction. N Engl J Med 358:2218–2230

Stone GW et al (2011) Heparin plus a glycoprotein IIb/IIIa inhibitor versus bivalirudin monotherapy and paclitaxel-eluting stents versus bare-metal stents in acute myocardial infarction (HORIZONS-AMI): final 3-year results from a multicentre, randomised controlled trial. Lancet 377:2193–2204

The EPIC investigators (1994) Use of a monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty. N Engl J Med 330:956–961

The PARAGON Investigators (1998) International, randomized, controlled trial of lamifiban (a platelet glycoprotein IIb/IIIa inhibitor), heparin, or both in unstable angina. Circulation 97:2386–2395

The Platelet Receptor Inhibition in Ischemic Syndrome Management (PRISM) Study Investigators (1998) A comparison of aspirin plus tirofiban with aspirin plus heparin for unstable angina. N Engl J Med 338:1498–1505

The Platelet Receptor Inhibition in Ischemic Syndrome Management in Patients Limited by Unstable Signs and Symptoms (PRISM- PLUS) Study Investigators (1998) Inhibition of the platelet glycoprotein IIb/IIIa receptor with tirofiban in unstable angina and non-Q-wave myocardial infarction. N Engl J Med 338:1488–1497

The PURSUIT Trial Investigators (1998) Inhibition of platelet glycoprotein IIb/IIIa with eptifibatide in patients with acute coronary syndromes. N Engl J Med 339:436–443

The RISC Group (1990) Risk of myocardial infarction and death during treatment with low dose aspirin and intravenous heparin in men with unstable coronary artery disease. Lancet 336:827–830

Theroux P et al (1988) Aspirin, heparin, or both to treat acute unstable angina. N Engl J Med 319:1105–1111

Topol EJ et al (2001) Comparison of two platelet glycoprotein IIb/IIIa inhibitors, tirofiban and abciximab, for the prevention of ischemic events with percutaneous coronary revascularization. N Engl J Med 344:1888–1894

Topol EJ et al (2003) Randomized, double-blind, placebo-controlled, international trial of the oral IIb/IIIa antagonist lotrafiban in coronary and cerebrovascular disease. Circulation 108:399–406

von Beckerath N et al (2005) Absorption, metabolization, and antiplatelet effects of 300-, 600-, and 900-mg loading doses of clopidogrel: results of the ISAR-CHOICE (Intracoronary Stenting and Antithrombotic Regimen: Choose Between 3 High Oral Doses for Immediate Clopidogrel Effect) Trial. Circulation 112:2946–2950

Wallentin L et al (2009) Ticagrelor versus clopidogrel in patients with acute coronary syndromes. N Engl J Med 361:1045–1057

Wiviott SD et al (2007) Prasugrel versus clopidogrel in patients with acute coronary syndromes. N Engl J Med 357:2001–2015

Yusuf S et al (2001) Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med 345:494–502

Zeymer U et al (2010) Randomized comparison of eptifibatide versus abciximab in primary percutaneous coronary intervention in patients with acute ST-segment elevation myocardial infarction: results of the EVA-AMI Trial. J Am Coll Cardiol 56:463–469

Antiplatelet Therapy in Cerebrovascular Disorders

Ralph Weber, Johannes Brenck, and Hans-Christoph Diener

Contents

R. Weber • J. Brenck • H.-C. Diener (✉)
Department of Neurology and Stroke Center, University Duisburg-Essen, Hufelandstr. 55, 45122 Essen, Germany
e-mail: hans.diener@uni-duisburg-essen.de

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_21,

Abstract Antiplatelet treatment is a mainstay in acute and long-term secondary stroke prevention. Aspirin is still most widely used worldwide, however, there is increasing evidence from small randomised trials that dual antiplatelet therapy combining aspirin with dipyridamole or clopidogrel might be more effective in the acute and early chronic post-ischemic phase (i.e. first 90 days). Both clopidogrel and the combination of aspirin and extended-release dipyridamole are recommended by current guidelines in long-term secondary stroke prevention in patients who are at high risk for a recurrent ischemic stroke, since they are more effective compared with aspirin monotherapy.

Antiplatelet agents are the therapy of choice in patients with ischemic stroke due to intracranial stenosis and patent foramen ovale. In contrast, oral anticoagulation is clearly superior to single or double antiplatelet therapy in patients with cardioembolic stroke, mainly caused by atrial fibrillation.

Concerning newer antiplatelet agents, only cilostazol appears to be a promising therapeutic option in patients with ischemic stroke in the near future, but so far, only studies in Asian stroke patients have been performed.

Keywords Antiplatelets • Anticoagulation • Stroke • Secondary prevention • Arterial dissection • Patent foramen ovale • Aspirin resistance • Atrial fibrillation • Aspirin • Dipyridamole • Clopidogrel • Cilostazol • Triflusal • Ticlopidine

1 Introduction

Ischemic stroke is the third leading cause of mortality and the leading cause of disability in developed countries (Lopez et al. 2006). Up to 15 % of patients suffer a recurrent stroke in the first year. Recurrent strokes are more severe than first strokes and the risk of stroke recurrence or stroke following transient ischemic attack (TIA) is highest in the first days thereafter (Giles and Rothwell 2007). As a consequence, secondary stroke prevention should be started immediately after a preceding TIA or stroke. Treatments shown to be effective in large-scale randomised trials include antithrombotic agents (antiplatelets and oral anticoagulation), carotid endarterectomy or stenting for severe symptomatic carotid stenosis, and medical treatment of increased blood pressure and high lipoprotein density cholesterol.

Aspirin was the first antiplatelet agent to be tested in randomised secondary stroke prevention trials in the mid-1970s. Nowadays, several antiplatelet agents are available for secondary stroke prevention, but aspirin is still most widely used

worldwide. The latest meta-analysis by the Antithrombotic Trialist's Collaboration in 2002 included 287 trials with 135,000 patients randomised to antiplatelet therapy versus control and 77,000 patients randomised to different antiplatelet regimens (Antithrombotic Trialists' Collaboration 2002). Overall, antiplatelets reduced the relative risk of serious vascular events (non-fatal myocardial infarction, non-fatal stroke or vascular death) by about 25 %. In 23,020 patients with a previous TIA or ischemic stroke, antiplatelet agents were able to prevent 36 cardiovascular events among 1,000 patients treated for 2 years and the benefit substantially outweighed the risk of major bleeding.

In contrast to myocardial infarction, which is mainly caused by atherothrombotic disease of one or more cardiac arteries, ischemic stroke is caused by various etiologies. The three main causes are large artery atherosclerosis of the brain supplying extra- and intracranial blood vessels, cardiac embolism (mainly from atrial fibrillation) and cerebral microangiopathy of the small penetrating brain arteries. This etiological heterogeneity of ischemic stroke/TIA is of upmost importance as long-term antithrombotic treatment (antiplatelet or anticoagulation) is dependent upon the underlying pathology. Furthermore, brain blood vessels are unique as they are part of the blood–brain barrier. The blood–brain barrier is altered by the cerebral ischemia itself, reperfusion and therapeutic agents administered in acute stroke treatment such as recombinant tissue plasminogen activator. These changes can result in an increased blood–brain barrier permeability which increases the risk of intracerebral haemorrhage which has also been taken into account when choosing an antithrombotic treatment in secondary stroke (Eisert and Schlachetzki 2008).

This chapter will review the current evidence of use of antiplatelet agents in acute and long-term secondary stroke prevention and discuss gaps of knowledge as well as future perspectives.

2 Antiplatelets in Acute Secondary Stroke Prevention

Intravenous thrombolytic treatment with recombinant tissue plasminogen activator (rt-PA), initiated within 4.5 h after the onset of stroke symptoms, is the only approved medical therapy available for acute ischemic stroke to date (Lees et al. 2010). However, only about 10–15 % of patients with acute ischemic stroke are eligible for rt-PA treatment due to the restricted time window and contraindications. Thus, prevention of a recurrent ischemic stroke with antiplatelets in the acute phase (i.e. first 24–48 h) after an ischemic stroke/TIA is one of the treatment goals to prevent further brain damage. Antiplatelet agents should be initiated immediately after brain haemorrhage has been ruled out by brain imaging. In patients who have been treated with intravenous thrombolysis antiplatelet treatment should be halted for 24 h due to an increased (intracerebral) bleeding risk and until after brain imaging has ruled out secondary brain haemorrhage.

Aspirin has been the only antiplatelet agent for long time that has been demonstrated to be modestly effective in stroke prevention in the acute post-ischemic

phase, but there is growing evidence that dual antiplatelet therapy with aspirin/dipyridamole or aspirin/clopidogrel might be superior to aspirin monotherapy (Dengler et al. 2010).

2.1 Aspirin Monotherapy

Early treatment with aspirin in patients with acute ischemic stroke was investigated in two large clinical trials in the 1990s, the International Stroke Trial (IST) and the Chinese Acute Stroke Trial (CAST). IST was an open trial that randomised 19,435 patients within 48 h of symptom onset to receive either aspirin (300 mg/daily), subcutaneous heparin (5,000 U/BID or 12,500 U/BID), both or placebo (International Stroke Trial Collaborative Group 1997). A further methodological limitation was the fact that not all patients received brain imaging before study entry to exclude haemorrhage. At 6 months, 62.2 % of patients treated with aspirin were dead or dependent compared with 63.5 % of untreated patients. Patients treated with aspirin had significantly fewer recurrent ischemic strokes within 14 days (2.8 % vs. 3.9 %) with no significant excess of hemorrhagic stroke (0.9 % vs. 0.8 %). Among aspirin-allocated patients, there were non-significantly fewer deaths within 14 days (9.0 % vs. 9.4 %).

In contrast to IST, CAST was a double-blind placebo-controlled trial that randomised 21,106 Chinese patients to receive either aspirin (160 mg/daily) or placebo for 4 weeks, starting within 48 h after symptom onset (Chinese Acute Stroke Trial Collaborative Group 1997). There was a significant reduction in mortality during the 4-week treatment period in aspirin-allocated patients (3.3 % vs. 3.9 %). Furthermore, there were significantly fewer recurrent ischemic strokes in patients treated with aspirin (1.6 % vs. 2.1 %). Treatment with aspirin was associated with a non-significant increase in hemorrhagic strokes (1.1 % vs. 0.9 %).

The prospectively planned combined analysis of these two large trials showed a modest but statistically significant benefit for aspirin over placebo, resulting in 9 fewer deaths or non-fatal strokes per 1,000 treated patients in the first weeks.

2.2 Aspirin and Extended-Release Dipyridamole

The randomised double-blind EARLY (Early treatment with aspirin plus extended-release dipyridamole for transient ischaemic attack or ischaemic stroke within 24 h of symptom onset) trial compared the combination of aspirin (25 mg BID) and extended-release dipyridamole (200 mg BID) with aspirin (100 mg/daily) in 543 patients with ischemic stroke or TIA started within 24 h of symptom onset (Dengler et al. 2010). 283 patients received aspirin/dipyridamole (early initiation group) and 260 received aspirin monotherapy (late initiation group) for the initial 7 days. Thereafter, all patients were treated with aspirin/ dipyridamole for 90 days. The primary end point was an excellent or good functional outcome at day 90, which was

assessed with the modified Rankin scale by a blinded investigator using a standardised telephonic interview. Vascular events and mortality were also assessed as a composite safety and efficacy end point. At day 90, 56 % of patients in the early initiation group and 52 % of patients treated with aspirin monotherapy during the first 7 days had an excellent or good outcome (difference 4.1 %, 95 % CI −4.5–12.6). The composite safety and efficacy end point occurred in 28 patients (10 %) in the early combination group and in 38 patients (15 %) in the aspirin monotherapy group (hazard ratio 0.73, 95 % CI 0.44–1.19). More patients in the late initiation group had a recurrent non-fatal stroke, but this difference also did not reach statistic significance (hazard ratio 0.61, 95 % CI 0.31–1.19). However, the EARLY trial was not powered to detect a significant difference in recurrent stroke rate.

A post hoc analysis of the PRoFESS (Prevention Regimen For Effectively avoiding Second Strokes) trial (see below for results of the main trial) that comprised 1,360 patients who were randomised within 72 h of ischemic stroke onset to either aspirin/dipyridamole (25 mg/200 mg BID) or clopidogrel (75 mg/daily) did not show a significant difference for recurrent stroke (1.64 % vs. 2.91 %; OR 0.56, 95 % CI 0.26–1.18) or death and dependency at 30 days between the two antiplatelet regimen (Bath et al. 2010).

2.3 Aspirin and Clopidogrel

Clopidogrel is not approved for treatment of patients with acute ischemic stroke in the first 7 days, but the combination of aspirin and clopidogrel has been compared with aspirin monotherapy in patients with minor ischemic stroke or TIA in the FASTER (Fast assessment of stroke and transient ischaemic attack to prevent early recurrence) pilot trial (Kennedy et al. 2007). Within 24 h of symptom onset, 392 patients were randomised to an initial loading dose of 300 mg clopidogrel followed by 75 mg daily or placebo on top of 180 mg aspirin daily. The primary outcome was an ischemic or hemorrhagic stroke within 90 days. Fourteen (7.1 %) patients on clopidogrel had a stroke within 90 days compared with 21 (10.8 %) patients on placebo (risk ratio 0.7, 95 % CI 0.3–1.2). Intracranial haemorrhage occurred in two patients in the clopidogrel group.

The combination of aspirin and clopidogrel was also compared to aspirin monotherapy in patients with a symptomatic $\geq$50 % carotid stenosis due to a TIA or stroke within the last 3 months. The Clopidogrel and Aspirin for Reduction of Emboli in Symptomatic Carotid Stenosis (CARESS) trial first screened 230 patients with transcranial ultrasound for the detection of microembolic signals (Markus et al. 2005). 107 patients with microembolic signals were subsequently randomised to clopidogrel (300 mg loading dose; 75 mg daily) or placebo and 75 mg aspirin daily. The primary end point was the frequency of microembolic signals: 43.8 % in the dual-therapy group had microembolic signals on day 7, as compared with 72.7 % of monotherapy patients (RRR 39.8 %; 95 % CI 13.8–58.0). There were four recurrent strokes and seven TIAs in the monotherapy group during the 1-week

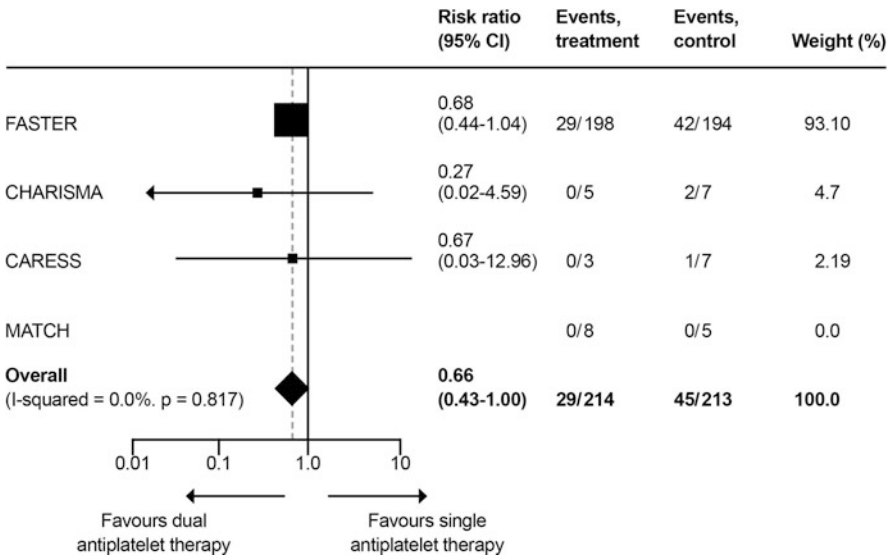


Fig. 1 Fixed-effects meta-analysis of 90-day risk of the combined outcome of stroke, TIA, acute coronary syndrome and all-cause death in stroke and TIA patients enrolled within 24 h of onset in the FASTER, CHARISMA, CARESS and MATCH trial. Note that *x*-axis is a logarithmic scale (Reprinted from The Lancet Neurol, 6: 961–969. Kennedy J, Hill MD, Ryckborst KJ, Eliasziw M, Demchuk AM, Buchan AM. Fast assessment of stroke and transient ischaemic attack to prevent early recurrence (FASTER): a randomised controlled pilot trial. Copyright (2007), with permission from Elsevier)

follow-up versus no stroke and four TIAs in the dual-therapy group that were treatment emergent and ipsilateral to the qualifying carotid stenosis. There was no significant difference in major bleeding (including intracerebral haemorrhage) between the two treatment groups.

A combined analysis of patients treated within 24 h in the FASTER, CARESS, CHARISMA (Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management and Avoidance) and MATCH (Management of ATherothrombosis with Clopidogrel in High-risk patients with recent TIA or ischemic stroke) trials showed a just non-significant 34 % RRR in patients on dual antiplatelet therapy with clopidogrel and aspirin vs. aspirin monotherapy (Fig. 1) (Kennedy et al. 2007): 13.5 % patients in the dual platelet therapy group and 14.4 % patients in the aspirin monotherapy group suffered a stroke (risk ratio 0.66, 95 % CI 0.34–1.00).

The MATCH trial had shown that major bleedings significantly increased the risk after about 90 days using the combination clopidogrel and aspirin in patients after ischemic stroke (Diener et al. 2004). Pooling data from the EXPRESS and FASTER studies, Geraghty and co-workers assessed bleeding risk under this combination in the first 90 days after stroke symptom onset (Geraghty et al. 2010). Major or life-threatening bleeding under aspirin plus clopidogrel occurred in 9 out of 241 aspirin-naive patients in 90 days versus in 1 out of 204 prior-aspirin patients ($p = 0.009$).

Therefore, the early bleeding risk under aspirin plus clopidogrel seems to be a cause for concern mainly in aspirin-naive patients.

In summary, patients with an acute TIA or minor ischemic stroke might benefit from a dual antiplatelet therapy for at least 30 days. However, one has to keep in mind that all aforementioned trials investigating the combination of aspirin and clopidogrel in acute stroke patients were small. There are three ongoing trials investigating the efficacy and safety of combination antiplatelet therapy (clopidogrel plus aspirin or clopidogrel plus aspirin/dipyridamole) within the first 48 h after symptom onset in patients with ischemic stroke or TIA (Rothwell et al. 2011) (Table 1). The results of these large trials will be required before definite recommendations can be made.

2.4 Glycoprotein IIb/IIIa Inhibitors

The phase III AbESTT (Abciximab in Emergency Treatment of Stroke Trial) II study had to be terminated prematurely after enrolment of 808 patients due to a significantly increased bleeding rate in the active treatment group (Adams et al. 2008). During the first 5 days of enrolment, 5.5 % of patients who had received intravenously administered abciximab within 5 h of onset of stroke had symptomatic or fatal intracranial haemorrhage versus 0.5 % of placebo-treated patients ($p = 0.002$). Thus, glycoprotein IIb/IIIa inhibitors are not recommended in acute secondary stroke prevention.

2.5 Antiplatelets Versus Anticoagulation in Acute Ischemic Stroke

There has been a long debate about the use of anticoagulants in acute (cardioembolic) ischemic stroke, but there is no evidence for their use in the first 48 h. A meta-analysis involving 4,624 patients with acute cardioembolic stroke (mainly due to atrial fibrillation), compared anticoagulants (unfractionated heparin, low-molecular-weight heparin, or heparinoids), started within 48 h, with other treatments (aspirin monotherapy or placebo) (Paciaroni et al. 2007). Anticoagulants were associated with a non-significant reduction in recurrent ischemic stroke within 7–14 days (3.0 % vs. 4.9 %, odds ratio 0.68, 95 % CI 0.44–1.06), a significant increase in symptomatic intracranial bleeding (2.5 % vs. 0.7 %, odds ratio 2.89, 95 % CI 1.19–7.01), and a similar rate of death or disability at final follow up (73.5 % vs. 73.8 %, odds ratio 1.01, 95 % CI 0.82–1.24).

In summary, antiplatelet agents are the treatment of choice in the first 48 h in *both* patients with an ischemic stroke of arterial and cardioembolic stroke. Full-dose heparin is recommended by some experts as an option in the acute post-ischemic phase in patients with cardiac sources of embolism with high risk of re-embolism

Table 1 Ongoing trials on combination antiplatelet therapy in patients with cerebrovascular disease

Trial	Treatment regimens	Included study population	Planned study size	Follow-up duration	Primary outcome parameter	Year started	Estimated study completion date
Platelet-Oriented Inhibition in New TIA and Minor Ischemic Stroke (POINT) (http://clinicaltrials.gov/ct2/show/NCT00991029) *NCT00991029*	Clopidogrel 75 mg/day plus aspirin (50–325 mg/day) versus placebo plus aspirin	Patients with TIA or minor ischemic stroke within 12 h from symptom onset	4,150 patients	90 days	Major ischemic vascular events (ischemic stroke, myocardial infarction, and ischemic vascular death)	2009	2016
COMbination of Clopidogrel and Aspirin for Prevention of Early REcurrence in Acute Atherothrombotic Stroke (COMPRESS) (http://clinicaltrials.gov/ct2/show/NCT00814268) *NCT00814268*	Aspirin 100 mg/day plus clopidogrel 75 mg/day versus Aspirin plus Placebo	Patients with ischemic stroke identified on DWI within 48 h from symptom onset	360 patients	90 days	Number of patients with new lesions on MRI (DWI/FLAIR) at day 30	2008	October 2011
Triple Antiplatelets for Reducing Dependency after Ischemic Stroke (TARDIS) trial (http://www.strokecenter.org/trials/TrialDetail.aspx?tid=959) *ISRCTN47823388*	Clopidogrel plus aspirin/dipyridamole versus Aspirin/dipyridamole	Non-cardioembolic ischemic stroke or TIA within 48 h from symptom onset	5,000 patients	3 years	Stroke severity at 90 days assessed by modified Rankin Scale	2009	2017

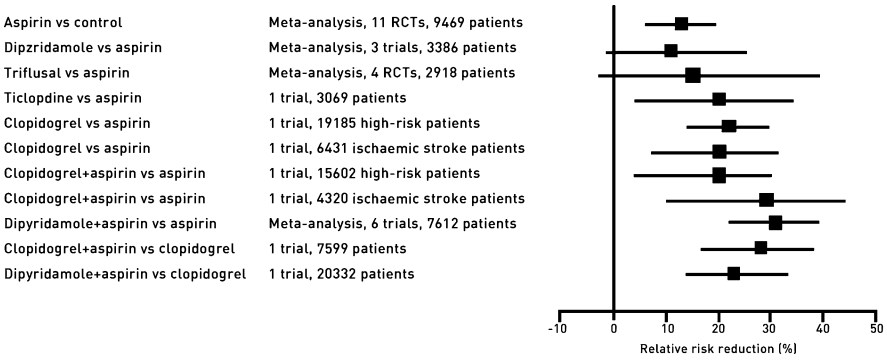


Fig. 2 Relative effects of antiplatelet regimen versus placebo, aspirin and clopidogrel in reducing the risk of stroke, myocardial infarction or vascular death (major vascular events). Point estimates and 95 % CIs are shown in comparison with placebo (zero), aspirin (13 %) or clopidogrel (22 %) (Reprinted from The Lancet Neurol, 9: 273–284. Antithrombotic drugs for patients with ischaemic stroke and transient ischaemic attack to prevent recurrent major vascular events. Copyright (2010), with permission from Elsevier)

(i.e. mechanical valve replacement), arterial dissection or a floating thrombus in the carotid artery prior to surgery (European Stroke Organisation (ESO) Executive Committee and ESO Writing Committee 2008).

3 Antiplatelet Therapy in the Long-Term Secondary Prevention of Non-cardioembolic Ischemic Stroke

Current guidelines of the American Heart Association/American Stroke Association and the European Stroke Organization recommend aspirin/dipyridamole or clopidogrel as first-line treatment in long-term secondary prevention of non-cardioembolic ischemic stroke (European Stroke Organisation (ESO) Executive Committee and ESO Writing Committee 2008; Furie et al. 2011). If these are not available or contraindicated, aspirin monotherapy, triflusal, or cilostazol in Asian patients are alternatives (see Fig. 2).

The efficacy of antiplatelet therapy beyond 4 years after the initial cerebrovascular event has not been studied in randomised trials. Theoretically, the treatment should continue lifelong, unless contraindications or severe bleeding complications emerge.

3.1 Aspirin

A meta-analysis of Algra and van Gijn published in 1999 included 11 randomised placebo-controlled trials investigating aspirin monotherapy in secondary stroke prevention (Algra and van Gijn 1999). Treatment with aspirin resulted in a RRR

of 13 % (95 % CI, 6–19 %) for the combined end point of stroke, myocardial infarction and vascular death. Additional trials comparing aspirin with placebo have not been published thereafter.

There is an ongoing debate about dosing of aspirin in secondary stroke prevention. However, there is no proven relationship between the dosage of aspirin and its efficacy in secondary stroke prevention (Patrono et al. 2005; Campbell et al. 2007). Studies directly comparing the effects of aspirin have not shown a difference in stroke recurrence between doses of 30 mg/daily and 283 mg/daily (The Dutch TIA Trial Study Group 1991), or 300 mg/daily and 1,200 mg/daily (Farrell et al. 1991). However, gastrointestinal side effects and bleeding complications are dose dependent and bleeding rates significantly increase with dosages exceeding 150 mg per day (Campbell et al. 2007).

3.2 Clopidogrel

Clopidogrel (75 mg/daily) was first compared with aspirin (325 mg/daily) for secondary stroke prevention in the randomised CAPRIE (Clopidogrel vs. Aspirin in Patients at Risk of Ischemic Events) trial in 19,185 patients with ischemic stroke, myocardial infarction or symptomatic peripheral arterial disease (CAPRIE Steering Committee 1996). The composite end point of vascular events (stroke, myocardial infarction and vascular death) was just significantly reduced by clopidogrel (RRR 8.7 %, 95 % CI 0.3–16.5 %) after a mean follow-up of 1.9 years (ARR of 0.5 %/year). The risks of gastrointestinal bleeds (2.0 % vs. 2.7 %) and gastrointestinal side effects (15.0 % vs. 17.6 %) were both significantly lowered with clopidogrel. For the subgroup of patients with ischemic stroke as qualifying event, the RRR was 7.3 % (95 % CI −5.7–18.7), which was not statistically significant.

3.3 Aspirin Plus Clopidogrel

The combination of clopidogrel (75 mg/daily) and aspirin (75 mg/daily) in long-term secondary stroke prevention has been compared with clopidogrel monotherapy in the MATCH trial (Diener et al. 2004). 7,599 high-risk patients with ischemic stroke or TIA in the previous 3 months and at least one additional vascular risk factor were treated for 18 months. The primary composite end point (ischemic stroke, myocardial infarction, vascular death, or rehospitalisation for acute ischemia) was reached by 15.7 % in the clopidogrel/aspirin group and 16.7 % in the clopidogrel monotherapy group, which was not statistically significant (RRR 6.4 %, 95 % CI –4.6–16.3). However, the combination therapy resulted in a significant increase of life-threatening bleeding complications (2.6 % vs.1.3 %; absolute risk increase 1.3 %, 95 % CI 0.6–1.9).

A non-prespecified post hoc analysis of the CHARISMA trial that included 9,478 patients with prior myocardial infarction, ischemic stroke or symptomatic peripheral

arterial disease showed a significantly lower rate of cardiovascular death, myocardial infarction or stroke in the clopidogrel plus aspirin arm than in the aspirin monotherapy arm (7.3 % vs. 8.8 %; hazard ratio 0.83, 95 % CI 0.72–0.96) (Bhatt et al. 2007).

3.4 Aspirin Plus Dipyridamole

Three large randomised trials investigated the combination of aspirin and dipyridamole in secondary stroke prevention. The Second European Stroke Prevention (ESPS-2) study included 6602 patients to receive aspirin monotherapy (25 mg BID), extended-release dipyridamole (200 mg BID), the combination of aspirin and extended-release dipyridamole, or placebo for a mean follow-up of 2 years (Diener et al. 1996). Aspirin/dipyridamole significantly reduced the primary end point stroke compared to aspirin monotherapy (RRR 23 %, 95 % CI 0.65–0.97) and to placebo (RRR 37 %, 95 % CI 0.48–0.73). Major bleeding complications were seen more frequently with aspirin and the combination of aspirin and dipyridamole, whereas dipyridamole monotherapy had a similar bleeding rate compared with placebo. Cardiac events occurred in similar frequency in the groups treated with dipyridamole compared to aspirin (Diener et al. 2001). A limitation of the ESPS-2 study was the poor study compliance of 71–80 % of the study population. The results of the ESPS-2 study could be replicated by the investigator-initiated ESPRIT (European/Australasian Stroke Prevention in Reversible Ischaemia) trial (Halkes et al. 2006). A total of 2,739 patients with non-cardioembolic TIA/stroke were randomised to unblinded treatment with aspirin (30–325 mg/daily) or the combination of aspirin and dipyridamole (200 mg BID) for a mean follow-up of 3.5 years. The primary end point was the combination of stroke, myocardial infarction, major bleeding complications or vascular death. Adjudication of outcome events was blinded to treatment allocation. The combination of aspirin/dipyridamole significantly reduced this end point (hazard ratio 0.80; 95 % CI 0.66–0.98). A meta-analysis of six randomised controlled trials with a total of 7,648 patients (including ESPS-2 and ESPRIT) found a significant reduction in the overall risk ratio in favour of aspirin plus dipyridamole for stroke alone (relative risk 0.77, 95 % CI 0.67–0.89) and a composite end point of stroke, myocardial infarction and vascular death (relative risk 0.85, 95 % CI 0.76–0.94) (Verro et al. 2008).

The Japanese Aggrenox Stroke Prevention versus Aspirin Programme (JASAP) Study compared extended-release dipyridamole (200 mg BID) plus aspirin (25 mg BID) versus aspirin monotherapy (81 mg/daily) over 1 year in 1,291 Japanese patients (Uchiyama et al. 2011). The primary end point of this study was the event rate of recurrent ischemic stroke. In contrast to ESPS-2 and ESPRIT, treatment with aspirin resulted in a non-significantly lower incidence of ischemic stroke (5.0 %) compared with aspirin/dipyridamole (6.9 %; hazard ratio 1.47, 95 % CI 0.93–2.31). The risks of major haemorrhage were found to be similar between the treatment arms. In comparison to ESPS-2 and ESPRIT, the follow-up period of 1 year was substantially shorter in the JASAP study. Furthermore, the number of included patients was smaller in the JASAP study, which resulted in a limited

statistical power to detect a difference between the two treatments. The results of the JASAP study highlight the importance of performing studies in different ethnic populations. The majority of included participants in ESPS-2 and ESPRIT were of Caucasian origin.

In all three aforementioned trials, addition of dipyridamole to aspirin significantly increased the risk of headache especially during the first days of intake, and resulted in a higher discontinuation rate. The pathophysiology of this dipyridamole associated headache is not exactly known but there are similarities with migraine headache (Diener and Davidai 2007). Dipyridamole increases the extracellular level of adenosine, which activates the enzyme adenylate cyclase and finally results in vasodilation. Furthermore, the enzyme phosphodiesterase is inhibited by dipyridamole which results in an increase of cyclic GMP by endothelium-derived vasodilation factors (i.e. nitric oxide). This vasodilation might be responsible for the increased headache rate. In clinical practice, the headache rate can be lowered by an initial titration phase with a lower starting dose of dipyridamole as shown in small randomised trials (Lindgren et al. 2004; Chang et al. 2006). In case of such a lower dose of dipyridamole, one has to add 50 mg of aspirin per day.

3.5 Aspirin Plus Dipyridamole Versus Clopidogrel

The PRoFESS trial directly compared the combination of aspirin (25 mg BID) and extended-release dipyridamole (200 mg BID) with clopidogrel (75 mg/daily) in 20,332 patients during a mean follow-up period of 2.5 years (Sacco et al. 2008). The primary outcome was a first recurrent stroke and occurred in 9.0 % of patients receiving aspirin/dipyridamole and in 8.8 % receiving clopidogrel (hazard ratio 1.01; 95 % CI 0.92–1.11). The secondary outcome was a composite of stroke, myocardial infarction or death from vascular causes and also did not differ between both treatment groups. Aspirin/dipyridamole resulted in significantly more intracranial haemorrhages (1.4 % vs. 1.0 %; hazard ratio 1.42; 95 % CI 1.11–1.83) and again a higher dropout rate due to headache compared with clopidogrel (5.9 % vs. 0.9 %). There was no subgroup of patients who had any benefit of one treatment regimen over the other.

3.6 Triflusal

Triflusal is a drug of the salicylate family but it is not a derivative of acetylsalicylic acid. A meta-analysis comprising 4 randomised trials with a total of 2,944 patients showed no significant difference between triflusal and aspirin in the risk of serious vascular events (OR 1.02, 95 % CI 0.83–1.26) (Costa et al. 2005). However, triflusal was associated with a lower risk of hemorrhagic complications, both minor (OR 1.60, 95 % CI 1.31–1.95) and major (OR 2.34, 95 % CI 1.58–3.46). Of note, triflusal is only available in some European countries (i.e. Spain).

3.7 Lotrafiban

The randomised BRAVO (Blockade of the Glycoprotein IIb/IIIa Receptor to Avoid Vascular Occlusion) trial compared the oral glycoprotein IIb/IIIa inhibitor lotrafiban (30 or 50 mg BID) with placebo in 9,190 patients with coronary artery disease or ischemic stroke (Topol et al. 2003). All patients additionally received aspirin (75–325 mg/daily). There was no significant difference in the primary composite end point of all-cause mortality, myocardial infarction, stroke, recurrent ischemia requiring hospitalisation and urgent revascularization (hazard ratio 0.94, 95 % CI 0.85–1.03), but serious bleeding complications were significantly more frequent in the lotrafiban arm (8.0 % vs. 2.8 %, $p < 0.001$).

3.8 Ticlopidine

The first-generation thienopyridine, ticlopidine, has been first investigated in the placebo-controlled Canadian American Ticlopidine Study (CATS) in 1,072 ischemic stroke patients in the mid-1980s (Gent et al. 1989). CATS included 1,072 patients with a thromboembolic stroke between 1 week and 4 months before randomisation. There was a 30.2 % RRR (95 % CI 7.5–48.3 %) of the composite vascular end point (stroke, myocardial infarction or vascular death) during a follow-up of 2 years. Ticlopidine (250 mg BID) has been compared with aspirin (650 mg BID) among 3,069 stroke patients, but did not significantly reduce serious vascular events during a mean follow-up of 3 years (OR 0.93, 95 % CI 0.79–1.09) (Hass et al. 1989). Likewise, the African American Antiplatelet Stroke Prevention Study (AAASPS) found no statistically significant difference between ticlopidine (500 mg daily) and aspirin (650 mg daily) in the prevention of recurrent stroke, myocardial infarction or vascular death during a 2-year follow-up period in 1,809 blacks (hazard ratio 1.22, 95 % CI 0.94–1.57) (Gorelick et al. 2003). Ticlopidine is rarely used any more due to an increased risk of thrombotic thrombocytopenic purpura, neutropenia, skin rashes and diarrhoea (Sudlow et al. 2009).

3.9 Practical Implications for Clinicians: Which Antiplatelet to Choose in Which Stroke/TIA Patient for Long-Term Secondary Stroke Prevention?

Both clopidogrel and the combination aspirin/extended-release dipyridamole are more effective compared to aspirin. However, aspirin is worldwide available and affordable, is familiar to the patient, has an acceptable side-effect profile and single-day dosing. Thus, especially patients at high risk should preferably receive a more potent secondary prevention therapy to derive the greatest benefit in terms of absolute risk reduction. To this aim, several risk stratification scores have been validated. The

Table 2 Essen stroke risk score (ESRS)

Risk factor	Points
Age 65–75 years	1
Age >75 years	2
Arterial hypertension	1
Diabetes mellitus	1
Previous MI	1
Other cardiovascular disease (except MI and atrial fibrillation)	1
Peripheral artery disease	1
Current smoker	1
Previous TIA or ischemic stroke in additionto qualifying event	1
Maximum ESRS score	10

Patients with an ESRS score ≥3 have a recurrent annual stroke risk >4 % and are considered to be at high risk

MI myocardial infarction, *TIA* transient ischemic attack

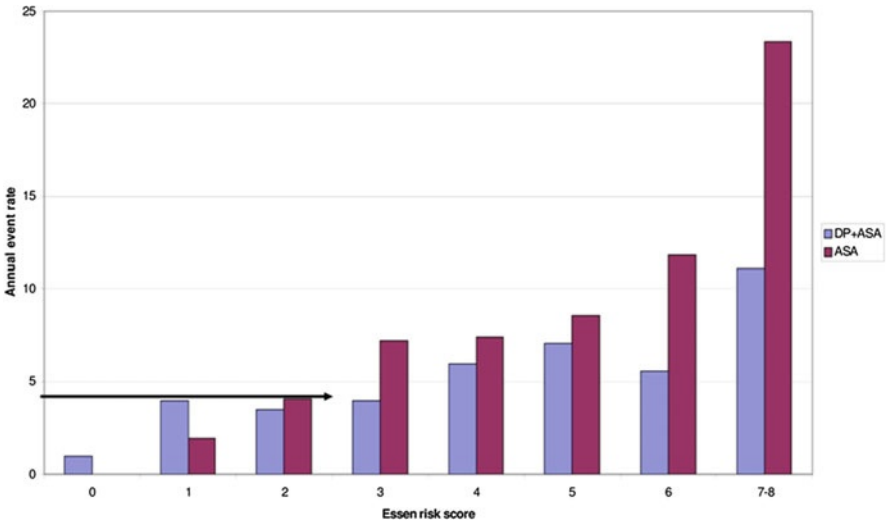


Fig. 3 Annual event rate for stroke (in %) with aspirin (ASA) and the combination of dipyridamole and aspirin (DP + ASA) in the Second European Stroke Prevention (ESPS-2) study stratified by the Essen Stroke Risk Score

Essen stroke risk score (ESRS) was developed from the data subset of 6,431 cerebrovascular patients from the CAPRIE trial and subsequently validated in patients with acute ischemic stroke as well as stable cerebrovascular outpatients (Weimar et al. 2009, 2010). The ESRS is an easy-to-use 10-point scale and predicts 1-year risk of recurrent stroke and combined cardiovascular events (Table 2) (Fitzek et al. 2011). Patients with an ESRS ≥ 3 have a recurrent annual stroke risk > 4 % and thus should be considered to receive either the combination of aspirin and dipyridamole or clopidogrel (Fig. 3). In contrast, low-dose aspirin (85–150 mg/day) is recommended in patients with an ESRS < 3. It is of utmost importance to start any antiplatelet treatment as soon as possible after TIA/stroke to prevent especially recurrent strokes in the acute post-ischemic phase where the risk is highest.

4 Antiplatelets in Patients with Symptomatic Intracranial Stenosis

Symptomatic intracranial stenoses are at very high risk for recurrent stroke and are predominantly prevalent in Asian stroke patients (Weber et al. 2010). Antiplatelet treatment with aspirin has been compared with oral anticoagulation in the WASID (Warfarin-Aspirin Symptomatic Intracranial Disease) trial in patients with symptomatic, angiographically proven intracranial stenosis, and has shown a superiority of aspirin (Chimowitz et al. 2005). WASID included a total of 569 patients to receive a high dose of aspirin (1,300 mg/daily) or warfarin (target INR 2.0–3.0). The trial was stopped prematurely due to safety concerns after a mean follow-up period of 1.8 years. There were significantly less deaths (4.3 % vs. 9.7 %, hazard ratio 0.46, 95 % CI 0.23–0.90), major haemorrhages (3.2 % vs. 8.3 %, hazard ratio 0.39, 95 % CI 0.18–0.84) and myocardial infarctions in the aspirin treatment group (2.9 % vs. 7.3 %, hazard ratio, 0.40, 95 % CI 0.18–0.91). The primary end point (a composite of ischemic stroke, brain haemorrhage or death from vascular causes other than stroke) was not statistically different in both treatment groups (22.1 % with aspirin vs. 21.8 % with warfarin; hazard ratio 1.04, 95 % CI 0.73–1.48).

Best medical treatment alone including antiplatelet therapy has also been shown to be superior to the combination of best medical treatment with angioplasty/stenting of symptomatic intracranial stenoses. The National Institute of Neurological Disorders and Stroke (NINDS) has stopped enrollment in the Stenting and Aggressive Medical Management for Preventing Recurrent Stroke in Intracranial Stenosis (SAMMPRIS) prematurely in April 2011 due to a higher risk of stroke and death in the angioplasty/stenting group (http://members.asnr.org/misc/NINDS_Clinical_Alert.pdf). This randomised multicenter trial enrolled patients with a TIA/stroke that was attributed to an intracranial arterial stenosis within 30 days. Aggressive medical management in both arms consists of aspirin 325 mg/daily for the entire follow-up, clopidogrel 75 mg/daily for 90 days after enrollment, treatment of blood pressure and cholesterol, and a lifestyle modification programme. Within the first 30 days, 14 % of patients treated within the angioplasty/stenting group experienced a stroke or died compared with 5.8 % of patients treated with medical therapy alone, a difference which was highly significant. There were five stroke-related deaths in the stenting arm compared with one in the medical arm within 30 days after enrollment (NCT00576693).

5 Antiplatelet Therapy in Long-Term Prevention of Cardioembolic Ischemic Stroke

Several trials have compared long-term oral anticoagulation with vitamin K antagonists (target INR 2.0–3.0) with single or dual antiplatelet therapy for primary and secondary stroke prevention in patients with atrial fibrillation (AF), which is by

far the most common cause of cardioembolic stroke. Patients with a prior TIA or ischemic stroke and AF are at high risk for stroke ($CHADS_2$ score of at least ≥ 2) and clearly benefit from oral anticoagulation as shown in a meta-analysis by Hart and co-workers. Warfarin reduces the overall risk of stroke by 64 % (95 % CI 49–74 %) compared with placebo in non-valvular AF, while antiplatelets (mostly aspirin) reduce the risk by 22 % (95 % CI 6–35 %). Warfarin led to a small absolute increase in major extracranial haemorrhage of ≤ 0.3 % on the basis of this meta-analysis (Hart et al. 2007).

Warfarin also proved to be superior in older patients with AF and when compared with dual antiplatelet therapy. The BAFTA (Birmingham Atrial Fibrillation Treatment of the Aged) study compared warfarin (target INR 2.0–3.0) with aspirin (75 mg/daily) in 973 patients aged over 75 years (Mant et al. 2007). The primary end point was stroke, intracranial haemorrhage or other arterial embolism. After a mean follow-up of 2.7 years this end point was significantly reduced in patients assigned to warfarin (relative risk 0.48, 95 % CI 0.28–0.80). The ACTIVE W (Atrial Fibrillation Clopidogrel Trial with Irbesartan for Prevention of Vascular Events) trial randomised 6,706 patients with AF (15 % with a history of prior TIA or stroke) to receive warfarin or clopidogrel (75 mg/daily) plus aspirin (75–100 mg/daily) (Connolly et al. 2006). The study was stopped prematurely after a median follow-up of 1.3 years due to a significant reduction in the primary end point of stroke, myocardial infarction, non-CNS systemic embolism or death from vascular causes in patients treated with warfarin (annual risk 3.9 % under warfarin vs. 5.6 % under clopidogrel/warfarin, relative risk 1.44, 95 % CI 1.18–1.76). There was no significant difference in major bleeding between warfarin (2.2 %/year) and the combined antiplatelet therapy (2.4 %/year; relative risk 1.10, 95 % CI 0.83–1.45), but there was a non-significant trend towards a higher intracranial bleeding rate with oral anticoagulation.

Two trials compared vitamin K antagonists (INR target of 2.5–4.0 and 2.0–3.5, respectively) with antiplatelets (aspirin 300 mg/daily or indobufen, a reversible platelet cyclooxygenase inhibitor, administered at a dose of 100 or 200 mg BID) specifically in patients with AF and a prior TIA/stroke. The combined analysis of these two trials confirmed that vitamin K antagonists were significantly more effective than antiplatelet agents both for all vascular events (OR 0.67, 95 % CI 0.50–0.91) and for recurrent stroke (OR 0.49, 95 % CI 0.33–0.72) in this patient group (Saxena and Koudstaal 2004). The risk for major extracranial haemorrhage was significantly higher with anticoagulant therapy (OR 5.16, 95 % CI 2.08–12.83), but there was no difference in the rate of intracranial haemorrhage.

Although oral anticoagulation with vitamin K antagonists is one of the most effective treatments in primary and secondary stroke prevention, about 40–50 % of patients with AF who are at moderate or high risk for stroke do not receive vitamin K antagonists in developed countries, because of the need for frequent laboratory testing and dose adjustments, interactions with food and other drugs, fear of bleeding complications, and patients will (Nieuwlaat et al. 2006). Thus, the quest for alternatives for vitamin K antagonists is a major challenge in stroke prevention. Dual antiplatelet therapy (clopidogrel plus aspirin) was only slightly superior compared with aspirin monotherapy in 7554 AF patients in whom warfarin was

considered inappropriate by the treating physician or unsuitable due to an increased bleeding risk or the patient's preference not to take warfarin (Connolly et al. 2009). The ACTIVE A trial had a median follow-up of 3.6 years. There was a just statistically significant reduction of the composite vascular end point primary in patients receiving dual antiplatelet treatment (6.8 %/year vs. 7.6 %/year; relative risk 0.89, 95 % CI 0.81–0.98). The difference was primarily due to a reduction in the rate of stroke with clopidogrel and aspirin (2.4 %/year vs. 3.3 %/year; relative risk 0.72; 95 % CI 0.62–0.83). However, major bleeding complications were significantly more frequent in patients assigned to clopidogrel plus aspirin (2.0 %/year vs. 1.3 %/year under aspirin; relative risk 1.57, 95 % CI 1.29–1.92).

Several new direct thrombin or factor Xa inhibitors have been or are currently tested in large phase III trials in patients with AF (see Hankey and Eikelboom 2010 for review). These components have the advantage that they can be applied in a fixed-dose regimen, do not need monitoring of coagulation parameters and have only a very limited interaction with other drugs. To date, only the factor Xa inhibitor apixaban has been compared with aspirin (81–324 mg/daily) in a randomised trial in AF patients in whom therapy with vitamin K antagonists was considered unsuitable. The AVERROES (Apixaban vs. Acetylsalicylic Acid to Prevent Stroke in Atrial Fibrillation Patients Who Have Failed or Are Unsuitable for Vitamin K Antagonist Treatment) trial was terminated early after a mean follow-up period of 1.1 years because of a clear benefit in favour of apixaban (Eikelboom et al. 2010a). The primary outcome (stroke or systemic embolism) occurred significantly less often in patients assigned to apixaban (1.6 % vs. 3.7 % under aspirin, hazard ratio 0.45; 95 % CI 0.32–0.62). There was no significant difference in major bleeding (1.4 %/year under apixaban vs. 1.2 %/year under aspirin).

6 Unresolved Problems, Knowledge Gaps and Future Directions of Antiplatelet Therapy in Secondary Stroke Prevention

6.1 Aspirin Resistance

One of the most important questions which has to be addressed in future randomised trials is how to treat stroke/TIA patients who experience a second stroke or other vascular event while being on aspirin or other antiplatelets. There is long-lasting debate about aspirin resistance and its clinical impact (Eikelboom et al. 2010b; Alberts 2010). Present practice patterns include increasing the dose of aspirin (i.e. to 325 mg per day) or switching to another antiplatelet agent, which are not unreasonable approaches but neither has definitive supportive evidence to date.

There are numerous possible causes of laboratory and clinical aspirin resistance: patient non-compliance, drug interactions (i.e. with non-steroidal anti-inflammatory drugs), genetic polymorphisms of the enzyme cylcooxygenase-1 and other genes

involved in thromboxane production, increased biosynthesis of thromboxane by alternative sources (i.e. by cylcooxygenase-2 in macrophages or vascular endothelial cells) and increased platelet turnover (Patrono et al. 2005; Hankey and Eikelboom 2006). However, there is still no gold standard available for determining laboratory aspirin resistance such as monitoring international normalised ratio in patients treated with vitamin K antagonists (Harrison et al. 2008; Santilli et al. 2009). The Popular (Do Platelet Function Assays Predict Clinical Outcomes in Clopidogrel-Pretreated Patients Undergoing Elective PCI) study is the largest study so far, which has prospectively evaluated the capability of five different platelet function tests to predict clinical outcome (Breet et al. 2010). The primary end point was a composite of all-cause death, myocardial infarction, stent thrombosis and ischemic stroke. A total of 1,069 consecutive patients undergoing elective coronary stenting followed by dual antiplatelet therapy with aspirin and clopidogrel (maintenance doses for aspirin were 80–100 mg/daily and 75 mg/daily for clopidogrel) were followed for 1 year. Only three of the five platelet function tests were significantly associated with the primary end point, and none was able to predict ischemic stroke alone.

Given this lack of evidence, one should thoroughly reexamine stroke aetiology in every patient who suffers a second stroke being on aspirin or another antiplatelet treatment, because a significant portion might have had cardioembolic stroke (i.e. due to non-detected paroxysmal AF) (Stahrenberg et al. 2010).

6.2 Antiplatelets in Stroke Patients with Patent Foramen Ovale

A congenital patent foramen ovale (PFO) is found in about 25 % of the general population (Hagen et al. 1984), and is significantly more prevalent in patients with a stroke of unknown aetiology (cryptogenic stroke) (Overell et al. 2000; Handke et al. 2007). There is an ongoing debate about the optimal secondary stroke prevention regimen in this patient group. In our opinion, current evidence from observational and small randomised trials supports the use of aspirin monotherapy as initial treatment in patients with cryptogenic stroke and evidence of a PFO. A prospective observational study in France enrolled 581 consecutive patients (18–55 years old) who had had an ischemic stroke of unknown origin within the preceding 3 months to receive 300 mg aspirin daily (Mas et al. 2001). After 4 years, the risk of recurrent stroke was 2.3 % (95 % CI 0.3–4.3 %) among the patients with PFO alone, 15.2 % (95 % CI 1.8–28.6 %) among patients with both PFO and atrial septal aneurysm, and 4.2 % (95 % CI 1.8–6.6 %) among the patients with neither of these cardiac abnormalities. Although not sufficiently powered, the PICSS (Patent foramen ovale In Cryptogenic Stroke Study) study did not find any evidence for superiority of oral anticoagulation (target INR 1.4–2.8) in comparison with aspirin (325 mg/daily) in 203 cryptogenic stroke patients with PFO (Homma et al. 2002). Preliminary results of the randomised CLOSURE I trial which compared closure of the PFO with a device and best medical therapy (aspirin 325 mg/daily and/or warfarin) in patients aged 18–60 years did not show superiority of the percutaneous intervention in the prevention of recurrent strokes and TIAs (NCT00201461).

6.3 Antiplatelets in Dissections of Brain Supplying Arteries

Dissections of the extra- and intracranial brain supplying arteries are a rare cause of stroke especially in younger stroke patients. Potential stroke mechanisms include both hemodynamic impairment from reduced flow in stenotic or occluded arteries and arterial thromboembolism. As a consequence, antithrombotic treatment is required in patients with a recent non-hemorrhagic cervicocephalic arterial dissection, but the type of antithrombotic treatment—anticoagulants or aspirin—or treatment duration is still debated (Arnold et al. 2011). There is no randomised trial to date comparing anticoagulation with antiplatelet treatment in this patient group. A meta-analysis including all trials published up to April 2007 did not find evidence for superiority of anticoagulants over antiplatelet agents (Menon et al. 2008). Low-dose aspirin was the antiplatelet agent most often used in the included studies, but overall only 185 patients treated with antiplatelet agents were included in the small trials. A prospective observational study on 298 consecutive patients with spontaneous dissection of the cervical carotid artery showed a low frequency of new cerebral (ischemic stroke, 0.3 %; TIA, 3.4 %) and retinal ischemic events (1 %) after 3 months and no significant difference between patients treated with anticoagulants (5.9 %) and those treated with aspirin (2.1 %) (Georgiadis et al. 2009).

It has been estimated that 1,400 patients have to be enrolled in each treatment arm in a randomised trial comparing antiplatelet agents and anticoagulation. It is very unlikely that such a trial will be performed. Engelter and co-workers therefore suggested an individual treatment approach based on clinical and paraclinical characteristics (Engelter et al. 2007). Immediate anticoagulation is recommended in patients with free-floating thrombus, multiple ischemic events, detection of microemboli in transcranial ultrasound studies or arterial (pseudo)occlusion of the dissected artery. In all other patients, especially patients with intracranial dissections, initial antiplatelet therapy with aspirin is reasonable.

6.4 New Antiplatelet Drugs that Have Been Investigated in Secondary Stroke Prevention

Given the only modest treatment effects of aspirin, the combination of aspirin and dipyridamole, and clopidogrel, several new antiplatelet agents have been or are currently under investigation in secondary stroke prevention (Weber and Diener 2010).

6.4.1 Cilostazol

The antiplatelet agent cilostazol, a phosphodiesterase inhibitor, is a promising new antiplatelet candidate for secondary stroke prevention, which has been investigated only in Asian patients so far. Besides its action on platelet aggregation, cilostazol

promotes arterial vasodilation, suppressed smooth muscle cell proliferation and intimal hyperplasia in animal models. Cilostazol prevented the progression of symptomatic intracranial atherosclerotic stenosis of the middle cerebral artery when added to aspirin 100 mg/daily in a randomised, double-blind and placebo-controlled study (Kwon et al. 2005). A randomised pilot trial in 720 Chinese patients who had had an ischemic stroke within the previous 1–6 months compared aspirin (100 mg/daily) with cilostazol (100 mg BID) and did not show a significant difference in stroke recurrence during a follow-up period of 1 year (hazard ration 0.62, 95 % CI 0.30–1.26) (Huang et al. 2008). The largest trial to date, the Cilostazol Stroke Prevention Study (CSPS) 2, also investigated cilostazol (100 mg BID) vs. aspirin (81 mg/daily) in 2,672 Japanese stroke patients with a non-cardioembolic in the previous 6 months and followed them for a median of 29 months (Shinohara et al. 2010). The primary end point was the first occurrence of stroke (cerebral infarction, cerebral haemorrhage or subarachnoid haemorrhage) which occurred significantly lower in the cilostazol group (2.76 %/year vs. 3.71 %/year in the aspirin group; hazard ratio 0.74, 95 % CI 0.56–0·98). Hemorrhagic events (cerebral haemorrhage, subarachnoid haemorrhage or haemorrhage requiring hospital admission) were markedly reduced in the cilostazol group (0·77 % vs. 1.78 % in the aspirin group, hazard ratio 0.46, 95 % CI 0.30–0.71).

6.4.2 Terutroban

Terutroban, an oral selective antagonist of the thromboxane-prostaglandin A2 receptor, has also been shown to prevent atherosclerosis by reducing inflammation and proliferation and to cause vasodilation of peripheral arteries. Terutroban (30 mg/daily) has been compared with aspirin (30 mg/daily) in the recently published PERFORM (Prevention of cerebrovascular and cardiovascular Events of ischaemic origin with teRutroban in patients with a history oF ischaemic strOke or tRansient ischaeMic attack) trial (Bousser et al. 2011). PERFORM was stopped prematurely for futility after a total of 19,120 patients with a recent ischemic stroke ($\leq$3 months) or TIA ($\leq$8 days) had been followed-up for a mean of 28.3 months. The primary efficacy end point was a composite of fatal or non-fatal ischemic stroke, fatal or non-fatal myocardial infarction, or other vascular death, and occurred in 11 % of patients in both treatment groups (hazard ratio 1.02, 95 % CI 0.94–1.12).

6.4.3 Sarpogrelate

Sarpogrelate is a selective inhibitor of the 5-hydroxytryptamine receptor which is involved in platelet aggregation and vasoconstriction. Sarpogrelate (100 mg three times daily) has been compared to aspirin (81 mg/daily) in 1,510 Japanese patients with ischemic stroke in the previous 6 months in the S-ACCESS (Sarpogrelate-Aspirin Comparative Clinical Study for Efficacy and Safety in Secondary Prevention of Cerebral Infarction) trial (Shinohara et al. 2008). The primary efficacy end

point was recurrence of cerebral infarction. After a mean follow-up of 1.6 years, sarpogrelate was not able to show non-inferiority to aspirin (hazard ratio 1.25, 95 % CI 0.89–1.77). However, the incidence rate of bleeding was significantly lower in patients treated with sarpogrelate (11.9 % vs. 17.3 %).

6.4.4 SCH 530348

SCH 530348 (voraxapar) is a selective antagonist of the platelet PAR1-receptor which is involved in platelet aggregation. SCH 530348 is currently evaluated in the randomised placebo-controlled TRA 2°P-TIMI 50 (Trial to Assess the Effects of SCH 530348 in Preventing Heart Attack and Stroke in Patients with Atherosclerosis) trial, which started in 2007 and plans to enroll 27,000 patients with myocardial infarction, ischemic stroke of presumed arterial aetiology (time period $\geq$2 weeks and $\leq$12 months after the stroke) or peripheral artery disease. The primary end point is a composite of cardiovascular death, myocardial infarction, stroke or coronary revascularisation. Among the first 12,000 randomised patients, 16 % had an ischemic stroke as qualifying event (Morrow et al. 2009). Due to an excess in intracranial bleedings in patients randomised to SCH 530348, the Data and Safety Monitoring Committee of the TRA 2°P-TIMI 50 trial recommended suspending further treatment with study medication in those subjects with a history of ischemic stroke. The study was allowed to continue in the other two arms (Van de Werf 2011).

6.5 New Oral Anticoagulants in Acute Treatment of Stroke/TIA and Secondary Prevention of Non-cardioembolic Stroke

The efficacy of the oral anticoagulant warfarin was compared to aspirin in patients with an ischemic stroke/TIA of arterial origin in SPIRIT (INR target of 3.0–4.5), WARSS (INR target of 1.4–2.8) and ESPRIT (INR target of 2.0–3.0) (The Stroke Prevention in Reversible Ischemia Trial (SPIRIT) Study Group 1997; Mohr et al. 2001; Halkes et al. 2007). None of the trials found anticoagulants to be more effective than aspirin, whereas high-intensity warfarin was not safe because of an excess of major bleedings (The Stroke Prevention in Reversible Ischemia Trial (SPIRIT) Study Group 1997). The accompanying editorial to the ESPRIT paper therefore concluded that “the coffin should remain shut” until anticoagulants with less propensity to cause haemorrhage are found (Elkind 2007). The new thrombin and factor Xa inhibitors might be such candidates. The direct thrombin inhibitor dabigatran and the factor Xa inhibitors rivaroxaban and Apixaban had significantly reduced rates of intracranial haemorrhages in comparison with warfarin in patients with atrial fibrillation (Eikelboom et al. 2011; Patel et al. 2011). However, patients were not included in these trials in the first 14 days after their index ischemic stroke.

As a consequence, further trials comparing these agents with antiplatelet therapy are needed and desirable to assess their efficacy and safety in patients with acute TIA/ischemic stroke and patients with non-cardioembolic strokes.

Knowledge Gaps

- The most important and challenging knowledge gap at the moment is the treatment of patients who experienced a stroke while being on aspirin, clopidogrel or the combination of aspirin/extended-release dipyridamole. Should we change the antiplatelet agent, and if so, which antiplatelet drug is recommended?
- There is no data from adequately powered randomised trials at the moment, whether the combination of aspirin and clopidogrel is superior to aspirin monotherapy in the acute and early secondary stroke prevention (i.e. first 30–90 days). Ongoing trials addressing this question are underway.
- The antithrombotic treatment of choice (antiplatelets or anticoagulants) is not known in patients with dissections of the extra- and intracranial brain supplying arteries.
- There is no data available that individualised antiplatelet therapy based on the use of antiplatelet function testing is able to reduce aspirin or clopidogrel non-responsiveness in secondary stroke prevention.
- It is not known whether the new phosphodiesterase inhibitor cilostazol might also be an alternative to aspirin in secondary stroke prevention of non-Asian patients. Furthermore, there are no comparative trials between cilostazol and the combination of aspirin/extended-release dipyridamole and clopidogrel, respectively.
- It is not known whether the new thienopyridine prasugrel is more efficacious than, and at least as safe as, clopidogrel in secondary stroke prevention.
- It is not known whether the new oral direct thrombin or factor Xa inhibitors are also safe in the first 14 days after ischemic stroke or whether these agents are also superior to antiplatelets in patients with non-cardioembolic stroke.

Key Messages

- Antiplatelet therapy is the treatment of choice in acute secondary stroke prevention (first 48 h) both in patients with ischemic stroke of arterial origin and cardioembolic stroke.
- The combination of aspirin with extended-release dipyridamole or clopidogrel might be superior to aspirin monotherapy in patients with acute ischemic stroke of arterial origin, but more data from randomised trials are needed.
- Glycoprotein IIb/IIIa inhibitors are not recommended for acute and long-term secondary stroke prevention.
- Clopidogrel and the combination of aspirin/extended-release dipyridamole are superior to aspirin monotherapy in the long-term secondary prevention of non-cardioembolic ischemic stroke.
- The combination of aspirin and clopidogrel is not recommended for the long-term secondary prevention of non-cardioembolic ischemic stroke due to an increased bleeding rate.
- Anticoagulation is superior to antiplatelet therapy in the long-term stroke prevention of patients with cardioembolic stroke.
- Antiplatelet therapy is treatment of choice in patients with symptomatic intracranial stenoses and stroke patients with evidence of patent foramen ovale.
- It is unclear how to treat stroke/TIA patients who experience a recurrent stroke while being on aspirin or another antiplatelet agent.
- The new antiplatelet agent cilostazol might be a future option in secondary stroke prevention due to a lower bleeding rate, but has been investigated only in Asians so far.

References

Adams HP Jr, Effron MB, Torner J, Davalos A, Frayne J, Teal P et al (2008) Emergency administration of abciximab for treatment of patients with acute ischemic stroke: results of an international phase III trial: Abciximab in Emergency Treatment of Stroke Trial (AbESTT-II). Stroke 39:87–99

Alberts MJ (2010) Platelet function testing for aspirin resistance is reasonable to do: yes! Stroke 41:2400–2401

Algra A, van Gijn J (1999) Cumulative meta-analysis of aspirin efficacy after cerebral ischaemia of arterial origin. J Neurol Neurosurg Psychiatry 66:255

Antithrombotic Trialists' Collaboration (2002) Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. BMJ 324:71–86

Arnold M, Fischer U, Bousser MG (2011) Treatment issues in spontaneous cervicocephalic artery dissections. Int J Stroke 6:213–218

Bath PM, Cotton D, Martin RH, Palesch Y, Yusuf S, Sacco R et al (2010) Effect of combined aspirin and extended-release dipyridamole versus clopidogrel on functional outcome and recurrence in acute, mild ischemic stroke: PRoFESS subgroup analysis. Stroke 41:732–738

Bhatt DL, Flather MD, Hacke W, Berger PB, Black HR, Boden WE et al (2007) Patients with prior myocardial infarction, stroke, or symptomatic peripheral arterial disease in the CHARISMA trial. J Am Coll Cardiol 49:1982–1988

Bousser M-G, Amarenco P, Chamorro A, Fisher M, Ford I, Fox KM et al (2011) Terutroban versus aspirin in patients with cerebral ischaemic events (PERFORM): a randomised, double-blind, parallel-group trial. Lancet 377:2013–2022

Breet NJ, van Werkum JW, Bouman HJ, Kelder JC, Ruven HJ, Bal ET et al (2010) Comparison of platelet function tests in predicting clinical outcome in patients undergoing coronary stent implantation. JAMA 303:754–762

Campbell CL, Smyth S, Montalescot G, Steinhubl SR (2007) Aspirin dose for the prevention of cardiovascular disease: a systematic review. JAMA 297:2018–2024

CAPRIE Steering Committee (1996) A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). Lancet 348:1329–1339

Chang YJ, Ryu SJ, Lee TH (2006) Dose titration to reduce dipyridamole-related headache. Cerebrovasc Dis 22:258–262

Chimowitz MI, Lynn MJ, Howlett-Smith H, Stern BJ, Hertzberg VS, Frankel MR et al (2005) Comparison of warfarin and aspirin for symptomatic intracranial arterial stenosis. N Engl J Med 352:1305–1316

Chinese Acute Stroke Trial Collaborative Group (1997) CAST: randomised placebo-controlled trial of early aspirin use in 20,000 patients with acute ischaemic stroke. Lancet 349:1641–1649

Connolly S, Pogue J, Hart R, Pfeffer M, Hohnloser S, Chrolavicius S et al (2006) Clopidogrel plus aspirin versus oral anticoagulation for atrial fibrillation in the Atrial fibrillation Clopidogrel Trial with Irbesartan for prevention of Vascular Events (ACTIVE W): a randomised controlled trial. Lancet 367:1903–1912

Connolly SJ, Pogue J, Hart RG, Hohnloser SH, Pfeffer M, Chrolavicius S et al (2009) Effect of clopidogrel added to aspirin in patients with atrial fibrillation. N Engl J Med 360:2066–2078

Costa J, Ferro JM, Matias-Guiu J, Alvarez-Sabin J, Torres F (2005) Triflusal for preventing serious vascular events in people at high risk. Cochrane Database Syst Rev CD004296

Dengler R, Diener HC, Schwartz A, Grond M, Schumacher H, Machnig T et al (2010) Early treatment with aspirin plus extended-release dipyridamole for transient ischaemic attack or ischaemic stroke within 24 h of symptom onset (EARLY trial): a randomised, open-label, blinded-endpoint trial. Lancet Neurol 9:159–166

Diener HC, Davidai G (2007) Dipyridamole and headache. Future Neurol 2:279–283

Diener HC, Cunha L, Forbes C, Sivenius J, Smets P, Lowenthal A (1996) European Stroke Prevention Study. 2. Dipyridamole and acetylsalicylic acid in the secondary prevention of stroke. J Neurol Sci 143:1–13

Diener HC, Darius H, Bertrand-Hardy JM, Humphreys M (2001) Cardiac safety in the European Stroke Prevention Study 2 (ESPS2). Int J Clin Pract 55:162–163

Diener HC, Bogousslavsky J, Brass LM, Cimminiello C, Csiba L, Kaste M et al (2004) Aspirin and clopidogrel compared with clopidogrel alone after recent ischaemic stroke or transient ischaemic attack in high-risk patients (MATCH): randomised, double-blind, placebo-controlled trial. Lancet 364:331–337

Eikelboom JW, O'Donnell M, Yusuf S, Diaz R, Flaker G, Hart R et al (2010a) Rationale and design of AVERROES: apixaban versus acetylsalicylic acid to prevent stroke in atrial fibrillation patients who have failed or are unsuitable for vitamin K antagonist treatment. Am Heart J 159:348.e1–353.e1

Eikelboom JW, Emery J, Hankey GJ (2010b) The use of platelet function assays may help to determine appropriate antiplatelet treatment options in a patient with recurrent stroke on baby aspirin: against. Stroke 41:2398–2399

Eikelboom JW, Wallentin L, Connolly SJ, Ezekowitz M, Healey JS, Oldgren J et al (2011) Risk of bleeding with 2 doses of dabigatran compared with warfarin in older and younger patients with atrial fibrillation: an analysis of the randomized evaluation of long-term anticoagulant therapy (RE-LY) trial. Circulation 123:2363–2372

Eisert W, Schlachetzki F (2008) Vascular endothelium and the blood-brain barrier. Handb Clin Neurol 92:197–214

Elkind MS (2007) Anticoagulation for secondary stroke prevention: another nail in the coffin? Lancet Neurol 6:97–99

Engelter ST, Brandt T, Debette S, Caso V, Lichy C, Pezzini A et al (2007) Antiplatelets versus anticoagulation in cervical artery dissection. Stroke 38:2605–2611

European Stroke Organisation (ESO) Executive Committee, ESO Writing Committee (2008) Guidelines for management of ischaemic stroke and transient ischaemic attack 2008. Cerebrovasc Dis 25:457–507

Farrell B, Godwin J, Richards S, Warlow C (1991) The United Kingdom transient ischaemic attack (UK-TIA) aspirin trial: final results. J Neurol Neurosurg Psychiatry 54:1044–1054

Fitzek S, Leistritz L, Witte OW, Heuschmann PU, Fitzek C (2011) The Essen Stroke Risk Score in one-year follow-up acute ischemic stroke patients. Cerebrovasc Dis 31:400–407

Furie KL, Kasner SE, Adams RJ, Albers GW, Bush RL, Fagan SC et al (2011) Guidelines for the prevention of stroke in patients with stroke or transient ischemic attack: a guideline for healthcare professionals from the american heart association/american stroke association. Stroke 42:227–276

Gent M, Blakely JA, Easton JD, Ellis DJ, Hachinski VC, Harbison JW et al (1989) The Canadian American Ticlopidine Study (CATS) in thromboembolic stroke. Lancet 1:1215–1220

Georgiadis D, Arnold M, von Buedingen HC, Valko P, Sarikaya H, Rousson V et al (2009) Aspirin vs anticoagulation in carotid artery dissection: a study of 298 patients. Neurology 72:1810–1815

Geraghty OC, Kennedy J, Chandratheva A, Marquardt L, Buchan AM, Rothwell PM (2010) Preliminary evidence of a high risk of bleeding on aspirin plus clopidogrel in aspirin-naive patients in the acute phase after TIA or minor ischaemic stroke. Cerebrovasc Dis 29:460–467

Giles MF, Rothwell PM (2007) Risk of stroke early after transient ischaemic attack: a systematic review and meta-analysis. Lancet Neurol 6:1063–1072

Gorelick PB, Richardson D, Kelly M, Ruland S, Hung E, Harris Y et al (2003) Aspirin and ticlopidine for prevention of recurrent stroke in black patients: a randomized trial. JAMA 289:2947–2957

Hagen PT, Scholz DG, Edwards WD (1984) Incidence and size of patent foramen ovale during the first 10 decades of life: an autopsy study of 965 normal hearts. Mayo Clin Proc 59:17–20

Halkes PH, van Gijn J, Kappelle LJ, Koudstaal PJ, Algra A (2006) Aspirin plus dipyridamole versus aspirin alone after cerebral ischaemia of arterial origin (ESPRIT): randomised controlled trial. Lancet 367:1665–1673

Halkes PH, van Gijn J, Kappelle LJ, Koudstaal PJ, Algra A (2007) Medium intensity oral anticoagulants versus aspirin after cerebral ischaemia of arterial origin (ESPRIT): a randomised controlled trial. Lancet Neurol 6:115–124

Handke M, Harloff A, Olschewski M, Hetzel A, Geibel A (2007) Patent foramen ovale and cryptogenic stroke in older patients. N Engl J Med 357:2262–2268

Hankey GJ, Eikelboom JW (2006) Aspirin resistance. Lancet 367:606–617

Hankey GJ, Eikelboom JW (2010) Antithrombotic drugs for patients with ischaemic stroke and transient ischaemic attack to prevent recurrent major vascular events. Lancet Neurol 9:273–284

Harrison P, Segal H, Silver L, Syed A, Cuthbertson FC, Rothwell PM (2008) Lack of reproducibility of assessment of aspirin responsiveness by optical aggregometry and two platelet function tests. Platelets 19:119–124

Hart RG, Pearce LA, Aguilar MI (2007) Meta-analysis: antithrombotic therapy to prevent stroke in patients who have nonvalvular atrial fibrillation. Ann Intern Med 146:857–867

Hass WK, Easton JD, Adams HP Jr, Pryse-Phillips W, Molony BA, Anderson S et al (1989) A randomized trial comparing ticlopidine hydrochloride with aspirin for the prevention of stroke in high-risk patients. Ticlopidine Aspirin Stroke Study Group. N Engl J Med 321:501–507

Homma S, Sacco RL, Di Tullio MR, Sciacca RR, Mohr JP (2002) Effect of medical treatment in stroke patients with patent foramen ovale: patent foramen ovale in Cryptogenic Stroke Study. Circulation 105:2625–2631

http://clinicaltrials.gov/ct2/show/NCT00814268. Accessed on 09-04-2011

http://clinicaltrials.gov/ct2/show/NCT00991029. Accessed on 09-04-2011

http://www.strokecenter.org/trials/TrialDetail.aspx?tid=959. Accessed on 09-04-2011

Huang Y, Cheng Y, Wu J, Li Y, Xu E, Hong Z et al (2008) Cilostazol as an alternative to aspirin after ischaemic stroke: a randomised, double-blind, pilot study. Lancet Neurol 7:494–499

International Stroke Trial Collaborative Group (1997) The International Stroke Trial (IST): a randomised trial of aspirin, subcutaneous heparin, both, or neither among 19435 patients with acute ischaemic stroke. Lancet 349:1569–1581

Kennedy J, Hill MD, Ryckborst KJ, Eliasziw M, Demchuk AM, Buchan AM (2007) Fast assessment of stroke and transient ischaemic attack to prevent early recurrence (FASTER): a randomised controlled pilot trial. Lancet Neurol 6:961–969

Kwon SU, Cho YJ, Koo JS, Bae HJ, Lee YS, Hong KS et al (2005) Cilostazol prevents the progression of the symptomatic intracranial arterial stenosis: the multicenter double-blind placebo-controlled trial of cilostazol in symptomatic intracranial arterial stenosis. Stroke 36:782–786

Lees KR, Bluhmki E, von Kummer R, Brott TG, Toni D, Grotta JC et al (2010) Time to treatment with intravenous alteplase and outcome in stroke: an updated pooled analysis of ECASS, ATLANTIS, NINDS, and EPITHET trials. Lancet 375:1695–1703

Lindgren A, Husted S, Staaf G, Ziegler B (2004) Dipyridamole and headache–a pilot study of initial dose titration. J Neurol Sci 223:179–184

Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJ (2006) Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. Lancet 367:1747–1757

Mant J, Hobbs FD, Fletcher K, Roalfe A, Fitzmaurice D, Lip GY et al (2007) Warfarin versus aspirin for stroke prevention in an elderly community population with atrial fibrillation (the Birmingham Atrial Fibrillation Treatment of the Aged Study, BAFTA): a randomised controlled trial. Lancet 370:493–503

Markus HS, Droste DW, Kaps M, Larrue V, Lees KR, Siebler M et al (2005) Dual antiplatelet therapy with clopidogrel and aspirin in symptomatic carotid stenosis evaluated using doppler embolic signal detection: the Clopidogrel and Aspirin for Reduction of Emboli in Symptomatic Carotid Stenosis (CARESS) trial. Circulation 111:2233–2240

Mas JL, Arquizan C, Lamy C, Zuber M, Cabanes L, Derumeaux G et al (2001) Recurrent cerebrovascular events associated with patent foramen ovale, atrial septal aneurysm, or both. N Engl J Med 345:1740–1746

Menon R, Kerry S, Norris JW, Markus HS (2008) Treatment of cervical artery dissection: a systematic review and meta-analysis. J Neurol Neurosurg Psychiatry 79:1122–1127

Mohr JP, Thompson JL, Lazar RM, Levin B, Sacco RL, Furie KL et al (2001) A comparison of warfarin and aspirin for the prevention of recurrent ischemic stroke. N Engl J Med 345:1444–1451

Morrow DA, Scirica BM, Fox KA, Berman G, Strony J, Veltri E et al (2009) Evaluation of a novel antiplatelet agent for secondary prevention in patients with a history of atherosclerotic disease: design and rationale for the Thrombin-Receptor Antagonist in Secondary Prevention of Atherothrombotic Ischemic Events (TRA 2 degrees P)-TIMI 50 trial. Am Heart J 158:335.e3–341.e3

Nieuwlaat R, Capucci A, Lip GY, Olsson SB, Prins MH, Nieman FH et al (2006) Antithrombotic treatment in real-life atrial fibrillation patients: a report from the Euro Heart Survey on Atrial Fibrillation. Eur Heart J 27:3018–3026

Overell JR, Bone I, Lees KR (2000) Interatrial septal abnormalities and stroke: a meta-analysis of case-control studies. Neurology 55:1172–1179

Paciaroni M, Agnelli G, Micheli S, Caso V (2007) Efficacy and safety of anticoagulant treatment in acute cardioembolic stroke: a meta-analysis of randomized controlled trials. Stroke 38:423–430

Patel MR, Mahaffey KW, Garg J, Pan G, Singer DE, Hacke W et al (2011) Rivaroxaban versus warfarin in nonvalvular atrial fibrillation. N Engl J Med 365(10):883–891

Patrono C, Garcia Rodriguez LA, Landolfi R, Baigent C (2005) Low-dose aspirin for the prevention of atherothrombosis. N Engl J Med 353:2373–2383

Rothwell PM, Algra A, Amarenco P (2011) Medical treatment in acute and long-term secondary prevention after transient ischaemic attack and ischaemic stroke. Lancet 377:1681–1692

Sacco RL, Diener HC, Yusuf S, Cotton D, Ounpuu S, Lawton WA et al (2008) Aspirin and extended-release dipyridamole versus clopidogrel for recurrent stroke. N Engl J Med 359:1238–1251

Santilli F, Rocca B, De Cristofaro R, Lattanzio S, Pietrangelo L, Habib A et al (2009) Platelet cyclooxygenase inhibition by low-dose aspirin is not reflected consistently by platelet function assays: implications for aspirin "resistance". J Am Coll Cardiol 53:667–677

Saxena R, Koudstaal P (2004) Anticoagulants versus antiplatelet therapy for preventing stroke in patients with nonrheumatic atrial fibrillation and a history of stroke or transient ischemic attack. Cochrane Database Syst Rev CD000187

Shinohara Y, Nishimaru K, Sawada T, Terashi A, Handa S, Hirai S et al (2008) Sarpogrelate-Aspirin Comparative Clinical Study for Efficacy and Safety in Secondary Prevention of Cerebral Infarction (S-ACCESS): a randomized, double-blind, aspirin-controlled trial. Stroke 39:1827–1833

Shinohara Y, Katayama Y, Uchiyama S, Yamaguchi T, Handa S, Matsuoka K et al (2010) Cilostazol for prevention of secondary stroke (CSPS 2): an aspirin-controlled, double-blind, randomised non-inferiority trial. Lancet Neurol 9:959–968

Stahrenberg R, Weber-Kruger M, Seegers J, Edelmann F, Lahno R, Haase B et al (2010) Enhanced detection of paroxysmal atrial fibrillation by early and prolonged continuous holter monitoring in patients with cerebral ischemia presenting in sinus rhythm. Stroke 41:2884–2888

Sudlow CL, Mason G, Maurice JB, Wedderburn CJ, Hankey GJ (2009) Thienopyridine derivatives versus aspirin for preventing stroke and other serious vascular events in high vascular risk patients. Cochrane Database Syst Rev CD001246

The Dutch TIA Trial Study Group (1991) A comparison of two doses of aspirin (30 mg vs. 283 mg a day) in patients after a transient ischemic attack or minor ischemic stroke. N Engl J Med 325:1261–1266

The Stroke Prevention in Reversible Ischemia Trial (SPIRIT) Study Group (1997) A randomized trial of anticoagulants versus aspirin after cerebral ischemia of presumed arterial origin. Ann Neurol 42:857–865

Topol EJ, Easton D, Harrington RA, Amarenco P, Califf RM, Graffagnino C et al (2003) Randomized, double-blind, placebo-controlled, international trial of the oral IIb/IIIa antagonist lotrafiban in coronary and cerebrovascular disease. Circulation 108:399–406

Uchiyama S, Ikeda Y, Urano Y, Horie Y, Yamaguchi T (2011) The Japanese Aggrenox (Extended-Release Dipyridamole plus Aspirin) Stroke Prevention versus Aspirin Programme (JASAP) Study: a randomized, double-blind, controlled trial. Cerebrovasc Dis 31:601–613

Van de Werf F (2011) Inhibitors of the platelet thrombin receptor: will they live up to their promises? Circulation 123:1833–1835

Verro P, Gorelick PB, Nguyen D (2008) Aspirin plus dipyridamole versus aspirin for prevention of vascular events after stroke or TIA: a meta-analysis. Stroke 39:1358–1363

Weber R, Diener HC (2010) Controversies and future perspectives of antiplatelet therapy in secondary stroke prevention. J Cell Mol Med 14:2371–2380

Weber R, Kraywinkel K, Diener HC, Weimar C (2010) Symptomatic intracranial atherosclerotic stenoses: prevalence and prognosis in patients with acute cerebral ischemia. Cerebrovasc Dis 30:188–193

Weimar C, Diener HC, Alberts MJ, Steg PG, Bhatt DL, Wilson PW et al (2009) The Essen stroke risk score predicts recurrent cardiovascular events: a validation within the REduction of Atherothrombosis for Continued Health (REACH) registry. Stroke 40:350–354

Weimar C, Benemann J, Michalski D, Muller M, Luckner K, Katsarava Z et al (2010) Prediction of recurrent stroke and vascular death in patients with transient ischemic attack or nondisabling stroke: a prospective comparison of validated prognostic scores. Stroke 41:487–493

Antiplatelet Therapy in Peripheral Artery Disease

Francesco Violi, Stefania Basili, Jeffrey S. Berger, and William R. Hiatt

Contents

Abstract Peripheral artery disease (PAD) is a term that relates to atherosclerosis and narrowing of the arteries in the lower extremities. The prevalence of PAD is approximately 12% of the adult population. Despite the low rate of peripheral complications and amputation, PAD is complicated by a high rate of cardiovascular events including myocardial infarction, stroke, and vascular death with an annual incidence of about 5%.

The detection of PAD is initially based on the appearance of typical symptoms (claudication and critical limb ischemia) related to peripheral arterial insufficiency. However, PAD may also be present in the absence of clinical symptoms (asymptomatic PAD). Accordingly, asymptomatic disease may occur in up to 50% of all patients with PAD. Ankle brachial index (ABI) is a diagnostic test used to evaluate

F. Violi (✉) • S. Basili
Prima Clinica Medica, Sapienza-University of Rome, Rome, Italy
e-mail: Francesco.Violi@uniroma1.it

J.S. Berger
NYU Langone Medical Center, New York, NY, USA

W.R. Hiatt (✉)
University of Colorado School of Medicine and CPC Clinical Research, Aurora, CO, USA
e-mail: Will.Hiatt@cpcmed.org

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_22,

the presence of PAD, defined by an ABI $\leq$0.90. The ABI is also demonstrated to be useful in the assessment of vascular risk in asymptomatic and symptomatic patients. Antiplatelet therapy remains a key intervention to reduce cardiovascular risk in PAD. Data from Antithrombotic Trialists' Collaboration showed that antiplatelet treatment was associated with a 23% risk reduction of vascular events in overall population with PAD. However, closer scrutiny of these data reveals that nonaspirin antiplatelet drugs, including ticlopidine, clopidogrel, picotamide, and dipyridamole largely drove the benefits in the PAD subgroup. It remains an open issue if PAD represents an atherosclerotic clinical model where aspirin, differently from coronary heart disease, is less effective in reducing atherosclerotic progression. Based on the reported results further trials with aspirin should be done in asymptomatic (ABI $\leq$0.90) and symptomatic PAD patients. Finally, the role of new antiplatelet drugs such as prasugrel and ticagrelor has not yet been studied in PAD.

Keywords Peripheral Artery Disease • Ankle Brachial Index • Antiplatelet Agents • Aspirin • Thienopyridines

1 Epidemiology and Classification of PAD

Peripheral artery disease (PAD) is a term that relates to atherosclerosis and narrowing of the arteries in the lower extremities. The spectrum of PAD encompasses acute limb ischemia and chronic limb ischemia, which is further divided to asymptomatic, claudication, and critical limb ischemia (Fig. 1) (Creager et al. 2008; Hiatt et al. 2008). PAD is a problem of substantial public health importance. It has been estimated that approximately 27 million persons in North America and Europe are afflicted with PAD (Rosamond et al. 2008; Belch et al. 2003). The prevalence of PAD is approximately 12% of the adult population (Fig. 2), with men being affected slightly more than women (Criqui et al. 1985; Kannel and McGee 1985). PAD is increasingly prevalent in the aging population. Findings from a national cross-sectional survey of PARTNERS (PAD Awareness, Risk, and Treatment: New Resources for Survival) found that PAD afflicts 29% of patients, aged $>$70 years, or have risk factors of diabetes or smoking (Hirsch et al. 2001).

PAD is an important hallmark of generalized atherosclerosis involving, in particular, coronary and cerebral circulation (Bhatt et al. 2006). Thus patients with PAD are at high risk of suffering from myocardial infarction, stroke, and vascular death with an annual incidence of about 5% (Steg et al. 2007). Nevertheless, perception of the specific risks associated with PAD is generally poor compared with other atherothrombotic settings, such as coronary artery disease or cerebrovascular disease. In fact, PAD represents a growing problem for internists since it is not just a localized disease, but it has serious systemic complications (Stehouwer et al. 2009; Pande et al. 2011).

Despite the strikingly high prevalence of PAD, this disease is under-diagnosed because it often presents with atypical symptoms or no ischemic symptoms related to

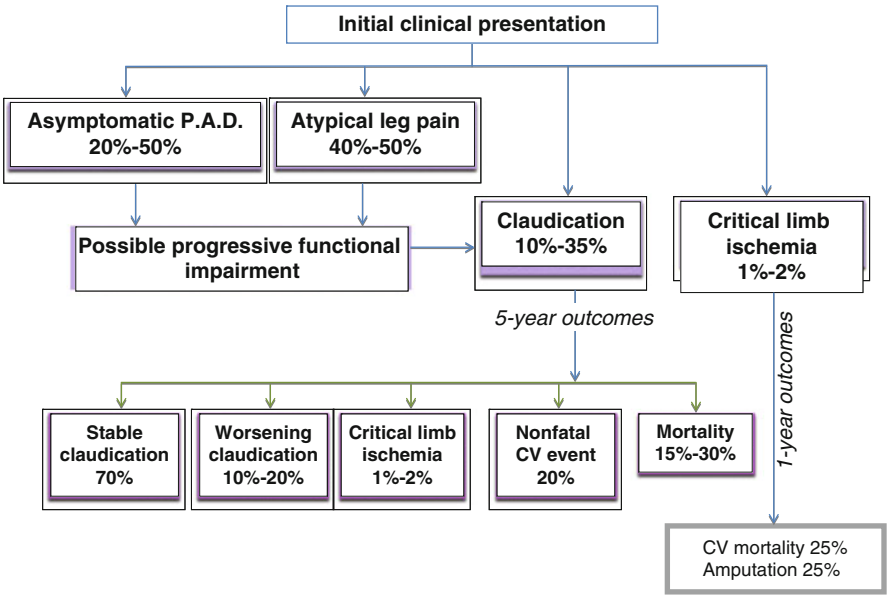


Fig. 1 Natural history of patients with peripheral artery disease

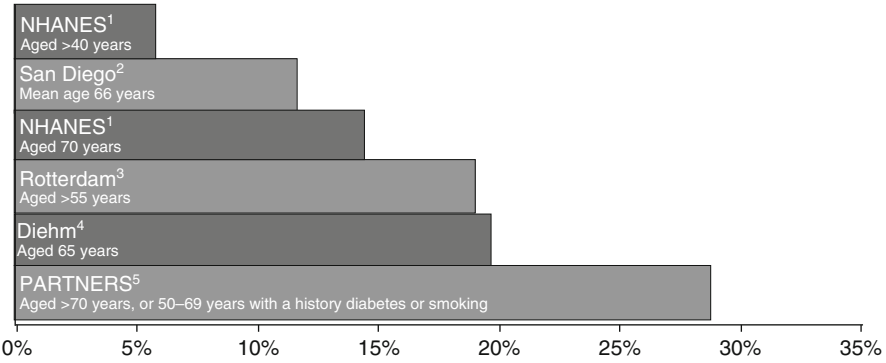


Fig. 2 Prevalence of peripheral artery disease

the legs at all as showed by the Walking and Leg Circulation Study (McDermott et al. 2002). Accordingly, asymptomatic disease may occur in up to 50% of all patients with PAD (Fig. 1) (Hirsch et al. 2006a). In the Rotterdam Study there was a 19.1% prevalence of PAD but claudication was reported in only 6.3% (Meijer et al. 1998).

Ankle brachial index (ABI) is the ratio of the ankle to brachial systolic blood pressure (White 2007). This simple, inexpensive noninvasive test is widely accepted as a diagnostic test used to evaluate the presence of lower extremity peripheral artery disease, defined by an ABI $\leq$ 0.90 in patients with symptoms of claudication or rest ischemia (Fig. 3) (Norgren et al. 2007a; Criqui et al. 2008).

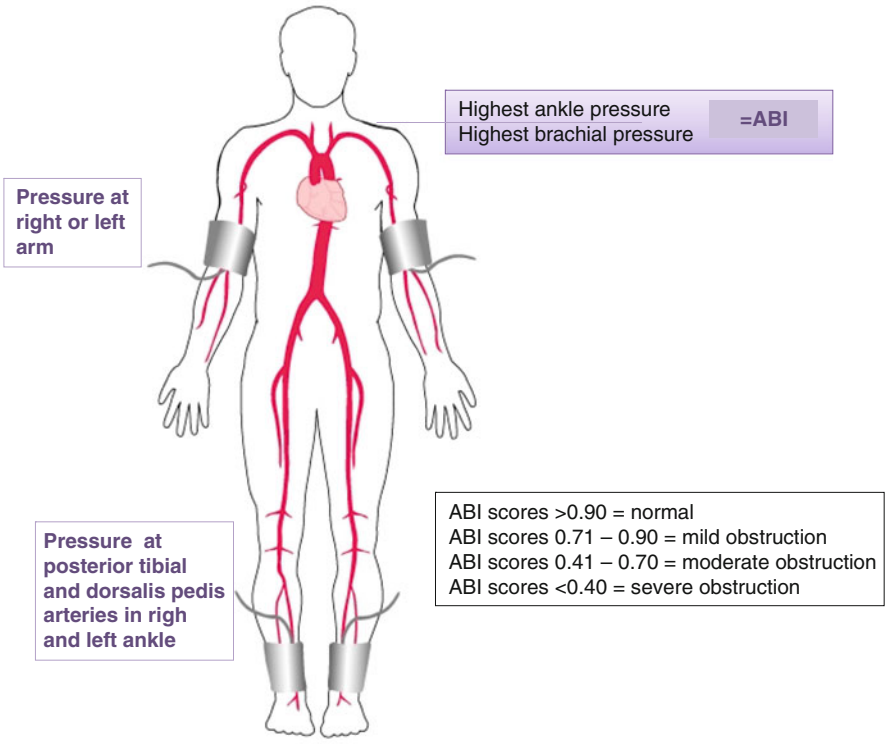


Fig. 3 Ankle brachial index

The ABI is also demonstrated to be useful in the assessment of vascular risk in asymptomatic and symptomatic patients. Screening for PAD in asymptomatic individuals should be considered in terms of cardiovascular risk and not merely of limb outcomes (Beckman et al. 2006).

Numerous studies have demonstrated that an abnormal ABI correlates with a significantly increased risk of coronary heart disease, stroke, and cardiovascular death. Most recently, a 2008 meta-analysis demonstrated that a low ABI ($\leq$0.90) was associated with approximately twice the 10-year total mortality, cardiovascular mortality, and major coronary event rate compared with the overall rate in each Framingham Risk Score category. Including ABI in cardiovascular risk stratification using Framingham Risk Score would result in reclassification of the risk category and modification of treatment recommendations in approximately 19% of men and 36% of women (Fig. 4) (Ankle Brachial Index Collaboration et al. 2008).

However, an ABI $\leq$ 0.90 in population-based cohort studies seems to have (Doobay and Anand 2005) high specificity but not enough sensitivity for predicting cardiovascular disease. Combining the population-based cohorts and the high-risk cohorts, the overall prevalence of ABI $\leq$ 0.90 is 13.2% (Doobay and Anand 2005). Because of its low sensitivity (yet high specificity), the ABI cannot be used as a

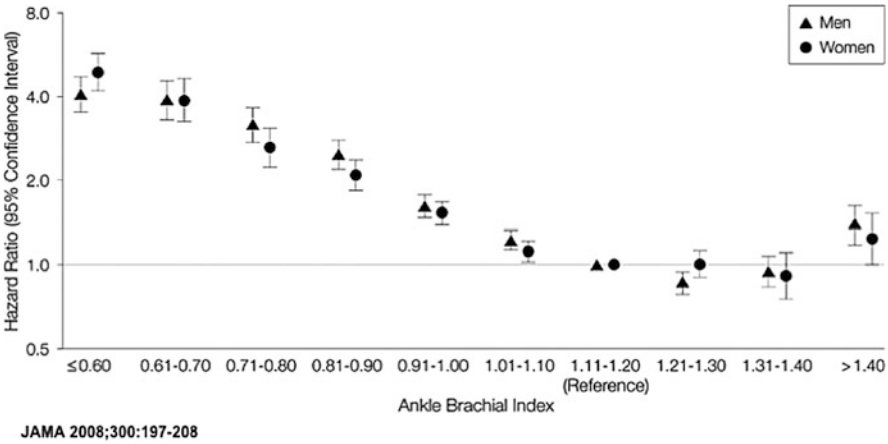


Fig. 4 Hazard ratios for total mortality in men and women by ankle brachial index at baseline for all studies combined in the ABI collaboration

generic screening test. Rather, it must be used in a focused manner, choosing individuals for whom the yield of the test is expected to be higher. The sensitivity of a low ABI to predict all-cause mortality is much higher in the high-risk cohorts compared with the population-based cohorts (85.0% vs. 31.2%). The sensitivity and specificity of a low ABI to predict incident coronary heart diseases were 16.5% and 92.7%, for incident stroke were 16.0% and 92.2%, and for cardiovascular mortality were 41.0% and 87.9%, respectively. Recently, a meta-analysis of 16 population cohort studies was performed to determine if the ABI provides information on the risk of cardiovascular events and mortality independently of the Framingham risk score (FRS) and if it can improve risk prediction (Ankle Brachial Index Collaboration et al. 2008). During 480,325 person-years of follow-up of 24,955 men and 23,339 women, the hazard ratios (HRs) for death for different levels of ABI compared with an ABI reference of 1.11 to 1.20 (low risk), a reverse J-shaped curve for both men and women was detected. For levels of ABI below 1.11, the HRs increased consistently with decreasing ABI as well as for an ABI of greater than 1.40 in both men and women. Conversely, no differences were found for levels of ABI from 1.11 to 1.40. In nearly all the studies in men, the HRs for total mortality were statistically significantly higher in individuals with an ABI of 0.90 or less compared with individuals with normal ABI values of 1.11 to 1.40. Likewise, significantly increased HRs were found in men and in women both for cardiovascular mortality and for major coronary events.

Adjustment of the HRs for individuals with an ABI of 0.90 or less relative to an ABI of 1.11 to 1.40 for FRS reduced the HRs that, however, were still significantly elevated. Thus, the authors concluded that ABI provided independent risk information compared with the FRS and, when combined with the FRS, low ABI (<0.90) was associated with approximately twice the 10-year total mortality, cardiovascular mortality, and major coronary event rate across all Framingham risk categories.

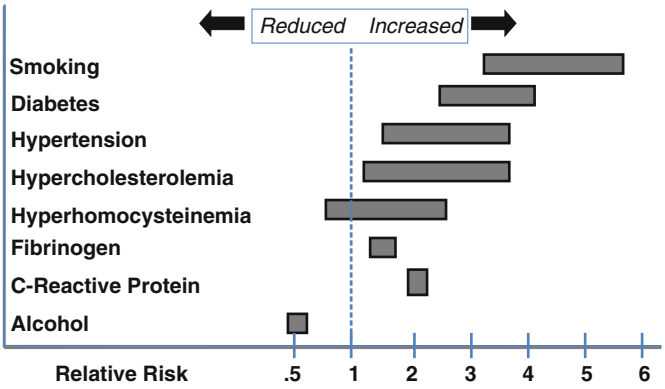


Fig. 5 Risk factors for peripheral artery disease

These findings confirmed the previously published results of the Strong Heart Study (Resnick et al. 2004), Cardiovascular Health Study (O'Hare et al. 2006), and Multi-ethnic Study of Atherosclerosis (McDermott et al. 2005) that showed the relationship between ABI and cardiovascular disease is nonlinear and varies across the range of ABI.

2 Management of PAD

The risk factors for PAD are similar to the risk factors for atherosclerosis elsewhere (Fig. 5); the most strongly associated with PAD, in addition to age, are cigarette smoking and diabetes mellitus (DM) (Norgren et al. 2007b; Haugen et al. 2007; Aronow 2010). Thus, smoking should be stopped, and hypertension, diabetes mellitus, and dyslipidemia should be treated. In addition antiplatelet therapy remains a key intervention to reduce cardiovascular risk in PAD. Exercise rehabilitation programs and cilostazol help to relieve symptoms and improve exercise performance (Aronow 2010).

Platelets play an important role in the process of atherothrombosis via release of molecules that are injurious for vascular walls and by precipitating thrombosis at the site of plaque injury. There is evidence indicating that platelets are activated in patients with PAD. Platelet activation as assessed by urinary excretion of 11-deydro-TxB_2 has been studied in patients with PAD and matched controls (Davì et al. 1997). This study demonstrated enhanced values of urinary 11-deydro-TxB_2 excretion in PAD patients compared with controls. Such differences, however, seemed to be attributable essentially to the coexistence of risk factors for atherosclerotic disease such as hypercholesterolemia, diabetes, or smoking. Thus, in PAD patients without such risk factors, urinary excretion of 11-deydro-TxB_2 was similar to that in controls, suggesting that the risk factors rather than atherosclerotic disease, per se, are responsible for enhanced production of TxA_2. Analysis of other markers of platelet activation confirmed the

existence of platelet activation in PAD. In particular a significant association between mean platelet volume (MPV) and PAD (as defined by a screening ABI < 0.9 even after multivariable adjustment) and high levels of soluble CD40 ligand and P selectin have been detected in patients with PAD and critical leg ischemia (Berger et al. 2010; Blann et al. 2005). However, there was no relationship between the severity of arteriopathy, defined as the presence of resting pain, and platelet activation. Therefore, it is still unclear if platelet activation is a reflection of peripheral atherosclerosis or of the coexistence of risk factors such as diabetes, dyslipidemia, and smoking that activate, per se, platelet function.

3 Antiplatelet Drugs in PAD

Antiplatelet agents are a therapeutic cornerstone in the secondary prevention of cardiovascular events in patients with established atherosclerosis. The essential role of antiplatelet therapy has been well established in serial publications of the Antithrombotic Trialist's Collaboration (ATC) including over 130,000 patients and 50 trials (Antithrombotic Trialists' Collaboration 2002). This meta-analysis clearly demonstrates a cardioprotective benefit of antiplatelet therapy in a broad population of patients with clinical evidence of atherosclerosis. Specifically, this high-risk population has been defined as those with a history of myocardial infarction, acute coronary syndrome, transient ischemic attacks or stroke, as well as coronary or carotid revascularization procedures. These are generally patients who have had ischemic events or interventions for symptomatic disease. In the subgroup of patients with stable peripheral arterial disease (PAD), similar benefits of antiplatelet therapy were observed (Antithrombotic Trialists' Collaboration 2002). Thus, antiplatelet treatment was associated with a 23% risk reduction of vascular events including MI, stroke, and vascular death in overall population with PAD. However, closer scrutiny of these data reveals that the benefits in the PAD subgroup were largely driven by nonaspirin antiplatelet drugs including ticlopidine, clopidogrel, picotamide, and dipyridamole (Antithrombotic Trialists' Collaboration 2002).

4 Aspirin and PAD

Aspirin is the most used antiplatelet drug to prevent cardiovascular events in patients with cardiovascular disease. Aspirin inhibits platelet COX1, thereby preventing the formation of thromboxane (Tx) A_2, a potent aggregating and vasoconstrictor molecule (FitzGerald 1991).

At variance with other circulatory territories such as coronary and cerebral trees, the effect of aspirin in peripheral atherosclerosis has been less well investigated and results are inconclusive. The effect of aspirin has been examined in a post hoc analysis of the Physicians' Health Study where aspirin was ineffective in preventing

claudication deterioration, while it seemed to reduce the incidence of peripheral surgical interventions (Goldhaber et al. 1992). However, these data are difficult to interpret due to the retrospective nature of the study. Therefore, evidence for any benefit of aspirin treatment in patients with PAD should be considered insufficient. Nevertheless, the American College of Chest Physicians (Sobel et al. 2008) and the ACC/AHA (Hirsch et al. 2006b) practice guidelines recommended aspirin treatment for patients with PAD.

Three more recent studies, the CLIPS (Critical Leg Ischaemia Prevention Study (CLIPS) Group et al. 2007), the POPADAD (Belch et al. 2008), and the AAA trials (Fowkes et al. 2010) specifically examined the role of aspirin in PAD (Table 1).

In the Critical Leg Ischaemia Prevention Study (CLIPS), a study with aspirin in PAD, anticipated randomizing 2,000 patients, the enrolment was discontinued prematurely after including only 210 PAD patients. The reasons for stopping early were feasibility and not due to encountering any predefined stopping rule. Patients were randomized to placebo or 100 mg aspirin and followed up for 2 years. Throughout the study major cardiovascular events, including fatal and nonfatal cerebral ischemia and myocardial infarction and critical leg ischemia, were monitored. During the follow-up there were seven major cardiovascular events in the aspirin-treated group and twenty in the placebo-treated group, with a hazard ratio of 0.35, 95% CI 0.15–0.82. However this benefit was entirely driven by a decrease in nonfatal events, as there were seven deaths on aspirin and four in patients randomized to the control group. Despite such interesting findings, the relatively few number of events and imbalance in deaths did not permit us to reach definite conclusions on the clinical efficacy of aspirin in the PAD population.

In the POPADAD study (Belch et al. 2008), Belch et al. investigated whether aspirin and antioxidants given together or separately can reduce MI and death in patients with diabetes and PAD. In their study, 1,276 patients with diabetes and evidence of asymptomatic PAD (as determined by a lower-than-normal ankle brachial pressure index of 0.99 or less, but no symptoms) over 40 years of age were randomized to receive either aspirin 100 mg or placebo, an antioxidant or placebo, or aspirin and an antioxidant or double placebo and followed over 8 years. There were two hierarchical composite primary endpoints: death from coronary heart disease or stroke, nonfatal MI or stroke, or amputation above the ankle for critical limb ischemia; and death from CHD or stroke. Overall, the researchers found no benefit from either aspirin or antioxidant treatment. Patients in the aspirin groups had 116 primary events compared with 117 in the placebo group [hazard ratio (HR) 0.98 (95% CI 0.76–1.26); $p = 0.86$]. There were 43 deaths from CHD or stroke in the aspirin group compared with 35 in the nonaspirin group [HR 1.23 (0.79–1.93); $p = 0.36$].

It is important to recognize that POPADAD is smaller than most of the other aspirin trials, with fewer events and that it is possible that small effects may be shown with larger trials continued for a longer time.

In the 2010, the results of AAA trial were published (Fowkes et al. 2010). The Aspirin for Asymptomatic Atherosclerosis (AAA) trial, conducted from April 1998 to October 2008, was an intention-to-treat, double-blind, randomized controlled

Table 1 Studies specifically examining the role of aspirin in PAD

Trial name (Reference)	Main outcomes (composite primary endpoint)	Aspirin dosage (AT)	Average duration (years)	Total patients	Patients		Vascular events		HR (95 %a)
					AT	CTRL	AT	CTRL	
CLIPS (Critical Leg Ischaemia Prevention Study (CLIPS) Group et al. 2007)	MACE + CLI	100	2	181	91	90	7	20	0.35 (0.15–0.82)
POPADAP (Belch et al. 2008)	MACE + CLI; CVD or STROKEDEATH	100	6.7	1,276	638	638	116;43	117;35	0.98 (0.76–1.26)
AAA (Fowkes et al. 2010)	MACE + RP	100 (EC)	8.2	3,350	1,675	1,675	134	136	1.03 (0.84–11.279)

trial of once daily low-dose *enteric-coated* aspirin (100 mg) vs. placebo. It involved 28,980 men and women, aged 50–75 years, who were free of clinical cardiovascular disease, recruited from a community Scottish health registry. All recruited patients had an ABI screening test. Of those, 3,350 with a low ABI (defined as an ABI value equal to or less than 0.95) entered the trial. *The trial was designed for 80% power to detect 25% proportional risk reduction in events*. Enrolled patients were randomly assigned in 1:1 ratio to low-dose aspirin (100 mg/daily) or to placebo groups. The primary endpoint of this study was a composite of fatal or nonfatal coronary event or stroke or revascularization. *The follow-up had to be extended from the originally planned 5 years to 9.5 years*. After a mean (SD) follow-up of 8.2 (1.6) years, aspirin was no more effective than placebo in reducing the primary endpoint (13.7 events per 1,000 person-years in the aspirin group vs. 13.3 events per 1,000 person-years in the placebo group; HR, 1.03; 95% CI 0.84–1.27). Moreover, aspirin treatment had no significant effect on any of the secondary endpoints. Aspirin therapy was, however, associated with a nonsignificant increased risk of major hemorrhage (2.0% vs. 1.2%; HR = 1.71, 95% CI 0.99–2.97). Intracranial hemorrhage occurred in 11 participants (including 3 fatal subarachnoid/subdural hemorrhages) in the aspirin group and 7 in the placebo group. *These results are similar to the findings of the aspirin primary prevention trials*. Based on these findings, the authors concluded that in asymptomatic PAD patients aspirin had no clinical benefit and may be harmful.

Additional findings were achieved by a recent meta-analysis performed by Berger et al. (2009) to assess the benefit of aspirin in treating both symptomatic and asymptomatic PAD patients including 18 prospective, randomized controlled trials of aspirin alone or in combination with other antiplatelet drugs. The meta-analysis was designed to test the null hypothesis that aspirin was not different from placebo or control in reducing the risk of the combined primary endpoint of nonfatal MI, nonfatal stroke, and cardiovascular death. Among 5,269 participants included in the analysis, cardiovascular events were experienced in 251 (8.9%) of 2,823 patients taking aspirin (alone or with dipyridamole) and in 269 (11.0%) of 2,446 in the control group. The pooled RR reduction of 12% in cardiovascular event rates was not statistically significant. Moreover, in the subset of 1,516 participants taking aspirin monotherapy compared to control, aspirin was associated with a nonsignificant reduction in cardiovascular events [8.2% vs. 9.6%; RR = 0.75 (95% CI 0.48–1.18)] and in all-cause or cardiovascular mortality, MI, or major bleeding.

Almost 20 years ago, the United States Food and Drug Administration (FDA) was asked to extend the labeling of aspirin to include PAD patients (Anonymous 1998). In its deliberations, the FDA could not find substantive evidence to support the role of aspirin for this indication. The implication of this deliberation was that patients with PAD who do not have overt clinical manifestations of atherosclerosis in other arterial beds might not be responsive to aspirin chemoprophylaxis.

Absent additional contradictory evidence, one should reserve aspirin therapy for PAD patients who have suffered coronary or cerebral ischemic events. This information could be of some importance in the design of additional placebo-controlled trials of aspirin in larger PAD population.

5 Nonaspirin Drugs and PAD Inhibiting the Platelet Arachidonic Acid Pathway

Picotamide, a derivative of methoxy-isophtalic acid, is an antiplatelet drug whose pharmacological properties consist in inhibiting both thromboxane A_2 receptors and TxA_2 synthase. As concentrations of the molecule needed to inhibit both pathways are almost equivalent (Modesti et al. 1989, 1994; Violi et al. 1988; Gresele et al. 1989; Berrettini et al. 1990; Cattaneo et al. 1991), picotamide may exert a dual pharmacological action in vivo and be potentially useful in various clinical settings characterized by atherosclerotic disease. Two prospective studies have been performed in PAD populations with different risks for cardiovascular events: the ADEP and DAVID trials.

The first large randomized trial (ADEP trial) on picotamide investigated its clinical usefulness in patients with PAD (Balsano and Violi 1993). For this study 2,304 patients were consecutively enrolled, allocated to either placebo or picotamide (300 mg t.i.d.) and followed up for 18 months. Endpoints of the study were major events (i.e., cardiovascular death, myocardial infarction, stroke, or amputation) and minor events (unstable angina, transient ischemic attack, hypertension, renal failure, deterioration of PAD). The "intention-to-treat analysis" showed a risk reduction (18.9%) in the combined endpoints, major plus minor events, in the picotamide group compared with the controls, which, however, did not reach statistical significance. Conversely, "on treatment analysis" showed a higher and statistically significant reduction (22.8%) in the same endpoints. Side effects such as bleeding were almost identical in the two groups. As the authors suggested, the lack of any beneficial effects of picotamide against major events could have been related to the low occurrence of these events during the follow-up. This phenomenon may be related to a bias in patient selection, which excluded high-risk patients. The capacity of picotamide to prevent vascular complications was, however, magnified when claudicant patients affected by diabetes were taken into account. Thus, a substudy of the ADEP trial retrospectively analyzed 438 diabetic patients and observed a risk reduction of 45.2% of combined major and minor events in those treated with picotamide compared with those treated with placebo (Milani et al. 1996). On the basis of this post hoc analysis, another randomized trial (the DAVID trial) was specifically designed for diabetic patients with PAD (Neri Serneri et al. 2004). Thus, 1,209 patients were enrolled and randomly assigned to picotamide (600 mg/bid) or aspirin (320 mg/day) and followed up for 2 years. The primary endpoint was overall mortality and the secondary one was the combined incidence of death and major cardiovascular events. Mortality was significantly lower in picotamide-treated patients than in those treated with aspirin, showing a relative risk of reduction of 45%. Furthermore, the incidence of gastrointestinal bleeding was much lower in the picotamide group than in the aspirin group. The secondary endpoint did not show any significant difference between the two populations, showing only a nonsignificant trend in favor of patients taking picotamide. As pointed out by the authors, a possible bias relative to the high

proportion of patients (about 20% in each group) who discontinued the trial because of nonfatal events may have underestimated the real incidence of the secondary endpoints. Moreover, it is possible that the sample size of the study was insufficient to detect any difference in these endpoints between the two groups. Comparison of the results achieved by the ADEP and DAVID trials also raises the question as to whether the differences seen are dependent on the fact that TxA_2 production is more relevant for atherosclerotic progression in PAD patients with diabetes compared with PAD without diabetes, or whether the different dosage of picotamide (600 mg vs. 1,200 mg in ADEP and DAVID, respectively) had a different impact on clinical outcome. Further studies are therefore necessary to explore the relationship between picotamide dosage and TxA_2 inhibition in vivo. In conclusion, activation of platelet arachidonic plays an important role in the pathogenesis of cardiovascular events in PAD, but the clinical trials with picotamide should be considered inconclusive and warrant further investigation.

6 Nonaspirin Drugs Inhibiting Platelet ADP Receptors and PAD

Thienopyridine is a drug category that inhibits platelet aggregation by interfering with platelet ADP receptors, i.e., $P2Y_1$ and $P2Y_{12}$.

Ticlopidine was the first drug of this class investigated in patients with PAD with the aim of evaluating the benefit on claudication or the cardiovascular events that complicate the clinical course of PAD. As far as the claudication is concerned, a prospective Italian study performed in 151 patients with claudication demonstrated that ticlopidine (250 mg/bid) increased walking distance compared to placebo-treated patients (Balsano et al. 1989). In the same trial, ticlopidine-treated patients' ABI did not worsen compared to placebo-treated patients, indicating that such antiplatelet treatment may favorably influence atherosclerotic *progression. As far as* the cardiovascular events are concerned, a Swedish trial (Janzon et al. 1990) investigated if ticlopidine (250 mg/bid), compared to placebo, was able to reduce cerebro- and cardiovascular events in a population with claudication. A total of 687 patients were included in the trial and followed up for 5 years. There was no difference in the clinical endpoints between the two groups. However, the secondary endpoint of mortality was lower in the ticlopidine group, and in an on treatment analysis, there were fewer ischemic events in the ticlopidine-treated group.

The effect of thienopyridine on cardiovascular events was later examined in the CAPRIE trial, which compared the clinical efficacy of clopidogrel (75 mg/day), which is a derivative of ticlopidine, with aspirin (325 mg/day) in a population at different risks of cardiovascular events (CAPRIE Steering Committee 1996). One-third of this population was affected by PAD. During 3 years of follow-up, clopidogrel was marginally significantly superior to aspirin in reducing cardiovascular events in the

Table 2 Meta-analysis of 29 clinical randomized trials on antiplatelet therapy for prevention of vascular death, myocardial infarction, and stroke in 10,735 peripheral artery disease patients (Basili et al. 2010)

	Thienopyridines	Picotamide	Aspirin	Other
Number of trials included	14	2	4	9
Global number of patients included in the trials	5,326	2,324	1,900	1,185
Odds ratio	0.779	0.785	0.847	1.213
95% CI	0.639–0.950	0.495–1.243	0.653–1.997	0.791–1.860
P value	0.014	0.302	0.208	0.376

Results according to the type of antiplatelet treatment (thienopyridines, picotamide, aspirin, and other antiplatelet drugs)

entire population (however the overall difference was insufficient to convince regulatory authorities that clopidogrel was indeed superior to aspirin). *A post-hoc analysis of the CAPRIE trial performed in the PAD population showed* a significant risk reduction of 24% for cardiovascular events in the clopidogrel-treated group compared to the aspirin group suggesting that the inhibitors of ADP receptors may be particularly efficacious in PAD patients.

In 2007, a publication of a post-hoc analysis of CHARISMA (Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance) trial (Bhatt et al. 2007) included 2,838 patients who had symptomatic PAD. Patients with PAD had a median time from diagnosis to the primary endpoint [cardiovascular death (including hemorrhagic death), myocardial infarction, or stroke (from any cause)] of 23.6 months in mean. The overall rate of cardiovascular death, MI, or stroke in PAD cohort was 8.7% in the placebo plus aspirin arm and 7.6% in the clopidogrel plus aspirin arm (HR = 0.869; 95% CI 0.671–1.125). Nevertheless, although the risk reduction in the PAD subgroup appeared similar to that observed in patients with prior MI or prior stroke it did not reach statistical significance.

Together these data indicate the clinical usefulness of thienopyridines in PAD to prevent major cardiovascular events. Given the level of evidence from the CAPRIE study, the FDA approved the use of clopidogrel in symptomatic patients with an ABI < 0.85 or PAD patients with a prior history of limb revascularization. Moreover, the use of clopidogrel monotherapy or the use of clopidogrel plus aspirin and in PAD should be further assessed by PAD-specific interventional trials.

7 Comparison of Efficacy of Antiplatelet Treatments in PAD

Although patients with PAD warrant life-long antiplatelet therapy, an important issue relates the potentially different impact of antiplatelet drug categories in the clinical progression of PAD. To investigate if specific antiplatelet treatment had

a different impact on clinical outcomes, a meta-analysis has been performed in patients with claudication and/or ABI $\leq$ 0.99 (Basili et al. 2010). Twenty-nine clinical randomized trials on antiplatelet therapy for prevention of vascular death, myocardial infarction, and stroke in 10,735 peripheral artery disease patients have been included in the analysis.

The authors found 1,900 (17.70%) patients in trials with aspirin, 5,326 (49.61%) in those with thienopyridines, 2,324 (21.65%) in those with picotamide, and 1,185 (11.04%) in those with others antiplatelet drugs (including three studies where the active drugs were aspirin and/or dipyridamole). The results of this meta-analysis showed that in patients with claudication, antiplatelet treatment is efficacious in reducing vascular outcomes with a risk reduction of 17%. Of note, analyzing separately each drug category conclusive results were achieved by trials with thienopyridines, that reduced the risk of cardiovascular events by 22% ($p = 0.014$); a trend to a reduction was observed with aspirin (-15%, $p = 0.208$) or picotamide ($-21\%, p = 0.302$) but these changes were not statistically significant (Table 2). One of the limitations of this meta-analysis included the fact that it has been done in patients with stable PAD and could not, therefore, be extrapolated to nonmedical PAD series. Another limitation of the study was the lack of a direct comparison between two antiplatelet treatments that should require more interventional trials.

8 Conclusions

Antiplatelet therapy is indicated in patients with PAD to reduce the risk of major cardiovascular events. In patients with PAD who have clinical evidence of concomitant coronary or cerebrovascular disease, aspirin would be first-line.

It remains an open issue if PAD represents an atherosclerotic clinical model where aspirin, differently from coronary heart disease, is less effective in reducing atherosclerotic progression. Based on the reported results further trials with aspirin should be done in symptomatic PAD patients. In these same patients, clopidogrel would be an acceptable alternative. However the data supporting the use of antiplatelet drugs in patients with PAD, who do not have a history of other cardiovascular disorders, are still inconclusive and further study is necessary to explore this issue.

Knowledge Gaps

- It remains an open issue if PAD represents an atherosclerotic clinical condition where response to aspirin is different from that in coronary heart disease.
- The role of new antiplatelet drugs (prasugrel and ticagrelor) has not been studied in PAD.

Key Messages

- Patients with symptomatic PAD are at high risk of cardiovascular events including myocardial infarction, stroke, and vascular death.
- Antiplatelet drugs reduce the risk of cardiovascular events in patients with peripheral artery disease.
- Aspirin appears to have marginal benefits for reducing initial cardiovascular events when used for PAD patients without clinically evident coronary artery disease.
- Clopidogrel significantly reduced cardiovascular events in the setting of peripheral arterial disease and would be an acceptable alternative to aspirin.

References

Ankle Brachial Index Collaboration, Fowkes FG, Murray GD et al (2008) Ankle brachial index combined with Framingham Risk Score to predict cardiovascular events and mortality: a meta-analysis. JAMA 300:197–208

U.S. Food and Drug Administration (1998) Internal analgesic, antipyretic, and antirheumatic drug products for over-the-counter human use; final rule for professional labeling of aspirin, buffered aspirin, and aspirin in combination with antacid drug products. Fed Regist 63: 56802–56819

Antithrombotic Trialists' Collaboration (2002) Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. BMJ 324:71–86

Aronow WS (2010) Office management of peripheral arterial disease. Am J Med 123:790–792

Balsano F, Violi F (1993) Effect of picotamide on the clinical progression of peripheral vascular disease. A double-blind placebo-controlled study. The ADEP Group. Circulation 87:1563–1569

Balsano F, Coccheri S, Libretti A et al (1989) Ticlopidine in the treatment of intermittent claudication: a 21-month double-blind trial. J Lab Clin Med 14:84–91

Basili S, Raparelli V, Vestri AR et al (2010) Comparison of efficacy of antiplatelet treatments for patients with claudication: a meta-analysis. Thromb Haemost 103:766–773

Beckman JA, Jaff MR, Creager MA (2006) The United States Preventive Services Task Force recommendation statement on screening for peripheral arterial disease: more harm than benefit? Circulation 114:861–866

Belch JJ, Topol EJ, Agnelli G et al (2003) Critical issues in peripheral arterial disease detection and management: a call to action. Arch Intern Med 163:884–892

Belch J, MacCuish A, Campbell I et al (2008) The prevention of progression of arterial disease and diabetes (POPADAD) trial: factorial randomised placebo controlled trial of aspirin and antioxidants in patients with diabetes and asymptomatic peripheral arterial disease. BMJ 337:a1840

Berger JS, Krantz MJ, Kittelson JM et al (2009) Aspirin for the prevention of cardiovascular events in patients with peripheral artery disease: a meta-analysis of randomized trials. JAMA 301:1909–1919

Berger JS, Eraso LH, Xie D et al (2010) Mean platelet volume and prevalence of peripheral artery disease, the National Health and Nutrition Examination Survey, 1999–2004. Atherosclerosis 213:586–591

Berrettini M, De Cunto M, Parise P et al (1990) "In vitro" and "ex vivo" effects of picotamide, a combined thromboxane A2-synthase inhibitor and-receptor antagonist, on human platelets. Eur J Clin Pharmacol 39:495–500

Bhatt DL, Steg PG, Ohman EM et al (2006) International prevalence, recognition, and treatment of cardiovascular risk factors in outpatients with atherothrombosis. JAMA 295:180–189

Bhatt DL, Flather MD, Hacke W et al (2007) Patients with prior myocardial infarction, stroke, or symptomatic peripheral arterial disease in the CHARISMA trial. J Am Coll Cardiol 49:1982–1988

Blann AD, Tan KT, Tayebjee MH et al (2005) Soluble CD40L in peripheral artery disease. Relationship with disease severity, platelet markers and the effects of angioplasty. Thromb Haemost 93:578–583

CAPRIE Steering Committee (1996) A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). Lancet 348:1329–1339

Cattaneo M, Tenconi PM, Lecchi A et al (1991) In vitro effects of picotamide on human platelet aggregation, the release reaction and thromboxane B2 production. Thromb Res 62:717–724

Creager MA, White CJ, Hiatt WR et al (2008) Atherosclerotic Peripheral Vascular Disease Symposium II: executive summary. Circulation 118:2811–2825

Criqui MH, Fronek A, Barrett-Connor E et al (1985) The prevalence of peripheral arterial disease in a defined population. Circulation 71:510–515

Criqui MH, Alberts MJ, Fowkes FG et al (2008) Atherosclerotic Peripheral Vascular Disease Symposium II: screening for atherosclerotic vascular diseases: should nationwide programs be instituted? Circulation 118:2830–2836

Critical Leg Ischaemia Prevention Study (CLIPS) Group, Catalano M, Born G et al (2007) Prevention of serious vascular events by aspirin amongst patients with peripheral arterial disease: randomized, double-blind trial. J Intern Med 261:276–284

Davì G, Gresele P, Violi F et al (1997) Diabetes mellitus, hypercholesterolemia, and hypertension but not vascular disease per se are associated with persistent platelet activation in vivo. Evidence derived from the study of peripheral arterial disease. Circulation 96:69–75

Doobay AV, Anand SS (2005) Sensitivity and specificity of the ankle-brachial index to predict future cardiovascular outcomes: a systematic review. Arterioscler Thromb Vasc Biol 25: 1463–1469

FitzGerald GA (1991) Mechanisms of platelet activation: thromboxane A2 as an amplifying signal for other agonists. Am J Cardiol 68:11B–15B

Fowkes FG, Price JF, Stewart MCW et al (2010) Aspirin for prevention of cardiovascular events in a general population screened for a low ankle brachial index. JAMA 303:841–848

Goldhaber SZ, Manson JE, Stampfer MJ et al (1992) Low-dose aspirin and subsequent peripheral arterial surgery in the Physicians' Health Study. Lancet 340:143–145

Gresele P, Deckmyn H, Arnout J et al (1989) Characterization of N, N′-bis(3-picolyl)-4-methoxy-isophtalamide (picotamide) as a dual thromboxane synthase inhibitor/thromboxane A2 receptor antagonist in human platelets. Thromb Haemost 61:479–484

Haugen S, Casserly IP, Regensteiner JG et al (2007) Risk assessment in the patient with established peripheral arterial disease. Vasc Med 12:343–350

Hiatt WR, Goldstone J, Smith SC Jr et al (2008) Atherosclerotic Peripheral Vascular Disease Symposium II: nomenclature for vascular diseases. Circulation 118:2826–2829

Hirsch AT, Criqui MH, Treat-Jacobson D et al (2001) Peripheral arterial disease detection, awareness, and treatment in primary care. JAMA 286:1317–1324

Hirsch AT, Haskal ZJ, Hertzer NR et al (2006a) ACC/AHA 2005 Practice Guidelines for the management of patients with peripheral arterial disease (lower extremity, renal, mesenteric, and abdominal aortic): a collaborative report from the American Association for Vascular Surgery/Society for Vascular Surgery, Society for Cardiovascular Angiography and Interventions, Society for Vascular Medicine and Biology, Society of Interventional Radiology, and the ACC/AHA Task Force on Practice Guidelines (Writing Committee to Develop Guidelines for the Management of Patients With Peripheral Arterial Disease): endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation; National Heart, Lung, and Blood Institute; Society for Vascular Nursing; TransAtlantic Inter-Society Consensus; and Vascular Disease Foundation. Circulation 113:e463–e654

Hirsch AT, Haskal ZJ, Hertzer NR et al (2006b) ACC/AHA 2005 guidelines for the management of patients with peripheral arterial disease (lower extremity, renal, mesenteric, and abdominal

aortic): executive summary a collaborative report from the American Association for Vascular Surgery/Society for Vascular Surgery, Society for Cardiovascular Angiography and Interventions, Society for Vascular Medicine and Biology, Society of Interventional Radiology, and the ACC/AHA Task Force on Practice Guidelines (Writing Committee to Develop Guidelines for the Management of Patients With Peripheral Arterial Disease) endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation; National Heart, Lung, and Blood Institute; Society for Vascular Nursing; TransAtlantic Inter-Society Consensus; and Vascular Disease Foundation. J Am Coll Cardiol 47:1239–1312

Janzon L, Bergqvist D, Boberg J et al (1990) Prevention of myocardial infarction and stroke in patients with intermittent claudication; effects of ticlopidine. Results from STIMS, the Swedish Ticlopidine Multicentre Study. J Intern Med 227:301–308

Kannel WB, McGee DL (1985) Update on some epidemiologic features of intermittent claudiation: the Framingham Study. J Am Geriatr Soc 33:13–18

McDermott MM, Greenland P, Liu K et al (2002) The ankle brachial index is associated with leg function and physical activity: the Walking and Leg Circulation Study. Ann Intern Med 136:873–883

McDermott MM, Liu K, Criqui MH et al (2005) Ankle brachial index and subclinical cardiac and carotid disease: the Multi-Ethnic Study of Atherosclerosis. Am J Epidemiol 162:33–41

Meijer WT, Hoes AW, Rutgers D et al (1998) Peripheral arterial disease in the elderly: The Rotterdam Study. Arterioscler Thromb Vasc Biol 18:185–192

Milani M, Longoni A, Maderna M (1996) Effects of picotamide, an antiplatelet agent, on cardiovascular, events in 438 claudicant patients with diabetes: a retrospective analysis of the ADEP study. Br J Clin Pharmacol 42:782–785

Modesti PA, Colella A, Abbate R et al (1989) Competitive inhibition of platelet thromboxane A2 receptor binding by picotamide. Eur J Pharmacol 169:85–93

Modesti PA, Cecioni I, Colella A et al (1994) Binding kinetics and antiplatelet activities of picotamide, a thromboxane A2 receptor antagonist. Br J Pharmacol 112:81–86

Neri Serneri GG, Coccheri S, Marubini E et al (2004) Picotamide, a combined inhibitor of thromboxane A2 synthase and receptor, reduces 2-year mortality in diabetics with peripheral arterial disease: the DAVID study. Eur Heart J 25:1845–1852

Norgren L, Hiatt WR, Dormandy JA et al (2007a) Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). J Vasc Surg 45:S5–S67

Norgren L, Hiatt WR, Dormandy JA et al (2007b) Inter-society consensus for the management of peripheral arterial disease. Int Angiol 26:81–157

O'Hare AM, Katz R, Shlipak MG et al (2006) Mortality and cardiovascular risk across the ankle-arm index spectrum: results from the Cardiovascular Health Study. Circulation 113:388–393

Pande RL, Perlstein TS, Beckman JA et al (2011) Secondary Prevention and Mortality in Peripheral Artery Disease. National Health and Nutrition Examination Study, 1999 to 2004. Circulation 124:17–23

Resnick HE, Lindsay RS, McDermott MM et al (2004) Relationship of high and low ankle brachial index to all-cause and cardiovascular disease mortality: the Strong Heart Study. Circulation 109:733–739

Rosamond W, Flegal K, Furie K et al (2008) Heart disease and stroke statistics—2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 117:e25–e146

Sobel M, Verhaeghe R, American College of Chest Physicians et al (2008) Antithrombotic therapy for peripheral artery occlusive disease: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest 133:815–843

Steg PG, Bhatt DL, Wilson PW et al (2007) One-year cardiovascular event rates in outpatients with atherothrombosis. JAMA 297:1197–1206

Stehouwer CD, Clement D, Davidson C et al (2009) EFIM Vascular Medicine Working Group. Peripheral arterial disease: a growing problem for the internist. Eur J Intern Med 20:132–138

Violi F, Ghiselli A, Iuliano L et al (1988) Inhibition by picotamide of thromboxane production in vitro and ex vivo. Eur J Clin Pharmacol 33:599–602

White C (2007) Clinical practice. Intermittent claudication. N Engl J Med 356:1241–1250

Primary Prevention of Ischaemic Cardiovascular Disorders with Antiplatelet Agents

Tom Meade

Contents

T. Meade (✉)
Department of Non-Communicable Disease Epidemiology, London School of Hygiene & Tropical Medicine, London, UK
e-mail: Tom.Meade@lshtm.ac.uk

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5_23,

Abstract In those who have already survived myocardial infarction (MI) or stroke, or have had a transient ischaemic episode (TIA), daily low dose aspirin (ASA) reduces the risk of recurrences by an amount that greatly exceeds the risk of serious bleeding (secondary prevention). ASA is therefore recommended for these people.

However, in primary prevention—reducing risk in those so far free of clinically manifest episodes—the benefit is of the same order as the bleeding hazard, (which is much the same in both primary and secondary prevention contexts). The use of other effective agents such as statins further emphasises the even balance between benefit and hazard in primary prevention. Six primary prevention trials are reviewed, first singly and then in a meta-analysis based on individual patient data. ASA reduced non-fatal myocardial infarction by about 25%. However, death from coronary heart disease (CHD) was not significantly reduced (by 5%), nor was any vascular death (3%). There was a non- significant reduction in strokes of 5%, this being the net result of an 8% reduction in non-fatal stroke and a 21% increase in stroke death (mainly from haemorrhagic events), both effects being non-significant. Serious vascular events (MI, stroke or vascular death) were significantly reduced by 12%, mainly due to the large effect on non-fatal MI. About 1650 people would need to be treated with ASA for a year to avoid one serious vascular event, which contrasts with the 10-20 events avoided in secondary prevention by treating 1,000 patients for a year. Other primary prevention trials not included in the meta-analysis have also reported no benefits in MI or stroke, but the findings of still unpublished trials are awaited. Recently, however, encouraging results have come from meta-analyses of the effects of ASA on cancer incidence and mortality and on its effects on cancer metastasis, particularly for adenocarcinomas. Typically, reductions in these measures have been around 30% following treatment for four or five years, but more in several instances. These results alter the balance in primary prevention between benefit and hazard as it appears for arterial events alone, tipping it towards the use of ASA. Consequently, new guidelines on advice and decisions on ASA in primary prevention are now needed. Low dose ASA, eg. 75 mg daily is as effective as higher doses for all the vascular and cancer benefits established in the meta-analyses, and it causes less serious bleeding than higher doses.

Keywords Ankle brachial index • Aspirin • Cancer • Clopidogrel • Coronary heart disease (CHD) • Diabetes • Myocardial infarction (MI) • Primary prevention • Stroke • Transient ischaemic attack (TIA)

1 Background

1.1 Introduction

The primary prevention of ischaemic cardiovascular disorders is the prevention, or more precisely, reduction of the risk of first events in those who have not had previous episodes. With one exception, the randomised controlled trials (RCTs) of antiplatelet agents in this context have all assessed the value of aspirin, so this account deals only with this agent. The exception is a trial of mainly secondary prevention but also with some data on primary prevention that evaluated clopidogrel with or without aspirin. Other agents not evaluated in RCTs are not considered.

Table 1 Antithrombotic Trialists Collaboration (2002); meta-analysis of randomised trials of antiplatelet therapy in high risk patients (secondary prevention): approx. proportional (%) reductions in recurrent events

	% Reduction
Serious vascular events	25
Non-fatal myocardial infarction	33
Non-fatal stroke	25
Vascular mortality	16

1.2 Early Work

Interest in the antithrombotic potential of aspirin was largely stimulated by the demonstration in laboratory studies that it inhibits platelet aggregation (Weiss and Aledort 1967). Soon after this, the first RCTs (Elwood et al. 1974; Elwood and Williams 1979) suggested that aspirin might reduce recurrent events in those who had had a myocardial infarction (MI), i.e. secondary prevention. However, these trials were small and the results were not statistically significant, so that only limited attention was paid to them at the time. In the following years, however, a growing number of RCTs (mostly but not exclusively of aspirin alone or aspirin combined with other agents) gave similar results, the proportional reductions in different vascular events in those who had already experienced them being shown in Table 1 (Antithrombotic Trialists' Collaboration 2002). The absolute reduction in serious vascular events (SVEs) among patients with a previous history of MI, for example, was 36 per 1,000 treated for 2 years. Furthermore, benefits greatly outweighed the risks of serious bleeding so that aspirin soon became routine treatment for those in the early stages of MI or who had recovered from it, and for many stroke patients.

1.3 Six Primary Prevention Trials

The clear value of aspirin in secondary prevention raised the obvious question—what would its value be in primary prevention? Six primary prevention RCTs dealing only with aspirin have been published. These are first considered singly, and their collective evidence is then considered in a meta-analysis (see Sect. 10). Three of these trials were in men alone, two in men and women and one in women only, and their main design features are summarised in Table 2. All but one of these six trials were factorial, i.e. were of simultaneous evaluations (in 2×2 designs or, in one case, a 3×2 design) of another non-platelet-active agent in addition to aspirin. Three primary prevention trials not included in the meta-analysis are also described.

Table 2 Main features of six trials of aspirin in primary prevention of vascular events (Antithrombotic Trialists' Collaboration 2002)

	Dates of recruitment	Participating countries	Year of main publication	Number of participants	Mean duration of follow-up (years)	Target population	Eligible age range (years) at entry	Aspirin regimen	Randomised factorial comparison	Placebo control
British Doctors' Study (Peto et al. 1988)	Nov 1978–Nov 1979	UK	1988	5,139	5–6	Male doctors	19–90	500 mg daily	None	No
US Physicians Health Study (1989)	Aug 1981–Apr 1984	USA	1988	22,071	5–0	Male doctors	45–73	325 mg Alternate days	β carotene vs. placebo	Yes
Thrombosis Prevention Trial (1998)	Feb 1989–May 1994	UK	1998	5,085	6–7	Men with risk factors for CHD	45–69	75 mg daily	Warfarin vs. placebo	Yes
Primary Prevention Project (Meade and Brennan 2000)	June 1993–Apr 1998	Italy	2001	4,495	3–7	Men and women with one or more risk factors for CHD	45–94	100 mg daily	Vitamin E vs. open control	No
Hypertension Optimal Treatment Trial (Collaborative Group of the Primary Prevention Project (PPP) 2001)	Oct 1992–May 1994	Europe, North and South America, Asia	1998	18,790	3–8	Men and women with DBP 100–115 mmHg	50–80	75 mg daily	Three blood pressure regimens	Yes

(continued)

Table 2 (continued)

	Dates of recruitment	Participating countries	Year of main publication	Number of participants	Mean duration of follow-up (years)	Target population	Eligible age range (years) at entry	Aspirin regimen	Randomised factorial comparison	Placebo control
Women's Health Study (Hansson et al. 1998)	Sep 1992–May 1995	USA	2005	39,876	10–0	Female health professionals	≥45	100 mg Alternate days	Vitamin E vs. placebo	Yes

Reproduced by kind permission of The Lancet

1.4 Statistical Methods

All six trials in the meta-analysis recorded their results by intention to treat. Some stated that Cox proportional hazards regression models were used, and they may have been used in other trials as well. The publications describing the principal results of each trial should be consulted for fuller details of statistical methods. In discussing the results for individual trials and the meta-analysis, confidence intervals (CIs) are usually given in the text only if they are not shown in tables or figure. Unless otherwise stated, results are shown for main effects, i.e. for all those on aspirin (whether or not on the other agent in a factorial design) compared with all those not on aspirin (whether or not on the other agent). The numerical results in the tables and figure are either exactly as shown in the original publications or are based on them. "Data not shown" generally means not shown in this review, but shown in original publications.

2 British Doctors Aspirin Trial

2.1 Trial Characteristics

During 1978, the British Doctors Aspirin Trial (BDAT) recruited a total of 5,139 male British doctors, half under the age of 60 (all born in the1900s) (Peto et al. 1988). They had originally replied to a questionnaire about their smoking habits sent to them in 1951. In all, 20,000 doctors were approached. Most would have liked to take part but were ineligible either because they were taking aspirin already for various reasons, while others could not take it because they had peptic ulcers, for example, or had already had episodes of definite MI. Some two-thirds (3,429) of the doctors were randomly allocated to take 500 mg aspirin, either in "ordinary" soluble or effervescent preparations or, later on, 300 mg enteric-coated aspirin. The remaining one-third (1,710) were randomly allocated to avoid aspirin in any form unless some specific indication for it developed. The trial was therefore was not blind. Treatment lasted until 1984, the mean duration of follow-up being 5.6 years. Doctors completed a brief questionnaire every 6 months about their health, particularly the occurrence of possible MIs, strokes or transient ischaemic attacks (TIAs), and the doctors who had treated them were also asked to provide details. Fatal events were ascertained through information from relatives, the records of the General Medical Council and through the Central Register of the National Health Service.

2.2 Results

There were no important differences in baseline characteristics between those in the aspirin and non-aspirin groups, apart from a 1 mmHg higher mean systolic blood pressure in those taking aspirin. Although nearly 20% of those allocated to aspirin

Table 3 British Doctors Aspirin Trial (Peto et al. 1988); numbers of events/subject years of observation

Non-fatal events	Aspirin (n = 3,429; subject years = 18,820)	Controls (n = 1,710; subject years = 9,470)
Non-fatal myocardial infarction:		
Confirmed myocardial infarction	42.5	43.3
Possible myocardial infarction	11.7	4.2
Non-fatal stroke:		
Probably haemorrhagic	1.6	2.1
Probably occlusive	6.9	4.2
Unknown aetiology	23.9	22.2
Possible stroke	3.2	3.2
Transient ischaemic attack:		
Confirmed transient ischaemic attack	15.9*	27.5*
Possible transient ischaemic attack	5.3*	14.8*
Bleed, not cerebral	10.6	7.4
Peptic ulcer	46.8*	29.6*
Non-fatal malignant neoplasm	63.2	61.2
Fatal events		
Myocardial infarction	47.3	49.6
Haemorrhagic stroke	5.3	4.2
Occlusive stroke	4.3	3.2
Stroke, unknown aetiology	6.4	5.3
Other vascular and related causes		
Gastric Haemorrhage	0.5	0
Peptic ulcer (haemorrhagic)	0	3.2
Peptic ulcer (perforated)	1.1	0
Unknown	1.1	1.1
Cancer of upper digestive tract	5.8	5.3
Cancer of lung	7.4	11.6
Other neoplasms	26.6	31.7
Total (all causes)	143.5	159.5

*2$p < 0.05$

Reproduced by kind permission of The British Medical Journal

stopped taking it during the first year, with some 5% stopping each subsequent year, half-way through the trial about 70% of those who had been allocated to aspirin were still taking it on most days. The main reason for discontinuation was gastro-intestinal symptoms. About 2% or so of those allocated to avoid aspirin began to use it each year for various reasons.

The main results are shown in Table 3, expressed in terms of 10,000 man years to take account of the two-thirds/one-third allocation. (The actual numbers of controls developing particular conditions are numerically similar to the rate per 10,000 years as tabulated.) There were no significant differences in the rates of fatal or definite non-fatal MI. However, there were only 137 heart disease deaths and 121 confirmed non-fatal MIs, and the 95% confidence interval for the effect of aspirin on total MIs ranged from 24% more MIs to about 27% fewer. Aspirin significantly reduced confirmed TIAs by about half ($p < 0.05$). There was a small but non-significant

excess of confirmed strokes in those taking aspirin, with similar findings for fatal and non-fatal events. Again, the confidence interval was wide, from a 25% reduction to a 50% increase in the incidence of stroke due to aspirin. However, a significant excess of fatal or disabling strokes was described as "disturbing" (19.1 and 7.4 per 10,000 man years in the aspirin and no aspirin groups respectively ($p < 0.05$) (data not shown). Non-vascular deaths were 15% (non-significantly) lower in the aspirin group than in the control group. This was mainly due to fewer deaths from respiratory disease and to some extent also from cancer, the latter being of relevance bearing in mind results to be discussed later. Non-fatal peptic ulcer was reported significantly more often by those taking aspirin.

2.3 Comment

The trial's findings suggested no benefit attributable to aspirin so far as MI and stroke are concerned, while it may have reduced the occurrence of TIAs. However, the numbers of events were generally small, perhaps partly reflecting the greater attention to their health of the doctors who were taking part. The confidence intervals around the estimates of effects were very wide, both excluding and including the possibility of benefit.

3 US Physicians' Health Study

3.1 Trial Characteristics

As its name implies, the US Physicians' Health Study (1989) was also carried out among doctors, again all male. Those with a prior history of MI, stroke or TIA, cancer, current liver or renal disease, peptic ulcer and contraindications arising from current use of aspirin or other relevant medications were excluded. During recruitment from 1981 to 1984, a total of 22,071 participants entered the trial. Their ages were between 45 and 73. The trial was factorial, double blind and placebo controlled. The aspirin treatment was 325 mg on alternate days, and the other component was 50 mg β-carotene, also taken on alternate days. There were 11,037 participants in the aspirin group and 11,034 in the placebo group. By 1988, the consumption of aspirin or other platelet-active agents was 85.7% in the aspirin group and 14.2% in the placebo group. Some 1,269 physicians asked for enteric-coated aspirin. The mean duration of follow-up was 5.0 years. Every 6 months, participants were sent a brief questionnaire asking about their compliance with treatment and the occurrence of possibly relevant events. Diagnoses of cardiovascular disease or death were considered to have been confirmed only after medical records had been reviewed and adjudicated by an End Points Committee.

3.2 Early Termination of Trial

In December 1987, the Data Monitoring Board recommended early termination of the aspirin component of the trial. First, there was a highly significant reduction in the risk of total MI (see Sect. 3.3) due to aspirin. Second, it seemed that no effect of aspirin on cardiovascular mortality could be detected until 2000 or later because of the very low cardiovascular death rate among those participating. Finally, aspirin was subsequently prescribed for more than 85% of participants who experienced non-fatal cardiovascular events, which would clearly have made findings about cardiovascular mortality difficult to interpret.

3.3 Results

There were no differences between the aspirin and placebo groups in baseline characteristics. The main findings of the trial are shown in Tables 4, 5 and 6. Table 4 shows a 44% reduction in MI among those on aspirin compared with those on placebo ($p < 0.0001$). An apparent 22% increase in strokes of any kind was not statistically significant, and was the net result of a (presumably unexpected) 11% increase in ischaemic strokes in the aspirin group and a doubling in haemorrhagic strokes. The difference between the aspirin and placebo groups in the latter was of borderline significance ($p < 0.06$). Table 5 shows that the total of cardiovascular deaths was virtually identical in the two groups (81 and 83 in the aspirin and placebo groups, respectively). There were 22 sudden deaths in those on aspirin, compared with 12 in the placebo group. If these sudden deaths are considered along with data in Table 4 to have been fatal MIs, which seems quite likely, there would therefore have been 32 fatal MI or coronary related events in the aspirin group and 38 in the placebo group, in which case the finding for fatal MI would be non-significant. Combining acute MI deaths with all other ischaemic heart disease deaths and with sudden deaths, there were 56 deaths in the aspirin group and 65 in the placebo group, a non-significant reduction of 14%. To achieve a risk/benefit figure, the combined end point of non-fatal MI, non-fatal stroke and death from cardiovascular disease gave 307 events among those on aspirin compared with 370 on those on placebo, a risk reduction of 18% ($p = 0.01$). Those on aspirin experienced a significant excess of bleeding problems (Table 6), easy bruising, melena and nose bleeds being the main reasons.

3.4 Sub-group Analyses

Sub-group analyses suggested that the benefit of aspirin was apparent only in those aged 50 or older and in those with low rather than high levels of cholesterol. However, these were apparently data dependent findings not previously specified for analysis and of only borderline significance, so therefore of limited if any clinical significance.

Table 4 US Physicians' Health Study Research Group (1989); main results; numbers of participants

	Aspirin group	Placebo group	Relative risk	95 % Confidence interval	*p* value
Myocardial infarction					
Fatal	10	26	0.34	0.15–0.75	0.007
Non-fatal	129	213	0.59	0.47–0.74	<0.00001
Total	139	239	0.56	0.45–0.70	<0.00001
Person-years of observation	54,560.0	54,355.7	–	–	–
Stroke					
Fatal	9	6	1.51	0.54–4.28	0.43
Non-fatal	110	92	1.20	0.91–1.59	0.20
Total	119	98	1.22	0.93–1.60	0.15
Person-years of observation	54,650.3	54,635.8	–	–	–

Reproduced by kind permission of The New England Journal of Medicine

Table 5 US Physicians' Health Study Research Group (1989); confirmed deaths, according to treatment group; numbers of participants

	Aspirin group	Placebo group	Relative risk	95 % Confidence interval	*p* value
Total cardiovascular deaths	81	83	0.96	0.60–1.54	0.87
Acute myocardial infarction	10	28	0.31	0.14–0.68	0.004
Other ischaemic heart disease	24	25	0.97	0.60–1.55	0.89
Sudden death	22	12	1.96	0.91–4.22	0.09
Stroke	10	7	1.44	0.54–3.88	0.47
Other cardiovascular	15	11	1.38	0.62–3.05	0.43
Total non-cardiovascular deaths	124	133	0.93	0.72–1.20	0.59
Total deaths with confirmed cause	205	216	0.95	0.79–1.15	0.60
Total deaths	217	227	0.96	0.80–1.14	0.64
Person-years of observation	54,894.6	54,864.2	–	–	–

Reproduced by kind permission of The New England Journal of Medicine

Table 6 US Physicians' Health Study Research Group (1989); side effects; numbers (%) of participants

	Aspirin group	Placebo group	
	Number/percent		*p* value
Bleeding problems	2,979 (27.0)	2,248 (20.4)	<0.0001
Easy bruising	1,587 (14.4)	1,027 (9.3)	<0.0001
Haematemesis	38 (0.3)	28 (0.3)	0.22
Melena	364 (3.3)	246 (2.2)	<0.00001
Non-specific gastrointestinal bleeding	440 (4.0)	422 (3.8)	0.55
Epistaxis	862 (7.8)	640 (5.8)	<0.0001
Other bleeding	724 (6.6)	596 (5.4)	0.0004

Reproduced by kind permission of The New England Journal of Medicine

3.5 Comment

In contrast to the British Doctors' trial, there was a large reduction in non-fatal MIs attributable to aspirin. If anything, aspirin increased strokes, both occlusive and haemorrhagic, and also gastrointestinal and other types of bleeding. It has been argued that the trial should not have been ended prematurely despite the clear reduction in non-fatal MI, obviously an important finding. However, many perhaps most patients will be more concerned about the value of aspirin (if any) in reducing the risk of fatal episodes. Aspirin did not reduce the total of cardiovascular deaths. It is, of course, possible to over-interpret the results of combining the more specific findings on coronary heart disease (CHD) events shown in Tables 4 and 5. However, the implications are that patients should probably be told that aspirin does not appear to reduce fatal CHD.

4 Thrombosis Prevention Trial

4.1 Trial Characteristics

Between 1989 and 1994 the Thrombosis Prevention Trial (TPT) (1998) in the UK recruited 5,085 men aged 45–69 who were at increased risk for major CHD events, but who had not so far experienced them. The trial was carried out through the Medical Research Council's General Practice Research Framework in 108 general practices throughout the UK. After excluding those who were not eligible because of a history of peptic ulceration, previous MI or stroke or medication incompatible with trial treatment for example, 61,422 were invited to screening clinics. Their risk was determined according to a score derived from weightings in the Northwick Park Heart Study (Meade et al. 1986) for smoking and family history, body mass index, blood pressure and blood levels of total cholesterol, plasma fibrinogen and plasma factor VII coagulant activity. Those in the top 20% of the risk scores were considered to be at relatively high risk. Of the 10,557 high risk men eligible for the trial, 5,499 (52%) entered it. (The trial began as one of warfarin or placebo. The 5,085 men whose results are considered here were those recruited when aspirin or placebo was added shortly afterwards.) The trial was placebo controlled, double blind and factorial in design. Men were randomly allocated to 75 mg enteric-coated aspirin daily or identical placebo, and also to low intensity oral anticoagulation with warfarin to an International Normalised Ratio (INR) of about 1.5, or to identical placebo treatment. A trial to demonstrate a 30% reduction in major CHD events (coronary death and MI) significant at the 1% level and with 90% power would have required 4,500 men followed for 5 years, so more men were entered than actually needed according to this specification. In order to maximise power, however, those recruited initially as well as later on all continued until a predetermined finishing date in September 1997. Men were followed-up for an average of 6.8 years. Possible cardiovascular events were reported

Table 7 Thrombosis Prevention Trial (1998); main results; numbers (%) of participants, % reduction and absolute reduction

	Aspirin			
	Yes (n = 2,545)	No (n = 2,540)	% Proportional reduction (95 % CI)	Absolute reduction/1,000 person years (95 % CI)
Person years	16,229	16,113		
CHD				
All	154 (9.5)	190 (11.8)	20 (1,35)	2.3 (0.1,4.5)
Fatal	60 (3.7)	53 (3.3)	−12 (−63,22)	−0.4 (−1.7,0.9)
Non-fatal	94 (5.8)	137 (8.5)	32 (12,48)	2.7 (0.9,4.5)
Stroke				
All	47 (2.9)	48 (3.0)	3 (−45,35)	0.1 (−1.1,1.3)
Thrombotic	21 (1.3)	33 (2.0)	35 (−16,64)	0.7 (−0.2,1.6)
Haemorrhagic	9 (0.6)	1 (0.1)	>100 %	−0.5 (−1.0,0.0)
Fatal	14 (0.9)	6 (0.4)	>100 %	−0.5 (1.1,0.1)
Death				
CHD or stroke (first event)	74 (4.6)	59 (3.7)	−25 (−78,13)	−0.9 (−2.3,0.5)
Other cardiovascular	10 (0.6)	12 (0.7)	17 (−109,68)	0.1 (−0.3,0.5)
Cancer	87 (5.4)	104 (60.5)	17 (−11,38)	1.1 (−0.6,2.8)
Other deaths	28 (1.7)	20 (1.2)	−39 (−160,24)	0.5 (−1.4,0.4)
Death, all causes	216 (13.0)	205 (12.2)	−7 (−30,12)	−0.71 (−3.1,1.7)

N.B. Some estimates have been made to complete data on % and absolute reductions not included in original paper (q.v.). % reductions for haemorrhagic and fatal strokes not shown in view of very large effects

Reproduced by kind permission of The Lancet

by doctors and nurses in the practices as they occurred, and by annual searches of practice notes by the nurses. Deaths were automatically notified through the National Health Service Central Register. Clinical reports from hospitals and general practitioners and autopsy reports were obtained, and case summaries submitted to an independent assessor, blind to the treatment allocation group, for decisions according to standard criteria on diagnoses or causes of death.

4.2 Results

Some 50% of men withdrew from trial treatment, the proportions at 1 year, 3 and 5 years being 14%, 29% and 42% respectively. More of these withdrawals were attributable to warfarin than aspirin. Overall, trial treatment was taken for about two-thirds of the trial's total person years. Those who did withdraw from trial treatment were still followed-up for end point events until the end of the trial. The main results for the aspirin component of the trial are shown in Table 7. While there was a 32% reduction in non-fatal MI attributable to aspirin, there was if anything an

Table 8 Thrombosis Prevention Trial (1998); bleeding episodes; numbers of participants

	Aspirin	Placebo
Major		
Gastrointestinal		
Upper	5[a]	1[a] (1)
Lower		1
Indeterminate	1	
Underlying renal-tract cancer	1	
Other	1 (1)	2 (1)
Total	8	4
Indeterminate		
Gastrointestinal	16	8
Genitourinary	16	7
Respiratory	1	
Nasal/throat	4	4
Ocular	6	5
Skin/locomotor	4	9
Miscellaneous	1	
Total	48	33
Minor		
Nose bleed	210**	162
Pink/red urine	52	57
Rectal bleeding	127*	96
Bruising	237***	166
Any minor bleed	484***	398

1[a]underlying gastric cancer
Compared with placebo: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (fatal events in parentheses)
Reproduced by kind permission of The Lancet

increase in fatal events, the net effect being a significant 20% reduction in all events of CHD. There were almost identical numbers of strokes of any kind in the two groups, the result of a small decrease in thrombotic strokes and an increase in haemorrhagic strokes. (Seven of the nine haemorrhagic strokes occurred in those on both active treatments with raised blood pressure (Meade and Brennan 2000). There was no evidence that warfarin affected the results for aspirin in any other outcomes.) There were in fact rather more deaths from CHD or stroke in those on aspirin compared with those who were not (74 and 59 events respectively). Again of interest and discussed later, there were 87 deaths from cancer in those in the aspirin group compared with 104 deaths in those on placebo. The results for bleeding (other than cerebral i.e. haemorrhagic stroke) are shown in Table 8 for the separate aspirin only and placebo only groups alone (i.e. omitting those in the two other groups with participants taking warfarin alone or warfarin with aspirin). Although the (fortunately small) total numbers of major bleeding events in the aspirin and placebo groups did not differ significantly, there were more upper gastrointestinal bleeding events in those on aspirin compared with placebo. Similarly, there were more intermediate events in those on aspirin compared with those not, and also significantly more minor events.

4.3 Comment

Overall, the results of TPT were similar to those in the US Physicians' Health Study, showing a large reduction in non-fatal MIs and no effect or a possible increase in fatal events, and no effect on all strokes.

5 Primary Prevention Project

5.1 Trial Characteristics

The Primary Prevention Project (PPP) (2001) was carried out through general practices in Italy from 1993 to 1998, when 4,495 patients were entered, of whom 2,583 were women. Participants were aged between 45 and 94 at entry, the mean age being 64.4 years. Eligibility was considered when patients attended their general practitioners for any reason. A few patients were recruited among those with hypertension attending hospital units. All those recruited had one or more of the risk factors of hypertension, hypercholesterolemia, diabetes, obesity, family history of premature MI or advanced age. The trial was factorial but not placebo controlled, the treatments being aspirin 100 mg enteric-coated daily and 300 mg vitamin E daily. Clinical visits were scheduled yearly and outcome events recorded. Follow-up was for 3.7 years. Reported events were assessed by an ad hoc committee of clinicians. The main end point was the sum of cardiovascular death, non-fatal MI and non-fatal stroke, but predefined analyses also included cardiovascular deaths alone, total deaths, and total cardiovascular events (cardiovascular death, non-fatal myocardial infarction, non-fatal stroke, angina, TIAs, peripheral arterial disease and revascularisation). Cardiovascular death was further sub-divided into different categories, e.g. sudden, or after a documented MI. At the end of the trial, vital status information was available for 99.3% of the trial population and 92.3% had had a clinical follow-up.

5.2 Power Calculation

Prior calculations had indicated that 7,500 participants followed-up for 5 years would be needed to be able to detect a 25% reduction in the cumulative end point with 90% power and at the 5% level of significance. At the second interim analysis in July 1998, however, a decision was taken to terminate the trial early because of emerging evidence from other trials of the value of aspirin, and also because of the likelihood of no effect for vitamin E unless the follow-up period was greatly extended.

5.3 Results

There were more participants with hypercholesterolemia (41%) in those taking aspirin than in those not (36%), but the two groups were otherwise well balanced on baseline characteristics. At the end of the trial, just over 19% of participants "had stopped taking the treatment." Among those not randomised to aspirin, 7.2% were taking it. The main results are shown in Table 9. The combined end point (cardiovascular death, non-fatal MI and non-fatal stroke) was reduced by 29% in those taking aspirin, but this effect (implausibly greater than the more realistic effect indicated by the meta-analysis—see Sect. 10 below) was not significant, nor was the 31% reduction in non-fatal MI. However, there was a marginally significant reduction of 44% ($p = 0.049$) in cardiovascular deaths. The group of outcomes comprising the main end point together with angina, TIAs, peripheral vascular disease and revascularisation procedures, was reduced significantly by 23% ($p = 0.014$). The relative risk reductions in all strokes and non-fatal strokes were 33% and 16% respectively in those taking or not taking aspirin, but neither of these effects were statistically significant. TIAs were 29% less in those taking aspirin than those not, though this reduction was also not statistically significant. The results on bleeding are shown in Table 10. There were 17 episodes of gastrointestinal bleeding in those on aspirin compared with five episodes in those not.

5.4 Comment

Although the relative risk reduction for non-fatal MI was not significant, the results were generally similar to those in TPT and the US Physicians Health Study in possibly demonstrating a reduction. There was no significant effect of aspirin in reducing stroke, although the point estimates suggested there might have been a beneficial effect.

6 Hypertension Optimal Treatment Trial

6.1 Trial Characteristics

As its name suggests, the Hypertension Optimal Treatment (HOT) (Hansson et al. 1998) trial was mainly concerned with the management of raised blood pressure, aiming to assess the optimum target diastolic blood pressure for individual patients with hypertension. The trial also assessed the potential benefit of low-dose aspirin. Between 1992 and 1994, a total of 18,790 men and women with diastolic blood pressures in the range 100–115 mmHg, aged between 50 and 80 (mean 61.5 years), were recruited in centres in Europe, North and South America and Asia. As expected, many participants were at increased risk of vascular events on account of blood pressure in particular, the mean systolic pressure being 170 mmHg at

Table 9 Primary prevention Project (Collaborative Group of the Primary Prevention Project (PPP) 2001); main results; numbers (%) of participants and relative risks

	Aspirin (n = 2,226)	No aspirin (n = 2,269)	Relative risk (95 % CI)
Main combined end point (cardiovascular death, non-fatal myocardial infarction, and non-fatal stroke)	45 (2.0 %)	64 (2.8 %)	0.71 (0.48–1.04)
Total cardio events or diseases	141 (6.3 %)	187 (8.2 %)	0.77 (0.62–0.95)
All deaths	62 (2.8 %)	78 (3.4 %)	0.81 (0.58–1.13)
Cardiovascular	17 (0.8 %)	31 (1.4 %)	0.56 (0.31–0.99)
Non-cardiovascular	45 (2.0 %)	47 (2.0 %)	0.98 (0.65–1.46)
All myocardial infarction	19 (0.8 %)	28 (1.2 %)	0.69 (0.38–1.23)
Non-fatal myocardial infarction	15 (0.7 %)	22 (1.0 %)	0.69 (0.36–1.33)
All stroke	16 (0.7 %)	24 (1.1 %)	0.67 (0.36–1.27)
Non-fatal stroke	15 (0.7 %)	18 (0.8 %)	0.84 (0.42–1.67)
Angina Pectoris	54 (2.4 %)	67 (3.0 %)	0.82 (0.58–1.17)
Transient ischaemic attack	28 (1.3 %)	40 (1.8 %)	0.71 (0.44–1.15)
Peripheral-artery disease	17 (0.8 %)	29 (13 %)	0.60 (0.33–1.08)
Revascularisation procedure	20 (0.9 %)	29 (1.3 %)	0.70 (0.40–1.24)

Reproduced by kind permission of The Lancet

Table 10 Primary prevention Project (2001); adverse events; number of patients

	Aspirin (n = 2,226)	No aspirin (n = 2,269)
Cancer	86	80
Bleeding		
Gastrointestinal	17	5
Intracranial (not parenchymal)	2	0
Ocular	1	1
Epistaxis	2	0
Other	2	0
Gastrointestinal disease (except bleeding)	8	3
Other events	36	21
Total	154	110

Reproduced by kind permission of The Lancet

entry. About 16% were smokers and 8% diabetic. The trial was factorial and placebo controlled, participants being allocated to one of the three target diastolic blood pressure regimens, $\leq$90 mmHg, $\leq$85 mmHg or $\leq$80 mmHg. The main antihypertensive treatment was with the long-acting calcium antagonist felodipine 5 mg daily. Additional pressure lowering treatments were introduced as necessary. Patients were also randomly allocated to aspirin 75 mg daily (9,399 patients) or placebo (9,391 patients) (so that the trial was 3 $\times$ 2 factorial in design). The necessary sample size was estimated at 40,000 patient years. Major cardiovascular events were defined as all MIs (fatal and non-fatal), all strokes (fatal and non-fatal)

Table 11 Hypertension Optimal Treatment Trial (Hansson et al. 1998); numbers of events, events/1,000 patient years and relative risks

Events	Number of events	Events/1,000 patient years	Relative risk (95 % CI)	*p*
Major cardiovascular events				
Acetylsalicylic acid	315	8.9		
Placebo	368	10.5	0.85 (0.73–0.99)	0.03
Major cardiovascular events, including silent myocardial infarction				
Acetylsalicylic acid	388	11.1		
Placebo	425	12.2	0.91 (0.79–1.04)	0.17
All myocardial infarction				
Acetylsalicylic acid	82	2.3		
Placebo	127	3.6	0.64 (0.49–0.85)	0.002
All myocardial infarction including silent cases				
Acetylsalicylic acid	157	4.4		
Placebo	184	5.2	0.85 (0.69–1.05)	0.13
All stroke				
Acetylsalicylic acid	146	4.1		
Placebo	148	4.2	0.98 (0.78–1.24)	0.88
Cardiovascular mortality				
Acetylsalicylic acid	133	3.7		
Placebo	140	3.9	0.95 (0.75–1.20)	0.65
Total mortality				
Acetylsalicylic acid	284	8.0		
Placebo	305	8.6	0.93 (0.79–1.09)	0.36

Reproduced by kind permission of The Lancet

and all other cardiovascular deaths. (A fatal event was one occurring within 28 days of the clinical onset of the episode.) Silent MIs were documented by electrocardiograms at randomisation and at the final visit. Follow-up was for an average of 3.8 years. Classification of events took account of information from hospital and physicians' records, death certificates and autopsy reports.

6.2 Results

Findings according to aspirin allocation are summarised in Table 11. Aspirin significantly reduced major cardiovascular events by 15% ($p < 0.03$), although the benefit fell to 9% when silent MIs were included in the analysis. All MIs were 36% less frequent in those on active treatment ($p < 0.002$), but again the benefit was reduced, to 15%, when silent MIs were included. There were no differences in strokes, cardiovascular mortality or total mortality between the two groups. Table 12 shows that fatal episodes of bleeding were similar in the two groups, while non-fatal major bleeds were significantly more frequent in those taking aspirin ($p < 0.001$), as were minor bleeds.

Table 12 Hypertension Optimal Treatment Trial (Hansson et al. 1998); bleeding episodes; numbers of patients

	Acetylsalicylic acid (n = 9,399)	Placebo (n = 9,391)
Fatal bleeds		
Total	7	8
Gastrointestinal	5	3
Cerebral	2	3
Other		2
Non-fatal major bleeds		
Gastrointestinal	72	34
Cerebral	12	12
Nasal	22	12
Other	23	12
Minor bleeds		
Total	156	87
Gastrointestinal	30	18
Nasal	66	24
Purpura	45	25
Other	15	20

Reproduced by kind permission of The Lancet

6.3 Comment

What value should be attached to the data on silent MIs, either as a category on their own or included with clinically evident MIs has been controversial.

7 Women's Health Study

7.1 Trial Characteristics

Between 1992 and 1995 the Women's Health Study (WHS) (Ridker et al. 2005) recruited 39,876 healthy women aged 45 or more into a placebo-controlled, factorial trial evaluating low-dose aspirin and vitamin E. Initially, invitation letters were sent to more than 1.7 million women health professionals, of whom just over 450,000 completed questionnaires and 65,000 were willing and eligible to take part. Women were eligible if they were 45 years of age or more, had no history of CHD, cerebrovascular disease, cancer (other than non-melanoma skin cancer) or other major illnesses, no history of side effects to the study medications, and were not taking regular or frequent aspirin or non-steroidal inflammatory medications, anticoagulants or steroid agents, or regular vitamin supplements. The trial was initially designed with a statistical power of 86% to detect a 25% reduction in major cardiovascular events. The treatments were 100 mg aspirin, or matching placebo, and vitamin E 600 IU, both on alternate days.

Table 13 Women's Health Study (Ridker et al. 2005); main results; numbers of events and relative risks

	Aspirin (n = 19,934)	Placebo (n = 19,942)	Relative risk (95 % CI)	p value
Major cardiovascular event	477	522	0.91 (0.80–1.03)	0.13
Stroke	221	266	0.83 (0.69–0.99)	0.04
Ischaemic	170	221	0.76 (0.63–0.93)	0.009
Haemorrhagic	51	41	1.24 (0.82–1.87)	0.31
Fatal	23	22	1.04 (0.58–1.86)	0.90
Non-fatal	198	244	0.81 (0.67–0.97)	0.02
Myocardial infarction	198	193	1.02 (0.84–1.25)	0.83
Fatal	14	12	1.16 (0.54–2.51)	0.70
Non-fatal	184	181	1.01 (0.83–1.24)	0.90
Death from cardiovascular causes	120	126	0.95 (0.74–1.22)	0.68
Transient ischaemic attack	186	238	0.78 (0.64–0.94)	0.01
Coronary revascularisation	389	374	1.04 (0.90–1.20)	0.61
Death from any cause	609	642	0.95 (0.85–1.06)	0.32

Reproduced by kind permission of The New England Journal of Medicine

A three-month placebo treatment run-in period assessed compliance with long-term treatment, as a result of which 19,934 women were allocated to receive aspirin and 19,942 to receive placebo. Every 12 months, women were sent a year's supply of active or placebo tablets in calendar packs, along with questionnaires requesting information on compliance, side effects and accounts of possible end points of relevance, as well as risk factors. Medical records were obtained on all women in whom a cardiovascular end point was recorded, and reviewed blind to treatment group by an end points committee. Follow-up was for an average of 10 years (range 8.2–10.9 years), and was virtually complete for the primary end point of a first major cardiovascular event, i.e. non-fatal MI, non-fatal stroke or death from cardiovascular causes. The individual components of the composite definition of cardiovascular events were considered secondary end points. Pre-specified sub-group analyses were carried out according to major risk factors.

7.2 Results

The aspirin and placebo groups were well balanced in terms of baseline characteristics. A sensitivity analysis to assess compliance was performed in which data were censored when a woman appeared to have taken less than two-thirds of the study medication during the previous year. The main results are shown in Table 13. In all, 999 women had a first major cardiovascular event, 477 in the aspirin group and 522 in the placebo group, a non-significant reduction of 9%. There was a marginally significant reduction of 17% in the risk of strokes in women in the aspirin group, this being the net result of a significant 24% reduction in the large number of ischaemic strokes along with a non-significant increase in the much smaller number of haemorrhagic strokes. There was no

Table 14 Women's Health Study (Ridker et al. 2005); numbers (%) of bleeding episodes and relative risks

	Aspirin (n = 19,934)	Placebo (n = 19,942)	Relative risk (95 % CI)	p value
Gastrointestinal bleeding				
Any	910 (4.6)	751 (3.8)	1.22 (1.10–1.34)	<0.001
Requiring transfusion	127 (0.6)	91 (0.5)	1.40 (1.07–1.83)	0.02
Peptic ulcer	542 (2.7)	413 (2.1)	1.32 (1.16–1.50)	<0.001
Haematuria	3,039 (15.2)	2,879 (14.4)	1.06 (1.01–1.12)	0.02
Easy bruising	10,561 (53.0)	8,494 (42.6)	1.40 (1.37–1.45)	<0.001
Epistaxis	3,801 (19.1)	3,321 (16.7)	1.16 (1.11–1.22)	<0.001
Any report of gastric upset	11,856 (59.5)	11,915 (59.7)	0.99 (0.97–1.02)	0.59

Reproduced by kind permission of The New England Journal of Medicine

evidence that aspirin reduced the overall risk of fatal MI, non-fatal MI or death from cardiovascular causes. There was, however, a 22% reduction in the risk of TIA attributable to aspirin. The beneficial effect on stroke was seen early in the trial and persisted throughout, while there was no benefit of aspirin on MI at any time during the follow-up. Aspirin was more beneficial in reducing major cardiovascular events among former smokers and those who had never smoked, with an apparently increased risk among current smokers (interaction term between treatment and smoking $p < 0.001$). Women aged 65 or more derived most benefit, the risk of major cardiovascular events being reduced by 26% due to aspirin (RR 0.74; 95% CI 0.59–0.92) ($p = 0.008$). These women were also the only ones in whom aspirin may have reduced the risk of MI (RR 0.66; 95% CI 0.44–0.97) ($p = 0.04$). Neither menopausal status nor the use of hormone replacement therapy modified the effect of aspirin. Analyses to take account of compliance with treatment suggested that aspirin reduced the risk of major cardiovascular events by 13%, the risk of stroke by 26% and the risk of ischaemic stroke by 33%, but that it did not affect the risk of MI. Table 14 shows the effects of aspirin on bleeding. Gastrointestinal episodes were significantly more common among women in the aspirin group compared with those in the placebo group. Thus, there were 127 episodes of gastrointestinal bleeding needing transfusion compared with 91 in the placebo group (RR 1.40, 95% CI 1.07–1.83) ($p = 0.02$). Less serious bleeding occurred in both groups, with significant excesses among those taking aspirin. Peptic ulcer occurred more frequently in those taking aspirin.

7.3 Gender Differences

In analyses according to gender, combined data on women from WHS, HOT and PPP showed that aspirin conferred a 19% reduction in stroke (RR 0.81, 95% CI 0.69, 0.96) ($p = 0.01$) but no reduction in MI (RR 0.99, 95% CI 0.83,1.19) ($p = 0.95$). In the aggregate data (cited in the WHS results) in men in BDAT, the

US Physicians' Health Study, TPT, HOT and PPP aspirin conferred a reduction of 32% in MI (RR 0.68, 95% CI 0.54, 0.86) ($p = 0.001$) and a non-significant increase in stroke (RR 1.13, 95% CI 0.96, 1.33) ($p = 0.15$). The differences between men and women were significant at the 0.01 level for MI and at the 0.005 level for stroke.

7.4 Comment

In summary, the WHS results differ from the other trials, carried out entirely or mostly in men, in finding a reduction in the risk of stroke but without any effect on MI or death from cardiovascular causes. The authors point out that the WHS results on ischaemic stroke are of substantial importance, since compared with men, women have a relatively greater proportion of strokes than MIs.

8 General

Two general points arising from the six trials need consideration. First, the primary outcomes were defined in most of the trials as a composite of several individual outcomes, e.g. non-fatal MI, non-fatal stroke and cardiovascular death; or major CHD which includes fatal and non-fatal events; or mortality from cardiovascular disease which also includes a number of manifestations. Rigid distinctions between "primary" and "secondary" end points, especially when the former is a composite of two or three individual outcomes, may risk paying too little attention to clear results on individual outcomes and differences between separate manifestations of vascular disease in their response to aspirin (provided a "secondary" end point has emerged as more than marginally significant and seems biologically plausible). Thus, non-fatal MI was not defined as a "primary" end point on its own in most of the trials, but is the outcome most strikingly reduced by aspirin in men. Second, the consistency between the five trials in men in finding no effect, or even harm, due to aspirin for stroke supports the reality of this observation (in addition to the findings of the meta-analysis to be discussed). There is also a degree of consistency in a reduction of TIAs due to aspirin.

9 CHARISMA (Clopidogrel Trial)

9.1 Trial Characteristics

This trial, known by its acronym CHARISMA (Bhatt et al. 2006), differs from the six trials already discussed, first, in including another antiplatelet agent, clopidogrel, as well as aspirin as a treatment. Second, although some patients

were free of clinical episodes of vascular disease (primary prevention), most had had clinical events (secondary prevention). Participants were aged 45 or more and about 30% were women. Of the 3,284 participants at high risk but free of prior events, some 80% were diabetic, while about 6% had ankle brachial indices (ABIs) <0.9 and about 20% had evidence of carotid artery plaques, some with evidence of stenosis. "Minor" risk factors in this group included systolic blood pressure $\geq$150 mmHg in spite of treatment, hypercholesterolemia, smoking more than 15 cigarettes a day or age $\geq$65 years for men or $\geq$70 years for women. There were 12,193 patients with established cardiovascular conditions. These patients included about 48% with documented CHD, 36% with cerebrovascular disease and 23% with peripheral arterial disease (some presumably with more than one condition). Because of the much larger numbers in the second group, the trial was primarily one of secondary prevention.

9.2 Design

The trial was double blind and compared the effects of clopidogrel 75 mg daily plus low dose aspirin 75–162 mg daily with those of placebo plus low dose aspirin for a median follow-up period of 28 months. The primary end point was a composite of MI, stroke or death from cardiovascular causes. Follow-up assessments occurred at 1, 3 and 6 months and every 6 months thereafter until the end of the trial, the date of which was based on the pre-specified target of 1,040 primary end points being reached. This number of events would be needed to detect a 20% relative risk reduction in the primary end point with 90% power at the 0.05 level of significance, assuming an annual event rate of 3.1% in the control group and between 18 and 42 months of follow-up. Secondary end points were the first occurrence separately of MI, stroke, death from cardiovascular causes, hospitalisation for unstable angina, TIA or revascularisation procedures. There was a common study end date so that some participants were in the trial longer than others. Several pre-specified sub-groups were defined for analyses that might show particular benefits or hazards in certain groups of patients.

9.3 Recruitment

Recruitment of 15,603 patients was carried out at 768 sites in 32 countries between October 2002 and November 2003. There were 7,802 receiving clopidogrel and aspirin, and 7,801 receiving placebo plus aspirin. Treatment was discontinued by 20.4% of those in the clopidogrel/aspirin group compared with 18.2% in the placebo/aspirin group ($P < 0.001$), just under 5% of patients in each group stopping treatment because of adverse events. Information on primary end points was available for over 99% of participants.

Table 15 CHARISMA Trial (Bhatt et al. 2006); composite and individual primary and secondary and bleeding end points; numbers (%) of patients and relative risks

	Clopidogrel plus aspirin ($n = 7,802$)	Placebo plus aspirin ($n = 7,801$)	Relative risk (95 % CI)*	p value
Efficacy end points				
Primary efficacy end point	534 (6.8)	573 (7.3)	0.93 (0.83–1.05)	0.22
Death from any cause	371 (4.8)	374 (4.8)	0.99 (0.86–1.14)	0.90
Death from cardiovascular causes	238 (3.1)	229 (2.9)	1.04 (0.87–1.25)	0.68
Myocardial infarction (non-fatal)	146 (1.9)	155 (2.0)	0.94 (0.75–1.18)	0.59
Ischaemic stroke (non-fatal)	132 (1.7)	163 (2.1)	0.81 (0.64–1.02)	0.07
Stroke (non-fatal)	150 (1.9)	189 (2.4)	0.79 (0.64–0.98)	0.03
Secondary efficacy end point	1301 (16.7)	1395 (17.9)	0.92 (0.86–0.995)	0.04
Hospitalisation for unstable angina, transient ischaemic attack, or revascularisation	866 (11.1)	957 (12.3)	0.90 (0.82–0.98)	0.02
Safety end points				
Severe bleeding	130 (1.17)	104 (1.3)	1.25 (0.97–1.61)	0.09
Fatal bleeding	26 (0.3)	17 (0.2)	1.53 (0.83–2.82)	0.17
Primary intracranial haemorrhage	26 (0.3)	27 (0.3)	0.96 (0.56–1.65)	0.89
Moderate bleeding	164 (2.1)	101 (1.3)	1.62 (1.27–2.08)	<0.001

Reproduced by kind permission of The New England Journal of Medicine

9.4 Results

The two groups were well balanced in terms of personal and medical histories and for non-trial treatments. Table 15 shows the main results. Primary end points occurred in 6.8% of those on clopidogrel plus aspirin compared with 7.3% among those on placebo plus aspirin—a non-significant reduction of 7%. Non-fatal MIs were 6% less, also non-significantly, in those on combined treatment. There were small, marginally significant benefits attributable to combined treatment for stroke and for other events such as hospitalisation for unstable angina, etc. (as shown in Table 15). Severe bleeding was somewhat though not significantly more frequent in those on clopidogrel plus aspirin, while results for fatal bleeding and primary intracranial haemorrhage were almost identical. Moderate bleeding did occur significantly more frequently in those on clopidogrel plus aspirin. Among the pre-specified subgroups, there was a slight but non-significant increase in primary events in those taking clopidogrel in the "asymptomatic" patients (those with risk factors but without documented clinical disease) whereas there was a marginally significant reduction with clopidogrel among those in the "symptomatic" group (i.e. who had had clinically manifest events). The interaction term between treatment and group was significant at $p = 0.045$, but in view of the overlap there must have been in many respects between

the two groups in the likely pathological involvement of the vasculature, it is probably wrong to attach much importance to the difference.

9.5 Other Clopidogrel Trials

In the secondary prevention CAPRIE trial (1996) (Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events) clopidogrel alone was found to be superior to aspirin on its own in reducing major vascular events. Four further large trials (Sabatine et al. 2005; Chen et al. 2005; The Clopidogrel in Unstable Angina to Prevent Recurrent Events Investigators 2001; Steinhubl et al. 2002) supported the use of dual antiplatelet therapy in patients with acute coronary syndromes and in those undergoing percutaneous coronary interventions. CHARISMA was thought to be "the logical next step" for evaluating combined treatment in those with established vascular disease or multiple risk factors. However, the trial concluded that the combination of clopidogrel and aspirin is not significantly more effective than aspirin alone, while the risk of moderate to severe bleeding was increased in those on clopidogrel plus aspirin.

10 Meta-analysis of Six Trials

10.1 Rationale

By combining the results of all the RCTs of an agent such as aspirin, meta-analyses, by virtue of randomisation, avoid the biases and confounding which may, and often do, occur in observational (i.e. non-experimental) studies. Randomisation of adequate numbers, which meta-analyses usually achieve, ensures that the groups being compared—active and placebo treatment groups, for example—should be virtually identical in all respects apart from the treatment under investigation. Any difference in outcome between the two groups can therefore only be due to the treatment. (This ideal is not always achieved and the design and analyses of individual trials need to be considered to identify and allow for any departures from it.) The large number of outcome events arising from the combination of several trials results in narrower confidence intervals and therefore more reliable conclusions on treatment effects than small, individual trials which may give conflicting results. On the other hand, critics of meta-analysis point out that trials differ in the characteristics of the populations they recruit including gender (as in the case of aspirin), in other than trial treatments participants have received, in proportions of smokers, and so on. It is therefore considered inappropriate to combine them. The individual trials contributing to the meta-analysis have each been described in the previous sections so that their similarities and differences can be appreciated. However, the balance is strongly in favour of meta-analysis, especially if (as with the trials concerned), individual participant data (rather than grouped data in publications) are available,

since it is then possible to see if particular groups, e.g. diabetics or smokers, derive more or less benefits (or hazards) than other groups.

10.2 Methods

In 2009, the Antithrombotic Trialists (ATT) Collaboration (Baigent et al. 2009) published a collaborative meta-analysis of individual participant data from both primary and secondary prevention trials concerned with vascular disease. The six primary prevention trials described in previous sections were only eligible if they were randomised comparisons of aspirin compared with no aspirin, no other antiplatelet agent being used in either group. Although individuals with histories of clinically manifest occlusive disease at entry were meant to be excluded, it later became apparent that 2% did in fact have some evidence of previous vascular disease. They have been included in all analyses apart from those estimating absolute effects of aspirin. Trials had to have recruited at least 1,000 non-diabetic participants with at least 2 years of treatment. Individual participant data were provided from all six published trials. Unpublished trials were sought by electronic searches and discussions, but none were identified. (The clopidogrel trial described in Sect. 9 was not included in the meta-analysis.)

10.3 Outcomes

Pre-specified analyses of the six trials were all by intention-to-treat for first events during the scheduled treatment period in those allocated aspirin compared with those allocated control or no medication. The main outcomes were (1) SVEs defined as myocardial infarction (MI), stroke or death from vascular causes, including sudden death, pulmonary embolism and haemorrhage, (2) major coronary events (MI, coronary death or sudden death), (3) any stroke classified by type if known, (4) death from any cause and (5) major extracranial bleeding episodes (mainly gastrointestinal). MIs and strokes were classified as fatal or non-fatal according to definitions of intervals between onset and death used for this classification in each individual trial. Five of the trials classified strokes on the basis of either clinical examination or CT imaging. Eleven pre-specified sub-groups were defined to establish those in whom treatment might be particularly beneficial or harmful. These sub-groups were according to age, gender, previous vascular disease (see Sect. 10.2), previous diabetes, smoking, previous hypertension, systolic and diastolic pressures separately, cholesterol level, body mass index and predicted 5-year risk of CHD. The meta-analysis used a fixed effects method for assessing outcomes according to aspirin treatment, and the main publication should be consulted for full details of statistical methods.

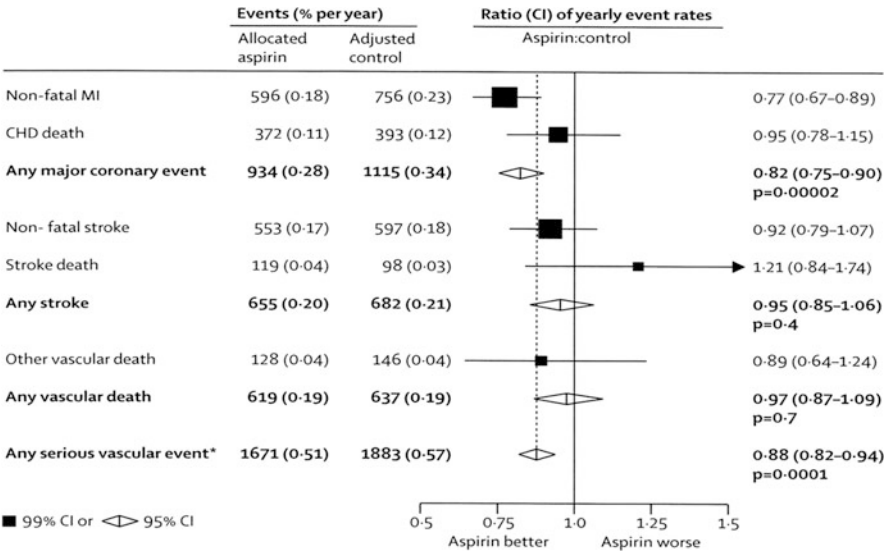


Fig. 1 Actual numbers for aspirin-allocated trial participants, and adjusted numbers for control allocated trial participants, are presented, together with the corresponding mean yearly event rate (in parentheses). Rate Ratios (RRs) for all trials are indicated by squares and their 99 % CIs by horizontal lines. Subtotals and their 95 % CIs are represented by diamonds. Squares or diamonds to the left of the solid line indicate benefit. *Myocardial infarction, stroke, or vascular death. Reproduced by kind permission of The Lancet

10.4 Results

Data were available for 95,000 people who experienced 3,554 SVEs. The main results are shown in Fig. 1. There were 1,671 SVEs during 330,000 person years in those allocated aspirin, corresponding to a rate of 0.51% per annum, compared with 1,883 events in those allocated control (0.57% per annum), a significant reduction of 12% ($p = 0.0001$). There was no significant heterogeneity affecting SVEs between any of the pre-specified sub-groups in their response to aspirin. The difference in absolute rates of SVEs (0.06% per annum) indicates that about 1,650 people would have to be treated with aspirin for a year to avoid one SVE. (This figure is in marked contrast with secondary prevention, in which recurrence rates are considerably higher than rates for first events. Thus, in secondary prevention, some 10–20 non-fatal events would be avoided by treating 1,000 people for a year.) Other vascular deaths were reduced by about 11%, though not significantly. Any vascular death was reduced by only 3%, again a non-significant result.

As major coronary events and strokes accounted for a large proportion of SVEs, the effects of aspirin on each outcome were analysed separately. Figure 1 also shows that there was an 18% proportional reduction in major coronary events. Most of the benefit was due to a 23% proportional reduction in non-fatal MI ($p < 0.0001$), while there was no definite reduction in CHD mortality. For major coronary events, tests for trend or

heterogeneity among the 11 sub-groups gave only one characteristic, gender, with a different finding on aspirin i.e. no significant effect in women (as reported in WHS) and a significant benefit for men, but this difference was of marginal significance only ($p = 0.03$, or 0.33 after allowing for multiple comparisons). There was a suggestion that aspirin benefited those with diabetes, but this was not statistically significant. As for major coronary events, there was an apparent difference in the response to aspirin between men and women for stroke, with women experiencing a marginally significant greater risk reduction ($p = 0.08$), but this was non-significant ($p = 0.88$) after allowing for multiple comparisons (data not shown). Aspirin increased the incidence of haemorrhagic stroke (RR 1.32, 95% CI 1.00–1.75), while it may have reduced the incidence of ischaemic stroke (RR 0.86, 95% CI 0.74–1.00) ($p = 0.05$). There was no beneficial effect on strokes as a whole. Apart from any effects on cerebral bleeding shown in earlier sections for the individual trials, aspirin increased major gastrointestinal and other extracranial bleeds by about half (0.10% vs. 0.07% per annum, RR 1.54, 95% CI 1.30–1.82) ($p < 0.0001$). The excess risk was mainly of non-fatal episodes and in fact there were fewer fatal episodes in those taking aspirin than in controls (9 vs. 20), though this difference based on such small numbers could well have been due to chance.

10.5 Interpretation

There are some apparent contrasts between the primary and secondary prevention trials over the effects of aspirin on different outcomes. Thus, in the primary prevention trials aspirin conferred no significant reductions in fatal CHD, stroke or vascular deaths, whereas in the secondary prevention trials these outcomes were all significantly less in those allocated to aspirin compared with those in no aspirin groups. For stroke, the contrast may depend on the proportions of all strokes that were haemorrhagic and on the outcome, fatal or non-fatal, of these and ischaemic strokes. The meta-analysis argues that the extent of some of the confidence intervals around estimates of effects means that the findings in primary and secondary prevention are compatible with one another. The arterial pathology of vascular disease is on a continuum of severity so that it is hard to imagine a point at which, in primary prevention, aspirin confers no benefit, but then, in secondary prevention, starts to be beneficial. On the other hand, the numbers on which the meta-analysis results for both primary and secondary prevention are based are not trivial, so that the possibility of real differences in responses to aspirin cannot be dismissed. Attention has already been drawn to the consistency of the results on stroke in the individual primary prevention trials, showing no effect or even harm due to aspirin. The overview findings do not convincingly confirm the WHS results in women of a value for aspirin in reducing stroke but not MI.

10.6 Modification of Benefits

The meta-analysis points out that the absolute risk of an SVE in primary prevention may only be about twice as much as the absolute increase in bleeding. Furthermore,

the risk of thrombotic vascular events may be further reduced, perhaps by as much as a half, by statins and other preventive agents besides aspirin. At the same time, the main bleeding hazards probably remain of the same order as in the secondary prevention trials. Thus, any reduction in thrombotic events due to aspirin in primary prevention may be almost equally offset by an increase in the risk of bleeding. Since in secondary prevention the absolute risk of thrombotic events greatly exceeds the risk of bleeding, aspirin confers a clear net benefit. Thus, in those who have previously experienced clinical thrombotic events, aspirin should be and in fact has become more or less routine treatment (unless, of course, there are contraindications to aspirin such as peptic ulceration). The US Preventive Services Task Force (USPSTF) (2009) gives useful guidelines about the points at which benefit outweighs hazard in primary prevention. As a useful working summary, the annual risk of CHD should be at least 2% to justify aspirin, although the USPSTF figures give a range of risks depending on gender and age. Following the meta-analysis, commentaries in both professional and lay publications suggested that those who have so far not experienced SVEs should not take aspirin unless shown to be at sufficiently high risk. Those self-medicating through over the counter purchases may be doing themselves more harm than good.

11 Two Further Primary Prevention Trials

At least three trials of aspirin have been published that, for different reasons, are not included in the meta-analysis considered earlier.

12 Japanese Primary Prevention of Atherosclerosis with Aspirin for Diabetes (JPAD)

12.1 Trial Characteristics

Between December 2002 and May 2005, JPAD (Ogawa et al. 2008) recruited 2,539 patients with type II diabetes aged between 30 and 85 years (mean age 65 years) one through hundred and sixty-three institutions throughout Japan. Just over half the patients were men. It was considered necessary to recruit 2,450 patients to detect a 30% relative risk reduction in "atherosclerotic disease".

12.2 Eligibility and treatment

Electrocardiographic changes and clinical histories of CHD or cerebrovascular disease, atrial fibrillation, pregnancy, use of antiplatelet agents and a history of severe gastric, liver or renal disease or allergy to aspirin were among the reasons for

exclusion. Randomisation was to 81 or 100 mg aspirin daily, or to a no aspirin group, the trial being open label, i.e. not blind. Hazard ratios (HRs) were estimated using a Cox model. Predetermined sub-group analyses were undertaken according to gender, age, hypertensive status, smoking status and lipid status.

12.3 Outcomes

Patients were followed-up until April 2008, the mean follow-up period being 4.37 years. The primary end point was a composite of sudden death, death from coronary, cerebrovascular and aortic causes, non-fatal acute MI, unstable angina, recent onset angina, non-fatal ischaemic and haemorrhagic stroke, TIA or non-fatal aortic and peripheral disease. Secondary end points were each primary end point and combinations of components of primary end points, and death from any cause.

12.4 Results

There were rather more current and past smokers in the aspirin compared with the no aspirin group. Otherwise, the two groups were generally well balanced. By the end of the trial, 123 patients (10%) in the aspirin group had stopped taking the trial treatment. Since aspirin treatment was allowed in the no aspirin group, six patients had taken aspirin and three patients had taken other antiplatelet medications. The main results are shown in Table 16. A total of 154 atherosclerotic events had occurred. The incidence of the primary end point was not significantly different between the aspirin and no aspirin groups. There was a significant difference suggesting a 90% reduction due to aspirin for the combination of coronary and cerebrovascular mortality. However, the numbers of these deaths were very small, one and 10 in the aspirin and no aspirin groups respectively, and the 95% CI for the reduction was very wide, ranging from 0.01 to 0.79. Among the other individual components of the composite primary end point and also for the secondary end points, the numbers of events were also generally small and there were no significant differences in outcomes for them. There were very few fatal MIs. There were slightly more non-fatal MIs in the aspirin group. There were no significant differences for any of the manifestations of cerebrovascular disease. There were 15 and 19 deaths from malignant disease in the aspirin and no aspirin groups respectively. In older patients (65 years or more), the incidence of "atherosclerotic" events was significantly lower in the aspirin than the non-aspirin group (HR 0.68, 95% CI 0.46, 0.99) ($p = 0.047$) (data not shown), but a formal test of interaction between treatment and age was not significant ($p = 0.27$). There were no significant differences according to the other pre-specified sub-groups. There were few bleeding episodes, marginally more in the aspirin than the non-aspirin group. There were, however, 17 reports of gastric ulcer in the aspirin group compared with three in the no aspirin group.

Table 16 Japanese Primary Prevention of Atherosclerosis with Aspirin for Diabetes (Ogawa et al. 2008); numbers (%) of participants; hazard ratios (95 % CI)

	Aspirin group	Non-aspirin group	Hazard ratio	
	No. (%)	No. (%)	(95 % CI)	*p* value
Primary end point: all atherosclerotic events	68 (5.4)	86 (6.7)	0.80 (0.58–1.10)	0.16
Coronary and cerebrovascular mortality	1 (0.08)	10 (0.8)	0.10 (0.01–0.79)	0.0037
CHD events (fatal & non-fatal)	28 (2.2)	35 (2.7)	0.81 (0.49–1.33)	0.40
Fatal MI	0	5 (0.4)		
Non-fatal MI	12 (1.0)	9 (0.7)	1.34 (0.57–3.19)	0.50
Cerebrovascular disease fatal & non-fatal)	28 (2.2)	32 (2.5)	0.84 (0.53–1.32)	0.44
Fatal stroke	1 (0.08)	5 (0.4)	0.20 (0.024–1.74)	0.15
Non-fatal stroke				
Ischaemic	22 (1.7)	24 (1.9)	0.93 (0.52–1.66)	0.80
Haemorrhagic	5 (0.4)	3 (0.2)	1.68 (0.40–7.04)	0.48
Transient Ischaemic attack	5 (0.4)	8 (0.6)	0.63 (0.21–1.93)	0.42

Reproduced by kind permission of The Journal of the American Medical Association

12.5 Comment

Numbers of outcome events were small, including the analysis giving the apparently large and significant difference in favour of aspirin for the sum of coronary and cerebrovascular mortality. Otherwise, no clear benefits of aspirin were found. In particular, the numbers of fatal or non-fatal MIs were too small to draw firm conclusions.

13 Aspirin for Asymptomatic Atherosclerosis Trial

13.1 Trial Characteristics

The Aspirin for Asymptomatic Atherosclerosis (AAA) trial (Fowkes et al. 2010) was a double blind comparison of 100 mg aspirin (enteric coated) or placebo in participants with an ABI of $\leq$0.95. Between April 1998 and October 2008, a total of 28,980 men and women aged between 50 and 75 years living in central Scotland and free of clinically manifest cardiovascular disease were recruited from a community health registry, and underwent ABI screening. Exclusion criteria were a history of MI, stroke, angina or peripheral arterial disease, current use of aspirin or other antiplatelet or anticoagulant agents, severe dyspepsia, chronic liver or kidney disease or contraindications to aspirin.

13.2 Methods

After 5 min rest in a supine position, right and left brachial, posterior tibial and dorsalis pedis systolic pressures were measured using a sphygmomanometer and Doppler probe. ABI was calculated as the ratio of the lowest ankle pressure to the higher pressure in either arm. The 3,350 participants recruited were in accordance with the sample size required to give 80% power at the 5% level of significance to detect a reduction in the event rate from 12 to 9%. Participants were followed after 3 months, 1 year and 5 years at special clinics and annually by telephone and also through a midyear letter enquiring about any problems. The primary end point was a composite of the earliest fatal or non-fatal coronary event, stroke or revascularisation. Secondary end points were of all initial vascular events, i.e. a composite of a primary event or angina, intermittent claudication or TIA and, finally, all cause mortality. The mean duration of follow-up was 8.2 years. Since the eventual outcome rate was 60% of that predicted during the trial, follow-up was extended for a further 4.5 years. Subsequently, and on advice from the Data Monitoring Committee, the trial was stopped 14 months early because of the improbability of finding a difference in the primary end point, accompanied by an increase in major bleeding due to aspirin. However, despite the early stopping, the required number of events had occurred. A trained nurse assessed self-reported adherence to treatment, and participants were followed till the end of the trial irrespective of whether they experienced an end point or an adverse event. Deaths were notified through the NHS Central Registry and (for non-fatal as well as fatal events) linkage to databases of deaths and hospital discharges at NHS National Services Scotland. Independent confirmation of events was carried out. A Cox proportional hazards regression model was used to assess treatment effectiveness. Analyses were also carried out on pre-determined sub-groups, i.e. according to gender and above or below the age of 62. Post hoc, additional analyses were carried out according to different ABI cut points and excluding diabetics.

13.3 Results

By contrast with most of the other aspirin studies, 72% of participants were women. Of all those recruited, 33% were current smokers. The mean ABI was 0.86. Physician or self-prescribed aspirin at 5 years was reported by 15% in the placebo group. Trial medication was taken for about 68% of person years with more than 85% of participants taking it for 6 months or more. The main results are shown in Table 17. Primary end point events occurred in 357 participants with no significant difference between the groups, nor were there any differences found for individual primary end points. All-cause mortality did not differ significantly between the two groups (176 and 186 deaths in the aspirin and placebo groups, respectively). Event rates were similar in the pre-specified and also ABI sub-groups. Initial major haemorrhagic events occurred in 34 participants in the aspirin group, compared with 20 in the placebo group (HR 1.71,

Table 17 Aspirin for Asymptomatic Atherosclerosis Trial (Fowkes et al. 2010); numbers (%) of participants and 95 % CI

	Aspirin group (n = 1,675)	Placebo group (n = 1,675)	Hazard ratio 95 % (CI)
Primary end point event	181 (10.8) [9.4–12.4]	176 (10.5) [9.1–12.1]	1.0 (0.8–1.3)
Fatal			
Coronary event	28 (1.7) [1.2–2.4]	18 (1.1) [0.7–1.7]	1.6 (0.9–2.8)
Stroke	7 (0.4) [0.2–0.9]	12 (0.7) [0.4–1.2]	0.6 (0.2–1.5)
Non-fatal			
Myocardial infarction	62 (3.7) [2.9–4.7]	68 (4.1) [3.2–5.1]	0.9 (0.6–1.3)
Stroke	37 (2.2) [1.6–3.0]	38 (2.3) [1.7–3.1]	1.0 (0.6–1.5)
Coronary revascularisation	24 (1.4) [1.0–2.1]	20 (1.2) [0.8–1.8]	1.2 (0.7–2.2)
Peripheral revascularisation	23 (1.4) [0.9–2.1]	20 (1.2) [0.8–1.8]	1.2 (0.6–2.1)

Reproduced by kind permission of The Journal of the American Medical Association

95% CI 0.99, 2.97). Of these events, 11 in the aspirin group and seven in the placebo group were of intracranial bleeding. There were 14 reports of gastrointestinal ulcer in the aspirin group, compared with eight in the placebo group.

13.4 Comment

Just over 70% of participants were women, partly because men responded to the screening invitation at slightly lower rates and because the population distribution of ABI is lower in women than in men. However, the gender imbalance along with the lower CHD rates in women than men were thought unlikely to have affected the results significantly. The results did not formally exclude the possibility of a risk reduction of up to 16% due to aspirin (or increased risk of up to 27%). However, the absence of a clear significant benefit adds to the case for considering that those who have not yet experienced clinically manifest episodes of CHD should not take aspirin, unless their risks of events are high, taking account of all risk factors besides ABI.

14 POPADAD

14.1 Trial Characteristics

This trial, referred to as POPADAD (Belch et al. 2008), was a 2 × 2 factorial trial assessing aspirin and antioxidants. The trial recruited 1,276 adults age 40 or more who had type I or type II diabetes and an ankle brachial pressure index (ABPI) of 0.99 or less, but otherwise no symptomatic cardiovascular disease. There were

1,670 potentially eligible participants, of whom 1,276 gave consent and were randomised, 320 to both active aspirin and antioxidants, 318 to active aspirin and placebo antioxidants, 320 to placebo aspirin and active antioxidants and 318 to receive both placebo treatments. Besides those with symptomatic cardiovascular disease, people were excluded if they were using aspirin or antioxidant therapy, had peptic ulceration or severe dyspepsia, a bleeding tendency, serious physical illness such as cancer or psychiatric illness, had congenital heart disease or if they could not give informed consent. Participants came from 16 hospital centres in Scotland.

14.2 Methods

The trial was double blind and placebo controlled. The treatments were daily aspirin 100 mg (or placebo) or antioxidant (or placebo) capsule daily (the active antioxidant capsule containing α- tocopherol 200 mg, ascorbic acid 100 mg, pyridoxine hydrochloride 25 mg, zinc sulphate 10 mg, nicotinamide 10 mg, lecithin 9.4 mg and sodium selenite 0.8 mg). Participants were followed-up every 6 months, when outcome events were recorded along with any adverse events or interventions. An end point committee classified deaths. Electrocardiograms were carried out at baseline and annually thereafter. The median length of follow-up was 6.7 years. Two "hierarchical composite primary end points" were defined: one was death from CHD or stroke, non-fatal MI or stroke or above ankle amputation for critical limb ischemia: the other, death from CHD or stroke. Secondary end points were all-cause mortality, non-fatal MI or other vascular events. Based on a 4% annual event rate for end points in the Edinburgh Artery Study (Fowkes et al. 1991) in those with asymptomatic peripheral arterial disease, and taking account of an event rate suggested by the literature for patients with diabetes of between two and threefold compared to the population without diabetes, it was originally planned to recruit 1,600 participants and follow each person up for 4 years. However, recruitment was slower than expected and event rates were lower than anticipated. Eventually 1,276 patients were recruited and the final power calculations in 2003 indicated that if follow-up continued until June 2006 the trial would have 73% power to detect a 25% relative risk reduction, and 89% power to detect a 30% reduction if only one treatment were effective. A Cox proportion hazards model was the primary method of analysis. Sub-group analyses were carried out according to age, gender and ABPI.

14.3 Results

The four treatment groups were similar for baseline characteristics. The cumulative proportion stopping trial treatment was 14% by the end of 1 year and 50% at 5 years. Negligible numbers were lost to follow-up. In all, 233 participants developed the composite primary end point of death from CHD or stroke, non-fatal MI or stroke or above ankle amputation for critical limb ischemia. Table 18 shows the main results for the comparison of aspirin treatment with placebo. Effect estimates were not significant for either of the primary end points or for any of the secondary end points, nor according to the sub-groups defined. There were also no significant effect estimates

Table 18 POPADAD (Belch et al. 2008); comparison between aspirin and no aspirin groups in number (percentage) of patients with diabetes who experienced primary end points, secondary end points, and specific adverse events

	Aspirin ($n = 638$)	No aspirin ($n = 638$)	Effect estimate (95 % CI)	p value
Primary end points:				
Composite end point	116 (182.2)	117 (18.3)	0.98 (0.76–1.26)	0.86
Death from coronary heart disease or stroke	43 (6.7)	35 (5.5)	1.23 (0.79–1.93)	0.36
Secondary end points:				
Death (any cause)	94 (14.7)	101 (15.8)	0.93 (0.71–1.24)	0.63
Coronary heart disease death	35 (5.5)	26 (4.1)	1.35 (0.81–2.25)	0.24
Stroke death	8 (1.3)	9 (1.4)	0.89 (0.34–2.30)	0.80
Non-fatal myocardial infarction	55 (8.6)	56 (8.8)	0.98 (0.68–1.43)	0.93
Non-fatal stroke	29 (4.6)	41 (6.4)	0.71 (0.44–1.14)	0.15
Above ankle amputation for critical limb ischaemia	11 (1.7)	9 (1.4)	1.23 (0.51–2.97)	0.64
Transient Ischaemic attack	14 (2.2)	20 (3.1)	0.70 (0.36–1.39)	0.31
Gastrointestinal bleeding	28 (4.4)	31 (4.9)	0.90 (0.53–1.52)	0.69
Gastrointestinal symptoms including dyspepsia	73 (11.4)	94 (14.7)	0.77 (0.55–1.08)	0.081

Reproduced by kind permission of The Lancet

for antioxidant treatment. However, by comparison with the expected result based on age and sex specific gender rates for Scotland, there may have been a significant increase in the number of deaths (from any cause) in the antioxidant treatment group. Interactions between the aspirin and antioxidant treatments were not statistically significant for either of the composite end points, although there were significant interactions for two of the secondary end points. There were no statistically significant differences between the aspirin and no aspirin groups for adverse events, including gastrointestinal bleeding and other gastrointestinal symptoms, including dyspepsia.

14.4 Comment

While aspirin might have been expected to reduce SVEs in diabetics with a degree of peripheral vascular insufficiency, this proved not to be the case and the authors concluded that they found "no evidence to support the use of either aspirin or antioxidants in the primary prevention of cardiovascular events and mortality in people with diabetes." The observation on deaths in the antioxidant treatment group may have reflected better survival than expected of those who did not receive antioxidants. The EDTRS Trial (EDTRS Investigators 1992) have also reported no significant results in diabetics. Two larger trials are in the process of recruiting only patients with diabetes (ASCEND Study 2008), (ACCEPT-D 2007). Results from two further trials in moderately high risk individuals but not confined to diabetics are awaited (Nelson et al. 2003), (ARRIVE Study 2009).

15 Cancer

15.1 Background

Caution about using aspirin in the primary prevention of vascular disease (Sects. 10.7 and 13.7) was and would have remained the appropriate advice until recently. Attention has already been drawn to the primary prevention trials in which results for cancer were commented on.

15.2 Findings

In 2010, Rothwell et al. (2010a) reported on the incidence and mortality of colorectal cancer in four RCTs of aspirin comparing aspirin with placebo or no aspirin, two of primary prevention, the British Doctors' Aspirin Trial (Peto et al. 1988) and the TPT (1998), and two of secondary prevention, the Swedish Aspirin Low-dose Trial (1991) and the UK-TIA Aspirin trial (Farrell et al. 1991). The mean duration of scheduled treatment was 6.0 years, and 391 (2.8%) of 14,033 patients had colorectal cancer during a median follow-up of 18.3 years. Aspirin reduced the 20-year incidence hazard of colon cancer by 24% (hazard ratio 0.76, 95% CI 0.60, 0.96) ($p = 0.02$) and mortality by 35% (HR 0.65, 95% CI 0.48, 0.88) ($p = 0.005$), but not rectal cancer. The benefit was confined to the proximal colon (HR 0.45, 95% CI 0.28, 0.74) ($p = 0.001$) for incidence, and by 66% for mortality (0.34, 95% CI 0.18, 0.66) ($p = 0.001$). Benefit increased with duration of treatment, so that allocation to aspirin for 5 years or more reduced the risk of proximal colon cancer by about 65% and also, with prolonged treatment, the risk of rectal cancer by about 42%. Benefit did not increase with aspirin dose. The reduction in proximal cancers is noteworthy because these tumours are not easily prevented effectively by screening by sigmoidoscopy, in particular, and while colonoscopy visualises the proximal colon, it is a more elaborate and, for many people, a less acceptable procedure. Very recently, a trial (Burn et al. 2011) of aspirin 600 mg daily in patients with Lynch syndrome, with colon cancer (rather than vascular disease) as the end point, has shown a reduction attributable to aspirin of up to 60% after nearly 5 years from the start of treatment, although numbers of outcome cases were not large. Nevertheless, the results support the finding from the much larger vascular trials.

Later in 2010, Rothwell et al. (2010b) reported on mortality from a number of individual cancers and all cancers in eight trials (25,570 patients, 674 cancer deaths). The mean scheduled trial treatment period was at least 4 years, and ranged for up to 20 years. Based on published results (i.e. not individual participant data) and regardless of duration of follow-up, aspirin reduced death due to cancer as a whole by 21% (odds ratio (OR) 0.79, 95% CI 0.68, 0.92) ($p = 0.003$). Individual participant data were available from seven trials and the main results are shown in Table 19. Benefit was only seen after 5 years follow-up, mortality from all cancers being reduced by 34% with reductions in all gastrointestinal cancers of 54%, and (considering individual cancer sites) significant reductions in pancreatic and, as expected

Table 19 Meta-analysis of the effect of aspirin on risk of death due to cancer by duration of follow-up (Rothwell et al. 2010b); hazard ratios (95 % CI)

		0–5 Years' follow-up		≥5 Years' follow-up	
	n	HR (95 % CI)	*p* Value	HR (95 % CI)	*p* Value
Site of primary cancer					
Gastrointestinal					
Oesophagus	23	0.78 (0.27–2.23)	0.64	0.43 (0.11–1.72)	0.23
Pancreas	45	0.88 (0.44–1.77)	0.73	0.25 (0.07–0.92)	0.04
Colorectal	54	0.78 (0.39–14.56)	0.48	0.41 (0.17–1.00)	0.05
Stomach	36	1.85 (0.81–4.23)	0.14	3.09 (0.64–14.91)	0.16
Other	24	0.67 (0.23–1.99)	0.47	0.20 (0.04–0.91)	0.04
All	182	0.96 (0.67–1.38)	0.81	0.46 (0.27–0.77)	0.003
Non-gastrointestinal					
Lung	198	0.92 (0.65–1.30)	0.65	0.68 (0.42–1.10)	0.11
Prostate	37	0.70 (0.29–1.73)	0.44	0.52 (0.20–1.34)	0.17
Bladder and kidney	31	1.04 (0.44–2.47)	0.93	1.28 (0.36–4.54)	0.70
Other solid	93	0.86 (0.52–1.44)	0.57	1.01 (0.51–1.98)	0.98
All	359	0.90 (0.69–1.16)	0.41	0.76 (0.54–1.08)	0.12
Unknown Primary	36	0.56 (0.28–1.15)	0.12	0.56 (0.09–3.38)	0.53
All solid cancers	577	0.88 (0.72–1.08)	0.22	0.64 (0.49–0.85)	0.002
Histological type					
Adenocarcinoma	247	0.86 (0.62–1.18)	0.34	0.53 (0.35–0.81)	0.003
Non-adenocarcinoma	224	0.89 (0.65–1.23)	0.48	0.79 (0.50–1.24)	0.30
Unknown	106	0.91 (0.58–1.44)	0.70	0.69 (0.34–1.43)	0.32
Haematological	50	0.82 (0.44–1.54)	0.53	0.34 (0.09–1.28)	0.11
All cancers	657	0.86 (0.71–1.04)	0.11	0.66 (0.50–0.87)	0.003

from the previous study, colorectal cancers. The 20-year risk of cancer death remained lower in the aspirin groups than in the control groups by some 20% (data not shown). Benefit increased with scheduled duration of trial treatment. The latent period before there was an effect on deaths was about 5 years for oesophageal, pancreatic, brain and lung cancer and longer for stomach, colorectal and prostate cancers. Benefit was unrelated to aspirin dose (75 mg daily or more), gender or smoking, but increased with age. Most recently, Rothwell et al. (2012) have shown that aspirin almost certainly reduces metastases, especially of adenocarcinomas.

15.3 Further Work

There had been indications from an earlier RCT (Flossmann and Rothwell 2007) and from observational studies (Elwood et al. 2009) of a protective effect of aspirin in colorectal cancer, but the overviews summarised here provided the first evidence from RCTs for a number of different cancers. Further work and analyses are needed to meet some of the still unanswered questions on aspirin and cancer (especially breast cancer for which there are so far inadequate numbers, as the trials have so far been mainly in men). Only trials using aspirin on a daily basis were included in the

two cancer overviews (Rothwell et al. 2010a, b), and while it may be that daily treatment is necessary for benefit, the possibility should not be overlooked that alternate day treatment also does so, though perhaps not to the same extent. Nevertheless, the evidence on aspirin and cancer is so far consistent and does suggest worthwhile benefit, especially if treatment goes on for at least 5 years.

16 Policy on Aspirin in Primary Prevention

Deciding about the benefits of aspirin in primary prevention therefore no longer depends only on considerations about vascular disease alone, but needs to take account of effects on cancer mortality, and incidence in the case of colorectal cancers (and possibly incidence of other cancers, yet to be established). The balance has moved in favour of treatment compared with any conclusions on vascular disease alone. As with the primary prevention of vascular events, the risks of bleeding remain the same. Formal estimates for guidance on benefit/risk ratios taking account of both vascular and cancer effects are awaited. As for vascular disease, people considering aspirin for reducing cancer risk need to accept a commitment to long-term treatment. Until more formal guidance is available, they and their physicians need to take account of individual risk factors for both vascular disease and different cancers in reaching decisions.

Knowledge Gaps

- It is surprising that there has so far apparently been no large trial assessing the combined effect of aspirin and a lipid-modifying agent, since this regimen would be attempting to modify the two main processes in CHD, i.e. atheroma and the superimposed thrombotic element. However, the results of ACCEPT-D, evaluating low-dose aspirin in patients with diabetes treated with statins, may help to fill this gap.
- Still unresolved is whether aspirin is beneficial in people with diabetes who have not yet experienced major cardiovascular episodes.
- Another question is whether it is feasible to detect those with “aspirin resistance” in numbers sufficient for clinical (as distinct from research) purposes.
- If so, might increasing the daily dose of aspirin, for example, confer protection for these people, who may at present not derive any benefit from lower aspirin doses?
- As yet, there are no specific guidelines for taking account of both vascular and malignant disease, though these may become clearer over the next few years with the availability of further evidence.

Key Messages

- Those who have already experienced episodes of cardiovascular disease such as MI or stroke should take regular daily aspirin (unless there are specific contraindications such as peptic ulceration or allergy to aspirin).
- The findings on cancer mean that recommendations about aspirin in primary prevention probably need revising.
- The mounting evidence that aspirin has a role in preventing the onset and progression of several cancers may alter the balance towards favouring its use in those at lower risk than so far considered appropriate for vascular events only.

Acknowledgements I am grateful to Professor Colin Baigent for his advice on the meta-analysis, to Liz Turner for her help with further information on Table 7 and Professor Gerry Fowkes and Dr. Izzy Butcher for further information on Table 15. Professor Bianca De Stavola gave statistical assistance. Professor Peter Rothwell kindly commented on the sections dealing with cancer. I am particularly grateful to Donna Davoren for her help with the manuscript.

References

A Study of Cardiovascular Events in Diabetes (ASCEND) Study (2008) http://www.ctsu.ox.ac.uk/ascend/. Accessed 23 April 2009

Antithrombotic Trialists' Collaboration (2002) Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. BMJ 324(7329):71–86

Aspirin to Reduce Risk of Initial Vascular Events (ARRIVE) Study (2008) http://www.arrive-study.com/EN/study.cfm. Accessed 23 April 2009

Baigent C, Blackwell L, Collins R, Emberson J, Godwin J, Peto R et al (2009) Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. Lancet 373(9678):1849–1860

Belch J, MacCuish A, Campbell I, Cobbe S, Taylor R, Prescott R et al (2008) The prevention of progression of arterial disease and diabetes (POPADAD) trial: factorial randomised placebo controlled trial of aspirin and antioxidants in patients with diabetes and asymptomatic peripheral arterial disease. BMJ 337:a1840

Bhatt DL, Fox KA, Hacke W, Berger PB, Black HR, Boden WE et al (2006) Clopidogrel and aspirin versus aspirin alone for the prevention of atherothrombotic events. N Engl J Med 354(16):1706–1717

Burn J, Gerdes AM, Macrae F, Mecklin JP, Moeslein G, Olschwang S et al (2011) Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: an analysis from the CAPP2 randomised controlled trial. Lancet 378(9809):2081–2087

CAPRIE Steering Committee (1996) A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). Lancet 348:1329–1339

Chen ZM, Jiang LX, Chen YP, Xie JX, Pan HC, Peto R et al (2005) Addition of clopidogrel to aspirin in 45,852 patients with acute myocardial infarction: randomised placebo-controlled trial. Lancet 366(9497):1607–1621

Collaborative Group of the Primary Prevention Project (PPP) (2001) Low-dose aspirin and vitamin E in people at cardiovascular risk: a randomised trial in general practice. Lancet 357(9250):89–95

De BG, Sacco M, Evangelista V, Filippi A, Giorda CB, Tognoni G et al (2007) Aspirin and Simvastatin Combination for Cardiovascular Events Prevention Trial in Diabetes (ACCEPT-D): design of a randomized study of the efficacy of low-dose aspirin in the prevention of cardiovascular events in subjects with diabetes mellitus treated with statins. Trials 8:21

Elwood PC, Williams WO (1979) A randomized controlled trial of aspirin in the prevention of early mortality in myocardial infarction. J Royl Coll GPs 29:413–416

Elwood PC, Cochrane AL, Burr ML, Sweetnam PM, Williams G, Welsby E et al (1974) A randomized controlled trial of acetyl salicylic acid in the secondary prevention of mortality from myocardial infarction. BMJ 1(905):436–440

Elwood PC, Gallagher AM, Duthie GG, Mur LA, Morgan G (2009) Aspirin, salicylates, and cancer. Lancet 373(9671):1301–1309

ETDRS Investigators (1992) Aspirin effects on mortality and morbidity in patients with diabetes mellitus. Early treatment diabetic retinopathy study report 14. JAMA 268(10):1292–1300

Farrell B, Godwin J, Richards S, Warlow C (1991) The United Kingdom transient ischaemic attack (UK-TIA) aspirin trial: final results. J Neurol Neurosurg Psychiatry 54(12):1044–1054

Flossmann E, Rothwell PM (2007) Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. Lancet 369(9573):1603–1613

Fowkes FGR, Housley E, Cawood EHH, MacIntyre CCA, Ruckley CV, Prescott RJ (1991) Edinburgh Artery Study: prevalence of asymptomatic and symptomatic peripheral arterial disease in the general population. Int J Epidemiol 20:384–392

Fowkes FG, Price JF, Stewart MC, Butcher I, Leng GC, Pell AC et al (2010) Aspirin for prevention of cardiovascular events in a general population screened for a low ankle brachial index: a randomized controlled trial. JAMA 303(9):841–848

Hansson L, Zanchetti A, Carruthers SG, Dahlof B, Elmfeldt D, Julius S et al (1998) Effects of intensive blood-pressure lowering and low-dose aspirin in patients with hypertension: principal results of the Hypertension Optimal Treatment (HOT) randomised trial. Lancet 351 (9118):1755–1762

Meade TW, Brennan PJ (2000) Determination of who may derive most benefit from aspirin in primary prevention: subgroup results from a randomised controlled trial. BMJ 321:13–17

Meade TW, Brozovic M, Chakrabarti RR, Haines AP, Imeson JD, Mellows S et al (1986) Haemostatic function and ischaemic heart disease: principal results of the Northwick Park Heart Study. Lancet 2:533–537

Medical Research Council's General Practice Research Framework (1998) Thrombosis prevention trial: randomised trial of low-intensity oral anticoagulation with warfarin and low-dose aspirin in the primary prevention of ischaemic heart disease in men at increased risk. Lancet 351:233–241

Nelson M, Reid C, Beilin L, Donnan G, Johnston C, Krum H et al (2003) Rationale for a trial of low-dose aspirin for the primary prevention of major adverse cardiovascular events and vascular dementia in the elderly: Aspirin in Reducing Events in the Elderly (ASPREE). Drugs Aging 20(12):897–903

Ogawa H, Nakayama M, Morimoto T, Uemura S, Kanauchi M, Doi N et al (2008) Low-dose aspirin for primary prevention of atherosclerotic events in patients with type 2 diabetes: a randomized controlled trial. JAMA 300(18):2134–2141

Peto R, Gray R, Collins R, Wheatley K, Hennekens C, Jamrozik K et al (1988) Randomised trial of prophylactic daily aspirin in British male doctors. BMJ 296:313–316

Ridker PM, Cook NR, Lee IM, Gordon D, Gaziano JM, Manson JE et al (2005) A randomized trial of low-dose aspirin in the primary prevention of cardiovascular disease in women. N Engl J Med 352(13):1293–1304

Rothwell PM, Wilson M, Elwin CE, Norrving B, Algra A, Warlow CP et al (2010) Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials. Lancet 376(9754):1741–1750

Rothwell PM, Fowkes FG, Belch JF, Ogawa H, Warlow CP, Meade TW (2011) Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. Lancet 377(9759):31–41

Rothwell PM, Wilson M, Price JF, Belch JF, Meade TW, Mehta Z (2012) Effect of daily aspirin on risk of cancer metastasis: a study of incident cancers during randomised controlled trials. Lancet 379(9826):1591–1601

Sabatine MS, Cannon CP, Gibson CM, Lopez-Sendon JL, Montalescot G, Theroux P et al (2005) Addition of clopidogrel to aspirin and fibrinolytic therapy for myocardial infarction with ST-segment elevation. N Engl J Med 352(12):1179–1189

Steinhubl SR, Berger PB, Mann JT III, Fry ET, DeLago A, Wilmer C et al (2002) Early and sustained dual oral antiplatelet therapy following percutaneous coronary intervention: a randomized controlled trial. JAMA 288(19):2411–2420

The Clopidogrel in Unstable Angina to Prevent Recurrent Events Investigators (2001) Effects of Clopidogrel in Addition to Aspirin in Patients with Acute Coronary Syndromes Without ST-Segment Elevation. The Clopidogrel in Unstable Angina to Prevent Recurrent Events Investigators. N Engl J Med 345:494–502

The SALT Collaborative Group (1991) Swedish Aspirin Low-dose Trial (SALT) of 75 mg aspirin as secondary prophylaxis after cerebrovascular ischaemic events. Lancet 338:1345–1349

The Steering Committee of the Physicians' Health Study Research Group (1989) Final report on the aspirin component of the ongoing physicians' health study. N Eng J Med 321:129–135

U.S. Preventive Services Task Force (2009) Aspirin for the prevention of cardiovascular disease: U.S. Preventive Services Task Force recommendation statement. Ann Intern Med 150:396–405, Ref type: Generic

Weiss HJ, Aledort LM (1967) Impaired platelet/connective-tissue reaction in man after aspirin ingestion. Lancet 2:495–497

Index

P. Gresele et al. (eds.), *Antiplatelet Agents*, Handbook of Experimental Pharmacology 210, DOI 10.1007/978-3-642-29423-5,