Platelet Activation in Vascular Disease
from Animal Studies to Clinical Consequences

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Platelet Activation in Vascular Disease:
from Animal Studies to Clinical Consequences

Plaatjesactivatie in vaatziekte:
van studies in proefdieren naar klinische consequenties

(met een samenvatting in het Nederlands)

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Chapter 1

General introduction and outline of the thesis

Atherosclerosis is a chronic systemic inflammatory disease and the main cause of disease in the western world. In atherosclerosis the composition of the arterial wall of medium size and large arteries changes[1]. It develops as a response to injury of the vessel wall and is induced by endothelial dysfunction as a result of cigarette smoking, diabetes mellitus, hypercholesterolemia, hyperlipidemia, hypertension, obesity, physical inactivity and stress. Endothelial injury changes the permeability of the arterial wall, leading to an influx of low-density lipoprotein (LDL), which elicits an inflammatory response in the vascular wall. Subsequent binding of monocytes and T-cells to the endothelial cells results in increased migration of the cells into the intima of the arterial wall, where the monocytes differentiate into macrophages. Macrophages are able to take up modified lipoproteins, which transform them into foam cells. In response to this process macrophages produce several cytokines and proteases. This vicious circle of lipid driven inflammation can lead to narrowing of the vessel's lumen without clinical consequences.
Clinical manifestations of advanced atherosclerotic disease are caused by destabilization of the atherosclerotic plaque[2]. The first recognizable lesion of atherosclerosis is the fatty streak, which consists of the above described foam cells and T-lymphocytes in the intima. Further development of the lesion leads to the intermediate lesion, composed of layers of macrophages and smooth muscle cells. In the more advanced stage, called the vulnerable plaque, the plaque with a large lipid core is covered by a thin fibrous cap. This cap separates the lipid contents of the plaque from the circulating blood. The vulnerable plaque is prone to rupture, resulting in the formation of a thrombus on the site of disruption. Alternatively, the thrombus can be superimposed on plaque erosion without clear signs of plaque rupture. The formation of a superimposed thrombus on a disrupted atherosclerotic plaque (already narrowing the lumen of the artery), leads to an acute occlusion of the vessel and hypoxia of the downstream tissue. Depending on the location of the atherosclerotic plaque this will cause a myocardial infarction, stroke or peripheral vascular disease.

The role of platelets in arterial thrombosis

Formation of a thrombus on a ruptured plaque is the product of a complex interaction between coagulation factors in the plasma and platelets. After endothelial damage, tissue factor (TF) is released by the subendothelium. TF induces a cascade of activation of coagulation factors ultimately leading to the formation of thrombin. Thrombin cleaves fibrinogen to fibrin, which assembles into a mesh that supports the platelet aggregates. Platelets are anucleated, discoid shaped cell fragments, originating from megakaryocytes in the bone marrow tissue[3]. They have a lifespan of 7-10 days. Their network of internal membranes forms the dense tubular system and the open canalicular system (OCS). The plasma membrane is an extension of the OCS, thereby greatly increasing the surface area of the platelet. The dense tubular system is comparable to the endoplasmatic reticulum in other cell types and is the main storage place of the majority of the platelet's Ca^{2+}. Three types of secretory granules exist in platelets: the dense granules and α-granules and the lysosomes. In the dense granules serotonin, adenosine diphosphate (ADP) and Ca^{2+} are stored. The α-granules contain P-selectin, fibrinogen, thrombospondin, von Willebrand Factor, platelet factor 4 and platelet derived growth factor.
In circulation, platelets are kept in a resting state by endothelial cell derived prostacyclin

(PGI_2) and nitric oxide (NO). PGI_2 increases cyclic adenosine monophosphate (cAMP), the most potent platelet inhibitor. Several agonists can activate platelets; ADP, collagen, thromboxane A2 (TxA_2), epinephrin, serotonine and thrombin, which lead to the following pattern of activation: platelet shape change followed by aggregation and granule secretion. Upon activation the discoid shape changes into a spherical form with pointed protrusions (pseudopods). The integrin receptor $\alpha_{IIb}\beta_3$ plays a vital role in platelet aggregation. The platelet agonists induce a conformational change of the $\alpha_{IIb}\beta_3$ receptor and exposition of binding domains for fibrinogen and von Willebrand Factor. This allows cross-linking of platelets and the formation of aggregates. In addition to shape change and aggregation, the membranes of the α- and dense granules fuse with the membranes of the OCS. This causes the release of their contents and the transportation of proteins embedded in their membrane to the plasma membrane. Activation of platelets is increased by two positive feedback loops (figure 1). In the first, arachidonic acid is cleaved from phospholipids and transformed by cyclooxygenase (COX) to prostaglandin G2 and H2, followed by the formation of TxA_2, a potent platelet agonist. The second feedback loop starts with of the secretion of ADP by the dense granules, resulting in activation of the ADP receptor $P2Y_{12}$. This causes inhibition of cyclic AMP and sustained aggregation.

This complex interaction between endothelial cells, clotting factors, platelets and other factors and cells can be studied in both *in vitro* and *in vivo* model systems. The disadvantage of *in vitro* assays is that it studies the role of a certain protein or cell in isolation. Given the large number of participants and the complex interactions of thrombus formation there is need to study thrombosis and hemostasis in intact living animals, with all the components important for thrombus formation - a vessel wall and flowing blood - present.

Animal models to study arterial thrombosis

Mice and rat can be used as a model to study thrombosis and hemostasis. Their platelets and clotting factors resemble those of human, but mice and rat are not an ideal genetic screening tool. Genetic screens are logistically difficult because of high costs and resource requirements.

Caenorhabditis elegans and *Drosophila* have established their position in genetic research. However, components of hemostasis in *C. elegans* or *Drosophila* do not resemble the factors found in humans. Although hematologically important signaling pathways are present in *Drosophila*, the hematologic system differs from vertebrates[4]. Adaptive immunity is absent and at a cellular level *Drosophila* hematology is primitive compared to mammals. Therefore these models cannot be used as a genetic screening model to study hemostasis and thrombosis.

The zebrafish (*Danio rerio*) was first introduced as an animal model to study thrombosis in the late nineties of the last century[5]. It has several advantages compared to other animal models, which made it possible to screen for novel genes involved in thrombosis and to study these genes in their natural complex *in vivo* environment.

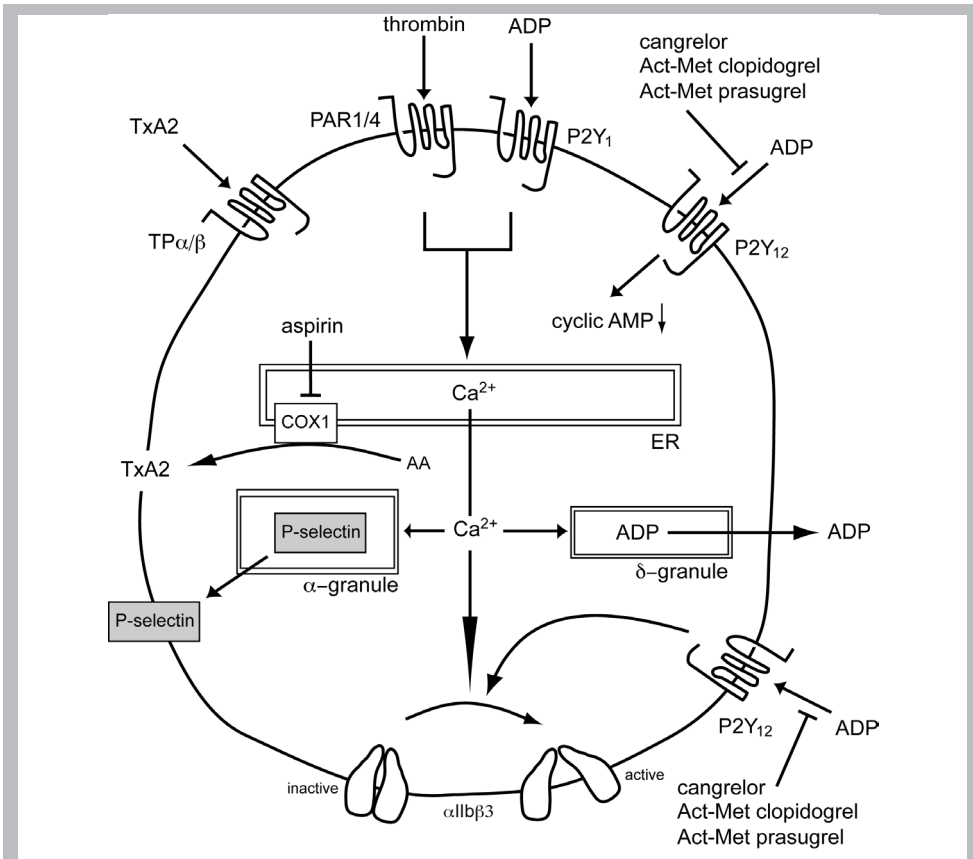

Figure 1. Schematic overview of platelet activation.
Initial platelet activation occurs via binding of several agonists, like thrombin, ADP and collagen (not shown in this picture) to their receptor on the platelet surface. This will lead to a rapid efflux of Ca^{2+} from the endoplasmatic reticulum (ER) to the platelet's cytosol and a conformational change of the $\alpha_{IIb}\beta_3$ receptor. This results in shape change, aggregation and secretion of the contents of the granules. Dense granules secrete ADP, which will activate a positive feedback loop by binding to the $P2Y_{12}$ receptor. Activation of this receptor leads to a decrease of the platelet inhibitor cAMP. A second positive feedback loop occurs via the formation of the potent platelet agonist TxA_2, a process regulated by cyclo-oxygenase (COX). Both positive feedback loops can be successfully inhibited by the COX inhibitor aspirin or the $P2Y_{12}$ antagonist clopidogrel.

Part A: The zebrafish as a model to study platelet function

The zebrafish as an animal model

Hamilton-Buchanan, a chief surgeon, who served Lord Wellesley in British India in 1803, first discovered the zebrafish. He was assigned to gather information on fisheries in India[6]. This resulted in more than 100 native fish species in the river Ganges, which arises from the glaciers of the Himalaya, among which are the zebrafish. George Steisinger used the zebrafish as a genetic tool to study vertebrate development[7]. Compared to other animal models it had the advantages of the ease of breeding, high productiveness, and its diploid nature. Living embryos can be screened for mutant phenotypes easily

with a simple dissecting microscope, since all major organs are visible during the first days of development, due to transparency of the embryo. The embryonic development proceeds rapidly with all the primary organs being formed within 24 hours, embryonic development being completed in 72 hours and a generation time of approximately 3 months.

Large-scale mutant screens were developed by Christiane Nusslein Volhard[8], and resulted in screens for phenotypes in, heart[9], hematopoiesis[10] and blood vessels[11]. In such a screen (also referred to as a forward genetic screen) point mutations are induced in the germline of an adult male zebrafish. These males are crossed with wild-type females. Each individual in the F1 progeny is heterozygous for a different mutagenized genome. These heterozygous F1 fish are crossed in to create a F2 progeny. Fifty percent of the F2 progeny are heterozygous for the same mutation. Mating these heterozygous F2 fish will lead to a phenotype in 25% of the embryos. This phenotype can be studied and cloning techniques can identify the mutation responsible for the phenotype.

Also reverse genetic approaches were developed. Protein levels of specific genes can temporarily be reduced by antisense oligonucleotides (morpholinos)[12]. These morpholinos were targeted against the splice-sites of the pre-mRNA or the start codon of a specific gene, leading to inadequate splicing or haltering translation and a knockdown of a gene. The disadvantage of this technique is that it only temporarily reduces the protein level of the gene of interest and that in most cases there is not a full knockout.

This problem has been solved and the first zebrafish knockout was described in 2002 in a study by Wienholds *et al*, who created a library of randomly mutagenized F1 males[13]. From these fish, genomic DNA was isolated and screened for mutations by nested PCR amplification of the target gene and subsequent DNA sequence analysis. After a certain mutation was identified, in vitro fertilization was performed to recover the carriers of the mutation. These could be bred to homozygosity and the phenotypes analyzed.

Hematopoietic development in the zebrafish

Recent studies have shown the presence of erythrocytes, granulocytes, monocytes, lymphocytes and thrombocytes in peripheral blood smears of zebrafish (figure 2)[14]. Marker gene expression, immunohistochemistry, fluorescent transgenic lines and physical properties in single cell suspensions of kidney marrow or whole embryos identified these specific blood cell types.

Erythrocytes and myeloid cells

Zebrafish erythrocytes are elliptical (typically 7x10 μm) and nucleated. As in mammals, erythrocytes serve similar functions of oxygen transport. Adult erythropoiesis occurs primarily in the interstitium of the anterior and posterior kidneys and is responsive to signalling via the erythropoietin receptor. The adult spleen functions as a reservoir of erythrocytes and a site of their destruction.

In literature zebrafish granulocytes, macrophages and lymphocytes have been described. The zebrafish blood contains two sorts of granulocytes: the neutrophil and the eosinophil. In adult zebrafish the neutrophil has a two to three lobular nucleus. Maturation occurs in the interstitium of the kidney. The first granulocytes appear in the second day of zebrafish development. Neutrophils participate in acute inflammation and show very reserved phagocytic activity. The zebrafish eosinophilic granulocytes

carry much larger and more amorphous granules. Their function in zebrafish has not been demonstrated.
Macrophages are the first leukocytes appearing in the embryo. They participate in response to wounding and exhibit motility and phagocytosis for cellular debris, bacteria and foreign particles. Embryonic macrophages migrate and nest in specific tissues. The similarities and differences between embryonic and adult macrophages are still unclear. Lymphopoiesis in the early zebrafish is initiated at day 4 in the thymus. Both T- and B-lymphocytes develop and can be differentiated by expression of markers.

Thrombocytes
Zebrafish thrombocytes are first detected at day 2 post-fertilization in the caudal hematopoietic tissue, by the expression of the thrombocyte specific CD41 (glycoprotein IIb, the product of the *itga2b* gene) and enter circulation at day 3 post-fertilization[15]. They are nucleated and easy to distinguish by the dense nuclear chromatin, cytoplasmic projections and aggregation in peripheral blood smears. In thrombocyte aggregations pseudopodia-like projections are visible comparable to human platelets. Studies in the transgenic *cd41:EGFP* zebrafish have shown two populations of fluorescent cells; the mature thrombocytes which are more fluorescent ($CD41^{high}$ cells) and cells with a more undifferentiated morphology which express less fluorescence ($CD41^{low}$ cells). It is suggested that the $CD41^{low}$ cells represent the prothrombocytes, since zebrafish do not have megakaryocytes.
The zebrafish thrombocyte is the equivalent of the mammalian platelet and expresses glycoprotein Ib, agglutinates without secretion in response to ristocetin and adheres to the endothelium after injury[16]. It can be activated by the agonists collagen, ADP and arachidonic acid. In zebrafish both COX-1 and COX-2 are present[17]. Inhibition of COX by aspirin and indomethacin inhibits thrombocyte activation. Specific antagonism of the ADP receptor $P2Y_1$ inhibits activation. The role of the $\alpha_{IIb}\beta_3$ complex has not been demonstrated in zebrafish thrombocytes. In an *in vivo* model of arterial thrombosis, which uses laser irradiation to damage endothelial cells of the blood vessels, it was shown that young thrombocytes ($CD41^{low}$ cells) initiate thrombus formation, followed by mature thrombocytes ($CD41^{high}$ cells)[18].

The zebrafish as a model to study platelet function, coagulation and thrombosis
As described earlier, arterial thrombus formation is the result of a complex interaction between platelets, endothelial cells and coagulation factors. Zebrafish possess all the major proteins of the coagulation system, including coagulation factors, anticoagulant factors, and the fibrinolytic pathway. The genes of the blood coagulation pathway have been cloned[19,20] and, although some of the genes are duplicated in the zebrafish genome, all of the coagulation factors in humans are present in zebrafish. Micro assays have been developed for prothrombin time and partial thromboplastin time[21]. Warfarin, an inhibitor of the coagulation pathway in mammals, also inhibits the coagulation pathway of zebrafish[22,23]. This demonstrated that vitamin K dependent pathways are important in zebrafish. For these reasons zebrafish are currently studied by numerous research groups.

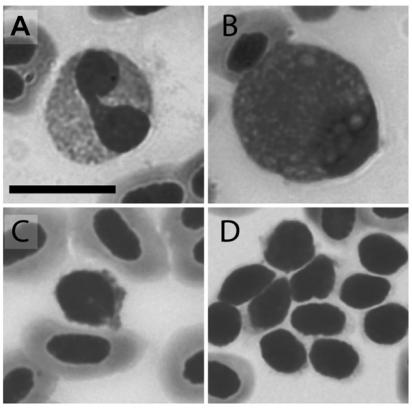

Figure 2. Morphology of adult zebrafish blood cells. Peripheral blood smears showing: (A) bilobed neutrophil, (B) eosinophil, (C) lymphocyte and nucleated erythrocytes and (D) aggregate of thrombocytes with visible cytoplasmic projections.
Adapted from Carradice, D. and Lieschke, G.J.[14]

Part B: Prevention of atherothrombosis by platelet inhibiting drugs

Platelet inhibiting drugs: aspirin and clopidogrel

Patients, who have suffered from a cardiovascular event, are prevented from having a second attack by different classes of drugs. Hypercholesterolemia, hypertension and diabetes mellitus are treated with respectively statins, different classes of anti-hypertensive drugs and oral blood glucose lowering agents or insulin. As described earlier, acute thrombus formation on a ruptured vulnerable atherosclerotic plaque is the cause for the symptoms of a cardiovascular event. Platelets play an important role in the formation of this thrombus and therefore several drugs inhibiting the aggregation of platelets have been developed. Two of the most famous are aspirin and clopidogrel.

Mechanism of action of aspirin

The oldest platelet aggregation inhibitor is aspirin. The French chemist Charles Frederic Gerhardt first prepared aspirin, also known as acetylsalicylic acid in 1853[24]. The British pharmacologist John Robert Vane, by then employed by the Royal College of Surgeons in London, discovered that aspirin suppressed the production of prostaglandins and thromboxanes in 1971, for which he was awarded the Noble Price in Physiology and Medicine in 1982[25]. This suppression of prostaglandins and thromboxanes is due to aspirin's ability to irreversibly inhibit the COX-1 and COX-2 enzymes. This blocks the production of TxA_2 in platelets. TxA_2 is synthesized and released from platelets in the response to different agonists, and induces irreversible aggregation after binding to its receptor, the TxA_2 receptor. Aspirin blocks this positive feedback loop[26]. Aspirin is rapidly absorbed in the stomach and small intestine and reaches peak levels in blood 30 to 40 minutes after ingestion. It has a plasma half-life of 20 minutes[27]. Despite this rapid clearance from circulation it has anti-platelet effects for the life of a platelet (7-10 days) because of irreversible inactivation of COX[28]. Therefore a once-a-day regimen is sufficient for anti-platelet therapy.

Activation of platelets by the ADP receptors; mechanism of action of clopidogrel

ADP activates platelets via the G-protein coupled receptors $P2Y_1$ and $P2Y_{12}$[29]. Upon ligand binding, $P2Y_1$ signals through the α-unit of the GTP-binding protein G_q and eventually activates the fibrinogen receptor $\alpha_{IIb}\beta_3$ and starts granule secretion. Release of ADP and TxA_2 enhances platelet activation through extracellular feedback. The target for the active metabolite (AM) of clopidogrel is $P2Y_{12}$, which signals via the GTP-binding protein G_i. The α-unit inhibits adenylyl cyclase, reducing formation of cAMP. Potent platelet activators such as thrombin induce platelet functions while decreasing the level of cAMP through ADP secretion and $P2Y_{12}$ activation[30]. The βγ-unit of G_i signals to phosphatidylinositol 3-kinase and activates PKB. Both results facilitate signalling by $P2Y_1$ and other activating receptors inducing activation of $\alpha_{IIb}\beta_3$, fibrinogen binding and aggregation[31].

Clopidogrel AM binds irreversibly to $P2Y_{12}$ by forming a disulfide bridge between the reactive thiol group of AM and a cysteine of $P2Y_{12}$[32]. $P2Y_{12}$ activity is limited to acceleration of functions induced by $P2Y_1$ ligand and other platelet activating agents[33]. *In vivo*, $P2Y_{12}$ is blocked by clopidogrel, cangrelor and the recently introduced prasugrel (figure 1). Clopidogrel is an inactive thienopyridine prodrug and converted to its AM by hepatic cytochrome P450[34]. The efficiency is poor (< 15%), the major product being an inactive, second metabolite. Compared with aspirin, clopidogrel induces a 10% better reduction in incidence of severe vascular events[35]. However, there remains an annual risk of 5.32% of ischemic stroke, myocardial infarction or vascular death[36]. Combinations of aspirin and clopidogrel reduce risk of cardiovascular death and myocardial infarction further by 30%[37].

Correlation between type 2 diabetes mellitus and arterial thrombosis: hypersensitive platelets

Patients with type 2 diabetes mellitus (T2DM) suffer from an absolute or relative defect in insulin function (insulin resistance). Patients with type 2 diabetes (T2D) have a 2- to 8–fold higher chance of developing atherosclerosis and coronary artery disease[38,39]. The increased risk is caused by abnormalities in the vessel wall and blood constituents including platelets[40,41]. Platelet abnormalities are (1) an increased turnover[42], (2) an increased cytosolic Ca^{2+} level[43], and (2) up-regulation of the $P2Y_{12}$ pathway[44]. This results in hypersensitive platelets that respond to stimuli that are normally not activating platelets, leading to pathologic occlusions in arteries. T2DM patients also tend to be more resistant to clopidogrel than non-diabetics, which is most likely due to a decreased inhibition of the $P2Y_{12}$ pathway by insulin receptor substrate-1 (IRS-1)[30,44].

Outline of the thesis

The zebrafish is an *in vivo* model system originally used to study development of vertebrates. Over the last years it has gained a more important role in studies focusing on diseases such as cancer and thrombosis. Platelets play an important role in thrombosis and therefore drugs inhibiting platelet enzymes and receptors are developed to prevent thrombosis. The most effective drugs inhibit the COX enzymes (aspirin) or the platelet activating receptor $P2Y_{12}$ (clopidogrel among others). Lack of clarity exists about the $P2Y_{12}$ receptor. In T2DM patients, there is speculation that there is less inhibition by

antagonists. Also the current insight in $P2Y_{12}$ signaling is limited to suppression of production of cAMP (a platelet inhibitor) and activation of protein kinase B/Rap1b (which stimulate aggregation).

Aims of the thesis:

(1) To evaluate the position of the zebrafish as a model system in research on platelet function and thrombus.

(2) Discuss the main problems the currently most used anti-platelet drugs (aspirin and clopidogrel) possess.

(3) To study the inhibition of the $P2Y_{12}$ receptor in diabetes patients and to demonstrate which secondary signalling molecules are activated by $P2Y_{12}$

In **chapter 2** we show the presence of Mlck1a in platelets and study its role in platelet activation using a recently introduced *in vivo* thrombosis assay in zebrafish embryos. **Chapter 3** describes the prevalence of symptoms suggestive for aspirin intolerance in a cohort of 1000 patients who are prescribed this drug to prevent them from having a second atherothrombotic event. **Chapter 4** focuses on the biological and clinical perspectives of the large variation in patients' response to clopidogrel, with extreme examples of poor responsiveness known as clopidogrel resistance. In **chapter 5** we study the inhibition of platelet activation by clopidogrel in T2DM patients compared to healthy individuals. **Chapter 6** addresses which other signaling molecules are activated by $P2Y_{12}$ by a high throughput micro-array approach and reveals $P2Y_{12}$ signalling to the protein tyrosine kinase EphA4. In **chapter 7** the findings of the preceding chapters are discussed in a broader context and implications for future studies are elaborated.

PART A

The zebrafish as a model to study platelet function

Chapter 2

Mlck1a is expressed in zebrafish thrombocytes and an essential component for thrombus formation

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We have used the advantages of the zebrafish model system to demonstrate which of the vertebrate Myosin Light Chain Kinase (*MLCK*) genes is expressed in thrombocytes and important for thrombus formation. Here we report that Mlck1a is an essential component for thrombus formation. Phylogenetic data revealed four zebrafish orthologous for three human *MLCK* genes. To investigate expression of the zebrafish *mlck* genes in thrombocytes we, compared GFP-tagged platelets with other cells by microarray, and showed that *mlck1a* expression was 4.5 fold enriched in platelets which were 8.7 fold enriched in the platelet specific gene *cd41*. Futhermore, *mlck1a* and *cd41* co-localized in thrombi. Expression of other *mlck* subtypes was lower in GFP-tagged platelets (*mlck1b*; 0.77 fold enriched) and absent in thrombi (*mlck1b, -2, -3*). To investigate the role of Mlck1a in thrombus formation, we knocked down *mlck1a* by two morpholinos. This resulted in impaired morphology changes of platelets adhering on fibrinogen. In a thrombosis model, in which thrombocytes adhere to a by laser irradiation damaged vessel wall, thrombus formation was slowed down. We conclude that Mlck1a is the subtype of MLCK that contributes to platelet shape change and thrombus formation.

Introduction

Platelets contribute to arterial thrombosis by formation of a platelet plug, release of platelet activating and vasoconstrictive components and generation of a pro-coagulant surface. Following adhesion to the damaged vessel wall, platelets undergo a profound change in morphology. Two signalling pathways leading to platelet shape change have been described[45]. First, there is the Ca^{2+}-independent route, initiated by thrombin, thromboxane A_2 and lysophosphatidic acid that triggers myosin phosphorylation through Rho-kinase (ROCK) and inhibition of myosin phosphatase. Second, there is a Ca^{2+} dependent route initiated by ADP, thrombin and collagen that activates Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK). MLCK induces phosphorylation of myosin light chain resulting in cytoskeletal rearrangements, folding of membrane surfaces and the contractile wave that centralizes the secretory granules[46,47]. Myosin light chain phosphorylation starts before shape change and can be detected in the aggregometer. Once started, the extent of shape change correlates strongly with the phosphorylation of myosin light chain[48,49] and with the association of myosin to actin[50,51].

Mammals express three genes for MLCK[52]. *MLCK1* (also named smooth muscle *MLCK* or *MYLK1*) is ubiquitously expressed in various tissues. *MLCK2* (or skeletal muscle *MLCK* or *MYLK2*) is expressed in skeletal and cardiac muscle tissue. *MLCK3* (or cardiac *MLCK* or *MYLK3*) is expressed in cardiac muscle tissue and may play a role in cardiogenesis[53]. MLCK phosphorylates the regulatory light chain of myosin on Ser 19 at the N-terminus[52]. The only myosin isoform expressed in platelets is myosin IIa[54]. Phosphorylation of myosin IIa leads to assembly of filaments, which mediate interaction with actin, forming a contractile unit similar to actomyosin in smooth muscle cells[55]. Contractile force is generated by the movement of myosin along actin, a process that requires

phosphorylation of the myosin regulatory light chain[56]. Phosphorylation of the myosin heavy chain inhibits filament formation[57]. Mutations in the myosin IIa gene (*MYH9*) cause thrombocytopenia with large platelets and a mild bleeding tendency. These platelets aggregate normally but fail to undergo shape change[58-61]. A similar but more severe phenotype is seen in mice with a megakaryocyte-restricted *MYH9* disruption. This leads to absent shape change and clot retraction and a dramatic increase in the bleeding time[62]. Platelets adhering to fibrinogen form normal lamellopodia but do not make stress fibers. In a carotid artery thrombosis model, coverage of the injured area is incomplete and thrombus stability is impaired leading to strong embolization.
The zebrafish is an attractive model to study platelet proteins involved in thrombus formation because of transparency of the embryos, labeling of thrombocytes with a GFP tag under the control of the *cd41* promoter and accessibility of gene interference by morpholinos[12,15]. The zebrafish has a coagulation cascade that is very similar to that of humans. Their platelets, although possessing a nucleus, express the fibrinogen receptor $\alpha_{IIb}\beta_3$ and the von Willebrand Factor receptor Glycoprotein Ib and aggregate upon stimulation with collagen, ADP and von Willebrand Factor /ristocetin[16,19,63]. In the present study we made use of these advantages to identify the MLCKs involved in platelet shape change and thrombus formation. We found zebrafish orthologues for all three human *MLCK* genes and show exclusive expression of *mlck1a* in zebrafish thrombocytes. Knock down of *mlck1a* by reverse genetics greatly impaired spreading of thrombocytes on a fibrinogen surface and leads to retarded vessel occlusion in an *in vivo* thrombosis model.

Materials and Methods

Zebrafish husbandry and lines
Embryos were obtained by mass matings of adult TL or *Tg(cd41:GFP)* fish and raised at 28°C. The transgenic line *Tg(cd41:GFP)*[15] was a kind gift from R. Handin (Boston, MA). In this line the sequence for green fluorescent protein (GFP) is fused to the promotor elements of the platelet specific gene *CD41*, to generate fluorescent thrombocytes.

A thrombosis model in zebrafish
Zebrafish larvae 3-5 days post-fertilization (dpf) were anaesthetized in MS222 solution (Sigma, St. Louis, MO) and immobilized in 1% low-melting agarose (Invitrogen, UK) on a microscope slide. A thrombus was induced by delivering a pulsed laser light pumped through coumarin 440 dye (445 nm) (MicroPoint Laser System, Photonic Instruments Inc., St. Charles, Illinois) at 7 pulses per second for 5 seconds through a 10x objective on a Zeiss Axioscope microscope (Carl Zeiss Light Microscopy, Göttingen, Germany). Laser damage was induced in the endothelial layer of the posterior cardinal vein and the dorsal aorta, as indicated, at the position of somite 5 posterior to the cloaca. Thrombus formation was recorded with a Hamamatsu ORCA-ER C4742-80 digital camera (Hamamatsu Photonics, Herrsching am Ammersee, Germany). Time to occlusion (TTO) was defined as the time between the start of laser irradiation and complete occlusion of blood flow.

Morpholino injections
Morpholinos (MOs) were obtained from Gene Tools (http://www.gene-tools.com) and diluted in water containing 0.2% phenol red. One cell stage embryos were injected (maximum volume of 2 nL) as described[12]. Embryos were injected with *prothrombin* or *mlck1a* specific morpholinos (8 ng/embryo each). Control embryos were injected with a *p53* morpholino (8 ng/embryo each). Morpholino sequences were:
$MO_{prothrombin}$: 5'-GTTTGGCTCCCATCCTTGAGAGTGA-3'; $MO1_{mlck1a}$: 5'-TATGCAAGTGTTCATACTCACCGAC-3';
$MO2_{mlck1a}$: 5'-TGATATACTCACGTGCCCAGTCGG-3'; MO_{p53}: 5'-GCGCCATTGCTTTGCAAGAATTG-3'

Microarray gene expression profiling
Three thousand *cd41*–GFP$^+$ (3 dpf) cells were sorted by fluorescence-activated cell sorter (FACS) analysis and sorting from single cell lysates from 3-4 day old embryos. Propidium iodide (PI; Sigma) was added as a marker (1 µg/mL) to indicate dead cell and debris. Cell sorting was performed based on PI exclusion, forward scatter, side scatter and GFP fluorescence using a FACS Vantage Flow Cytometer (Becton Dickinson, San Jose, CA). All cell populations were sorted twice to optimize cell purity. These were compared with 3000 *cd41*-GFP$^-$ cells (a mixture of other cells of the embryos). Total RNA was purified and analyzed using Affymetrix zebrafish gene chips, as described[64].

Whole-Mount in situ hybridization
In situ hybridizations were performed essentially as described[65]. Embryos were mounted in glycerol and documented with a Zeiss Axioplan mounted with a Leica DFC 480 Camera.

Spreading of *cd41*-GFP positive cells on fibrinogen coated glass
For analysis of static adhesion, glass slides (Menzel Gläser 18x18 mm) were cleaned overnight with bromic acid and rinsed with distilled water. Slides were coated with 100 µl of 100 µg/ml fibrinogen (Kordia, Leiden, the Netherlands) for 60 minutes at 22 °C. Coverslips were blocked with 1% BSA in PBS (30 minutes, 22 °C) and washed with Hepes/Tyrode buffer. *cd41*-GFP positive larvae (4 dpf) were anesthetized by MS222 solution and placed on the fibrinogen-coated slide in a drop of Hepes/Tyrode solution. Blood was withdrawn by an incision through the inflow tract of the heart. After withdrawal of the blood, the embryos were removed from the cover slip. The coverslip was covered with a second coverslip (fibrinogen-free) and incubated (30 minutes, 22 °C). *cd41*-GFP$^+$ cells were scored for morphologic features. Different cell morphologies were: (1) round cells, absence of initiation of cell shape change (2) cells that were fully spread on fibrinogen. The number of round cells was measured.

Statistical analysis
Results are expressed as means ± SEM with number of experiments. Statistical comparisons of two groups were by students t-test, using Graphpad Prism 4.0 software. Differences were considered significant at $p<0.05$.

Results

A thrombosis model in zebrafish

We used a thrombosis model in zebrafish and confirmed the contribution of the coagulation system by a knock down approach of the *prothrombin* gene. *Tg(cd41:GFP)* larvae were irradiated and the accumulation of GFP$^+$ thrombocytes was recorded. Approximately five seconds after inducing endothelial injury, the first thrombocytes adhered to the vessel wall and formed a thrombus. The thrombus rapidly grew in size and after about 45 seconds had occluded the vessel and blood flow came to a stand still (figure 1A-G). To investigate the contribution of the coagulation system, we knocked down *prothrombin* expression by introducing a morpholino targeted against the translation start site ($MO_{prothrombin}$). Three days after fertilization laser damage was induced in the posterior cardinal vein and the time to occlusion (TTO) was measured. Knockdown of thrombin expression increased the TTO from 34 ± 6 to 132 ± 12 s (n = 28 embryos per group) (figure 1H). These data confirm that the coagulation mechanism contributes to thrombus formation in a zebrafish, similarly as in humans.

Expression of *mlck* genes in thrombocytes.

We identified four zebrafish orthologues for the three human *MLCK* genes (supplemental Figure 1). To assess which of these zebrafish *mlck* genes is expressed in zebrafish thrombocytes, FACS analyses was performed on a single cell suspension of *Tg(cd41:GFP)* larvae three days post fertilization. A CD41$^+$ cell population was detected

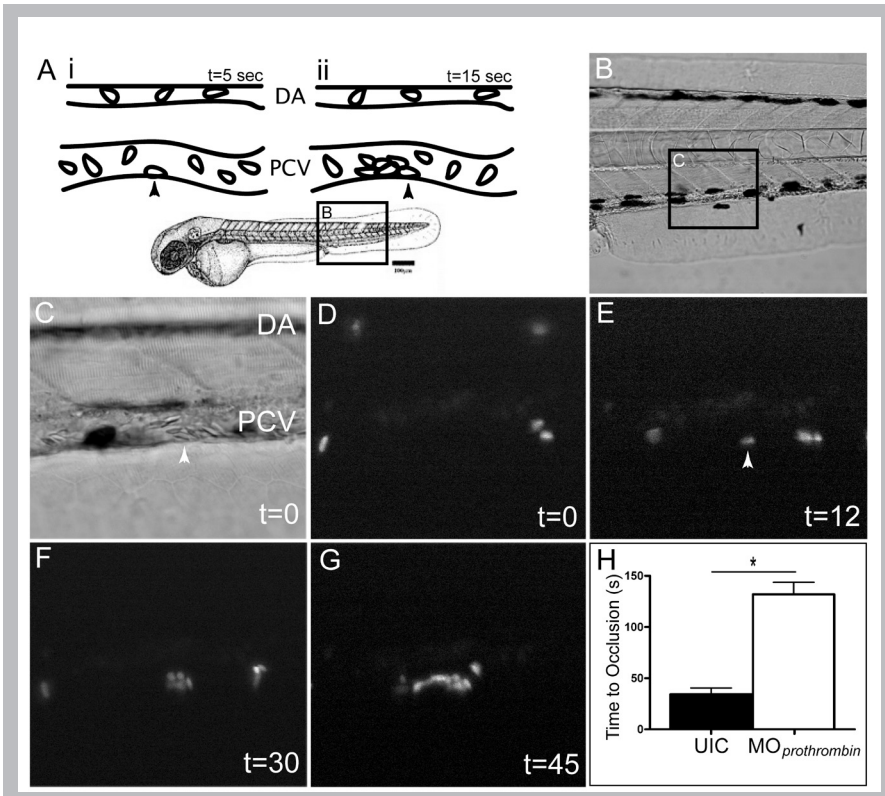


Figure 1. Laser induced thrombosis in Tg(*cd41:GFP*) zebrafish larvae (5 dpf) and the effect of the knockdown of prothrombin.
(A) Endothelial cells in the pericardinal vein were damaged by laser irradiation. Approximately five seconds after endothelial injury the first blood cells adhered to the vessel wall and started forming a thrombus (Ai), which grew rapidly within the next seconds (Aii). (B) To keep variation between fish as low as possible the same point (5 somites posterior to cloaca) was taken as a target for laser irradiation of the endothelium. (C-G) Induction of a thrombus in Tg(*cd41:GFP*) resulted in fluorescent cells adhering to the injured vessel wall. These cells were identified as zebrafish thrombocytes. The arrowhead indicates the location of laser injury. Panels D-G display different intervals after laser injury. (H) The average time to occlusion after knockdown of prothrombin by an ATG-morpholino ($MO_{prothrombin}$) compared to uninjected controls (UIC). A significant difference is indicated by *.

in this transgenic line, which represented about 0.18 % of total cell number (figure 2Ai). The *cd41*-GFP$^+$ population was enriched by two rounds of sorting to a purity of 83.8%. Then, approximately 4000 cells GFP$^+$ cells (figure 2Aii) were compared with 4000 GFP$^-$ cells (figure 2Aiii) by micro-array analysis. Expression of the thrombocyte marker *cd41* was 8.7 fold higher in GFP$^+$ cells than in GFP$^-$ cells, confirming the specificity of this marker. Two probe sets were present for *mlck1a* on the micro-array chip. Both showed 4.5 times higher expression of *mlck1a*, evidence for thrombocyte-specific importance of *mlck1a*. In contrast, for *mlck1b* this number was 0.77 indicating lower expression of this gene (figure 2B). Unfortunately, the array did not contain probe sets for *mlck2* and *mlck3* genes. Together, these data indicate that zebrafish thrombocytes express *mlck1a* and not *mlck1b*.

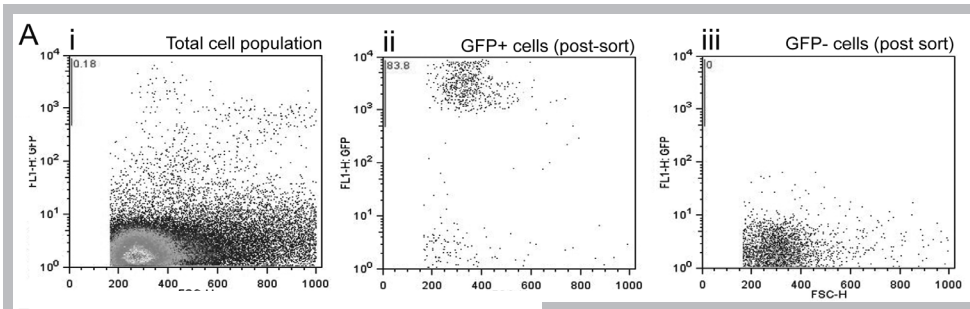
Expression of *mlck* subtypes in a zebrafish thrombus.

To confirm differences in expression of *mlck1a* and *mlck1b* and search further for expression of *mlck2* and *-3*, we performed whole-mount in situ hybridization in zebrafish laser-irradiated at the posterior cardinal vein. Embryos were fixed and incubated with probes specific for *mlck1a, -1b, -2 and -3.* A probe for *cd41* was used as a positive control. In line with the array data, *mlck1a* was expressed at the site of thrombus formation (figure 3A). Individual cells were visible in the posterior cardinal vein at the site of thrombus formation and intersomitic vessels. Staining for *mlck1a* strongly overlapped staining for *cd41*, confirming expression in thrombocytes. Probes for *mlck1b, -2* and *-3* showed no staining of blood cells in the posterior cardinal vein both on whole-mount and cross-sections (figure 3B). These findings confirm that *mlck1a* is the major *mlck* subtype (and possibly the only one) expressed in zebrafish thrombocytes.

***cd41*-GFP⁺ cells with knocked-down *mlck1a* show a decreased spreading on fibrinogen.**

To clarify the role of *mlck1a* in thrombocyte function and thrombus formation, we knocked down gene expression by designing two splice site morpholinos upstream of the protein kinase domain. Morpholino 1 ($MO1_{mlck1a}$) blocked the splicedonor site of exon 6 and morpholino 2 ($MO2_{mlck1a}$) that of exon 9 (figure 4A). Injection with MO_{p53} served as a control. Injection of 8 ng of morpholinos did not change the wild-type appearance of the embryo (data not shown).

The efficiency of the morpholinos was determined by RT-PCR with *mlck1a* specific primers of cDNA extracted from embryos of the same experiment and compared with MO_{p53} injected embryos (figure 4B). Injection of $MO1_{mlck1a}$ induced the appearance of a second band of smaller size. Knockdown of *mlck1a* with $MO2_{mlck1a}$ induced two extra bands. These bands were cut out of the gel and cloned. For $MO1_{mlck1a}$, 3 out of 17 clones



B

Gene	Affymetrix ID	Fold change (upregulation)
cd41	DrAffx.1.8.S1_at	8.7
mlck1a	Dr.12326.1_A1_s_	4.5
	Dr.7877.1.A1_at	4.5
mlck1b	Dr.22326.1.A1_at	0.77

Figure 2. Expression of mlck genes in thrombocytes.

(A) A single cells suspension of about thousand 3 day old *Tg(cd41:GFP)* zebrafish embryos was sorted on GFP expression by FACS. Of the total cell population (Ai) 0.18% of the cells showed positive GFP expression (Aii). Four thousand GFP⁺ cells (purity of 83.8% after two rounds of sorting) were sorted from these embryos and compared to 4000 GFP⁻ cells (Aiii) by microarray analysis.

(B) Micro-array analysis of RNA of four thousand GFP⁺ cells compared to four thousand GFP- cells. *Cd41* was used as a positive control. The data show 4.5 times higher expression of *mlck1a* (no probes were available for *mlck2* and *mlck3*).

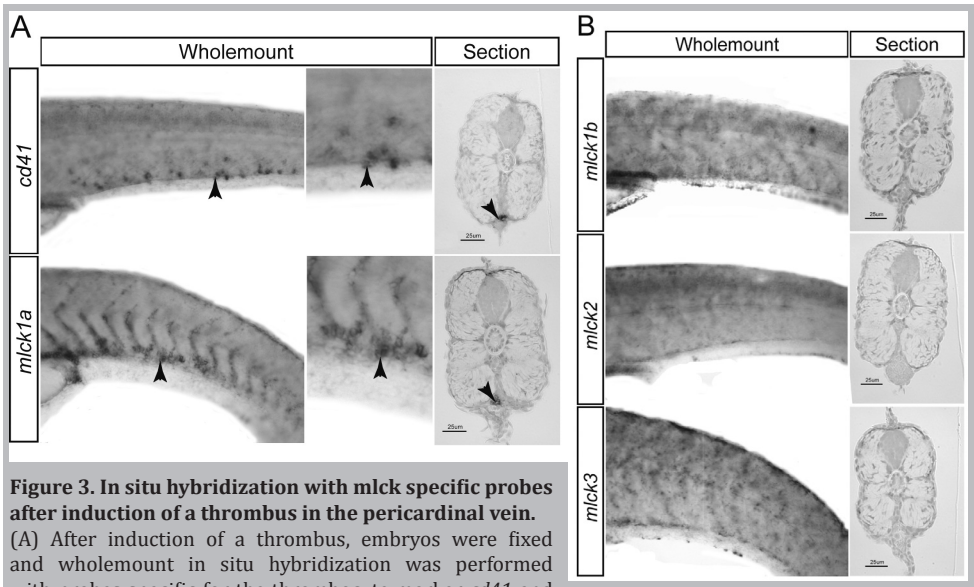


Figure 3. In situ hybridization with mlck specific probes after induction of a thrombus in the pericardinal vein.
(A) After induction of a thrombus, embryos were fixed and wholemount in situ hybridization was performed with probes specific for the thrombocyte marker *cd41* and *mlck1a*. The probe for *cd41* was used as a positive control and showed staining of single cells on the site of thrombus formation. Sections of these embryos showed staining at the location of the pericardinal vein. The probe for *mlck1a* clearly displayed a similar staining pattern as the *cd41* probe on wholemount and sectioned embryos.
(B) Probes for *mlck1b*, *mlck2*, and *mlck3* showed no staining at the site of thrombus formation.

showed a wild type sequence (supplemental figure 2A); 14/17 showed a 36 nucleotide deletion. The protein sequence of this deletion showed a high level of orthology with human, mouse and rat (supplemental figure 2B). For $MO2_{mlck1a}$, 4/18 colonies showed a normal boundary for exon 8 - 9, while in 14/18 colonies either exon 9 (6/18) or both exon 9 and 10 (8/18) were spliced out (supplemental figure 2C). Both splice forms resulted in a frameshift and premature termination of the transcript. These data show that both MOs severely reduce the level of functional protein.
The impact on thrombocyte function was measured in a static adhesion assay. Thrombocytes were isolated and spread on a fibrinogen-coated surface. *cd41*-GFP$^+$ thrombocytes were scored for changes in morphology, as defined (figure 4C-D). In the control, the MO_{p53}-treated zebrafish, the number of thrombocytes that completely preserved the resting, round morphology was 12.0 ± 3.3% of CD41-GFP$^+$ cells. This number increased to 32.5 ± 2.1% and 31.4 ± 4.4% (n = 3 experiments; $p < 0.05$) in thrombocytes from zebrafish treated with $MO1_{mlck1a}$ and $MO2_{mlck1a}$ respectively. This data indicates that the thrombocytes' ability to undergo a morphology change upon contact with fibrinogen depends on *mlck1a*.

Knockdown of *mlck1a* retards thrombus formation
In the MO_{p53} injected embryos, laser damage of the dorsal aorta endothelium induced thrombus formation and complete occlusion of the vessel after 31 ± 2 seconds (n = 19; figure 5). Knockdown of *mlck1a* induced a 70% fold increase in occlusion time in $MO1_{mlck1a}$ injected embryos and a 120% fold increase in $MO2_{mlck1a}$ injected embryos ($p<0.005$). These data show that in zebrafish, platelet Mlck1a is important for formation

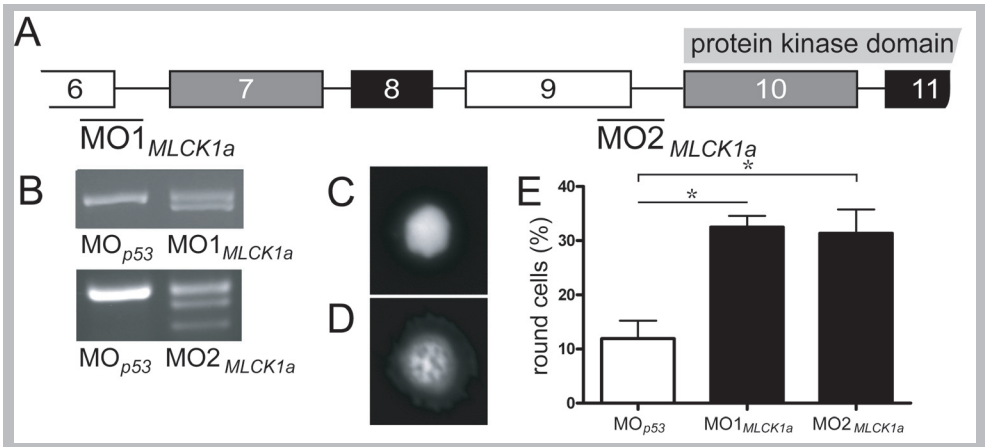


Figure 4. Knockdown of *mlck1a* attenuates spreading of *cd41*-GFP$^+$ cells on fibrinogen.
(A) Target sites of the 2 splice site morpholinos (MO1$_{mlck1a}$ and MO2$_{mlck1a}$) for *mlck1a*. Both were designed to target an exon-intron boundary upstream of the 5' end of the protein kinase domain.
(B) Expression by RT-PCR of *mlck1a* in the embryos injected with a *p53* targeting morpholino showed a normal sized band. Injection of MO1 resulted in two bands, a wildtype band and a shorter band. Injection of MO2 resulted in three bands: a wildtype band and two shorter bands.
(C-D) Fluorescent thrombocytes on fibrinogen coated slides at room temperature. Images of fluorescent channel of the different morphologic features of spreading *cd41*-GFP$^+$ cells on fibrinogen. After 30 minutes, mainly two different cells morphologies were seen: (C) round cells, absence of initiation of cells shape change (D) cells that were fully spread on fibrinogen.
(E) Number of thrombocytes that preserved their round morphology in knockdowns of *mlck1a* by MO1$_{mlck1a}$ and MO2$_{mlck1a}$ compared to the control situation (MO$_{p53}$).

of a thrombus. In PCR experiments using the human megakaryocytic CHRF-288-11 cell-line, we demonstrated that *MLCK1* is also the mainly expressed *MLCK* in humans. The MLCK inhibitor ML-7 inhibits collagen-induced aggregation by human platelets, which suggests that the role for Mlck1a established in zebrafish might be similar in human individuals (supplemental figure 3).

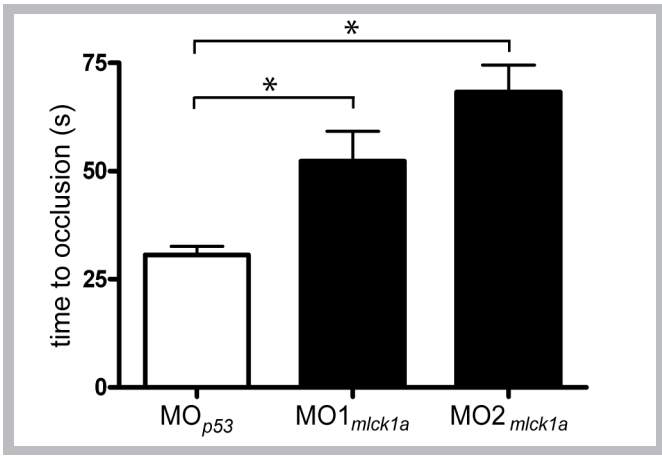


Figure 5. Knockdown of *mlck1a* increases the time to occlusion.
Time to occlusion was measured in knockdown of *mlck1a* by two (MO1$_{mlck1a}$ and MO2$_{mlck1a}$) morpolinos and in controls (MO$_{p53}$). Time to occlusion was defined as the time between the start of laser irradiation and complete arrest of blood flow.

Discussion

This study addressed the question which *MLCK* gene contributes to platelet shape change and thrombus formation, using the zebrafish as a tool for rapid gene inactivation. Transient knockdown of *mlck1a* by a reverse genetic approach decreased the ability of thrombocytes to spread on fibrinogen and impaired thrombus formation in an *in vivo* thrombosis model. These data indicate that *mlck1a* is the subtype that drives the phosphorylation of myosin light chain and thereby the change in platelet morphology. *Mlck1b* and *mlck2* and *-3* were not involved.

Activation of *Mlck1a* is a late step in a pathway that links receptor activation with phosphorylation of myosin IIa. A signaling protein upstream of *Mlck1a* is calmodulin. In human platelets stimulated with the glycoprotein VI ligand convulxin, inhibition of calmodulin by W-7 caused a 50% fall in shape change[66]. Inhibition of myosin by blebbistatin strongly decreased aggregation/secretion, impaired the formation of stress fibers on a collagen surface and increased thrombus embolization *in vitro*[67]. Thus, signaling though *Mlck1a* is vital for platelet morphology changes and thrombus formation.

Activation of the *Mlck1a* pathway appears a general property of platelet activating agents with some stimulating through Gq and phospholipase Cβ, e.g. thrombin and ADP, and others through phospholipase Cγ, e.g. collagen[45]. Toth-Zsamboki[68] *et al*, showed that platelet shape change can also be induced by P2X1- mediated Ca^{2+} influx and Ca^{2+}/CaM-dependant initiation of myosin IIa phosphorylation. MLCK activity is enhanced by P2X1 mediated ERK2 activation which leads to amplified platelet secretion. Aggregation was completely inhibited by the MLCK inhibitor ML-7. cAMP-dependent protein kinase phosphorylates MLCK, which interferes with binding of calmodulin and causes overall inhibition[69]. In our study MLCK-depleted thrombocytes showed impaired spreading on fibrinogen. We have demonstrated that inhibition of Mlck1a results in an impaired thrombus formation *in vivo.* It is possible that this impaired thrombus formation is due to unstable thrombi and increased embolization, which results in increased occlusion times.

A parallel route that controls the phosphorylation state of myosin light chain is through Rho-kinase (ROCK) and inhibition of myosin light chain phosphatase. Inhibition of ROCK by Y27632 hardly affected myosin light chain phosphorylation and shape change after stimulation with ADP or collagen related peptide[45] and inhibition of ROCK had a minor effect on static adhesion to different surfaces[70-72]. Mice treated with Y27632 were unable to form stable thrombi and displayed fast embolization in an *in vivo* thrombosis model[67]. Activation of this pathway is restricted to thromboxane A_2, thrombin and lysophosphatidic acid whose receptors in addition to G_q also activate G12/13 and signaling to ROCK[45].

Mammals express three genes for MLCK. *MLCK1* (also named smooth muscle *MLCK* or *MYLK1*) expresses three transcripts due to alternate promoters[52]. The first transcript codes for the short isoform (130 kDa) and contains a kinase domain, three immunoglobin domains, a fibronectin domain and an actin binding domain and is ubiquitously expressed in adult tissue with the highest concentration in smooth muscle cells[73]. The second transcript codes for the long isoform (also referred to as 210-kDa

MLCK), contains an extra six immunoglobin domains and two actin-binding domains in addition to the sequence of the short isoform and is expressed in embryonic smooth muscle cells and non-muscle cells[74, 75,76]. A third transcript results in the expression of the 17 kDa protein telokin and only contains the C-terminal Ig domain[77]. Telokin plays a role in Ca^{2+} desensitization of smooth muscle force by cyclic nucleotides[78]. Mice with blocked expression of the three *MLCK1* transcripts developed to full size, but died within 1-5 hours after birth[79].
A mammalian platelet expresses *MLCK1* (and a zebrafish thrombocyte its orthologue *mlck1a*), which is activated by Ca^{2+}/calmodulin, although *MLCK2* also contains a Ca^{2+}/calmodulin binding site[52]. The actin-binding sequence in 130 kDa MLCK1 was found necessary for high affinity binding[80]. The function of the other domains in the different MLCK's is unclear and is remains uncertain why certain cells express a specific *MLCK* gene. We found that the zebrafish transcribes four genes for Mlck (supplemental figure 2). The difference with other vertebrates is caused by the expression of two genes for *MLCK1*, *mlck1a* and *mlck1b* in zebrafish. Although the zebrafish genome has orthologues for the *mlck2* and *mlck3* genes, their thrombocytes only express *mlck1*a.

A recent study in neutrophils suggests that the function of MLCK1 might extend beyond the regulation of myosin light chain kinase. In a lung injury model, mice lacking the long, 210 kDa MLCK1 isoform showed impaired attachment of neutrophils to the endothelium and further migration[81]. The defect was caused by impaired activation of β_2 integrins, revealing a role of this MLCK1 isoform in integrin regulation in normal mouse neutrophils. This effect was surprisingly independent of myosin II activity. The myosin light chain could be phosphorylated in *MLCK1* depleted neutrophils and inhibition of MLCK and myosin II led to contradictory effects on neutrophil adhesion and actin polymerization. Platelets express several β1-integrins (α2β1, α5β1 and α6β1) and β3 integrins (αIIbβ3 and αVβ3), but no β2- integrins. Future studies are needed to demonstrate a similar effect of MLCK signaling to integrins in platelets.

Acknowledgements

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Supplemental Information

Supplemental Figures 1,2,3 and 4 are found on the website of the Journal of Thrombosis and Haemostasis; http://www.journalth.com/

PART B

Prevention of atherothrombosis by platelet inhibiting drugs

Chapter 3

The prevalence of intolerance for low dose acetylsalicylacid in the secondary prevention of atherothrombosis

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Daily low dose acetylsalicylacid (ASA) is prescribed to patients with atherothrombosis frequently to prevent vascular complications. In reports on complications and side-effects of low-dose ASA use in the literature there is a range of definitions. We explored the incidence, characteristics and consequences of symptoms suggestive of ASA intolerance in patients on low dose ASA. General practitioners and specialists in 105 centers were asked to review their patient files for the last ten consecutive patients who were prescribed ASA. Participating patients completed a questionnaire about their current ASA use (doctors completed the questionnaire together with the patients), use of co-medication and symptoms suggestive of ASA intolerance. A total of 947 patients were included in this study. Sixty patients (6.6%) had ceased ASA treatment, predominantly because of the occurrence of side-effects suspected to be caused by ASA use. A quarter of the patients concomitantly used an anti-acid agent. 271 patients (30.6%) indicated symptoms during ASA intake. The most common symptoms were related to the gastrointestinal tract (25.1%). In patients prescribed a low dose of ASA monotherapy, side-effects suggestive of intolerance are common. More awareness should be created to detect and treat these symptoms, because the occurrence of side-effects is the most important reason for patients to discontinue ASA treatment.

Introduction

The benefits of ASA in the prevention of atherothrombosis have been well described in literature[82]. However, little is known about the incidence of symptoms and side-effects of low-dose ASA use. Different studies have studied the efficacy and adverse effects of ASA, but used a higher dose of ASA[83,84] or reported only bleeding complications[85,86]. In a study by Silagy *et al*[87], the adverse effects of low-dose aspirin (100 mg daily) were studied in elderly without pre-existing major vascular diseases in a double-blind, randomized, placebo-controlled trial. Gastrointestinal symptoms were reported by 18% of participants receiving aspirin (compared to 13% in the placebo group). Clinically evident gastrointestinal bleeding occurred in 3% of subjects receiving aspirin and none receiving placebo.

Definitions used for this phenomenon are: hypersensitivity, allergy, intolerance and interaction. Studies have estimated the prevalence between 5 to 36 percent depending on the definition used[88,89]. Signs and symptoms of intolerance include dyspepsia and allergic rhinitis and more serious side-effects such as gastric hemorrhage and/or perforation, bronchospasm and the exacerbation of asthma[90,91]. The onset of side-effects varies from days (skin rash) to months or even years (asthma or dyspepsia) after initiation of ASA therapy. Discontinuation of ASA therapy is associated with an increased risk of atherothrombotic complications[92]. In contrast to patients on a high dosage of ASA, the association between symptoms and low dose of ASA might be underestimated by both patients and doctors.

We performed an observational study to estimate the prevalence of ASA intolerance in patients with a low dose (≤ 120 mg) ASA monotherapy. The secondary goals of

this study were to examine the signs and symptoms experienced by ASA intolerant patients, the severity and duration of these signs and symptoms and the consequences. To investigate this in a large population we approached patients over 40 years of age in 105 outpatient practices of vascular surgeons, neurologists, internists and general practitioners.

Material and Methods

From December 2006 until January 2008 investigators (vascular surgeons, neurologists, internists or general practitioners) in 105 centers in the Netherlands were asked to search their patient files for the last ten patients known to be prescribed low dose ASA (figure 1). Ten consecutive patients were selected who satisfied inclusion and no exclusion criteria. The consecutive method was chosen to prevent patient selection bias. Patients were invited to visit the practice, during which visit the doctor interviewed the patient and completed the questionnaire. The start date of ASA use was documented. The doctor could include both chronic and recent users of ASA. Only patients who were already prescribed ASA treatment according to their files were included, which means that only patients were included of which the participating doctors were convinced that they could tolerate ASA at the start of prescription.

Inclusion and exclusion criteria
Inclusion criteria for study participation were: (1) age over 40 years (2) use of ASA or carbasalate calcium in a dosage of 30 - 120 mg per day. Patients were excluded from the study if ASA or carbasalate calcium was used in combination with other platelet aggregation inhibitors such as clopidogrel or dipyridamole.

ASA use
Information about duration of ASA use and daily dosage was obtained. Patients were asked if they were still taking ASA. If not, the following reasons to end ASA intake were asked: occurrence of side effects; increase of complaints; no effect of treatment; unclear necessity for ASA use; patient thinks ASA use is not required; other reasons to cease ASA use.

Contra-indications for ASA use
Contra-indications screened were: history of stomach problems or symptoms of stomach ache after previous ASA intake; history of peptic ulcer disease or gastro-intestinal bleeding; allergy to salicylic acid or prostaglandin synthesis inhibitors; severe renal or hepatic insufficiency; history of hemorrhagic diathesis or coagulopathy.

Use of other medication
The use of comedication was screened for the following drugs: antacids; oral anticoagulants; non-steroidal anti-inflammatory drugs (NSAIDs).

General symptoms
Patients were asked for adverse effects during the full period of ASA use. When answered “yes”, these were further characterized by the following categories: symptoms of the upper respiratory tract; symptoms of blood and lymphoid system, skin or immune system and symptoms of the gastrointestinal tract.

Symptoms of the upper respiratory tract
Patients were screened for the existence of symptoms of the upper respiratory tract: nose bleedings; rhinitis; bronchospasms; asthma; other symptoms of the upper respiratory tract. If present, these symptoms were further characterized by duration, course and intensity.

Symptoms of the blood and lymphoid system, skin or immune system
Patients were screened for the existence of symptoms in blood and lymphoid system (thrombocytopenia; anaemia), skin (rash; urticaria) and immune system (e.g. angio-edema; allergy). Occurrence of thrombocytopenia was documented.

Symptoms of the gastrointestinal tract
Subjects were screened for the following gastrointestinal symptoms: epigastric discomfort; epigastric pain; vomiting; constipation; diarrhoea; gastrointestinal bleeding; gastric/duodenal ulcer; epigastric burning / reflux; gastritis and other symptoms of the gastrointestinal tract.
To document these symptoms a questionnaire based on the ROME II criteria (Research Diagnostics Questions for functional gastrointestinal disorders), was used[93]. The questions in this list quantify the severity of the complaint, by measuring the period and intensity of its occurence.

Ethical considerations
The study did not necessitate any extra medical interventions. The investigators maintained all freedom to prescribe medication. No ethical approval of this study was needed according to Dutch law as subjects were not submitted to changes in medications or interventions. The data was collected anonymously. To protect the patient's privacy, only the year of birth was documented and a unique study number was assigned to the patient. The documented data can only be traced to the medical source data by the patients own doctor.

Data analysis
Data are presented by descriptive statistics, consisting of means with standard deviation, median and range for continuous variables and numbers and percentages for categorical variables.

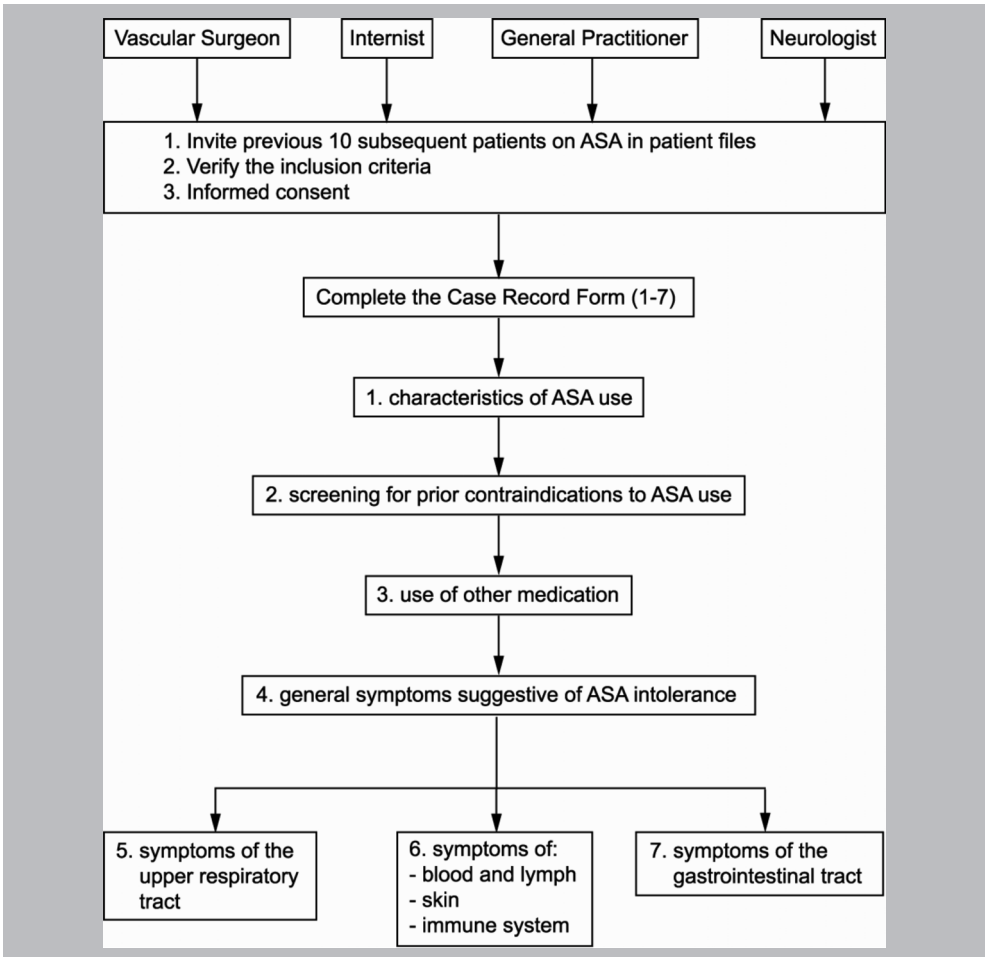

Figure 1. Profile of the study

Results

Patient characteristics

Table 1 shows the baseline characteristics of the study population. 957 patients were included from 105 centers. Although we chose the combination of ASA with another platelet aggregation inhibitor such as clopidogrel of dipyridamole as an exclusion criterium, 10 patients (1.1%) did not use ASA as monotherapy and were excluded from the analysis, which resulted in a total inclusion of 947 patients. The mean age of the patients was 68.3 years. More males than females were included (59.6% vs. 40.4% respectively). The median ASA dosage used was 100 mg/day (range 30-120 mg/day), for an average of 5.07 years. Sixty patients (6.6%) who were documented to use ASA according to their file, appeared to have cessated using ASA, mostly because of occurrence of side-effects (52%). Other reasons to cease ASA intake were increase of complaints (10%), no effect (3%), unclear necessity for ASA use (3%), the patient assumed ASA use is not required (3%) and other reasons to stop ASA (18%). Of these patients, 65% (39/60) terminated ASA use after thirty days.

Contra-indications of ASA and co-medication

The six questions considering contra-indications of ASA use indicated that 13.7% of the patients reported a history of pain in the stomach region (table 1). Other contra-indications to ASA, such as allergy to salicylic acid or prostaglandin synthesis inhibitors, severe kidney or liver insufficiency, intake of other platelet aggregation inhibitors and history of hemorrhagic diathesis or coagulopathy were uncommon. However, 7.1% of patients had a history of peptic ulcer disease or gastro-intestinal bleeding. A quarter of the patients using daily low-dose ASA concomitantly used an anti-acid agent. Concomitant use of oral anti-coagulants occurred in 5.2% of patients or and use of other NSAIDs occurred in 6.6% of patients.

Baseline characteristics	**%**	
Age (years)	68.3	*(±11.19)
Male (%)	59.6	
Female (%)	40.4	
Median dosage of ASA (mg/day)	100	†(30-120)
Mean duration of ASA use	5.07	*(± 5.2)
Contra-indications	**%**	
History of frequent stomach upset or gastric pain after previous ASA intake	13.7%	#(129/942)
History of peptic ulcer disease or gastro-intestinal bleeding	7.1%	#(67/942)
Allergy to salicylic acid or prostaglandin synthesis inhibitors	1.0%	#(9/942)
Severe renal or hepatic insufficiency	2.0%	#(19/939)
History of hemorragic diathesis or coagulopathy	0.1%	#(1/942)

Table 1. Baseline characteristics and contra-indications of ASA
*(± SD), †(range), #(number of patients with contra-indications / total number of patients (n = 947) minus the missing patients)

Occurrence of side-effects

Over thirty percent of the included patients using ASA indicated the occurrence of side-effects to the investigator. Twenty-nine patients (3.1%) experienced severe side-effects, as indicated by the highest score to their side-effects on the questionnaire considering asthmatic (severe) or gastrointestinal (severe or highly severe) symptoms.

Eighty-nine patients (9.4%) reported side-effects related to the upper respiratory tract (table 2). Asthma (23.6%) or nose bleedings (21.3%) formed a large part of this group, but most patients indicated other type of symptoms (39.3%) in this category. Almost half of these patients had side-effects for more than two years, but 30.1% only experienced problems shorter than three months. The side-effects occurred less than once a week in half of the cases. In our study severe asthma was reported to occur in 2 patients, which, expressed as a proportion of all respiratory patients, amounted to 3.5% (2/57). Side-effects considering the blood and lymphoid system were relatively rare (4.4%). Skin problems occurred in 70 patients (7.4%). In 38.6% of cases it concerned skin rash, but in most cases these symptoms were classified as other skin problems. Immune system side-effects were very rare (0.7%). Angio-edema occurred in 3 patients.

The results for gastrointestinal related side-effects are shown in table 3. These side effects were experienced in 25.1% of patients (238 of 947 patients). Heartburn was reported most often (54.2%). Epigastric discomfort, gastritis and epigastric pain occurred in 20% of the patients. Nearly forty percent of gastrointestinal side-effects existed for more than two years. Considering the frequency of side-effect occurrence, 61.0% of the patients experienced side-effects only seldom. The course of side-effects

Symptoms	%	n
Upper Respiratory Tract	**9.4%**	**89**
Nose bleeding	21%	19
Rhinitis	13%	12
Bronchospasm	15%	14
Asthma	23%	21
Other	39%	35
Blood and Lymph	**4.4%**	**42**
Thrombocytopenia	5%	2
Anemia	40%	17
Other	55%	23
Skin	**8.3%**	**79**
Rash	34%	27
Urticaria	25%	20
Other	41%	32
Immune system	**0.7%**	**7**
Angio edema	43%	3
Other	57%	4

Table 2. Symptoms of ASA intolerance per category

Symptoms	%	n
Gastrointestinal tract	**25.1%**	**238**
Epigastric discomfort	20.2%	48
Epigastric pain	17.2%	41
Vomiting	2.5%	6
Constipation	4.6%	11
Diarrhea	4.2%	10
Gastrointestinal bleeding	5.0%	12
Gastric ulcer	3.3%	8
Duodenal ulcer	4.6%	11
Epigastric burning / reflux	54.2%	129
Gastritis	19.3%	46
Other	18.1%	43
Period		**217**
0-4 weeks	14.3%	31
1-3 months	14.7%	32
3-6 months	10.6%	23
6-12 months	8.8%	19
1-2 years	12.0%	26
over 2 years	39.6%	86
Missing		*21*
Frequency		**218**
Less than 10% of time	61.0%	133
More than 25% of time	28.0%	61
More than 50% of time	8.3%	18
Almost 100% of time	2.8%	6
Missing		*20*
Course		**210**
Improvement in the last 3 months	19.5%	41
Worsened in the last 3 months	9.5%	20
No difference in the last three months	52.4%	110
No symptoms in the last three months	18.6%	39
Missing		*28*
Severity		**215**
Mild	52.6%	113
Moderate	41.9%	90
Severe	5.6%	12
Missing		*23*

Table 3. Characteristics of symptoms of ASA intolerance in the gastrointestinal tract

over time was stable. There was no difference in severity of gastro-intestinal side-effects throughout the last three months in more than half of the patients, and only 9.5% of patients reported that their situation had worsened. 52.6% of the population with gastrointestinal side-effects reported these as being mild. Twelve patients experienced severe side-effects. These patients suffered from gastrointestinal bleeding (2 patients), peptic ulcer (3 patients) and epigastric reflux/pain (7 patients).

Discussion

This multi-center observational study in 947 patients who were prescribed a low dose of ASA suggests that approximately thirty percent of this population shows at least one sign of ASA intolerance. The most common side-effects are associated with the gastrointestinal tract. Although most patients experienced mild symptoms, 5.6% of the patients with gastrointestinal side-effects considered them as severe. A review of randomized controlled trials on the gastrointestinal toxicity of aspirin revealed that the pooled odds ratios for categories of gastrointestinal bleeding were between 1.5 and 2.0[89]. The risk of peptic ulcer and upper gastrointestinal symptoms was increased (OR 1.3 and 1.7 respectively). The review included mainly trials in which a higher dose of ASA was used (over 500 mg/day). It showed that toxicity of ASA use was dose-related. Previous studies have demonstrated the benefit of a low-dose of ASA in the secondary prevention of atherothrombosis[82]. Our study quantifies signs of gastrointestinal side-effects in a population of patients prescribed a low dose of ASA. A recent study demonstrated that endoscopy in asymptomatic patients using a low dose of ASA showed hemorrhagic abnormalities in the duodenum or stomach in almost half of the cases[94]. Although most patients with gastrointestinal side-effects considered them to be mild, the clinical relevance of these symptoms becomes clear by the fact that a quarter of the patients on ASA also used an anti-acid agent. However, it remains unclear from our study if such an agent was given for treatment or prevention.

In our study, 9.4% of the cases presented with side-effects of the upper respiratory tract, such as asthma (23.6%). A large cohort study in Finland demonstrated that the risk of aspirin intolerance causing shortness of breath or asthma was 8.0 times higher in people with allergic rhinitis than without[91]. Bochenek *et al*[95] showed that atopy is related to adverse drug reactions to NSAIDs. The mechanism of aspirin-precipitated asthma is thought to be related to the inhibition of cyclo-oxygenase (COX) and generation of cysteinyl leukotrienes in the respiratory tract of sensitive patients[96].

In our population 60 patients had discontinued ASA treatment, more than half of them because of side-effects. Treatment cessation by the patient without prior medical advice, may be deleterious in patients at risk for atherothrombosis. In a meta-analysis, aspirin discontinuation or non-compliance was associated with a threefold higher risk of major cardiac events in patients with coronary artery disease[92]. This risk is even higher in patients with an intracoronary stent. Other platelet aggregation inhibitors have become available, with similar or even better results concerning the secondary prevention of atherothrombotic events[37,83]. It is necessary that future studies focus on the differences in side-effects of these agents compared to ASA. We could hypothesize

that less intolerance with equal or better efficacy may become a reason to favour a new medicine over ASA in the secondary prevention of atherothrombosis.

It can be assumed that doctors would screen their patients for the presence of contra-indications before ASA was prescribed. In our study 13.7% of patients reported gastric pain after previous use of ASA or a history of stomach complaints (table 1). In a study by Silagy *et al*[87], the adverse effects of low-dose aspirin (100 mg daily) in the elderly were studied over a 12-month period in a double-blind, randomized, placebo-controlled trial. 400 subjects were randomized to received low-dose aspirin (100 mg daily) or placebo. Gastrointestinal symptoms were reported by 18% (n = 36) of participants receiving aspirin (compared to 13% in the placebo group). The relatively high incidence may be due to the fact that the definition of the gastric contra-indication leaves room for a considerable number of gastric clinical entities. However, if we look at the contra-indication 'history of peptic ulcer disease or gastro-intestinal bleeding' (7.1%), we can conclude that for a well defined entity such as peptic ulcer disease or gastro-intestinal bleeding the reported incidence is still 7.1%. According to the prescribing information, these patients should never have started ASA use.
The concomitant use of antacids including proton pump inhibitors in this study was reported to be 25.0% (226/906). Two studies have compared aspirin to clopidogrel with antacid therapy. In the first study by Ng *et* al[97] patients with aspirin-induced peptic ulcer disease treated with omeprazole (20 mg/day) were randomized to receive clopidogrel (75 mg/day) or to continue with low-dose aspirin. 45% in the clopidogrel group and 42% in the aspirin group had a minor gastrointestinal bleed. The distributions of peptic ulcer disease were similar in the clopidogrel and aspirin groups. Using per protocol analysis, the treatment success rates of clopidogrel and aspirin were 94% (62/66) and 95% (57/60), respectively.
A second study by Chan *et al*[98] compared clopidogrel alone with aspirin plus esomeprazole on the prevention of recurrent ulcer bleeding. The cumulative incidence of recurrent bleeding during the 12-month period was 8.6 percent (95 percent confidence interval, 4.1 to 13.1 percent) among patients who received clopidogrel and 0.7 percent (95 percent confidence interval, 0 to 2.0 percent) among those who received aspirin plus esomeprazole (difference, 7.9 percentage points; 95 percent confidence interval for the difference, 3.4 to 12.4; p=0.001). A third group receiving clopidogrel plus esomeprazole was not included in this study. Both studies had great contributions to the knowledge about ASA treatment in combination with a proton pump inhibitor. However, in both studies patients were included with known peptic ulcer disease or gastric bleeding. On that aspect, patients in those studies differed from our study population.

Our study has several limitations. First, to show that the symptoms addressed by patients using ASA are specific for ASA it would be better to carry out a placebo-controlled trial. However, with a placebo controlled trial the patients in the placebo group would be withdrawn from essential medication (i.e. ASA) they need to prevent them from having a new atherothrombotic event. Placebo-controlled trials studying ASA intolerance would place patients potentially at risk, and are therefore unethical. The aim of the study was to explore the magnitude of the occurrence of side-effects of low dose ASA in daily clinical practice. Our study demonstrates that even a low-dose of ASA can induce

symptoms suggestive of intolerance. Secondly, we did not conduct detailed descriptions of possible side effects of ASA. We mainly focused on the symptoms that were most prevalent in our population and further studies are needed to investigate the cause of these side-effects. Thirdly, despite its use in clinical practice, the ROME II criteria were developed to be used in epidemiological studies and not designed for this purpose. At last, there might be an additive effect of NSAIDs on the occurrence of side effects, which may increase the prevalence of ASA intolerance beyond its strict magnitude. However, only a relatively small part (6.6%) of the patients used ASA and NSAIDs..

In conclusion, side-effects of low-dose daily ASA occur in a considerable number of patients. Patients and doctors need to be aware of a possible relationship between their symptoms and the use of ASA. This study shows that more attention to the occurrence of side effects after prescribing ASA may identify patients at risk. A proactive approach in the collection of information about adverse effects may be of benefit, because the occurrence of side-effects is the most important reason for patients to discontinue ASA treatment, with a possible increase in atherothrombotic events. When adverse effects are suspected doctors should either (1) treat the symptoms, e.g. by prescribing antacids when gastrointestinal side effects occur or (2) advise against the use of ASA and initiate another platelet aggregation inhibitor.

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Chapter 4

Causes of clopidogrel resistance

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Submitted

Since the introduction of clopidogrel in the secondary prevention of atherothrombosis, there is increasing interest in patients whose platelets are resistant to this treatment. Clopidogrel is a prodrug, which is metabolized by hepatic cytochrome P450 into an active metabolite that inhibits the platelet ADP receptor $P2Y_{12}$. Receptor occupancy by ADP enhances platelet functions induced by a second ADP receptor, $P2Y_1$, and by other platelet activating receptors. Resistance to clopidogrel may result from (i) impaired cytochrome P450 activity, (ii) increased signalling by $P2Y_{12}$ and (iii), enhanced signalling by platelet activating receptors that oppose modulation through $P2Y_{12}$. In healthy subjects there is a considerable inter-individual variation in platelet responsiveness to ADP that affects pharmacological blockade. Attempts to establish prevalence of this resistance, to adjust medication and to evaluate clinical outcome are hampered by lack of a general laboratory test, which defines clopidogrel resistance.

Introduction

The last few years have seen a rapid increase in the use of clopidogrel as an anti-thrombotic agent for secondary prevention of atherothrombosis. Clopidogrel is a receptor antagonist, which interferes with mechanisms in platelets that suppress the intracellular inhibitor cAMP and activate the fibrinogen receptor $\alpha_{IIb}\beta_3$. Compared with aspirin, clopidogrel induces a 10% better reduction in incidence of severe vascular events[35]. However, there remains an annual risk of 5.32% of ischemic stroke, myocardial infarction or vascular death[36]. Combinations of aspirin and clopidogrel reduce risk of cardiovascular death and myocardial infarction further by 30%[37]. Despite these impressive data, 4.5% of patients on dual therapy still suffer from cardiovascular death, myocardial infarction or require urgent revascularisation within 30 days of percutaneous coronary stent intervention (PCI).

Studies of clopidogrel's effect on secondary prevention of cardiovascular events have focused on PCI, carotid endarterectomy and peripheral arterial disease. There is a large variation in the response of patients to clopidogrel, with extreme examples of poor responsiveness known as clopidogrel resistance. Since clopidogrel resistance increases the risk of recurrent atherothrombosis, there is a need for (i) a clear definition of resistance, (ii) diagnostic tools to recognize resistant individuals and (iii) algorithms to adapt medication to the sensitivity of the patient's platelets.

Clopidogrel resistance: the biological perspective

Mechanism of action of clopidogrel

ADP activates platelets via the G-protein coupled receptors $P2Y_1$ and $P2Y_{12}$[29]. Upon ligand binding, $P2Y_1$ signals through the α-unit of the GTP-binding protein G_q to phospholipase (PL) $C_{\beta 2}$ and inositol 1,4,5 trisphosphate-dependent Ca^{2+} mobilization[99-101] and formation of diacylglycerol. Further signalling to protein kinases (PK) B, C and the GTPase Rap1b activates the fibrinogen receptor $\alpha_{IIb}\beta_3$ and starts granule secretion. Release of ADP and thromboxane A_2 (TxA_2) enhances platelets activation through extracellular feed

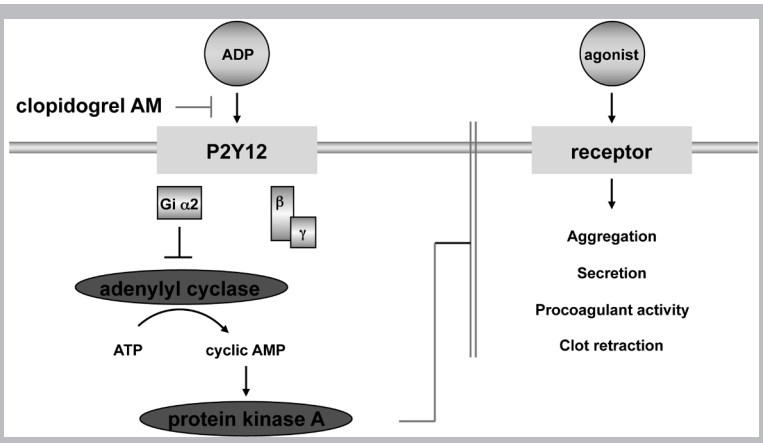

Figure 1. Platelet inhibition by clopidogrel active metabolite (AM). The ADP receptor $P2Y_{12}$ suppresses formation of the platelet inhibitor cyclic AMP through the inhibitory G-protein $G_i\alpha_2$, which inhibits adenylyl cyclase. A second signal supports activation of the fibrinogen receptor through the βγ subunits of the G-protein. cAMP activates protein kinase A, which inhibits numerous steps in the platelet activating machinery through phosphorylation. Clopidogrel AM interferes with ADP-$P2Y_{12}$ interaction.

back. The target for the active metabolite (AM) of clopidogrel is $P2Y_{12}$ which signals via the GTP-binding protein G_i (Figure 1). The α-unit inhibits adenylyl cyclase, reducing formation of cAMP. Potent platelet activators such as thrombin induce platelet functions while decreasing the level of cAMP through ADP secretion and $P2Y_{12}$ activation[30]. The βγ-unit of G_i signals to phosphatidylinositol 3-kinase and activates PKB. Both results facilitate signalling by $P2Y_1$ and other activating receptors inducing activation of the fibrinogen receptor (integrin $\alpha_{IIb}\beta_3$), fibrinogen binding and aggregation[31].

PKA is a kinase consisting of two catalytic and two regulatory subunits, each with several subtypes. Different subunits are targeted to different subcellular localizations by A-kinase anchoring proteins[102,103]. cAMP-dependent PKA inhibits multiple steps in the activation pathways initiated by ADP, thrombin, von Willebrand Factor, collagen and TxA_2. Examples are the thrombin receptor (PAR1)[104], the thromboxane A_2 receptor TPα[105], PLC_b but not PLC_g[106,107], the IP_3-receptor[108], β_3-endonexin, Glycoprotein Iba,b signalling, myosin light chain kinase (MLCK), G_{13}a and p38MAPK and p42MAPK[109]. The result is inhibition of platelet aggregation, secretion of granule contents and the generation of a pro-coagulant surface[109,110]. In addition, a high cAMP induces endocytosis of the collagen receptor Glycoprotein VI[111] (Figure 2).

Clopidogrel AM binds irreversibly to $P2Y_{12}$ by forming a disulfide bridge between the reactive thiol group of AM and a cysteine of $P2Y_{12}$[32]. Under laboratory conditions, activation of $P2Y_{12}$ inhibits ADP-$P2Y_1$-induced platelet shape change, Ca^{2+} increases and aggregation[112]. As this is one of the primary pathways for platelet activation, $P2Y_1$ is a poor target for pharmaceutical intervention since this would interfere with haemostasis. In contrast, $P2Y_{12}$ activity is limited to acceleration of functions induced by $P2Y_1$ ligand and other platelet activating agents[33]. *In vivo*, $P2Y_{12}$ is blocked by clopidogrel, cangrelor and the recently introduced prasugrel. Clopidogrel is an inactive thienopyridine prodrug and converted to its AM by hepatic cytochrome P450[34]. The efficiency is poor (< 15%), the major product being an inactive, second metabolite. An advantage of prasugrel over clopidogrel is its faster and more efficient AM formation[113-115]. For instance, AM formation from 60 mg prasugrel is 2.2-fold better than that from 600 mg clopidogrel[113].

The difference also shows in inhibition of ADP-induced aggregation which is 2.4 fold greater after administration of prasugrel (loading dose, LD: 60 mg, maintenance dose, MD: 10 mg/day) than clopidogrel (LD: 600 mg; MD: 75 mg/day)[116].

Inter-individual variation in responsiveness to ADP

Among healthy individuals, there is a large variation in platelet responsiveness to ADP (Figure 3). Causes are single nucleotide polymorphisms (SNPs) in the genes that code for key proteins in pathways signalling to aggregation, ADP secretion, TxA_2 formation and generation of a procoagulant surface. Examples are SNPs in genes coding for $P2Y_1$, $P2Y_{12}$ and integrin a_2 and b_3 subunits[117-119]. Especially SNPs that correlate with platelet hyperreactivity are of interest since they may increase the flux through pathways with which $P2Y_{12}$ signalling attempts to interfere. Patients with arteriothrombotic disease have a higher frequency of a_2 allele 1, which is associated with higher levels of the collagen receptor $\alpha_2\beta_1$. Upregulation of Glycoprotein VI correlates with increased procoagulant activity[120,121]. The large variation in ADP-induced fibrinogen binding (4 - 79%) and P-selectin expression (6 - 90%) in 500 healthy individuals illustrates the impact of these polymorphisms[122]. Hyperresponsiveness within this group is associated with SNPs in genes for the IP3 receptor and integrin subunit a_2. For fibrinogen binding alone, associations were found with SNPs for JAK2 (involved in growth factor signalling) and MAP2K2; for P-selectin expression alone, with SNPs in $P2Y_{12}$, VAV_3 (an GTP-exchange factor for RhoA), $G_z\alpha$ (a member of the G_i family) and MAPK14, among others[123,124](Figure 2).

Are all $P2Y_{12}$ receptors blocked by clopidogrel *in vivo*?

The extent of $P2Y_{12}$ inhibition by clopidogrel can be inferred from *ex vivo* incubation with cangrelor (AR-C69931MX)[125]. Patients receiving a clopidogrel regimen of LD 300 mg, MD 75 mg/day, 4 - 7 days have platelets which *ex vivo* show a 50% fall in ADP (30 mM)-aggregation upon addition of cangrelor (100 nM), illustrating that *in vivo* blockade of $P2Y_{12}$ is far from complete. Patients undergoing PCI receiving a low clopidogrel regimen (LD 600 mg; MD 75 mg/day) show a higher ADP (5 μM)-aggregation than those with a high clopidogrel regimen (LD: 600 mg; MD 150 mg/day) at thirty days after PCI (6512% versus 45±21%, n=60) indicating that *in vivo* $P2Y_{12}$ blockade can indeed be improved[126]. The cardiovascular outcome of different dosages is under current investigation[127,128]. The PRINCIPLE-TIMI 44 trial compared prasugrel (LD 60 mg; MD 10 mg/day) with clopidogrel (LD 600 mg; MD 150 mg/day). Six hours after LD, ADP (20 μM)-aggregation was about 25% in subjects with prasugrel and 69% in subjects with clopidogrel[129]. These data illustrate that prasugrel is a stronger aggregation inhibitor than clopidogrel.

Congenital causes of clopidogrel resistance

Association studies between sequence variation in the $P2Y_{12}$ gene and ADP-aggregation reveal three SNPs and one nucleotide insertion in complete linkage disequilibrium designated H1 (86% of the population) and H2 (14% of the population)[130]. The H2 haplotype is associated with a 2.4-fold higher ADP (2 μM)-aggregation and the result of a sequence variation in the promotor region, which might control transcription efficiency and $P2Y_{12}$ copy number. In a case control study with males with peripheral arterial disease, the H2 haplotype is present in 30% of the patients and 21% of matched

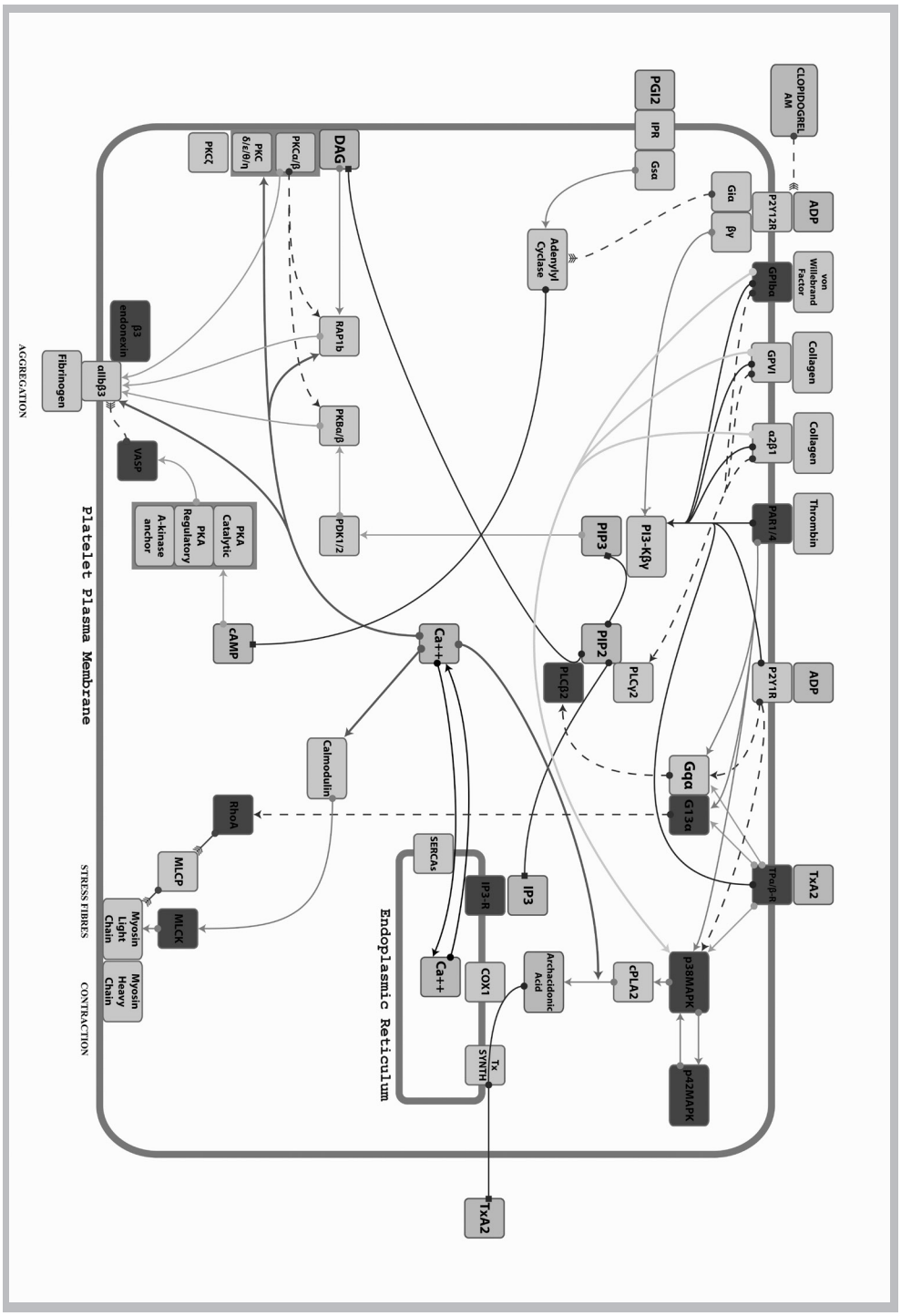
CLOPIDOGREL AM
ADP
P2Y12R
Gia
βγ
PGI2
IPR
Gsα
Adenylyl Cyclase
von Willebrand Factor
GPIbα
Collagen
GPVI
Collagen
α2β1
Thrombin
PAR1/4
PI3-Kβγ
PIP3
PIP2
PLCβ2
PLCγ2
ADP
P2Y1R
Gqα
G13α
TPα/β-R
TxA2
cPLA2
p38MAPK
p42MAPK
Archacidonic Acid
COX1
Tx SYNTH
TxA2
Ca++
Calmodulin
SERCAs
IP3-R
IP3
Ca++
DAG
PKCα/β
PKC δ/ε/θ/η
PKCζ
RAP1b
PKBα/β
PDK1/2
cAMP
PKA Catalytic
PKA Regulatory
A-kinase anchor
VASP
β3 endonexin
αIIbβ3
Fibrinogen
RhoA
MLCP
MLCK
Myosin Light Chain
Myosin Heavy Chain
Platelet Plasma Membrane
Endoplasmic Reticulum
AGGREGATION
STRESS FIBRES
CONTRACTION

Figure 2. Systems biology of P2Y12 interference with platelet activating pathways.
Activation of $P2Y_{12}$ by ADP induces signaling through the G-protein G_i to (i) adenylyl cyclase, cAMP and protein kinase A (PKA) and, (ii) PI3-K (phosphatidyl inositol 3-kinase) and the fibrinogen receptor, integrin $\alpha_{IIb}\beta_3$. Some intermediates consist of multiple subunits contributing to complexity in signal generation and the chance that genetic polymorphisms affect their activity. An example is PKA, which consists of two catalytic subunits (with α, β, γ subtypes) and two regulatory subunits (with α and β subtypes). PKA's couple to different subcellular localizations by A-kinase anchoring proteins. Targets for inhibition by PKA (in dark blue) are receptors for platelet activators (for thrombin: PAR1; for von Willebrand factor: Glycoprotein Ib, for thromboxane A2: TPα), PLCβ2 (phospholipase $C_{\beta 2}$), G_{13} (G protein), MAPKs (mitogen activated protein kinases), IP3-R (the receptor for inositol trisphosphate that releases Ca^{2+}), Rho (the GTPase involved in cytoskeleton re-arrangements), MLCK (myosin light chain kinase, involved in contractility), β3-endonexin (which controls the fibrinogen receptor) and especially VASP (vasodilator-stimulated phosphoprotein, which inhibits activation of the fibrinogen receptor).

controls. H2 haplotype does not correlate with platelet response to clopidogrel. Thus, polymorphisms in the *P2Y12* gene are not related to clopidogrel resistance[131-135].

Clopidogrel resistance in diabetes mellitus

Patients with type 2 diabetes (T2DM) have a 2- to 8–fold higher chance of developing atherosclerosis and coronary artery disease[38,39]. The increased risk is caused by abnormalities in the vessel wall and blood constituents including platelets[40,41]. Platelet abnormalities are (i) an increased turnover[42], (ii) an increased cytosolic Ca^{2+} level[43], and (iii) up-regulation of the $P2Y_{12}$ pathway[44]. The CAPRIE trial[36] demonstrated that in T2DM patients clopidogrel shows improved prevention of recurrent ischemic events than aspirin[136]. In healthy individuals, insulin interferes with the $P2Y_{12}$ mediated suppression of cAMP formation, thereby attenuating ADP- and thrombin induced Ca^{2+} increases, aggregation, secretion and the generation of a pro-coagulant surface[30]. The ligand-activated insulin receptor activates insulin-receptor substrate-1 (IRS-1), which associates with, and thereby inactivates, $G_i\alpha_2$. In T2DM platelets, $P2Y_{12}$ signalling is 2-3 fold faster than in controls and resistant against inhibition by insulin[44]. The loss of insulin signalling to $G_i\alpha_2$ is accompanied with impaired signalling to PKB, a second protein under control of IRS-1, pointing at a defect in IRS-1. As expected with an up-regulated pathway, $P2Y_{12}$ signalling is less responsive to a suboptimal concentration of cangrelor. At higher inhibitor concentrations the difference disappears suggesting that clopidogrel resistance in T2DM patients can be overcome by increasing dosage. In the OPTIMUS trial this possibility was investigated in patients on clopidogrel and aspirin[137]. Patients were considered clopidogrel resistant when during therapy ADP (20 μM)-aggregation was >50% and randomized to receive a MD of 75 or 150 mg/day for 30 days, when aggregation was re-assessed. Thereafter, all patients continued with the standard dose (75 mg/day) for 30 days. Aggregation was tested to investigate a return to the range typical for the standard dose. Forty out of 64 T2DM patients were clopidogrel resistant. Although the high MD induced a further reduction in the average aggregation, 60% of these patients preserved aggregations within the limits set for clopidogrel resistance. Thus, about half of clopidogrel resistant T2DM patients do not respond to an increase in MD.

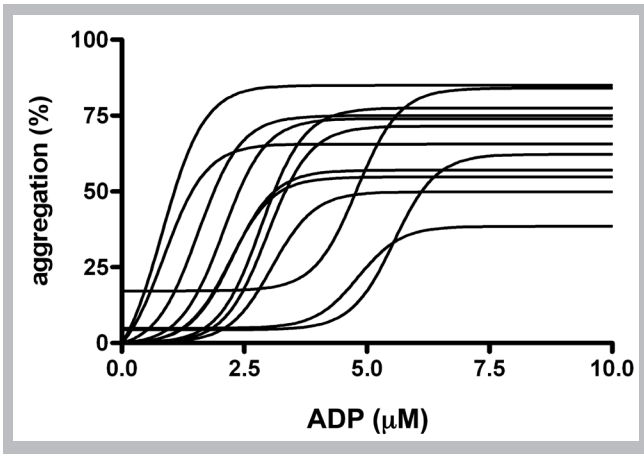

Figure 3. Interindividual variation in ADP-induced platelet aggregation.
Dose-response curve of ADP-induced aggregation in 12 healthy individuals, illustrating the strong inter-individual variation.

Variations in pharmacokinetics

Clopidogrel is an inactive prodrug, requiring several metabolization steps, mediated by cytochrome P450 isoforms. The most abundant clopidogrel metabolite is the carboxylic acid derivative SR26334, which reaches a plasma level of 43.0 ± 16.9 mg/mL at 4 hrs after an LD of 600 mg[138]. Taking SR26334 as a marker for clopidogrel metabolism, healthy males given an LD of 50 - 150 mg clopidogrel show a peak after 0.8-1.0 hr and a plasma $t_{1/2}$ of 7.2-7.6 hrs, independent of dose[139]. Poor aggregation inhibition correlates with low plasma AM, indicating that variations in absorption and metabolism contribute to the variability in clopidogrel response[140]. In patients with a recent history of stent thrombosis, plasma AM levels were 78% lower than in healthy controls, whereas levels of residual clopidogrel and inactive carboxyl metabolite levels were similar[138]. The reason is unclear.

The effect of clopidogrel metabolism on variability of response

Members of the cytochrome P450 (CYP) family involved in clopidogrel metabolism include CYP2C19, CYP1A2, CYP2B6, CYP2C9 and CYP3A4[141]. SNPs in the CYP2C19 gene lead to a cryptic splicing site and complete loss of enzyme activity[142]. This results in 39% lower AM levels and 20% less inhibition of ADP (20 mM)-aggregation than in controls[143] and is associated with an 3.8 fold higher risk of stent thrombosis after PCI[144]. Two recent studies showed that patients on clopidogrel carrying the loss of function phenotype have a higher chance of developing a cardiovascular event[145,146]. This loss of function allele is a very important determinant of the prognosis of young patients (under 45 years) receiving clopidogrel after a myocardial infarction[145].

SNPs associated with decreased CYP2C9 and CYP2C19 activity also result in decreased AM levels and a higher prevalence of clopidogrel resistance[147,148]. CYP2B6 is inhibited by clopidogrel in a dose dependent manner[149]. Different SNPs have been identified, with one inducing an increase in enzyme activity, but so far no associations with clopidogrel resistance have been reported[150-152]. The cholesterol lowering drug atorvastatin is a substrate of CYP3A4[141] and decreases platelet inhibition by clopidogrel[153]. CYP3A4 activity varies among individuals as does its effect on aggregation inhibition[154]. More than 30 SNPs in the CYP3A4 gene have been described, among which are five

nonsynonymous SNPs, six SNPs in the 5'-UTR, 13 SNPs in the introns and three SNPs in the 3'-UTR[155-157]. Carriers of the IVS10+12G>A allele, located in intron 10 of the CYP3A4 gene, showed reduced activation of the fibrinogen receptor $\alpha_{IIb}\beta_3$ by ADP and a better response to clopidogrel compared with non-carriers[158]. The polymorphism does not affect platelet aggregation, probably due to the limited sensitivity of the test.
In healthy individuals smoking >20 cigarettes/day, CYP1A2 activity increases 1.72 fold[159]. Theoretically, this would lead to more AM production, better inhibition of aggregation and better cardiovascular outcome. In patients undergoing PCI, smokers on chronic clopidogrel therapy showed lower aggregation than non-smokers[160].

Clopidogrel resistance: the clinical perspective

Prevalence of clopidogrel resistance

The large variability in clopidogrel AM formation, in signalling thought the $P2Y_{12}$ pathway and in responsiveness of platelet activating pathways for $P2Y_{12}$ signals has a great impact on antithrombotic therapy with clopidogrel. In a meta-analysis of 2574 patients undergoing PCI, study groups were defined on the basis of: (i) ADP concentration for aggregation (5, 20 mM), (ii) clopidogrel LD (300, 600 mg), (iii) time between LD and aggregation analysis (<24 hrs, 24-48 hrs, 2-7 days, >7 days) and, (iv) co-medication with aspirin (<100 mg, 101-299 mg, >300 mg)[161]. The unadjusted mean prevalence of clopidogrel resistance was 21%. Multivariate analysis adjusted for ADP-aggregation, LD, time between loading and aggregation and use of aspirin demonstrated a prevalence of 14%.

Laboratory diagnosis of clopidogrel resistance

Although ADP-aggregation detected by light transmittance is now considered the gold standard for measurement of clopidogrel resistance, there is uncertainty about [1] optimal ADP concentration, [2] optimal time of analysis, [3] adjustment for aspirin use, [4] threshold that separates a normal from an abnormal response, and [5] effect of the LD.

[1 & 2] Optimal ADP concentration and time of analysis

$P2Y_{12}$ is rapidly desensitized by G protein-coupled receptor kinase and the time between blood collection and start of aggregation studies should be short and kept constant[162]. The 2006 update of the ACC/AHA/SCAI 2005 guideline for PCI recommends a LD of 300 mg clopidogrel at least 6 hrs before PCI, followed by a MD of 75 mg/day for 1 - 12 months depending on the type of stent implanted[163]. Studies published so far report great variations in ADP concentration and time of analysis. In a study by Gurbel *et al,* patients undergoing PCI had preloading ADP-aggregations of 62% (5 μM ADP) and 83% (20 μM ADP)[164]. Clopidogrel resistance was defined as <10% difference between baseline and post-treatment and was determined at 2 hrs, 5 days and 30 days after the LD (300 mg). More resistance was seen with the low ADP concentration (63%, 31% and 15% at the respective analyses) than with the high ADP concentration (53%, 32% and 21%) and an ADP (5 μM)-aggregation performed 2 hrs after LD detected the highest number of resistant patients. The clinical outcome remains to be evaluated.

In the PREPARE POST-STENTING study, ADP (20 μM)-aggregation was measured 24 hours after PCI[165]. Cardiovascular events determined 6 months later were almost completely absent in patients with <50% aggregation. However, about 50% of events occurred in patients within the 25th to 75th percentile of the aggregation data suggesting that aggregation analysis shortly after PCI has limited sensitivity for detecting individuals at risk of cardiovascular events.
In patients with myocardial infarction with ST-segment-elevation undergoing PCI, aggregation was tested before LD and during five subsequent days[166]. In the group with the highest pre-medication ADP (5 μM)-aggregation, responses showed a 60% decrease at day 1. After 6 days aggregations returned to the range found before clopidogrel administration. Patients were divided in quartiles based on aggregation-inhibition at day 5. Fourty percent of those in the highest quartile suffered from a secondary cardiovascular event within the next 6 months. In the second quartile this number was 6.7%.

[3] Adjustment for aspirin use
Aspirin is an irreversible inhibitor of the formation of thromboxane A_2, which strongly enhances the 2nd phase of ADP-induced platelet aggregation and differences in aspirin intake might therefore disturb comparisons between clopidogrel effectiveness and aggregation. The 2006 update of the ACC/AHA/SCAI 2005 guideline for PCI recommends that patients should take 75 - 325 mg of aspirin at least 2 hrs before PCI followed by the same dose on a daily basis after PCI[163]. These dosages are well above the threshold of 30 mg/day for complete blockade of thromboxane A_2 synthesis[167,168], indicating that -with rare exceptions- reported studies on clopidogrel resistance include patients with complete arrest of thromboxane A_2 formation. Thus, comparisons between clopidogrel treatments based on aggregometry are not disturbed by variations in aspirin intake.

[4] The threshold that separates a normal from an abnormal response
Clopidogrel-resistance has been defined as <10% fall between pre- and post-treatment ADP (20 μM)-aggregation[154,164,169]. Resistant patients had a high aggregation after treatment (78±12%) whereas responders showed low aggregation curves afterwards (39±11%)[170]. A high pre-treatment platelet activity was not a predictor of clopidogrel resistance. PCI patients in the highest quartile of post-treatment ADP-aggregation had the highest risk of recurrent cardiovascular events (OR 22.4)[171].

[5] Effect of the LD
In patients with suspected or documented coronary artery disease, LD's of 300 and 600 mg clopidogrel reduced ADP (5 mM)-aggregation by 23% and 34% after 4 hrs[172]. Increasing the LD to 900 mg failed to increase plasma AM levels and aggregation inhibition, probably due to limits in intestinal absorption. Assuming that the pharmacokinetics of clopidogrel metabolite (peak after 1 hr; $t_{1/2}$ about 8 hours)[139] resemble those of clopidogrel AM, interference of LD with MD will have disappeared 24 hrs later. However, since clopidogrel AM irreversibly blocks $P2Y_{12}$, interference with platelet aggregation will last much longer and is primary determined by platelet survival (about 9 days). The effect of repeated doses of clopidogrel on aggregation and its tolerance were assessed in two randomized, double-blind studies in healthy male

adults[173]. After day 6, aggregation-inhibition reached steady state at 30, 46, 53 and 73% with 25, 50, 100, and 150 mg clopidogrel. After treatment, significant inhibition persisted for up to 8 days. Recovery of bleeding time was observed within 7-8 days.

Does clopidogrel resistance affect clinical outcome?

In the meta-analysis referred to above, eight studies on patients undergoing PCI were combined to calculate mean cardiovascular outcome in terms of OR's[161]. Outcome measurements were cardiovascular events, stent thrombosis and myonecrosis. The studies differed in detection method, time of analysis, aspirin dose and LD. The OR of all cardiovascular outcomes was 8.0, for stent thrombosis 7.0 and for cardiovascular events 12.0 compared with non-resistent individuals**.** A second study using myonecrosis as outcome measurement found an OR of 2.2[174]. In patients with ST-segment-elevation myocardial infarction (STEMI) undergoing PCI, 40% of those in the highest quartile of baseline ADP-aggregation developed a second cardiovascular event. This is a clear indication that clopidogrel resistance increases the risk of recurrent atherothrombotic events[166].

Despite the well-established benefit of aspirin-treatment, 2-5% of patients undergoing carotid endarterectomy suffer from ischemic stroke in the peri-operative period[175,176]. Aggregation (0.5–4 μM ADP) correlated with postoperative embolization[177]. Patients with a high sensitivity to ADP had greater numbers of postoperative emboli than low responders, which suggests that these patients might benefit from peri-operative clopidogrel administration. Patients undergoing carotid endarterectomy on routine aspirin therapy were randomized to receive either a single dose of 75 mg clopidogrel or placebo on the night before surgery[178]. The subjects on clopidogrel showed a small but significant decrease in ADP (1 μM) induced platelet activation assessed by fibrinogen binding by flow cytometry and a 10-fold reduction in relative risk of >20 emboli in the postoperative period. Embolization was used as an endpoint because of the low incidence of stroke after carotid surgery. Platelet responsiveness to ADP, measured by aggregation and flow cytometry, showed the largest decrease by clopidogrel in those with the highest baseline response to ADP[179]. The patients' weight correlated negatively with clopidogrel response.

The CAPRIE study showed that especially patients with peripheral arterial disease benefit from clopidogrel[36]. The average event rate of cardiovascular events was 3.71% in the clopidogrel-treated (aspirin-free) group compared to 4.86% in the aspirin-treated group, a relative risk reduction of 24%. In patients with claudicatio undergoing endovascular revascularization, the effect of aspirin alone was compared with combined aspirin/clopidogrel[180]. ADP (10 μM)-platelet activation was inferred from fibrinogen binding and P-selectin expression measured 1 hr, 24 hrs and 30 days after endovascular intervention. The combination of aspirin and clopidogrel reduced ADP-induced platelet activation by 50%, whereas aspirin alone left aggregation unchanged. ADP (10 μM)-induced fibrinogen binding decreased from 75% at baseline to 30% after 24 hrs and increased to 34% after 30 days. At 12 hrs, 6 out of 61 patients showed no reduction of platelet activation[181].

Conclusion

Resistance to clopidogrel increases the risk of atherothrombosis. Causes can be congenital abnormalities in (i) the mechanisms that synthesize the AM from the drug, (ii) transducers of signals of the platelet $P2Y_{12}$ pathway and (iii) steps in the platelet activating machinery with which $P2Y_{12}$ signalling interferes. Causes can also be acquired especially when platelets become insulin-resistant which lowers the sensitivity of $P2Y_{12}$ signalling to clopidogrel AM. For the diagnosis of resistance, ADP-induced platelet aggregation appears to be the gold standard, although there is little consensus about optimal time of analysis and ADP concentration. In patients undergoing PCI, a high baseline aggregation has no predictive value for clopidogrel resistance, but a too small difference between pre- and post-treatment aggregation as well as aggregations in the highest quartile post-treatment correlate with secondary cardiovascular events. The tests may be a basis for dose adjustment or application of more potent platelet inhibitors *e.g.* prasugrel[129,182] or elinogrel during further treatment. In patients with carotidendarterectomy who generally receive aspirin, ADP-aggregation correlates with embolization and both decrease upon additional treatment with clopidogrel. More studies are required before risk reduction by clopidogrel administration can be based on the outcome of ADP-aggregation measurements. This also applies to patients with peripheral arterial disease. It is clear that optimal treatment by clopidogrel should be based on accurate assessment of platelet reactivity of the individual patient[183].

Chapter 5

The consequences of hypersensitive platelets in type 2 diabetes mellitus patients for the treatment with clopidogrel; a pilot study

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Type 2 diabetes mellitus patients are insulin resistant and suffer from atherothrombotic complications due to hypersensitivity of their platelets. This hypersensitivity is caused by up-regulation of the $P2Y_{12}$ pathway. The $P2Y_{12}$ receptor is inhibited by the platelet aggregation inhibitor clopidogrel. We investigated whether type 2 diabetes mellitus patients show a decreased response to clopidogrel compared to matched controls. Platelet activation was measured by P-selectin expression at baseline (t=0) and after 7 days of clopidogrel 75 mg/day in 8 type 2 diabetes mellitus patients and 8 matched controls. Platelets activation was induced by the agonists ADP and collagen related peptide (CRP-XL). Platelets of patients showed a higher P-selectin expression compared to healthy platelets, while being in a resting state. At t=0 diabetic platelets showed an increased response to ADP and CRP-XL, expressed as a higher P-selectin expression. After one week of clopidogrel, diabetes patients had a higher response to both agonists compared to the control group. Additive *in vitro* $P2Y_{12}$ inhibition decreased platelet reactivity further. Platelets of type 2 diabetes mellitus patients show a higher basal level of activation and post-clopidogrel-treatment platelet reactivity. This might be reduced by more potent $P2Y_{12}$ antagonists, which can lead to a decreased risk of recurrent atherothrombotic events.

Introduction

Patients with type 2 diabetes mellitus (T2DM) suffer from an absolute or relative defect in insulin function (insulin resistance). They have a higher chance of developing atherosclerosis and cardiovascular events[38,39]. Besides changes in the vascular wall in T2DM, the platelets are hypersensitive and respond to stimuli at concentrations that normally do not activate platelets, leading to pathologic occlusions in arteries.

One of the main pathways of platelet activation occurs via the $P2Y_{12}$ receptor. Upon binding of the platelet agonist ADP to the $P2Y_{12}$ receptor, this receptor clusters and activates the GTP binding protein G_i. This G-protein dissociates in an α-unit inhibiting adenylate cyclase, reducing the synthesis of cyclic AMP (cAMP). A low level of the platelet inhibitor cAMP in platelets favors aggregation[184,185]. The $\beta\gamma$-unit activates the enzyme phosphatidylinositide-3-kinase (PI3-kinase), activating RAP1 and AKT1/2. This leads to a conformational change of the fibrinogen receptor $\alpha_{IIb}\beta_3$ from an inactive to an active formation, enhancing fibrinogen binding and aggregation[31].

Insulin binds to the insulin receptor, which activates the insulin receptor substrate-1 (IRS-1). This initiates activation of PI3-kinase, AKT1/2 and the uptake of glucose. However, IRS-1 has the ability to bind to G_i, thereby preventing its activation. Therefore, insulin is an inhibitor of aggregation in healthy individuals[30]. Platelets from T2DM patients, on the other hand, have lost their sensitivity to insulin, probably due to a defect in IRS-1. Therefore the inhibition of the $P2Y_{12}$ pathway, by the binding of IRS-1 to G_i, is lost favouring platelet activation. T2DM platelets have become hypersensitive compared to the platelets from non-diabetics[44,186].

This defect in T2DM patients may influence the response these patients have to $P2Y_{12}$ inhibiting drugs like clopidogrel. Clopidogrel is prescribed to prevent patients

suffering from atherothrombosis from having a secondary event. Clopidogrel first has to be metabolized in the bowel and liver to its active metabolite. This metabolite binds irreversibly to the $P2Y_{12}$ receptor. The antagonism of the $P2Y_{12}$ receptor by clopidogrel is not complete, a phenomenon described as non-responsiveness or resistance to clopidogrel. This occurs in 20-30% of the patients and is associated with a higher risk of occurrence of atherothrombotic events[166]. Studies have shown clopidogrel resistance to be even higher in T2DM patients[137,166]. T2DM patients show a hypersensitive $P2Y_{12}$ signaling and a reduced sensitivity to *in vitro* $P2Y_{12}$ antagonism[44].
We aimed to investigate whether T2DM also show a reduced response to *in vivo* antagonism of the $P2Y_{12}$ receptor by clopidogrel and what causes this reduced response. We show that the baseline level of platelet activation in T2DM patients is higher compared to non-diabetics. Platelets from T2DM patients also show higher reactivity to ADP and collagen. This hypersensitivity of diabetic platelets contributes to the reduced response to clopidogrel in T2DM patients

Materials and Methods

Study population and study design
The study population consisted of male T2DM patients treated with oral blood glucose lowering drugs (biguanides or sulfonylurea derivates). These were compared to age and sex matched controls. Before entering the study, subjects were asked for bleeding disorders in the family, bleeding complications after prior surgery, and easiness of bruising or bleeding. Examinations included the body-mass index (BMI), waist circumference, blood pressure and inspection for hematomas. Blood was drawn for the determination of thrombocyte count, HbA1c, serum cholesterol and triglyceride levels. Exclusion criteria were: use of anti-epileptics, use of acetylsalicylic acid or other non-steroidal anti-inflammatory drugs (NSAIDs), GPIIbIIIa-antagonists, heparin, thrombolytics or oral anticoagulants, known diabetic retinopathy, smoking, severe liver-function disorder, history of pathologic bleeding like for example intra-cranial hemorrhage or peptic ulcer or a planned surgical procedure 7 days prior to or after the study. The local ethics commission of the University Medical Center Utrecht, the Netherlands, approved the study. All study subjects approved by written informed consent.
Blood was drawn at two time points: t=0 days and t=7 days. In the period between t=0 and t=7, subjects took 75 mg/day of clopidogrel.

	Control group	T2DM patients	p
Age (years)	59 ± 2	63 ± 3	0.673
Length (meter)	1.8 ± 0.02	1,7 ± 0.02	0.164
Weight (kg)	80 ± 4	94 ± 5	0.037
BMI (kg/m^2)	24.2 ± 0.8	30.1 ± 1.4	0.005
Waist (cm)	93 ± 3	108 ± 5	0.006
Systolic Blood Pressure (mmHg)	139 ± 8	146 ± 5	0.487
Diastolic Blood Pressure (mmHg)	86 ± 5	81 ± 2	0.297
HbA1c (%)	5.4 ± 0.1	8.1 ± 0.6	0.003
Cholesterol (mmol/L)	5.8 ± 0.3	4.1 ± 0.3	0.004
Triglycerides (mmol/L)	1.4 ± 0.2	2.0 ± 0.6	0.438
Creatinine (µmol/L)	94 ± 6	98 ± 6	0.519
Trombocytes (10^9/L)	213 ± 14	225 ± 18	0.908

Table 1. Baseline characteristics of the study population.

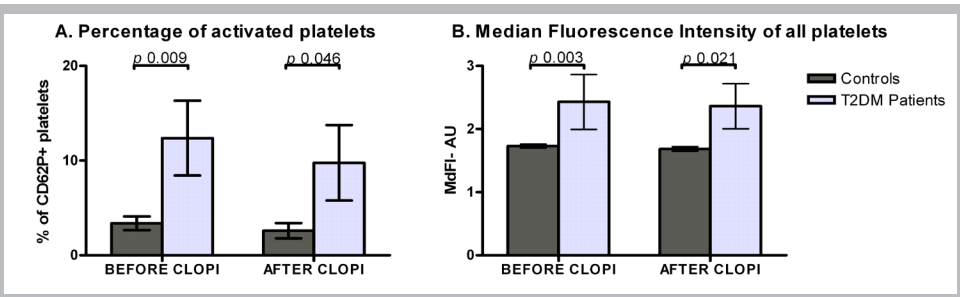

Figure 1. Basal level of platelet activation.
(A) Percentage of P-selectin positive platelets and (B) median fluorescence intensity (MdFI) in resting platelets of T2DM patients and matched controls measured by flow cytometry. Platelets of T2DM patients show a higher level of activation in the resting state.

Reagents
Agents used were ADP (Sigma, St. Louis, MO) CRP-XL (cross linked collagen related peptide; a kind gift from R.W. Farndale, Cambridge, UK), AR-C69931MX (a kind gift from Astra Zeneca, Loughborough, UK) and PE-anti-CD62P and isotype control (both from BD Biosciences, Franklin Lakes, NJ).

Whole blood flow cytometry
Blood was collected into 0.1 volume of 130 mmol/L Na_3citrate and was processed within 15 minutes of venepuncture. Whole blood was incubated with indicated concentrations of the $P2Y_{12}$ inhibitor AR-C69931MX. 5 mL of citrated whole blood was added to 50 mL of HBS (HEPES buffered saline: 10 mM HEPES; 150 mM NaCl; 1 mM $MgSO_4$; 5 mM KCl; pH 7.4). Samples were incubated for 20 minutes at room temperature with ADP (1 μM) or CRP-XL (0.01 μg/mL) in the presence of PE-anti-CD62P. The reaction was stopped by hundredfold dilution in formyl saline (0.2% formaldehyde in 0.9% NaCl). Negative controls for the P-selectin antibody were set using an appropriate isotype control. Flow cytometric analysis was carried out on a Becton Dickinson FACSCalibur (BD Biosciences; San Jose; CA) counting 3000 platelets per sample. Data were recorded as percentage of positive platelets above the treshold (set with the isotype control) and the median fluorescence intensity (MdFI) of all platelets. The MdFI is an indication of the number of p-selectin receptors on the cell surface of a single platelet.

Statistical analysis
Data are expressed as mean ± S.E.M. Percentages of p-selectin positive platelets and Median Fluorescence Intensity of all platelets were compared between T2DM patients and controls by the Mann-Whitney U test. Descriptive statistics were used to describe patient characteristics. For all analyses, a two-tailed p-value of less than 0.05 was considered to indicate statistical significance. Statistical analysis was performed using the Statistical Package for Social Science (version 15; SPSS; Chicago, IL, USA).

Results

Eight T2DM patients were included in the study. Table 1 represents the baseline characteristics of the population. There were significant differences in weight, BMI, waist circumference, HbA1c levels and cholesterol levels.
We determined the basal level of platelet activation by measuring P-selectin expression on the platelet's surface by flow cytometry (figure 1). At baseline (t=0) in healthy controls, 3.4 ± 0.7% of the platelets showed expression of P-selectin above the threshold, compared to 12.4 ± 3.9% of the platelets of T2DM patients (p = 0.009) (figure 1A). The MdFI, was also higher in T2DM platelets (figure 1B). This shows that in T2DM patients, resting platelets are in a more activated state. After one week of treatment with clopidogrel (t=7), platelets of both T2DM patients and controls showed lower surface expression of P-selectin, although significant differences in basal percentage of

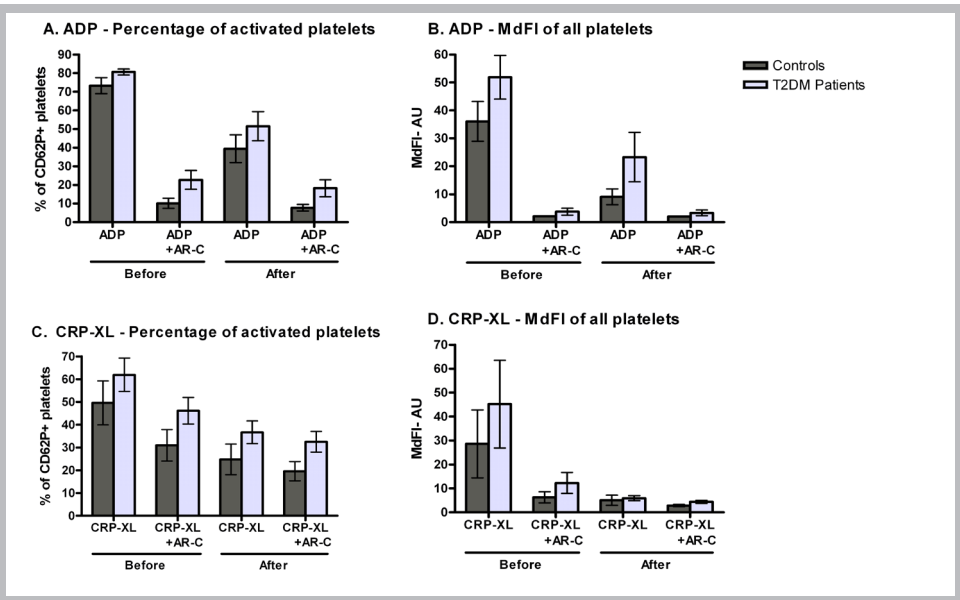

Figure 2. Platelet reactivity after activation by ADP and CRP-XL.
(A+B) Percentage of p-selectin positive platelets and MdFI of platelets after activation by ADP 1 μM at baseline and after one week of clopidogrel 75 mg/day. To investigate whether $P2Y_{12}$ inhibition is complete, blood was incubated with AR-C69931MX (1 μM) prior to activation.
(C+D) Percentage of p-selectin positive platelets and MdFI of platelets after activation by CRP-XL 1 μg/mL at baseline and after one week of clopidogrel 75 mg/day.

P-selectin positive platelets and in MdFI between both groups remained present (figure 1).

We stimulated platelets with the agonits ADP and CRP-XL. At t=0, platelets of T2DM patients demonstrated a higher level of ADP-induced activation, illustrated by more platelets (73.3 ± 4.3% in the control group vs. 80.6 ± 1.6% in the T2DM group; p = 0.208) that express P-selectin (figure 2A). The MdFI of the platelets of controls after stimulation with 1 μM ADP was 36.1 ± 7.1 compared to 51.9 ± 7.8 in T2DM patients (p = 0.074)(figure 2B). After one week of clopidogrel both T2DM patients and controls showed reduced platelet reactivity, but the T2DM patients remained higher. Inhibition by clopidogrel 75 mg/day was not complete for both groups, since the addition of 1 μM AR-C69931MX to the blood prior to activation, reduced platelet reactivity to ADP further.

We found similar results for CRP-XL induced platelet aggregation (figure 2C and 2D). T2DM patients had more platelets expressing P-selectin (49.6 ± 9.7% for the control group vs. 61.9 ± 7.3% for the T2DM group; p = 0.406) and the platelets expressed more P-selectin receptors at the surface after stimulation. After a week of clopidogrel platelets reactivity to CRP of both groups is decreased, but the T2DM platelets had a smaller decrease in reactivity to CRP. Addition of AR-C69931MX did not further decrease platelet reactivity to CRP.

Discussion

Here we demonstrate that platelets of T2DM patients show a higher level of activation while being in a resting state than platelets of matched controls without diabetes. Platelets of T2DM patients respond to a stimulus by expressing more P-selectin receptors on their surface. After one week of clopidogrel, T2DM platelets still show a higher agonist induced P-selectin expression.

Findings in earlier studies have shown that resting platelets of diabetics also have a 2-fold higher basal level of Ca^{2+} than healthy platelets[44]. The disturbed Ca^{2+} homeostasis is unresponsive to inhibition by insulin. The same study showed increased $P2Y_{12}$ signalling in T2DM patients. The diabetic platelets were also less sensitive to *in vitro* inhibition of the $P2Y_{12}$ receptor. We investigated if this is also true for inhibition of the $P2Y_{12}$ receptor *in vivo*. At t=0, the diabetic platelets respond to ADP by bringing more P-selectin to their surface than the healthy controls, i.e. the diabetic platelet is more activated than the healthy platelet. And although the platelets of diabetics and matched controls show a similar amount of inhibition after one week of clopidogrel administration, the difference in P-selectin expression remained different between both groups. *In vitro* additive inhibition of the $P2Y_{12}$ receptor decreases platelet reactivity to ADP further, but the differences between diabetic and healthy platelets are persistent. Platelets of T2DM are hypersensitive to stimulation by agonists, which might have implications for treatment by clopidogrel. High post clopidogrel treatment platelet reactivity in PCI patients is correlated with an increased risk of recurrent cardiovascular events[171]. Platelet reactivity in our study is higher in diabetics, which implicates that they are at risk for having a new atherothrombotic event.

The additive inhibitory effect of AR-C69931MX in both groups, suggests that administration of one week of clopidogrel does not completely inhibit the $P2Y_{12}$ receptor. This implicates that a higher dose of clopidogrel results in an enhanced $P2Y_{12}$ inhibition at possibly less recurrence of cardiovascular events. The OPTIMUS trial[137] showed that in T2DM patients with a decreased response to clopidogrel a dose of 150 mg/day induced a further reduction in the average aggregation, but that 60% of these patients preserved a weak response to clopidogrel.

Recently, several new $P2Y_{12}$ receptor antagonists have appeared on the market, which showed a more potent platelet inhibitory effect[182,187,188]. Future studies need to show if these agents have a stronger effect in diabetes patients.

Chapter 6

ADP activation of platelet protein tyrosine kinases reveals $P2Y_{12}$ signalling to Ephrin A2/4 receptor

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Submitted

ADP activates platelets via the G-protein coupled $P2Y_1$- and $P2Y_{12}$-receptors. $P2Y_{12}$ is the target for the platelet inhibitor clopidogrel, which is an effective anti-atherothrombotic agent. Current insight in $P2Y_{12}$ signalling is limited to suppression of the production of cAMP (a platelet inhibitor) and activation of protein kinase B/Rap1b (which stimulates aggregation). We investigated whether other signalling molecules are activated by $P2Y_{12}$ by a high through-put micro-array approach based on 144 Protein Tyrosine-kinase (PTK) substrates. ADP stimulates Tyr-phosphorylation of 28 peptide substrates representing surface receptors, 2nd messenger enzymes, and cytoskeletal proteins. Strong phosphorylation is found in peptides representing members of the Ephrin-receptor family. Blockade of $P2Y_{12}$ inhibits phosphorylation of peptides derived from EphA2 and EphB1. Immunoblots confirm ADP- and TRAP-induced Tyr-phosphorylation of EphA2/4 especially in stirred suspensions. The 2,5-dimethylpyrrolyl benzoic acid derivative that inhibits EphA2/4 Tyr-phosphorylation in HT22 neuronal cells, inhibits ADP-induced Tyr-phosphorylation of platelet EphA2/4, 2nd phase aggregation, dense- and α-granule secretion and thrombus formation at 1600 s^{-1} on a collagen surface. The results suggest that EphA2/4 is under control of $P2Y_{12}$ and contributes to granule secretion and thrombus stabilization.

Introduction

ADP activates platelets via the P2 purinergic receptors on the plasma membrane $P2Y_1$ and $P2Y_{12}$. $P2Y_1$ is coupled to the G-protein G_q and essential for shape change, aggregation and mobilization of Ca^{2+} from the endoplasmic reticulum. $P2Y_1$ also contributes to responses induced by suboptimal concentrations of other agonists such as the thrombin receptor (PAR1) activating peptide TRAP[189-191]. $P2Y_1$ null mice show impaired aggregation by ADP and mice overexpressing $P2Y_1$ exhibit hyperactivity, illustrating the importance of $P2Y_1$ signalling for platelet aggregation. $P2Y_{12}$ augments signals generated by ADP-occupied $P2Y_1$ and other agonist-receptor complexes resulting in secretion, integrin $\alpha_{IIb}\beta_3$ activation and thromboxane A_2 formation. Patients with dysfunctional $P2Y_{12}$ suffer from a mild bleeding tendency and an impaired response to ADP and $P2Y_{12}$ null mice have a strongly reduced ADP-aggregation[189]. Polymorphisms in the human *P2Y12* gene associate with increased P-selectin surface expression and fibrinogen binding[192], indicating that abnormalities in $P2Y_{12}$ signalling may lead to platelet hyperactivity.

$P2Y_{12}$ is the target of the anti-atherothrombotic drug clopidogrel, which reduces the relative risk of the development of ischaemic stroke, myocardial infarction or vascular death by 8.7% compared to aspirin[36]. Active clopidogrel metabolite (clopidogrel-AM) interferes with the $P2Y_{12}$ induced activation of G_i, the G-protein which through $G_i\alpha$-2 inhibits adenylyl cyclase and formation of the platelet inhibitor cAMP and through G_i-$\beta\gamma$ activates protein kinase B and Rap1b which contribute to $\alpha_{IIb}\beta_3$ activation[189]. Under laboratory conditions, the action of clopidogrel-AM is mimicked by AR-C69931MX, which reveals the significant contribution of $P2Y_{12}$ signalling in aggregation, secretion and the generation of a pro-coagulant surface induced by ADP and other platelet activating agents[110].

To increase our insight in signalling pathways initiated by ADP, we applied Protein Tyrosine Kinase (PTK) activity profiling using microarrays with 144 peptides each representing a 13 amino acids Tyr-phosphorylation motif derived from know phosphorylation sites in different signalling molecules. We found strong Tyr-phosphorylation of peptides representing different members of the erythropoietin-producing hepatoma receptor (Eph) family. This family of transmembrane receptors consists of 16 kinase receptors divided in 10 EphA's and 6 EphB's based on sequence homology of the extracellular domain. In general, EphA's bind to type A ephrins, which are tethered to the cell membrane by a glycosylphosphatidylinositol anchor. EphB's bind type B ephrins which have a transmembrane domain and a short cytoplasmic region. An exception is EphA4 which binds A-type ephrins but also certain B-type ligands albeit with a lower affinity[193-196]. Receptor-ligand interaction is made possible through cell-cell contact with one cell providing the Eph-receptor and the other the ephrin ligand. The result is Tyr-phosphorylation of cytosolic tails in receptor and ligand, which become targets for signalling proteins that control the actin cytoskeleton and strengthen cell-cell interaction. Eph-receptor ephrin-ligand mediated anchorage contributes to a wide spectrum of biological effects e.g. spinal neurulation[197], osteoclastogenesis[198], tumorigenesis[194], glucose-stimulated insulin secretion[199] and arterial-venous segregation during blood vessel formation[200].

Platelets express EphA4, EphB1 and ephrinB1[201-206] but not ephrinA3[201]. Forced clustering of EphA4 and ephrinB1 by self-oligomerizing GST-fusion proteins containing the exodomains of ephrinA4 and EphB1 induce adhesion to fibrinogen, α-granule secretion and cytoskeletal reorganization[201]. ADP-induced aggregation is accompanied by binding of EphA4 to the Src kinases Fyn and Lyn inducing a signalling cascade that promotes thrombus stability. Clustering of EphA4 and ephrinB1 alone is insufficient to induce binding of soluble fibrinogen but potentiates aggregation induced by ADP in platelet suspensions by outside-in signalling through integrin $\alpha_{IIb}\beta_3$[202,203].

The present findings show that ADP-stimulated platelets contain PTKs capable of inducing Tyr-phosphorylation of multiple Eph-derived phosphorytation sites in the absence of cell-cell contact. Findings obtained by PTK activity profiling and confirmed by immunoblotting reveal that EphA2/4 is a downstream target of $P2Y_{12}$ signalling. Pharmacological interference with EphA2/4 suppresses ADP- and ligand-induced EphA2/4 Tyr-phosphorylation and leads to a strong reduction in the secretion of granule contents. The finding that EphA2/4 is under control of $P2Y_{12}$ reveals a novel, ephrin-independent mechanism through which thrombus stabilization is maintained.

Methods

Chemicals

Agents used (with sources) were ADP, ATP, A3P5PS, collagen type I, protease inhibitor mixture, sodium vanadate (all from Sigma, St.Louis, MO), BSA (Bovine Serum Albumin, Fraction V, Calbiochem, La Jolla, CA), Horm collagen (derived from equine tendon; Nycomed, Munich, Germany), AR-C69931MX (a kind gift from Astra Zeneca, Loughborough UK), human fibrinogen (Kordia, Leiden, The Netherlands), iloprost (Schering-Plough, Kenilworth, NJ), the EphA2/4 receptor blocker 2,5-dimethylpyrrolyl benzoic acid derivative (compound 2 in Noberini *et al*[207]) and the same, but inactive, compound lacking one hydroxyl group (compound 30 in Noberini *et al*[207]; Matrix Scientific, Colombia, STATE), pentasacharide (Arixtra, Glaxo Smith Kline, Greenford, UK), PPACK (H-D-Phe-Pro-Arg-chloromethylketone, Bachem, Torrence, CA), PY-BSA, (MP biomedicals, OH), protein G-Sepharose (Amersham, Uppsala, Sweden), and PP2 (Invitrogen, Carlsbad, CA,

USA). Thrombin receptor (PAR-1)-activating peptide SFLLRN (TRAP) was synthesized with a semiautomatic peptide synthesizer (Labortec AG SP650; Bubendorf, Switzerland).
Antibodies used were against Tyr-phosphorylated proteins (4G10, Upstate Biothechnology, Bucks, UK), EphA2 (Novus Biologicals, Littleton, CO, USA), EphA2 (H-77), EphA4 (S21) (both from Santa Cruz, St.Louis, MO), EphA4 (BD Biosciences Franklin Lakes, New Jersey USA); antibodies against Platelet Factor 4 (PF-4) for coating (antibody mouse anti human PF-4) and for detection (goat PF-4) were from R&D systems, Minneapolis, MI. Renaissance Chemiluminescence Western blot reagent was from PerkinElmer Life Sciences (Boston, MA).

Preparation of washed platelets and incubations.
Freshly drawn venous blood obtained from healthy, medication-free subjects was collected into 0.1 volume of 130 mM trisodium citrate according to procedures approved by the local Medical Ethical Review Board. Washed platelets were prepared as described[208]. The final platelet concentration was adjusted to $2.0x10^{11}$ cells/L. Prior to experiments, platelets were kept at 20°C for 45 minutes to ensure a resting state. For perfusion assays, whole blood was anticoagulated with 0.1 volume 500 mM PPACK and 200 U/mL pentasacharide in 0.9% NaCl. To evaluate signalling by ADP receptors, washed platelets were incubated at room temperature with $P2Y_1$ antagonist (500 μM A3P5PS, 5 minutes), $P2Y_{12}$ antagonist (100 nM AR-C69931MX, 1 minute) and inhibitors of Src-kinase family members (1 μM PP2, 5 minutes), adenylyl cyclase (50 μM SQ22536, 5 minutes), phosphatidyl inositol 3-kinase (0.5 μM wortmannin, 10 μM LY294002, 5 minutes), unless indicated otherwise.

Protein Tyrosine Kinase activity profiling
Unstirred, washed platelets were stimulated with ADP for indicated times and concentrations at 37°C. Incubations included 10 μM iloprost for complete arrest of platelet activation, ADP in combination with 100 μM A3P5PS to block $P2Y_1$ signalling and 100 nM AR-C69931MX to block $P2Y_{12}$ signalling. Platelet suspensions (200 μL) were lysed in 200 μL M-PER mammalian extraction buffer with Halt Phospatase Inhibitor cocktail and Halt Protease Inbititor cocktail (Pierce, Rockford, IL) and stored at -80°C prior to analysis. A quantity of 9 μL was used for the PamChip procedure. Micro-array experiments were performed using PamChip® peptide microarrays run on a PamStation instrument (PamGene, 's Hertogenbosch, the Netherlands). Temperature-controlled peptide chips were run in parallel by pumping the sample up and down through a 3-dimensional porous chip. Data was captured by real-time imaging of the fluorescence signal by CCD imaging. The Tyr kinase PamChip peptide microarrays comprise 144 different peptides, each consisting of a 15 amino acid sequence, of which 13 residues are derived from known Tyr-phosphorylation sites in Swissprot and Phosphobase databases. Two N-terminal residues link the phosphosite sequence to the solid support of the 3-dimensional chip. The kinase reaction mixtures consisted of Abl Reaction Buffer (50 mM Tris–HCl pH 7.5, 10 mM MgCl2, 1 mM EGTA, 2 mM dithiothreitol, 0.01% Brij 35; New England BioLabs, Ipswich, MA), 1 mg/mL bovine serum albumin, 400 μM ATP, and 12.5 μg/mL of monoclonal, FITC-conjugated anti-phospho-Tyr antibody (Exalpha Biologicals, Inc., Maynard, MA); 31 μL was added to each sample lysate containing 2–5 μg protein. Prior to application of the sample, the chips were blocked with 2% BSA and washed 2 times with kinase reaction buffer. During 60 minutes incubation at 30°C, real time images were taken automatically every 5 minutes. Images were analysed by BioNavigator software (PamGene, 's Hertogenbosch, the Netherlands). The fluorescence intensities were expressed as arbitrary units. Data were expressed as means from quadruplicates; variation coefficients generally ranged between 4 and 25 % for significant signals. Results were obtained from 4 healthy donors; only results which differed significantly from iloprost-treated samples are shown ($p<0.05$).

Immunoblots
Washed platelets were incubated at 20 °C without and with stirring, as indicated, with agonist and mixed (1:10 v/v) with ice-cold lysis buffer consisting of 10% (v/v) Nonidet P-40, 5% (w/v) octylglucoside, 50 mM EDTA, 1% (w/v) SDS, supplemented with 5 mM $NaVO_3$ and 10% (v/v) protease inhibitor mixture. Immunoprecipitation (IP) was performed with anti-EphA2 (H-77) directed against aa 423–498, which is for 24 % homologous to aa 424-504 of EphA4. Immune complexes were incubated with protein G-Sepharose (3 hours, 4°C), washed, solubilized in 2x reducing Laemmli buffer, separated by SDS-PAGE on 10% gels followed by westernblotting using PVDF membranes blocked with PY-BSA in TBS-Tween. Membranes were incubated (16 hours, 4°C) with 4G10 antibody against Tyr-phosphorylated proteins and anti-EphA2 (Novus) directed against aa 927-969, which is for 40 % homologous to aa 934–976 of EphA4. A few experiments were performed with anti EphA4 directed against the C-terminus of EphA4 for IP. Antibody binding was detected using peroxidase-linked secondary antibodies and visualized by the enhanced chemiluminescence reaction.

Platelet aggregation, secretion and thromboxane A_2 production
Washed platelets were prepared as described[208], and the platelet concentration adjusted to $2.0x10^{11}$/L. Aliquots of 0.5 mL were pre-warmed to 37°C for 5 minutes and stimulated with 20 μM ADP, 10 μM TRAP and 1 μg/mL Horm collagen. To all samples, fibrinogen (100 μg/mL, f.c.) was added. Platelet aggregation was monitored continuously for 15 minutes at 900 rpm in an optical aggregometer (model 570 VS, Chrono-Log Corporation, Havertown, PA). ATP secretion was measured by luminescence in the aggregometer by adding 100 μL Chrono Lume.

Platelet Factor-4
Washed platelets were incubated at 20°C with stirring and stimulated for 5 minutes. Then, samples were supplemented with 10 ng/mL PGI_2 (f.c.), put on ice and centrifuged at 3000 x g for 3 minutes (4°C). The supernatant was collected and PF-4 was measured by elisa. Normal pool serum was used as a reference.

Real Time Perfusion
Collagen type I was immobilized on glass coverslips (24×50 mm, Menzel Gläser) by 1 hour incubation at room temperature with 350 mL collagen type I suspension (100 mg/mL) per coverslip. Coverslips were incubated with 1% human albumin in PBS overnight at 4°C. Perfusions were performed in a laminar perfusion chamber combined with an inverted optical microscope (Carl Zeiss Axio observer. Z1, Oberkochen, Germany) and CCD camera (Zeiss AxioCam MR3, Oberkochen, Germany), as described[209,210]. Whole blood was pre-warmed to 37°C, and then incubated with vehicle (0.1% ethanol), EphA2/4 blocker or control (400 _M) and AR-C 69931MX (100 nM, all final concentrations) for 2 minutes at 37°C. Blood was perfused over a collagen type I-coated surface for 4 minutes at a wall shear rate of 1600 s^{-1}. During perfusion, DIC images were continuously recorded for off-line analysis with the CCD camera under an objective of 40x (EC plan-Neofluar 40x/0.75 M27 Zeiss, Germany) with a frequency of 1 frame/2 s. Thrombus size was measured by confocal microscopy in the z-direction.

Results

PTK activity profile of ADP-stimulated platelets

The PTK activity profile of ADP-stimulated platelets was analyzed in unstirred suspensions stimulated for 10 minutes with increasing concentrations of ADP and with 20 _M ADP stimulation for 5, 10 and 20 minutes (figure 1). In the absence of ADP, some peptides already showed a slight Tyr-phosphorylation indicative for activation during sample preparation. A suspension with iloprost was therefore included to completely arrest platelet activation and the Tyr-phosphorylation data were taken as reference for those from stimulated platelets. Stimulation with ADP induced a dose- and time-dependent increase in the Tyr-phosphorylation of 28 peptides. Rate and extent differed between peptides and some showed significant phosphorylation in all four donors and others only in three or two (Table 1). Peptides that were Tyr-phosphorylated were those representing surface receptors, 2nd messenger enzymes and cytoskeletal proteins, among others. The group of surface receptors included receptors for platelet derived growth factor, vascular endothelial growth factor and fibroblast growth factor. Also the peptide representing aa 361-373 of the erythropoietin receptor was found positive, but since this receptor is absent in platelets, this data probably reflects a kinase that Tyr-phosphorylates the thrombopoietin receptor. Of specific interest was the Tyr-phosphorylation of peptides representing Ephrin receptors. ADP-induced phosphorylation of peptides representing EphA2 and EphB1 (in 4 out of 4 donors), EphA7 (3/4) and EphA1 (2/4). The region of EphA2 **765**EDDPEATYTTSGG**777** is for 85% homologous with the EphA4 region **772**EDDPEAAYTTRGG**784**, suggesting cross reactivity with EphA4. Earlier work has demonstrated ligand-induced EphA4 Tyr-

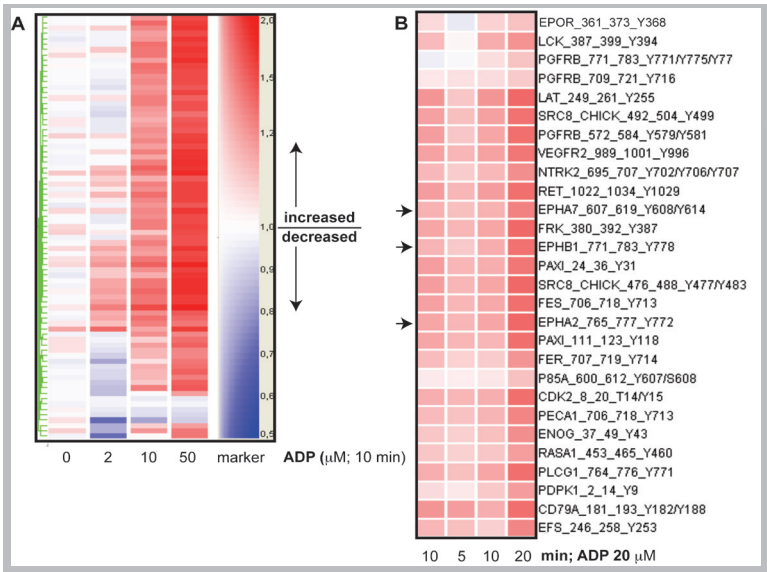


Figure 1. Protein Tyr Kinase activity profiling.
(A) Heatmap of ADP-induced Tyr-phosphorylation of peptides mimicking phosphorylation sites of known proteins. Platelets were incubated with ADP at indicated concentrations and times in a static suspension. Lysates were prepared in the presence of a cocktail of Tyr-phosphatase inhibitors and incubated with chips containing 144 different peptides each consisting of a 13 amino acids region analogous to the Tyr-phosphorylation sites of proteins in Swissprot and Phosphobase databases. (B) Tyr-phosphorylation of 28 peptides induced by kinases in ADP-stimulated platelets. Numbers refer to amino acid notations in the intact molecules with indicated Tyr phosphorylation site(s). Arrowheads indicate bands corresponding to Ephrin receptors.

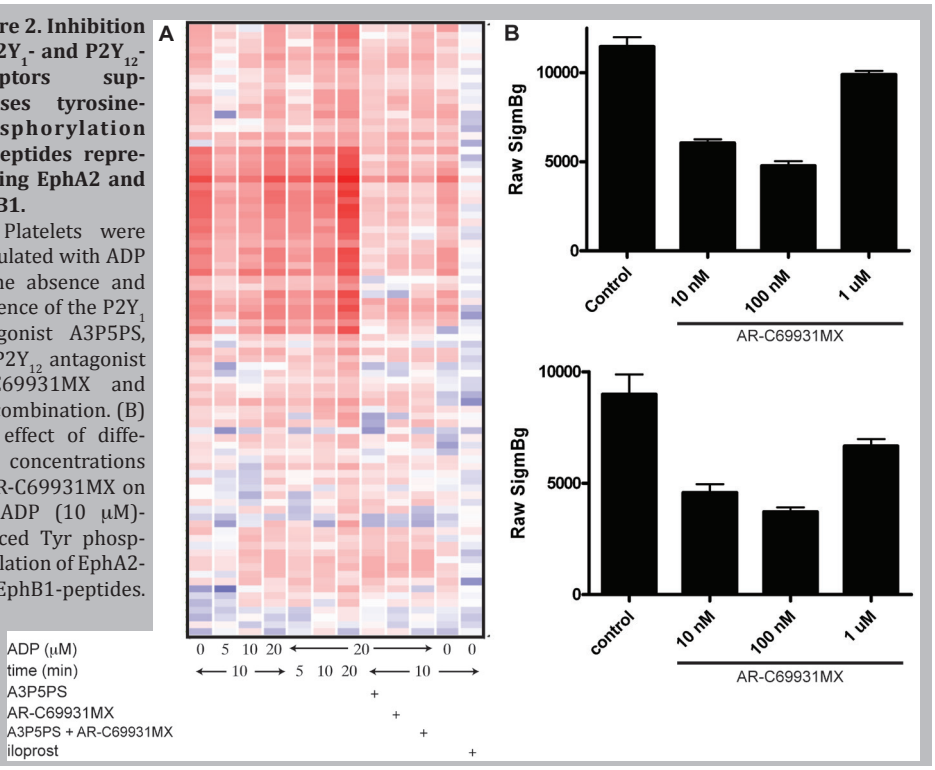


Figure 2. Inhibition of $P2Y_1$- and $P2Y_{12}$-receptors suppresses tyrosine-phosphorylation of peptides representing EphA2 and EphB1.
(A) Platelets were stimulated with ADP in the absence and presence of the $P2Y_1$ antagonist A3P5PS, the $P2Y_{12}$ antagonist AR-C69931MX and the combination. (B) The effect of different concentrations of AR-C69931MX on the ADP (10 μM)-induced Tyr phosphorylation of EphA2- and EphB1-peptides.

Table 1. ADP-induced tyrosine-phosphorylation of PTK peptide substrates in 4 donors.
Platelets were stimulated without stirring with 10 μM ADP for 10 minutes and the activity of Protein Tyrosine Kinases was measured by analyzing the phosphorylation of peptides mimicking Tyr-phosphorylation sites in 144 proteins. Data were compared with iloprost-treated inactive platelets.

	4 donors	3 donors	2 donors	
Surface Receptors	EphA2	EphA7	EphA1	
	EphB1	EPO-R	progesterone-R	
		PDGF-R		
		VEGF-R		
		FGF-R		
2nd messengers		fyn	PDK-1	cyclin-dependent K
		FER-TK	PLCγ	lymfocyte TK
			JAK-1	z-chain ass. prot. K
			LAT	neurotrophic TK
			ras	p21 GTPase act. prot
Cytocortical proteins		cortactin	paxillin	
		cadherin ass. prot. B1		
Fibrils		keratin II	myelin basic protein	
Immunoglobin Rs			pecam-I	
Glycolytic enzymes			enolase2	
Oncogenes	feline sarc. onc.	erythrobl. leuk. onc.	ret. proto-oncogene	
Rest		ery membrane prot. 1	paired related homeobox2	
			annexin1	

Abbreviations: R: receptor; Eph, Ephrin-R; EPO: erythropoietin, PDGF: platelet-derived growth factor; VEGF: vascular/endothelium growth factor; FGF: fibroblast growth factor, FER: P-protein NCP94; PLC: phospholipase; ery: erythrocyte; onc: oncogene; ass: associated; TK: Tyr-kinase; prot: protein

phosphorylation in platelets following addition of GST-ephrinA4[203]. The PTK activity profile established in the present study indicates that EphA2 Tyr-phosphorylation might also occur in a ligand-independent manner upon platelet stimulation by ADP.
To clarify the individual contribution of $P2Y_1$ and $P2Y_{12}$ signalling to Tyr-phosphorylation of the peptides, platelets were stimulated with ADP without and with inhibitors of $P2Y_1$ (A3P5PS) and $P2Y_{12}$ (AR-C69931MX) signalling. Figure 2A illustrates the activation by sample preparation that occasionally showed up and the strong inhibition by iloprost. The separate inhibitions as well as the combination of $P2Y_1$ and $P2Y_{12}$ blockade induced a sharp fall in Tyr-phosphorylation of a number of peptides (Table 2). $P2Y_1$ blockade reduced phosphorylation of peptides representing platelet derived growth factor, vascular endothelial growth factor, and in addition peptides representing phospholipase Cγ1, cortactin and fes (c-fes/fps protein), among others. $P2Y_{12}$ blockade reduced Tyr-phosphorylation of cortactin- and fes peptide. The combination also reduced these peptides and in addition inhibited Tyr-phosphorylation of cyclin-depent kinase and the Src kinase fyn. Tyr- phosphorylation of EphA2- and EphB1-peptide was inhibited by both receptor antagonists, suggesting that these Ephrin receptors are downstream of $P2Y_1$ and $P2Y_{12}$. Inhibition of $P2Y_{12}$ was dose-dependent showing an optimum at 100 nM (Figure 2B). These data illustrate that $P2Y_{12}$ blockade has a strong impact on the PTKs that phosphorylate EphA2 and EphB1.

$P2Y_1$-R block	$P2Y_{12}$-R block	$P2Y_1$-R + $P2Y_{12}$-R block
EphA2	EphA2	EphA2
EphB1	EphB1	EphB1
PDGF-R	cortactin	PDGF-R
VEGF-R	fes	PLCγ1
PLCγ1		cortactin
cortactin		ret
ret		fes
fes		CD79A
CD79A		cyclin-dependent K
		fyn

Table 2. Reduction of tyrosine-phosphorylation of PTK peptide substrates by $P2Y_1$- and $P2Y_{12}$ blockade.
Platelets were stimulated without stirring with 10 μM ADP for 10 minutes without and with $P2Y_1$ antagonist (100 μM A3P5PS, 10 minutes), $P2Y_{12}$ antagonist (100 nM AR-C69931MX, 10 minutes) or both. Data were compared with iloprost-treated inactive platelets.

Detection of EphrinA2/4 tyrosine-phosphorylation by immunoblotting

To confirm results obtained by PTK activity profiling, incubations were repeated in suspensions stimulated by TRAP and ADP and analyzing EphA2- and EphA4 Tyr-phosphorylation by immunoblotting. In stirred platelet suspensions treated with iloprost, EphA2 phosphorylation was absent but stimulation with TRAP induced a strong phosphorylation (Figure 3A, B). $P2Y_{12}$ blockade with AR-C69931MX led to a strong reduction, but blockade of $P1Y_1$ with A3P5PS had little effect. Certain derivatives of 2,5-dimethylpyrrolyl benzoic acid inhibit ligand-induced EphA2/4 phosphorylation in HT22 neuronal cells through binding to the ligand binding pocket thereby interfering with ligand-induced receptor Tyr-phosphorylation. Compound 2 in Noberini *et al*[207] (EphA2/4 blocker, in short) did not change TRAP-induced EphA2 phosphorylation and also its inactive control (Compound 2 lacking a single hydroxyl group; compound 30 in Noberini *et al*[207], EphA2/4 control, in short) had little effect. In a static suspension, TRAP induced a much weaker EphA2/4 phosphorylation than in a stirred suspension, indicating that cell-cell contact contributed to this response. Again there was inhibition by $P2Y_{12}$ blockade and neither the EphA2/4 blocker nor the control changed the phosphorylation. A general inhibitor of Src kinases (PP2) also failed to change EphA2 phosphorylation (figure 3B). Stimulation with ADP induced a much weaker EphA2 phosphorylation than TRAP. Here, a slight but reproducible interference by EphA2/4 blocker was observed. Again, the phosphorylation was inhibited by the agonist of $P2Y_{12}$ but not by the agonist of $P2Y_1$. These data suggest that Tyr-phosphorylation of EphA2 is primarily controlled by $P2Y_{12}$ signalling. Similar results were seen in immunoblots for EphA4 with Tyr-phosphorylation induced by ADP and inhibited by AR-C69931MX (Figure 3C).

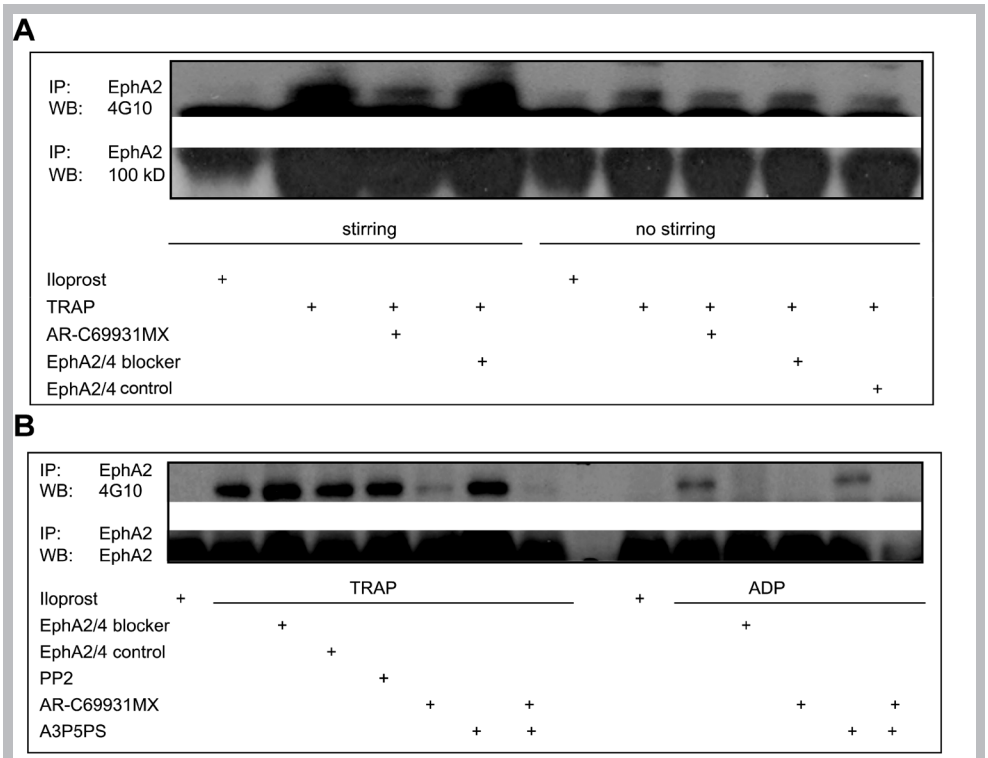


Figure 3. EphA2 tyrosine-phosphorylation by TRAP and ADP.
(A) Effect of platelet-platelet contact on EphA2 Tyr phosphorylation induced by TRAP (20 μM) in the absence and presence of the $P2Y_{12}$ antagonist AR-C69931MX, EphA2/4 blocker and its inactive control. Stirring increases EphA2 Tyr-phosphorylation; the $P2Y_{12}$ antagonist inhibits EphA2 Tyr-phosphorylation.
(B) EphA2 Tyr-phosphorylation in stirred suspensions treated with iloprost, TRAP (20 μM) and ADP (10 μM) in the absence and presence of EphA2/4 blocker (40 μM) and its control, the Src-kinase family blocker PP2, the $P2Y_{12}$ antagonist AR-C69931MX and the $P2Y_1$ antagonist A3P5PS. The $P2Y_{12}$ antagonist inhibits EphA2 Tyr phosphorylation in TRAP-and ADP-stimulated platelets.
(C) EphA4 Tyr-phosphorylation induced by 10 μM ADP in the absence and presence of AR-C69931MX. ADP induces EphA4 Tyr phosphorylation, which is inhibited by AR-C69931MX.

Role of EphA2/4 in aggregation and secretion

The finding that Tyr-phosphorylation of platelet EphA2/4 strongly depends on $P2Y_{12}$ signalling and that the EphA2/4 blocker interferes with this reaction made it possible to clarify the role EphA2/4 in platelet functions that critically depend on $P2Y_{12}$. The EphA2/4 blocker but not its inactive control induced a dose-dependent inhibition of aggregation induced by ADP with an optimum at about 40 μM (figure 4A). Epinephrine lowers cAMP through G_z and together with traces of ADP and thrombin induces biphasic aggregation[211,212] and again EphA2/4 blockade inhibited aggregation. Interestingly,

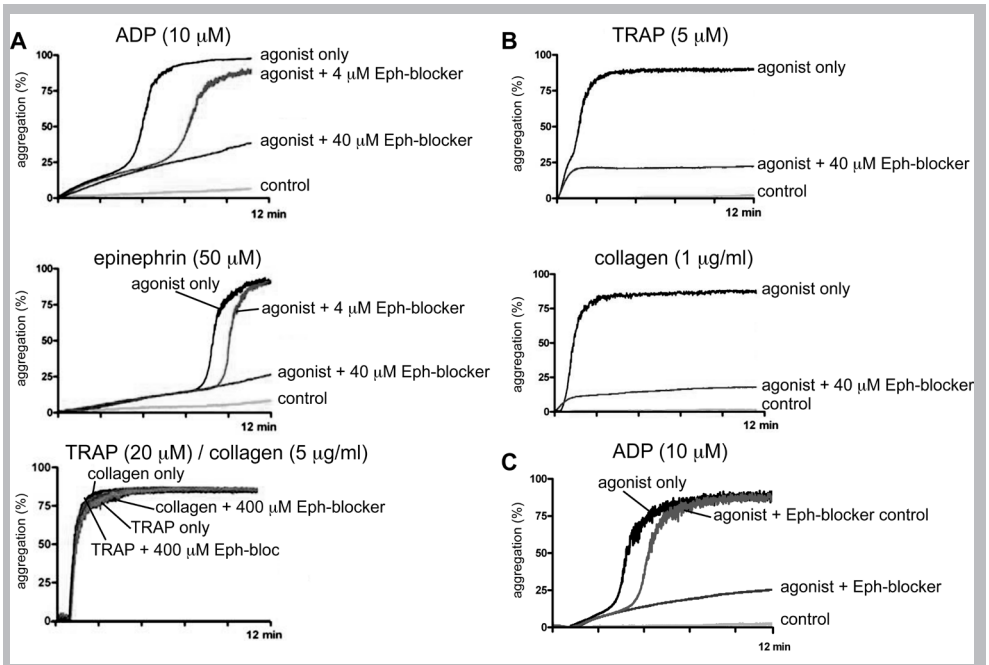


Figure 4. The EphA2/A4 blocker inhibits secondary platelet aggregation.
(A) Stirred platelet suspensions were stimulated by ADP (10 μM) and epinephrine (50 μM) at 37°C in the absence and presence of EphA2/4 blocker (compound 2 in Noberini *et al*[207]) at indicated concentrations. EphA2/4 blocker inhibits 2nd phase aggregation. EphA2/4 blocker does not change aggregation induced by optimal concentrations of TRAP and collagen. (B) EphA2/A4 blocker inhibits aggregation by suboptimal concentrations of TRAP and collagen. (C) The control for EphA2/4 blocker (compound 2 lacking a single hydroxyl group, compound 30 in Noberini *et al*[207]) has no effect.

the inhibition was restricted to the 2nd phase of the aggregation response, which is known to depend on secretion of granule ADP and $P2Y_{12}$-mediated acceleration of platelet activating sequences. A 10-fold higher inhibitor concentration did not change aggregation induced by a high concentration of TRAP and collagen, which is known to occur independently of $P2Y_{12}$ signalling. At lower concentrations, however, aggregation was disturbed by the blocker such in line with the contribution of $P2Y_{12}$ signalling under these conditions (figure 4B). The finding that the EphA2/A4 control did not show this inhibition illustrates the specificity of the EphA2/A4 blocker (figure 4C). Under the conditions of ADP-induced aggregation, EphA2/A4 blocker did not interfere with the production of thromboxane A_2 (data not shown). These data suggest that the EphA2/4 blocker is a specific antagonist of platelet EphA2/4 and that activation of EphA2/4 contributes significantly to 2nd phase aggregation, possibly by stimulating the secretion of ADP. To investigate this possibility, aggregation studies were repeated in the lumi-aggregometer which monitors the release of ATP as a substitute for ADP. In stirred suspensions, the EphA2/4 blocker greatly reduced ATP release induced by ADP (figure 5A). The same inhibition was observed when secretion of the α-granule marker platelet factor 4 was measured (figure 5B). These findings suggest that EphA2/4 contributes to 2nd wave aggregation by stimulating the secretion response.

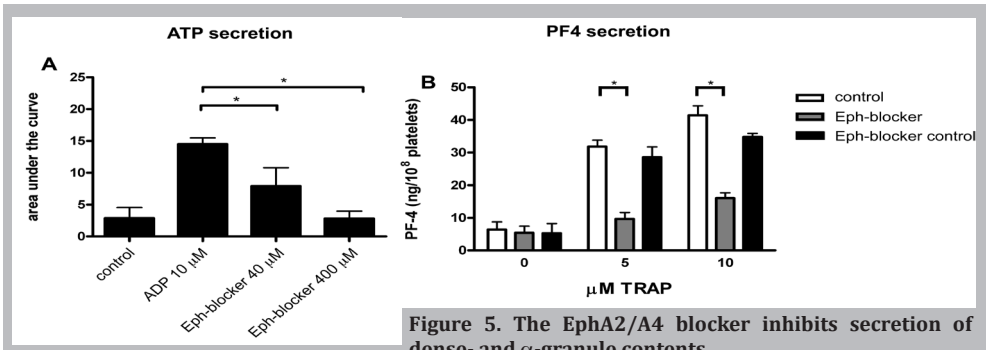


Figure 5. The EphA2/A4 blocker inhibits secretion of dense- and α-granule contents.

(A) Stirred platelet suspensions were stimulated by ADP (10 μM) at 37°C without and with EphA2/4 blocker and the secretion of δ-granule ATP was measured in a lumi-aggregometer. (B) Stirred platelet suspensions were stimulated by TRAP (5, 10 μM) and the secretion of the α-granule marker platelet factor 4 (PF4) was measured. Data are means ± SD, n=3 (* $p<0.05$).

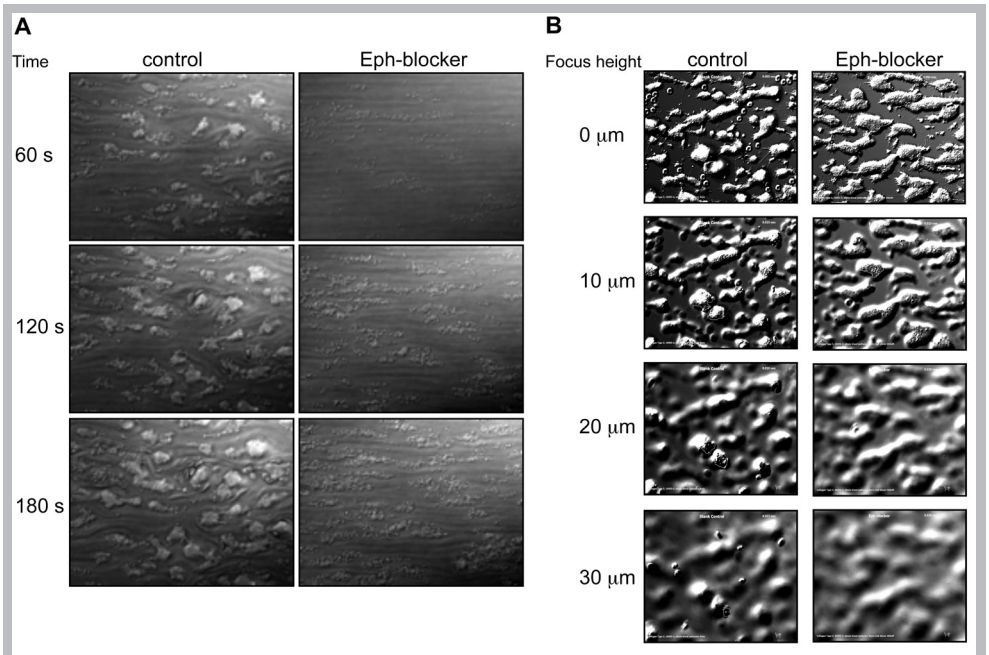


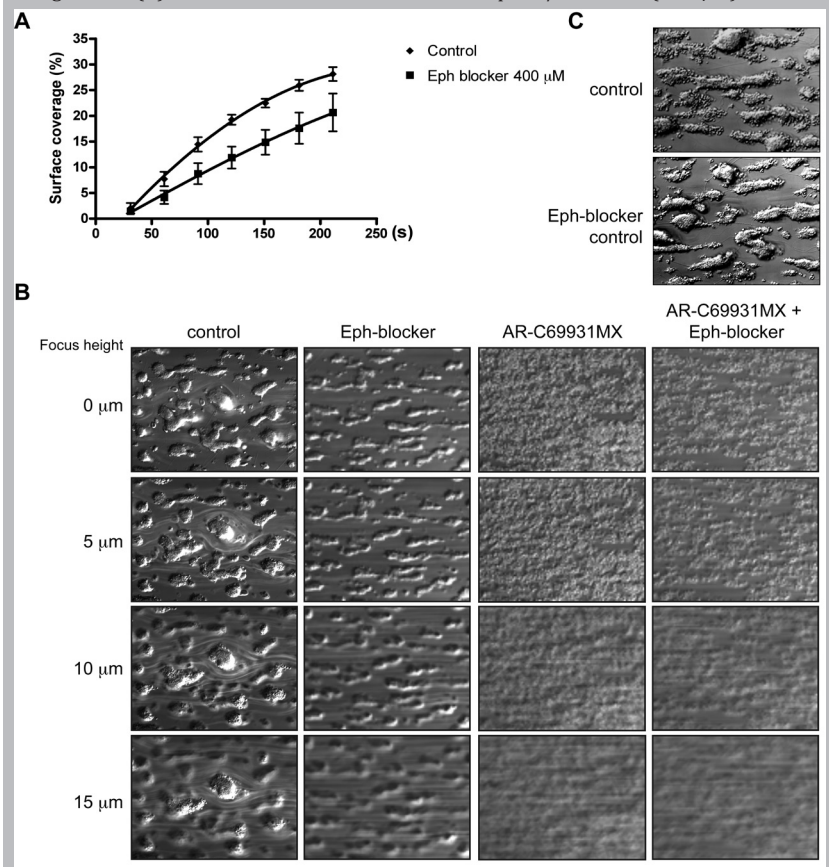
Figure 6. The EphA2/A4 blocker inhibits thrombus growth on collagen under flow.

(A) Whole blood was perfused over a collagen type-I coated surface at a shear of 1600 s^{-1} and adhesion and thrombus formation were measured real-time. Pictures show adhesion and thrombus formation during the first three 60 s intervals for blood treated with ethanol vehicle and EphA2/4 blocker (400 μM). EphA2/A4 blockade attenuates platelet adhesion and interferes especially with thrombus growth. (B) Thrombus height was determined by confocal analysis in the z-direction.

Effects of EphA2/4 inhibition on thrombus growth

Platelet adhesion and thombus formation on a collagen-coated surface in a flow chamber is reduced by 30% in $P2Y_{12}$-deficient platelets, indicating that in normal platelets $P2Y_{12}$ signalling contributes to adhesion and thrombus growth[213]. At concentrations used in aggregation studies with washed platelets, the EphA2/4 blocker had no effect but in the perfusion studies with whole blood a 10-fold higher concentration strongly interfered with platelet-collagen interaction. In the presence of inhibitor, thrombus growth (figure 6A) and thrombus hight (figure 6B) were strongly reduced by EphA2/4 blockade, but surface coverage was only slightly lower than in untreated suspensions (figure 7). Since Tyr phosphorylation of EphA2/4 is downstream of $P2Y_{12}$ receptor signalling, a difference between $P2Y_{12}$- and EphA2/4 blockade might be expected. Indeed, whereas the EphA2/4 blocker mainly interfered at later stages of thrombus growth, $P2Y_{12}$ blockade interfered early resulting in many small thrombi. A combination of both blockers induced the appearance of $P2Y_{12}$ blockade alone, as expected when $P2Y_{12}$ is upstream of EphA2/4 activation (figure 7B,C).

Figure 7. The EphA2/A4 blocker inhibits thrombus growth on collagen under flow.
(A) Whole blood was perfused over a collagen type-I coated surface at a shear of 1600 s^{-1} and adhesion and thrombus formation were measured real-time. Surface coverage after 200 s in the flow experiments shown in figure 6A. (B) Whole blood was incubated with EphA2/4 blocker (400 μM), AR-C69931MX (100 nM) and the combination. Thrombus height was determined by confocal analysis in the z-direction. (C) Results of the flow experiments of blood treated with the EphA2/A4 blocker control, which show thrombus formation similar as in blood treated with the ethanol vehicle.



Discussion

The present findings are best explained by assuming a model in which a resting platelet expresses $P2Y_{1/12}$ receptors on the plasma membrane together with EphA2/4 associated with integrin $\alpha_{IIb}\beta_3$[202] (figure 8). Contact with ADP activates $P2Y_1$ which by signalling through G_q induces Ca^{2+} mobilization, exposure of ligand bindingsites on $\alpha_{IIb}\beta_3$ and - when suspensions are stirred - aggregation. ADP also activates $P2Y_{12}$ which lowers the level of the platelet inhibitor cAMP and activates protein kinase B/Rap1b initiating secretion and more $\alpha_{IIb}\beta_3$ activation[189]. The PTK activity profile suggest that ADP induces Tyr phosphorylation of EphA2/4 in a static platelet suspension. Apparently, the Tyr-phosphorylation is weak and below detection limits of conventional blotting techniques. Under the same static conditions, TRAP induces a stronger phosphorylation which can be detected on immunoblots. This Tyr-phosphorylation is almost fully inhibited by $P2Y_{12}$ blockade indicating that it is a product of ADP signaling. In contrast, $P2Y_1$ inhibition has little effect on EphA2/4 Tyr phosphorylation. The finding that ADP and TRAP induce EphA2/4 Tyr-phosphorylation in the absence of stirring implies that the receptor is phosphorylated without making contact with ephrin-ligands on adjacent platelets.

Stirring strongly increases EphA2/4 Tyr phosphorylation by TRAP and makes ADP-induced phosphorylation detectable on immunoblots. Apparently, the ligand-induced receptor activation synergistically enhances the weak responses by TRAP and ADP. This agrees with the concept that Eph-ephrin association induce phosphorylations of cytosolic regions in receptor and ligand, which become initiation sites for further signaling[195]. EphA2/4 phosphorylation by ADP is inhibited by the EphA2/4 blocker, which binds to a cavity in the high affinity ephrin binding channel[214] and inhibits ligand-induced EphA2 Tyr-phosphorylation in HT22 neuronal cells[207]. This suggests that the inhibitor affects the cytosolic part of EphA2/4 making it less accessible to PTKs. With strong activation by TRAP, no interference by EphA2/4 blocker is seen suggesting that the effect of the inhibitor is relatively weak compared with the strong activation of PTKs by TRAP.

Interference by EphA2/4 blocker has important functional consequences as it leads to complete blockade of secretion of ATP and PF-4. In the aggregometer, EphA2/4 blocker strongly inhibits the 2nd phase of an optical aggregation curve, leaving the primary wave undisturbed. A similar disturbance is observed with platelets deficient in _-granule ADP[215] or $P2Y_{12}$ receptors[216,217] and in line with the proposed role of $P2Y_{12}$ signalling to secretion in which EphA2/4 appears a crucial intermediate. The same interference with EphA2/4 Tyr- phosphorylation leads to a strong reduction in thrombus growth on a collagen-coated surface under flow.

Earlier, Prevost and colleagues showed that platelets express EphA4 and EphB1 together with ephrinB1 ligand. Clustering of Eph receptors triggered adhesion to fibrinogen, surface expression of the α-granule marker P-selectin, but failed to initiate aggregation[201-203]. Ligand-occupied EphA4 signalled to the Src family kinases Fyn and Lyn but this response required pre-activation by ADP. The present findings are in line with these observations and show that pre-activation through Tyr-phosphorylation induced by ADP and TRAP enhances responses by ligand-occupied EphA2/4. The pre-activation of EphA2/4 is not in agreement with the classical concept that Eph-ephrin binding

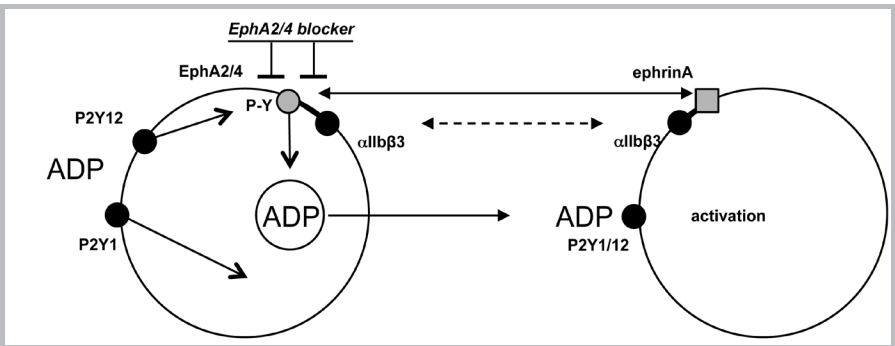


Figure 8. Schematic overview of $P2Y_{12}$ signalling to EphA2/A4.
See Discussion for details.

between two adjacent cells is a requirement for signal generation. Recent studies reveal other deviations from classical Eph-ephrin interaction[194]. Ligand independent EphA2 Tyr- phosphorylation has been demonstrated in tumor cells in vitro[194]. Biologicall active antibodies induce EphA2 phosphorylation and subsequent degradation in malignant cells[218]. Alternatively, Eph receptors might bind ephrin ligands on the same cell through the Eph fibronectin-III domain or might be localized separately in secretion granules and on the plasma membrane coming together during extrusion of granule contents[195]. A novel finding in this study is the predominant role of $P2Y_{12}$ in this response. Although the PTK activity profiles suggest that both $P2Y_1$ and $P2Y_{12}$ activate kinases that signal to EphA2/4 receptors, conventional blotting shows that this activation is almost exclusively mediated through $P2Y_{12}$. The finding that $P2Y_{12}$ is an upstream activator of EphA2/A4 and that blockade of this receptor halts secretion, suggest that the many pathways initiated by activated Eph receptors such as signalling to Fyn/Lyn, Rap1b and outside-in signaling through $\alpha_{IIb}\beta_3$ activate mechanisms that release the contents of δ- and α-granules. In addition to the strengthening of platelet-platelet interaction by Eph-ephrin interaction, these pathways may contribute to mechanisms that control thrombus growth. The interference by EphA2/4 blocker with these extracellular reactions might explain the strong reduction in thrombus formation in a flow model. These findings also imply that interference with $P2Y_{12}$ signalling by clopidogrel-AM and related drugs slows down these signaling sequences regulating thrombus growth and stability.

The high throughput screening of PTKs in stimulated cells has many advantages over the laborious analysis of Tyr-phosphorylated proteins by convential immunoblotting. Is also has limitations. The peptide substrates for activated kinases reflect the Tyr-phosphorylation sites of the native proteins only to a limited extend and the kinases work in a non-physiological environment. Both factors might compromise specificity. This is illustrated by the Tyr-phosphorylation of the peptide representing the erythropoietine receptor, which probably acts as a substrate for the PTK for the thrombopoietin receptor. The same may be true for EphA2, which does not show up in proteomic approaches and probably reflects the phosphorylation of EphA4. The cross-reactivity of anti EphA2 and anti EphA4 antibodies supports this conclusion. Despite these disadvantages, the technique offers a basis for the rapid screening of Tyr-phosphorylated proteins and might complement studies that associate gene polymorphisms with abnormalities in platelet functions.

Chapter 7

General Discussion

The formation of an arterial thrombosis is the result of a complex interplay between the proteins and enzymes in the coagulation cascade and platelets and the disturbed vessel. It results in a blood clot that can completely occlude an artery, leading to ischemia. Usually such a thrombus is superimposed on a part of the vessel wall affected by atherosclerosis, a systemic disease weakening the arterial wall. The formation of an arterial thrombus is best studied *in vivo*, because all the components for thrombus formation are present there. *In vivo* arterial thrombus formation can be studied in animal models like mice, rat and zebrafish and in man, obviously.

Because platelets play such an important role in atherothrombosis, drugs inhibiting their activation by blocking different platelet receptors have been developed. These are successful in preventing patients from having a second cardiovascular event. Particularly the $P2Y_{12}$ receptor is a target for development of new drugs, demonstrated by the recent appearance of prasugrel on the market.

This thesis aimed to evaluate the role of the zebrafish as a model system in research on platelet function and thrombus formation. Furthermore we have discussed the main problems the currently most used anti-platelet drugs (aspirin and clopidogrel) possess. Lack of clarity exists about the $P2Y_{12}$ receptor, the receptor antagonized by clopidogrel. In T2DM patients, there is speculation that there is less inhibition by antagonists. Also the current insight in $P2Y_{12}$ signalling is limited to suppression of production of cAMP (a platelet inhibitor) and activation of protein kinase B/Rap1b (which stimulate aggregation). We aimed to study the inhibition of the $P2Y_{12}$ receptor in diabetes patients and to demonstrate which secondary signalling molecules are activated by $P2Y_{12.}$

The zebrafish as an *in vivo* model to study platelet function and thrombus formation

The zebrafish (*Danio rerio*) is a vertebrate, originally used to study development. Over the years, it has gained popularity as a disease model. It has the advantages of fast development and the ease of genetic manipulation. This can make it an ideal instrument to study the genetic aspects of platelet development, function and thrombus formation. Their transparency during the first days of development makes it possible to study the formation of a thrombus in real-time. As in mammalian blood, zebrafish blood is composed of erythrocytes, granulocytes, monocytes, lymphocytes and thrombocytes and the proteins of the coagulation cascade. The vessels are lined with endothelial cells, which can be damaged by laser irradiation, a technique we used to induce atherothrombosis (chapter 2). This technique, first described by Jagadeeswaran *et al*[219], induces a thrombus that occludes the lumen of the dorsal aorta within one minute. The time to occlusion can be used as an outcome measurement to compare the function of platelets in a certain mutant with the wild-type situation. Via reverse genetics a specific gene of interest can be knocked out, where after the function of this gene in platelet function can be studied. This was done in a study by the Bloodomics Consortium[220,221] in which comparative transcript analysis of platelets and megakaryocytes, endothelial cells and erythroblasts revealed several novel platelet membrane proteins. Their role in thrombus function was studied by knocking the genes for these proteins down by morpholinos. This led to an altered thrombus formation in 4 out of 5 genes (2 proteins stimulating thrombosis and 2 proteins inhibiting thrombosis). We compared transcript levels of embryonic zebrafish thrombocytes with a mixture of all other cells of the

embryo (chapter 2). This generated a list of genes that showed an increased expression in thrombocytes, among which was *mlck1a*. This list encloses more novel genes that might be important for platelet development and function. Novel platelet membrane receptors can be used as targets for the development of new drugs. Modern drug development can be divided in three components; target identification, target validation and drug screening, and the zebrafish can have a role in all three[222]. Target identification refers to the process of identifying gene products that can be adapted by a drug. In the validation process, the ability of the target to be modulated by drugs is investigated. Large-scale mutagenesis screens, comparative transcript analysis and proteomic approaches make the zebrafish an ideal tool for identifying new targets. These targets can be validated through the rapid analysis of gene function. Knockdown of the gene of interest by antisense morpholinos provides information about the function of the gene in development. The small size of the embryos and larvae and the fact that they live in an aqueous milieu makes them ideal for drug screening. They can be arrayed in 96-wells plates and can survive for several days in approximately 50 μL of water. Antithrombotic drugs currently used in humans are effective in preventing clot formation induced by ADP in living zebrafish larvae[222]. Further studies are needed to determine whether the effects compounds have in zebrafish are similar in humans.

Besides the discovery of novel genes in hemostasis and platelet function, mutants with a bleeding phenotype can be further analyzed. The zebrafish mutant *redhead* reveals hemorrhage in the brain region[223]. This was caused by a mutation in the gene *p21-activated kinase 2a* (*pak2a*), a gene important for angiogenesis, regulation of cytoskeletal structure, endothelial cell migration and contractility. Further analysis of the mutant's hemostasis by studying the *in vivo* thrombus formation exhibited no difference in time to occlusion between mutants and their siblings, which suggested that the cerebral hemorrhages were not the result of a global defect in hemostasis. The Pak2a deficient vessels appeared to be prone to rupture and loss of junctional integrity, without evidence for a general increase in leakiness or permeability. It showed that also genes essential for endothelial cell structure and integrity can be studied using the *in vivo* thrombosis assay.

In the future the zebrafish will become an even more popular research tool. Since the report on the first knockout[13], more knockout fish have been described. With their short generation time it is possible to obtain knockout fish relatively fast and their high productiveness will result in a large group of study animals.

We compared the mRNA levels of zebrafish thrombocytes with a mixture of all other cells in the embryo. The thrombocytes were divided from the rest of the embryo by fluorescent activated cell sorting (FACS) in embryos 3-4 days post fertilization. However, comparative transcript analyses can be done at different time points in the development of the embryo and might reveal novel genes important for thrombocyte maturation. Also stable fluorescent fish lines exist for other blood cells, such as erythrocytes[224] and neutrophils[225]. Comparing these different blood cell lineages might exhibit novel genes specific for thrombocytes. The function in thrombocytes can then be established and compared to that of human platelets.

The troubles with the current anti-platelet drugs aspirin and clopidogrel

Nowadays, acetylsalicylic acid (also known as aspirin) is the most prescribed platelet aggregation inhibitor. It is recommended to patients suffering from a cardiovascular event to prevent them from having another attack. In patients who suffered from an ischemic stroke, myocardial infarction or atherosclerotic peripheral arterial disease, aspirin reduced the risk of stroke, myocardial infarction or vascular death by 25%[226]. However, patients on aspirin still have an annual risk of ischemic stroke, myocardial infarction or vascular death of 5.83%[36]. Furthermore, aspirin has some potentially serious adverse effects such as gastrointestinal discomfort and bleeding. We have shown that 30.6% of patients on low-dose aspirin complain about symptoms suggestive for aspirin intolerance (chapter 3). Most common symptoms were related to the gastrointestinal tract. Also, 6.6% of the patients had ceased aspirin treatment, predominantly because side-effects occurred.

Clopidogrel is a platelet aggregation inhibitor that showed to be a little more potent than aspirin. The occurrence of side-effects is similar to that of aspirin, although severe gastrointestinal hemorrhage occurred less frequently. On the other hand the frequency of severe rash was higher in patients on clopidogrel. Still, patients on clopidogrel have an annual risk of 5.32% of developing a secondary atherothrombotic event. In the Netherlands, clopidogrel monotherapy is indicated to patients with established occlusive vascular disease, who are intolerant to aspirin. The combination of aspirin and clopidogrel is prescribed to patients suffering from acute coronary syndrome or to patients undergoing percutaneous coronary intervention (PCI). Combinations of aspirin and clopidogrel reduce risk of cardiovascular death and myocardial infarction further by 30%[37]. Despite this, 4.5% of patients on dual therapy still suffer from cardiovascular death, myocardial infarction or require urgent revascularisation within 30 days of PCI.

The fact that patients may experience recurrent events while on antiplatelet drugs can be explained by the multifactorial nature of atherothrombosis[227]. This failure of treatment is often referred to as 'resistance'. There is discussion about the existence of 'aspirin resistance'. Agonist induced platelet aggregation is used to quantify the antiplatelet effect of aspirin, but it variably reflects the aspirin sensitive TXA_2-dependent component of platelet aggregation[228]. In a recent study in healthy individuals, it was shown that 100 mg/day aspirin for 1-8 weeks uniformly and persistently suppressed platelet COX-1 activity, exhibited by serum TXB_2 levels[229]. This was not reflected by functional assays, such as platelet aggregation measurements.

For clopidogrel, a large variation in the response of patients has been described. Clopidogrel is an inactive prodrug and converted to its active metabolite by hepatic cytochrome P450[34]. The efficiency is poor (< 15%), the major product being an inactive, second metabolite. Recently more evidence has become available that this ineffective active metabolite formation is the main reason for the decreased response to clopidogrel. Patients on clopidogrel carrying the loss of function phenotype for the cytochrome P450 family member CYP2C19, have a higher chance of developing a cardiovascular event[145,146]. But also T2DM patients show a decreased response to clopidogrel, possibly explained by an upregulated $P2Y_{12}$ pathway[44]. We demonstrated that T2DM platelets in their resting state are more activated and that they are more sensitive to ADP and collagen stimulation (chapter 5). This difference between T2DM platelets and healthy platelets remains even after a week of clopidogrel. Angiolillo *et al* earlier showed that

about half of clopidogrel resistant T2DM patients do not respond to an increase in clopidogrel dosage to 150 mg/day[137].

A new kid in town: prasugrel

The recently introduced $P2Y_{12}$ antagonist prasugrel has to be, like clopidogrel, converted to its active metabolite. This is also modulated by the cytochrome P450, but it is more efficient and faster than the conversion of clopidogrel. This leads to higher plasma levels of the active metabolite and the peak level is reached faster, which results in a better inhibition of ADP-induced platelet aggregation. A randomized controlled trial comparing prasugrel with clopidogrel found a reduction in the incidence of myocardial infarction, but also an increase in major bleeding with prasugrel[182]. In the prasugrel group the rate of life-threatening bleeding was higher (1.4% vs. 0.9%; p=0.01). The risk of major bleeding is higher in people with an enhanced response to antiplatelet therapy[230]. Guidance of antiplatelet treatment based on platelet function testing is suggested to avoid bleeding complications. In case of bleeding complications, aspirin, clopidogrel and prasugrel have the major disadvantage of being irreversible inhibitors of their target receptor. This means that platelets remain inhibited throughout their lifespan (7-10 days) and that it will take several days until enough fresh, uninhibited platelets have formed.

Reversible $P2Y_{12}$ antagonists

The problems of irreversibility of $P2Y_{12}$ antagonists in case of bleeding complications seems to be solved with the appearance of the reversible $P2Y_{12}$ inhibitors cangrelor and ticagrelor. These also have advantages for people undergoing surgery. Clopidogrel and aspirin are stopped routinely several days before the surgical procedure, because they can increase the risk of postoperative bleeding. During these days of discontinuation of aspirin and clopidogrel, the patients have a higher risk of developing a secondary atherothrombotic event. Reversible antiplatelet drugs can solve this problem. Cangrelor, formerly known as AR-C69931MX, is a potent inhibitor of the $P2Y_{12}$ receptor. It does not require conversion to an active metabolite, but needs to be administered intravenously. Therefore it might only be interesting to reduce ischemic events during an intervention (for example PCI). The CHAMPION PCI trial investigated the value of periprocedural cangrelor during PCI[231]. Enrollment was stopped when an interim analysis concluded that the trial would be unlikely to show superiority for the primary end point, the composite of death, myocardial infarction, or ischemia-driven revascularization at 48 hours after PCI.

Ticagrelor, previously known as AZD6140 is a selective reversible $P2Y_{12}$ antagonist that is orally available. Dose-finding and safety studies showed that ticagrelor 400 mg per day rapidly and nearly completely inhibited ADP-induced platelet aggregation[232]. However, treatment with ticagrelor was associated with an incidence of dyspnea of 10-20%. In the very recently described PLATO trial, ticagrelor was compared to clopidogrel in 18.624 patients[188]. At 12 months, death from vascular causes, myocardial infarction, or stroke had occurred in 9.8% of patients receiving ticagrelor as compared with 11.7% of those receiving clopidogrel (hazard ratio, 0.84; 95% confidence interval,

0.77 to 0.92; P<0.001). It showed no increase in the rate of overall major bleeding, but patients on ticagrelor had an increased rate of non-procedure-related bleeding (such as fatal intracranial bleeding).

The efficacy of platelet aggregation inhibitors: to monitor or not to monitor?

The ideal platelet aggregation inhibitor would not need monitoring. However, a suboptimal response to clopidogrel is one of the main problems the drug possesses (chapter 4). On the other hand, people with an enhanced response to antiplatelet therapy have an increased risk of bleeding. The recently introduced agents seem to be more potent than aspirin and clopidogrel. This also comes at a cost of an increased bleeding risk. Reversible antiplatelet drugs have the benefit that discontinuation of the agent leads to a fast return to normal platelet function. Monitoring of a patient's response to an antiplatelet drug seems to be compulsory. Bedside assays have been described, but so far none have been able to replace aggregometry as the golden standard. Aggregometry is time-consuming and can be logistically demanding. Measurement of agonist induced P-selectin expression by flow cytometry is a simple, fast assay, measured in whole blood (chapter 5). It needs only small amounts of blood and the procedure can be automated. Future studies are needed to compare this assay to the golden standard.

In a study by Jones *et al*, using a high-throughput functional genomic approach, it was shown that single nucleotide polymorphisms in genes with a function in platelet signaling pathways are associated with this variance in response[123]. In a similar manner, we have studied the $P2Y_{12}$ signalling pathway via a high-throughput micro-array approach, based on 144 Protein Tyr-kinase (PTK) substrates and show that EphA2/4 is under control of the $P2Y_{12}$ receptor and contributes to granule secretion and thrombus stabilization (chapter 6). In the future, functional genomic and proteomic approaches might reveal novel genes important for variance to platelet inhibiting drugs.

To keep the haemostatic balance stable, optimal treatment by antiplatelet drugs should be based on accurate assessment of platelet reactivity of the individual patient[183]. This can only be done when there is consensus about the method used, optimal time of analysis and agonist concentration.

Subgroups of patients on antiplatelet drugs might show a decreased response to antiplatelet drugs, like for example T2DM patients. Monitoring the efficacy of platelet aggregation inhibitors is even more important in these patients. Future studies can demonstrate that they benefit from a more potent $P2Y_{12}$ inhibitor.

Chapter 8

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Nederlandse Samenvatting
List of Publications
Dankwoord
Curriculum Vitae

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Nederlandse Samenvatting

Atherosclerose (slagaderverkalking) is een chronische ontstekingsziekte en een belangrijke oorzaak van overlijden in de Westerse Wereld. Atherosclerose zorgt voor een verandering van de samenstelling van de wand van bloedvaten. Het ontstaat als reactie op schade aan de vaatwand door bijvoorbeeld roken, diabetes mellitus, obesitas en hypercholesterolemie. De cellen die normaal de vaatwand vormen worden vervangen door ontstekingscellen en lipoproteïnen. Dit lijdt tot een vernauwing van het lumen van het bloedvat. Klinische manifestaties van atherosclerose worden veroorzaakt door destabilisatie en het scheuren van de atherosclerotische plaque. Hierdoor scheurt ook het endotheel (de binnenbekleding van het bloedvat). Het circulerende bloed komt in aanraking met de cellen onder het endotheel, wat ervoor zorgt dat bloedplaatjes en het stollingssysteem worden geactiveerd. Dit resulteert in het ontstaan van een thrombus op de plaats van de scheur van de plaque, een occlusie van het lumen van het vat en hypoxie van het achterliggende weefsel. Afhankelijk van de plaats van de atherosclerotische plaque ontstaat een myocard infarct, beroerte of een perifeer arteriële vaatafsluiting. De vorming van een thrombus in een slagader wordt ook wel atherothrombose genoemd.

Een belangrijke rol in de vorming van een thrombus is weggelegd voor de bloedplaatjes (in het vervolg plaatjes genoemd). Dit zijn celfragmenten die worden gevormd uit megakaryocyten in het beenmerg. In circulatie worden ze in een rustende staat gehouden door het door endotheelcellen gevormde prostacycline (PGI_2) en stikstofmonoxide (NO). Plaatjes worden geactiveerd door verschillende agonisten zoals thromboxaan A_2 (TXA_2), ADP, thrombine, collageen en epinephrine. Dit leidt tot een vormverandering, de vorming van aggregaten en secretie van de inhoud van de granula. De integrine receptor $\alpha_{IIb}\beta_3$ speelt een belangrijke rol in plaatjesactivatie. De verscheidene agonisten zorgen voor een conformatieverandering van inactief naar actief van deze receptor, waardoor de $\alpha_{IIb}\beta_3$ receptor kan binden aan fibrine (het eindproduct van de stollingscascade) en von Willebrand Factor. Plaatjesactivatie wordt versterkt door twee positieve feedbackloops. In de eerste feedbackloop wordt TXA_2 gevormd uit arachidonzuur. Deze feedbackloop wordt geremd door aspirine. De tweede feedbackloop begint met de secretie van ADP uit de dense-granules, wat resulteert in de activatie van de ADP-receptor $P2Y_{12}$. Deze feedbackloop wordt geremd door de $P2Y_{12}$ remmer clopidogrel.

Deel A: De zebravis als model om thrombusvorming te bestuderen

De complexe interactie tussen endotheelcellen, stollingsfactoren, plaatjes en andere factoren en cellen kan bestudeerd worden in *in vitro* en *in vivo* onderzoeksmodellen. *In vitro* modellen hebben als nadeel dat een eiwit of cel geïsoleerd bestudeerd wordt. Omdat zoveel factoren van invloed zijn op thrombusvorming is het noodzakelijk om deze in levende organismen te bestuderen. Hiervoor kunnen verschillende organismen gebruikt worden. De zebravis (*Danio rerio*) werd eind jaren negentig voor het eerst gebruikt om thrombose en hemostase te bestuderen. De zebravis heeft verschillende voordelen ten opzichte van andere soorten proefdieren. Ze planten zich makkelijk voort, waarbij ze veel embryo's produceren. Fenotypes kunnen makkelijk gescoord worden,

omdat de embryo's gedurende de eerste dagen van de ontwikkeling transparant zijn. Deze ontwikkeling gaat snel. Binnen 24 uur worden alle primaire organen gevormd en na drie maanden kunnen de opgegroeide vissen zichzelf voortplanten. Tevens is de zebravis gemakkelijk genetisch te manipuleren. Recente studies hebben de aanwezigheid van alle belangrijke hematologische cellen in zebravisbloed aangetoond. Tevens zijn alle voor thrombose belangrijke cellen en eiwitten aangetoond in zebravisembryo's en volwassen vissen. De eiwitten van de stollingscascade zijn vergeleken met die van de mens, waaruit bleek dat deze erg op elkaar lijken. Ook zijn bloedplaatjes aangetoond die reageren op vaatschade en die te activeren zijn door de agonisten ADP, collageen en arachidonzuur.

Wij hebben de zebravis gebruikt om de rol van het eiwit Mlck1a in plaatjes aan te tonen. Mlck, oftewel Myosin Light Chain Kinase, is een eiwit dat belangrijk is voor het ondergaan van vormverandering bij verschillende soorten cellen. In mensen bestaan 3 vormen van MLCK; MLCK1, MLCK2 en MLCK3. In zebravissen is Mlck1 gedupliceerd en bestaan er dus vier vormen van Mlck (Mlck1a, Mlck1b, Mlck2 en Mlck3). Omdat bloedplaatjes na activatie een vormverandering ondergaan, was onze hypothese dat een van de vormen van MLCK hierbij betrokken zou kunnen zijn. In hoofdstuk 2 hebben we aangetoond dat in de plaatjes van zebravissen alleen Mlck1a aanwezig is. Als dit Mlck1a wordt uitgeschakeld kunnen de plaatjes minder goed van vorm veranderen. Tevens wordt er, na het induceren van vaatschade in een arterie van de zebravis, minder snel een thrombus gevormd als Mlck1a is uitgeschakeld.

Deel B: De preventie van atherothrombose door plaatjesaggregatieremmers

Patiënten die eerder een atherothrombose doorgemaakt hebben, krijgen verschillende soorten medicijnen om een herhaling te voorkomen. Omdat plaatjes zo'n belangrijke rol spelen in de vorming van de thrombus, worden de plaatjes van deze patiënten geremd door verschillende soorten medicijnen. De meest gebruikte zijn aspirine en clopidogrel. Aspirine werd voor het eerst gemaakt in 1853, maar de werking van aspirine werd pas ontdekt in 1971. Aspirine remt de vorming van TxA_2 en daarmee een van de twee aanwezige positieve feedbackloops van plaatjesactivatie. Het is de meest voorgeschreven plaatjesaggregatieremmer. Het belangrijkste nadeel van aspirine betreft de bijwerkingen. In de literatuur worden verschillende percentages genoemd van intolerantie voor aspirine, waarschijnlijk door een verschil in definitie tussen de verschillende studies.

In hoofdstuk 3 onderzochten we de prevalentie van intolerantie voor aspirine in 947 patiënten door middel van een vragenlijst die de deelnemende patiënten samen met hun behandelend arts invulden. Deze vragenlijst behandelde vragen over het huidige aspirinegebruik, het gebruik van andere medicatie en het voorkomen van symptomen die suggestief waren voor bijwerkingen van aspirine. Van de 947 patiënten waren er inmiddels 60 (6.6%) gestopt met het gebruik van aspirine, voornamelijk door het optreden van bijwerkingen. Tevens gaf 30.6% van de patiënten aan last van bijwerkingen te hebben. De meest voorkomende bijwerkingen betroffen klachten van het maagdarmstelsel. Een kwart van de patiënten gebruikte een maagzuurremmer. Het lijkt er dus op dat een groot gedeelte van de patiënten die aspirine gebruiken last

heeft van hinderlijke bijwerkingen. Er moet meer aandacht worden geschonken aan het behandelen van die bijwerkingen, omdat het de meest voorkomende oorzaak is van het stoppen met medicatie. Hierdoor worden patiënten blootgesteld aan een verhoogd risico op een herhaling van een atherothrombose.

Clopidogrel wordt sinds de jaren negentig voorgeschreven aan patiënten die een atherothrombose doormaakten. Clopidogrel remt de $P2Y_{12}$ receptor en daarmee de tweede positieve feedbackloops van plaatjesactivatie. Alvorens clopidogrel de $P2Y_{12}$ receptor kan remmen moet het eerst worden omgezet in een actief metaboliet door de darmen en lever. Deze omzetting is echter niet erg effectief, waardoor slechts 15% van de hoeveelheid clopidogrel uiteindelijk wordt omgezet in actief metaboliet. Dit fenomeen wordt ook wel clopidogrelresistentie genoemd.

Hoofstuk 4 behandelt de verschillende oorzaken van clopidogrel resistentie, zoals de verminderde werking van het cytochroom P450 enzymsysteem (de enzymen die clopidogrel moeten omzetten in het actief metaboliet) en een verhoogde $P2Y_{12}$ signalering. Het onderzoek naar de prevalentie van clopidogrelresistentie wordt bemoeilijkt door het gebrek aan een eenduidige laboratoriumtest, met duidelijke definities van clopidogrelresistentie.

Patiënten met type 2 diabetes mellitus (T2DM; in de volksmond “ouderdomsdiabetes” genoemd) lijken ook minder goed te reageren op clopidogrel. De plaatjes van T2DM patiënten zijn insuline resistent en hypersensitief. Deze hypersensitiviteit wordt veroorzaakt door een verhoogde gevoeligheid van de $P2Y_{12}$ signaleringsroute. In hoofdstuk 5, onderzochten we of de plaatjes van T2DM patiënten een verminderde respons op clopidogrel hebben vergeleken met een controlegroep zonder T2DM. Plaatjesactivatie werd getest op twee verschillende tijdstippen. Tussen deze twee tijdstippen namen de patiënten clopidogrel. Voor inname van clopidogrel hebben de T2DM patiënten een verhoogde gevoeligheid voor stimulantia. De respons op clopidogrel is ook lager in T2DM patiënten. De remming van de $P2Y_{12}$ receptor is nog wel te versterken door de *in vitro* toevoeging van de $P2Y_{12}$ remmer AR-C69931MX. Het zou dus kunnen dat sterkere $P2Y_{12}$ remmers beter werken in T2DM patiënten.

De huidige kennis van de $P2Y_{12}$ signaleringsroute is beperkt tot de suppressie van cAMP (een belangrijke plaatjesremmer) en de activatie van proteïne kinase B (welke aggregatie stimuleert). We onderzochten of andere signaleringsmoleculen worden geactiveerd door $P2Y_{12}$ in hoofdstuk 6. Hierbij hebben we gebruik gemaakt van een micro-array gebaseerd op 144 proteine tyrosine kinases (PTKs) substraten. ADP stimuleert de tyrosine phosphorylering van 28 peptide substraten. We vonden een sterke phosphorylering van substraten die de verschillende leden van de Eph-receptorfamilie vertegenwoordigen. Remming van $P2Y_{12}$ zorgt voor een inhibitie van de phosphorylering van de substraten van EphA2 en EphB1. Een remmer van EphA2/A4 phosphorylering resulteert in een verminderde ADP geïnduceerde aggregatie, secretie en thrombusvorming. Het lijkt erop dat EphA2/A4 onder controle staat van $P2Y_{12}$ en daarmee bijdraagt aan secretie en thrombusstabilisatie.

Nieuwe plaatjes aggregatieremmers zijn recent op de markt verschenen of zullen dit in de nabije toekomst doen. Deze lijken sterker te zijn dan de huidige medicijnen. Hiermee neemt ook de kans op bloedingen toe. Voor de patiëntengroepen die een verminderde respons op plaatjesaggregatie hebben (zoals T2DM patiënten) lijken de nieuwe remmers een uitkomst. Toekomstige studies moeten het resultaat in deze patiënten aantonen. Om te voorkomen dat de balans tussen te weinig en te veel remming doorslaat lijkt het monitoren van patiënten noodzakelijk. Het ontbreekt echter nog aan een snelle test die de aggregometrie als gouden standaard kan vervangen.

List of Publications

Tournoij E, Koekman CA, Boss M, Verhoef S, Du V, Roest M, Houkes L, Pijnenburg D, Ruijtenbeek R, Moll FL, Akkerman JW.
ADP activation of platelet protein tyrosine kinases reveals P2Y12 signalling to Ephrin A2/4 receptor.
Submitted for publication

Tournoij E, de Bono B, Moll FL, Akkerman JW.
Causes of clopidogrel resistance.
Submitted for publication

Tournoij E, Weber GJ, Akkerman JW, de Groot PG, Zon LI, Moll FL, Schulte-Merker S.
Mlck1a is expressed in zebrafish thrombocytes and is an essential component for thrombus formation.
J Thromb Haemost. 2010 *in press*

Tournoij E, Peters RJ, Langenberg M, Kanhai KJ, Moll FL.
The prevalence of intolerance for low-dose acetylsalicylacid in the secondary prevention of atherothrombosis.
Eur J Vasc Endovasc Surg. 2009 May;37(5):597-603

Tournoij E, Slisatkorn W, Prokop M, Verhagen HJ, Moll FL.
Thrombus and calcium in aortic aneurysm necks: validation of a scoring system in a Dutch cohort study.
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Surg Endosc. 2007 Aug;21(8):1413-7

Draaisma WA, Gooszen HG, **Tournoij E**, Broeders IA.
Controversies in paraesophageal hernia repair: a review of literature.
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Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 30 juli 1981 in Eindhoven. In 1999 behaalde hij cum laude zijn atheneum diploma aan het Stedelijk College Eindhoven. Dat zelfde jaar werd gestart met de studie Geneeskunde aan de Universiteit van Utrecht. Gedurende zijn studie participeerde hij in een onderzoek naar de waarde van Virtual Reality simulatoren voor het trainen van laparoscopische operaties onder begeleiding van prof. dr. I.A.M.J. Broeders (afdeling Heelkunde). Tevens verrichtte hij een onderzoek naar de validiteit van een scoringssysteem om de nek van een aneurysma te beoordelen (onder begeleiding van prof. dr. F.L. Moll; afdeling Vaatchirurgie). Na het laatste co-schap op de spoedeisende hulp van het Cris Hani Baragwanath Hospital in Johannesburg, behaalde hij in 2005 zijn artsexamen. Direct hierna startte hij met zijn promotieonderzoek op de afdeling Vaatchirurgie van het Universitair Medische Centrum Utrecht. Dit onderzoek werd begeleid door prof. dr. F.L. Moll, prof. dr. S. Schulte-Merker (Hubrecht Instituut) en prof. dr. J.W.N. Akkerman (afdeling Klinische Chemie en Hematologie). Voor een gedeelte van het in dit proefschrift beschreven onderzoek ontving hij in 2009 de AIO Wetenschapsprijs van de Nederlandse Vereniging voor Thrombose en Hemostase. Op dit moment werkt hij als arts-assistent chirurgie in het Meander Medisch Centrum te Amersfoort (dr. A.J. van Overbeeke en dr. E.C.J. Consten).